PharmaNest
Princeton, NJ, USA

Biomarker Qualification Letter of Intent

ATTN: CDER-Biomarker Qualification Program
Email: CDER-BiomarkerQualificationProgram@fda.hhs.gov

Subject: Biomarker Qualification Letter of Intent (LOI)

1 Administrative Information

1. **SUBMISSION TITLE:** ***Digital Pathology Biomarker to aid in assessing fibrosis severity in adult patients with MASH***

2. **REQUESTING ORGANIZATION:**

Name or Organization: PharmaNest Inc.

Organization Address: 100 Overlook Center, Princeton, NJ 08540, USA
Phone: +1 609 375 2003
Email: info@pharmanest.com
www.pharmanest.com

Type of Organization: Industry, U.S. Small Business Entity

Primary Contact: Name: Mathieu M. Petitjean, Ph.D.
Title: CEO
Phone: +1 973-699-1579
Email: mathieu.petitjean@pharmanest.com

Alternative Contact: Name: Vernessa T. Pollard
Title: Partner, McDermott Will & Emery LLP
Phone: +1 202 756 8181
Email: vpollard@mwe.com

3. **SUBMISSION DATE : October 13, 2023**

2 Drug development Need Statement

Currently, there are no approved therapies for non-alcoholic steatohepatitis (NASH), recently renamed Metabolic dysfunction associated steatohepatitis (MASH). Pathology plays a critical role in NASH/MASH clinical trials with histology being the current reference method to determine inclusion in trials. Manual histological review[1,2] is complex, subjective, and prone to inter- and intra-reader variability and error [3–6]. Existing pathology scoring systems and practices show only moderate to fair reproducibility, limiting their utility for clinical research and practice. Thus, there is a critical need for tools that would improve upon histological interpretation to achieve greater consistency and standardization[7].

Digital Pathology methods of Quantitative Image Analysis for Tissue Biomarker use[8] such as the one presented in this Letter of Intent offer the significant advantage of full compatibility with standard histological methods (staining, WSI FDA approved scanners). They generate automated, scalable (worldwide delivery via the cloud) and continuous scores for fibrosis severity with an analytical and diagnostic performance that is superior to histological paradigms. These assessments will be used in this COU to assist the pathologist with consistent and reproducible staging of liver fibrosis in conjunction with readings from either standard glass or digital slides. In this COU there will not be quantitative information, but there will be a cut point to discern a NASH/CRN score of less than or equal to a fibrosis score of stage 2 OR a fibrosis score of greater than or equal to stage 3.

In the future, as more data on interpretation of the of quantitative data is obtained and compared to clinical outcomes, a paradigm shift in statistical analyses in therapeutic trials for liver diseases from changes in semi-quantitative, categorical stages towards quantitative scores reflecting mean changes from baseline may be possible. Because fully quantitative scores should have higher sensitivity to detect early changes, and an adjustable, lower detection threshold, these methods will provide more comprehensive information on the antifibrotic potency of tested drug candidates. This information will be submitted in future LOIs. See Section 10.

3 Biomarker Information and Interpretation

3.1 Biomarker name, Definition

We are proposing a Digital Pathology biomarker that is measured from the Digital Pathology WSI Image of a liver biopsy FFPE section stained with Masson's Trichrome using high resolution quantitative image analysis.

Biomarker Name	**FibroNest Phenotypic Fibrosis Composite Score**
Acronym	FibroNest Ph-FCS
Type of Biomarker	Histologic based, Digital, Quantitative Image Analysis, Imaging modality
DDT Type	**Biomarker**
BEST Category	**Diagnostic Biomarker**
Biomarker Definition	A quantitative, normalized (no unit) and continuous composite score that aggregates quantitative histological features of fibrosis severity measured by high resolution quantitative image analysis. For this COU a cut point of < 5 or ≥ 5 will determine if patients have a fibrosis score of ≤ stage 2 or a fibrosis score of ≥ stage 3 (NASH/CRN fibrosis staging system)

Liver Disease nomenclature changes: Liver diseases nomenclature has changed very recently. It has been established that non-alcoholic steatohepatitis (NASH) and Metabolic dysfunction associated steatohepatitis (MASH) can be used

interchangeably in the context of noncirrhotic patient. For the sake of simplicity and consistency with already presented data, the acronym NASH is used in this Letter of Intent.

3.2 Analytical Methods Summary

3.2.1 Phenotypic analytical hypothesis

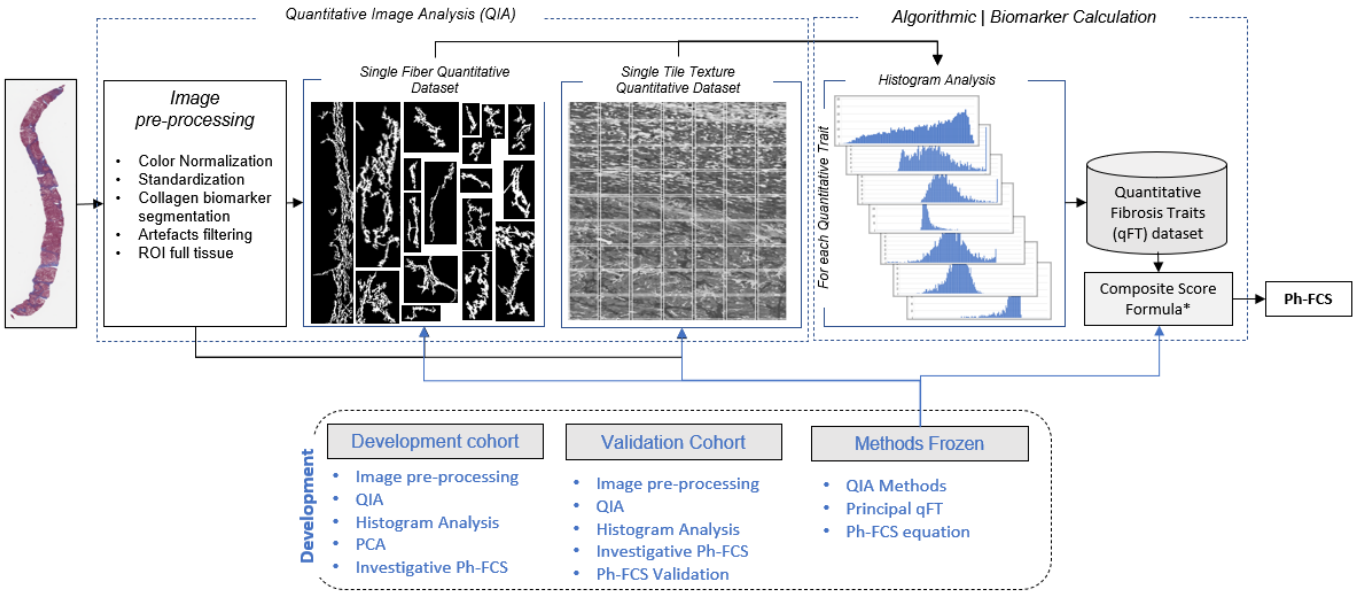
The hypothesis supporting the FibroNest method is that the histological phenotype of fibrosis changes as fibrosis severity progresses. These changes affect the overall aspect of the collagen distribution, the morphometry of collagen fibers and the architecture of fibrosis. These histological aspects are accounted for in the traditional histological methods used to describe liver fibrosis severity whether for NASH (NASH CRN / Kleiner-Brunt[1]), hepatitis C (METAVIR/ P. Bedossa [9], ISHAK / K. Ishak[10], Primary Biliary Cholangitis (NAKANUMA / Y. Nakanuma[11]), or cirrhosis (LAENNEC and BEIJIN scores[12,13]) but also for other fibrotic conditions such as Idiopathic Pulmonary fibrosis (ASHCROFT / T. Ashcroft[14]).

While maintaining these core insights, the FibroNest method introduces truly quantitative capabilities (measurements) that are currently lacking from traditional histological analyses.

The histogram-based description of phenotypic traits of fibrosis is an effective computational solution to account for elements of fibrosis progression and distortion.

3.2.2 Flowchart and summary table

The Inputs, Quantitative Image Analysis (QIA) measurement description, Imaging Tissue Assay development, single fiber parameters definitions and algorithm outputs (FibroNest Ph-FCS) are summarized in the flowchart and table below.



Units of Measurement	Unitless
Source	A Digital Pathology (WSI) Image of a liver biopsy FFPE section stained with Masson's Trichrome acquired with a FDA approved Whole Slide Imager at 40X, and in .SVS format.

Computational Infrastructure	The FibroNest high resolution quantitative image analysis algorithms are running on the NSF-funded cloud platform for bio-image Semantic Query User Environment (BiSQue) analysis management[15] and hosted by the OnDemand Computational infrastructure of Amazon Web Services.
Quantitative Image Analysis (QIA) measurement	• A pre-processing step of the WSI image addresses color normalization, color standardization, artifacts filtering (e.g., dust, rinsing, scanning stripes etc.) and automated full tissue ROI (Region of Interest) selection. • Measurements are made by high resolution / native resolution Digital Pathology Quantitative Image Analysis (QIA). • Individual fibers are detected, and their multiple physical properties (traits) are measured (QIA parameters). • Fibers are also classified into two groups based on the complexity of their skeleton ("Fine" and "Assembled" fibers classes). • Texture Analysis is applied on the segmented collagen image layer to generate additional architectural QIA parameters. • Histogram Analysis of the QIA parameters distribution generates quantitative Fibrosis traits, qFT.
Method development	• Principal component analysis (PCA) was used in the development phase to select the qFTs (principal qFTs) that account for fibrosis severity and find the optimal formula that generates the Ph-FCS. • There are about 100 principal qFTs that form the Ph-FCS
Method validation	The performance of the Ph-FCS is validated in the context of its performance as a diagnostic Test of early vs advanced fibrosis. When accepted / validated all the methods (QIA Method, list of principal qFTs and composite score formula) are frozen to form a static Ph-FCS Biomarker method.
Biomarker Calculation and Algorithm output	From a digital pathology WSI image, the principal qFTs are measured and combined to form the Ph-FCS from the pre-established and validated formula.
Detailed Methods, definitions, algorithms flowcharts	Please refer to US Patent US 11,430,112 of August 30, 2022 and extensions for: • The itemized definitions of the QIA and qFT parameters • Comprehensive Algorithmic Methods and flowcharts

3.3 Fibrosis Ph-FCS Biomarker Interpretation

3.3.1 Biological plausibility

It has been observed that the histological features of collagen present in tissue sections stained for collagen are strongly related to fibrosis severity, and the risk of liver related events in patients diagnosed with NASH (Kleiner-Brunt[1], Sanyal-NASH CRN[16], Angulo-Kleiner[17]. These histological features are used in semiquantitative systems to quantify aspects or the overall collagen distribution, morphometry of collagen fibers and architecture of fibrosis. FibroNest's Quantitative Image Analysis (QIA) measurement

While maintaining these core insights, the FibroNest method introduces Quantitative Image Analysis (QIA) measurements that are currently lacking from traditional histological analyses.

3.3.2 High resolution, single fiber Quantitative Image Analysis

Figure 1 visualizes some of the QIA parameters measured from a Digital Pathology image, and their visual difference between Early and Advanced fibrosis. The full tissue analysis of WSI images at the native resolution (~0.25µm/pixel or less at 40X) is performed in 5 to 10 minutes thanks to the scalable on-demand computational architecture of the FibroNest engine on AWS. The full list of parameters and their definition is available here.

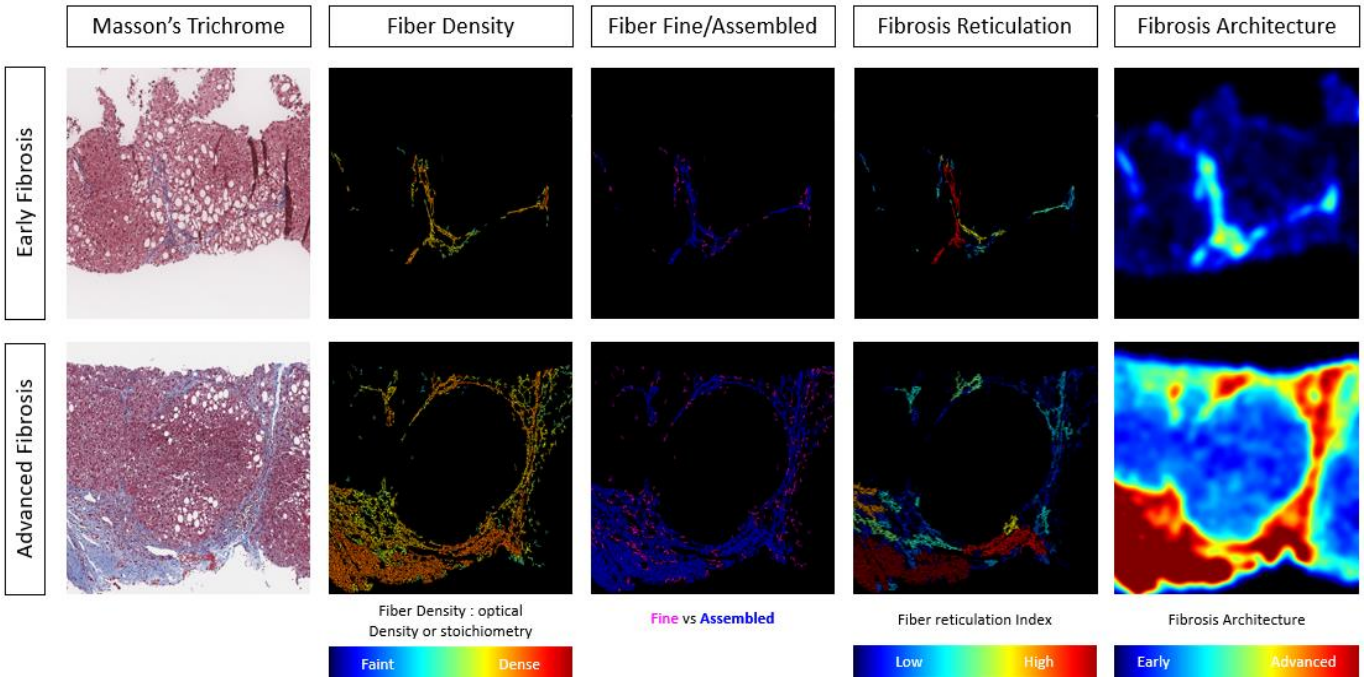
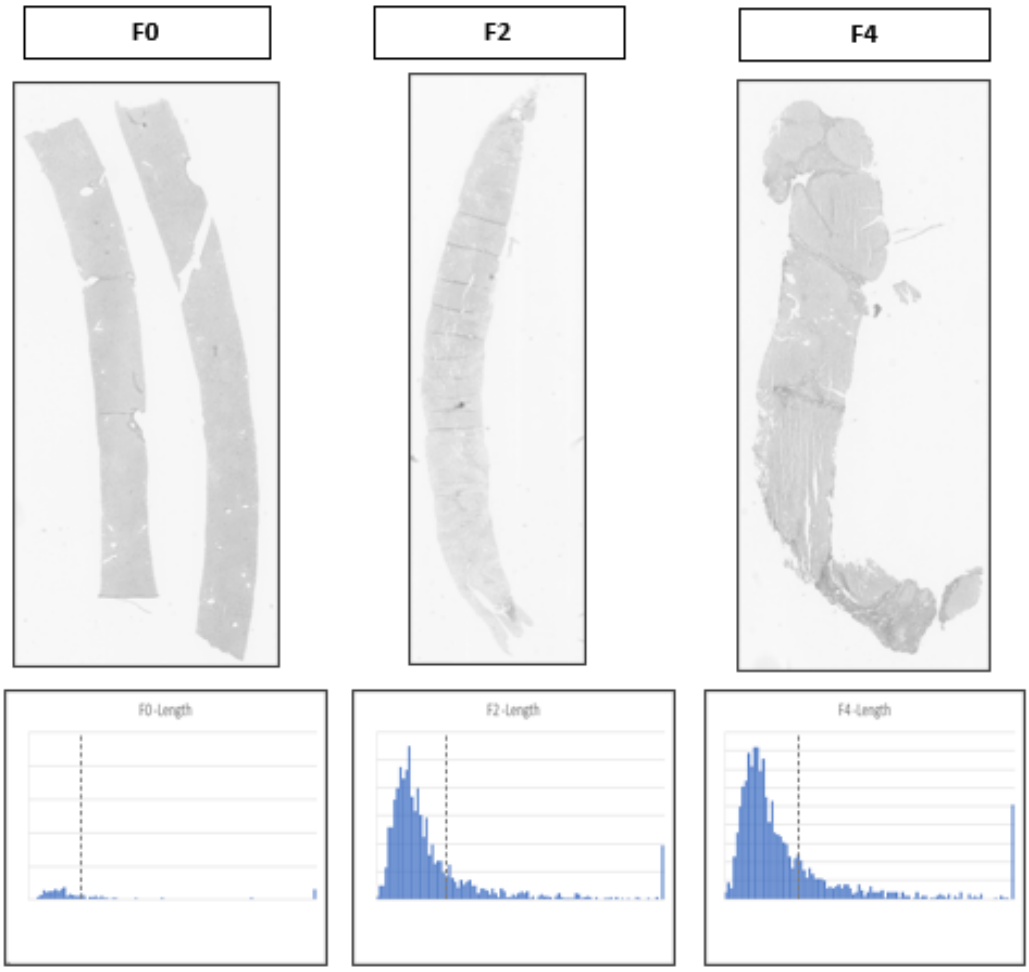


Figure 1: *Examples of collagen QIA parameters measured by FibroNest high resolution quantitative image analysis.*

3.3.3 Interpretation of Single-fiber Fibers phenotypic dimension and related QIA Parameters

Figure 2 illustrates the change in one of the 13 morphometric features (or traits) used to describe collagen fibers. There are about 6,000 fibers in an adequate F2 liver biopsy and the distribution of their traits are illustrated by histograms (see figure 2, as an illustration for the "length" of the fibers). Histogram analysis derives statistical parameters such as means and medians that are sensitive to progression, standard deviation, skewness, and kurtosis that are sensitive to distortion. FibroNest uses pre-defined cut-off values to isolate specific phenotypes of disease severity such as short and long fibers. These quantitative parameters are the qFTs. While qFTs have different dynamic ranges according to the progression of fibrosis (F0 to F2 or F2 to F4), others might not be related to severity (see "median" in figure 2).



LENGTH	Value
Short	30.9/mm2
Long	31.8/mm2
Mean	63.2px
Median	31.0px
Std Dev	123.3px
Skewness	6.6
Kurtosis	55.0

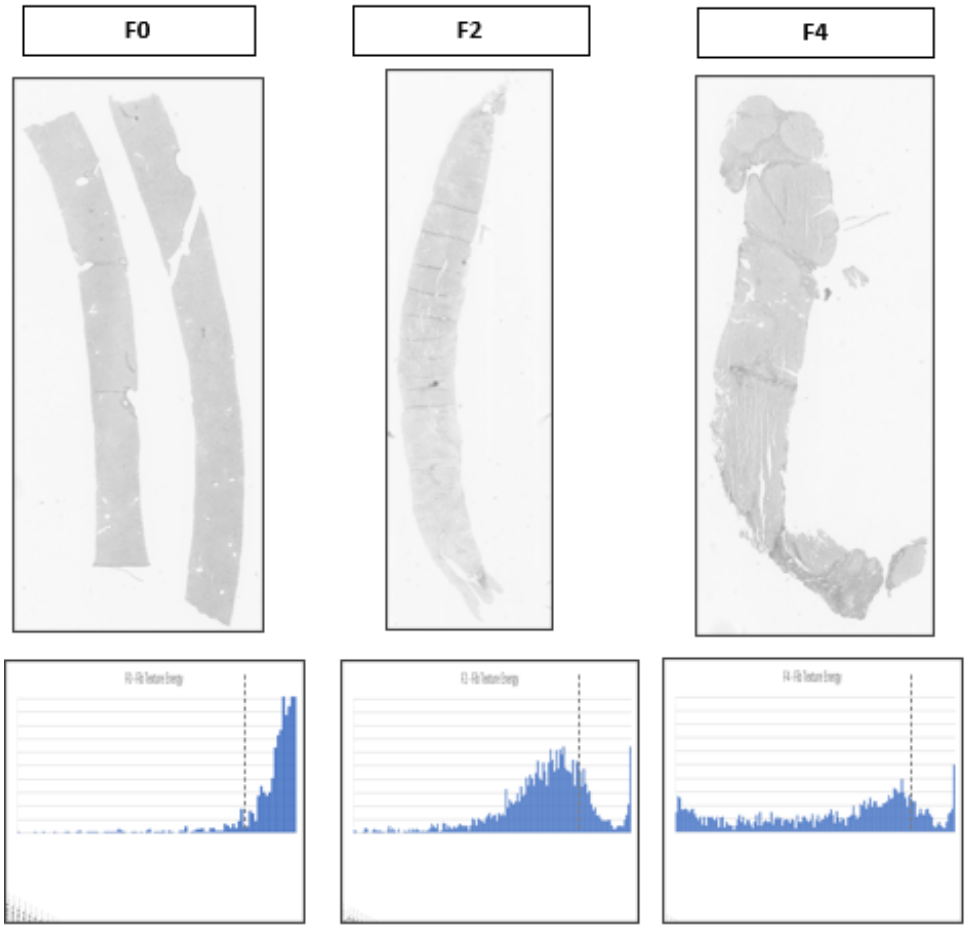
LENGTH	Value
Short	101.2/mm2
Long	94.5/mm2
Mean	49.31px
Median	30.0px
Std Dev	104.9px
Skewness	14.3
Kurtosis	271.11

LENGTH	Value
Short	185.4/mm2
Long	197.3/mm2
Mean	61.1px
Median	31.00px
Std Dev	183.85px
Skewness	15.78
Kurtosis	290.71

Figure 2: *Examples of the changes in the collagen fiber length QIA parameter as fibrosis severity progresses. The qFT are derived from the Histogram Analysis. Full nomenclature here)*

3.3.4 Interpretation of Fibrosis Architecture Phenotypic dimension and related QIA Parameters

Figure 3 illustrates the change in one of the seven Architectural QIA features used to describe the architecture of fibrosis. The Architecture QIA parameters are calculated applying GLCM texture analysis methods[18–21] to small computational windows that map the entire biopsy image. There are about 10,000 computational windows per image. Histograms of such GLCM features[20] reveals the coexistence of healthy tissue and emerging complex architectural features in the F2 range, which are fully expressed in the F4 range.



ENERGY	Value
Blurry	59.5/mm2
Resolved	197.5/mm2
Mean	0.92
Median	0.95
Std Dev	0.11
Skewness	-3.38
Kurtosis	13.74

ENERGY	Value
Blurry	233/mm2
Resolved	22.81/mm2
Mean	0.69
Median	0.972
Std Dev	0.16
Skewness	-1.02
Kurtosis	1.95

ENERGY	Value
Blurry	483/mm2
Resolved	48.9/mm2
Mean	0.57
Median	0.65
Std Dev	0.28
Skewness	-0.48
Kurtosis	-1.04

***Figure 3**: Examples of the changes in one fibrosis Architecture QIA parameter as fibrosis severity progresses. The qFT are derived from the Histogram Analysis. (Full nomenclature here)*

3.3.5 Principal component analysis and Phenotypic Fibrosis Composite Score interpretation and utility

We have illustrated above how QIA parameters and their histogram analysis generate 385 qFTs. Not all of them (as illustrated in Figures 2 and 3) are useful to describe fibrosis severity.

The qFTs that account for variance in severity (principal qFTs) have been identified by principal component analysis during the development phase of the FibroNest QIA Tissue Assay and using a cohort including both early fibrosis and severe fibrosis groups. Recent results (presented at the EASL meeting 2023) have shown that the qFT identified by this method overlap (90%) with qFTs identified using clinical outcomes (liver related events) from a cohort of 404 patients with 18 years of follow up[22] (see section 8.1.3).

Once identified, the principal qFTs are normalized and combined in a composite score that recapitulates the quantification of the in the bulk, morphometric and architectural phenotypes of fibrosis severity.

3.3.6 Phenotypic Fibrosis Composite Score interpretation

The Phenotypic Fibrosis Composite Score Ph-FCS interpretation derives from the aggregated quantification of histological traits that account for the change and distortion of the fibrosis phenotype as severity progresses. Heat charts from principal qFTs (Fig 4.1) and their relative trajectories (Fig 4.2) support this interpretation paradigm.

The Ph-FCS is a continuous score for severity of Fibrosis in NASH liver biopsies.

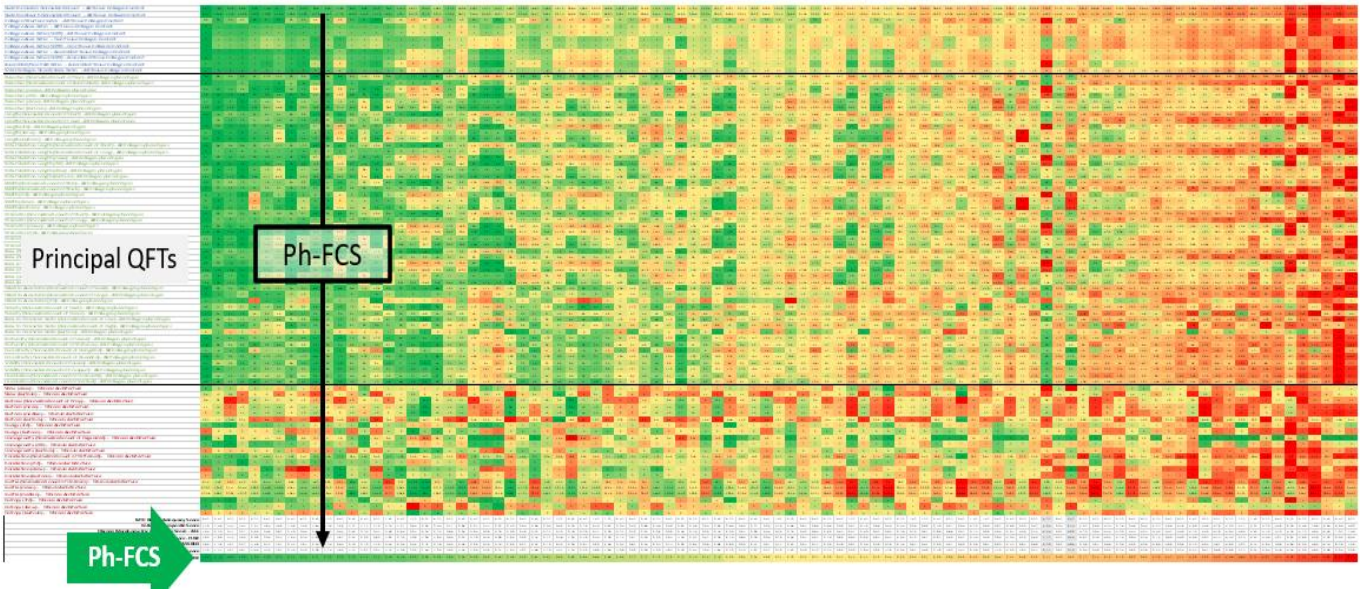
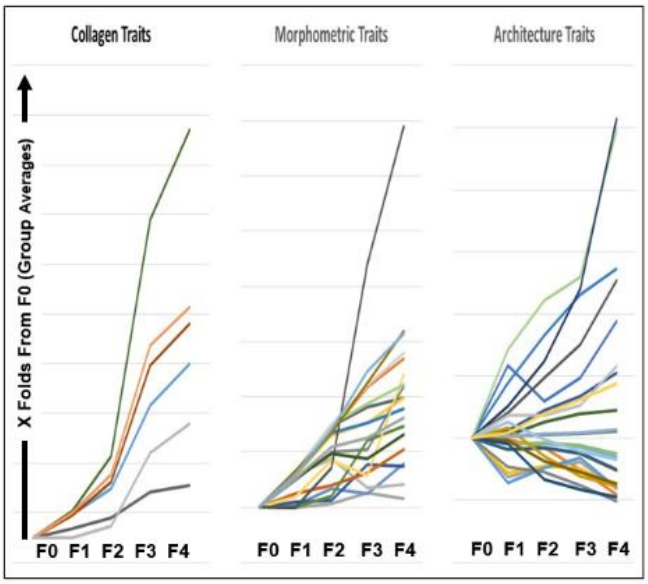


Figure 4.1 *Phenotypic heat chart describes the liver severity progression (one patient per column) using individual principal qFT (rows) across three phenotypic layers and how their aggregation yields to the Ph-FCS Fibrosis severity score*[23].



To provide additional insights on the detailed phenotypes of fibrosis severity progression, each principal trait (or row in a heat chart as shown in figure 4.1) can be individually followed. Such "trajectories" (figure 4.2) demonstrate that all the changes in the fibrosis phenotype are not linear, with some of them drastically changing in the F2-3 spectrum as has been reported semi-quantitatively by pathologists.

Figure 4.2 *In each phenotypic layer, Principal qFTs trajectories (normalized folds) describe the continuous changes of histological traits as fibrosis severity progresses.*[24]

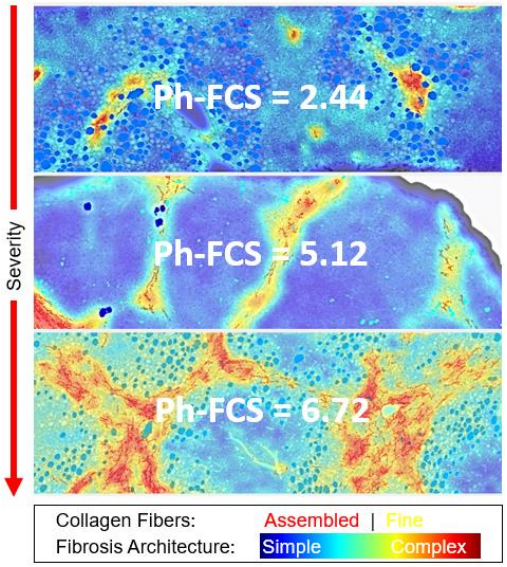
3.4 Clinical Interpretive Criteria

Ph-FCS ability to classify based on diagnostic performance (sensitivity, specificity, PPV, NPV):

- ICD10-K74.01: Hepatic Fibrosis, Early Fibrosis (=< NASH CRN F2),
- ICD10-K74.02: Hepatic Fibrosis, Advanced Fibrosis (>=NASH CRN F3),

Reference Biomarker: NASH-CRN Fibrosis Stages. The performance of the Ph-FCS might be affected by the accuracy of the reference biomarker.

3.5 Augmented Pathology visualization for Biomarker Interpretation and Utility



The FibroNest Fibrosis Biomarker and its QIA features can be visualized by the pathologists as an aid to their assessment as illustrated in Figure 5.

Figure 5: *Augmented Digital Pathology images using the FibroNest Digital Pathology QIA analysis, Fibrosis Severity Scores*[23]

4 Context of Use statement (COU)

A **diagnostic biomarker** to assist the pathologist in the assessment of NASH-CRN fibrosis stage from adequate Digital Pathology Whole Slide Images (WSI) of stained liver biopsies FFPE sections as part of the histopathologic evaluation for enrollment of adult patients (>18 years old) in pre-cirrhotic Non-Alcoholic Steatohepatitis (NASH)/ Metabolic Dysfunction Associated Steatohepatitis (MASH) clinical trials.

5 Conditions of Use

Population for use	• Patients enrolled in a NASH clinical trial who have had or will have a liver biopsy performed. • Adults, 18 years and older
Tissue and Histology for use	• Formalin-fixed, paraffin-embedded (FFPE) liver biopsy tissue section (~ 4mm) • Stained for collagen with Masson's Trichrome using certified Reagents and their according package insert. • Appropriately Quality Checked for absence of defects, such as sectioning artifacts, coverslip bubbles, uneven staining, etc.

Digital Image Acquisition, Digital Pathology Whole Slide Imaging (WSI) Systems for Use	• FDA Approved WSI Scanners, that meet and exceed FDA's Guidance document for the Technical Performance Assessment of Digital Pathology Whole Slide Imaging Systems (April 2016) • Appropriately maintained per Manufacturer's User Manual • Used per Manufacturer's User's Manual • 40X Magnification • .SVS image file format • Appropriately Quality Checked for absence of defects, such as scanning stripes and out of focus areas.

6 Analytical Considerations

6.1 Summary of information provided above.

Analytical Methods summary	See 3.2 Analytical Methods Summary
Analytical and Computational Approach	See section 3.3.2
Principal component analysis and Phenotypic Fibrosis composite score	See section 3.3.3
Biomarker interpretation and Utility	See section 3.3.4

6.2 Pre-Analytical Factors and Quality Assurance/Quality Control

See Section 5, Conditions of Use

6.3 High Resolution Digital Pathology Quantitative Image Analysis Tissue Assay Development

6.3.1 Approach and Workflow

The Digital Pathology Quantitative Image Analysis Tissue Assay Development process is summarized in the flow-chart below. A development cohort is used to define the analytical methods, including pre-processing steps, quantitative image analysis methods, QIA parameter histogram analysis, qFT principal component analysis, and composite scores mathematical formulas for the Ph-FCS. Then the method is applied automatically to images from study cohorts.

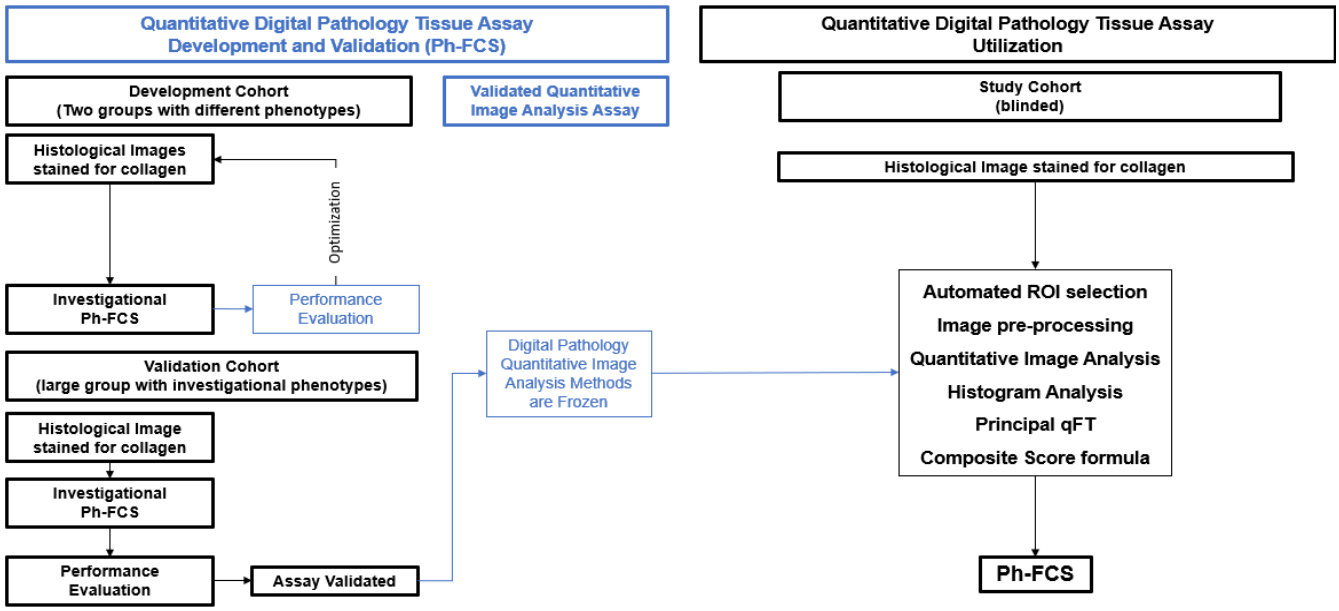
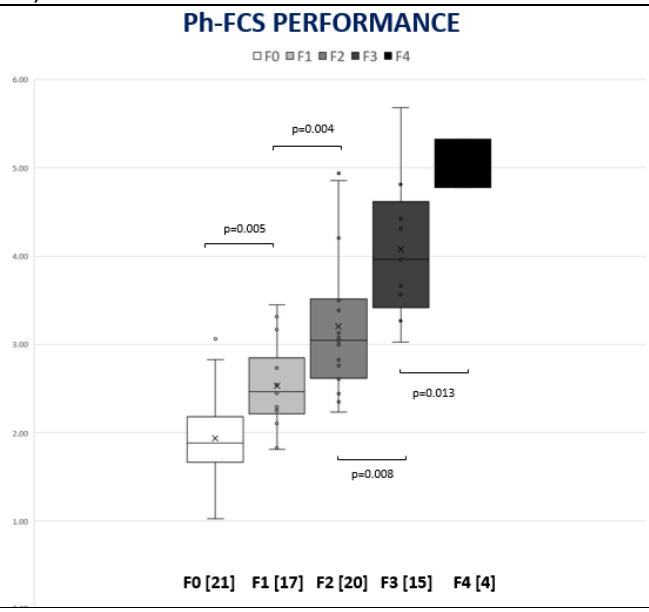


Figure 6: *FibroNest Digital Pathology Quantitative Image Analysis Tissue Assay Development process including development steps (Left) and Validated Assay execution (right)*

6.3.2 Development and validation cohorts

Development Cohort	A balanced cohort of Patient with NASH, (N=77), F0 (N= 21), F1 (N=17), F2 (N=20), F3 (N=15), F4 (N=4)
Results	
Validation criteria for the investigative Ph-FCS	Ability to classify based on diagnostic performance (sensitivity, specificity) • ICD10-K74.01: Hepatic Fibrosis, Early Fibrosis (=< NASH CRN F2), • ICD10-K74.02: Hepatic Fibrosis, Advanced Fibrosis (>=NASH CRN F3),
Validation Acceptance criteria for the investigative Ph-FCS	Sensitivity, specificity greater than 0.7 for an optimized cut off value of the Ph-FCS
Publication	**Evaluation of a novel histology-based fibrosis phenotypic composite score and its correlation with NASH-CRN Fibrosis scores in patients with NASH** Li Chen Michael Lung, Cynthia Behling, Arun J. Sanyal, Mathieu Petitjean EASL 2020 (Poster Link), Ref [24,25]
Validation Cohorts	A large dataset (N=846) from Phase 2 NASH Clinical Studies including: Ph2b FALCON 1 (F2/F3) (NCT348699)[26] Ph2b FALCON 2 (Cirrhosis) (NTC03486912)[27]
Results for the validation cohorts	See section 7, Clinical Considerations

6.4 High Resolution Digital Pathology Quantitative Image Analysis Tissue Assay Implementation

Once validated, the Digital Pathology Quantitative Image Analysis Tissue Assay methods are frozen and become part of a specific "Materials and Methods" controlled SOP in PharmaNest's Quality Management System. 100% of the methods are parametrized and frozen in the FibroNest engine. The Assay is deployed in production and each image analysis transaction is associated to an audit trail. All the analysis and their datasets are securely stored on AWS servers.

There are minimal, yet necessary operator interventions associated with:

- Verification of the automatic detection of biopsy tissue (ROI). Corrections may be needed when biopsy artifacts are present, such as fragments of the liver fibrotic capsule at the end of the biopsy.
- Verification (and correction if needed) of the automated image pre-processing and corrections for tissue processing, such as significant defects to pre-analytical specifications, rinsing artifacts, coverslip bubbles, scanning stripes.
- All the subsequent steps are automated.

6.5 Current Analytical Validation Results

6.5.1 Sensitivity to intra-liver tissue variability

6.5.1.1 Early to advanced Fibrosis: Not evaluated.

The FibroNest method and the Ph-FCS biomarker are prone to intra-liver biopsy variability, as are all liver biopsy specimens.

We are actively searching for a rare retrospective cohort (~20 NASH patients with moderate fibrosis and with multiple biopsies obtained at the same time) that would allow us to evaluate this parameter. We are currently in discussion with Pr. Vlad Ratziu, MD who performed a dual biopsy study in patients with NASH[28]. For the Qualification Plan we would like to analyze these paired liver biopsy specimens in non-cirrhotic NASH to obtain better sensitivity data to support the qualification.

6.5.1.2 Cirrhosis

In Cirrhotic patients (N=20 patients, each with 5 biopsies performed at the same time of transplant) the average intra-liver coefficient of variability of the Ph-FCS is 16% [29], down from 47.3% when evaluated using collagen Proportionate Area[30].

6.5.2 Sensitivity to staining variability and sectioning variability.

To simulate common tissue preparation and staining variability, the FFPE bloc of a unique adequate NASH biopsy (mid fibrosis severity) with 5 segments is processed using consecutive sections at 2, 3 and 4 microns. The stain bath duration is varied from 10 to 60 minutes by increments of 10 minutes. The Ph-FCS is calculated on each biopsy segment of the FFPE section, then averaged.[28]

Ph-CFS varies from 1% to 6% when tissue thickness varies from 3 to 4 um and staining bath time from 20 to 30 mins for a mid-fibrosis severity tissue[24].

6.5.3 Repeatability

Repeatability measures the variation in measurements taken by a single instrument and same person under the same conditions. Three (3) digital Pathology images of liver biopsies with low (F0-F1), mid (F2-low F3) and high (highF3-low F4) fibrosis severity are evaluated by the same operator using the same methods. The analyses are repeated each day for one week (5 different days). The Coefficient of Variation % (100* Standard deviation/mean) is calculated for each level of fibrosis severity.

Repeatability - Coefficient of Variation % (Std/Mean)

Fibrosis Severity	Low	Mid	High
Ph-FCS	0.47%	0.21%	0.29%

These results show that the test has excellent performance on repeatability of measurements, with a Coefficient of Variation less than 1% in each range of fibrosis. A typical Phase 2 study can be conducted by one operator in one week.

6.5.4 Reproducibility

Reproducibility represents the extent to which consistent results are obtained when an experiment is repeated. The test is conducted in three different laboratories with three different trained operators and different environmental conditions. There is no standardization on the methods as would be done in the context of a

controlled clinical study. For each operator, the analyses are repeated each day for one week (5 different days). The Coefficient of Variation % (100* Standard deviation/mean) is calculated for each level of fibrosis severity.

Reproducibility - Coefficient of Variation % (Std/Mean)

Fibrosis Severity	Low	Mid	High
Ph-FCS	11.53%	4.95%	3.33%

These results show excellent reproducibility of the test across different laboratories and operators, with a Coefficient of Variation less than 12% in the low range and less than 5% in the mid and high fibrosis ranges.

Furthermore, in the conduct of a large (Phase 3) Clinical Trial, workflows, processes and methods will be standardized across one or multiple operators and laboratories, to deliver a reproducibility that will be significantly superior.

6.5.5 Accuracy

The evaluation of the accuracy of the method was not possible due to the lack of **analytical** standards as the NASH CRN scale is based on **clinical** utility and no analytical standards are possible. See clinical validation vs NASH-CRN (Section 7.1) and vs Liver Related Outcomes (section 8.1.3)

6.5.6 Comparison with Stain-Free Imaging methods

Unstained FFPE sections of Biopsies from a cohort of 7 patients with histological diagnosis of NASH and fibrosis stages 0 to 4 {F0 (N= 21), F1 (N=17), F2 (N=20), F3 (N=15), F4 (N=4)} were imaged using Second Harmonic Generation (20X, 0.37 μm/pixel, Genesis200, Histoindex, Singapore). The same sections were then stained and scanned using an FDA approved Aperio AT2 WSI system (20X, 0.50 μm/pixel). The Ph-FCS was applied to both image formats (Figure 7). The two methods shown a very high level of correlation and similar correspondence with histological staging[25], demonstrating that chromogenic stains are correctly interpreted by the FibroNest methods. In fact, there is a better performance at low fibrosis stages using chromogenic stains as the pixel intensity of the WI image is derived from the exponential nature of the Beer Lambert transmission law[31] rather than the linear response of SHG as a function of collagen stoichiometry in the sample.

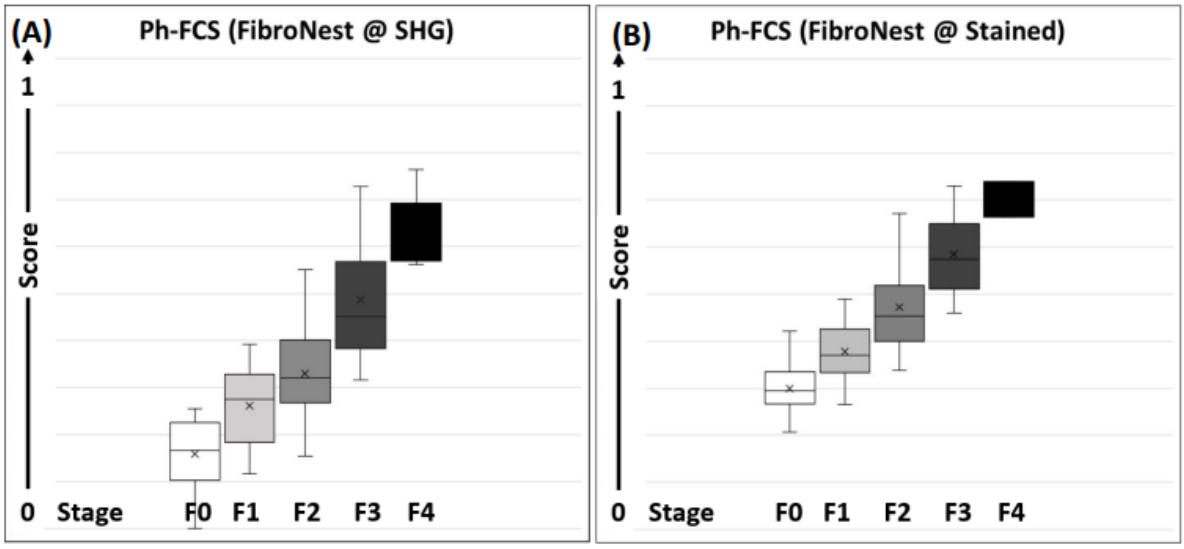


***Figure 7**. Comparison of the FibroNest Ph-FCS obtained some the same slides imaged with SHG or WSI[25].*

7 Clinical Considerations

7.1 Current Clinical performance of the FibroNest Ph-FCS Biomarker as a diagnostic test

The correspondence of Ph-FCS with NASH-CRN stages adjudicated by trained pathologists in the context of four studies including two Phase 2 NASH studies (described in Table B) is reported in Figure 8. The performance of the Ph-FCS biomarker as a diagnostic test is summarized in the Receiving Operating Curve of Figure 8 with an AUC=0.926, and in Table A using a cut-off value of 5 (Ph-FCS>=5 vs NASH CRN>=F3, Ph-FCS<5 vs NASH CRN <= F2) For clinical trials that exclude patients with cirrhosis, other histopathological and clinical criteria will be used to exclude these patients.

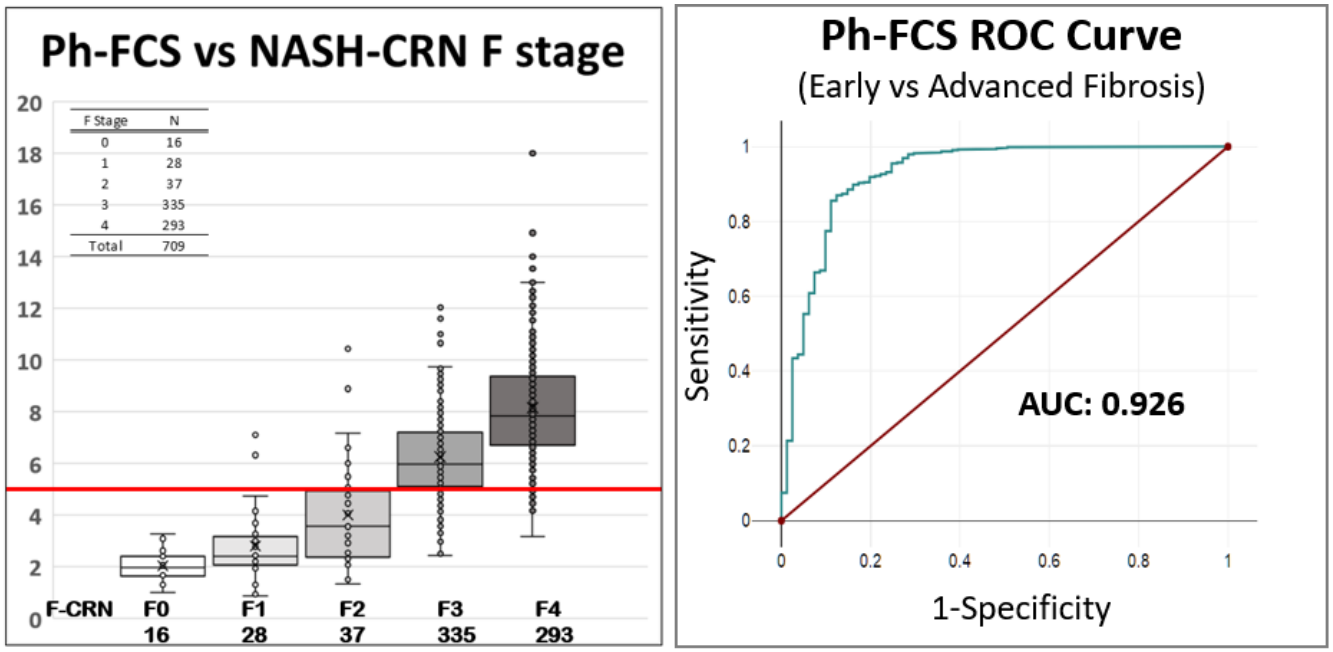


Figure 8: *Left: Correspondence of Ph-CFS with NASH-CRN stages adjudicated in the context of several Studies (see Table B). Receiver Operating Curve (ROC) of the Ph-FCS as a diagnostic tool to classify Early from Advanced Fibrosis*

	N	Ph-FCS Cut Off Value	Sensitivity (True Posivite Rate)	Specificity (True Negative Rate)	Precision / Positive Predictive Value (PPV)	Negative Predictive Value (NPV)*
Ph-FCS as Diagnostic Biomarker for Advanced (vs Early) Hepatic Fibrosis	709	5	86.62%	87.65%	98.19%	45.81%

Table A: *Performance of the Ph-FCS biomarker as a diagnostic test. Reference Biomarker: NASH-CRN Fibrosis Stages.*

****The performance of the Ph-FCS might be affected by the accuracy of the reference biomarker.***

Trial	Number of patients	Brief description of pop.	Available Adequate Biopsies with NASH-CRN Stages
Phase 2b FALCON1 study (NCT0348699) [26]	N=197	Eligible adults of 18-75 years of age with NASH and stage 3 fibrosis diagnosed by histologic assessment of liver biopsy according to NASH CRN criteria. During the 48-week double-blind treatment period, patients received subcutaneous 10mg, 20mg, or 40mg PGBF or placebo once weekly. Liver biopsies were obtained up to six months prior to or during screening and at week 24	336
Phase 2b FALCON2 study (NTC03486912) [27]	N=145	Eligible adults of 18-75 years of age with NASH diagnosed by histologic assessment of liver biopsy according to NASH CRN criteria and stage 4 fibrosis, defined as Cirrhosis, During the 48-week double-blind treatment period, patients received 10mg, 20mg, or 40mg pegbelfermin subcutaneous or placebo once weekly.	209
Investigational Cirrhosis Cohort [29]	N=100	20 hepatitis C (HCV) patients undergoing liver transplantation. 5 core biopsies were taken from five segments of the liver immediately after explantation.	91
Development Cohort	N=77	A balanced cohort of Patient with NASH, (N=77), F0 (N= 21), F1 (N=17), F2 (N=20), F3 (N=15), F4 (N=4)	74

Table B: *Performance of the Ph-FCS biomarker as a diagnostic test. Reference Biomarker: NASH-CRN Fibrosis Stages.*

With the exception of the Investigational Cirrhosis Cohort, the patient population was determined by the enrollment criteria required for pre-cirrhotic NASH/MASH with moderate to severe fibrosis. The value of this Investigational Cirrhosis Cohort was to determine the full dynamic range of the Ph-FCS.

One of the limitations of the data presented is that the distribution of patients per Fibrosis Stage does not reflect the natural distribution of patients[32]. We propose to address this gap in our full qualification plan as a result of our collaboration with the LITMUS consortium.

A general limitation of the validation method is that the accuracy of the reference biomarker (NASH-CRN stages for fibrosis) is poor.

7.2 Ph-FCS utility in drug development or Noncirrhotic NASH / MASH clinical trials

In the context of Noncirrhotic NASH/MASH clinical studies and as illustrated in the simplified workflow of Figure 9, the Ph-FCS Fibrosis biomarker will:

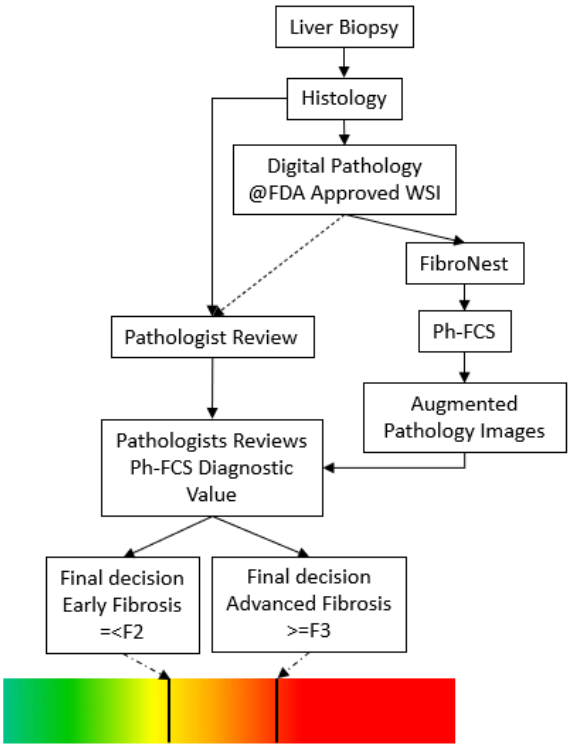


Figure 9: *Proposed workflow to use the Ph-FCS in the context of a NASH clinical study.*

- **Improve the quality of the NASH Studies baseline assessment of liver fibrosis stage with less variability in histopathologic interpretation.**
- **Aid pathologists adjudicate NASH-CRN Fibrosis stages in the F2-F3 transition zone.**
- **Assist in enrollment of a F3 population that will be more likely to develop clinical outcomes.**
- **Generate an exploratory continuous outcome for fibrosis severity for potential future use to better assess small differences in baseline fibrosis and changes in fibrosis secondary to treatment.**

7.3 Benefits and risks of applying the Ph-FCS in drug development or Clinical Trials.

Benefits

- Established from the same slide / WSI image as reviewed by the Pathologist for evaluation of liver fibrosis stage (≤ 2 vs ≥3 NASH/CRN fibrosis staging system).
- Compatible with Digital Pathology WSI scanners.
- The development of the Ph-FCS is directly based on the measurement and detection of histological phenotypes of severity, and not based on annotations from pathologists.
- Provides quantitative reference for Pathologists to help adjudicate histological fibrosis stages.
- The Ph-FCS is fully quantitative and continuous.
- The reproducibility and repeatability of the Ph-FCS is excellent.
- Helps recruit robust patient cohorts with advanced hepatic fibrosis (F>=3) in pre-cirrhotic NASH studies
- Does not change the current enrollment criteria for NASH/MASH clinical trials based on the NASH/CRN Brunt/Kleiner Fibrosis Scoring system.

Risks

- There are no significant risks to this method which will only assist the pathologist in staging the liver fibrosis according to the NASH/CRN scale in a consistent way.

8 Supporting information

8.1 Summary of Existing preclinical or clinical data to support biomarker development

Ph-FCS detects the effect of investigational compounds in pre-clinical models.

Extensive pre-clinical documentation of the effect of anti-fibrotic and antisteatotic compounds, including known agents, using quantification by the FibroNest scores with a higher performance than conventional histological readings ([33–38]).

8.1.1 Ph-FCS detects the effect of investigational compounds in Phase 2 NASH Studies.

8.1.1.1 Results from the Aramchol Phase 2b NASH Study (NCT02279524)

Results from the Aramchol Phase 2b NASH Study demonstrate that FibroNest Ph-FCS and ranked paired reading identify fibrosis improvement with Aramchol missed by conventional histological staging (Ref[23] Manuscript is under review).

8.1.2 [26]Ph-FCS predicts Liver Related Clinical Events outcomes.

Results from a retrospective cohort show that the Ph-FCS can predict first liver-related events (LRE, N=404, 18 years of follow-up) with a performance of AUC=0.675 (Figure 11). The Ph-FCS predicts LRE with a sensitivity of 66.7 and specificity of 74.5 (Cut off =3)[22]. This proof-of-concept data[22] established from the multicentric, European, Hepatic OuTcomes and SURvival Fatty Liver Registry (HOTSURFR) study retrospective cohort demonstrates the potential of the FibroNest method and the related Ph-FCS to generate evidence to qualify the Ph-FCS as a likely-surrogate endpoint for Fibrosis histological severity.

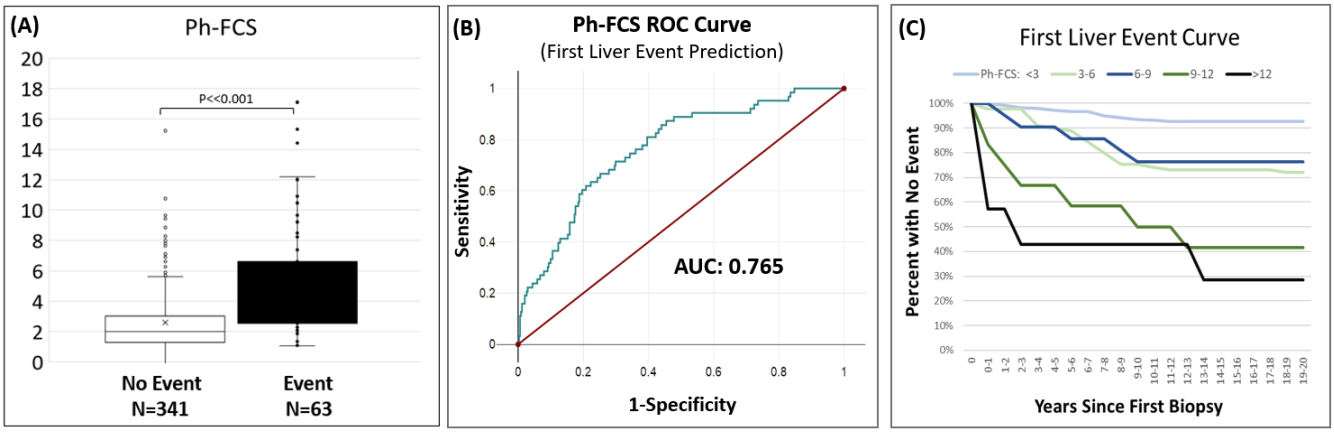


Figure 11: *Performance of the Ph-FCS as a predictor of liver events. Mean age was 53.5 yrs, 56% were males, mean BMI 30.6 kg/m², 39% had diabetes and 62% arterial hypertension. The proportion of histological fibrosis stages were: 0/1/2/3/4, 53%/17%/8%/14%/8% with N=404, respectively. Median follow-up was 11.4 yrs (IQR 4.7). 63 pts (15%) had at least one Liver Related Event (LRE). The Ph-FCS predicts LRE with a sensitivity of 66.7 and Specificity of 74.5 (Cut of =3)*[22]

8.2 Data generation plan to support further Validation data for FibroNest Ph-FCS

The FibroNest method is compatible with existing well curated cohorts of stained slides and digital images, as well as prospective studies. While the cut-point was established and validated in previous cohorts, see Section 6.3.2, in this context, the following table summarizes the data generation plan and partnerships (to date) to further support the analytical and clinical validation of the FibroNest Ph-FCS.

Study	Partnership With	Objectives	Steering Committee
PharmaNest 2023 POC Outcome Study	The multicentric, European, Hepatic OuTcomes and SURvival Fatty Liver Registry (HOTSURFR)	PharmaNest will use the data generated in its 2023 Liver Outcome Study (N=404) patients (data shown above) to enrich the Ph-FCS Validation cohort with 214 F0, 69 F1 and 32 F2 patients and evaluate the clinical performance of the Ph-FCS on a more balanced cohort.	Pr Vlad Ratziu and HOT SURF Registry
NIMBLE Phase 2	Foundation for the National Institutes of Health Biomarker Consortium Letter of Intent here	PharmaNest will participate (2024 and beyond) in the NIMBLE biomarker consortium to generate the Ph-FCS from the same images used to establish the categorical NASH-CRN stages and explore its value alone and in conjunction with the development and validation of novel non-invasive biomarkers for NASH including NIS4, PRO-C3 and Siemens (ELF) Well described long term liver outcomes might be included.	Arun Sanyal, MD Roberto Calle, MD
LITMUS	Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS)	PharmaNest will participate (late 2023 – 2024) in the LITMUS biomarker consortium and will generate the Ph-FCS as an alternative to categorical NASH-CRN states and explore its value alone and in conjunction with the development and validation of novel non-invasive biomarkers for NASH including NIS4, PROC3 and Siemens (ELF) including: • The LITMUS Metacohort (1a) with 1000 patient cases with a RNASeq cohort ~2000 patients and a Proteomics cohort ~600 patients. • LITMUS Study cohort (1b) – with 2,000 well characterized patients	Quentin M Anstee, MD, Patrick M Bossuyt, MD, Vlad Ratziu, MD
PharmaNest 2024-25 Outcome Study	To be announced	PharmaNest plans to recruit (2024) a composite, international, multicenter retrospective cohort of NASH patients (N~ 1700 to 2000) with well curated and standardized Liver Related Outcomes to (i) replicate the performance of the Ph-FCS as a prognostic of Liver events (N~400, (ii) fully establish the performance of Ph-FCS as a diagnostic biomarker to assess severity of liver fibrosis in baseline liver biopsy specimens for patients entering clinical trials	To be announced

Furthermore, the validation of the FibroNest Phenotypic scores in Pediatric Studies are ongoing[39] and the Pediatric Ph-FCS (Ped-Ph-FCS) will be adapted to pediatric studies where different phenotypes of NASH have already been reported.[40,41]

8.3 Other applications and fibrosis composite scores interpretation and utility

Because the FibroNest's Phenotypic analytical hypothesis and approach does not require annotations from Pathologists, it can be applied to multiple phenotypic questions for the study of liver fibrosis as summarized in the table below, showcasing the robustness of the approach, interpretation, and its translational relevance, including in pre-clinical models.

Fibrosis Phenotypic question	Authors	Reference
Is the histological phenotype of Fibrosis different between LEAN and OBESE NASH patients	Michihiro Iwaki, Vincent Wai-Sun Wong et al. Department of Gastroenterology and Hepatology, Yokohama City University School of Medicine, Japan, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong	[42] AASLD 2021 Poster here
Etiology-independent liver fibrosis severity scoring by quantitative digital pathology image analysis.	Michael Pavlides et Al. Medical Sciences Division, University of Oxford, Oxford, UK, Translational Gastroenterology Unit, Nuffield Department of Medicine, University of Oxford, Oxford, UK	[43] EASL 2022 Poster here Manuscript Sub'ed
An autocrine signaling circuit in hepatic stellate cells underlies advanced fibrosis in nonalcoholic steatohepatitis.	Wang (Icahn Mount Sinai), Scott Friedman, Neil C. Henderson, Maria I. Fiel et al.	[34] 10.1126/scitranslmed.add3949
Advanced quantitative phenotypic fibrosis and steatosis scoring is markedly superior to histology-based conventional staging in NASH animal models.	Scott Friedman et Al. Division of Liver Diseases, Icahn School of Medicine at Mount Sinai New York, NY, USA	[33] EASL/ILC 2021 Poster here
Automated fibrosis phenotyping of liver tissue from non-tumor lesions of patients with and without hepatocellular carcinoma after liver transplantation for non-alcoholic fatty liver disease	Nakamura Y, Miyaaki H, Miuma S, et al Nagasaki University, Nagasaki, Japan	[44] Doi:10.1007/s12072-022-10340-9
Continuous staging of NASH Patients at low (F1) Fibrosis Severity: Evaluation of the performance of a novel histology-based fibrosis phenotypic composite score and predictive AI tools.	Cynthia Behling et Al. University of California, San Diego, NAFLD Research Center, Division of Gastroenterology.	[45] AASLD 2021 Poster here
Semaglutide Has Beneficial Effects on Non-Alcoholic Steatohepatitis in Ldlr-/-.Leiden Mice.	José A. Inia et Al. Metabolic Health Research, The Netherlands Organization for Applied Scientific Research (TNO), 2333 BE Leiden, The Netherlands	[35] doi.org/10.3390/ijms24108494

9 Previous Interactions with FDA

- PharmaNest is an FDA Medical Device Establishment: 3025584441, operator 10086910
- PharmaNest has obtained FDA's Small Business Certification.
- PharmaNest image data system is listed as a Medical Device (product code OUG), listing number D491537.

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