Research Article

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Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: Implications for prognosis and clinical management

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Background and Aims: There remains uncertainty about the natural history of non-alcoholic fatty liver disease (NAFLD). The spectrum of NAFLD includes non-alcoholic fatty liver (NAFL; steatosis without hepatocellular injury) and steatohepatitis (NASH; steatosis with hepatocyte ballooning degeneration ± fibrosis). Our aim was to assess the histological severity of NAFLD in a cohort with serial biopsy data, and determine factors predicting progression.

Methods: Patients with two liver biopsies more than a year apart were identified. Clinical and laboratory data were collected from the time of liver biopsy.

Results: 108 patients had serial biopsies (median interval 6.6 years, range 1.3–22.6). 81 (75%) patients had NASH and 27 had NAFL. Overall, 45 (42%) patients had fibrosis progression, 43 (40%) had no change in fibrosis, while 20 (18%) had fibrosis regression. Importantly, no significant difference in the proportion exhibiting fibrosis progression was found between those with NAFL or NASH at index biopsy (37% *vs.* 43%, $p = 0.65$). Progression to NASH was seen in 44% of patients with baseline NAFL. Of 10 patients with NAFL who had fibrosis progression, 3 progressed by 1 stage, 5 by 2 stages and 2 by 3 stages; all had NASH on follow-up biopsy. Of concern, 6 of 27 (22%) patients with baseline NAFL, reached stage 3 fibrosis at follow-up biopsy. Among the patients with NAFL, 80% of those having fibrosis progression were diabetic at the follow-up liver biopsy compared with 25% of non-progressors ($p = 0.005$).

Conclusions: Contrary to current dogma, this study suggests that steatosis can progress to NASH and clinically significant fibrosis.

Keywords: NAFLD; NASH; Steatohepatitis; Fibrosis; Cirrhosis.
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Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of a metabolic syndrome and is currently the most common cause of liver disease in many developed countries worldwide [1–3]. Studies suggest that approximately 30% of the general population have radiological evidence of steatosis and 8% have raised transaminases due to NAFLD, although many go unrecognised [4–7]. NAFLD is defined as steatosis affecting >5% of hepatocytes in the absence of excessive significant alcohol consumption, other liver disease or the consumption of steatogenic drugs. The histological spectrum of NAFLD includes non-alcoholic fatty liver (NAFL; steatosis without hepatocellular injury), steatohepatitis (NASH; steatosis with inflammation and hepatocyte ballooning degeneration), fibrosis and ultimately cirrhosis [8]. Those patients who progress to cirrhosis are at risk of potentially life threatening liver-related complications such as portal hypertension, hepatic failure and hepatocellular carcinoma [9–13].

There remains considerable uncertainty about the natural history and prognosis of NAFLD. Despite its high prevalence, only a minority of NAFLD patients progress to significant fibrosis or experience associated morbidity [3]. This variability is in part due to subtle individual genetic differences that modify response to environmental factors and lifestyle, and so determine disease phenotype [14–16]. Few studies, together totalling approximately 400 patients, have examined the histological evolution of steatosis, steatohepatitis and fibrosis in NAFLD patients using paired biopsies [17–26]. In general, it is thought that fibrosis progression in patients with NAFL is uncommon, whereas NASH progresses more frequently [18,19,21–25]. However, two small studies have recently challenged this dogma, suggesting that NAFL can evolve to NASH with advanced fibrosis, which would imply that it may not be an entirely benign condition [20,26].

The aim of the DELTA study was to assess the change in histological severity and evolving natural history of NAFLD using serial liver biopsies in a histologically characterised patient cohort, and to determine which clinical factors were predictors of progressive hepatic fibrosis.

Materials and methods

Patients

Patients with 2 or more liver biopsies, taken at least 1-year apart, were identified from a sub-specialist tertiary NAFLD clinic at the Freeman Hospital, Newcastle upon Tyne, UK. Index liver biopsies were performed between 1991 and 2011 as part of investigation of abnormal liver function tests, or to stage disease severity, in patients with radiological evidence of NAFLD. Follow-up liver biopsies were conducted between 2001 and 2013 to assess disease progression or as an entry requirement for inclusion in a clinical trial. Due to the uncertain natural history of NAFLD, it was our usual practice to perform a follow-up liver biopsy at 5-yearly intervals to monitor for disease progression in pre-cirrhotic patients aged <65-years who failed lifestyle intervention. For patients with >2 liver biopsies, the first and last biopsies were used, unless the patient had participated in a therapeutic clinical trial where the pre-trial biopsy was used (therefore no patients received trial medications during the study period). Patients with alternate liver diagnoses or evidence of coexistent liver disease (haemochromatosis, viral hepatitis, Wilson's disease, alpha-1-antitrypsin deficiency or autoimmune liver disease) were excluded. Patients who consumed more than 30 g of alcohol per day for males or more than 20 g per day for females prior to the first biopsy or during the follow-up period were also excluded. Five patients with type 2 diabetes and NASH were receiving pioglitazone during the study period, but none received vitamin E. Fig. 1 summarises study recruitment as a CONSORT flow diagram. Clinical and laboratory data were collected from the time of liver biopsy. For patients who had liver biopsies prior to 1999, data was collected retrospectively from the time of liver biopsy or within 6 months, but data was collected prospectively since 1999. Relevant clinical details such as gender, age, weight, height and average current and previous alcohol intake (g/day) were obtained from all patients at the time of liver biopsy. The body mass index (BMI) was calculated by the formula: weight (kg)/height^2 (m^2). Patients were identified as having type 2 diabetes (T2DM) if they were receiving dietary, oral hypoglycaemic drug or insulin treatment for diabetes, or had fasting blood glucose >7.0 mmol/L or glucose >11.1 mmol/L following an oral glucose tolerance test. Blood tests taken at the time of liver biopsy or within 6-months were used to calculate the FIB-4 and Non-alcoholic fatty liver fibrosis scores (NAFLD fibrosis score) as previously described [27–29].

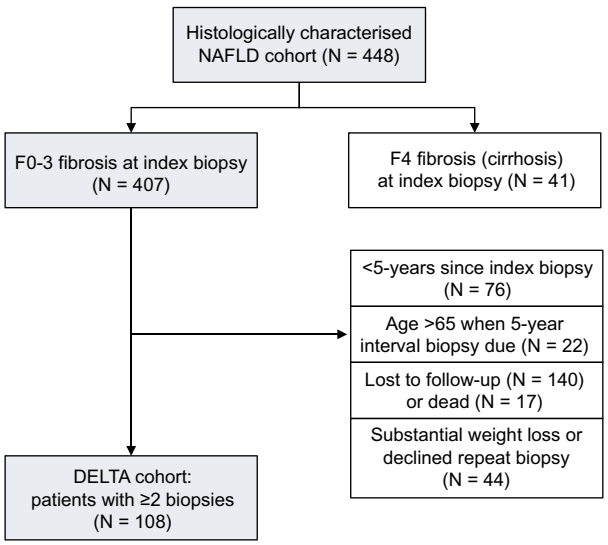

Fig. 1. DELTA study CONSORT diagram.

Histological assessment

Percutaneous liver biopsies were performed using a Menghini needle or an 18G BioPince liver biopsy system (Medical Devices Technologies, Gainesville, Florida, USA). Liver biopsies were all >15 mm in length and were read by an experienced hepatopathologist (ADB). Histological scoring was performed according to the NASH Clinical Research Network criteria (NASH CRN) [30]. The NAFLD activity score (NAS) was graded from 0 to 8 including scores for steatosis (0–3), lobular inflammation (0–3) and hepatocellular ballooning (0–2). Fibrosis was staged from 0 to 4. 'NASH' was defined as steatosis with hepatocyte ballooning degeneration and inflammation +/− fibrosis [31]. 'NAFL' was defined as steatosis only, or steatosis with mild inflammation without hepatocyte ballooning degeneration. 'Bland steatosis' was defined as steatosis, but no hepatocyte ballooning, inflammation or fibrosis. 'Steatosis and mild inflammation' was defined as steatosis with mild lobular inflammation with ⩽stage 1 fibrosis, but no hepatocyte ballooning. Three patients with significant liver injury (1 patient with steatosis and stage 3 fibrosis and 2 patients with steatosis, lobular inflammation and stage 2 fibrosis) were classified as ''NASH'' despite hepatocyte ballooning degeneration being absent. In these patients the cause of the significant liver injury was believed to be due to NASH and it is likely that sampling error played a role in the finding of absence of hepatocyte ballooning degeneration. The rate of fibrosis was calculated by: (last biopsy fibrosis stage – first biopsy fibrosis stage)/time between biopsies (years).

Statistical analysis

All statistical analyses were performed using SPSS software version 21.0 (SPSS Inc, Chicago, USA). Continuous normally distributed variables were represented as mean ± standard deviation (SD). Categorical and non-normal variables were summarised as median and range. Chi squared test or Fisher's exact test were used to determine the distribution of categorical variables between groups. To compare the means of normally distributed variables between groups the Student's *t* test was performed. To determine differences between groups for continuous non-normally distributed variables, medians were compared using the Mann-Whitney U test. Longitudinal changes in continuous variables were assessed by paired *t* test or Wilcoxon rank-sum test. Binary logistic regression was performed to identify factors associated with progression of fibrosis. Significant factors on univariate analysis were included in the multivariate analysis. The diagnostic performance of non-invasive tests was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUROC) was used as an index to compare the accuracy of tests. The sensitivity and specificity for relevant cut-offs were also displayed.

Results

As summarised in Fig. 1, from a total of 448 patients with histologically confirmed NAFLD, 108 patients were identified with at least 2 liver biopsies, more than 1-year apart, that met the inclusion/exclusion criteria. These patients comprised the DELTA study cohort that was used in subsequent analyses.

Cohort characteristics at baseline

Patient characteristics are summarised in Table 1. Two-thirds (71) of the cohort was male. At the time of the index biopsy, the mean age was 48 ± 12 years and 52 (48%) had T2DM.

Histological assessment determined that one-quarter (n = 27) of the patients had NAFL, of whom 17 had 'bland steatosis' and 10 had 'steatosis with mild inflammation' but without evidence of ballooning hepatocyte degeneration. Three-quarters (n = 81) of the patients had NASH with a median NAS of 4 (range: 1–8). Table 2 shows a comparison between subjects with NAFL and NASH at index biopsy. Compared with subjects with NAFL on index biopsy, patients with NASH were significantly older (p <0.001), more likely to be diabetic (p = 0.004) and had higher serum IgA levels (p = 0.01), AST/ALT ratio (p = 0.01), FIB-4 score

Table 1. Comparison of the clinical and demographic factors at baseline and follow-up liver biopsy.

Characteristic	Baseline	Follow up	*p* value
Age (years)	48 ± 12	55 ± 12	<0.001*
Gender (% male)	66%	66%	-
BMI (kg/m^2)	33.9 ± 5.0	34.9 ± 5.2	0.004*
T2DM	48%	65%	<0.001#
ALT (IU/L)	112 ± 80	79 ± 66	<0.001*
AST (IU/L)	73 ± 48	57 ± 35	0.01*
GGT (IU/L)	117 ± 105	148 ± 195	0.11*
Platelets (x10^9/L)	244 ± 67	230 ± 62	<0.001*
IgA (g/L)	2.88 ± 1.36	3.26 ± 1.50	0.06^
IgG (g/L)	12.7 ± 12.9	10.9 ± 3.1	0.24^
Ferritin	281 ± 536	194 ± 218	0.17*
AST/ALT ratio	0.7 ± 0.27	0.81 ± 0.30	<0.001*
FIB-4 score	1.5 ± 1.0	1.79 ± 1.39	0.036*
NAFLD score	-1.49 ± 1.42	-0.77 ± 1.38	<0.001*
NAS	4 (1-8)	4 (1-7)	0.64^
Steatosis	2 (1-3)	2 (1-3)	
Inflammation	1 (0-3)	1 (0-3)	
Ballooning	1 (0-2)	1 (0-2)	
Fibrosis stage	2 (0-3)	2 (0-4)	<0.001^
0	23 (21%)	23 (21%)	
1	29 (27%)	19 (18%)	
2	33 (31%)	19 (18%)	
3	23 (21%)	33 (31%)	
4	0 (0%)	13 (12%)	
Steatosis/NASH	27 (25%)/ 81 (75%)	21 (19%)/ 87 (81%)	0.14#

*Paired *t* test.
^Wilcoxon signed rank test.
#Chi Square test.
BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase; NAS, non-alcoholic fatty liver disease activity score; T2DM, type 2 diabetes mellitus.

Table 2. Comparison between patients with NAFL and those with NASH at baseline and follow-up liver biopsies.

Characteristic	NAFL N = 27	NASH n = 83	*p* value
Age (years)	41 ± 11	50 ± 12	<0.001*
Gender (% male)	67%	65%	0.91#
BMI (kg/m^2)	32.9 ± 5.2	34.1 ± 4.9	0.33*
T2DM	21%	56%	0.004#
ALT (IU/L)	119 ± 97	109 ± 75	0.64*
AST (IU/L)	64 ± 46	75 ± 49	0.40*
GGT (IU/L)	151 ± 113	109 ± 102	0.13*
Platelets (x10^9/L)	250 ± 80	243 ± 64	0.67*
IgG (g/L)	11.2 ± 2.1	13.1 ± 14.4	0.57*
IgA (g/L)	2.17 ± 1.02	3.05 ± 1.39	0.01*
AST/ALT ratio	0.54 ± 0.17	0.74 ± 0.28	0.01*
FIB-4 score	0.89 ± 0.42	1.62 ± 1.01	<0.001*
NAFLD score	-2.15 ± 1.49	-1.37 ± 1.38	0.07*
Baseline NAS	2 (1-4)	5 (1-8)	<0.001^
Baseline fibrosis	0 (0-1)	2 (0-3)	<0.001^
0	19 (70%)	4 (5%)	
1	8 (30%)	21 (26%)	
2	0	33 (41%)	
3	0	23 (28%)	
4	0	0	
Follow up NAS	3 (1-6)	4 (1-7)	0.001^
Follow up fibrosis	0 (0-3)	2 (0-4)	<0.001^
0	15 (56%)	8 (10%)	
1	4 (15%)	16 (20%)	
2	2 (7%)	17 (21%)	
3	6 (22%)	27 (33%)	
4	0	13 (16%)	
Fibrosis progressor	37%	43%	0.65#
Fibrosis rate (stage/yr)	0.067 ± 0.11	0.084 ± 0.29	0.67*
Time between biopsies	8 (1.7-22.6)	6.4 (1.3-18.5)	0.34^

*Student's *t* test.
^Mann-Whitney U test.
#Fisher's exact test.
BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase; NAS, non-alcoholic fatty liver disease activity score; T2DM,type 2 diabetes mellitus.

(p <0.001), NAS (p <0.001) and NASH CRN fibrosis stage (p <0.001).

Fibrosis stage on index biopsy ranged from 0 to 3 with each of these fibrosis stages well represented within the cohort. At the index biopsy stage of fibrosis was significantly associated with the NAS (p <0.001), NAFLD fibrosis score (p = 0.004; Fig. 2A), FIB-4 score (p <0.001; Fig. 2B), age (p = 0.006), BMI (p = 0.034) and the presence of diabetes (p <0.001).

Metabolic and histological evolution of disease during follow-up

The median time between the index and follow-up liver biopsy was 6.6 years (IQR: 3.8–9.0; range: 1.3–22.6 years). 73 (68%) of the patients had liver biopsies more than 5-years apart. Overall, at the time of follow-up liver biopsy, patient adiposity measured as BMI had significantly increased (mean 34.9 ± 5.0 *vs.* 33.9 ± 5.2 kg/m^2; p = 0.004). Patients were more likely to have T2DM (65% *vs.* 48%, p <0.001) and in general exhibited more advanced hepatic fibrosis (p <0.001). A detailed comparison of the clinical characteristics of the cohort at the time of index and follow-up biopsies is shown in Table 1.

Changes from baseline histological grade (activity of steatohepatitis) are shown in Table 3. Of the 81 patients with NASH on index biopsy, 75 (93%) still had NASH at follow-up while 6 had regressed to NAFL. Among the 27 patients with NAFL as base-

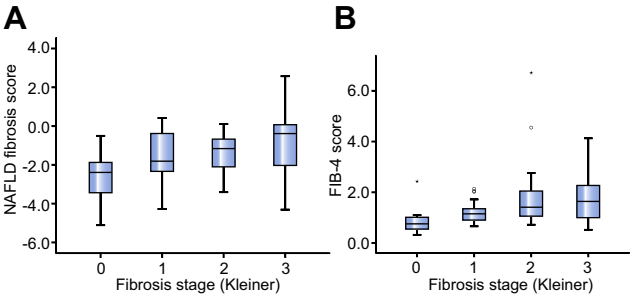

Fig. 2. Relationship between fibrosis stage and simple non-invasive fibrosis scores. Relationship between stage of fibrosis and (A) the NAFLD fibrosis score, and (B) the FIB-4 score.

Table 3. Distribution of histological disease activity at index and follow-up liver biopsies.

Baseline disease activity	Follow-up disease activity			
	Bland steatosis	Steatosis and mild inflammation	NASH	Total
Bland steatosis	7	**6**	**4**	17
Steatosis and mild inflammation	2	0	**8**	10
NASH	5	1	75	81
Total	14	7	87	108

Numbers in **bold** indicate progression of disease activity.

line, 12 (44%) patients had progressed to NASH at follow-up biopsy. The principal factor associated with significant changes in NAS between liver biopsies was change in BMI ($r_s = 0.23$, $p = 0.026$), with increased BMI potentially conferring an increased risk of steatohepatitis.

During the interval between biopsies 45 (42%) patients had progression of fibrosis, 43 (40%) patients had no change in their fibrosis scores, while 20 (18%) patients had regression of fibrosis. The distribution of changes in fibrosis stage between baseline and follow-up is shown in Table 4. Of the patients whose fibrosis progressed, 26 progressed by 1 stage, 15 by 2 stages and 4 by 3 stages. Overall, the mean rate of fibrosis was 0.08 ± 0.25 stages/year, increasing to 0.29 ± 0.24 stages/year when only those with disease progression were considered. For patients who had fibrosis regression, 17 patients regressed by 1 stage and 3 patients by 2 stages.

At follow-up liver biopsy 46 (43%) patients had advanced fibrosis (33 had stage 3 and 10 had cirrhosis (stage 4). These patients were more likely to have T2DM (89% *vs.* 47%, $p < 0.001$), and as expected exhibited significantly higher follow-up NAFLD fibrosis score ($p < 0.001$) and FIB-4 score ($p = 0.001$) than patients with less advanced fibrosis (stage 0–2). There was a significant relationship between the change in fibrosis stage between biopsies and the change in both NAFLD fibrosis score ($r_s = 0.24$, $p = 0.035$) and FIB-4 score ($r_s = 0.24$, $p = 0.033$). The NAFLD fibrosis score identified patients with advanced fibrosis on follow-up liver biopsy with reasonable accuracy (AUROC: 0.83, CI: 0.74–0.92, $p < 0.001$; 91% sensitivity and 46% specificity at a score of −1.455, and 28% sensitivity and 98% specificity at a score of 0.676), whereas the FIB-4 score had modest accuracy (AUROC: 0.72, CI: 0.62–82).

Table 4. Distribution of histological fibrosis at index and follow-up liver biopsy.

Baseline fibrosis	Follow-up fibrosis stage					
	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Total
Stage 0	16	**4**	**1**	**2**	**0**	23
Stage 1	6	7	**3**	**11**	**2**	29
Stage 2	1	7	11	**11**	**3**	33
Stage 3	0	2	4	9	**8**	23
Stage 4	0	0	0	0	0	0
Total	23	20	19	33	13	108

Numbers in **bold** indicate fibrosis progression.

Subgroup comparison between fibrosis progressors and non-progressors

To identify factors that were associated with progressive fibrosis, patients with histological evidence of increasing fibrosis stage (*progressors*) were compared to subjects whose fibrosis remained stable or regressed (*non-progressors*). As is shown in Table 5, although no significant difference in histological factors including NAS ($p = 0.19$), presence of steatohepatitis (78% *vs.* 73%, $p = 0.65$) or stage of fibrosis ($p = 0.90$) was evident between groups on index biopsy, *progressors* did have a significantly lower platelet count ($p = 0.04$), a higher AST/ALT ratio ($p = 0.04$) and higher FIB-4 score ($p = 0.02$) at that time. By the time that follow-up biopsies were performed, *progressors* continued to exhibit significantly lower platelet count ($p = 0.001$), higher AST/ALT ratio ($p = 0.01$,) FIB-4 score ($p = 0.001$). In addition, NAFLD fibrosis score ($p < 0.001$) was also significantly raised compared with *non-progressors*. At the time of follow-up biopsy, T2DM was significantly more common in patients with progressive fibrosis ($p < 0.001$) as was histological evidence of steatohepatitis (100% *vs.* 67%, $p < 0.001$) and grade of inflammation measured by NAS ($p < 0.001$).

Identification of factors at baseline predicting subsequent fibrosis progression

A multivariate analysis incorporating platelet count, AST/ALT ratio and FIB-4 score was conducted to identify clinical factors at the index biopsy that would predict subsequent progression of fibrosis. The FIB-4 score was the only significant baseline factor that predicted fibrosis progression (OR: 2.1, CI: 1.1–3.9, $p = 0.019$). However, the AUROC of the FIB-4 score for predicting progression of fibrosis was only 0.63 (CI: 0.51–0.76, $p = 0.036$).

Identification of factors at follow-up indicating presence of fibrosis progression

To identify clinical factors at the time of follow-up liver biopsy that were indicative of fibrosis progression, a multivariate analysis was conducted incorporating presence of T2DM, platelet count, GGT, AST/ALT ratio, FIB-4 score, NAFLD fibrosis score. The presence of T2DM (OR: 6.25, CI: 1.88–20, $p = 0.003$), and FIB-4 score (OR: 3.1, CI: 1.4–6.8, $p = 0.004$) at the time of follow-up liver biopsy were significant indicators of presence of fibrosis progression.

Histological evolution of patients with NAFL on the index liver biopsy

An important and as yet unresolved clinically relevant question is whether patients without evidence of active steatohepatitis may subsequently develop progressive liver disease. Among the 27 patients with NAFL on the index biopsy, 12 (44%) had evidence of NASH on the follow-up liver biopsy over a median follow-up period of 8 years (range: 1.7–22.6 years; IQR: 6.4–10 years). At the follow-up liver biopsy, 10 (37%) patients with NAFL had progression of fibrosis by ⩾1 stage of fibrosis (3 patients by 1 stage, 5 by 2 stages and 2 by 3 stages). The time between biopsies for the patients who progressed by 3 stages were 15.6 and 19.2 years. For the patients who progressed by 2 stages the interval ranged from 6.4 to 22.6 years. 6 (22%) of the patients with NAFL at baseline had stage 3 fibrosis at the follow-up biopsy,

but none were cirrhotic. There was a trend towards more patients with steatosis and mild inflammation having fibrosis progression than patients with bland steatosis (60% *vs.* 24% respectively, $p = 0.07$).

Among the patients with NAFL at baseline, 80% of those who had fibrosis progression were diabetic at the follow-up liver biopsy (including 5 patients with new onset diabetes), compared with 25% of the non-progressors ($p = 0.005$). Patients with NAFL who had fibrosis progression also had significantly higher baseline NAS (2.5 (2–3) *vs.* 1 (1–4), $p = 0.007$) and follow-up NAS scores (4.5 (3–6) *vs.* 2 (1–4), $p < 0.001$) compared with those NAFL patients that did not progress.

Of the 17 patients with bland steatosis at the index biopsy, 4 (24%) patients had fibrosis progression (2 subjects with stage 1 fibrosis after 5 and 15 years, 1 patient with stage 2 fibrosis after 5 years and 1 stage 3 fibrosis after 5 years). In these patients, baseline and follow-up NAS scores were significantly higher in *progressors* than *non-progressors* (2 (2–3) *vs.* 1(1–2), $p = 0.01$ and 4.5 (3–5) *vs.* 2 (1–4), $p = 0.006$ respectively).

Discussion

Due to the high prevalence of NAFLD in the community, determining the natural history of this disease is vitally important. The main aim of this study was to assess the histological evolution of NAFLD in our cohort of patients with sequential liver biopsies to look for clinical factors that predict disease progression. Overall, we found that NAFLD has a very variable natural history with 42% of patients having progression of fibrosis and 18% having regression of fibrosis over a median follow-up period of 6.6 years, which is in agreement with previous studies [17–26]. It is generally believed that few patients with NAFL develop progressive liver fibrosis, whereas NASH has significant fibrogenic potential and frequently progresses. One of the key findings of the present study is that, in contrast to current dogma, 44% of the patients with NAFL at the index liver biopsy progressed to NASH and 37% had progression of fibrosis, including 6 (22%) patients who developed advanced (stage 3) fibrosis.

Interestingly, three other recent studies have also demonstrated that NAFL has the potential to progress to NASH with fibrosis. The first study was conducted by Wong *et al.* and prospectively performed liver biopsies at 3 years in a cohort of 52 patients with NAFLD, 29 of whom had a baseline NAS <3. In that study, fibrosis progression was demonstrated in 28% of patients with a NAS <3 at baseline, and increases in NAS were seen in 58% [20]. More recently a retrospective French study that included 25 patients with NAFL found that 16 (64%) of these patients developed NASH and 6 (24%) progressed to bridging fibrosis after a mean follow-up period of 3.7 years [26]. A meta-analysis has now been reported looking at rates of fibrosis progression in 411 patients with NAFLD who had paired liver biopsies [17]. In that study, fibrosis progression was also seen in patients with NAFL as baseline, although the rates of progression were slower than in patients who had NASH at index biopsy. This meta-analysis did have some limitations; in particular, it included a heterogeneous group of studies that had different histological definitions of NAFL and NASH, and 7 of the studies included had less then 25 patients. However, taken together with our results, these studies clearly demonstrate that NAFL can progress to NASH with significant fibrosis. These findings suggest that current guidelines on the management of NAFLD [8] should be revised to indicate that NAFL has the potential to progress. Although we have demonstrated that fibrosis progression can occur in patients with NAFL, it remains unknown whether this will have an impact or liver-related morbidity and mortality, and large long-term prospective studies are required to answer this question.

Even in the absence of NASH, lobular inflammation has been identified as a predictor of fibrosis [22] or progression of fibrosis [26]. In our cohort of patients with NAFL, some had "steatosis with mild inflammation", so it could be argued that the presence of inflammation in these patients puts them at risk of the fibrosis progression. However, we also found progression of fibrosis in patients with bland steatosis on the index biopsy. Interestingly, in all those patients with bland steatosis who had fibrosis progression, steatosis evolved to steatohepatitis and the majority were diabetic or became diabetic at follow-up. This suggests that rather than being distinct entities, steatosis and steatohepatitis represent different stages in the evolution of NAFLD, and that increases in insulin resistance might be a key factor in the progression from steatosis to steatohepatitis with fibrosis.

One of the aims of the present study was to identify clinical factors that can identify patients with fibrosis progression. Unfortunately none of the baseline factors assessed in this study has sufficient accuracy to be useful clinically to predict fibrosis progression. However, at the follow-up liver biopsy the presence of diabetes was an independent predictor of fibrosis progression. T2DM is a well-recognised risk factor for progression of NASH [23], and has been shown to be an independent risk factor for mortality in patients with NAFLD [32]. Therefore, targeting patients with NAFLD and T2DM for regular liver reassessments to identify subclinical progression seems logical as they are at highest risk of developing progressive disease.

Identification of patients with advanced fibrosis due to NASH is important so that they can be screened for liver-related complications such as hepatocellular carcinoma or portal hypertension. Previous studies have shown that simple non-invasive scores for fibrosis, such as the NAFLD fibrosis score and FIB-4, are effective in identifying patients with advanced fibrosis in NAFLD [28,33–36]. In the present study, there was a significant relationship between the change in NAFLD fibrosis score and change in fibrosis stage, and the NAFLD fibrosis score was quite effective at identifying patients with advanced fibrosis on the follow-up liver biopsy (AUROC: 0.83). Therefore, until a more accurate non-invasive test is widely available, the NAFLD fibrosis score might offer a simple score that could be used to monitor patients, and identify those requiring further investigations to confirm advanced fibrosis/cirrhosis. Interestingly, a recent study has shown that the NAFLD fibrosis score is also effective in predicting liver-related complications and death in patients with NAFLD [37].

This study does have some weaknesses. Firstly, this was a retrospective study, and therefore there was a potential for selection bias that might have influenced the observed rates of progression/regression. However, due to the uncertain natural history of NAFLD, it was our usual unit practice to perform follow-up liver biopsies at approximately 5-year intervals in patients who had failed lifestyle interventions. Even with the potential selection bias the finding that NAFL can evolve to NASH with significant fibrosis holds true. Secondly, as with any study using liver biopsy as a standard, sampling error might have led to miss-diag-

Table 5. Clinical factors at baseline and follow-up biopsy for all patients and a comparison between patients with fibrosis progression and those without progression.

Characteristic	All patients (n = 108)	No progression of fibrosis (n = 63)	Progression of fibrosis (n = 45)	*p* value
Results at baseline biopsy				
Age (years)	48 ± 12	47 ± 12	49 ± 13	0.39*
Gender (% male)	66%	67%	64%	0.81#
BMI (kg/m^2)	33.9 ± 5.0	33.4 ± 4.1	34.5 ± 5.8	0.28*
Change in BMI (kg/m^2)	1.0 ± 3.4	0.92 ± 2.6	1.1 ± 4.2	0.77*
T2DM	48%	43%	53%	0.30
ALT (IU/L)	112 ± 80	113 ± 82	110 ± 77	0.89*
AST (IU/L)	73 ± 48	65 ± 40	84 ± 57	0.06*
GGT (IU/L)	117 ± 105	112 ± 91	124 ± 122	0.55*
Platelets (x10^9/L)	244 ± 67	255 ± 69	229 ± 60	0.04*
IgA (g/L)	2.88 ± 1.36	2.87 ± 1.48	2.88 ± 1.19	0.96*
IgG (g/L)	12.7 ± 12.9	11.7 ± 2.6	14.2 ± 20	0.36*
Ferritin	281 ± 536	322 ± 700	230 ± 187	0.40*
AST/ALT ratio	0.7 ± 0.27	0.65 ± 0.22	0.78 ± 0.32	0.04*
FIB-4 score	1.5 ± 1.0	1.26 ± 0.57	1.85 ± 1.31	0.02*
NAFLD score	-1.49 ± 1.42	-1.71 ± 1.23	-1.18 ± 1.62	0.11*
NAS	4 (1-8)	4 (1-8)	4 (2-7)	0.19^
Steatosis	2 (1-3)	2 (1-3)	2 (1-3)	0.08^
Inflammation	1 (0-3)	1 (0-3)	1 (0-3)	0.89^
Ballooning	1 (0-2)	1 (0-2)	1 (0-2)	0.08^
Fibrosis stage	2 (0-3)	2 (0-3)	1 (0-3)	0.90^
0	23 (21%)	16 (25%)	7 (16%)	
1	29 (27%)	13 (21%)	16 (36%)	
2	33 (31%)	19 (30%)	14 (31%)	
3	23 (21%)	25 (24%)	8 (18%)	
4	0 (0%)	0 (0%)	0 (0%)	
Steatosis/NASH	27 (25%)/81 (75%)	17 (27%)/46 (73%)	10 (22%)/35 (78%)	0.65#
Results at follow up biopsy				
BMI (kg/m^2)	34.9 ± 5.2	34.4 ± 4.7	35.6 ± 5.9	0.27*
T2DM	65%	51%	84%	<0.001
ALT (IU/L)	79 ± 66	82 ± 77	76 ± 48	0.63*
AST (IU/L)	57 ± 35	52 ± 34	63 ± 36	0.13*
GGT (IU/L)	148 ± 195	109 ± 143	202 ± 239	0.03*
Platelets (x10^9/L)	230 ± 62	248 ± 51	208 ± 69	0.001*
IgA (g/L)	3.26 ± 1.50	2.95 ± 1.32	3.7 ± 1.65	0.05*
IgG (g/L)	10.9 ± 3.1	11.2 ± 3.3	10.5 ± 2.7	0.4*
Ferritin	194 ± 218	199 ± 205	187 ± 237	0.81*
AST/ALT ratio	0.81 ± 0.30	0.74 ± 0.29	0.89 ± 0.29	0.01*
FIB-4 score	1.79 ± 1.39	1.36 ± 0.62	2.33 ± 1.69	0.001*
NAFLD score	-0.77 ± 1.38	-1.35 ± 1.08	-0.07 ± 1.40	<0.001*
NAS	4 (1-7)	3 (1-6)	5 (3-7)	<0.001
Fibrosis stage	2 (0-4)	1 (0-3)	3 (1-4)	<0.001^
0	23 (21%)	23 (37%)	0 (0%)	
1	19 (18%)	16 (25%)	4 (9%)	
2	19 (18%)	15 (24%)	4 (9%)	
3	33 (31%)	9 (14%)	24 (53%)	
4	13 (12%)	0 (0%)	13 (29%)	
Steatosis/NASH	25 (23%)/83 (77%)	21 (33%)/42 (67%)	0 (0%)/44 (100%)	<0.001#
Time between biopsy (yr)		6.7 ± 3.5	7.5 ± 5	0.35

Student's *t* test.
^Mann-Whitney U test.
#Fisher's exact test.
BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase; NAS, non-alcoholic fatty liver disease activity score; T2DM, type 2 diabetes mellitus.

nosis of disease activity and fibrosis stage at either biopsy [38]. The potential for sampling error in this study was minimised by including only patients with biopsies >15 mm. Moreover, with our sample size, the proportion of patients with upstaging or downstaging of fibrosis or disease activity should equal out, such that our conclusions are valid. Thirdly, as this was not a controlled trial the impact of potentially confounding factors such as glycaemic control or prescribed medications (e.g. anti-hypertensives or anti-diabetic agents) on the natural history of NAFLD could not be assessed.

In conclusion, this study demonstrates that NAFLD has a variable natural history. Importantly and contrary to current dogma, we have demonstrated that NAFL has the potential to progress to NASH with advanced fibrosis, particularly in patients who have or develop diabetes.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

SM, QMA, CPD: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. ADB, EH, and TH: acquisition of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content.

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