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Use of orcein as an adjunct stain in the evaluation of advanced fibrosis

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Aims: Advanced liver fibrosis can regress following the elimination of causative injuries. Trichrome (TC) stain has traditionally been used to evaluate the degree of fibrosis in liver, although it is rarely helpful in assessing quality of fibrosis (i.e. progression and regression). Orcein (OR) stain highlights established elastic fibres, but its use in examining fibrosis is not well recognised. This study assessed the potential utility of comparing OR and TC staining patterns to evaluate the quality of fibrosis in various settings of advanced fibrosis.

Methods and results: The haematoxylin and eosin and TC stains of 65 liver resection/explant specimens with advanced fibrosis caused by different elements were reviewed. Twenty-two cases were scored as progressive (P), 16 as indeterminate (I) and 27 as regressive (R) using TC stain based on the Beijing criteria. The OR stains confirmed 18 of 22 P cases. The remaining P cases showed either stable fibrosis or mixed P and R. Of the 27 R cases, 26 were supported by OR stain, with many showing thin perforated septa typically seen in adequately treated viral hepatitis cases. The 16 I cases showed a variety of OR staining patterns, which allowed for further subclassification than using TC stain alone. Viral hepatitis cases were enriched for regressive features (17 of 27).

Conclusions: Our data demonstrated the utility of OR as an adjunctive stain to evaluate the changes in fibrosis in cases of cirrhosis.

Keywords: fibrosis, orcein, progression, regression, trichrome

Introduction

Advanced liver fibrosis is a dynamic process that can regress following the elimination of causative injuries, such as cessation of alcohol intake, treatment of hepatitis B (HBV) or C (HCV) viral infection, resulting in a sustained virological response (SVR).[1,2] Regression of cirrhosis eventually leads to significant improvements in clinical outcomes.[3]

Multiple systems have been proposed to evaluate the degree of liver fibrosis. The Batts–Ludwig system was originally developed for chronic hepatitis, with a staging scale of 0–4.[4] The Laennec system uses a similar 0–4 scale, but expands the cirrhosis category into 4A, 4B and 4C.[5,6] The Beijing criteria take into account the quality of fibrosis in cases of chronic HBV and further classify cirrhosis into three categories: predominantly progressive (P: broad septa composed of loose collagen), predominantly regressive (R: thin, perforated septa composed of densely compacted collagen) or indeterminate (I: roughly balanced numbers of broad and thin septa).[7,8] The Beijing and Laennec scoring systems have been shown to be comparable, with significant overlap between the Beijing P and Laennec 4C categories and the Beijing R and Laennec 4A categories.[9,10] The Beijing criteria also include a three-tier system for grading necroinflammatory activity.[8,11]

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Trichrome (TC) stain is routinely used to evaluate the quantity and extent of fibrosis in liver specimens, and is suggested to assign a score accurately in any of the above systems. Other histochemical stains that can be used include reticulin (which stains reticulin fibres), as well as Verhoeff's van Gieson (EVG) and orcein (OR), both of which stain elastic fibres. They can be helpful in differentiating between the regenerative nodules in necrosis and cirrhotic nodules in established fibrosis.[12] However, OR is more sensitive than EVG for the identification of thin, delicate elastic fibres in cirrhosis.[12] Reticulin can outline residual hepatic plate architecture in necrotic zones, but it showed inferior performance compared to orcein in distinguishing scar from necrotic zones.[12]

TC and OR have varying staining patterns in recent versus remote fibrosis (Table 1). Elastic fibres have been observed to accumulate later compared to trichrome during progression of liver fibrosis and slowly degrade during regression.[13–15] In the recent fibrosis setting, both TC and OR stains tend to show pale and thin collagen and elastic fibres, respectively, with elastic fibres not readily observed until collagen has been deposited and forms bundles. In contrast, in cases of remote fibrosis, TC stain will show dense, darker blue staining of collagen bands in the fibrous zones and septa, while OR stain can show dense and dark bands of elastic fibres in variably thick or thin septa depending on the degree of regression in cirrhosis. OR stain also highlights bundles of elastic fibres in areas of parenchymal extinction of advanced cirrhosis, but does not demonstrate elastic fibres in zones of recent necrosis/injury.[12,16] Large bundles of elastic fibres may also stain pale blue on TC, and thus could be confused with the collagen in early fibrosis; however, OR can be used to confirm that these zones are elastic fibres.[12] By comparing TC and OR stains, we surmised that they could assist a pathologist in determining the quality of fibrosis (i.e. progression versus regression). We chose OR rather than EVG because of the previous finding that elastic fibres within the regions of fibrosis were better visualised by OR in all cirrhotic cases.[12]

In cases of progressive fibrosis we hypothesise that there would be more TC than OR, as collagen tends to accumulate more quickly than elastic fibres. In contrast, we hypothesise that a pattern of equivalent OR and TC in the setting of thin, perforated septa would suggest regression. This study thus aimed to examine whether OR stain in addition to TC stain can be used to more accurately and reproducibly determine the quality of advanced fibrosis caused by different factors.

Materials and Methods

We examined 65 liver specimens (eight resections and 57 explants) with advanced fibrosis caused by different factors collected at UCSF during 2018–22 (Table 2). Thirty-eight explants and all eight resection specimens contained mass lesions. These generally consisted of treated or viable hepatocellular carcinoma. Some cases

Table 1. General utility of trichrome and orcein stains

	Remote fibrosis	Recent fibrosis
Trichrome (TC)	Dense fibrous septa	Pale, less dense staining of collagen
Orcein (OR)	1. Dark staining of thick broad scars	Pale or lack of staining for elastic fibres
	2. Compact elastic fibres in thin septa (regression)	

Table 2. Patient demographics

Age (years)	Gender	Race/ethnicity	Cause of cirrhosis
Average 61.7 Range 30–81	45 (69%) male 20 (31%) female	31 (46%) Caucasian 18 (28%) Asian 9 (14%) Hispanic 1 (2%) Black 1 (2%) Native American 5 (8%) Other	29 HBV and/or HCV 28 NASH/ASH 8 Viral and NASH/ASH

ASH, Alcoholic steatohepatitis; HBV, Hepatitis B; HCV, Hepatitis C; NASH, Non-alcoholic steatohepatitis.

Table 3. Addition of orcein stains for Interpretation of Beijing scheme

Beijing criteria (n = 65)	OR versus TC	n	Interpretation aided by OR stains (n)	Reclassified n (%)	New interpretation with help of OR stain
Regression (R; n = 27)	OR = TC	17	R (17/17)	0 (0%)	Regression (36)
	OR ~ TC with focal OR < TC	8	R (4/8), R > P (4/8)	0 (0%)	
	OR < TC	2	R > P (1/2), R ~ P (1/2)	1 to I (50%)	
Indeterminate (I; n = 16)	OR = TC	5	R > P (3/5), stable fibrosis with focal R (2/5)	5 to R (100%)	Indeterminate (7)
	OR ~ TC, with focal OR < TC	4	R > P (4/4)	4 to R (100%)	
	OR < TC	7	P > R (4/7), R > P (1/7), R ~ P with non-linear remodelling (2/7)	4 to P, 1 to R (71%)	
Progression (P; n = 22)	OR = TC	3	Stable fibrosis with minimal remodelling (3/3)	3 to I (100%)	Progression (22)
	OR ~ TC, with focal OR < TC	2	P > R (2/2)	0 (0%)	
	OR < TC	11	P (5/11); P > R (5/11), P ~ R with non-linear remodelling (1/11)	1 to I (9%)	
	OR < < TC	6	Rapid P (6/6)	0 (0%)	

I, Indeterminate: OR, Orcein; P, Progression; R, Regression; TV, Trichrome.

also contained simple cysts, bilomas and macroregenerative nodules.

The study cohort included 45 males (69%) and 20 females (31%), comprising Caucasian (46%), followed by Asian (28%) and Hispanic (14%) participants. The mean patient age was 61.7 years (range = 30–81). Various factors caused advanced fibrosis, including viral infection (chronic HBV/HCV, 29 cases), concurrent viral infection and non-alcoholic steatohepatitis (NASH)/alcoholic steatohepatitis (ASH) (eight cases), and NASH/ASH alone (28 cases). Of the 37 cases with viral causes, 12 were either untreated or treated with incomplete response while the remaining 25 cases had SVR. Patient demographics are summarised in Table 2.

In each case, haematoxylin and eosin (H&E) and TC stains representative of the overall pattern of fibrosis were evaluated by at least two liver pathologists (K.W./L.D.F./S.E.U.) and scored using the Beijing P-I-R criteria.[7,8] We chose partial resection and explant specimens, which provided more contiguous tissue in each section for a thorough evaluation. In order to avoid potential peritumoral inflammatory reaction/fibrosis secondary to mass effect, sections were chosen to represent cirrhotic non-neoplastic liver at a distance from mass/lesion. After each case was scored using the Beijing criteria with H&E and TC, a modified Beijing score was generated using an OR stain evaluated on each section to compare the extent and pattern of OR versus TC staining.

To correlate necroinflammatory activity with the degree of progression/regression, we designed a three-point grading system modified from the Beijing criteria, with minor modifications to account more accurately for the variety of disease processes included in this study (Supporting information, Methods S1).

Binomial statistical and χ^2 tests were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). This study was approved by the UCSF IRB.

Results

QUALITY OF FIBROSIS (P-I-R)

Of the 65 cases that were scored with the Beijing criteria based on H&E and TC staining, 22 cases were classified as P, 16 as I and 27 as R (Table 3). EVG staining was performed on 12 cases and reticulin staining was performed on eight cases; these stains were not superior, and in some cases less sensitive compared to OR in highlighting elastic fibres, as previously shown by Ferrell *et al.*[12]

Seventeen of 27 R cases showed comparable OR and TC staining with diffusely thin, perforated septa (Figure 1A,B). Eight cases showed OR approximately equivalent to TC with focal areas of less OR staining than TC, and these cases remained in the R category. Two cases showed OR < TC; while one was still predominantly regressive, the other was reclassified as I because of evidence of R and P to a similar extent. Overall, 26 of 27 cases (96%) remained as R following the addition of OR.

Six of 22 P cases showed thick bands of fibrosis with TC, but significantly less OR than TC to suggest more rapid progression (Figure 1C,D). Eleven cases showed OR < TC and remained as predominantly P, with the exception of one case which showed both P and R, and was reclassified as I. Two of five remaining cases showed OR ~ TC with focal OR < TC, and remained as predominantly P. The remaining three cases showed OR = TC with stable fibrosis and were reclassified as I (Figure 1E,F). Overall, 18 of 22 cases (82%) remained as P following the addition of OR.

Five of 16 I cases were reclassified as R (OR = TC). Four cases showed OR ~ TC with focal OR < TC and were reclassified as predominantly R. Seven cases showed OR < TC, four of which were reclassified as predominantly P, one as predominantly R and the original interpretation of I did not change in the remaining two cases, as they showed evidence of non-linear remodelling. In these cases, TC and OR stains demonstrated a mixture of thick fibrous bands in addition to thin, regressive-appearing septa highlighted by both stains (Figure 1G,H). The addition of OR stain was particularly helpful in I cases, allowing reclassification of 88% of cases (14 of 16) as either predominantly P or R.

CIRRHOSIS/ADVANCED FIBROSIS CAUSES

We then categorised the various causes of advanced fibrosis into P, I and R (Table 4). Cases due to chronic viral causes were enriched for regressive features (18 of 29; 62%); specifically, with SVR, 15 of 18 cases were classified as R (83%). NASH or ASH cases showed no clear pattern of fibrosis (P/predominantly P in 11 cases, I in five cases, and R/predominantly R in 12 cases) (Table 4).

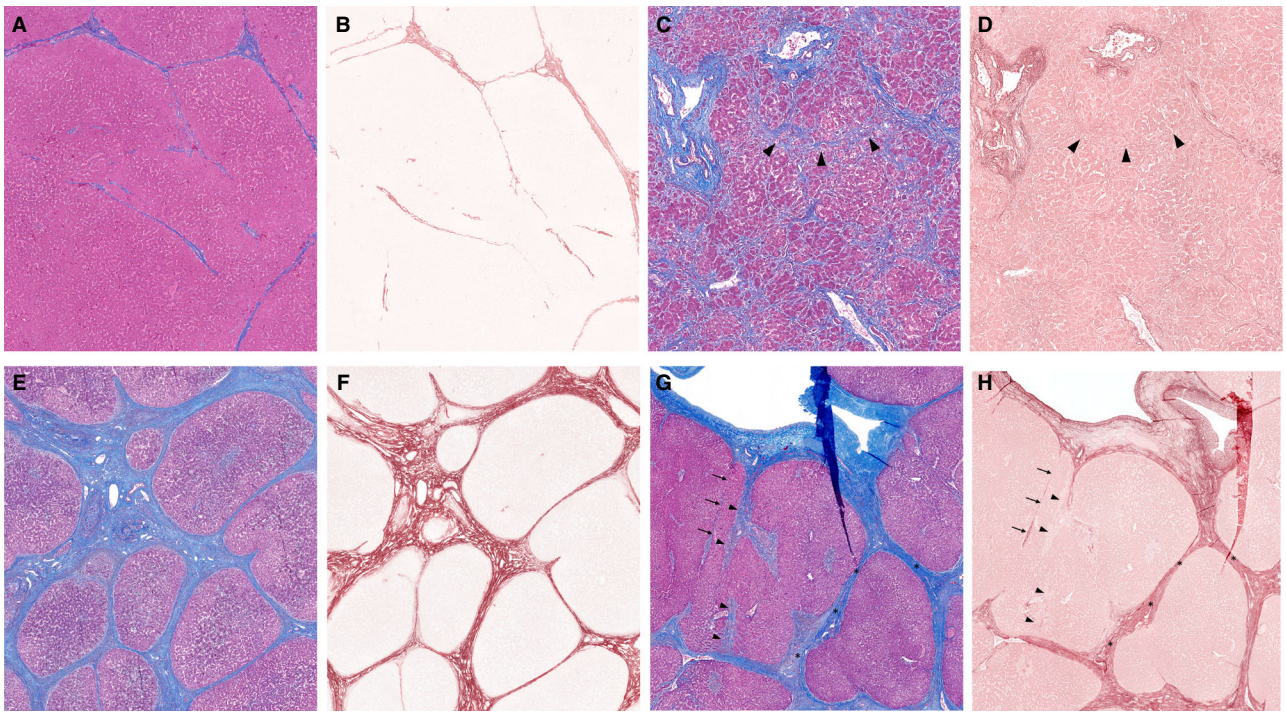

Figure 1. Evaluation of the quality of advanced fibrosis by comparing orcein (OR) and trichrome (TC) stains in resections. A, B, Linear regression (TC = OR) in hepatitis C virus (HCV)-related cirrhosis with a sustained virological response (SVR). C, D, Rapid progression (P) of fibrosis in a case of alcoholic steatohepatitis (ASH) cirrhosis; ▲: areas of P with more TC than OR staining. E, F, stable cirrhosis in a non-alcoholic steatohepatitis (NASH) case with equivalent TC and OR staining. G, H, Non-linear P/R of fibrosis in a case of ASH; ↑: R fibrous septa; ▲: areas of P; *: highlight well-established fibrosis. A, C, E, G, TC stains. B, D, F, H, OR stains.

Table 4. Qualitative changes in fibrosis by orcein and trichrome interpretation

	Viral	Viral + NASH/ASH	NASH/ASH
P or P > R	9	2	11
I (R ~ P)	2	0	5
R or R > P	18	6	12
Total	29	8	28

ASH, Alcoholic steatohepatitis; I, Indeterminate: NASH, Non-alcoholic steatohepatitis; P, Progression; R, Regression.

Table 5. Relationship of necroinflammatory activity to causes of cirrhosis

	Grade 1	Grade 2	Grade 3
P or P > R	5	12	5
I (R ~ P)	1	5	1
R or R > P	31	4	1
Viral	21	7	1
Viral + NASH/ASH	4	3	1
NASH/ASH	12	11	5
Total	37	21	7

ASH, Alcoholic steatohepatitis: I, Indeterminate; NASH, Non-alcoholic steatohepatitis; P, Progression; R: Regression.

NECROINFLAMMATION IN RELATION TO FIBROSIS QUALITY

Using our three-point grading system to score necroinflammatory activity, 37 of 65 cases demonstrated grade 1/minimal necroinflammatory activity and 21 showed grade 2/mild activity. Seven cases were scored as grade 3/severe activity (Table 5, Figure 2). The grade 1 cases were predominantly R (31 of 37; 84%). The majority of these cases (25 cases) were due to chronic viral hepatitis (Table 5; Figure 4). In the remaining six grade 1 cases (five P and one I), four were due to chronic viral infection (one case of incompletely treated HBV, one case of treated HBV and two cases of treated HCV), and two were due to ASH. The grade 2 (12 of 21; 57%) and grade 3 (five of seven; 71%) cases were predominantly P. Twenty-one of 29 cases (72%) due to chronic viral hepatitis had low necroinflammatory activity/grade 1, but a similar trend was not seen in cases of NASH/ASH ($P < 0.05$, Fisher's exact test; Table 5, Figure 3).

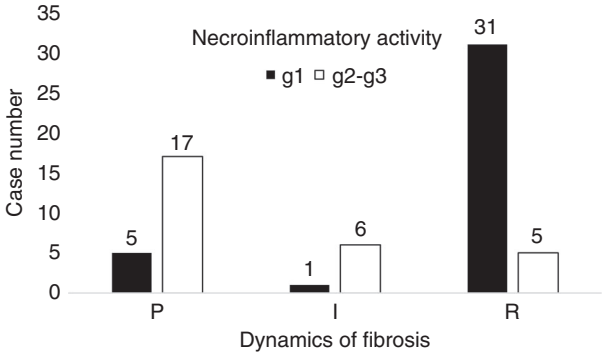

Figure 2. Distribution of necroinflammatory activity grades among qualitative changes in liver fibrosis. P: progressive, I: indeterminate, R: regressive, following reclassification by orcein (OR) and trichrome (TC) stains.

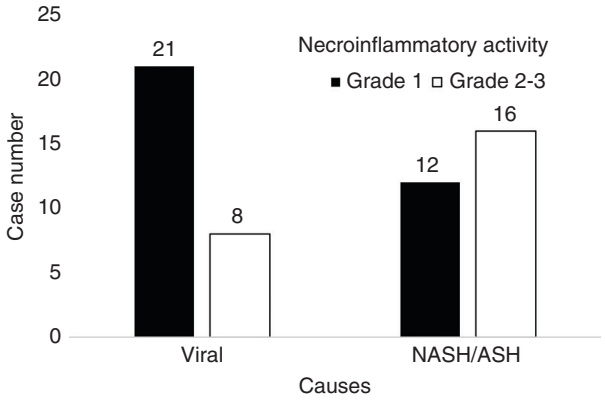

Figure 3. Distribution of necroinflammatory activity grades by different causes of cirrhosis. Cases with mixed causes not plotted. $P < 0.05$.

Discussion

This study identified a number of situations in which OR aids in understanding the quality of fibrosis to a greater extent than with TC alone. In cases of developing liver disease, a pattern of significantly more TC than OR was often observed. This finding is most probably reflective of progressive fibrosis, as collagen tends to accumulate more quickly than elastic fibres in cases of recent fibrosis (Figure 4A). Such cases were most common in NASH/ASH. Cases with a pattern of roughly equivalent OR and TC with focally increased TC were interpreted as minimal progression. Interestingly, in three cases that would be classified as P using the Beijing scheme, we noted a pattern of equivalent OR and TC with predominantly thick septa, probably representing an equilibrium state between deposition and resorption of the

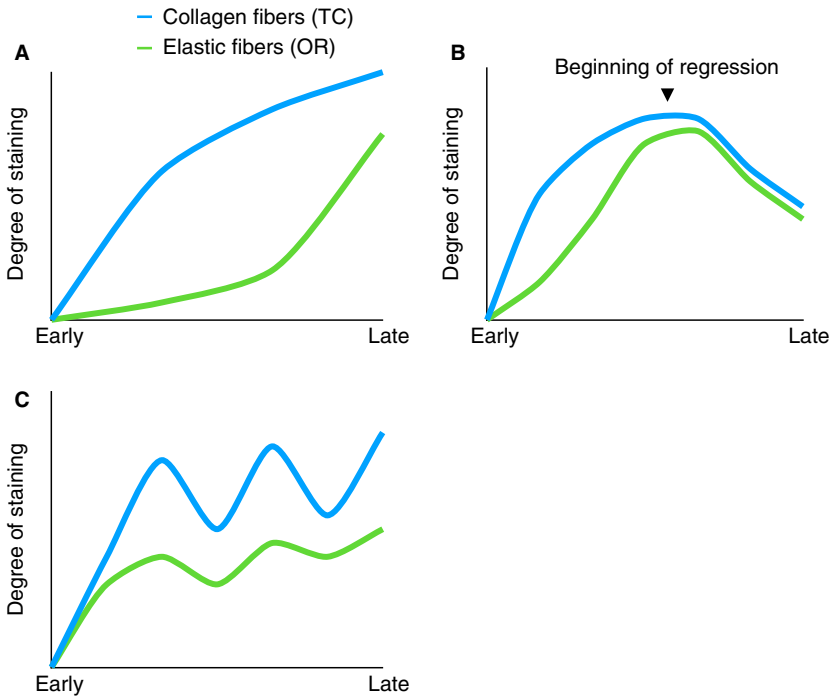

Figure 4. Patterns of trichrome (TC, collagen fibres) and orcein (OR, elastic fibres) staining in cirrhotic liver. A, Staining quality of TC and OR in progressive cirrhosis. B, Increase then decrease in staining quality in regressed cirrhosis. C, Non-linear quality of staining in incompletely treated cirrhosis (e.g. relapsed alcoholic cirrhosis).

extracellular matrix, and thus did not show a clear direction of fibrosis.[17] Such cases were reclassified as I.

With the help of OR, R cases increased from 42 to 55%. Adding OR was helpful in the Beijing I category[7,8] as the I cases decreased from 25 to 11%, with most of the I cases reclassified as (predominantly) R (63%). This suggests that OR can add to our ability to identify regressed features in cirrhosis. Cases with a pattern of equivalent OR and TC with thin, perforated septa suggest linear/continuous R.[1,7] This pattern was most common in cases with viral causes, especially in treated viral hepatitis. As the most recent practice guidelines for HBV/HCV include antiviral treatment prior to potential liver transplant,[18,19] it is not surprising that the specimens evaluated in this study (the majority of which had reached SVR) showed regressive features. Following full treatment of the viral infection, the virus-induced necroinflammatory activity should cease, allowing for the regression of cirrhosis (Figure 4B).

More variable patterns of P and R were seen in cases of ASH/NASH cirrhosis. In alcohol-related cirrhosis, regression could be indicative of cessation of alcohol intake prior to transplant surgery. Conversely, despite the development of biochemical markers of abstinence,[20] it is possible that some patients with ASH cirrhosis may have had multiple periods of abstinence with intervening periods of alcohol consumption, leading to a non-linear 'waxing-and-waning' pattern. Accordingly, the elimination of causes of cirrhosis would be less definitive than the treatment of viral infection (Figure 4C). We anticipate that a focused study on ASH/NASH cirrhosis cases of confirmed cessation of alcohol intake or strict diet restriction might demonstrate more definitive regressive fibrosis, as observed in successfully treated chronic HBV/HCV.

Various active and chronic injuries stimulate inflammatory responses that lead to liver fibrosis, and may further progress to cirrhosis. Our analysis showed a correlation of necroinflammation with the quality of fibrosis (Figures 2 and 3).[8,21] Both regressed cirrhosis and chronic viral infection were associated with minimal/grade 1 inflammation, while progressive cirrhosis was associated with more severe (≥ grade 2) inflammation. NASH/ASH cases did not show a significant association with the degree of inflammation. This is reminiscent of the more variable fibrotic patterns of R and P seen in cases of NASH/ASH, and is probably a consequence of the waxing and waning disease course.

We examined liver resection/explant specimens in contrast to the Beijing scheme,[8] which was developed for use on liver biopsies in chronic HBV. We found that many septa were difficult to fully evaluate on biopsy cores, and focal features could be easily missed. Nevertheless, our approach of using OR as an adjunctive stain to TC should be applicable to liver biopsy specimens, including those with more limited samples. Prior studies of regressed cirrhosis were limited, consisting of liver biopsy specimens from patients with cirrhosis due to viral disease: chronic HBV patients in China[7] or chronic HCV patients.[9] In contrast, the current study included a variety of liver resection specimens from an ethnically diverse patient population in a tertiary/quaternary academic centre with multiple causes of cirrhosis, including HBV, HCV, NASH and ASH.

A major limitation of this study is that it consisted of a significant number of explant specimens. As a result, the fibrotic and inflammatory aspects of these disease processes were evaluated at relatively late stages. At end-stage fibrosis, characteristic pathological features such as fat, ballooning and inflammation can disappear, resulting in histology that may be paradoxically pauci-inflammatory. Furthermore, abnormal vascular flow/ischaemic changes and cholestasis are more common in advanced fibrosis, and their associated inflammation may not be due to the original causes. These factors could have confounded our data. Additionally, this study did not examine patients with regressed cirrhosis who did not proceed to resection/explant. Examination of liver specimens at earlier stages of fibrosis could shed further light upon the bidirectional changes in fibrosis. Use of OR on liver biopsy specimens could allow for comparison of OR and TC during the course of disease for patients who had had multiple biopsies performed. As a proof-of-concept, we examined a total of nine liver needle core biopsy specimens with advanced fibrosis and found that similar histochemical patterns (i.e. TC >> OR, TC > OR, TC = OR with thin septa) could be recapitulated in various cases (Supporting information, Figure S1 and Table S1).

Other future studies may compare each disease category (viral hepatitis, autoimmune hepatitis, ASH and NASH) with different histopathological patterns, haemodynamics (hepatic vascular flow, ascites, varices) and clinical follow-up. In summary, OR stain can help to determine the quality of fibrosis in cases of advanced liver fibrosis, in particular with cases classified as 'indeterminate' by the Beijing criteria. The distinction of progressive and regressive fibrosis can be potentially useful to track and prognosticate patients' liver disease.

Acknowledgements

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Conflicts of interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Evaluation of the quality of advanced fibrosis by comparing orcein (OR) and trichrome (TC) stains in biopsies. A, B: Linear regression (TC = OR) in a non-alcoholic steatohepatitis (NASH) case. C, D: Progression of fibrosis in a case of NASH cirrhosis. A, C: TC stains. B, D: OR stains.

Table S1. Addition of Orcein Stains for Interpretation of Beijing Scheme in Small Liver Biopsies. Abbreviations: OR: orcein; TV: trichrome; R: regression; P: progression.

Data S1. Supporting Information.