

Article

Identification of Unrecognized Hepatitis B, C, and D Infections Through the Private Laboratory-Based RE-LINK Screening Project in Romania: A Micro-Elimination Initiative

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Abstract

Background/Objectives: Chronic hepatitis B (HBV) and C (HCV) remain major public health challenges in Romania despite vaccination and antiviral therapy. Understanding infection patterns in different healthcare settings is essential for targeted elimination strategies. **Methods:** We conducted the prospective screening phase of the RE-LINK project (January–June 2025) through two nationwide private laboratory networks. Adults undergoing routine testing were screened for HBsAg and anti-HCV. HBsAg-positive samples were further analyzed for HBV DNA, HBeAg, anti-HBe, anti-HDV, and HDV RNA, while anti-HCV-positive cases were tested for HCV RNA. Risk factors were assessed using chi-square and logistic regression analyses. **Results:** Among 9149 individuals (66.6% women with a median age of 53 years), HBsAg prevalence was 2.9%, and anti-HCV was 1.3%, both increasing significantly with age ($p < 0.001$). Of all HBsAg-positive individuals, 12.5% had undetectable HBV DNA, 70.4% had low viremia (<2000 IU/mL), and 17.1% had high viral loads. Anti-HDV antibodies were detected in 2.3% of HBsAg-positive subjects, all with detectable HDV RNA (range 1250–680,000 IU/mL). Significant risk factors for HBsAg positivity were male sex, older age, urban residence, physician-indicated testing, neuropsychiatric comorbidity, family or parental hepatitis, and institutional/orphanage care, while HBV vaccination and moderate alcohol use were protective. Anti-HCV positivity correlated with older age, cardiovascular disease, elevated transaminases, transfusions, surgery, and HIV co-infection. Only 20.2% of anti-HCV-positive individuals were viremic. **Conclusions:** Private-laboratory screening reveals residual low-replicative HBV and declining viremic HCV, while community programs uncover HDV and advanced disease in vulnerable groups. A coordinated approach integrating private, community, and hospital-based pathways can accelerate elimination efforts and ensure that HDV is not overlooked.

Keywords: hepatitis B; hepatitis C; hepatitis D; screening; Romania



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1. Introduction

Chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV) continues to represent a major global public health challenge. These viral infections are leading causes of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), accounting for substantial morbidity and mortality worldwide. According to the World Health Organization (WHO), an estimated 254 million people are chronically infected with HBV, and about 50 million were infected with HCV as of 2022, while approximately

15–20 million individuals globally are co-infected with HDV. In the WHO European Region alone, viral hepatitis is responsible for approximately 53,000 deaths annually, exceeding the combined mortality attributed to HIV and tuberculosis. The majority of these deaths are related to complications of chronic HBV, HCV, and HDV infection, such as decompensated cirrhosis and HCC [1–4].

In recent years, the landscape of viral hepatitis management has changed substantially. Universal HBV vaccination programs have led to a decline in the prevalence of HBV infection in many regions. Moreover, the advent of direct-acting antivirals has revolutionized the treatment of HCV, achieving cure rates above 95% with short, highly effective, and well-tolerated regimens. For HDV, novel therapies such as Bulevirtide offer promising perspectives for disease control. However, despite these advances, a large proportion of infected individuals remain undiagnosed or untreated, especially in countries with intermediate to high endemicity [5,6].

Over the last two decades, Romania reported one of the highest prevalences of HBV and HCV infection in Europe [7,8]. HBV/HDV co-infection still represents a leading indication for liver transplantation in our country, contrasting with trends observed in Western Europe, where alcohol-related disease predominates. The burden of chronic viral hepatitis in Romania is further characterized by regional and sociodemographic disparities. Data from previous screening programs, including the LIVE(RO)2 project, revealed a high prevalence of viral hepatitis infection in rural populations, often facing barriers, such as low awareness, limited healthcare access, and socioeconomic vulnerability [9,10].

International studies highlight the complex interplay between geography, social determinants of health, and the epidemiology of viral hepatitis. For example, a retrospective analysis from Turkey [11] found no significant difference in HBsAg and anti-HCV prevalence between urban and rural settings but noted higher anti-HBs positivity in urban populations, likely reflecting increased vaccination coverage. Beyond biomedical factors, psychosocial determinants also influence outcomes. A recent cross-sectional study from Yozgat et al. [12] showed that patients with chronic HBV or HCV infections living in urban environments in Turkey reported higher levels of stigma and negative self-image, which can hinder participation in screening programs and treatment adherence. This leads to undiagnosed cases and ongoing transmission, undermining global efforts to eliminate viral infections by 2030, as outlined by the WHO. To accelerate progress towards the WHO 2030 elimination targets, the screening strategy must be calibrated to reach underserved, vulnerable populations. Large-scale, nationwide initiatives must be complemented by micro-elimination approaches targeting specific high-risk groups or geographic regions [13], especially relevant in Romania, where vulnerable populations such as migrants, individuals with a history of intravenous drug use or incarceration, and persons with comorbidities (e.g., diabetes, chronic kidney disease, or hematological disorders) are often underdiagnosed and undertreated. The concept of micro-elimination refers to targeted elimination efforts within specific populations, healthcare systems, or regions, rather than at the national level, and is endorsed by the WHO and EASL as a pragmatic, scalable pathway to achieving broader elimination goals [14,15]. In our case, we applied this approach by utilizing the infrastructure of private laboratory chains, which allowed us to reach a nationwide and heterogeneous population. By identifying new or unlinked cases and referring them to hepatology services, our project contributes to the cascade of hepatitis care, with the goal of improving individual outcomes and public health impact.

In this context, the current analysis focuses on the prospective component of the RE-LINK project, which evaluated risk-based screening for HBV and HCV within private laboratory networks in Romania. By leveraging the laboratories' infrastructure to test individuals presenting for routine medical analyses, this study aimed to assess the feasibility

and diagnostic yield of this strategy, as well as to characterize the demographic and clinical risk profiles associated with HBV and HCV infections. Ultimately, the findings are intended to inform targeted interventions and support national and regional efforts toward the micro-elimination of viral hepatitis in Romania, in alignment with global public health goals.

2. Materials and Methods

2.1. Study Design

We conducted a prospective screening phase of the RE-LINK study (study number: IN-US-987-7284; Ethics Committee Approval: 797/C/11.10.2024), consisting of a 6-month active screening campaign (January–June 2025) targeting consecutive individuals presenting for routine check-ups or incidental laboratory investigations at two private laboratory chains covering the entire country. All study procedures were conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent covering the screening tests, reflex virological analyses, and the epidemiological questionnaire was obtained from all participants prior to inclusion; data were processed in an anonymized form for analysis in accordance with GDPR.

2.2. Study Population and Eligibility

The target population comprised adults (≥ 18 years) residing in Romania who presented at two private laboratory chains (Synevo and Affidea, Romania) that cover the whole of Romania for testing for any other tests, either by themselves or sent by physicians during the stated time frame. The inclusion criteria were age ≥ 18 years, no previous known viral HBV or HCV infection, provision of informed consent for participation, completion of a questionnaire for risk factors, testing, and follow-up in order to link them to therapy.

The study was conducted within two major private laboratory networks in Romania: Synevo, operating over 150 collection centers and more than 20 regional laboratories; and Affidea, which offers broad national coverage, including both urban and small-town areas across all 41 counties and Bucharest. These laboratories provide diagnostic services through self-paid testing, employer-sponsored medical subscriptions, and physician referrals. As such, they are commonly accessed by economically active adults with stable employment and better healthcare engagement, who seek preventive care or follow-up testing. Although data on education level or income were not collected, the population tested is generally not socioeconomically disadvantaged and represents a medically relevant subgroup often under-represented in public screening campaigns. The extensive infrastructure and standardized protocols of these networks ensured consistent data collection across geographic regions and facilitated the inclusion of a diverse urban and semi-urban population. This complements ongoing national efforts that primarily target vulnerable or underserved groups through community and public healthcare channels.

2.3. Screening and Testing Procedures

All eligible participants underwent blood sampling and serological testing for hepatitis B surface antigen (HBsAg) and anti-HCV antibodies (anti-HCV) using validated electrochemiluminescence immunoassays (ECLIA) on the Cobas e801 analyzers (Roche Diagnostics GmbH, Mannheim, Germany). For anti-HCV, we used the Elecsys Anti-HCV II kit (third-generation ECLIA), based on the sandwich principle, with high analytical sensitivity across all six major genotypes. Participants who tested HBsAg-positive were further assessed with the full panel of HBV serological markers: HBeAg, anti-HBe, anti-HBc (total), and anti-HBs, using Elecsys ECLIA kits (Roche Diagnostics). HBV DNA quantification was performed using real-time PCR on the COBAS 8800 system (Roche Diagnostics), with a lower limit of detection of 10 IU/mL and an analytical sensitivity of 2.7 IU/mL.

HBsAg-positive individuals were also tested for anti-HDV antibodies using the LIAISON® XL Murex Anti-HDV assay (CLIA, DiaSorin, Italy), with reactive samples (≥ 1 AU/mL) undergoing reflex HDV RNA testing via real-time PCR (LoD: 200 copies/mL, equivalent to ~ 80 IU/mL). All individuals with anti-HCV positivity were referred for HCV RNA quantification by real-time RT-PCR (COBAS 8800, Roche Diagnostics), with a lower limit of detection of 15 IU/mL. It is important to note that in our protocol, Western blot confirmation was not routinely performed; instead, direct HCV RNA quantification followed a positive anti-HCV result. This approach may explain a subset of anti-HCV-positive individuals with undetectable HCV RNA, which is discussed further in the manuscript. All testing was conducted at Synevo Laboratories, following manufacturers' instructions and internal validated procedures. All individuals with confirmed chronic viral hepatitis were referred to specialized hepatology centers for further evaluation, fibrosis staging, and initiation of antiviral treatment in accordance with national and international clinical guidelines.

2.4. Data Collection

The following variables were collected by a structured questionnaire administered at inclusion: age, sex and area of residence (urban/rural), ethnicity, history of blood transfusions, previous surgical or dental interventions, history of intravenous/intranasal drug use, alcohol abuse, frequent hospitalizations (≥ 2 /year), incarceration, known HBV/HCV/HDV infection in relatives, and history of comorbidities (diabetes mellitus, chronic kidney disease/dialysis, neurological or psychiatric disorders, cancer, and previous chemotherapy or radiotherapy). Participants were also asked to report whether they had been vaccinated against hepatitis B; vaccination status was self-reported and not confirmed through immunization records or anti-HBs titers. Laboratory results (HBsAg, anti-HCV, HBV DNA, HBeAg, anti-HBe, anti-HBs, anti-HDV, and HCV RNA) were systematically recorded.

2.5. Statistical Analysis

Descriptive statistics were used to summarize baseline characteristics. Continuous variables were presented as mean, median, standard deviation, interquartile range, and extreme values. Categorical variables were expressed as absolute frequencies and percentages. Crude prevalence rates for chronic HBV, HCV, and HDV infections were calculated overall and stratified by risk factors. Associations between HBsAg or anti-HCV seropositivity and risk factors were assessed using the chi-square test or Wilcoxon rank-sum test, as appropriate. To estimate the strength of association, univariate logistic regression models were constructed for each risk factor. Results were reported as odds ratios (ORs) with 95% confidence intervals (CIs). A two-sided p -value < 0.05 was considered statistically significant.

3. Results

Study Population

A total of 9149 individuals were tested for HBsAg and anti-HCV antibodies in the two private laboratory chains across Romania. Women represented 66.6% of the cohort, and most participants resided in urban areas (85.2%). The median age of the tested individuals was 52 years for females and 55 years for males. The prevalence of HBsAg positivity was 2.9% ($n = 264$), while the prevalence of anti-HCV positivity was 1.3% ($n = 120$). There was a significant trend of age-associated increase in prevalence for both HBV and HCV infection ($p < 0.001$) (Figure 1).

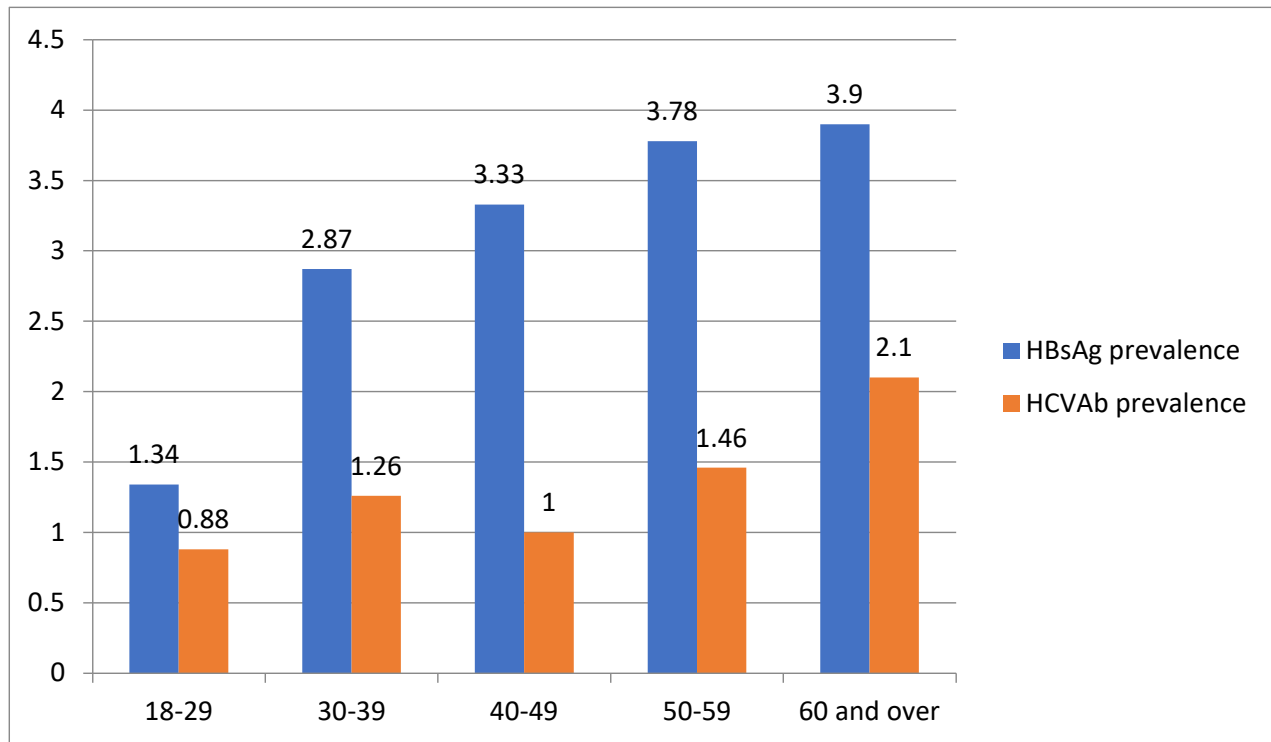


Figure 1. Prevalence of HBsAg and anti-HCV positivity by age group. Legend: The figure displays age-specific prevalence rates (%) of HBsAg (blue bars) and anti-HCV (orange bars) among individuals tested in the RE-LINK project, based on routine laboratory testing across two private diagnostic chains in Romania. A clear age-related increase in prevalence was observed for both HBV and HCV infections ($p < 0.001$). The absolute number of positive cases and tested individuals per age group is provided as follows: 18–29 years: 10/738 HBsAg-positive and 6/738 anti-HCV-positive; 30–39 years: 35/1205 HBsAg-positive and 15/1205 anti-HCV-positive; 40–49 years: 62/1852 HBsAg-positive and 19/1852 anti-HCV-positive; 50–59 years: 86/2264 HBsAg-positive and 33/2264 anti-HCV-positive; 60 and over: 121/3090 HBsAg-positive and 65/3090 anti-HCV-positive.

In the analysis of risk factors for HBsAg positivity, several demographic, clinical, and familial variables showed significant associations (see Table 1). Men were more frequently positive than women, and positive individuals were older on average, indicating that hepatitis B infection was more commonly identified in older adults. Urban residence was also associated with a higher prevalence compared with rural areas. Patients tested at the recommendation of a physician demonstrated a higher likelihood of positivity. A significant association was found with neuropsychiatric disorders. However, diabetes and cardiovascular diseases were not associated with HBsAg positivity, while elevated transaminases and low platelet counts showed only nonsignificant trends toward higher prevalence. Cancer history did not influence the likelihood of positivity. Vaccination against HBV emerged as a clear protective factor, reducing the risk of infection. Family history, particularly the presence of hepatitis in relatives or parents, was one of the strongest predictors of HBsAg positivity. These findings are most likely attributable to confounding or selection biases and should be interpreted cautiously. Dialysis, imprisonment, HIV infection, and a history of sexually transmitted infections showed nonsignificant but noteworthy trends toward increased prevalence. In contrast, transfusions, surgical interventions, tattoos or piercings, multiple sexual partners, and intravenous drug use were not significantly associated with HBsAg positivity. Overall, the most consistent determinants of HBsAg positivity were male sex, older age, urban residence, physician-indicated testing, neuropsychiatric

comorbidity, and especially family history of hepatitis, while HBV vaccination remained a protective factor.

Table 1. Univariable analysis of risk factors associated with HBsAg positivity.

Factor Analyzed	HBsAg Prevalence (with Factor)	HBsAg Prevalence (Without Factor)	OR (95% CI)	<i>p</i> -Value
Age (Years)	Mean 55.9 vs. 52.5	–	–	<0.001
Sex (Male)	3.6%	2.5%	1.44 (1.11–1.87)	0.004
Urban area	2.6%	1.6%	1.65 (1.13–2.41)	<0.001
Ethnicity (Romanians)	2.9%	1.3%	1.93 (0.61–6.07)	0.36
Physician-indicated testing	3.9%	2.9%	1.33 (1.01–1.75)	0.006
Diabetes	2.6%	2.9%	0.92 (0.59–1.44)	0.81
Neuro/psychiatric disorders	4.2%	2.8%	1.53 (1.02–2.30)	0.04
Cardiovascular disease	2.6%	3.0%	0.89 (0.66–1.20)	0.48
Elevated transaminases	3.9%	2.8%	1.42 (0.94–2.13)	0.11
Low platelets	3.6%	2.9%	1.27 (0.62–2.60)	0.65
Cancer history	3.0%	2.9%	1.03 (0.65–1.62)	0.91
HBV vaccination	1.3%	3.0%	0.42 (0.19–0.92)	0.03
Family history of hepatitis (HBV/HCV)	6.3%	2.7%	2.47 (1.71–3.57)	<0.001
Parental HBV/HCV hepatitis	5.1%	2.8%	1.85 (1.05–3.26)	0.03
Blood-risk profession	1.5%	3.0%	0.48 (0.28–0.82)	0.007
Transfusions	2.1%	2.9%	0.72 (0.39–1.33)	0.36
Dialysis/CKD	7.9%	2.9%	2.91 (0.89–9.51)	0.09
Past surgery	3.0%	2.5%	1.18 (0.88–1.59)	0.29
Tattoos/Piercings	2.1%	2.9%	0.72 (0.42–1.24)	0.28
Past imprisonment	6.9%	2.9%	2.47 (0.99–6.20)	0.059
Multiple sexual partners	2.6%	2.9%	0.88 (0.49–1.59)	0.78
IV/IN drug use	2.1%	2.9%	0.71 (0.10–5.20)	0.98
STI history	3.3%	2.9%	1.14 (0.36–3.61)	0.75
Abortions (women)	2.8%	2.9%	0.97 (0.45–2.07)	0.99
Known HIV	7.7%	2.9%	2.81 (0.36–21.7)	0.32
Frequent hospitalizations (≥ 2 /year)	0.9%	3.0%	0.29 (0.09–0.93)	0.04
Immigrant	0.8%	3.0%	0.28 (0.04–2.00)	0.04
Orphanage/institutional care	9.4%	2.9%	3.54 (1.05–11.9)	0.004
Alcohol use (≥ 14 drinks/week men; ≥ 7 women)	1.1%	3.1%	0.34 (0.16–0.75)	0.04

Among individuals who tested positive for HBsAg, 12.5% had undetectable HBV DNA, 70.4% had detectable but low levels (<2000 IU/mL), and 17.1% exhibited high viral loads (>2000 IU/mL). Within this HBsAg-positive group, 11.5% also presented protective anti-HBs titers (>10 IU/mL), 2.6% were HBeAg positive, and 90.1% were positive for anti-HBe antibodies. Overall, self-reported HBV vaccination rates in the entire study cohort were low (5.9%) but showed significant variation by age group ($p < 0.0001$). The highest

vaccination rates were observed in younger adults: 7.0% in those aged 18–29 and 10.2% in the 30–39 age group. In comparison, the rates declined with increasing age: 7.7% in the 40–49 group, 6.4% in the 50–59 group, and only 2.6% among those aged ≥ 60 years.

In the overall HBsAg-positive cohort, 2.3% had detectable anti-HDV antibodies. All anti-HDV-positive individuals were confirmed to have detectable HDV RNA, with a median viral load of 188,000 IU/mL (range: 1250–680,000 IU/mL).

Table 2 shows the risk factors associated with the presence of anti-HCV antibodies. The prevalence of HCV antibodies was slightly higher in urban areas compared with rural areas (1.35% vs. 1.02%), but the association was not statistically significant. HCV antibody positivity in this screening cohort was predominantly detected in older individuals. No significant difference between sexes was observed. Patients tested upon physician recommendation also showed a markedly higher prevalence, consistent with targeted testing in individuals with clinical suspicion. Elevated transaminase levels and thrombocytopenia were both significant predictors. Cardiovascular comorbidities were also linked to higher prevalence, possibly indicating overlapping risk factors or increased healthcare exposure. Transfusions and previous surgical interventions emerged as important determinants, consistent with recognized iatrogenic transmission routes. In addition, HIV co-infection was associated with anti-HCV positivity. Other factors demonstrated only nonsignificant trends. Dialysis, neuropsychiatric disorders, intravenous or intranasal drug use, and a history of abortions suggested higher prevalence but did not reach statistical significance, likely due to small subgroup sizes. Conversely, variables typically considered high risk, such as tattoos or piercings, multiple sexual partners, or prior sexually transmitted infections, did not show significant associations in this dataset. Detection of hepatitis in the family and parental infection also did not emerge as a significant risk factor, in contrast to hepatitis B. Vaccination against HBV appeared to be associated with a lower risk for HCV infection. Notably, alcohol consumption was not associated with increased prevalence of HCV antibodies. Overall, the typical HCV-antibody-positive patient in this cohort was an older adult, often with biochemical or hematologic abnormalities, cardiovascular disease, or a history of transfusion or surgical procedures, with HIV co-infection as an additional marker of elevated risk.

Table 2. Univariable analysis of risk factors associated with anti-HCV positivity.

Factor Analyzed	Anti-HCV Prevalence (with Factor)	Anti-HCV Prevalence HCV (Without Factor)	OR (95% CI)	<i>p</i> -Value
Age	Mean 63.8 vs. 52.4 years	–	–	<0.001
Sex (Male)	1.24%	1.35%	0.92 (0.62–1.36)	0.68
Urban area	1.35%	1.02%	1.33 (0.69–2.55)	0.39
Ethnicity (Romanians)	1.34%	0.7%	1.96 (0.63–6.15)	0.43
Physician-indicated testing	1.05%	1.40%	0.74 (0.46–1.21)	0.006
Diabetes	1.7%	1.3%	1.35 (0.76–2.41)	0.57
Neuro/psychiatric disorders	2.0%	1.3%	1.62 (0.91–2.90)	0.08
Cardiovascular disease	2.0%	1.1%	1.76 (1.20–2.57)	0.01
Elevated transaminases	2.9%	1.2%	2.50 (1.54–4.07)	<0.001
Low platelets	2.7%	1.3%	2.16 (0.94–4.96)	0.04
Cancer history	1.4%	1.3%	1.08 (0.56–2.08)	0.87
HBV vaccination	0.9%	1.3%	0.69 (0.28–1.69)	0.71

Table 2. Cont.

Factor Analyzed	Anti-HCV Prevalence (with Factor)	Anti-HCV Prevalence HCV (Without Factor)	OR (95% CI)	p-Value
Family history of hepatitis (HBV/HCV)	1.8%	1.3%	1.41 (0.73–2.71)	0.45
Parental HBV/HCV hepatitis	2.0%	1.3%	1.53 (0.60–3.88)	0.65
Blood-risk profession	1.1%	1.3%	0.78 (0.41–1.49)	0.68
Transfusions	4.3%	1.1%	3.89 (2.43–6.23)	<0.001
Dialysis/CKD	2.6%	1.3%	2.04 (0.28–15.01)	0.41
Previous surgery	1.5%	0.7%	2.22 (1.31–3.77)	0.026
Tattoos/Piercings	0.8%	1.3%	0.56 (0.23–1.37)	0.77
Past imprisonment	1.4%	1.3%	1.05 (0.14–7.58)	0.87
IV/IN drug use	2.1%	1.3%	1.61 (0.22–11.74)	0.35
Multiple sexual partners	0.6%	1.3%	0.47 (0.15–1.49)	0.45
STI history	1.1%	1.3%	0.83 (0.11–5.97)	0.33
HIV	7.7%	1.3%	6.31 (0.81–48.9)	0.045
Abortions (women)	2.4%	1.3%	1.89 (0.83–4.35)	0.05
Immigrant	1.7%	1.3%	1.29 (0.31–5.28)	0.32
Orphanage/institutional care	0%	1.3%	0.00 (0.00–1.8)	0.27
Frequent hospitalizations (≥ 2 /year)	1.7%	1.3%	1.33 (0.58–3.04)	0.28
Alcohol use (≥ 14 drinks/week men; ≥ 7 women)	0.7%	1.3%	0.54 (0.07–3.87)	0.22

Among 120 individuals with anti-HCV positivity tested for HCV RNA, only 20.2% had detectable viremia. The mean viral load among viremic cases was 4.5×10^5 IU/mL (range 0– 1.21×10^7 IU/mL).

4. Discussion

In this nationwide, risk-based screening initiative conducted through two private laboratory networks, we found intermediate prevalences of HBsAg (2.9%) and anti-HCV (1.3%) among predominantly urban, middle-aged adults, with a clear age gradient for both infections. Among HBsAg-positive individuals, most had either undetectable or low HBV DNA (<2000 IU/mL) and predominantly HBeAg-negative infection. Anti-HDV seropositivity was low (2.3%) but all replicative. Only a minority of anti-HCV-positive participants showed an active viremic HCV infection (20.2%). Collectively, these findings suggest that the population currently accessing private testing in Romania includes a reservoir of mostly inactive or low-replicative HBV, alongside a high proportion of anti-HCV-positive individuals with either spontaneously resolved infection or HCV RNA levels below the assay's limit of detection, both well-recognized explanations for seropositivity in the absence of detectable viremia. Compared to other national datasets, our HBsAg prevalence (2.9%) is higher than the community-based, vulnerability-focused LIVE(RO)2 screening (overall 1.67% across ~320,000 people), and it is higher in urban than rural settings (1.79% vs. 1.62%), which is consistent with our urban-skewed sample and clinician-directed testing that enriches for risk (and disease) (LIVE(RO)2). Our anti-HDV rate among HBsAg-positive individuals (2.3%) is lower than the 4.87% anti-HDV prevalence reported in the vulnerable HBV cohort of LIVE(RO)2 (with 75.6% RNA positivity among anti-HDV-positive) and far below estimates from tertiary clinics in Romania [9,10,16]. This suggests

that our study population represents different risk strata compared to community programs and referral centers. While the differences between testing contexts (community, tertiary centers, and private labs) have been discussed throughout, we now more clearly emphasize that the observed variation in prevalence reflects not only epidemiological distribution but also who becomes tested, under what circumstances, and with what clinical intent. This difference can be partly explained by access to treatment and the implementation of various screening programs in recent years, which may have influenced the profiles of the populations undergoing testing in different settings. Other large Romanian laboratory datasets [17] also report higher HDV co-infection proportions among HBsAg-positive patients (11.3% HDV-Ab across 2018–2023), again highlighting that population, access, and referral patterns shape observed co-infection rates. Our comparatively low HDV detection likely reflects a more general screening of individuals in private labs (older $\approx$ 50 years, insured, urban), whereas LIVE(RO)2 targeted socially disadvantaged groups with higher HBV/HDV burden and more advanced liver disease. For HCV, the modest anti-HCV prevalence (1.3%) together with the low prevalence of viremic patients (20.2%) mirrors the declining HCV epidemic and treatment scale-up in the region. Romanian laboratory surveillance shows a falling HBV/HCV co-infection signal since 2020, consistent with broad direct-acting antivirals uptake and improved prevention [18–21].

Male sex, older age, urban residence, physician-indicated testing, neuropsychiatric comorbidity, and family clustering (hepatitis in relatives/parents) were the most consistent correlates of HBsAg positivity, aligning with LIVE(RO)2 patterns that emphasize sociodemographic gradients and intrafamilial transmission. The seemingly “protective” associations observed for certain healthcare-related exposures, such as blood-risk professions or frequent hospitalizations, are best explained by selection bias. Since individuals with a known history of HBV or HCV infection were excluded, those from higher-risk groups who had already been diagnosed through routine screening were not represented in the analysis. Similarly, although individuals with a known history of HCV were excluded, unrecognized, or unreported prior successful direct-acting antiviral therapy cannot be fully ruled out as an explanation for some anti-HCV-positive/HCV-RNA negative results. As a result, only undiagnosed or recently infected individuals remained, leading to artificially lower prevalence in these categories. In addition, people in blood-risk professions are more likely to undergo regular testing and to have been vaccinated against HBV. In Romania, universal newborn HBV vaccination began in 1995 [22], but adult vaccination is limited to specific groups and often lacks documentation. This may explain why some participants reporting vaccination were still HBsAg positive, likely due to incomplete immunization or unverified protection. Nonetheless, the strong protective association observed reinforces the need to maintain and expand HBV vaccination coverage, as emphasized in national prevention strategies [23–26].

Our private laboratory-based model efficiently reached a large number of adults already engaged with the healthcare system. Because a significant proportion of tests were performed at the physician’s request, the screened population was enriched with older individuals and those with comorbidities, resulting in higher positivity rates compared to the general population. This approach complements community-based micro-elimination strategies, such as the LIVE(RO)2 program, which focuses on socially vulnerable and underserved groups, revealing a substantial burden of HDV co-infection and advanced liver fibrosis, particularly in disadvantaged regions. Although not included in the present analysis, hospital-based initiatives like the HepC ALERT study [13], conducted in over 20 Romanian institutions and enrolling more than 25,000 hospitalized or ambulatory adults, also contribute to mapping national hepatitis prevalence across different care settings. These programs reported comparable anti-HCV positivity rates ($\sim$ 1.39%) and underscore the

added value of structured, clinician-initiated testing within hospitals. Taken together, these complementary efforts support a mixed-model screening strategy in Romania: sustained outreach and linkage to care for high-risk vulnerable populations, opportunistic testing in private laboratories targeting insured and urban individuals, and hospital-based screening pathways capable of identifying new cases early and ensuring referral to specialized care.

The distribution of HBV DNA levels, predominantly undetectable or below 2000 IU/mL, combined with the very low rate of HBeAg positivity, suggests that a large proportion of individuals are in the HBeAg-negative inactive carrier phase. This highlights the need for careful risk stratification and ongoing longitudinal follow-up. Nevertheless, the proportion of patients with viremia > 2000 IU/mL is clinically relevant and warrants timely evaluation for treatment eligibility. Given the substantial clinical impact of HDV co-infection and the LIVE(RO)2 evidence of meaningful HDV prevalence in vulnerable HBsAg-positive persons, routine anti-HDV testing for all HBsAg-positive individuals, with reflex HDV RNA where positive, should be standard, coupled with access to novel agents such as bulevirtide.

Strengths of our study include a large, real-world cohort with standardized serologic workflows and reflex testing. Key limitations of this study include the predominantly urban population, clinician-directed testing bias, and limited behavioral data that restrict causal inference regarding some risk factors. The generalizability of our findings may also be influenced by selection bias, as the study population consisted mainly of adults accessing private laboratory services, individuals who are typically insured, employed, and more engaged in preventive healthcare. This group differs from populations targeted by public screening campaigns, which focus on vulnerable and underserved communities. Although data on income and education level were not available, the setting implies a degree of financial and healthcare system access. The lack of information on test refusal rates is also noted as a limitation, though it reflects the real-world nature of the laboratory workflow, where data were anonymized, and the testing occurred as part of routine diagnostics without formal enrollment procedures.

These conclusions direct our focus toward the rural population of Romania, which still comprises a substantial share of the population (about 45%) [27] and which faces well-documented barriers, including limited access to testing, lower health literacy, deficient awareness, and education. In addition, vulnerable persons with restricted access to healthcare (due to insurance status, poverty, or social marginalization) deserve special attention. Since these rural and vulnerable subgroups in LIVE(RO)2 did not show elevated prevalence compared to urban or tertiary-clinic cohorts, it would be useful to explore regional epidemiological pockets (micro-hotspots) as focal points for targeted detection and elimination strategies.

5. Conclusions

In Romania, the epidemiology of HBV and HCV varies across healthcare settings. Our findings from private laboratory-based screening highlight a substantial reservoir of undiagnosed HBV infection, predominantly in inactive or low-replicative phases, as well as a declining pool of viremic HCV cases, particularly among older, health-engaged adults. In contrast, community-based programs have identified higher burdens of HDV co-infection and advanced liver disease among vulnerable and underserved populations. Hospital-based screening initiatives further contribute by capturing cases within structured clinical workflows. A coordinated, mixed-model screening approach, integrating community outreach, opportunistic testing in private settings, and systematic in-hospital programs, can effectively reach diverse at-risk subgroups. This strategy not only advances national HBV

and HCV elimination goals but also ensures HDV co-infection is not overlooked among HBsAg-positive individuals.

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Abbreviations

The following abbreviations are used in this manuscript:

HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis D virus
HCC	hepatocellular carcinoma
WHO	World Health Organization
HIV	Human Immunodeficiency Virus
HBsAg	hepatitis B surface antigen
Anti-HCV	anti-HCV antibodies
PCR	polymerase chain reaction
ORs	odds ratios
CI	confidence intervals
ED	emergency-department

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