Second harmonic generation microscopy can quantify and subclassify early stages of NASH fibrosis progression: data from a screen-failure cohort of a NASH phase 2 study

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INTRODUCTION

- Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease with an estimated global prevalence of approximately 25%[1]
- NAFLD comprises a spectrum of liver injury which can vary in severity, with non-alcoholic steatohepatitis (NASH) representing the progressive subtype of NAFLD[2]
- Assessment of liver biopsies with the grading and staging systems proposed by the non-alcoholic steatohepatitis (NASH) clinical research network (CRN)[3] is widely used in clinical trials due to regulatory authority guidance[2]
- Conventional microscopy with histopathologist scoring using the NASH CRN criteria is the most common method which provides a semiquantitative assessment of NASH severity
- More recently, digital quantification of liver histology features with artificial intelligence analyses is becoming increasingly recognised as a key approach in quantifying NASH[4,5], as these techniques have the potential to provide novel analyses of liver histology, aiding our understanding of natural history and therapeutic response
- The use of second harmonic generation/two-photon excitation fluorescence (SHG/TPEF) microscopy with computer-assisted analyses may provide a standardised and reproducible approach for precise quantitative assessment of NASH histology

AIM

- The aim of this exploratory study was to apply SHG/TPEF microscopy with computer-assisted analyses for precise quantification of liver fibrosis in patients with NAFLD, and determine its added benefit when used in parallel to conventional liver histology assessment in evaluating liver biopsies

METHOD

- This study analysed liver biopsies from patients with NAFLD who failed screening for a Phase 2, randomised, double blind, placebo-controlled trial, which assessed the combination of tropifexor and cenicriviroc in patients with NASH (TANDEM, NCT03517540)
- SHG/TPEF microscopy was used to assess liver fibrosis on a continuous scale (qFibrosis); these scores were also converted into categorical scores (qF0–qF4) using cut-offs which have previously been reported[6]
- The Genesis-®200 SHG/TPEF imaging system (Histoindex, Singapore) is an objective and reproducible imaging technique which can quantify collagen fibres present in multiple areas of the liver including the overall area, central vein, portal tract and perisinusoidal regions[6]
- Collagen parameters for qFibrosis were measured in portal tract, peri-portal, pericentral, central vein and perisinusoidal regions in zones 1, 2 and 3, where zones 1 and 3 are defined as 100 µm away from the end of collagen connected to vessel structures
- Independently, all biopsies were assigned a NASH CRN fibrosis stage (F0–F4)[3] by an expert central pathologist
- Two-sample t-tests were performed to assess the statistical differences in the zonal fibrosis quantification when comparing the F1 and F2 patient cohorts according to (a) NASH CRN, and (b) qFibrosis staging. Further, Spearman's correlation coefficient was used to assess the correlation between NASH CRN fibrosis and qFibrosis. Significance was set at $p < 0.05$

RESULTS

- A total of 138 patients with NAFLD were included in this study
- Overall, qFibrosis scores correlated moderately with NASH CRN scores (continuous [r = 0.59]; categorical [r = 0.58])
- The main difference in staging between SHG/TPEF microscopy and NASH CRN was in patients with qF1/F1 (qF1 [n = 25] vs F1 [n = 61]), qF2/F2 (qF2 [n = 42] vs F2 [n = 7]) and qF3/F3 (qF3 [n = 29] vs F3 [n = 12]) fibrosis, while smaller differences were observed in patients with qF0/F0 (qF0 [n = 30] vs F0 [n = 36]) and qF4/F4 (qF4 [n = 12] vs F4 [n = 10]) fibrosis (**Table 1**)
- SHG/TPEF staging classified 60.1% of patients with qF2–qF4 fibrosis while this proportion was markedly lower (23.0%) based on CRN staging of the same cohort, highlighting that SHG/TPEF staging may identify patients with more advanced fibrosis in NAFLD compared to CRN staging
- The proportion of patients in each fibrosis stage based on NASH CRN staging and SHG/TPEF microscopy staging is shown in **Figure 1**

Table 1. Fibrosis staging based on SHG/TPEF microscopy (qF0–qF4) and NASH CRN (F0–F4).

Fibrosis stage	SHG/TPEF microscopy staging, n (%)	NASH CRN staging, n (%)
qF0/F0	30 (22)	36 (29)
qF1/F1	25 (18)	61 (48)
qF2/F2	42 (30)	7 (6)
qF3/F3	29 (21)	12 (10)
qF4/F4	12 (9)	10 (8)
Total	**138**	**126***

*Note that NASH CRN staging was not available for 12 biopsies.
CRN, clinical research network; F, fibrosis stage; qF, qFibrosis stage; n, number of patients in group; NASH, non-alcoholic steatohepatitis; SHG, second harmonic generation; TPEF, two-photon excitation fluorescence.

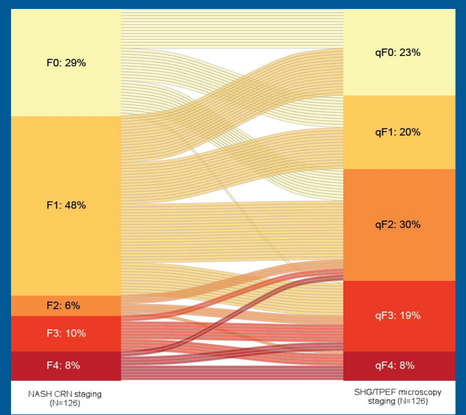

Figure 1. Classification of fibrosis based on NASH CRN staging and SHG/TPEF microscopy staging.

A Sankey diagram showing the proportion of patients in each fibrosis stage based on NASH CRN staging and SHG/TPEF microscopy staging. The lines between each staging system detail how patients are classified using each system. Percentages detail the proportion of the total cohort in each fibrosis stage.
CRN, clinical research network; N, number of patients with available data; NASH, non-alcoholic steatohepatitis; SHG, second harmonic generation; TPEF, two-photon excitation fluorescence.

- SHG/TPEF microscopy identified significant differences in portal ($p = 0.004$), periportal ($p < 0.001$) and portal+periportal+zone 1 ($p = 0.001$) fibrosis areas between qF1 and qF2 stages, as well as differences between qF1 and qF2 stages in zone 1 fibrosis areas alone ($p = 0.005$) (**Table 2**)
- When fibrosis was assessed on a continuous scale in the F1 and F2 patient cohorts (using staging based on SHG/TPEF microscopy), the data suggested that the NASH CRN staging system identified significant differences in the localization of fibrosis in portal ($p < 0.001$), periportal ($p = 0.030$) and portal+periportal+zone 1 ($p < 0.001$) fibrosis areas, but not in zone 1 fibrosis areas alone ($p = 0.211$), between F1 and F2 stages (**Table 2**)
- However, there were no significant differences in the mean fibrosis quantification in other acinar zones for both SHG/TPEF microscopy staging and NASH CRN staging

Table 2. Quantitative fibrosis measurements by SHG/TPEF in lobular regions based on staging by SHG/TPEF microscopy and NASH CRN. Periportal and pericentral areas were set at 100 µm from the portal tract and central vein, respectively

SHG/TPEF microscopy staging			
Lobular regions	**qF1, mean (n = 25)**	**qF2, mean (n = 42)**	**p value**
Portal	1.490	2.879	0.004
Periportal	0.659	1.242	<0.001
Perisinusoidal (zone 1)	0.080	0.161	0.005
Perisinusoidal (zone 2)	1.129	1.309	0.342
Perisinusoidal (zone 3)	0.093	0.096	0.881
Pericentral	0.292	0.312	0.688
Central vein	0.339	0.448	0.209
Portal+periportal+perisinusoidal (zone 1)	2.228	4.282	0.001
NASH CRN staging			
Lobular regions	**F1, mean (n = 61)**	**F2, mean (n = 7)**	**p value**
Portal	2.082	5.185	<0.001
Periportal	0.974	1.473	0.030
Perisinusoidal (zone 1)	0.119	0.170	0.211
Perisinusoidal (zone 2)	1.287	1.121	0.658
Perisinusoidal (zone 3)	0.096	0.082	0.678
Pericentral	0.318	0.372	0.549
Central vein	0.401	0.463	0.624
Portal+periportal+perisinusoidal (zone 1)	3.176	6.828	<0.001

CRN, clinical research network; F, fibrosis stage; qF, qFibrosis stage; n, number of patients in group; NASH, non-alcoholic steatohepatitis; SHG, second harmonic generation; TPEF, two-photon excitation fluorescence.

- **Figure 2** details the microscopic imaging produced by SHG/TPEF microscopy of key features of liver histology; qFibrosis is the overall output from assessment of fibrosis in the liver specimen comprising the quantitative readouts in different areas of the liver lobule (**Figure 2A**)
- The periportal and pericentral areas are set at 100 µm from the portal tract and central vein, respectively, and the region in between is the perinisinusoidal area (**Figure 2B**). The 100 µm is an approximation, based on a tenth of the average distance between the portal tract and central vein in a normal liver. Further expansion of the "peri" region to 200 µm and 400 µm were also considered and are shown below (**Figure 2C–D**)
- The demarcation of 100/200/400 µm for the peri region is, however, not part of the NASH CRN staging system, and different interpretations of this region would have an impact on the evaluation of F1/F2 stages. Hence, the number of patients being staged F1 or F2 may vary, which may explain the differences observed in **Table 2**

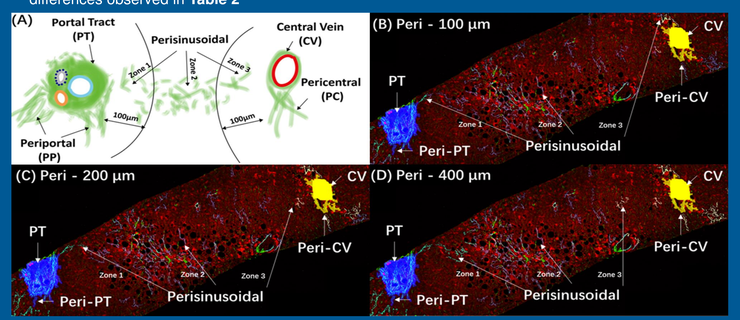

Figure 2. Key histologic areas of NASH liver biopsies using SHG/TPEF microscopy.

Figures detailing **(A)** diagrammatic images of zonal regions in the liver lobule; **(B)** SHG/TPEF image with periportal and pericentral areas set at 100 µm from the portal tract and central vein; **(C)** SHG/TPEF image with periportal and pericentral areas set at 200 µm from the portal tract and central vein; and **(D)** SHG/TPEF image with periportal and pericentral areas set at 400 µm from the portal tract and central vein.

CV, central vein; PC, pericentral; PP, periportal; PT, portal tract; SHG, second harmonic generation; TPEF, two-photon excitation fluorescence.

CONCLUSIONS

- SHG/TPEF microscopy with computer-assisted analyses is a sensitive and reproducible methodology that allows quantitation and categorisation of changes in liver fibrosis that might not be identified using the NASH CRN staging system
- SHG/TPEF microscopy can assess an increase in collagen fibres in perisinusoidal zone 1 fibrosis areas between stages qF1/F1 and qF2/F2 compared with NASH CRN, which is a key feature of differentiating between these fibrosis stages
- Further research is needed to determine the clinical significance of such changes and select the parameters to be used when evaluating clinical trial inclusion and efficacy of therapeutic interventions, including investigational medicines
- SHG/TPEF microscopy and digital pathology is a useful tool, in parallel with the NASH CRN scoring system, in gaining additional and detailed information and assessing liver fibrosis

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