In HDV sub-genotypes 1, the high degree of genetic variability can drive the selection of divergent genetic pathways modulating HDV replicative potential and cytolytic activity

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Introduction

- Both Hepatitis Delta Antigen isoforms (HDAg) are characterized by different functional domains interspersed by inter-domains (ID):
  - **RNA-Binding Domains (RBD):** Bind HDV RNA, crucial for replication.
  - **Coiled-Coil Sequence (CCS):** Supports protein-protein interactions and HDAg oligomerization.
  - **Nuclear Localization Sequence (NLS):** Directs HDAg to the nucleus for replication.
  - **Virus Assembly Signal (VAS):** Enables genome packaging and interaction with HBV envelope proteins.

- 18 Cytotoxic T lymphocytes (CTLs) domains are present on HDAg (*Kohsar et al. 2021, JHEP Reports)* and they stimulate host CD4+ and CD8+ cells. CD8+ T cells recognize infected hepatocytes via epitopes, leading to the destruction of infected cells and limiting viral replication. In chronic Infection, persistent HDV infection results in immune escape mutations in T cell epitopes.

- HDV genome is characterized by an auto-catalytic activity thanks to a 85 bp region of RNA named ribozyme characterized by four double-stranded domains (P1, P1.1, P2, P3, and P4), three single-stranded regions (J1/2, J1.1/4, and J4/2) and 2 loop regions (L3 and L4).

So far, paucity of information is available on the extent of HDV genetic diversification in HDAg domains and Cytotoxic T lymphocytes (CTL) epitopes across the different sub-genotypes 1 and their correlation with virological and biochemical parameters.

Aim

The aim of the study is to evaluate:

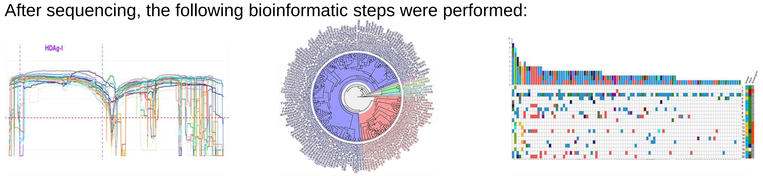
- the genetic diversity of HDV within sub-genotype 1 by assessing nucleotide and amino acid conservation in key regions of HDAg and the ribozyme,
- the conservation of CTL epitopes
- The correlation of genetic variability with biochemical markers such as ALT levels.

Method

We included **103** individuals with an available serum sample, from different Italian centers (Milan, Turin, Padua and Rome).
HDV full length sequences were obtained through the following steps:

1 RNA Extraction | 2 2 Step-PCR Amplification | 3 NGS Sequencing

After sequencing, the following bioinformatic steps were performed:



- NGS reads (median [IQR] reads/seq: 62045[30460-91899].
- Fastq cleaning
- Fasta preparation
- Mutations reports

4 NGS Data Analysis

- MAFFT alignment
- Iqtree for phylogenetic tree
- Maximum likelihood tree
- 1000 bootstraps
- Figtree for visualization

5 Phylogenetic Analysis

- Removal of 100% Prevalent Mutation
- 50% cutoff for the selected mutations
- Stratifying mutations based on sub-genotypes
- 95% cutoff to select conserved regions

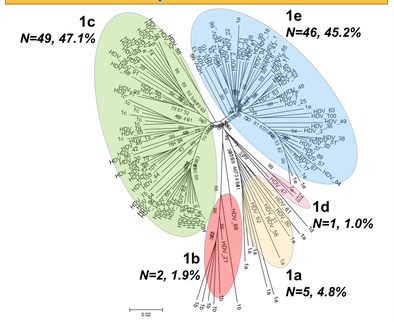
6 Mutational Analysis

Results

Characteristics of the study population

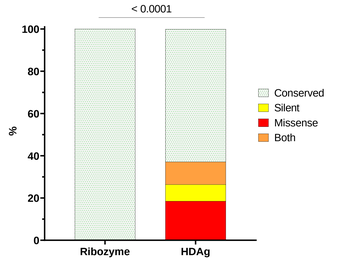
	N = 103
Age, *N (%)*	54 (44-60)
Male, *N (%)*	60 (61.9)
Nationality, *N (%)*	
Italian	42 (40.7)
East-European	49 (47.6)
African	4 (4.2)
Unknown	8 (7.7)
HBsAg (IU/mL), *median (IQR)*	8399 (3473-15000)
HDV-RNA (log IU/ml), *median (IQR)*	5.6 (4.9-6.2)
HBV-DNA <10/TND, *N (%)*	47 (45.6)
HBV-DNA ≥10, *N (%)*	38 (36.9)
HBV-DNA (IU/ml), *median (IQR)*	20 (0-84.5)
ALT, *median (IQR)*	94 (65-152)

Sub-genotype 1c and 1e are the most prevalent



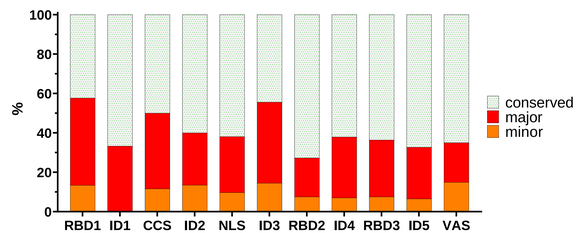
The HDV subgenotypes were attributed by phylogenetic tree constructed by Neighbor-Joining method according to bootstrap > 60%. Each color represent a different sub-genotype. The reference sequences for each subgenotype were retrieved from Karimzadeh et al. 2019. Branching order's reliability was assessed by bootstrap analysis of 1000 replicates.

The ribozyme region is more conserved compared to HDAg



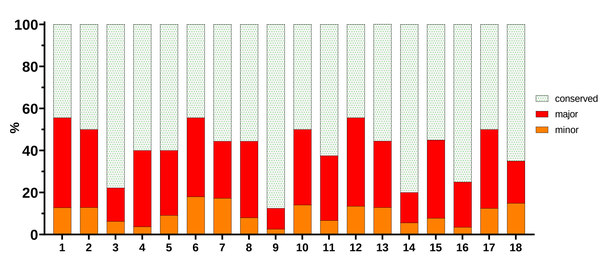
The graph reports the percentage of mutations in the region of ribozyme compared to the HDAg. Statistically significant differences have been calculated by Chi-squared test.

In HDAg mutation rate ranges from 27.3% in ID1 to 57.7% in ID3



The graph reports for each HDAg functional domain the rate of conserved (dotted green) and mutated (red and orange) aa positions. Red color indicates the rate of mutations with an intra-patient frequency ≥20% (major), while the orange of those with an intra-patient frequency <20% (minor). No significant differences have been highlighted.

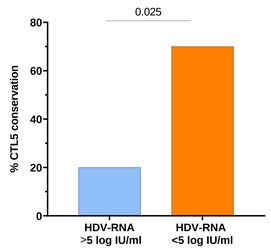
CTLs showed overall mutation rate <60%



The graph reports for each CTL domain the rate of conserved (dotted green) and mutated (red and orange) aa positions. Red color indicates the rate of mutations with an intra-patient frequency ≥20% (major), while the orange of those with an intra-patient frequency <20% (minor). No significant differences have been highlighted.

By comparing sub-genotypes 1c and 1e:
- **No differences in terms of mutational rates have been observed in the HDAg domains apart from ID5 (50% for 1c vs 20% for 1e, P=0.058).**
- **Regarding CTL domains, no differences have been observed apart from CTL 18 (20% for 1c vs 45.0% for 1e, P=0.09).**

Individuals with HDV-RNA >5 log IU/ml showed a lower conservation in CTL5 respect to lower viremic ones



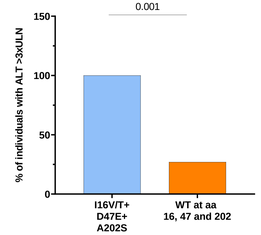
The graph reports the percentage of conserved aa positions of CTL5 in the groups stratified according to HDV-RNA levels higher than (light blue) or lower than (orange) 5 log IU/ml. Statistically significant differences have been calculated by Chi-squared test.

Sub-genotype 1c and 1e are characterized by specific divergent mutational pathways

Mut	1c (N=49)	1e (N=46)	HDAg domain	CTL domain	P-value
I16V/T	34 (69.4)	1 (2.0)	RBD1	-	<0.001
N22S	14 (28.6)	0 (0.0)	RBD1	-	<0.001
D47E	21 (42.9)	0 (0.0)	CCS	1, 13	<0.001
R112K	22 (44.9)	0 (0.0)	ID4	8, 11, 15	<0.001
T180A	15 (30.6)	0 (0.0)	ID5	-	<0.001
A202S	19 (38.8)	2 (4.1)	VAS	7, 18	<0.001
D29E	0 (0.0)	13 (26.5)	RBD1	12	<0.001
D46E	0 (0.0)	20 (40.8)	CCS	1, 13	<0.001
K113R	0 (0.0)	13 (26.5)	ID4	11, 15	<0.001
R131K	0 (0.0)	16 (32.7)	ID4	15, 16	<0.001
M171L	1 (2.0)	11 (22.4)	ID5	5, 15	<0.001
I188V	0 (0.0)	10 (20.4)	ID5	17	<0.001

The table reports the percentage of the different mutations in the groups of individuals with HDV subgenotype 1c (green) compared to those with genotype 1e (light blue). Statistically significant differences have been calculated by Chi-squared test.

Notably, co-presence of I16V, D47E, and A202S in 1c showed significantly increased levels of ALT levels >3ULN



The graph reports the percentage of individuals with ALT levels 3 times upper than normal limit (3xULN) in the groups of individuals with the HDAg mutations I16V/T+D47E+A202S (light blue) compared to those without this combination (orange). Statistically significant differences have been calculated by Chi-squared test.

Conclusions

- Sub-genotypes 1 are characterized by a conspicuous degree of genetic diversification that has led to the selection of divergent genetic signatures.
- The enrichment of mutations in HDAg functional domains and CTL epitopes could hamper HDV recognition by immune response and in turn enhance the viral replication.
- The co-presence of specific mutations (I16V/T, D47E, and A202S) in HDV sub-genotype 1c are strongly associated with elevated ALT levels (>3× ULN), suggesting that these mutations may enhance viral replication and contribute to increased liver inflammation, highlighting the need for targeted therapeutic and diagnostic approaches
- Overall, the role of the high degree of genetic variability in affecting the proper HDV detection by the currently available diagnostic assays deserves further investigation.

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