Original Article

Co-existing regeneration mechanisms in severe alcohol-related steatohepatitis

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Background: Keratin 7 positive (K7$^+$) cells are considered to be activated in case of impaired hepatocyte replication. Their exact role and their interaction with hepatocytes and macrophages also implicated in liver regeneration remain poorly characterized in humans. The aim of this study is to evaluate hepatocyte, K7$^+$ cells and macrophage populations in severe alcohol-related steatohepatitis (sASH) and to link them with liver injury and patients' outcomes.

Methods: Immunohistochemical and morphometric studies for total K7$^+$ cells, macrophages (CD68$^+$ cells), proliferative hepatocytes (Ki67$^+$ hepatocytes) and proliferative K7$^+$ cells (double K7$^+$ and Ki67$^+$) were

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performed on liver biopsies of patients with sASH recruited prospectively in 16 different centres. Patients were divided into improvers or non-improvers, according to mortality and model for end-stage liver disease (MELD) score change at 3 months.

Results: Fifty-seven cases were included for histological and morphometrical assessment. Liver total $K7^+$ cell expansion was positively correlated to the severity of the disease evaluated by the MELD score. A proportion of these $K7^+$ cells were proliferating. The number of proliferating $K7^+$ cells was less than the number of proliferating hepatocytes. Increased hepatocyte replication was correlated to a higher proliferative $K7^+$ cell count. A higher number of macrophages was associated with a higher proliferation of both hepatocytes and $K7^+$ cells. No difference of total $K7^+$ cells, proliferative $K7^+$ cells, proliferative hepatocytes or macrophages was observed between improvers and non-improvers.

Conclusions: In biopsy-proven cases of sASH, proliferation of hepatocytes and $K7^+$ cells occurs in parallel. This could suggest that liver progenitor cells begin to replicate even in the absence of massive hepatocyte senescence in humans, or that proliferating progenitor cells are capable of giving rise to hepatocytes with replicative skills. This is associated with macrophagic expansion, which is therefore considered beneficial. However, in this severe, life-threatening disease, these mechanisms remain insufficient to improve patient prognosis.

Keywords: Liver progenitor cell (LPC); cirrhosis; proliferation; macrophage; alcohol-related liver disease (ALD)

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Introduction

Background

The mechanisms of hepatic regeneration remain poorly understood, and many patients still die from the consequences of hepatocellular failure in the context of cirrhosis and/or severe alcohol-related hepatitis (AH). Severe AH, defined as a clinical syndrome characterized by the recent appearance of jaundice in a situation of chronic alcohol abuse (1,2) and a Maddrey discriminant function (MDF) ≥32 (2,3), induces a high short-term mortality (20% to 35% during the first month) (4,5). Even in the absence of mortality, recovery of good liver function is often critical, and can be measured by improvement in the model for end-stage liver disease (MELD) score at 3 months (6-8). Alcohol-related steatohepatitis (ASH) is the underlying disease entity of clinical AH and is defined by the presence of steatosis, lobular inflammatory neutrophil infiltrates (often arranged in a specific pattern called satellitosis) and hepatocyte ballooning on liver biopsy (9-12). The treatment of severe ASH is first of all based on alcohol abstinence (4). In terms of pharmacotherapy, corticosteroids are used for their anti-inflammatory effects providing a reduction of short-term mortality (13,14). However, the exact mechanism of action, the short-lived and modest benefits of this drug as well as its potential side effects (i.e., increased risk of infection) constitute major drawbacks of its use and several other potential therapies are under evaluation (4). Among them, enteral nutrition (15), inhibition of hepatocyte apoptosis (4), inhibition of tumour necrosis factor (TNF) alpha (16), administration of hematopoietic stem cells (6) … have been tested in addition to corticosteroids in randomized controlled trials, showing unfortunately no benefit of any strategy in this severe liver disease. The small sample size of patients included in these studies compared to the improving survival observed in recent years (i.e., for the placebo group) is probably one of the reasons for the lack of proven benefit from interventions (5). The incomplete understanding of the pathophysiological mechanisms leading to mortality in severe ASH also accounts probably for these negative results (4).

Rationale and knowledge gap

Mortality in severe ASH is mainly linked to the loss of hepatocytes and the resultant liver failure. Hepatocyte replication by mitosis is the most important source of regeneration in order to replace lost comrades (17) and can be detected by immunohistochemistry of the proliferation marker Ki67 (7,18,19). This hepatocyte proliferation is largely

responsible for the restoration of liver mass and function after partial hepatectomy and toxin-induced injury (20). However, in case of significant chronic destruction of the parenchyma, this mechanism becomes insufficient due to hepatocyte senescence rendering hepatocytes unable to proliferate (20). Liver progenitor cells (LPCs), which are a subgroup of biliary epithelial cells (BECs) located near the canals of Hering, are recruited in a process called ductular reaction (DR) (20-22). The expansion of BECs [identified by immunohistochemistry of keratin 7 (K7)] is associated with liver damage in several chronic liver diseases, such as hepatitis C (23,24), auto-immune cholangitis (25), metabolic dysfunction-associated steatotic liver disease (MASLD) (26,27) and AH (7,21,28). LPC-driven regeneration of hepatocytes has been demonstrated in mice with florescent cell tracking experiments (29-34). Importantly, yellow fluorescent protein positive (YFP^+) biliary cells contributing to DR and hepatocyte regeneration express well-described LPC markers such as K7 or K19 (33). However, to date, there is no direct proof of LPC transformation into hepatocytes in humans (35). There is only indirect evidence (20,25,36), notably from repeated liver biopsies stained for K7 in the same patient (37).

Resident macrophages or Kupffer cells (KCs) are also implicated in the pathogenesis of AH (38). They can be activated by different stimuli such as microbial dysbiosis, loss of intestinal barrier integrity, hepatocyte death or inter-organ crosstalk (38). Once activated, inflammatory macrophages can favor the development of fibrosis (39,40). On the other hand, macrophages can also produce cytokines that may play a role in promoting liver regeneration through hepatocyte and LPC proliferation (38,41,42). They are also capable of favouring LPC transformation into hepatocytes rather than into cholangiocytes (43,44). Key elements in this setting are the expression by macrophages of Wnt3a, resulting in Wnt signaling in LPC, promoting their specification to hepatocytes (43) and of TNF-related weak inducer of apoptosis (TWEAK), a known mitogen of LPC proliferation (20). Based on these observations, macrophage therapy is now explored as a new therapeutic strategy to stimulate liver regeneration (45,46).

Objective

Since proliferating hepatocytes, proliferating $K7^+$ cells and macrophages are still poorly described and controversial in humans, as well as their contribution to liver regeneration and thus to the improvement of liver function and survival of patients, our aim is to evaluate total $K7^+$ cells, macrophages, $K7^+$ cell proliferation and hepatocyte proliferation in severe ASH in a multicentre prospective trial and to compare those parameters with patient outcome. We present this article in accordance with the MDAR reporting checklist (available at https://tgh.amegroups.com/article/view/10.21037/tgh-24-92/rc).

Highlight box

Key findings

- In biopsy-proven cases of severe alcohol related steatohepatitis, proliferation of hepatocytes and keratin 7 positive ($K7^+$) cells occurs in parallel, which may suggest that liver progenitor cells start to replicate even in the absence of proliferative failure of hepatocytes in humans or that a beneficial $K7^+$ cell subtype give rise to hepatocytes with replicative potential.

What is known and what is new?

- Expansion of biliary epithelial $K7^+$ cells was usually described as a marker of poor prognosis, in a context of hepatocytes unable to proliferate.
- However, $K7^+$ cell regeneration of hepatocytes has been demonstrated in mice with cell tracking experiments.
- This study demonstrates that while the total number of $K7^+$ cells is related to the severity of the disease in humans, some of these $K7^+$ cells are proliferating, despite the simultaneous presence of proliferating hepatocytes and in the presence of hepatic macrophages.

What is the implication, and what should change now?

- It is important to differentiate between different types of $K7^+$ cells in humans, some of which are capable of proliferation, even in the presence of regenerating hepatocytes. Hepatic macrophages are associated with this phenomenon.
- Further studies should identify the characteristics of beneficial $K7^+$ cells and their dialogue with the microenvironment.

Methods

Patients and material

The patients, the liver tissue and the clinical and biological data were taken from a study on enteral nutrition in severe ASH (number: NCT01801332) (15). Briefly, for this initial trial, 136 patients treated in 20 different hospitals in Belgium and France were enrolled. Aged from 18 to 75 years, patients were all chronic alcohol consumers (>40 g/day) and had an onset of jaundice within the past 3 months. They all suffered from severe ASH confirmed by biopsy (according to the presence of ballooned hepatocytes, Mallory bodies

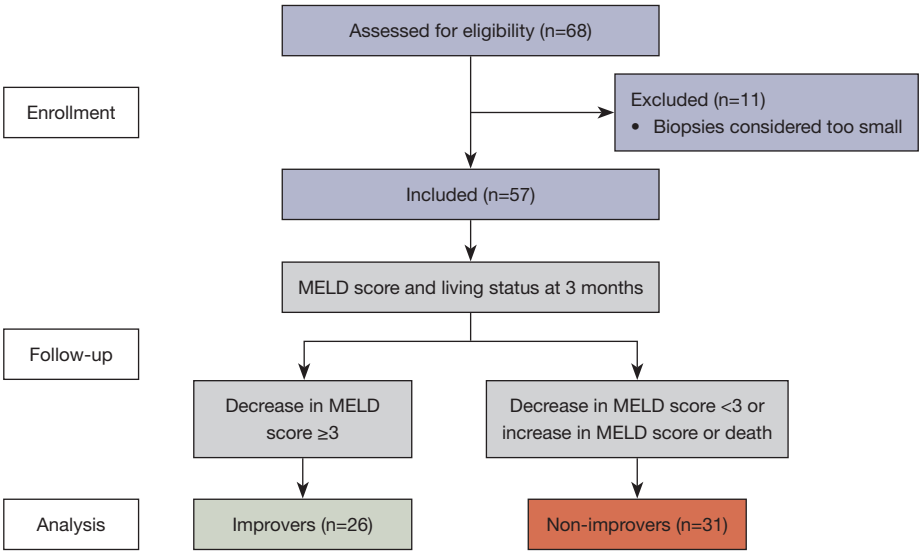

Figure 1 Study flowchart. Created with BioRender.com. MELD, model for end-stage liver disease.

and neutrophil infiltration on local pathology evaluation) and a MDF ≥32. Other causes of chronic liver diseases were excluded.

For the current study, all principal investigators were invited to participate in this sub analysis.

Ethical statements

The initial multicentre clinical trial performed in 18 Belgian and 2 French hospitals was approved by the ethical committee of Cliniques universitaires de Bruxelles (CUB) Hôpital Erasme (reference P2009/333) and by the local institutional review board or ethics committee at each participating hospital. All research was conducted in accordance with the Declarations of Helsinki (as revised in 2013). Written informed consent was obtained from all participants. The present additional retrospective study was approved by the Comité d'Ethique Hospitalo-Facultaire of Cliniques universitaires Saint-Luc (reference CEHF: 2016/01JUI/239).

Hepatocyte, progenitor cell and macrophage evaluation

The liver biopsy was performed after admission and prior to steroid therapy, using the transjugular or percutaneous route depending on the center's routine and the patient's clinical characteristics. Biopsy material was fixed in formaldehyde, paraffin-embedded and processed locally for light microscopy with standard stainings. For each case, locally stained slides (Masson's trichrome or picrosirius red; haematoxylin and eosin) and 2 unstained paraffin-embedded slides were sent to the pathology department of Cliniques universitaires Saint-Luc for central reading and additional stains as outlined below. To ensure a reliable histological evaluation, patients with biopsies' size of less than 5 microscopic fields at a ×400 magnification were excluded (*Figure 1*). Immunohistochemical staining was performed on those two serial paraffin sections. First, a double immunohistochemistry staining (Ki67 and K7) was performed on the same slide. After heating slides 36 min in CC1 buffer for antigen retrieval, a first Ki67 primary antibody (Dako, M7240, 1:100 dilution, Glostrup, Denmark) was detected by brown diaminobenzidine. Then horseradish peroxidase activity was heat-inactivated 8 min at 85 °C and sections were incubated with a second K7 primary antibody (Dako, M7018, 1:100 dilution) revealed by pink diaminobenzidine. The amount of total $K7^+$ cells was estimated by the percentage of total K7-marked area (proliferative or not) on the total liver surface measured by a morphometric program (Visiopharm®, Hoersholm, Denmark). The counting of proliferative hepatocytes was performed manually at ×400 magnification, as previously described (7). Proliferative hepatocytes were determined by the identification of K7 negative cells with the typical cuboid morphology of mature hepatocytes (not confirmed by Hep Par labelling), as achieved in other analyses (7,47,48). The total number of Ki67 positive hepatocytes was calculated and standardized according to the size of the liver biopsy (total number of fields studied). $K7^+$ cell proliferation was

also assessed by manually counting proliferative K7$^+$ cells (cells positive for both K7 and Ki67) then also related to the total number of fields. We classified the proliferative K7$^+$ cells in 3 categories, based on their immunohistochemical marking, global aspect and parenchymal localization: cells from the DR, intermediate progenitor cells (IPC) and intermediate hepatocytes (IH) were identified and manually counted, as previously described (7,47,48). Cells from the DR are cuboid adjacent K7 intense positive cells, smaller than hepatocytes, forming ductules. IPC are K7 positive cells (often isolated) located in the liver lobule and also smaller than hepatocytes. IH are large cuboid hepatocyte-like cells with a less intense K7 staining than DR and IPC. Second, immunostaining against CD68 (Dako, M0876, 1:100 dilution) revealed by brown diaminobenzidine on the second serial section to highlight macrophages. The extent of macrophage expansion was evaluated by Visiopharm®, calculating the percentage of CD68 positive area reported on the total liver surface, as described previously (7,49).

Outcomes

Patients were divided in two groups, improvers and non-improvers, according to the delta between the MELD-score at 3 months and the screening MELD score (7). The clinical improvement was considered significant for a reduction of 3 points or more of the MELD-score three months after admission, a cut-off already used in previous studies (6-8). Non-survivors at 3 months were included in the non-improver group (*Figure 1*).

Statistical analysis

The distribution of data for each criterion was first submitted to an Agostino-Pearson normality test. When the distribution of the data was normal, correlation was tested by linear regression, and the *T*-test used was the Student *t*-test. In case of non-normal distribution, the non-parametric Spearman rank correlation test and the Mann-Whitney test were used.

Results

Characteristics of the study population

Of the 20 centres initially involved in the initial study, sixteen investigators from 16 centres agreed to participate. Sixty-eight patients from the original study with biopsy-proven ASH were enrolled (*Figure 1*). Table S1 provides additional information about the participating centres, including location, investigator name, and number of patients enrolled per centre. Eleven patients were excluded because of insufficient biopsy quality according to the aforementioned criteria, leaving 57 patients (38 males and 19 females) to be included in the final analysis (*Figure 1*). Their baseline characteristics are depicted in *Table 1*. The average age was 50 years. Clinical and screening biological parameters confirmed severe liver disease: the majority of patients had ascites (65%), the median INR was 1.8, the median platelet count was 120,000/µL and the mean bilirubin level was 16.6 mg/dL. The mean MELD score was 23.5 and the median Maddrey score was 54. Patients were then classified according to their clinical and biological evolution at 3 months. Twenty-six patients were improvers and 31 non-improvers, the latter including 26 patients who died within 3 months after inclusion (*Figure 1* and *Table 2*).

Histological diagnosis of ASH

The timing of the biopsies is important for the reliability of the analyses. In this study, biopsies were performed on average 1.7 days before baseline evaluation data, and thus before corticosteroids administration. However, the timing since the patient's admission was variable and unfortunately not always reported (15). The diagnosis of ASH was histologically confirmed for the 57 included patients, on basis of the presence of satellitosis (n=48) and/or neutrophilic inflammation (n=57). Cirrhosis, defined by a Metavir score of 4, was formally established for 42 patients (*Table 1*).

Expansion of LPCs

While in normal healthy livers, K7 immunostaining is exclusively restricted to bile duct cells, the included patients had a clear expansion of K7$^+$ cells (*Figure 2A*,*2B*) with a mean surface of 4.83% (and a median surface of 2.59%, *Table 1*). The total surface of K7$^+$ cells had a significant correlation with the severity of the disease as evaluated by the MELD score [r=0.3416; 95% confidence interval (CI): 0.08113–0.5588; P=0.009], confirming that total K7$^+$ cell expansion is a marker of the severity of liver damage (*Figure 2B*).

Proliferation of K7$^+$ cells and hepatocytes

Quantification of proliferating K7$^+$ cells among each cell

Table 1 Clinical, biological and histological patient characteristics at screening (n=57)

Characteristics at screening	Values
Clinical	
Age (years)	50.03±8.65
Male sex	38 [67]
BMI (kg/m^2)	27.33±5.46
Ascites	37 [65]
Grade I	2 [4]
Grade II–III	35 [61]
Encephalopathy	16 [28]
Grade I	10 [18]
Grade II–III	6 [11]
Biological	
INR	1.80 (1.60–2.09)
Platelets (10^3/mL)	120 (82–201)
White blood cell count (/mL)	10,316 (7,033–12,460)
Bilirubin (mg/dL)	16.55±9.92
Creatinine (mg/dL)	0.74 (0.57–0.91)
Albumin (g/L)	26.00 (22.25–29.00)
Sodium (mEq/L)	134.30±5.31
AST (U/L)	126.50±52.72
GGT (U/L)	181.00 (139.30–342.50)
MELD score	23.49±4.41
MDF	54.00 (43.23–71.5)
Histological	
Biopsies length (mm)	12.87 (8.59–21.20)
Biopsies fields (×400)	21 (13–37.5)
Portal tracts on biopsy	4 (3–12)
Metavir fibrosis score	
Grade II	3 [5]
Grade III	12 [21]
Grade IV	42 [74]
K7 area (%)	2.59 (0.97–5.87)
Proliferative K7 by field	0.71 (0.10–2.06)
Proliferative K7 (DR) by field	0.27 (0–1.03)
Proliferative K7 (IPC) by field	0.16 (0–0.49)
Proliferative K7 (IH) by field	0.15 (0.01–0.49)
Proliferative hepatocytes by field	5.81 (1.32–9.75)
CD68 area (%)	1.55 (0.82–3.04)

Data are presented as mean ± standard deviation, n [%] or median (interquartile range, 25th–75th percentile). Data are presented for the entire cohort (n=57). Missing data for 3 patients for the BMI, 5 patients for the albumin level, 1 patient for the sodium level, 1 patient for the white blood cell count and 1 patient for the GGT level. BMI, body mass index; INR, international normalized ratio; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; MELD, model for end-stage liver disease; MDF, Maddrey discriminant function; K7, keratin 7; DR, ductular reaction; IPC, intermediate progenitor cells; IH, intermediate hepatocytes; CD68, cluster of differentiation 68.

subtype (*Figure 2C*) revealed similar levels of the different subpopulations throughout the cohort with less than one double-positive cell per field in the DR, IPC and IH subgroups (*Figure 2D*). While the rate of hepatocyte proliferation is almost zero in normal liver, the number of proliferating hepatocytes was high in ASH patients (average number of 5.81 positive cell/field), and significantly higher than the number of proliferating K7$^+$ cells (*Figure 2E*). Of note, the rate of proliferating hepatocytes strongly correlated with the rate of proliferating K7$^+$ cells (r=0.8112; 95% CI: 0.6942–0.8865; P<0.001) (*Figure 2F*).

Expansion of liver macrophages

We observed a significant correlation between the total amount of macrophages, estimated by the percentage of CD68 marked area (*Figure 3A*) and the total combined number of proliferative hepatocytes and proliferative K7$^+$ cells by field (r=0.3012; 95% CI: 0.03622–0.5266; P=0.02) (*Figure 3B*). This indicates that a higher number of macrophages was associated to a more important proliferation of both hepatocytes and K7$^+$ cells.

Clinical, biological and histological characteristics of improvers

We then compared patients considered improvers with patients considered non-improvers. Clinically, these two groups were comparable in terms of age, sex and presence

Table 2 Comparison of clinical, biological and histological data at screening between improvers and non-improvers based on mortality and MELD score evolution at 3 months

Characteristics at screening	Improvers (n=26)	Non-improvers (n=31)	P value
Clinical			
Age (years)	49.23±8.40	50.70±8.93	0.46
Male sex	16 [62]	22 [71]	0.58
BMI (kg/m^2)	25.80±4.27	28.75±6.11	0.046
Ascites	14 [54]	23 [74]	0.16
Ascites grade			0.02
Grade I	2 [8]	0 [0]	
Grade II–III	12 [46]	23 [74]	
Encephalopathy	6 [23]	10 [32]	0.56
Encephalopathy grade			0.47
Grade I	4 [15]	6 [19]	
Grade II–III	2 [8]	4 [13]	
Biological			
INR	1.72 (0.92–1.47)	1.90 (1.68–2.28)	0.02
Platelets (10^3/mL)	194 (108–228)	91 (71–135)	0.002
White blood cell count (/mL)	9,000 (6,160–12,300)	10,560 (7,670–12,650)	0.64
Bilirubin (mg/dL)	15.44±8.26	17.49±11.18	0.47
Creatinine (mg/dL)	0.65 (0.55–0.80)	0.80 (0.61–1.00)	0.051
Albumin (g/L)	25.50 (21.00–28.13)	26.00 (22.75–29.70)	0.66
Sodium (mEq/L)	135.80±4.42	133±5.73	0.06
AST (U/L)	129.10±59.01	124.10±47.70	0.90
GGT (U/L)	270.00 (160.50–482.50)	152.50 (82.50–284.50)	0.004
MELD score	22.06±3.11	24.69±4.93	0.02
MDF	46.00 (37.83–62.66)	62.24 (50.23–76.48)	0.02
Histological			
Biopsies length (mm)	14.40 (10.85–24.05)	11.66 (7.56–20.33)	0.20
Biopsies fields (×400)	23 (16–40)	20 (10–32)	0.08
Portal tracts on biopsy	6 (3–11)	4 (3–11)	0.55
Metavir fibrosis score			0.20
Grade II	2 [7.69]	1 [3.23]	
Grade III	7 [26.92]	5 [16.12]	
Grade IV	17 [65.38]	25 [80.65]	
K7 area (%)	1.89 (0.96–4.68)	3.72 (1.65–6.51)	0.22
Proliferative K7 by field	0.72 (0.29–1.64)	0.71 (0.06–2.22)	0.95

Table 2 (*continued*)

Table 2 (*continued*)

Characteristics at screening	Improvers (n=26)	Non-improvers (n=31)	P value
Proliferative K7 (DR) by field	0.33 (0.07–0.70)	0.20 (0.00–1.09)	0.50
Proliferative K7 (IPC) by field	0.17 (0.06–0.30)	0.16 (0.00–0.52)	0.84
Proliferative K7 (IH) by field	0.13 (0.05–0.32)	0.15 (0.01–0.56)	0.47
Proliferative hepatocytes by field	6.26 (1.50–9.64)	5 (1.58–10.27)	0.63
Total CD68 area (%)	1.84 (0.82–2.93)	1.24 (0.95–2.98)	0.71

Data are presented as mean ± standard deviation, n [%] or median (interquartile range, 25th–75th percentile). Data are presented for the entire cohort (n=57). Missing data for 3 patients for the BMI, 5 patients for the albumin level, 1 patient for the sodium level, 1 patient for the white blood cell count and 1 patient for the GGT level. Student t-test was used when the distribution of the data was normal and Mann-Whitney test in case of non-normal distribution. MELD, model for end-stage liver disease; BMI, body mass index; INR, international normalized ratio; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; MDF, Maddrey discriminant function; K7, keratin 7; DR, ductular reaction; IPC, intermediate progenitor cells; IH, intermediate hepatocytes; CD68, cluster of differentiation 68.

of encephalopathy (*Table 2*). However, the non-improvers had a significantly higher body mass index (BMI) and more severe ascites (*Table 2*). Biologically, non-improvers had higher INR at screening, more marked thrombocytopenia, lower gamma-glutamyl transferase (GGT) levels and higher MELD and Maddrey scores (*Table 2*). A visual comparison of the distribution of MELD scores between the two groups is provided (Figure S1A). There was no significant difference in bilirubin, white blood cell count, albumin, sodium, aspartate aminotransferase (AST) and creatinine levels (*Table 2*). Histologically, there were no significant differences between the two groups on the baseline biopsy (*Table 2*). The non-improvers were characterized by a non-significant increase in the total number of $K7^+$ cells. However, the rate of proliferating cells (hepatocytes or $K7^+$ cells) was the same in both groups (*Table 2*). The macrophage count was also lower in non-improvers than in improvers, but this difference was not statistically significant (*Table 2*). Taken together, these results reveal no histological difference between the two groups. We also performed the analysis based on 3-month mortality (Table S2). A visual comparison of the distribution of MELD scores between the two groups is provided (Figure S1B). Nor was there any difference between living and dead patients in terms of proliferating cells in the different compartments (Table S2).

Discussion

Key findings

The main conclusions of this study are that the total number of $K7^+$ cells is related to the severity of the disease, and that some of these $K7^+$ cells are proliferating, despite the simultaneous presence of proliferating hepatocytes and in the presence of hepatic macrophages (*Figure 4*).

Strengths and limitations

It was well documented that progenitor cell activation occurs in a wide range of human diseases, and is always linked to some degree of tissue damage and poor renewal capacity of resident cells, namely hepatocytes in the case of liver impairment (18,50,51). The strengths of this investigation were to analyse a subpopulation of these $K7^+$ cells and compare them with proliferating hepatocytes and hepatic macrophages in patients with severe and life-threatening liver disease. These regeneration data could then be assessed in relation to patient outcome. This was made possible by the combined histological, biological and clinical data from prospectively recruited patients (*Figure 4A*), a great value and strength for this study. The limitations of this study are the absence of cell tracing, which would prove the origin of the $K7^+$ cells and hepatocytes but which is impossible to perform in humans; the multicentre nature of the study with potential variations in clinical evolution linked to the different centres; and the variable timing of the biopsy in relation to the onset of the disease, as we know that these inflammation and regeneration mechanisms evolve over time.

Comparison with similar researches and explanation of findings

Previous findings on LPCs and hepatocytes showed that

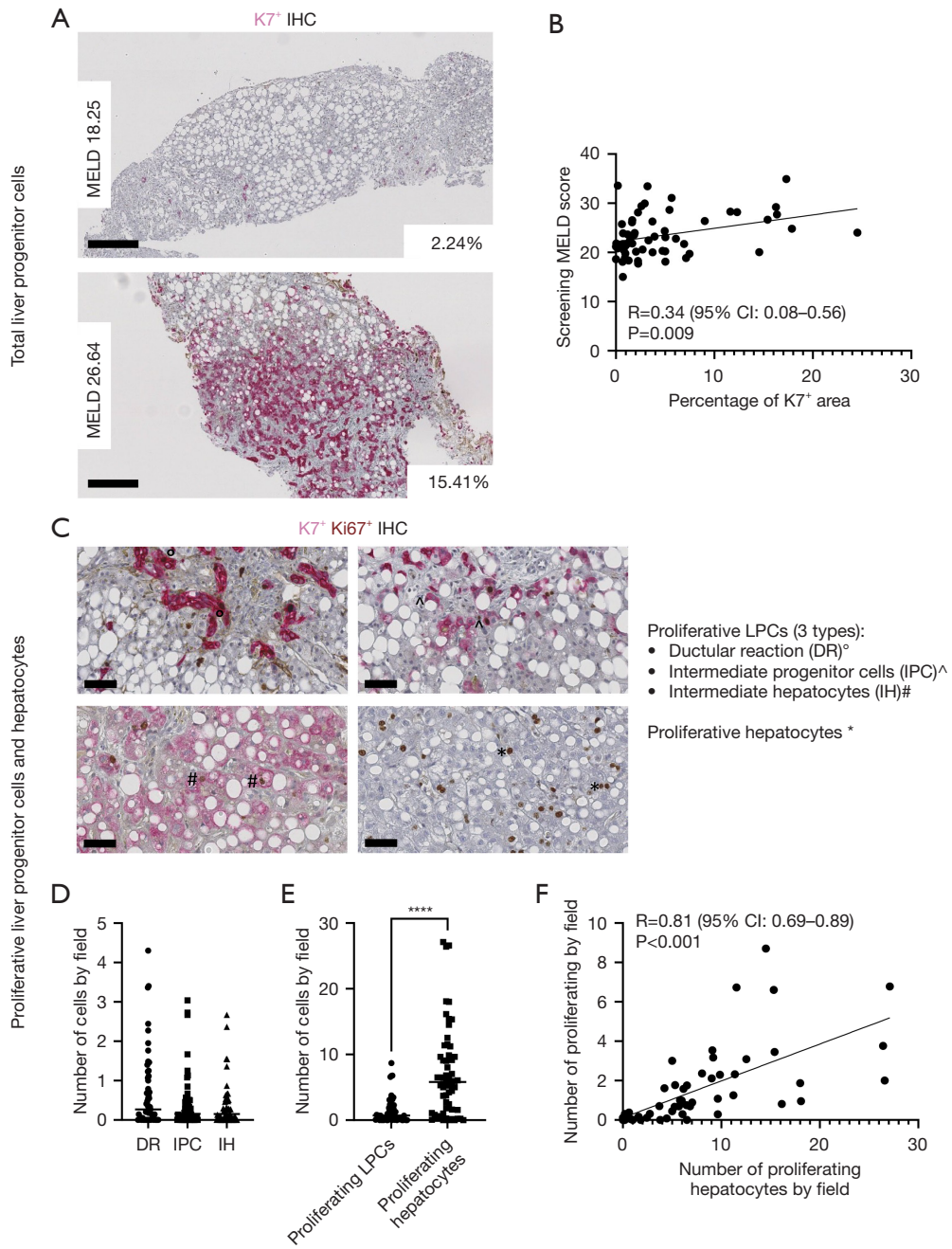

Figure 2 Evaluation and characterization of liver progenitor cells (total + proliferative) and proliferative hepatocytes. Low-magnification (×100, scale bar =200 µm) histological images of K7 IHC showing pink cell expansion calculated as a percentage in two patients with different MELD scores (A). Correlation plot between the percentage of K7-positive cells on the whole slide and the initial MELD score (B). High-magnification (×400, scale bar =50 µm) histological images of double immunohistochemistry K7 (cytoplasm in pink) and Ki67 (nucleus in brown) illustrating different cell populations and double-positive cells (C). Quantification of the mean number of different proliferating LPC subtypes per field examined (D), namely DR cells, IPC and IH. Comparison (E) and correlation (F) between the mean number of proliferating liver progenitor cells and the mean number of proliferating hepatocytes. ****, P<0.001. K7, keratin 7; IHC, immunohistochemistry; CI, confidence interval; MELD, model for end-stage liver disease; LPC, liver progenitor cell; DR, ductular reaction; IPC, intermediate progenitor cells; IH, intermediate hepatocytes.

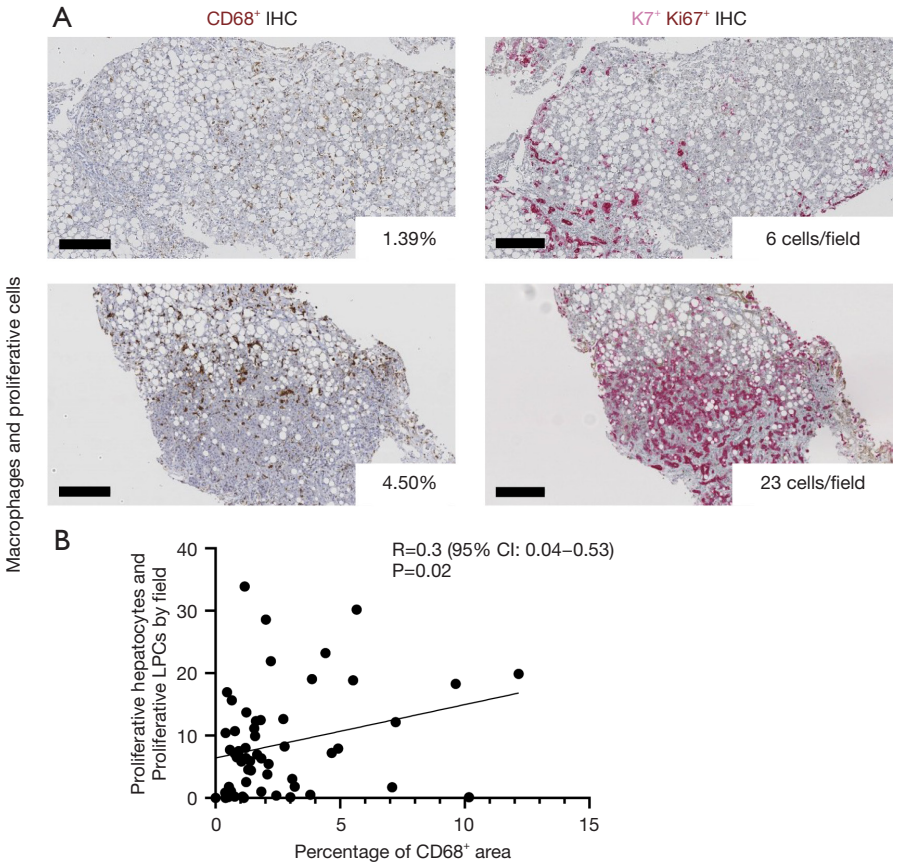

Figure 3 Evaluation of hepatic macrophages and comparison with proliferating hepatocytes and liver progenitor cells. Low-magnification (×100, scale bar =200 µm) IHC histological images showing CD68-positive macrophages quantified as a percentage over the entire section and proliferating LPCs quantified as the average number of Ki67-positive cells per field (A). Correlation plot between CD68-positive cell surface and number of proliferating cells (hepatocytes and LPC) (B). IHC, immunohistochemistry; CI, confidence interval; K7, keratin 7; LPC, liver progenitor cell.

the LPC-derived regeneration mechanisms of the liver are activated in ASH patients (7,21,52). Indeed, in healthy liver, K7$^+$ cells are not activated and their total number, estimated by the total K7 marked area, is close to zero, while it is increased in ASH patients (7,21). In our cohort, we confirm that the total number of K7$^+$ cells is increased in ASH and actually correlates with the severity of liver damage as assessed by the MELD score (*Figure 4B*).

However, we also demonstrate that a subpopulation of these K7$^+$ cells are proliferating. The latter K7 and Ki67 double-positive cells (*Figure 4C*) were already previously identified in a study of 58 AH patients as being associated with a better prognosis (7). Proliferation of these K7$^+$ cells is indeed associated with increased levels of hepatocyte growth factor, making them a potential therapeutic target (7,47).

We also show that the proliferation of K7$^+$ cells and hepatocytes is associated with greater macrophagic expansion (*Figure 4D*). This inflammation is considered beneficial and capable of stimulating regeneration (38,41,53). This may confirm previous findings supporting that macrophages are also capable of favouring the transformation of K7$^+$ cells into hepatocytes rather than cholangiocytes, as a result of the Wnt expression (43,44). Macrophages and Wnt signaling could therefore be the target of future treatments aiming to promote liver regeneration in ASH such as cell therapy or polarization treatments (4,46,54). Indeed, both resident liver macrophages, which play a role in tissue homeostasis, and recruited macrophages, which can be converted into prorestorative macrophages following the phagocytosis process in acute or chronic diseases, are now being presented as beneficial (54). The complexity of the nature and roles

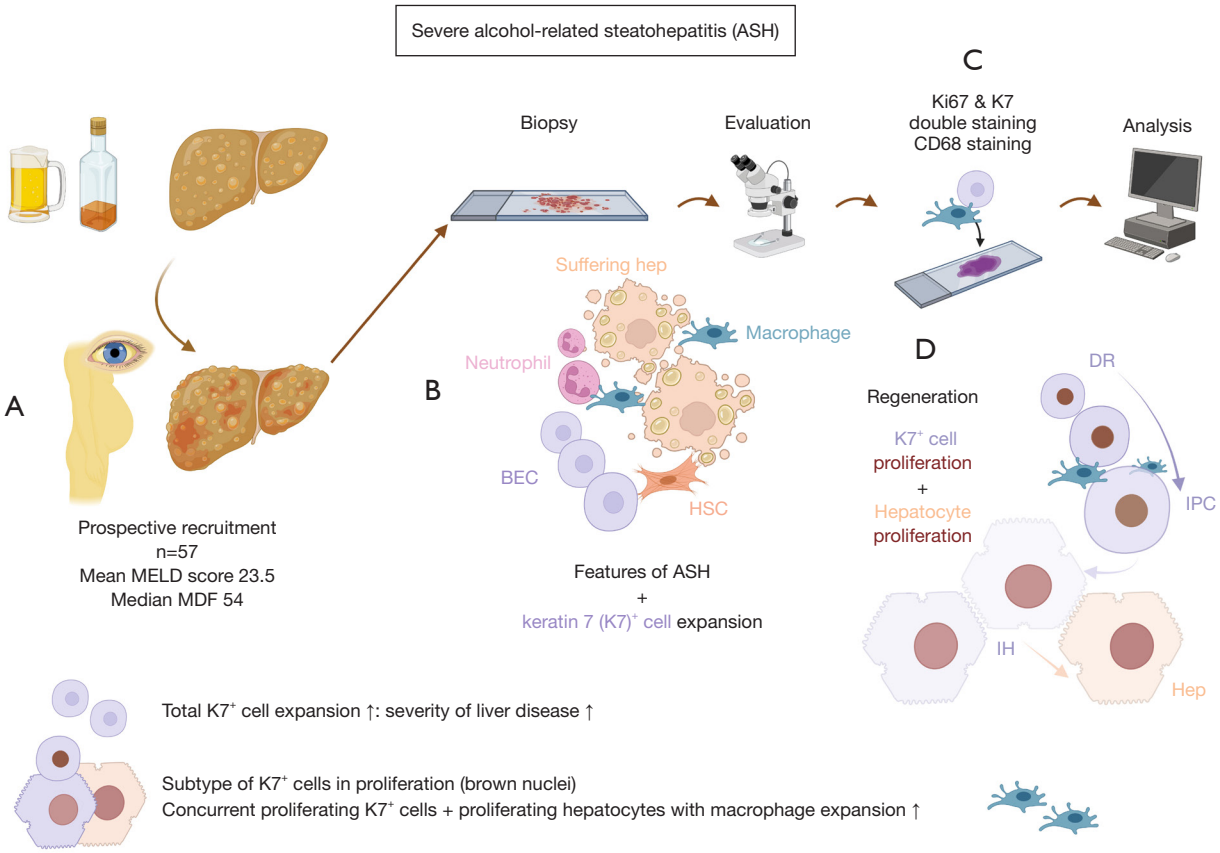

Figure 4 Main data and principal findings of this study. Among 57 patients evaluated with a mean MELD score of 23.5 and a median MDF of 54 (A), histological evaluation revealed classic signs of ASH and an expansion of total K7$^+$ cells associated with the severity of liver disease (B). Suffering steatotic hep are shown as large cells with lipid vacuoles. Recruited/active macrophages appear in blue, HSC are in orange, neutrophils are colored pink and shown with their multi-lobulated nuclei. BECs and K7$^+$ cells are shown in purple. Double immunohistochemistry for Ki67 and K7 as well as CD68 (C) revealed coexisting proliferation of both K7 cells and Hep, associated with macrophage expansion (D). Again, the BECs are purple and macrophage are shown in blue. Note the transition from smaller cells (DR) to larger cells (IPC then IH) and finally to Hep. IH are shown in light purple to indicate their possible origin from biliary epithelial K7$^+$ cells. Brown cell nuclei indicate cell proliferation, as are DAB-labelled Ki67$^+$ nuclei. Created with BioRender.com. ASH, alcohol-related steatohepatitis; MELD, model for end-stage liver disease; MDF, Maddrey discriminant function; BEC, biliary epithelial cell; HSC, hepatic stellate cell; K7, keratin 7; DR, ductular reaction; IPC, intermediate progenitor cells; IH, intermediate hepatocytes; Hep, hepatocytes; DAB, diaminobenzidine.

of different macrophage subsets therefore remains an important research topic (55). The potential beneficial role of neutrophils in ASH resolution through their effects on macrophage polarisation and liver regeneration is also a source of growing interest (53,56). Neutrophil infiltration on liver biopsy is indeed associated with favorable outcome (3-month survival) in patients with ASH (57).

Our results also clearly indicate that in severe ASH both proliferative K7$^+$ cells and proliferating hepatocytes are found (*Figure 4D*). This contradicts the current concept that when regeneration occurs in the liver, only one path is activated: either hepatocyte replication or K7$^+$ cell proliferation when hepatocyte proliferation is impaired (50,58). This concept is, however, based on animal experiments in which hepatocyte replication is selectively inhibited (59), or in models that mimic periportal damage (50). In the current human study of alcohol-related injury we found that both phenomena occur simultaneously: the activation of hepatocyte proliferation happens in parallel with proliferation of K7$^+$ cells. To the best of our knowledge, we are the first to investigate this

and demonstrate this particular aspect of regeneration in severe ASH.

Several hypotheses can help explaining this phenomenon. Firstly, animal data show that proliferating LPCs constitute a good prognostic niche capable of inducing more resistant hepatocytes with enhanced proliferative capacity (30). Hepatocytes identified as proliferating may therefore have proliferating LPCs as their source. Secondly, this phenomenon could be due to the simultaneous activation of LPC and hepatocyte proliferation in humans, if regeneration from hepatocytes is conserved but deemed insufficient. As mentioned above, the hepatic damage in human ASH does not block hepatocyte replication. $K7^+$ cells are nevertheless recruited to accelerate liver regeneration despite the presence of dividing hepatocytes. This has also been suggested in the context of chronic hepatitis C by Delladetsima *et al.* who identified a significantly higher number of $K7^+$ DR cells, but also $K7^+$ IPC in patients with proliferating hepatocytes compared to patients without proliferating hepatocytes (24). Some authors speculate that $K7^+$ cells are actually hepatocytes dedifferentiated into biliary cells (17). However, the pattern of arrangement of these cells, that seems to start near the portal tract with a DR, followed by IPC and then weakly $K7^+$ IH, suggests a phenomenon that occurs in the other direction (*Figure 4D*). Studies with serial biopsies showing a decrease in DR over time and the appearance of IHs a few days later also point in this direction (37).

Our study shows that the proliferation of both $K7^+$ cells and hepatocytes does not determine patient outcome. This contrasts with the previous study (7), which, however, involved less severe cases (3-month mortality rate of 10% compared with 46% in our study), who were biopsied very soon after admission and managed at a single expert centre. In our study, the timing from patient admission to hospital was not recorded and is probably longer, linked to the fact that some patients are referred from centres where transvenous biopsy was not available. The previous study showed that it was indeed on the first biopsy performed shortly after admission that cell proliferation was associated with patient prognosis (7). The association was no longer significant with the results on the second biopsy (7). The other factors that may explain the difference is the fact that in our study, all patients presented with sASH, and standard management was multicentric. It has been clearly identified that regenerative mechanisms may be insufficient in sASH, and that patient survival is influenced by standard centre-specific management (5). Finally, the quality of the biopsy procedures performed is also susceptible to vary in a multicentre project. The quality of the biopsies received was not always optimal. To reduce the risk of collecting unreliable data, patients with biopsies of less than 5 fields at ×400 magnification were excluded. Lower quality biopsies may be explained in part by the multicentric nature of our population, with different operators performing the biopsies, and by the fact that paraffine blocks had to be recut to obtain this additional analysis not included in the initial protocol.

Finally, our study also shows that improvers have higher GGT levels than non-improvers. This may seem surprising, given the association between higher GGT levels and mortality in the general population (60,61), which may be explained by greater alcohol consumption or more severe metabolic steatosis (62,63). The result of this biological test is not usually given in studies evaluating prognostic factors in AH (7,28,57,64,65). However, the powerful deleterious prognostic factor of a low GGT level has already been demonstrated in patients with cirrhosis (66), and can be explained by the fact that the GGT level is implicated in hepatic glutathione metabolism and therefore performs important roles in antioxidant mechanism. In our population, a decrease in GGT levels may thus reflect, in addition to defective liver function, a decrease in hepatic glutathione synthesis.

Implications and actions needed

ASH is a severe disease most often occurring in the setting of cirrhosis and coming with a high short-term mortality (1). Current treatment of severe cases consists of corticosteroid therapy, the mechanism of action of which is poorly understood in this indication, and the benefits of which are low (14). As the loss of liver function is linked to the loss of hepatocytes, the mechanisms of regeneration remain a target for investigation and treatment (4). A precise histological and molecular assessment of the potential mechanisms of liver regeneration is required in prospective cohorts, in order to identify, in human subjects, beneficial factors (cell to cell interaction and communication with the microenvironment) that could be the target of evaluations or interventions. Further studies should also identify the characteristics of beneficial $K7^+$ cells, macrophages and their dialogue with damaged liver tissue. This justifies continuing to perform liver biopsies as part of clinical studies to better understand the pathophysiology of this severe disease (12,64,67-69).

Conclusions

Regeneration mechanisms are activated in sASH and this study has therefore enabled us to better describe these pathways. Total $K7^+$ cell extension is linked to the severity of liver disease. A higher number of macrophages is associated to a more important proliferation of both hepatocytes and $K7^+$ cells and a higher hepatocyte replication is correlated to a higher proliferative $K7^+$ count, indicating that the two regeneration phenomena are interconnected.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tgh.amegroups.com/article/view/10.21037/tgh-24-92/rc

Data Sharing Statement: Available at https://tgh.amegroups.com/article/view/10.21037/tgh-24-92/dss

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The initial multicentre clinical trial performed in 18 Belgian and 2 French hospitals was approved by the ethical committee of Cliniques universitaires de Bruxelles (CUB) Hôpital Erasme (reference P2009/333) and by the local institutional review board or ethics committee at each participating hospital. All research was conducted in accordance with the Declarations of Helsinki (as revised in 2013). Written informed consent was obtained from all participants. The present additional retrospective study was approved by the Comité d'Ethique Hospitalo-Facultaire of Cliniques universitaires Saint-Luc (reference CEHF: 2016/01JUI/239).

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