Biomarker Qualification Letter of Intent (LOI)

ADMINISTRATIVE INFORMATION

1. **Submission Title**

   "Second Harmonic Generation (SHG) and Machine-Learning based Model for a Stain-free Measurement of Fibrosis (qFibrosis) in Non-Alcoholic Steatohepatitis (NASH) Clinical Trials"

2. **Requesting Organization**

   Histoindex Pte Ltd
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3. **Submission Date**

   March 11, 2022

DRUG DEVELOPMENT NEED STATEMENT

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of histological changes ranging from isolated fatty infiltration of the liver (isolated steatosis), to progressive liver damage with liver inflammation and fibrosis (steatohepatitis) and ultimately liver cirrhosis. NAFLD can be divided into three consecutive stages - nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH) and NASH with cirrhosis. NASH with liver fibrosis is a serious and life-threatening condition, which was confirmed in the recent publication by the FDA and its new Division of Hepatology and Nutrition.[1]

Currently, there are no approved therapies for NASH. Approximately 30 - 40% of patients who develop NASH will develop fibrosis to a varying degree, and 15 - 20% of those with fibrosis will go on to develop cirrhosis, which is in turn associated with an elevated risk for decompensated cirrhosis, hepatocellular carcinoma (HCC), requirement for liver transplantation and liver-related mortality.[2] Hence, its increasing prevalence has notable implications in terms of the clinical and economic burden of disease on national and global levels.

Assessment of liver histology plays a critical role in NASH clinical trials – for diagnosis and, histopathology scoring of NASH features is the current reference method to determine inclusion in trials and change in disease activity and fibrosis stage. Patients with noncirrhotic NASH coupled with significant and advanced liver fibrosis – fibrosis stage 2 (F2) and stage 3 (F3) respectively (NASH CRN staging system)[3] are eligible for enrolment in pre-cirrhotic clinical trials to evaluate new drugs for NASH treatment, following the accelerated approval pathway. However, conventional histological assessment requires staining of liver sections with subsequent review, which makes it complex, subjective, and prone to inter- and intra-reader variability and error. A recent interobserver study highlighted the discordance of assessment of all features of NASH, including fibrosis (linearly weighted kappa for fibrosis 0.609), with the implication of this being that trial entry criteria had only been met in 53.7% of biopsies re-read at the end of the study.[4] These reported levels of substantial inter- and intra-observer variations are a major concern as it affects study enrollment and assessment of drug efficacy, having a negative impact on drug development and preventing patient access to potential treatments.

There is a pressing need for reproducible, objective, and standardized evaluation of liver fibrosis to identify subjects who will fulfill the histopathologic criteria for NASH with fibrosis stage 2 or stage 3.[3] The use of qFibrosis as an aid to the pathologist will allow a reliable differentiation (or exclusion) of NAFLD patients with no fibrosis (F0) or minimal fibrosis (F1) at the one end of the spectrum, as well as patients with established cirrhosis (F4) at the other end of the spectrum.

BIOMARKER INFORMATION AND INTERPRETATION

1. Biomarker

Biomarker Name: qFibrosis

Type of Biomarker: Histology based quantitation of liver fibrosis using stain-free imaging modality

BEST Classification: Diagnostic biomarker

2. Analytical Methods

Imaging of the unstained slides are conducted using second harmonic generation (SHG) microscopy to visualize collagen. The samples were laser excited at 780 nm and SHG signals were recorded at 390 nm. Using the NASH CRN scoring system (Table 1) as the reference standard, automated measure of fibrosis was developed in the training group and validated in the validation group.

Fibrosis Stage	
0	None
1a	Mild, zone 3 perisinusoidal
1b	Moderate, zone 3 perisinusoidal
1c	Periportal sinusoidal fibrosis without accompanying zone 3 fibrosis
2	Zone 3 perisinusoidal and portal/periportal
3	Bridging fibrosis
4	Cirrhosis

Table 1. NASH-CRN developed Fibrosis Scoring System.[3]

The sequential procedure for establishing the algorithm includes (1) detection of collagen parameters in different regions of the lobules; (2) quantification of the architectural parameters characteristic of NASH fibrosis features; (3) selection of the most significant

parameters; and (4) model construction, combination of parameters into a single index for fibrosis.

Collagen parameters are measured as its physical properties such as length, width, intersections. A list of examples for these parameters are demonstrated in Table 2. A full list of parameters has been previously reported.[5]

No.	Abbreviation	Description
1	CollagenAreaAll	The area of collagen at overall region
2	FiberAreaAll	The area of fibers at overall region
3	FiberAWidthAll	The total average width of fibers at overall region
4	FiberMWidthAll	The total maximal width of fibers at overall region
5	FiberLengthAll	The total length of fibers at overall region
6	FiberPerimeterAll	The total perimeter of fibers at overall region
7	#FiberAll	The number of fibers at overall region
8	#LongFiberAll	The number of long fibers at overall region
9	#ShortFiberAll	The number of short fibers at overall region
10	#ThickFiberAll	The number of thick fibers at overall region
11	#ThinFiberAll	The number of thin fibers at overall region
12	AggAreaAll	The area of aggregated fibers at overall region
13	#IntersectionAll	The number of intersections at overall region

Table 2. List of collagen fiber parameters.

A total of 128 collagen parameters were quantified at the central vein (CV), portal tract (PT), and perisinusoidal (PS) regions, and the number of parameters required to optimally assess fibrosis was refined during the algorithm training process. Samples were assigned *a priori* using stratified randomization to either the training or the validation group.

The qFibrosis algorithm outputs a numerical index that indicates the severity of fibrosis with value ranging between 0 and 6.55 and based on its distribution relative to the NASH CRN categorical staging, cut-off values convert the numerical index to qFibrosis stages.

3. Measurements Units and Limits(s) of Detection:

Fibrosis features used in CRN system will be measured and quantified by the model to determine the final qFibrosis measurement of F0, F1, F2, F3, F4, and it has no unit.

4. Biomarker Interpretation and Utility:

Interpretation of qFibrosis measurement is similar to that of the CRN staging system i.e.,

F0, F1, F2, F3 and F4. The central pathologist will take into consideration the qFibrosis measurement when making the final fibrosis assessment.

CONTEXT OF USE STATEMENT

A diagnostic biomarker that is stain-free and AI-based, intended for use, in conjunction with clinical factors, to identify patients likely to have liver biopsy histopathologic findings of nonalcoholic steatohepatitis (NASH) and with a nonalcoholic fatty liver disease activity score (NAS) ≥4 and liver fibrosis stages 2 or 3 (NASH CRN system); and thus, appropriate for inclusion in liver biopsy-based NASH drug development clinical trials focused on pre-cirrhotic stages of NASH.

ANALYTICAL CONSIDERATIONS

Key fibrosis feature(s) according to the definition established by the CRN system was shown earlier in Table 1, where F1 is characterized by sinusoidal fibrosis, F2 is both sinusoidal and portal, bridging fibrosis in F3, and cirrhotic nodules in F4. Based on this, the qFibrosis algorithm is designed by identifying these collagen features in these regions as described by the CRN system in a machine learning-based manner to provide an objective fibrosis evaluation.

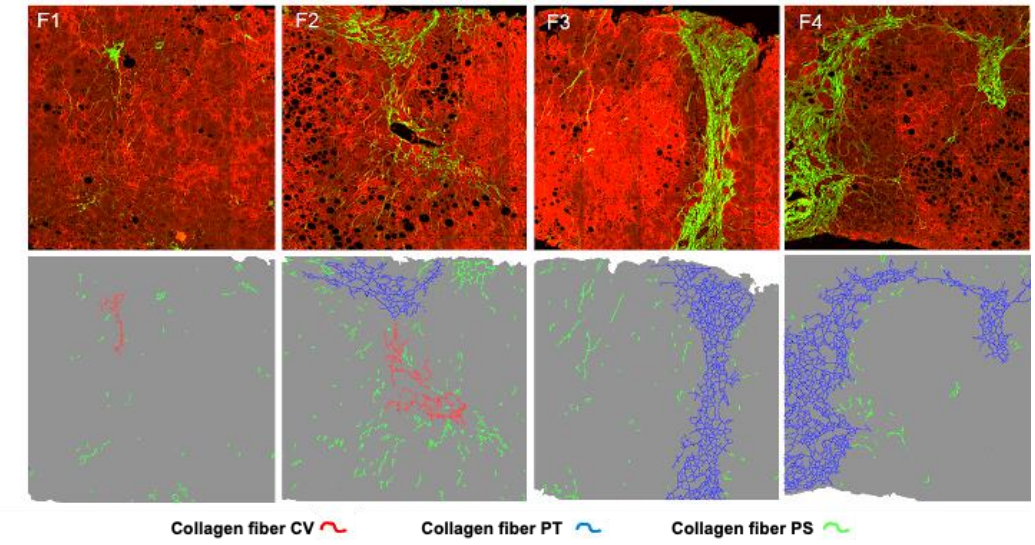

Figure 1: Examples of how AI can identify fibrosis stage specific features: isolate central vein fibrosis for F1; central vein and portal tract fibrosis in proximity for F2; extension of portal tract fibrosis to other portal tract for F3; extension of portal tract fibrosis in circular formation (nodule) with no/little perisinusoidal fibrosis inside the nodule.

As shown in Figure 1, we demonstrate that the qFibrosis algorithm can identify specific features as described in the CRN system. F1 shows only zone 3 CV fibrosis parameters, F2 shows both zone 3 central vein and portal tract fibrosis, F3 shows clear bridging fibrosis with collagen fibers connecting between 2 points (PT to PT or PT to CV), and F4 with clear nodular cirrhosis with negligible perisinusoidal fibrosis inside the nodule.

Spearman nonparametric method was used to estimate the correlation between qFibrosis and NASH CRN–defined semiquantitative pathological categories. The area under the receiver operating characteristic curve (AUROC) analysis was performed to evaluate the accuracy of the qFibrosis for prediction of the different stages of fibrosis. qFibrosis could accurately differentiate fibrosis stages of F≤1 versus F≥2 with an area under the curve (AUC) of 0.870 (95% CI 0.804 - 0.959), and of F≤3 versus F4 with an AUC of 0.951 (95% CI 0.905 - 0.996). (Table 3)

	AUROC	95% CI	*P* value	Sensitivity	Specificity	PPV	NPV
F0 vs F≥1	0.870	0.787-0.953	<0.001	94%	63%	84%	83%
F≤1 vs F≥2	0.881	0.804-0.959	<0.001	97%	58%	65%	96%
F≤2 vs F≥3	0.945	0.891-0.999	<0.001	96%	76%	66%	97%
F≤3 vs F4	0.951	0.905-0.996	<0.001	87%	91%	72%	96%

Table 3. AUROC analysis of the performance of qFibrosis and detailed breakdown of performance characteristics (sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV]).

CLINICAL CONSIDERATIONS

The review of liver histology by an expert hepatopathologist currently is and will always be the final determination in diagnostic screening. The decision tree for qFibrosis implementation in a biopsy-based clinical trial for diagnostic screening is shown. (Figure 2)

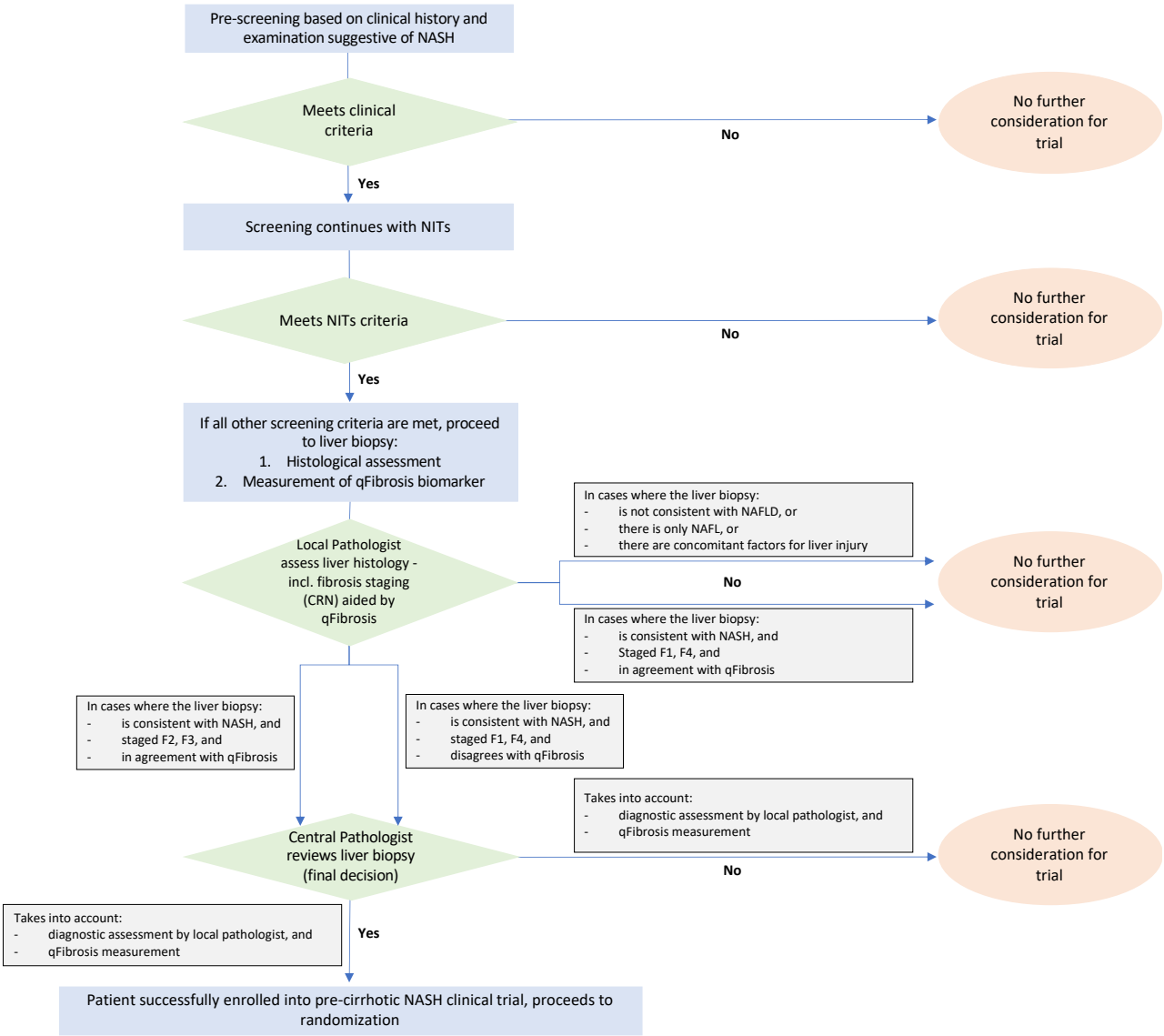

Figure 2: Decision tree for the implementation of qFibrosis diagnostic biomarker

1. Proposed Conditions of Qualified Use

According to the draft guidance document by the FDA, patients with a NASH activity score (NAS) ≥ 4 with at least 1 point each in inflammation and ballooning along with a NASH CRN fibrosis score greater than stage 1 fibrosis but less than stage 4 fibrosis should be

considered eligible for clinical trials investigating non-cirrhotic NASH.[6]

Hence, the proposed use for qFibrosis measurement will only be conducted for subjects who have met the pre-screening and non-invasive tests (NITs) criteria and are recommended to undergo a liver biopsy for diagnostic screening for inclusion into a non-cirrhotic NASH clinical trial.

Whole Slide Image Considerations for Clinical Trial Use:

- Formalin-fixed, paraffin-embedded (FFPE) liver biopsy tissue
- Unstained slides should be scanned by trained technicians on Genesis®200 imaging device according to a standardized operating procedure

2. General Clinical Validation Plan

qFibrosis will be validated on the following 2 aspects:

1. By comparing qFibrosis to consensus staging generated by an adjudication panel of expert board certified, liver pathologists to establish the concordance between qFibrosis and the ground truth results (Figure 3). To do so, the percentage agreement (PA) between qFibrosis and the consensus staging will be tabulated.
2. By establishing an improvement in the reproducibility of pathologists' fibrosis assessment when qFibrosis measurement is used as an aid to the pathologist during screening process (Figure 4). To do so, we will evaluate the weighted linear kappa of the inter- and intra-reader variability of the pathologists with and without the aid of qFibrosis measurement.

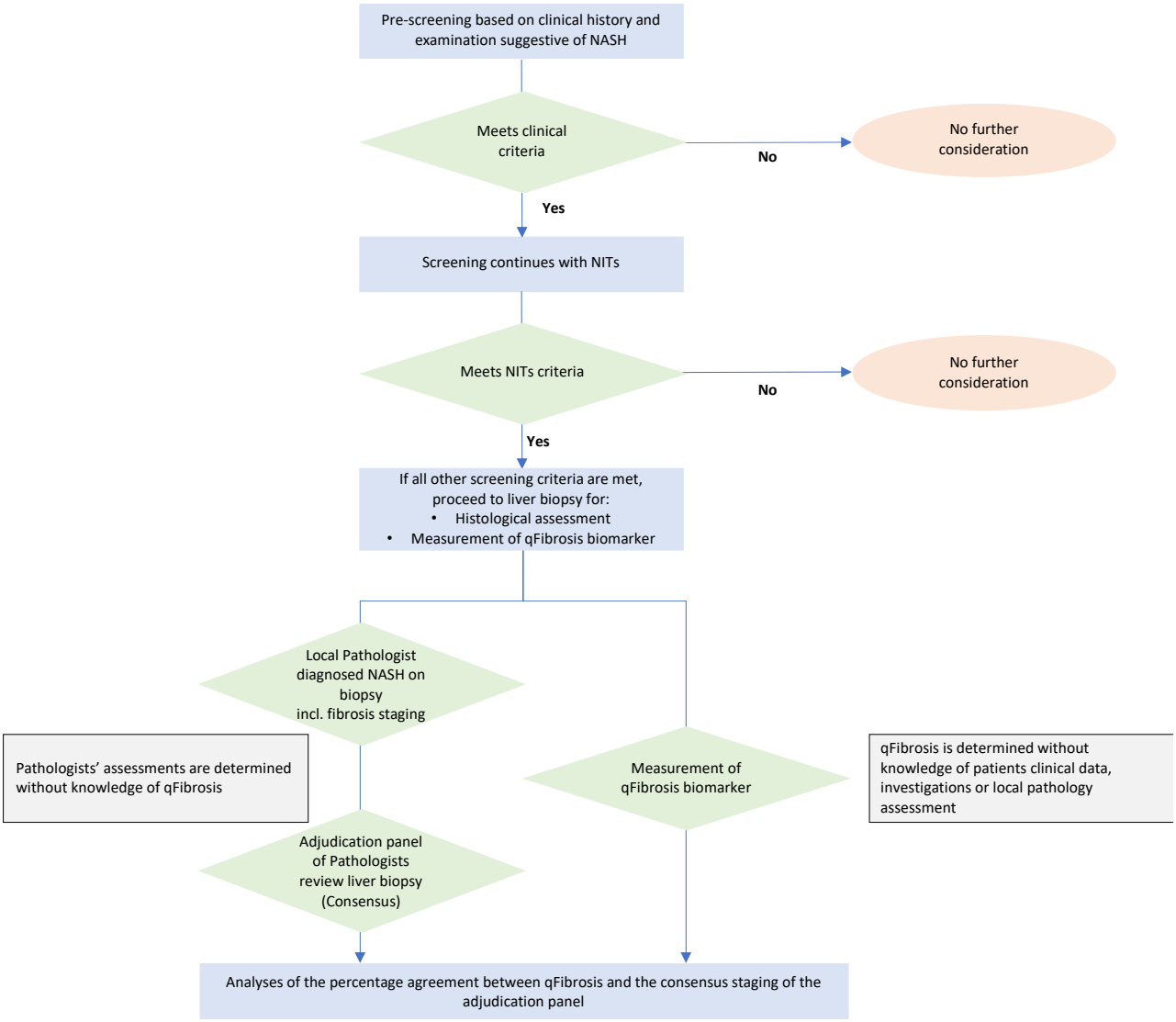

Figure 3: Validating qFibrosis measurement against the consensus read of an adjudication panel

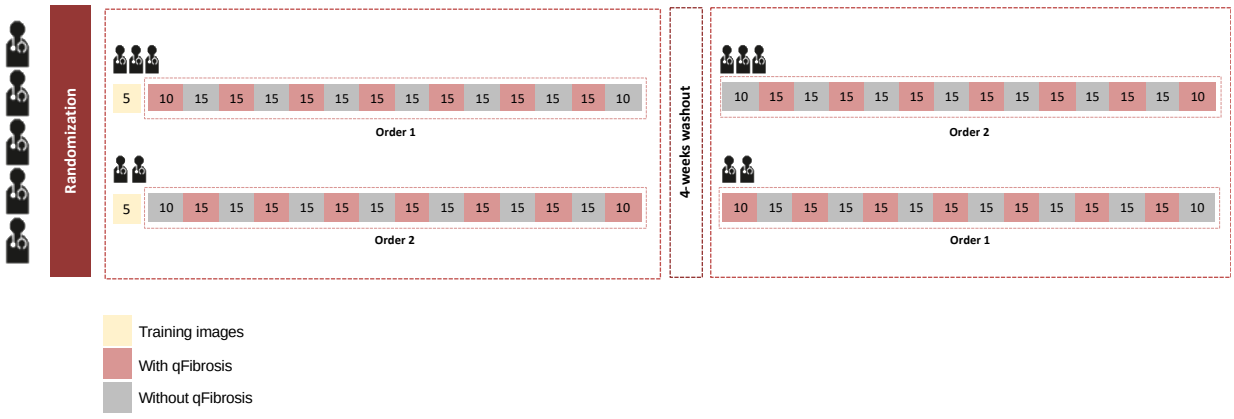

Figure 4: Schematic of the study design to evaluate the impact of qFibrosis on diagnostic performance of pathologists. Pathologists are randomized to one of the 2 orders. Each rectangle

indicates a set of images; the color of the rectangle indicates the mode (Assisted with qFibrosis or without qFibrosis), and the number in the rectangle indicates the number of images in that set. Note that the sample size will be calculated and finalized in the Qualification Plan.

To evaluate the impact of qFibrosis on the pathologists' inter- and intra-reader variability, we intend to conduct a multi-reader study as illustrated in Figure 4. The pathologists will assess fibrosis for all study images in both modalities (with qFibrosis assistance or without) in 2 sessions separated by a wash-out period of at least 4 weeks. To mitigate bias for possible performance differences at the beginning versus the end of a given session, the complete set of images was divided into blocks of 10 or 15 images, with each block containing a similar distribution of fibrosis staging. In addition, to reduce possible biases, the pathologists will be randomized into 2 groups and will begin the first session either with qFibrosis assistance or without qFibrosis. In either mode, the order and specific images reviewed were identical; the difference was solely in modality.

We hypothesize that with the assistance of the qFibrosis diagnostic biomarker, it aids the pathologists in correctly identifying eligible NASH patients i.e., patients with fibrosis staging F2-F3 and enrolling these into clinical trials for intervention, which in turns helps to reduce overall screen failure rate for NASH clinical trials. Additionally, we are also exploring the possibility of re-reading screen failed samples from existing NASH clinical trials with qFibrosis assisting the central pathologist to provide supporting clinical evidence on the impact of qFibrosis on improving screen failure rate.

qFibrosis has also been published in numerous peer-reviewed journals and presented at several conferences.[4,7-8] We are also currently involved in more than 15 NASH clinical trials and many NASH clinical studies. In terms of the maturity of qFibrosis, it has been tested for repeatability/reproducibility. Briefly, we used sample slide across different machines to evaluate the qFibrosis assessment, and the qFibrosis stage is consistent across all machines, with >95% agreement for our inter and intra-system repeatability.

The statistical analysis plan (SAP), including the statistical method and analysis as well as sample size calculations, for these analytical and clinical validation studies will be developed and finalized prior to the start of these studies and will be described in the Qualification Plan.

3. Overview of Risks and Benefits

The potential benefit of incorporating qFibrosis measurement into the screening strategy for entry into clinical trials lies in its ability to aid the pathologists at improving their inter- and intra-reader variability, enabling the correct identification of eligible" NASH patients i.e., enrichment of the trial population with correct fibrosis staging of F2-F3. And at the same time, distinguishing patients with F0 and F1 fibrosis reduces the risk for unnecessary treatment of this patient subset; and distinguishing patients with cirrhosis (F4) from those with earlier stages because the disease biology and clinical course are different.

Currently, a liver biopsy remains necessary for the review of liver histology to make the final determination in diagnosing NASH, hence there is no associated additional risk owing to qFibrosis measurement. At this point in the development, any other potential risks have not been identified.

4. Knowledge Gaps

At this point the knowledge gaps, limitations, and assumptions for utilization of the mentioned biomarker have not been defined.

SUPPORTING INFORMATION

Previous Oral / Poster Presentation:

1. AASLD The Liver Meeting (TLMdX) 2021, Oral Presentation. Parallel 10: Diagnostics and Biomarkers of NAFLD. **Digital Pathology with Artificial Intelligence Analyses (DP-AI) Overcomes the Limitations of Current Scoring Systems in Assessing Fibrosis Regression for NASH F3 Patients**. By Arun Sanyal.
2. AASLD The Liver Meeting (TLMdX) 2021, Oral Presentation. Parallel 21: NAFLD and NASH: Clinical Trials of Novel Therapeutics. **Safety and Efficacy of Tropifexor plus Cenicriviroc Combination Therapy in Adult Patients with Fibrotic NASH: 48 Week Results from the Phase 2b TANDEM Study**. By Quentin Anstee.
3. AASLD The Liver Meeting (TLMdX) 2021 ePoster. **Unique Fibrosis Progression and Regression Features in NAFLD, Validation of Concept in Animal and Human Studies Using Artificial Intelligence Analyses (DP-AI)**. By Dean Tai, Amon Asgharpour, Yayun Ren, Mulugeta Seneshaw, Faridoddin Mirshahi, Arun Sanyal.
4. AASLD The Liver Meeting (TLMdX) 2021 ePoster (Poster of Distinction). **Development of Machine Learning Histological Scores that Correlated with Portal Pressures and Development of Varices in NASH patients with Cirrhosis**. By Mazen Noureddin, Zachary D. Goodman, Dean Tai, Yayun Ren, Elaine Chng, Pol Boudes, Harold Shlevin, Stephen Harrison, Naga P. Chalasani.
5. AASLD The Liver Meeting (TLMdX) 2021 ePoster. **qFIBS Performance in Pediatric Non-Alcoholic Steatohepatitis**. By Feng Liu1*, Lai Wei*, Wei Qiang Leow, Shu-Hong Liu, Xiao-Xiao Wang, Xiao-He Li, Hui-Ying Rao, Rui Huang, Nan Wu, Aileen Wee, Jing Min Zhao.
6. AASLD The Liver Meeting (TLMdX) 2021 ePoster. **Second Harmonic Generation (SHG)/Two-Photon Excitation Fluorescence (TPEF) Microscopy Imaging and Quantitative Assessment of Septa Fibrosis in Choline Deficient High Fat Diet (CDHFD) Rat Model of Nonalcoholic Steatohepatitis (NASH)**. By Xiao Teng, Rohan Manohar, Hiroaki Yashiro, Qiang Yang, Alvin Leong, Gideon Ho.
7. AASLD The Liver Meeting (TLMdX) 2021 ePoster. **SHG/TPEF Microscopy Imaging in the Fibrosis and Steatosis Progression of Nonalcoholic Fatty Liver Disease (NAFLD) in Mouse Models**. Wang Xiaoxiao, Rui Jin, Xiao-He Li, Qiang Yang, Xiao Teng, Nan Wu, Hui-Ying Rao, Feng Liu.
8. Digital presentation at the 2020 *The Liver Meeting Digital Experience (TLMx).* **Safety and Efficacy of Tropifexor in Patients with Fibrotic Nonalcoholic Steatohepatitis: 48-week Results from Part C of the Phase 2 FLIGHT-FXR Study** by Arun Sanyal.
9. Poster presentation at the 2020 *Digital Paris NASH Meeting.* **Dynamic Fibrosis Features in Post-treatment Biopsies and its Interpretation** by Elaine Lay Khim Chng, Dean Tai, Yayun Ren and Pierre Bedossa.
10. Poster presentation at the 2020 *NASH-Tag*. **Concomitant Zonal Quantification of qSteatosis and qFibrosis in a Sub-study from EMMINENCE, a 12-month Phase 2b NASH study of MSDC-0602K** by Stephen Harrison, Dean Tai, Yayun Ren, Elaine Chng, and Howard Dittrich.
11. Poster presentation at the 2019 *The Liver Meeting (AASLD).* **qFIBS for the Automated Quantitative Evaluation of Histological Evolution in Pediatric Nonalcoholic Steatohepatitis** by Feng Liu, Jingmin Zhao, Leow Wei-Qiang, Ya-Yun Ren, Xiaoxiao Wang, Xiaohe Li, Huiying Rao, Wei Zhang, Aileen Wee and Lai Wei.

12. Poster presentation at the 2019 *The Liver Meeting (AASLD).* **Steatosis and Fibrosis Measured as Continuous Variables on Paired, Serial Liver Biopsies in the Resmetirom (MGL-3196) 36-week Phase 2 NASH Study** by Stephen A Harrison, Dean Tai, Ya-Yun Ren, Rebecca A. Taub, Mustafa Bashir.
13. Oral presentation at the 2018 *The Liver Meeting (AASLD).* **New Automated Evaluation Tool qFIBS - Quantitative Assessment for Fibrosis, Inflammation, Ballooning, and Steatosis in Patients with Non-Alcoholic Steatohepatitis** by Feng Liu, Boon Bee George Goh, Dina Tiniakos, Aileen Wee, Leow Wei-Qiang, Jingmin Zhao, Huiying Rao, Xiao-Xiao Wang, Qin Wang, Wei-Keat Wan, Kiat Hon Lim, Manuel Romero-Gomez, Salvatore Petta, Elisabetta Bugianesi, Chee-Kiat Tan, Stephen A. Harrison, Quentin M. Anstee, Jason Chang and Lai Wei.

PREVIOUS QUALIFICATION INTERACTIONS AND OTHER APPROVALS

This is the first regulatory interaction for this proposed biomarker.

REFERENCES

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7. Leow, W., Bedossa, P., Liu, F., Wei, L., Lim, K., Wan, W., . . . Goh, G. B. (2020). An improved Qfibrosis algorithm for Precise screening and Enrollment into Non-Alcoholic Steatohepatitis (NASH) clinical trials. Diagnostics, 10(9), 643. doi:10.3390/diagnostics10090643

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