Original research

# Clinical, histological and molecular profiling of different stages of alcohol-related liver disease

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## ABSTRACT

**Objective** Alcohol-related liver disease (ALD) ranges from never-decompensated ALD (ndALD) to the life-threatening decompensated phenotype, known as alcohol-related hepatitis (AH). A multidimensional study of the clinical, histological and molecular features of these subtypes is lacking.

**Design** Two large cohorts of patients were recruited in an international, observational multicentre study: a retrospective cohort of patients with ndALD (n=110) and a prospective cohort of patients with AH (n=225). Clinical, analytical, immunohistochemistry and hepatic RNA microarray analysis of both disease phenotypes were performed.

**Results** Age and mean alcohol intake were similar in both groups. AH patients had greater aspartate amino transferase/alanine amino transferase ratio and lower gamma-glutamyl transferase levels than in ndALD patients. Patients with AH demonstrated profound liver failure and increased mortality. One-year mortality was 10% in ndALD and 50% in AH. Histologically, steatosis grade, ballooning and pericellular fibrosis were similar in both groups, while advanced fibrosis, Mallory-Denk bodies, bilirubinostasis, severe neutrophil infiltration and ductular reaction were more frequent among AH patients. Transcriptome analysis revealed a profound gene dysregulation within both phenotypes when compare to controls. While ndALD was characterised by deregulated expression of genes involved in matrisome and immune response, the development of AH resulted in a marked deregulation of genes involved in hepatocyte reprogramming and bile acid metabolism.

**Conclusions** Despite comparable alcohol intake, AH patients presented with worse liver function compared with ndALD patients. Bilirubinostasis, severe fibrosis and ductular reaction were prominent features of AH. AH patients exhibited a more profound deregulation of gene expression compared with ndALD patients.

## Significance of this study

**What is already known on this subject?**

- Most forms of alcohol-related liver disease (ALD) are clinically silent, making early diagnosis challenging.
- Patients at any stage of ALD are at risk of presenting a decompensated alcohol-related hepatitis (AH) episode, a severe condition characterised by liver-related complications along with markedly dysregulated hepatic gene expression.
- Accumulation of hepatic progenitor cells has been associated with liver dysfunction and poor prognosis in AH.

## INTRODUCTION

Alcohol-related liver disease (ALD) is the leading cause of liver-related mortality worldwide.[1] Most patients are identified at late stages, when mortality is high despite ethanol cessation.[2 3] The paucity of studies and clinical programmes focused on early

## Significance of this study

**What are the new findings?**

- Daily alcohol consumption is similar in patients with never-decompensated ALD (ndALD) with preserved liver function and AH, so factors other than the daily amount of alcohol contribute to the development of severe forms of ALD.
- In patients with ndALD, ductular reaction is more prominent in those with advance fibrosis.
- ndALD is characterised by alteration of the expression of genes involved in the immune response and fibrogenesis, while the development of AH results in severe deregulation of genes involved in hepatocyte cell fate and bile acid metabolism. It is important to differentiate these two clinical entities molecularly in order to improve the quality of translational research in ALD and to design pathogenesis-based clinical trials.

**How might it impact on clinical practice in the foreseeable future?**

- This study might contribute to increased awareness of the analytical and histological features of early compensated forms of ALD.
- The molecular characterisation of ndALD could favour the development of novel pathophysiological-based targeted therapies.

stages of ALD is due to the lack of well characterised clinical, histological and molecular phenotypes.[3] Within the ALD spectrum, two of the main phenotypes include alcohol-related hepatitis (AH) and a phenotype that encompasses patients with never-decompensated ALD (ndALD) that can show different stages of fibrosis.[2] While AH is characterised by an acute worsening of liver function and clinical decompensations, ndALD is clinically silent.[4]

Although it has been assumed that ndALD is the harbinger of transition to severe AH, there are few studies that have assessed clinical and histological parameters of patients with this early phenotype.[2 5–8] In contrast, much more research attention has been paid to the severe form of ALD that is clinically manifested as AH,[9–12] which is one of the most common precipitant factors of acute-on-chronic liver failure (ACLF).[13]

While the transcriptome of AH patients has been described,[11 14 15] few studies have analysed the transcriptome of patients with ndALD.[16] Therefore, we hypothesised that the clinical, histological and molecular characterisation of ndALD and AH would elucidate critical biomarkers and pathways of disease progression that could then facilitate early diagnosis and identify novel drug targets. To test this hypothesis, we used two well-characterised cohorts of patients: one with definite (ie, biopsy-proven) AH and second, a cohort of patients with ndALD diagnosed clinically and histologically.

## METHODS

### Clinical, analytical assessment

Participating centres entered demographic, clinical, and histological data from trial patients (online supplemental table S1) into a comprehensive web-based de-identified database (design and keep by the biostatistics and clinical research core, Centre for Gastrointestinal Biology and Disease from University of North Carolina at Chapel Hill School of Medicine (https://cryoweb-prod.med.unc.edu/clash/login.php). Only patients whose clinical course and outcome data (eg, alive/death status and date of death) were available were included in the study. Other inclusion and exclusion criteria are reported below.

### Patient cohorts

The study group of patients with definite AH was obtained from two sources: The Liver Unit (Hospital Clinic, Barcelona, Spain) (Cohort A, n=121) and the InTeam Consortium observational study (ClinicalTrials.gov NCT0207591) (Cohort B n=104). The inclusion criteria for the InTeam study can be found in the online supplemental methods. The inclusion and exclusion criteria for the Barcelona cohort have been extensively described elsewhere.[9 17] In both cohorts, the histological diagnosis of AH was established by the presence of inflammatory infiltration and hepatocellular damage (ballooning and/or Mallory-Denk bodies (MDB) in the background of steatosis). Characteristics from both cohorts can be found in online supplemental tables S2 and S3.

The cohort of patients with biopsy-proven ndALD was obtained from nine centres in the US and Europe (n=110). All fulfilled the clinical criteria described in the online supplemental material. Histological diagnosis was established by the presence of inflammatory infiltration and hepatocellular damage (ballooning and/or MDB) in the background of steatosis. Finally, each participating centre obtained approval from their institutional ethics committee, (online supplemental figure S1).

### Histological analysis and keratin 7 staining quantification

Liver sections stained with H&E and Masson trichrome were analysed by specialised liver pathologists from all participating centres. To ensure reliability all pathologists received training material with index images before histological assessment, as described previously.[9] We next performed a concordance study for ndALD patients with eight fully scored cases (central pathologist-RM). Reliability between the central pathologist and satellite liver pathologists was reasonable with all the parameters above kappa coefficients of 0.5; only three parameters (grade of steatosis; polymorphonuclear cells (PMN) infiltration and presents of megamitochondria) showed kappa coefficient's categorised as discrete agreement (online supplemental table 4). Pathologists considered up to 10 morphological characteristics per sample (online supplemental table S1). Finally, progenitor cell proliferation was quantified as previously described.[18] Five paraffin-embedded liver sections from patients with AH, 3 from patients with ndALD and bridging fibrosis or cirrhosis and four from patients with ndALD without bridging fibrosis or cirrhosis were used for the cytokeratin 7 (KT7) assessment. Immunohistochemistry was semi-quantitatively assessed by M.EH using as previously described methodology[19 20] (online supplemental methods).

### Microarray studies

Hepatic transcriptome of 15 patients with AH and 8 patients with ndALD was analysed. Clinical and histological characteristics as well as criteria for patient selection are depicted in online supplemental tables S5 and S6. A comparative analysis between the subset of patients with AH used for microarray studies and the whole cohort is depicted in online supplemental table S7. Patients with malignancies or any other potential cause of liver disease were excluded. Patients with AH were previously characterised and published elsewhere.[11 14 21] Additionally, we included non-neoplasic liver tissue from control patients obtained from optimal cadaveric liver donors (n=3) or at the time of metastatectomy (n=4).[11 14 21] Fully compensated patients with ndALD

were obtained from Saint-Luc Academic Hospital (Brussels, Belgium) and Salem Medical Centre (Heidelberg, Germany) using the inclusion and exclusion criteria described above. The Ethics Committee of all the centres approved the protocol and only patients with signed informed consents were included.

### Bioinformatics and statistical analysis

Microarray studies were performed using Human Genome U133A plus 2.0 GeneChip (Affymetrix Hgu133plus, Santa Clara, CA, USA). Functional analysis was conducted by Gene Set Enrichment Analysis (GSEA) using ranked lists. This type of analysis is suitable for uncovering the biological functions that are behind a specific transcriptomic gene signature. The Molecular Signatures Database (MSigDB) is an up-to-date repository of gene sets that includes a complete collection of signatures from thousands of published microarray and sequencing studies. In our analyses, we included the 'Curated' gene sets (MSigDB C2), which includes gene sets from the BIOCARTA, Kyoto Encyclopaedia of Genes and Genomes (KEGG) and REACTOME pathway databases and the 'Hallmark' gene sets (MSigDB H). For visualisation of Hallmark gene set enrichment, bar graphs with normalised enrichment score (NES) were generated. In order to visualise the overlap between MSigDB-C2 gene sets found to be significantly enriched in the comparison between ASH and AH, we used Enrichment Map plug-in within Cytoscape environment (Cytoscape v3.6.1).[22 23] The Enrichment Map uses GSEA data and computes the existing overlap -similarity index and its significance- between the gene sets enriched in a particular condition by using hypergeometric statistics. Also, it allows an interpretable visualisation of the several overlaps (termed 'Edges') between gene sets (termed 'Nodes'). Only nodes with p value below 0.1 and edges with similarity above 0. 4 are presented. Heatmaps of top different expressed (DE) genes ($p<0.001$) within different enriched gene sets were generated using function *heatmap.2* of the *gplots* R-package, where genes were grouped based on the Euclidean distance of their expression and presented as a dendrogram. In order to visualise significant gene expression changes in specific KEGG pathways, the function pathview of the gauge R-package was used.[24] Results are presented as frequencies and percentages for categorical variables, means and SD for normal continuous variables and median, and percentile 25 and 75 for continuous variables without a normal distribution. For the microarray studies differential expression was assessed by using linear models and empirical Bayes moderated t-statistics. The Linear Models for Microarray Analysis (*limma*) R-package was used for the analysis of gene expression microarray data.[25] Group comparisons and determinations of false discovery rate (computation using Benjamini-Hochberg procedure)[26] were performed. Finally, signatures for immune infiltration were obtained from previous work,[27] for the calculation of Z score using Gene Set Variation Analysis. Signatures of TNF and IFN-related genes were obtained from HALLMARK collection of the MSigDB. Specifics for statistics analysis for clinical, histological and survival analysis are explained in online supplemental material.

## RESULTS

### Comparative clinical and analytical characteristics of patients with ndALD and Ah

We first compared the clinical and laboratory characteristics of patients with definite AH to a cohort of patients with ndALD (table 1). Patients with AH were younger but consumed the same amount of daily alcohol as those with ndALD. Although patients with AH had a higher body mass index (BMI), this was an artefact of the study design, as we excluded patients with BMI >30 from the ndALD cohort to exclude patients with NASH. At admission, patients with AH had lower systolic and diastolic blood pressures compared with those with ndALD. Only white, non-Hispanic populations were well represented across the two cohorts. Black and Asian populations were only present in the AH cohort (2.2% and 0.4%, respectively). As expected, only patients with AH presented with liver-related decompensation at inclusion (68% ascites, 19.1% variceal bleeding, 98.7% jaundice and 13.3% confirmed infection).

Analytically, patients presenting with AH had lower serum values for haemoglobin, platelets, sodium and albumin compared with the ndALD cohort. Conversely, AH patients had higher white blood cell counts and alkaline phosphatase than patients with ndALD. Due to the existence of liver failure in AH, serum bilirubin (11.9 mg/dL vs 1 mg/dL), international normalised ratio (INR) (1.7 vs 1.1) and albumin (2.6 vs 4.3) were profoundly altered in AH and normal in ndALD. AST levels (128 IU/L vs 99 IU/L) and the aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio (2A.7 vs 1.4) were higher in patients with AH compared with ndALD. There were no significant differences between the two groups in creatinine levels. The metabolic status was similar across both groups except for cholesterol levels, which were higher among ndALD patients. Finally, GGT levels, a classical biomarker of active alcohol consumption, was higher among the ndALD group. Interestingly, ndALD patients with different degrees of fibrosis (no or portal fibrosis, expansive fibrosis and bridging fibrosis) showed no anthropometric or clinical differences. Platelets, INR, albumin and cholesterol were lower in patients with bridging fibrosis group compared with the other groups (table 2). In summary, AH patients presented in a state of clinical decompensation with low blood pressure; overall indicating impaired liver function and haemodynamic derangement (table 1 and online supplemental table S8).

### Comparative histological parameters of patients with ndALD versus AH

A histological comparison between both phenotypes was performed next. Within AH samples, the percentage of bridging fibrosis or cirrhosis (81.8%) was double that of ndALD (43.6%), while 1/3 of the patients with ndALD exhibited isolated portal fibrosis or no fibrosis. The grade of steatosis was similar in both phenotypes (figure 1A,B). Both disease phenotypes were associated with high percentages of pericellular fibrosis (76% vs 82.7% AH vs ndALD, respectively) (figure 1C,D). Interestingly, the presence of megamitochondria, a marker of oxidative stress and progression to fibrosis, was slightly higher among ndALD patients. The percentage of severe neutrophil infiltration, the predominant inflammatory cell type in ALD, was higher among AH patients (figure 1E,F). The degree of hepatocyte injury, assessed by the presence of ballooning and MDB's, was also higher among AH patients. Bilirubinostasis (ie, presence of bile pigment as small granules in hepatocytes, plugs in dilated intercellular canaliculi or both) was different between both phenotypes, and was observed in 12% and 68% of patients with ndALD and AH, respectively ($p<0.001$) (figure 1G–I). Of note, severe neutrophil infiltration was not found in patients without fibrosis or with only portal fibrosis (table 2).

Finally, as the presence of a ductular reaction holds prognostic significance,[21] a focused analysis was conducted. Specimens with AH showed marked ductular reaction in some periportal/septal hepatocytes as highlighted by KT7 immunostain, in contrast

**Table 1** Anthropometric and clinical characteristics of patients with AH and ndALD

| Parameters | AH n=225 Median (P25–P75) | ndALD n=110 Median (P25–P75) | P value |
|---|---|---|---|
| Clinical parameters | | | |
| Age (years) | 50 (41–56) | 52 (46–58) | 0.02 |
| Gender (male), n (%) | 142 (63.1) | 81 (73.6) | 0.06 |
| EtOH consumption (g/day) | 100 (72–150) | 100 (69–150) | 0.3 |
| BMI | 25.8 (22.6–31) | 24 (21–27) | <0.001 |
| Race (white), n (%)* | 82.7% | 99.1% | <0.001 |
| Haemodynamic parameters | | | |
| Systolic BP (mm Hg) | 120 (108–133.5) | 128 (119–140) | 0.001 |
| Diastolic BP (mm Hg) | 71 (62–80) | 80 (70–85) | <0.001 |
| Laboratory parameters at baseline | | | |
| Haemoglobin (g/L) | 106 (88–122) | 138 (126–149) | <0.001 |
| Leucocyte count (x$10^9$/L) | 9.4 (6.5–14.1) | 6.6 (5.3–7.9) | <0.001 |
| Platelet count (x$10^9$/L) | 122 (80–173) | 172 (129–227) | <0.001 |
| INR | 1.7 (1.4–2) | 1.1 (1–1.1) | <0.001 |
| AST (IU/L) | 128 (88–186) | 99 (67–144) | <0.001 |
| ALT (IU/L) | 54.9 (34–69) | 65.5 (45.5–92) | <0.001 |
| AST/ALT ratio | 2.6 (2.1–3.5) | 1.5 (1.04–2.1) | <0.001 |
| Serum bilirubin (mg/dL) | 11.9 (6.8–20.7) | 1 (0.6–1.4) | <0.001 |
| Glucose (mg/dL) | 101 (86–127) | 97.6 (86.6–109.6) | 0.08 |
| Serum sodium (mmol/L) | 134 (130–136) | 140 (137–142) | <0.001 |
| Serum albumin (g/dL) | 2.6 (2.3–3) | 4.3 (3.8–4.6) | <0.001 |
| Serum creatinine (mg/dL) | 0.8 (0.6–1.1) | 0.78 (0.6–0.9) | 0.3 |
| Cholesterol (mg/dL) | 126 (86.6–183) | 209 (174-248) | <0.001 |
| Triglycerides (mg/dL) | 117.5 (84.8–173) | 110.5 (88.5–150.2) | 0.7 |
| GGT (IU/L) | 257 (96.8–852) | 419 (186–769) | 0.01 |
| Alkaline phosphatase (IU/L) | 277 (166–439) | 102 (78–162) | <0.001 |
| Severity scores | | | |
| MELD | 22.4 (18.8–26.3) | 6.9 (5–9.9) | <0.001 |
| MELD-Na | 25.7 (21.7–29.5) | 7.2 (4.5–10.3) | <0.001 |
| Histological features at baseline | | | |
| Fibrosis stage | | | |
| No fibrosis or portal fibrosis | 8 (3.6) | 36 (32.7) | <0.001 |
| Expansive periportal fibrosis | 33 (14.7) | 26 (23.6) | |
| Bridging fibrosis or cirrhosis | 184 (81.8) | 48 (43.6) | |
| Steatosis | | | |
| <33% | 76 (33.8) | 42 (38.2) | 0.50 |
| 33%–66% | 69 (30.7) | 32 (29.1) | |
| >66% | 80 (35.6) | 36 (32.7) | |
| Mallory-Denk bodies | | | |
| No or occasional | 23 (10.2) | 38 (34.6) | <0.001 |
| Many | 202 (89.8) | 72 (65.5) | |
| Bilirubinostasis† | | | |
| No | 72 (32) | 94 (87.9) | <0.001 |
| Hepatocellular | 62 (27.6) | 13 (12.1) | |
| Canalicular and/or ductular | 40 (17.8) | 0 | |
| Hepatocellular plus canalicular and/or ductular | 51 (22.7) | 0 | |
| Ballooning | | | |
| No/occasional | 116 (51.6) | 68 (61.8) | 0.03 |
| Many | 109 (48.4) | 42 (38.2) | |
| PMN infiltration | | | |
| None/Mild | 152 (67.6) | 103 (93.6)‡ | <0.001 |
| Severe | 73 (32.4) | 7 (6.4) | |
| Megamitochondria§ | | | |
| Yes | 48 (22) | 41 (37.3) | 0.004 |
| Pericellular fibrosis¶ | | | |
| Yes | 79 (76) | 91 (82.7) | 0.2 |

Per study design, patients with patients with BMI >30 were excluded from ndALD cohort, while we included those patients in the AH cohorts.
*Race was only assessed in AH patients from cohort B and ndALD patients.
†n=332.
‡Forty patients in the ndALD cohort did not have neutrophils.
§n=218.
¶Only patients from cohort B had data on pericellular fibrosis.
AH, alcohol-related hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; EtOH, ethanol; GGT, gamma-glutamyl transferase; INR, international normalised ratio; MELD, model for end-stage liver disease; Na, sodium; ndALD, never-decompensated alcohol-related liver disease; PMN, polymorphonuclear cell; P25-P75, percentile 25–percentile 75.

**Table 2** Anthropometric, clinical and histological characteristics of ndALD patients by the underlying fibrosis degree

| Parameters | No fibrosis or portal fibrosis ndALD n=36 Median (P25–P75) | Expansive periportal fibrosis ndALD n=26 Median (P25–P75) | Bridging fibrosis or cirrhosis ndALD n=48 Median (P25–P75) |
|---|---|---|---|
| Clinical characteristics | | | |
| Age (years) | 50.4 (44.5–56) | 50 (43.8–58.3) | 53 (48–58.8) |
| Gender (male), n (%) | 26 (72.2) | 17 (65.4) | 38 (79.2) |
| EtOH consumption (g/day) | 100 (80–147.5) | 120 (60–152.5) | 100 (72.5–145) |
| BMI | 27.7 (21.9–27) | 23.9 (21.9–27.6) | 24.4 (21–26.8) |
| Haemodynamic parameters | | | |
| Systolic BP (mm Hg)* | 130 (120–147.3) | 120 (111–130) | 130 (120–145) |
| Diastolic BP (mm Hg) | 80 (70–89) | 76 (70–80) | 80 (70–84) |
| Analytical parameters at baseline | | | |
| Haemoglobin (g/dL) | 14 (12.1–15) | 13.7 (12.3–14.9) | 13.7 (12.1–15) |
| Leucocyte count (x$10^9$/L) | 6.0 (5.5–7.5) | 6.7 (5–7.5) | 7.2 (5.2–9.5) |
| Platelet count (x$10^9$/L)* | 183 (138.3–223) | 196.5 (157.5–265) | 157.5 (106.5–276.2) |
| INR* | 1 (1–1.1) | 1 (1–1.1) | 1.1 (1–1.2) |
| AST (IU/L)* | 118 (73.5–181.5) | 121 (74.5–155.8) | 80(57-118) |
| ALT (IU/L)* | 84 (56–128) | 67 (46.5–93) | 51 (32.3–78.5) |
| AST/ALT ratio | 1.3 (0.9–1.9) | 1.5 (1–2) | 1.7 (1.1–2.3) |
| Serum bilirubin (mg/dL) | 0.9 (0.6–1.2) | 0.8 (0.6–1.5) | 1.2 (0.7–1.6) |
| Glucose (mg/dL) | 99(83–109) | 95(81-108) | 98(90-112) |
| Serum sodium (mmol/L) | 140 (137–142) | 141 (136.5–142.5) | 140(138-142) |
| Serum albumin (g/dL)* | 4.5 (4.1–4.7) | 4.1 (3.6–4.5) | 4.2 (3.7–4.5) |
| Serum creatinine (mg/dL) | 0.8 (0.7–1) | 0.7 (0.7–0.9) | 0.7 (0.6–1) |
| Cholesterol (mg/dL)* | 231 (188–270) | 210 (190–239) | 196(167–241) |
| Triglycerides (mg/dL) | 110 (79–148) | 92 (83–116) | 105(83–147) |
| GGT (IU/L) | 425 (185–959) | 421 (218–744) | 345(187–768) |
| Alkaline Phosphatase (IU/L) | 94 (77–120) | 108 (88–167) | 110(77–180) |
| Severity score MELD* | 6.5 (4.6–8) | 6 (4–8.6) | 8 (6–11) |
| MELD-Na | 6.9 (3.9–9.2) | 6.7 (2.6–8.9) | 8.4 (5.6–11.7) |
| Histological features at baseline | | | |
| Steatosis | | | |
| <33% | 13 (36.1) | 6 (23.1) | 23 (47.9) |
| 33%–66% | 11 (30.6) | 8 (30.8) | 13 (27.1) |
| >66% | 12 (33.3) | 12 (46.2) | 12(25) |
| Mallory bodies | | | |
| No or occasional | 14 (38.9) | 13(50) | 11 (22.9) |
| Many | 22 (61.1) | 13(50) | 37 (77.1) |
| Bilirubinostasis | | | |
| No | 24 (66.7) | 17 (65.4) | 27 (56.3) |
| Hepatocellular | 12 (33.3) | 9 (34.6) | 21 (43.8) |
| Canalicular and/or ductular | 0 | 0 | 0 |
| Hepatocellular plus canalicular and/or ductular | 0 | 0 | 0 |
| Ballooning | | | |
| No/occasional | 24 (66.7) | 17 (65.4) | 27 (56.3) |
| Many | 12 (33.3) | 9 (34.6) | 21 (43.8) |
| PMN infiltration | | | |
| None/Mild | 36(100) | 25 (96.2) | 42 (87.5) |
| Severe | 0 | 1 (3.8) | 6 (12.5) |
| Megamitochondria | | | |
| Yes | 10 (27.8) | 10 (38.5) | 21 (43.8) |
| Pericellular fibrosis | | | |
| Yes | 28 (77.8) | 23 (88.5) | 40 (85.1) |

*P<0.05 between the three groups. None of the histological parameters showed statistically significant differences between the three groups.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; EtOH, ethanol; GGT, gamma-glutamyl transferase; INR, international normalised ratio; MELD, model for end-stage liver disease; MELD-Na, MELD-sodium; ndALD, never-decompensated alcohol-related liver disease; PMN, polymorphonuclear cell; P25-P75, percentile 25-percentile 75.

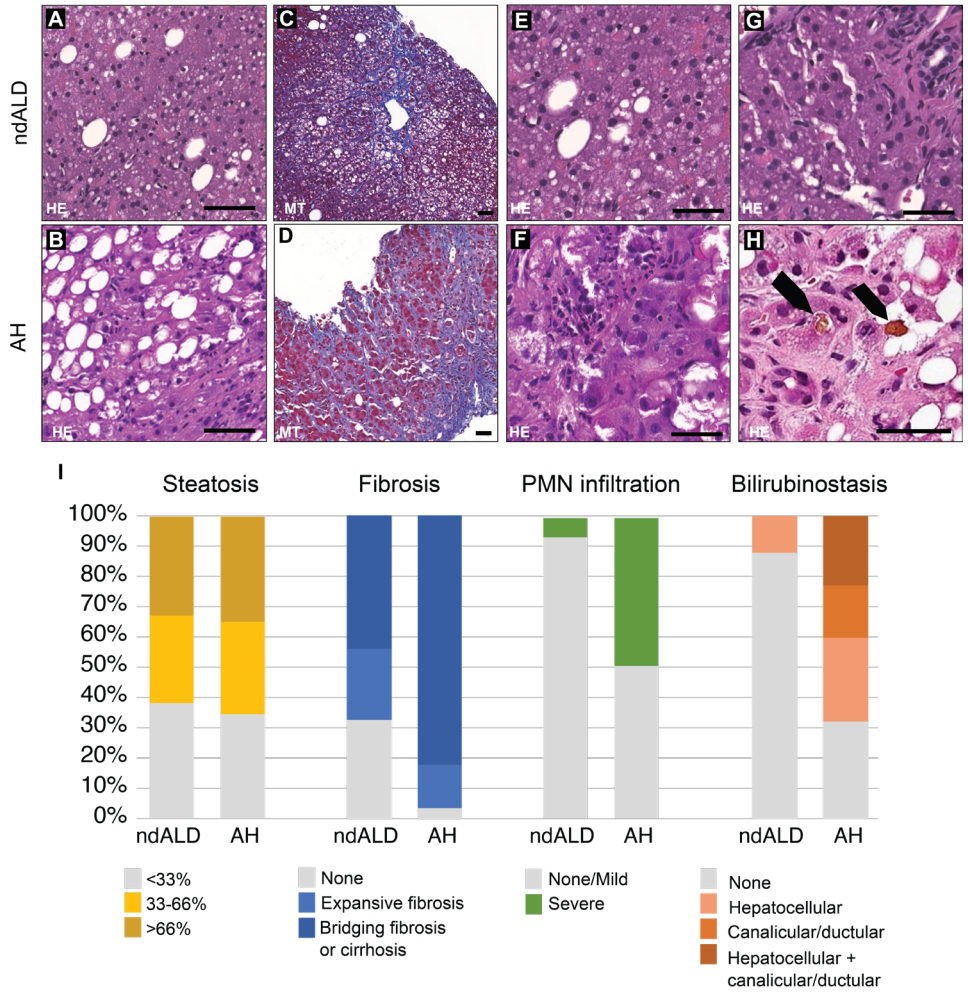


**Figure 1** Histological features of AH and ndALD patients. (A) Steatosis, ndALD patient H&E viewed using a ×20 objective (x20) (B) steatosis, AH patient, H&E, ×20. (C) Pericellular and perisinusoidal fibrosis in ndALD patient, Masson-Trichrome ×20. (D) Bridging fibrosis and pericellular fibrosis on AH patient, Masson-Trichrome ×10. (E) Structural preserved parenchyma area without inflammatory infiltration, ndALD patient, H&E, ×20. (F) PMN infiltration, AH patient, H&E, ×20. (G) Normal bile duct with preserved parenchymal architecture without bilirubinostasis, ndALD, H&E, ×20. (H) Hepatocanalicular bilirrubinostasis (black arrow) on AH patient, H&E, ×20. (I) Percentage of different grade of steatosis and fibrosis, presence of PMN infiltration and types of bilirrubinostatis by cohorts (ndALD and AH patients). Scale bars indicate 50 µm. AH, alcohol-related hepatitis; MT, Masson's trichrome; ndALD, never-decompensated alcohol-related liver disease; PMN, polymorphonuclear cell.

to patient samples with ndALD. Interestingly, KT7 score was more elevated in ndALD patients with more advanced fibrosis (figure 2A–E). In summary, patients with AH had liver histology notable for more severe fibrosis, neutrophils, MDB's, ductular reaction and bilirubinostasis than patients with ndALD (table 1).

### Survival analysis in patients with ndALD and AH

Transplant-free survival for the ndALD cohort was 100% at 28 days, 99.1% at 90 days, 93.5% at 1 year and 89.7% at 2 years. Short and medium-term transplant-free survival percentages within the AH groups were 81.1% (cohort A) and 94.6% (cohort B) at 28 days, and 66% (cohort A) and 64.3% (cohort B) at 90 days. Long-term survival was only available for cohort A with 50% of mortality at 1 year and 68% at 2 years. As expected, transplant-free survival percentages among AH patients were lower at all time points compared with ndALD patients ($p<0.05$). Of note the incidence of ACLF among AH patients was over 50% and the CLIF-C ACLF score strongly correlated with survival (online supplemental figure S2).

### Comparative liver transcriptome analysis between ndALD and AH

We next investigated the hepatic transcriptome differences between ndALD and AH. Compared with normal human livers, there was a greater number of differentially expressed genes in AH compared with ndALD. Specifically, 1327 genes were deregulated in ndALD (724 upregulated and 603 downregulated, $p<0.01$), while in AH, 4921 were differentially expressed (2402 upregulated and 2519 downregulated, $p<0.01$).

To further understand the transition from ndALD to AH, the transcriptome was directly compared between patients with ndALD and AH. Patients with AH had 865 differentially expressed genes when compared with ndALD patients (420 upregulated and 445 downregulated). Analysis of the driver genes known to regulate the 10 major pathophysiological processes involved in the hepatic response to injury[28] (ie, inflammation, fibrogenesis, progenitor cell expansion, lipid metabolism, etc) (figure 3A) was subsequently conducted. Genes related to hepatocyte biosynthetic functions were downregulated in patients with AH while conserved in patients with ndALD. In contrast, patients with AH

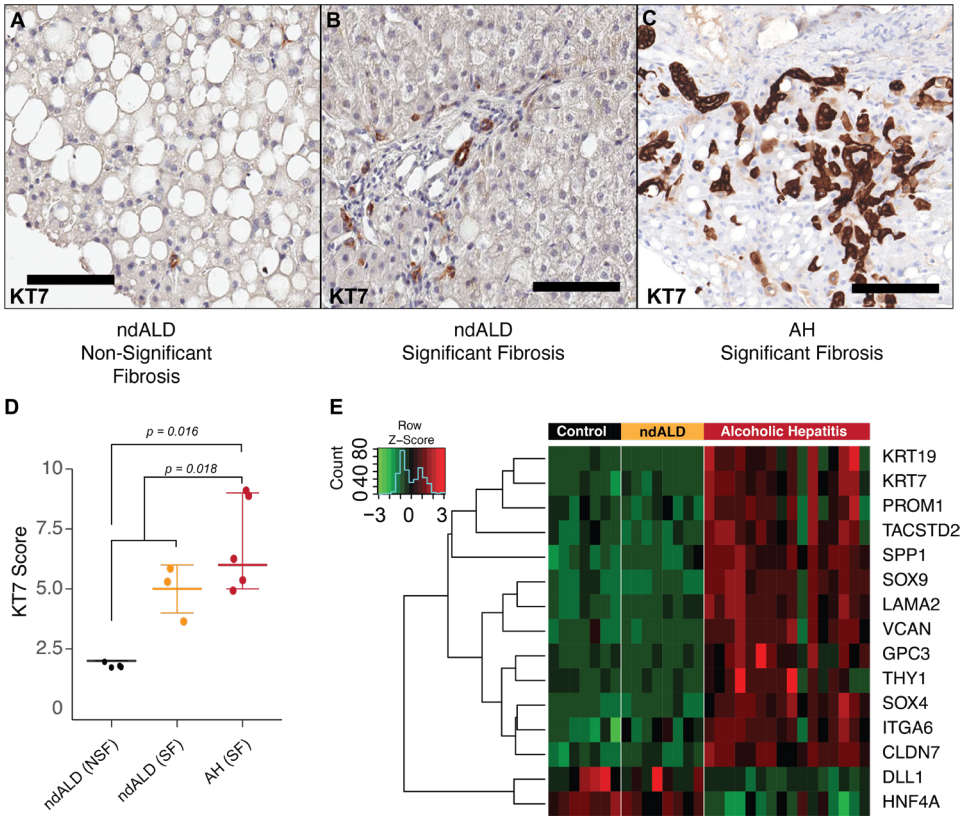


**Figure 2** Ductular reaction analysis in control, ndALD with and without underling significant fibrosis and AH patients. Immunohistochemistry (IHC) of KT7: (A) ndALD patient without significant fibrosis (no fibrosis or only portal fibrosis without septa or with incomplete septa), KT7 IHC observed with ×20 objective, (B) ndALD patient with significant fibrosis KT7 IHC and (C) decompensated AH patients with significant fibrosis, KT7 IHC. (D) Semiquantitative assessment of KT7 within the three phenotypes (ndALD without significant fibrosis n=4; ndALD with significant fibrosis n=3, AH n=5). More details about the semiquantitative assessment can be found in online supplemental methods (E) DNA microarray analysis of the most representative genes of ductular reaction within the three phenotypes is presented as a heat map. Scale bar indicate 100 µm. AH, alcohol-related hepatitis; KT7, cytokeratin 7; ndALD, never-decompensated alcohol-related liver disease; NSF, no significant fibrosis.

also had increased levels of transcripts related to cell proliferation and extracellular matrix (ECM) deposition.

To further delineate the dysregulated pathways between ndALD and AH, an unbiased GSEA of DE genes was completed. The most downregulated Hallmark gene set was the 'Bile Acid Metabolism' pathway, while the most upregulated gene set was the 'Epithelial to Mesenchymal Transition' (EMT) pathway (figure 3B). Interestingly, TNFα and IL6 signatures were only slightly up-regulated (figure 3B). Functional enrichment using the C2/canonical pathways collection revealed significant overlap of genes belonging to different gene sets. Enrichment mapping of these overlaps showed key nodes were related to biosynthetic metabolic functions of the hepatocyte, which were mostly downregulated, and to proliferation and ECM organisation, which were upregulated (figure 4). A focused analysis on genes involved in bile acid metabolism showed how the regulation of this pathway in hepatocytes was completely subverted (online supplemental figure S3A and B). As expected, FGF19 was upregulated, while its receptor β-Klotho and other mainstream regulators of bile acid synthesis (SHP, RXRα) together with important target genes (ie, AKR1D1, CYP7A1, CYP27A1) were downregulated. Bile acid transporters were also markedly altered in AH. While transporters located in the baso-lateral (SLC51A or OSTα and SLC51B or OSTβ) and canalicular (BSEP, MRP2, MRD3, AE2) membranes were upregulated, other transporters such as OATP were downregulated (online supplemental figure S3C–F). Importantly, the EMT gene set was the most upregulated one in patients with AH, while it was barely upregulated in ndALD (online supplmental figure S4). We found a close correlation between the expression of ETM-related genes and biomarkers indicative of synthetic liver dysfunction, suggesting a potential role in hepatocyte dedifferentiation and loss of synthetic functions (online supplemental figure S5).

Other pathways upregulated in patients with AH were related to cell proliferation, such as 'MYC targets' (Hallmark, online supplemental figure S6A,B) or cell cycle (Reactome, online supplemental figure S6C). Because the liver from patients with AH is characterised by a massive progenitor cell expansion along with decreased hepatocyte proliferation[19 29] these changes could reflect progenitor cell expansion. Gene sets related to apoptosis (online supplemental figure S7A) and angiogenesis (online supplemental figure S7B) were globally upregulated in patients with AH. Downregulated gene sets in livers of patients with AH included those related to additional biosynthetic functions, like drug, xenobiotic and lipid metabolism (online supplemental figure 8), Finally, we interrogated our microarray data for innate and adaptative immune signatures ndALD and AH were equally distributed in both low and high expression clusters (online supplemental figure S9A). Interestingly, patients with the higest Alcoholic Hepatitis Histologic Score (AHHS) were all part of the higher expression of immune inflammatory signatures (online supplemental figure S9B). In conclusion, patients with ndALD have a relatively conserved transcriptome, while late stages of ALD such as AH is characterised by

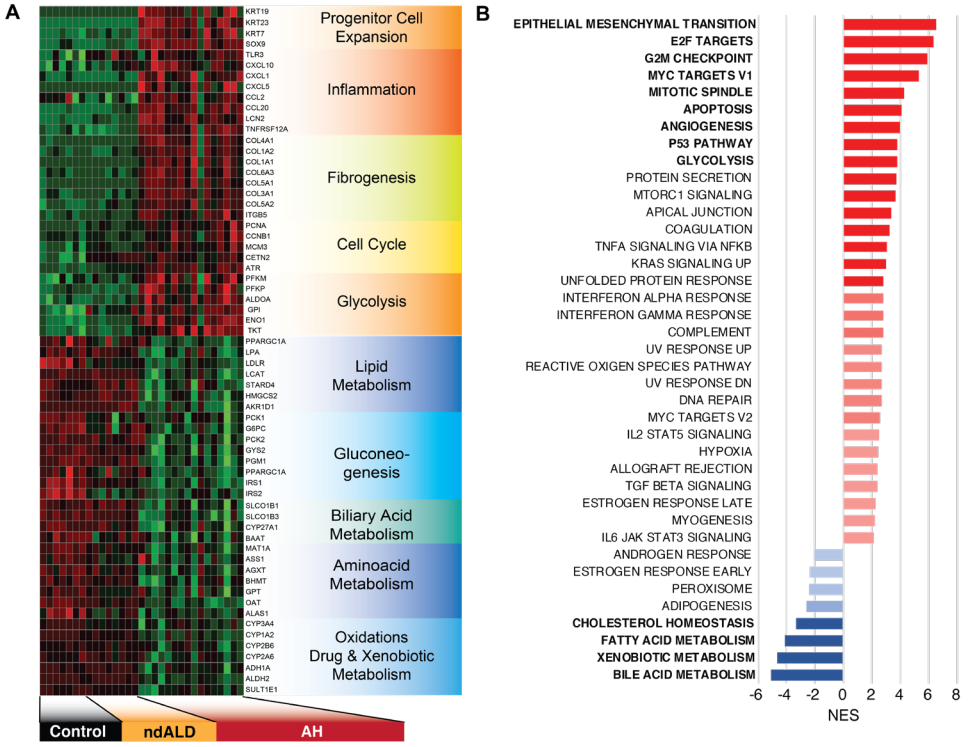


**Figure 3** Liver gene expression patterns of patients with ndALD and AH. (A) RNA microarray analysis is presented as a heatmap. The genes were manually selected by their role on hepatocyte function and the significant up or downregulation when comparing ndALD versus AH patients ($p<0.001$). (B) Gene Set Enrichment Analysis (GSEA) of differentially expressed genes comparing ndALD versus AH livers. Hallmark gene set was used and Normalised Enrichment Score (NES) is presented. Positive (red) and negative (blue) bars indicate up and downregulated gene sets in AH. AH, alcohol-related hepatitis; ndALD, never decompensated alcohol-related liver disease.

profound changes in genes related to hepatocellular function and hepatic response to injury.

## DISCUSSION

This study represents an international effort to perform a comprehensive clinical and molecular characterisation of never decompensated forms of ALD versus the life-threatening AH. While most attention has been paid to AH, few studies have characterised early stages of ALD,[30] which precludes the development of liver-related complications in the long-term.[31] There is no consensus regarding the nomenclature of different stages of ALD and there is an emerging movement among the experts in the field for revision. Moreover, the concept AH does not properly reflect neither the severity nor the histological changes of the disease nevertheless the current recommendation by liver association are to keep the terminology AH due to its large acceptance. Following persistent alcohol intake, a subset of patients with ndALD develop an episode of AH that is a common trigger of ACLF[13 32] and carries a bad short-term prognosis. The genetic and environmental determinants of the individual susceptibility to develop AH are not well known. There is no clear explanation why some patients develop this phenotype, nor what are the triggers. It has been speculated that this is due to an increased alcohol consumption, but this has never been demonstrated. This collaborative international study attempts to fill this knowledge gap.

In our study, clinical outcomes were very different between the two phenotypes. The most dramatic difference between both groups of patients was the survival status. Patients with AH showed 62% at 2 years, vs 10% in patients with ndALD. The presence of advanced fibrosis and continued alcohol consumption has been proved to be the main parameters associated with early mortality in patients with compensated forms of ALD.[2] Though it can be argued that our definition of ndALD is too wide and encompasses both early and advance disease, we have conducted a sub-analysis showing that although patients have different fibrosis degrees, they only differ in a few analytical parameters (table 2). These results highlight the importance of elucidate the underlying fibrosis degree within patients with never decompensated disease with either non-invasive test or biopsy if necessary. Finally, we try to avoid including overlapping NASH and ndALD by excluding patients with BMI >30.

Among patients with AH, the laboratory profile demonstrated impaired liver function, clearly differentiating this form from ndALD. Remarkably, patients with AH did not have a higher daily alcohol intake than patients with asymptomatic forms of the disease. This important finding suggests that factors other than the amount of alcohol influence the individual susceptibility to develop AH. Differences in disease progression despite similar alcohol histories may possibly be explained by unique genetic as well as epigenetic changes.[16 33] Finally, we found some interesting analytical findings. First, GGT levels were higher among patients with ndALD, probably reflecting improved preservation of hepatocyte mass and subsequent induction of CYP2E1-related genes. Second, the AST/ALT ratio was higher in patients with AH, partially due to lower ALT levels. This is clinically relevant, since many patients with AH show caloric-protein malnutrition and low vitamin B complex levels, which could contribute to the low serum ALT levels.[34]

Our study also performed a comparative histological analysis. This study expands our previous analysis[9] by comparing AH and ndALD. Both phenotypes show similar degrees of steatosis and

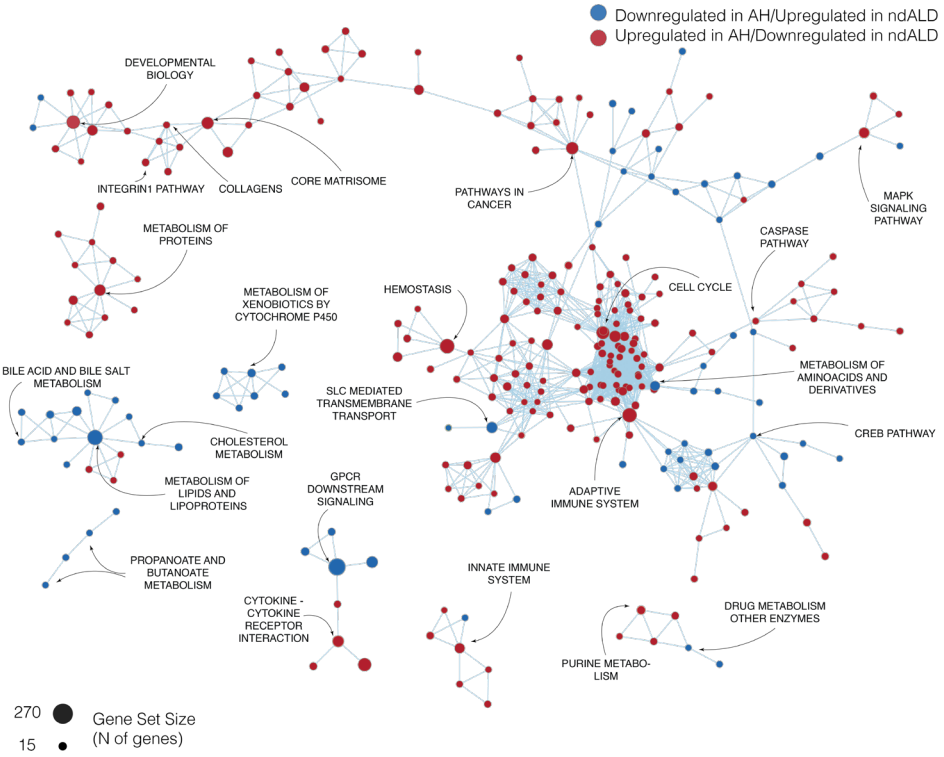


**Figure 4** Overlap between molecular signature of AH versus ndALD. Cytoscape enrichment map of Gene Set Enrichment Analysis was used to visualise overlap between Molecular Signatures Database of curated gene sets (C2) that were enriched in patients with AH vs patients with ndALD. Red nodes represent upregulated while blue nodes represent downregulated pathways in AH patients. Only nodes with p value below 0.1 and edges with similarity above 0.4 are shown. AH, alcohol-related hepatitis; CREB, c-AMP responsive element binding protein; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; ndALD, never decompensated alcohol-related liver disease; SLC, solute carrier family member.

pericellular fibrosis. These two histological attributes might be the first markers of histological damage induced by excessive alcohol intake and a potential target of future therapies for early phases of the disease. In our histological concordance study, steatosis was one of the three features with lower kappa coefficient. A tendency to overestimate steatosis has previously been described[35] and it translated into worse reliability index.

Although the precise histological parameters that define steatohepatitis need to be evaluated in prospective studies, our histological analyses yielded interesting data. As expected, bilirubinostasis was a typical feature in AH, while only a minority of patients with ndALD have the hepatocellular form. The fact that some patients with ndALD have some initial bilrubinostasis is extremely interesting. One could speculate that these patients have some degree of impairment in bile acid secretion and they are prone to develop an episode of AH. Although some very focused studies have not uncovered the relationship between specific genetic variants and predisposition to AH development.[36] Next generation sequencing technologies will allow for an unbiased scan of the whole coding and non-coding human genome to fully understand this intriguing question. Future studies should identify histological and molecular predictors of AH development in patients with silent forms of ALD. Finally, an important finding of our histological analysis was that AH is characterised by profound ductular reaction, which is almost inexistent in asymptomatic phases. This represents an inefficient regenerative response by hepatocytes.[19 29] We also showed in recent studies that ductular reaction in AH is associated with increased inflammation and fibrosis, suggesting that manoeuvres aimed at reducing this futile attempt to regenerate the liver could have beneficial effects in these patients.[14 37] Patients with ndALD forms and AH share many histological findings, therefore the clinical syndromes are basically diagnosed clinically. Further attention should be paid to patients with a clinical and analytical picture consistent with AH in whom the biopsy did not show signs of ASH and to those diagnosed of AH through the NIAAA criteria without a liver biopsy to determine whether histology should play a role in the clinical diagnosis or whether to use other criteria.

Compared with asymptomatic forms of ALD, AH patients showed an upregulation of genes related to the EMT, progenitor cell expansion and cell cycle along with decreased expression of genes involved in lipid metabolism, gluconeogenesis, drug/xenobiotic and bile acid metabolism pathways. This last pathway has been proven to be of key importance in AH patients and translates into a disbalance in bile acids towards FGF19 with an impairment on the novo bile acid synthesis.[38] Ductular reaction has been proposed as an important source of FGF19. Previous clinical data[39] as well as some of the current findings highlights the role of inflammation in AH in our cohort through the inhibition of CYP7A1. Finally, TNFα and IL6 signatures were only slightly upregulated. This could reflect the fact that other mechanisms different from proinflammatory pathways are behind the hepatic transcriptional reprograming in AH patients and perhaps the lack of success of anticytokine strategies in this clinical scenario. Single cell RNAseq studies might be key to uncover the keys of hepatocyte reporgraming and transdifferenciation.

These findings reflect profound cellular reprogramming in AH leading to jaundice and liver failure in alignment with previous findings.[16 18 40] By contrast, ndALD patients did not show such profound changes in cellular reprogramming and as severe inflammatory and profibrogenic phenotypes. The

transcriptomic analysis reflects gene expression throughout the liver, and the specific cells most responsible for changes in the liver transcriptome are uncertain. We are currently performing single cell transcriptomic, methylomics, proteinomics, metabolomics and ChIPseq analysis to elucidate this question and to identify novel druggable targets for future clinical trials.

Our study has some limitations. Patients from the ndALD cohort and AH cohort B were enrolled in multiple centres, and while this broadened the study population, it also introduced variability in the histological assessment. Enrolment over represented masculine Caucasian populations, making necessary the external validation of our results. We decided to exclude patients with metabolic syndrome criteria. Although there is growing evidence suggesting a synergistic effect of alcohol and metabolic syndrome, including patients with a dual aetiology might have biased the interpretation of the results, and therefore, studies focused on this population should be more appropriate from a methodological point of view

Finally, the follow-up of patients with ndALD was short and parameters associated with poor outcomes could not be identified. Despite the limitations, this is a comprehensive comparative study between ndALD and AH to date and could advance the field by providing specific analytical, histological and molecular profiling of these distinct disease stages.

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