Letter of Intent

LITMUS request for Biomarker Qualification Advice

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1. List of Abbreviations

AASLD	American Association for the Study of Liver Diseases
AE	Adverse event
ALT	Alanine aminotransferase
APASL	Asian Pacific Association for the Study of the Liver
AST	Aspartate aminotransferase
BQP	Biomarker Qualification Program
CAP	Controlled Attenuation Parameter
CE	Manufacturer's declaration that the product meets the applicable EC directives
CLD	Chronic liver disease
CLIA	Clinical Laboratory Improcement Amendments
COU	Context of Use
DAMP	release damage-associated molecular pattern
EASL	European Association for the Study of the Liver
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
EU	European Union
FAST	Name of the composite maker to be included in the final composite marker
FDA	U.S. Food and Drug Administration
HSC	hepatic stellate cells
IDE	Investigational Device Exemption
IFCC	International Federation of Clinical Chemistry
IFU	Information for use

IMI2	Innovative Medicines Initiative
IVD	in vitro diagnostic
LITMUS	Liver Investigation: Testing Marker Utility in Steatohepatitis
LOI	Letter of Intent
LSM	Liver Stiffness Measure
NAFLD	Non-Alcoholic Fatty Liver Disease
NAS	NAFLD Activity Score
NASH	Non-Alcoholic SteatoHepatitis
NASH-CRN	Non-Alcoholic SteatoHepatitis Clinical Research Network
NPV	Negative Predictive Value
NSE	Not substantial equivalent
PPV	Positive Predictive Value
QC	Quality Control
SAE	Serious Adverse event
SOP	Standard Operating Procedure
TMB	Tetramethylbenzidine
ROS	Reactive Oxygen Species
RUO	Research use Only
UK	United Kingdom
US	United States
VCTE	Vibration Controlled Transient Elastography

2. Administrative Information

2.1 Submission Title

To qualify a composite biomarker within the context of use (COU) Diagnostic screening, c.

2.2 Requesting Organization

LITMUS (www.litmus-project.eu) is an EU funded consortium within the Innovative Medicines Initiative 2 (IMI2) Program (www.imi.europa.eu).
The Project Coordinator is Prof Quentin M. Anstee from Newcastle University, UK
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Specific information on the LITMUS consortium can be found in **Attachment 1.**

2.3 Biomarker information and COU

This LOI includes a composite biomarker consisting of 2 individual biomarkers. The two biomarkers are planned to be validated individually first before validating the final composite marker. The biomarkers are:

1: PRO-C3, a serum/plasma collagen neo-epitope

2: The FAST score, which is based on two different imaging markers (measured using the FibroScan®) and a blood measure of aspartate aminotransferase, in this document defined as 'the FAST score (LSM and CAP (FibroScan®) and AST)'

3: The final composite biomarker, which will include both PRO-C3 as well as the FAST score (LSM and CAP (FibroScan®) and AST), in this document defined as 'the final composite marker'

A list of questions is included to ensure that our current proposal is meeting the expectations of the FDA Biomarker Qualification Program (BQP). At this stage we would like to ask questions in relation to the use of the Metacohort for definition of cut-off values, and the LITMUS study for validation of these cut-offs. As the clinical studies are ongoing, it is of utmost importance for the LITMUS consortium to get feedback on these questions in case any changes would be required. We would appreciate the opportunity to implement such changes as soon as possible.

References are available upon request.

2.4 Contact information

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2.5 Submission Date

December 3, 2019

3. Drug Development Need Statement

Describe the drug development need that the biomarker is intended to address, including (if applicable) the proposed benefit over currently used biomarkers for similar context of uses (COUs)

As recently acknowledged by the FDA[1]: *'NAFLD is the most common cause of chronic liver disease in North America. Currently, there are no approved drugs for the treatment of NASH. Given the high prevalence of NASH, the associated morbidity, the growing burden of end-stage liver disease, and limited availability of livers for organ transplantation, FDA believes that identifying therapies that will slow the progress of, halt, or reverse NASH and NAFLD will address an unmet medical need.'* and '*At this time, reliable diagnosis and staging of NASH can only be made by histopathological examination of a liver biopsy specimen. Liver biopsy, however, is an invasive procedure that is associated with occasional morbidity and, in rare circumstances, mortality. The use of liver biopsies in clinical trials poses significant logistical challenges (e.g., cost, availability of pathologists with specific expertise in NASH); in addition, some patients are reluctant or unwilling to undergo biopsy. Therefore, noninvasive biomarkers are needed (including imaging) to supplant liver biopsy and provide a comparable or superior ability to accurately diagnose and assess various grades of NASH and stages of liver fibrosis. Identification and validation of such biomarkers could significantly accelerate drug development in NAFLD. FDA encourages sponsors to consider biomarker development.'*

LITMUS is an EU funded consortium aiming to identify and validate biomarkers which can be qualified for use in the development of new therapies within NAFLD/NASH (see **Attachment 1**). Ultimately, the goal is to identify biomarkers that can decrease or eliminate the use of liver biopsy.

4. Biomarker Information and Interpretation

4.1 Biomarker names

4.1.1 PRO-C3

PRO-C3 is an in vitro diagnostic (IVD), assessing N-terminal propeptide fragments containing the neo-epitope generated by ADAMTS-2. This marker measures formation of type III collagen. The PRO-C3 ELISA is for Research use Only (RUO) in the US. See **Attachment 2**.

4.1.2 The FAST score (LSM and CAP (FibroScan®) and AST)

The FAST score combines two physical biomarkers, namely liver stiffness measurement (LSM) at 50 Hz shear wave frequency by Vibration Controlled Transient Elastography (VCTE) and Controlled Attenuation Parameter (CAP) together with the circulating biomarker aspartate aminotransferase (AST). The medical device used to measure the two physical biomarkers named FibroScan® (Echosens, Paris, France), is CE marked and FDA approved (510(k)). See **Attachment 3** and **4.**

4.2 Analytical methods

4.2.1 PRO-C3

The PRO-C3 ELISA employs the quantitative competitive ELISA technique. The target of recognition is the released N-terminal propeptide of type III collagen: N-^{145}PTGPQNYSP^{153}↓. Streptavidin pre-coated plates are coated with the biotinylated antigen: biotin-PTGPQNYSP. Unbound biotinylated antigens are washed away before standards, quality control (QC) specimens, and unknown samples in appropriate dilutions are pipetted into the wells followed by addition of the horseradish peroxidase-conjugated antibody (HRP-Ab) specific for the PRO-C3 neoepitope. After incubation, the wells are washed to remove unbound HRP-Ab and sample material and the substrate solution 3,3',5,5'-

tetramethylbenzidine (TMB) is added. TMB develops color inversely proportional to the concentration of PRO-C3 in the samples. Concentration of PRO-C3 is determined by colorimetric assessment.

4.2.2 FAST score (LSM and CAP (FibroScan®) and AST)

The FAST score combines the three following biomarkers:

1. LSM (Liver stiffness measurement) at 50Hz shear wave frequency by Vibration Controlled Transient Elastography (VCTE)
2. CAP (Controlled Attenuation Parameter)
3. Aspartate aminotransferase (AST)

LSM: Liver stiffness measurement by VCTE

VCTE[2] is the patented technology used by the FibroScan® medical devices (Echosens, Paris, France) to measure liver stiffness. The general principle of VCTE relies on the use of a vibrator to induce a 50 Hz shear wave within the liver. Ultrasound signals are used to track the shear wave and compute a shear wave propagation map from which the shear wave speed is derived. The shear wave speed is directly related to the liver stiffness (details are provided in **Attachment 3**).

CAP: Controlled Attenuation Parameter

The CAP technology[3,4] is a patented technology used by the FibroScan® medical devices to measure steatosis of the liver. The general principle of measurement of ultrasound attenuation coefficient relies on the measurement of the loss of energy as ultrasound propagates through the medium. As attenuation of ultrasound also depends on their frequency, CAP is provided at a fixed frequency of 3.5 MHz (details are provided in **Attachment 3**)

Aspartate aminotransferase

AST activity level must be assessed according to the International Federation of Clinical Chemistry (IFCC) guidelines from venous blood.

4.3 Measuring units and limits of detection

4.3.1 PRO-C3

The measuring range for serum and EDTA plasma samples is determined as the range from LLOQ to ULOQ for serum: 2.0-53.8 ng/mL.

4.3.2 FAST score (LSM and CAP (FibroScan®) and AST)

The FAST score output has no unit and ranges from 0.0 to 1.0.

4.3.3 The final composite marker

Not yet determined.

4.4 Biomarker Interpretation and utility

Post-analytical application/conversion of biomarker raw measure to the applied measure

4.4.1 PRO-C3

The raw biomarker measurement will be used to investigate the utility of PRO-C3. PRO-C3 is reported as ng/mL.

4.4.2 FAST score (LSM and CAP (FibroScan®) and AST)

The FAST score is based on the following logistic regression model which is about to be made public[5]:

$$FAST=\frac{e^{-1.65+1.07\times \ln(LSM)+2.66*10^{-8}\times CAP^{3}-63.3\times AST^{-1}}}{1+e^{-1.65+1.07\times \ln(LSM)+2.66*10^{-8}\times CAP^{3}-63.3\times AST^{-1}}}$$

where LSM is expressed in kPa, CAP is expressed in dB/m and AST in IU/L.

4.4.3 The final composite marker

Not yet determined.

4.5 Rationale for post-analytical elements

Describe rationale for post-analytical elements used as inputs in application or conversion of the raw biomarker measurement

4.5.1 PRO-C3

Not applicable.

4.5.2 FAST score (LSM and CAP (FibroScan®) and AST)

Optimal exploratory variables' transformations of the FAST score were selected using multivariable first degree fractional polynomials to optimize the model. In this method, first order fractional polynomials are formulated as a power transformation of the predictors taken from the set -2, -1, -0.5, 0, 0.5, 1, 2, 3. Optimal power is selected for each predictor (considered in order of decreasing statistical significance) using a backward stepwise selection procedure[5].

4.5.3 The final composite marker

Not yet determined.

4.6 Clinical Interpretive Criteria

Describe the cut-off values, cut-points/thresholds, boundaries/limits or other comparators used in the interpretation of the biomarker measurement or its applied/converted form to draw an actionable conclusion based on the biomarker result

The cut-off values, cut-points/thresholds, or boundaries/limits for PRO-C3, the FAST score (LSM and CAP (FibroScan®) and AST) score and the final composite marker are not yet determined. Optimal cut-offs for the biomarkers will be determined in the Metacohort Study (see **Attachment 5**) using the approach described in **Section 6** below, guided by statistical considerations reflected in the statistical analysis plan (see **Attachment 6**), and thereafter validated in the LITMUS Study (see **Attachment 7**).

5. Context of Use Statement (COU)

The final composite biomarker will be tested against the specific COU 'Diagnostic screening'. The biomarker should be a diagnostic screening biomarker, which, among individuals with non-alcoholic fatty liver disease (NAFLD) identifies those at high risk of having liver fibrosis stages 2 or 3 (based on the non-alcoholic steatohepatitis clinical research network (NASH-CRN) scoring system), and which could subsequently be included in NAFLD clinical trials for drug development after further confirmation of their diagnosis with liver biopsy. The approach is described in the statistical analysis plan (see **Attachment 6**) and by Angulo et al[6].

6. Analytical Considerations

General description of what aspect of the biomarker is being measured including Index scoring as appropriate

6.1 PRO-C3

Serum and/or plasma baseline concentration of PRO-C3 will be obtained for the COU diagnostic

screening.

6.2 The FAST score (LSM and CAP (FibroScan®) and AST)

For the purpose of the FAST score calculation, LSM and CAP are measured with a FibroScan® device (Echosens, Paris, France) in addition to AST. LSM and CAP will be measured using either the M or XL probe according to the device automated probe selection tool. Since LSM and CAP are measured concomitantly by the FibroScan® device during a single examination, both biomarkers should come from the same examination which should have at least ten valid measurements. Finally, there should be less than 6 months of time interval between, on one hand, the LSM and CAP assessments by FibroScan® and, on the other hand, the phlebotomy from which AST is assessed. Further details on how LSM by VCTE and CAP should be measured are provided in **Attachment 3.**

6.3 Description

6.3.1 Description of sample source, matrix (base material and any additives), stability and composition of biomarker

For PRO-C3 and the FAST score (LSM and CAP (FibroScan®) and AST) - both to be part of the final composite score - the sample source will be selected from the Metacohort (see **Attachment 5**) for definition of cut-off.
Samples for validation of the respective cut-offs will be based on samples from the LITMUS study (see **Attachment 7**). For LSM and CAP (FibroScan®) the validation of the respective cut-offs will be based on measurements according to the imaging protocol (see **Attachment 9).**
The biospecimens from the Metacohort have been stored at -80°C for up to 9 years; most have been thawed and refrozen one or two times. The prospectively collected samples from the LITMUS study have been stored at -80°C according to the sampling protocol (see **Attachment 8**).

6.3.2 PRO-C3 sample stability and robustness

To ensure sample stability and robust assessments of the biomarker PRO-C3 in serum, the current recommended pre-analytical requirements and storage conditions are listed below:

- Blood samples should be obtained by venipuncture and hemolysis should be avoided
- It is recommended to collect blood samples in spray-coated silica tubes to aid clotting and a polymer gel for improved serum separation
- Allow blood to clot for a minimum of 30 minutes at room temperature and centrifuge to collect serum within one hour from sampling
- After collection of serum, the sample shall be stored at 2-8°C if the test is not initiated within 4 hours. The serum samples can be stored at 2-8°C if testing is initiated within 24 hours, otherwise the samples shall be stored frozen at -20°C or below.
- While human serum samples can be stored up to 12 months at -20°C with no adverse effect, it is recommended to use -80°C for long-term storage.
- In addition, serum samples can undergo 3 freeze/thaw cycles with no adverse effect.

6.3.3 The FAST score (LSM and CAP (FibroScan®) and AST) sample stability and robustness

LSM by VCTE and CAP shall be measured in vivo according to the recommendations of FibroScan® manufacturer's as detailed in **Attachment 3**. In brief, operators must be trained and certified by the manufacturer or its local representative. Patients must be fasting for a minimum of 3 hours before undergoing the FibroScan® procedure. Operator shall choose the probe corresponding to the morphology of the patient. Ten valid measurements at the same measurement point must be obtained. AST activity level must be assessed according to the International Federation of Clinical Chemistry (IFCC) guidelines from venous blood.

6.3.4 The final composite marker

Will consist of PRO-C3 and the FAST score (LSM and CAP (FibroScan®) and AST), see above.

6.4 Description of factors and plans to preserve specimen integrity

6.4.1 Description of pre-analytical factors and quality assurance/quality control (QA/QC) plans to preserve specimen integrity

A standard operating procedure (SOP) for sample collection including timing and location that sample will be collected from, storage and test/assay methodology; reference or control samples.

For the LITMUS Study the quality of samples and measurements is ensured by collecting all data according to the protocol and sampling protocols (see **Attachment 7** and **8**). Furthermore, monitoring plans for both the LITMUS Study and the Imaging Study are in place (see **Attachment 10** and **11**), as well as a Data management plan (see **Attachment 12**). Moreover, measuring of PRO-C3 samples is done in a CLIA certified laboratory.
For the Metacohort Study, no specific monitoring plan has been in place, outside good laboratory practice (see **Attachment 5**). PRO-C3 levels from samples of the Metacohort study are analyzed in a CLIA certified laboratory. The PRO-C3 test will be performed according to manufactures IFU, the analysis is at present for RUO.

6.4.2 Analytical validation plan

Analytical validation plan: description of measurement tool and device calibrations

6.4.2.1 Technical performance validation PRO-C3

The PRO-C3 ELISA has been fully validated using human serum samples, in accordance with the White Paper released by the Biomarker Assay Collaborative Evidentiary Considerations Writing Group, Critical Path Institute (C-Path) describing scientific and regulatory considerations for the analytical validation of assays used in the qualification of biomarkers in biological matrices[7]. This includes evaluation of (1) Detection Limits, (2) Linearity, (3) Parallelism, (4) Reference Range, (5) High Dose Hook Effect, (6) Reproducibility, (7) Analytical Specificity, (8) Carry Over, (9) Sample Stability, (10) Reagent Characterization, and (11) Reagent Stability. Guidelines from Clinical and Laboratory Standards Institute (CLSI) have been adopted when available, and studies of Sample Stability and Reagent Stability are currently ongoing in a CAP/CLIA certified laboratory. Outline protocols for the 11 validation protocols above are included in **Attachment 13**.
The Metacohort Study will generate data for cut-off determination using the PRO-C3 ELISA technically validated at a level equivalent to the requirements of a CE labeling[8]. The PRO-C3 ELISA, which is technically validated according to the White Paper[7], will be used in samples from the LITMUS Study to validate the cut-off.

6.4.2.2 The FAST score (LSM and CAP (FibroScan®) and AST)

Not applicable.

6.4.2.3 The final composite score

See above - as this will consist of PRO-C3 and the FAST score (LSM and CAP (FibroScan®) and AST)

6.5 Validation of the final version of the measurements tool

Once the SOP and analytical validation plan is finalized, describe how you will use this process to validate the final version of the measurement tool

The results of the technical validation of the individual biomarkers as well as the clinical utility (cut-off defined in Metacohort study and confirmed in the LITMUS study) will be the basis for the final composite biomarker for qualification.

7. Clinical considerations

Describe how the biomarker measurement is used to inform drug development. Please provide a decision tree to guide how the biomarker information would be used in drug development or a clinical trial.

7.1 COU Diagnostic – Screening

The decision tree for diagnostic screening is shown in **Attachment 14**. Individuals are screened for the initial eligibility criteria. If the individual meets the eligibility criteria, the final composite score (consisting of PRO-C3 and the FAST score (LSM and CAP (FibroScan®) and AST)), will be measured in relevant blood samples and by using the FibroScan® device. If values are above the selected cut-offs, individuals are at high risk of having clinically significant fibrosis, and will be recommended to have a confirmatory liver biopsy performed. If histopathological assessment confirms the inclusion criteria, the individual is enrolled in the clinical trial. In this way, the diagnostic screening biomarker will reduce the number of biopsies required for patient screening.

Describe patient population or drug development setting in which the biomarker will be used.

Non-alcoholic fatty liver disease (NAFLD) is a common progressive disorder closely associated with the clinical features of metabolic syndrome. This chronic liver condition occurs by excessive accumulation of fatty acids within hepatocytes and also represents a range of alterations to the extracellular matrix[9–11]. NAFLD represents a wide spectrum of disease ranging from simple steatosis to NASH, which is characterized by hepatic steatosis, inflammation and hepatocyte injury with variable degrees of fibrosis in the absence of secondary causes of steatosis[10,12]. Changes in the architecture and composition of the liver have been shown to be associated with clinically relevant progression of the disease[13–15]. The biomarkers proposed herein reflect various aspects of the alterations taking place in the liver during disease progression.

In order to identify patients with NASH, NAS ≥ 4 and F ≥ 2, which represent a composite target, biomarkers related to steatosis, fibrosis, lobular inflammation and ballooning are needed. The rationale for the selection of the biomarkers to be included in the final composite score was the following:

PRO-C3 specifically assesses N-terminal pro-peptide fragments containing the neo-epitope generated by ADAMTS-2, and thus reflects the degree of type III collagen formation which has been shown to drastically increase with increasing liver fibrosis[16]. PRO-C3 has been demonstrated in various multi-center cross-sectional cohorts of patients with NAFLD to be a diagnostic marker for advanced fibrosis[17–24].

The FAST score is a simple combination of two physical biomarkers: LSM by VCTE and CAP together with AST, a circulating biomarker. In order to have a biomarker related to lobular inflammation and ballooning, it was *a priori* decided to use a simple and readily available biomarker from routine clinical practice known to be linked to liver damages. Aspartate aminotransferase (AST) is an intracellular enzyme present in a number of tissues including hepatocytes. Additional AST is released in the bloodstream when cells are damaged, causing levels of enzymes to rise. Very high levels are seen in acute hepatic injury and modest increases are seen in many types of liver diseases. Information on steatosis and fibrosis can be provided by CAP and LSM, respectively. Several publications assessed the performances of LSM and CAP in assessing liver fibrosis and liver steatosis, respectively, using concomitant liver biopsy as the reference in NAFLD/NASH patients (**Attachment 3**).

The recently released FDA NASH draft guidance states that the ideal inclusion criteria in NASH trials

is 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[1]. This is also reflected by the reflection paper by EMA[25].

The final composite marker is intended to identify individuals with NAFLD at high risk of having clinically significant fibrosis (i.e. stages 2 and 3, based on the NASH-CRN scoring system) who could subsequently be included in NAFLD clinical trials for drug development after further confirmation of their diagnosis with liver biopsy. This will reduce the number of biopsies required.

7.2 Clinical validation: provides information to support biological and clinical relevance of the biomarker as applied in the COU.

The clinical validation for each of the two biomarkers will be done utilizing two separate NASH/NAFLD study groups.

The Metacohort Study (see **Attachment 5**), will be used to define cut-offs for the components of the final composite marker (see **Attachment 6**).

The LITMUS study is performed under a protocol, patients will be included according to inclusion and exclusion criteria as described in the LITMUS Study protocol (see **Attachment 7**), using the technically validated biomarker PRO-C3 which is validated according to the white paper released by the Biomarker Assay Collaborative Evidentiary Considerations Writing Group, Critical Path Institute (C-Path)[7].
The FAST score will be validated as described in the imaging study protocol (substudy of the LITMUS study) see **Attachment 9**). Information on the technical validation of the measurement of liver stiffness by VCTE and steatosis by CAP is provided in the K123806 and K150949 510(k) submission, respectively. The FAST score will be computed on the web based calculator provided by Echosens (my FibroScan® app) which has been verified and validated against the logistic regression model provided in Newsome et al.[5]

The reference value will be liver biopsies which are collected both in the Metacohort and the LITMUS study (see **Attachment 5** and **7**).

Samples and biopsies will be collected at baseline and yearly usually at the time of routine clinical appointments. However, if this is not practical, patients may be asked to attend for sample collection on a mutually convenient separate occasion. Adverse events will be collected by the investigator according to the protocols.

11 countries in Europe will be involved in the LITMUS Study.

7.3 7.3 Describe how normal or other reference values are established, provide study design(s), analytical plan, etc.

7.3.1 Reference value

The reference for the biomarkers mentioned is the histological assessment of a liver biopsy which have been collected from all patients included in the Metacohort and will be collected in the LITMUS study and the imaging study (sub-study to the LITMUS study).
The LITMUS study and the Imaging sub-study will include data from NASH/NAFLD patients under the governance and processes of the LITMUS consortium (see **Attachment 1**).

The LITMUS Study and the Imaging sub study are prospective clinical studies for which data are collected in accordance with clinical protocols (see **Attachment 7** and **9**), quality is ensured by monitoring plans (see **Attachment 10** and **11**), a sampling protocol (see **Attachment 8**), a standardized procedure for obtaining, processing and assembly of a liver biopsy in the LITMUS study (see **Attachment 15**), and a data management plan (see **Attachment 12**). Furthermore, the imaging charter can be found in **Attachment 16**. Statistical analysis to be performed is described in the statistical analysis plan (see **Attachment 6).**

7.3.2 Benefit and Risk

Benefits and Risks of applying the biomarker in drug development or a clinical trial.

A liver biopsy has an inherent risk of discomfort, bleeding, and in very rare cases death. A qualified biomarker for the described COU diagnostic screening will decrease the number of biopsies to be performed in future NAFLD/NASH drug development clinical trials. As such, the biomarkers will allow for a more efficient and less burdensome development plan. At this point in the development the risks have not been identified.

7.3.3 Knowledge gaps

Describe any current knowledge gaps, limitations and assumptions in applying the biomarker in drug development or a clinical trial

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

8. Supporting Information

8.1.1 Provide underlying biological process or supporting evidence of association of the biological process with the biomarker

Liver fibrosis is biochemically complex but is orchestrated primarily by activated hepatic stellate cells (HSCs). Activated HSCs produce components of the extracellular matrix (ECM). The ECM includes an array of proteins involved in scar formation including fibronectin, laminin, collagens, hyaluronic acid (HA), and proteoglycans. Type I, III, IV, and V collagen are prominently expressed within the liver[26]. HA is an essential component of the ECM and is produced primarily by HSC[27]. The accumulation of deposited ECM progressively replaces the normal liver parenchyma, producing damage and scar tissue and ultimately disrupting hepatic architecture and function.

8.1.2 PRO-C3

Biology of PRO-C3

Liver fibrosis is the consequence of a protracted wound healing response due to chronic liver disease (CLD). The fibrotic scars that form within the liver are composed of a variety of ECM proteins such as collagens, elastin and proteoglycans[28]. The ECM composition of a fibrotic liver is qualitatively and quantitatively different from that of a healthy one[29,30]. Furthermore, a ten-fold increase in both collagenous and non-collagenous ECM components has been observed in fibrotic livers[30,31]. Myofibroblasts, derived from activated HSCs, are the primary source of ECM within fibrotic livers, although other cell types such as peri- and portal fibroblasts contribute to the formation of fibrosis[28]. Chronic injury to the liver releases a plethora of pro-fibrotic stimuli which activate quiescent HSCs; for example dying hepatocytes release damage-associated molecular patterns (DAMPs), reactive oxygen species (ROS) as well as pro-fibrotic cytokines such as transforming growth factor (TGF)-β[32].

Expression of type III collagen is restricted to soft tissues and correlates with the number of activated (myo-) fibroblasts in fibrotic tissue. Consequently, the accuracy for fibrotic processes is greater for type III collagen (and other, minor collagens), as compared to type I collagen. Type III (pro) collagen peptides are therefore good prognostic biomarkers for liver fibrosis[33–35]. The currently best validated

and most accurate biomarkers for the measurement of type III collagen formation are the N-terminal propeptide PIIINP and PRO-C3, a peptide located at the cleavage site of PIIINP[16], albeit with very important differences.
More details on the rationale of parameter selection are provided in **Attachment 2**.

8.1.3 The FAST score (LSM and CAP (FibroScan®) and AST)

Biology of LSM

All chronic liver diseases induce histological changes that ultimately lead to accumulation of excess scaring tissue (fibrosis) in the extracellular matrix. In soft tissues, the latter is the main driver tissue stiffness. There is therefore a direct link between liver stiffness and fibrosis content. Other factors, such as hepatitis (or hepatic inflammation, particularly in patients with serum alanine aminotransferase (ALT) greater than three times the upper limit of normal), mechanic cholestasis, liver congestion (that is, heart failure), cellular infiltrations, and deposition of amyloid have been shown in various chronic liver disease to affect LSM irrespective of the fibrosis stage[36]. However, in 373 patients undergoing a liver biopsy for suspicion of NAFLD/NASH, a multivariable analysis found that, when adjusted for fibrosis stage, there was no significant influence of steatosis grade, steatosis grade, ballooning grade, lobular inflammation and portal inflammation on LSM[37].

Biology of CAP

Ultrasound attenuation is the term used to account for loss of wave amplitude (or ''signal'') due to all mechanisms, including absorption and scattering. Presence of fat in the liver is known to induce a hyper echogenicity of the liver (increased scattering) which leads to an increased attenuation of ultrasound.

Biology of AST

Aspartate aminotransferase (AST) is an intracellular enzyme present in several tissues including hepatocytes. Additional AST are released in the bloodstream when cells are damaged, causing levels of enzymes to rise. Very high levels are seen in acute hepatic injury and modest increases are seen in many types of liver diseases.

More details on the rationale of parameter selection are provided in **Attachment 3**.

8.2 Summary of existing clinical data to support the biomarker in its COU (e.g. summaries of literature findings, previously conducted studies).

8.2.1 PRO-C3 – Clinical data as a Diagnostic for screening

All relevant literature on PRO-C3 are presented in **Attachment 2.**

PRO-C3, a marker of both fibrogenesis and type III collagen formation, has been demonstrated in various multi-center cross-sectional cohorts of patients with non-alcoholic fatty disease (NAFLD) to be a diagnostic marker for clinically significant and advanced fibrosis[18,20–22,38,39] (see **Table 1**). Similarly, PRO-C3 was also found to be highly related to disease activity within these NAFLD cohorts, as determined by the NAFLD activity score (NAS)[38]. Daniels *et al* found within a derivation cohort of 150 NAFLD patients that the optimal cut-off of PRO-C3 for the identification of patients with advanced fibrosis (F3-4) was 15.8 ng/mL. Within a validation cohort of 281 patients the optimal cut-off was determined to be 15.6 ng/mL[38]. The diagnostic performance of PRO-C3 within the study by Daniels *et al* can be found in **Table 2** below. PRO-C3 was further found to accurately identify patients with advanced fibrosis within a discovery cohort of 164 NAFLD patients (AUROC 0.74)[18]. The optimal cut-off for the detection of advanced fibrosis was found to be 20.9 ng/mL in the discovery cohort by Luo *et al* (sensitivity 57%, specificity 84%) and validated in a cohort of 41 patients (sensitivity 57%,

specificity 79%)[18]. Phase II and III studies are currently ongoing, however data from these studies cannot be presented at this time due to confidentiality agreements.
For clinical data from other COU for PRO-C3, see **Attachment 2**.

Table 1. Clinical data for PRO-C3 as a diagnostic screening tool

Lead Author or Sponsor	Citation	Clinical Trial Number	Composite data*	Context of Use	Condition	Etiology
Daniels et al.[38]	Hepatology. 2019 Mar;69(3):1075-1086.	NA	Yes	Diagnostic / Screening	Liver fibrosis	NAFLD/NASH
Luo et al.[18]	Scientific Reports 8, Article number: 12414 (2018).	NA	No	Diagnostic / Screening	Liver fibrosis	NAFLD/NASH
Boyle, M. et al.[39]	AASLD LiverLearning® 201278 (2017). [ABSTRACT 93]	NA	Yes	Diagnostic / Screening	Liver fibrosis	NAFLD/NASH
Leeming, D. J. et al.[20]	AASLD LiverLearning® 195639 (2017). [ABSTRACT]	NA	Yes	Diagnostic / Screening	Liver fibrosis	NAFLD/NASH
Leeming, D. J. et al.[21]	J. Hepatol. 66, S154 (2017). THU-351 [ABSTRACT]	NA	No	Diagnostic / Screening	Liver fibrosis	NASH
Leeming, D. J. et al.[22]	International Workshop on NASH Biomarkers 2017 - Program book [ABSTRACT 07]	Screening population from NCT02217475	No	Diagnostic / Screening	Liver fibrosis	NAFLD/NASH
Bril et al. [24]	Diabetes Care 2019 May; dc182578.	NA	Yes*	Diagnostic/ Screening	Liver Fibrosis	NAFLD/NASH

*Data on a composite score of PRO-C3 and clinical variables presented in the citation.

Table 2. Diagnostic performance of PRO-C3 in the study by Daniels et al[38]

	Derivation cohort	Validation cohort
n	150	281
% F3-4	22.0%	23.1%
AUROC	0.81 [0.743 - 0.873]	0.82 [0.775 - 0.867]
Cut-off (ng/mL)	15.8	15.6
Sensitivity	90.9 [75.7 - 98.1]	86.1 [75.3 - 93.5]
Specificity	63.2 [53.8 - 72.0]	70.4 [63.8 - 76.4]
PPV	41.1 [29.7 - 53.2]	46.7 [37.5 - 56.0]
NPV	96.1 [89.0 - 99.2]	94.4 [89.7 - 97.4]

Abbreviations: AUROC, area under the receiver operating curve; PPV, positive predictive value; NPV, negative predictive value.

8.2.2 The FAST score (LSM and CAP (FibroScan®) and AST) – Clinical data as a Diagnostic for screening

All relevant literature on LSM and CAP are presented in **Attachment 3**.

The FAST score has been subject to several oral and poster presentation in several international liver disease meetings such as:

- the 2018 AASLD Liver meeting in 2018 (San Francisco, CA, USA)
- the 2019 APASL annual meeting (Manilla, Philippines)
- the 2019 EASL International Liver Conference (Vienna, Austria)
- the 2019 International Conference on Fatty Liver (Berlin, Germany)
- the 2019 EASL NAFLD summit (Sevilla, Spain).

The pivotal paper on the development of this score has been accepted for publication in the Lancet Gastroenterology & Hepatology[5] and is summarized below.

A prospective, multicentre study of patients undergoing a liver biopsy for suspicion of NAFLD was conducted in England to derive this score. This was a pre-specified secondary outcome of a study for which the primary endpoints of which have already been reported. Liver stiffness measurement (LSM) by vibration-controlled transient elastography and controlled attenuation parameter (CAP) measured by FibroScan® device were combined with aspartate aminotransferase (AST), alanine transaminase (ALT) or AST:ALT ratio. To identify those patients with NASH, an elevated NAS and clinically significant fibrosis the best fitting multivariable logistic regression model was identified and internally validated using boot-strapping. Score calibration and discrimination performance were determined in both the derivation dataset (England) and seven independent international (France, USA, China, Malaysia, Turkey) histologically confirmed cohorts of patients with NAFLD (external validation cohorts). Between March 20th 2014 and January 17th 2017, 350 patients with suspected NAFLD attending liver clinics in England were prospectively enrolled in the derivation cohort. The most predictive model combined LSM, CAP and AST. Performance was satisfactory in the derivation dataset (C-statistic = 0.80, 95% confidence interval: 0.76-0.85 and was well calibrated). In external validation cohorts, calibration of the score was satisfactory and discrimination was good across the full range of validation cohorts (C-statistics ranging from 0.74 to 0.95, C-statistic = 0.85, 95% confidence interval: 0.83-0.87 in the pooled external validation patients' cohort; n=1026). Cut-offs for a sensitivity and a specificity ≥0.90 were 0.35 and 0.67 respectively in the derivation cohort and lead to a positive predictive value (PPV) of 0.83 (84/101) and a negative predictive value (NPV) of 0.85 (93/110). In the external validation cohorts, corresponding PPV ranged from 0.33 to 0.78 and NPV from 0.73 to 1.

8.2.3 The final composite score

Data on the final score has not yet been obtained.

9. Previous Qualification Interactions and Other

9.1 Qualification Interactions

An Innovation Task Force briefing meeting was held at the European Medicines Agency (EMA) on 10th October 2018. This was a general meeting at which the individual biomarkers were not discussed.

On June 15th 2019 a scientific advice was started on Qualification of the same markers as defined in this LOI for Diagnostic Screening and another scientific advice on Prognostic Enrichment. A meeting will be held on February 11th 2020 with the defined group at EMA premises, written feedback is expected December 6th 2019 and January 24th 2020, respectively.

On June 16th 2019 an LOI was submitted to FDA, which included 3 different COU in one LOI, and on July 17th 2019 a phone meeting was held with the FDA qualification team. At this meeting LITMUS was requested to send one COU per LOI, and informed that each LOI can only include one biomarker - however a biomarker which is a composite was mentioned as acceptable. The current LOI is written in response to this request.

9.2 Other regulatory interactions PRO-C3

Discussions with CDRH was previous performed under K14091 and Q151218 (these discussions were related to a different use of the PRO-C3 test in patients with hepatitis).
The PRO-C3 test is not approved for the US market or the European market.
The PRO-C3 test is currently a RUO test.

9.3 Other regulatory interactions The FAST score (LSM and CAP (FibroScan®) and AST)

9.3.1 Interactions with the FDA on LSM and CAP (FibroScan®)

Table 3. Regulatory interactions with the FDA on LSM and CAP (FibroScan®)

Date	Event	Summary
August 17 2004	pre-IDE Meeting	• Device recognized as Non Significant Risk (NSR) • FDA requests review of IDE • Endpoint of AUC = 0.85 was acceptable • Small inter-and intra-operability study sufficient • No predicate for 510(k) process • Device would be considered Class III PMA
February 2007	IDE follow-up	• Phase I study report accepted by FDA • Launch of Phase II study
September 24, 2007	Pre-IDE meeting	• Proposal to get approval with a tool claim • Response from FDA - IFU does not match open IDE - FDA will insist on clinical IFU - 510(k) De Novo –possible
February 26, 2009	IDE summary meeting	• FDA raising several concerns about the results of second phase of the IDE clinical study
July 7, 2009	510(k) submission	• K092055 • First 510(k) submission on the FibroScan® device based on the results of the IDE clinical study with the intend to to for a De Novo 510(k)
September 15, 2009	RFAI	• Pending NSE letter
October 27, 2009	NSE	• No predicate
November 11, 2009	De Novo 510(k) application	• Request for evaluation of automatic class III designation under section 513(f)(2)
June 9, 2010	Response to De Novo application	• Denial of De Novo 510(k) application • NTQ product code created • Class III • Used to diagnose cirrhosis • Intended for quantitative elastography • Indicated to provide information to the clinician that may be used adjunctively with other medical data obtained by a physician for the diagnosis of liver cirrhosis for patients with HBV or HCV chronic liver disease
August 1, 2011	IDE submission	• Second clinical trial proposal

August 31, 2001	Response to IDE submission	• several issues regarding the proposed study methodology • study classified as NSR and therefore not requiring FDA authorization
October 17, 2011	IDE supplement	• Response to FDA issues about study methodology
November 16, 2011	IDE close-out	• By FDA based on NSR status
February 7, 2012	Pre-IDE submission	• new package presenting the regulatory strategy envisioned based on the informal discussions which took place with new FDA lead reviewer during the end of 2011 and the beginning of 2012
December 12, 2012	510(k) submission	• K123806 • IFU: - The FibroScan® system is intended to provide 50 Hz shear wave speed measurements through the internal structures of the body. - FibroScan® is indicated for noninvasive measurement of shear wav speed at 50 Hz in the liver. The shear wave speed may be used as an aid to clinical management of patients with liver disease
January 25, 2013	RFAI to K123806	• Request for additional information from the FDA on 510(k) submission file
March 7, 2013	Response to RFAI	• Point by point response to all questions raised by the FDA
April 5, 2013	510(k) approval	• First 510(k) approval for FibroScan® • 502 Touch with M and XL probes • Common Name: Diagnostic Ultrasound System and Accessories • Classification: - Ultrasonic Pulsed Echo Imaging (21 CFR §892.1560) - code IYO - System Diagnostic Ultrasonic Transducer (21 CFR §892.1570) – code ITX
June 20, 2014	Pre-Sub	• Expansion of use for pediatric patients
July 11, 2014	Pre-Sub	• Controlled attenuation parameter (CAP) expansion of technical performance and indication for use
January 28, 2015	510(k) submission	• K150239 • Expansion of use for pediatric patients
March 20, 2015	RFAI on K150239	• Request for additional information on K150239 510(k) submission
March 31, 2015	510(k) submission	• K150949 • Controlled attenuation parameter (CAP) expansion of technical performance and indication for use
June 3, 2015	510(k) approval	• K150949 • IFU - The FibroScan® system is intended to provide 50 Hz shear wave speed measurements and estimates of tissue stiffness as well as 3.5 MHz ultrasound attenuation coefficient of attenuation (CAP: Controlled Attenuation Parameter) in internal structures of the body. - FibroScan® is indicated for noninvasive measurement in the liver of 50 Hz shear wave speed and estimates of stiffness as well as 3.5 MHz ultrasound coefficient of attenuation (CAP: Controlled Attenuation Parameter). The shear wave

		speed and stiffness, and CAP may be used as an aid to clinical management of adult patients with liver disease.
July 23, 2015	Supplement to 510(k) K150949	• Response for changes in the labelling requested by the FDA in K150949 approval letter
July 30, 2015	Response to RFAI	• Response to request for additional information on K150239 510(k) application
September 1, 2015	510(k) approval	• K150239 • IFU - The FibroScan® system is intended to provide 50 Hz shear wave speed measurements and estimates of tissue stiffness as well as 3.5 MHz ultrasound attenuation coefficient of attenuation (CAP: Controlled Attenuation Parameter) in internal structures of the body. - FibroScan® is indicated for noninvasive measurement in the liver of 50 Hz shear wave speed and estimates of stiffness as well as 3.5 MHz ultrasound coefficient of attenuation (CAP: Controlled Attenuation Parameter). The shear wave speed and stiffness, and CAP may be used as an aid to clinical management of adult patients with liver disease. - Shear wave speed and stiffness may be used as an aid to clinical management of pediatric patients with liver disease.
February 23, 2016	510(k) submission	• K160524 • New FibroScan® model 530 Compact with M+ and XL+ probes
March 14, 2016	RFAI on K160524	• Request for additional information on K160524 510(k) application
March 18, 2016	510(k) approval	• K160524 • New FibroScan® model 530 Compact with M+ and XL+ probes
October 7, 2016	Pre-Sub	• Q140760 • Change of IFU for FibroScan® product family product
November 22, 2016	Pre-Sub response	• Written response from FDA to Pre-Sub package
July 10, 2017	510(k) submission	• K172142 • New FibroScan® model 430 Mini+ with M+ and XL+ probes
July 31, 2017	RFAI on K172142	• Request for additional information on K172142 510(k) application
August 3, 2017	Response to RFAI	• Submission of responses to RFAI on K172142 510(k) application
September 13, 2017	510(k) approval	• K172142 • New FibroScan® model 430 Mini+ with M+ and XL+ probes
September 26, 2017	510(k) submission	• K173034 • Rewording of the IFU for all FibroScan® devices to introduce “diagnosis and monitoring of liver disease”
November 9, 2017	RFAI on K173034	• Additional questions for K173034
November 10, 2017	Response to RFAI	• Responses to additional questions on K172142 510(k)
November 14, 2017	510(k) approval	• K173034 • IFU - The FibroScan® Family of Products (Models: 502 Touch, 530 Compact, and 430 Mini+) is intended to provide 50Hz shear wave speed measurements and estimates of tissue stiffness as well as 3.5 MHz ultrasound

		coefficient of attenuation (CAP: Controlled Attenuation Parameter) in internal structures of the body. - FibroScan® Family of Products (Models: 502 Touch, 530 Compact, and 430 Mini+) is indicated for noninvasive measurement in the liver of 50 Hz shear wave speed and estimates of stiffness as well as 3.5 MHz ultrasound coefficient of attenuation (CAP: Controlled Attenuation Parameter). - The shear wave speed and stiffness, and CAP may be used as an aid to diagnosis and monitoring of adult patients with liver disease, as part of an overall assessment of the liver. - Shear wave speed and stiffness may be used as an aid to clinical management of pediatric patients with liver disease.
June 6, 2018	510(k) submission	• K181547 • S+ probe for FibroScan® model 530 Compact and 430 Mini+
June 18, 2018	RFAI on K181547	• Request for clarification for K181547
June 20, 2018	Response to RFAI	• Response to FDA's request for Clarification for K181547
July 3, 2018	RFAI on K181547	• Request for Changes for K181547
July 4, 2018	Response to RFAI	• Response to FDA's request for Changes for K181547
July 9, 2018	510(k) approval	• K181547 • S+ probe for FibroScan® model 530 Compact and 430 Mini+

9.3.2 Interactions with Notified bodies on LSM and CAP (FibroScan®)

Table 4. Regulatory interactions with the European Notified Body on LSM and CAP (FibroScan®)

EUROPEAN UNION **93/42/EEC Directive and 2017/745 Regulation**					
Device commercial reference	**Device class**	**Classification rationale**	**Evaluation of conformity**	**Market clearance**	**EC certificate**
FibroScan®630 (liver application)	IIa	**<u>Rule 10 of Annex IX of 93/42/CEE directive</u>** : "Active devices intended for diagnosis are in Class IIa: If they are intended to supply energy which will be absorbed by the human body, except for devices used to illuminate the patient's body, in the visible spectrum […]"	Annex II, excluding point 4 (directive 93/42/EEC)	August 13th, 2019	13635
FibroScan® 430 Mini+				December 14th 2017	
FibroScan® 430 Mini				July 6th 2016	
FibroScan® 530 Compact				February 15th 2016	
FibroScan® 502 Touch				October 9th 2014	

FibroScan® 402				July 9th 2010	
FibroScan® 502				December 2nd 2003	
Probes (S+, M+ and XL+)				December 2nd 2003	

Intended use

The FibroScan® device is intended to provide:

- Liver stiffness measurement at 50Hz shear wave frequency
- Liver ultrasound attenuation (CAP: Controlled Attenuation Parameter) at 3.5 MHz

Indications for use

- Liver stiffness and liver CAP are indicated as an aid to diagnosis and monitoring of adult patients as part of an overall assessment of liver disease.
- Liver stiffness is indicated as an aid to diagnosis and monitoring of pediatric patients as part of an overall assessment of liver disease.

9.3.3 Interactions with FDA/European Notified Body on FAST score

No regulatory interaction has been performed with the FDA or the European regulators about the FAST score.

10. Attachments

Attachment 1 = LITMUS charter
Attachment 2 = PRO-C3 information
Attachment 3 = The FAST score (LSM and CAP (FibroScan®) and AST)
Attachment 4 = 510(k) FibroScan® K123806
Attachment 5 = Metacohort Study description
Attachment 6 = Statistical analysis plan LITMUS
Attachment 7 = LITMUS Study protocol
Attachment 8 = Sampling protocol (Study Handbook)
Attachment 9 = Imaging protocol (sub study)
Attachment 10 = Monitoring plan for the LITMUS Study
Attachment 11 = Monitoring plan for the Imaging Study
Attachment 12 = Data management plan
Attachment 13 = Outline of CLSI protocols
Attachment 14 = Decision tree for diagnostic screening
Attachment 15 = Biopsy description
Attachment 16 = Imaging charter

11. Questions for BQP

Questions on clinical validation of biomarkers:

- Is the Metacohort Study (see **Attachment 5)** sufficient to be used for determination of cut-offs for the PRO-C3 and the FAST score (LSM and CAP (FibroScan®) and AST) (Note: PRO-C3 has been technically validated up to CE level before measuring the individual levels in this patient cohort)?
- The plan is to define the cut-offs based on data from the Metacohort Study (see **Attachment 5**) in approximately 100 NAFLD patients. Is this number considered sufficient for definition of cut-offs for the COU diagnostic screening.
- Do the protocols for the prospective LITMUS Study (see **Attachment 7**) and the Imaging Study (see **Attachment 9**), from which data will be collected for validation of the cut-offs, correctly define the primary objective, inclusion/exclusion criteria, SAE/AE reporting and sample collection, for a later qualification of the final composite biomarker for the COU diagnostic screening? (The PRO-C3 test to be used at this step has been validated according to the White paper[7]).
- **Plans, SOPs, SAP and monitoring of the prospective LITMUS study**:
    - All biomarkers will be compared to liver biopsies, is the biopsy description (see **Attachment 15**) correctly defined for a later qualification of the final composite biomarker?
    - Are the monitoring plans (**Attachment 10** and **11**) for the LITMUS Study and Imaging Study (**Attachment 7** and **9**) in accordance with FDA expectations for data that will be used for a later qualification of the final composite biomarker?
    - Is the data management plan (see **Attachment 12**) considered sufficient for a later qualification of the final composite biomarker?
    - Is the SOP for collection, processing, and storage of plasma, serum and frozen biopsies considered sufficient (**Attachment 8**) for a later qualification of the final composite biomarker?

12. References

1. FDA/CDER. Noncirrhotic Nonalcoholic Steatohepatitis With Liver Fibrosis: Developing Drugs for Treatment Guidance for Industry DRAFT GUIDANCE. (2018).
2. Sandrin, L. *et al.* Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med. Biol.* **29,** 1705–13 (2003).
3. Sasso, M. *et al.* Controlled Attenuation Parameter (CAP): A Novel VCTE™ Guided Ultrasonic Attenuation Measurement for the Evaluation of Hepatic Steatosis: Preliminary Study and Validation in a Cohort of Patients with Chronic Liver Disease from Various Causes. *Ultrasound Med. Biol.* **36,** 1825–1835 (2010).
4. Sasso, M. *et al.* Liver Steatosis Assessed by Controlled Attenuation Parameter (CAP) Measured with the XL Probe of the FibroScan: A Pilot Study Assessing Diagnostic Accuracy. *Ultrasound Med. Biol.* **42,** 92–103 (2016).
5. Newsome, P. N. et al. FibroScan based FAST (FibroScan-AST) score for the non-invasive identification of patients with non-alcoholic steatohepatitis (NASH) and significant activity and fibrosis: a prospective derivation and global validation study. *Lancet Gastroenterol. Hepatol.* **(in press),** (2019).
6. Angulo, P. *et al.* The NAFLD fibrosis score: A noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* **45,** 846–854 (2007).
7. Piccoli, S. *et al.* Points to Consider Document: Scientific and Regulatory Considerations for the Analytical Validation of Assays Used in the Qualification of Biomarkers in Biological Matrices. *Biomark. Assay Collab. Evidentiary Considerations Writ. Group, Crit. Path Inst.* 1–54 (2017).
8. EMA. Guideline on bioanalytical method validation. *EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2*** **44,** 1–23 (2012).
9. Kleiner, D. E. *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41,** 1313–1321 (2005).
10. EASL-EASD-EASO. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J. Hepatol.* **64,** 1388–1402 (2016).
11. Schuppan, D., Surabattula, R. & Wang, X. Y. Determinants of fibrosis progression and regression in NASH. *J. Hepatol.* **68,** 238–250 (2018).
12. Chalasani, N. *et al.* The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* **67,** 328–357 (2018).
13. Hagström, H. *et al.* Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD. *J. Hepatol.* **67,** 1265–1273 (2017).
14. Dulai, P. S. *et al.* Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. *Hepatology* **65,** 1557–1565 (2017).
15. Ekstedt, M. *et al.* Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* **61,** 1547–1554 (2015).
16. Nielsen, M. J. *et al.* The neo-epitope specific PRO-C3 ELISA measures true formation of type III collagen associated with liver and muscle parameters. *Am J Transl Res* **5,** 303–315 (2013).
17. Daniels, S. J. *et al.* ADAPT: An Algorithm Incorporating PRO-C3 Accurately Identifies Patients With NAFLD and Advanced Fibrosis. *Hepatology* **69,** 1075–1086 (2019).
18. Luo, Y. *et al.* An Evaluation of the Collagen Fragments Related to Fibrogenesis and Fibrolysis in Nonalcoholic Steatohepatitis. *Sci. Rep.* **8,** 12414 (2018).
19. Boyle, M. *et al.* Performance of the PRO-C3 Collagen Neo-Epitope Biomarker in Non-Alcoholic Fatty Liver Disease. *JHEP Reports* **0,** (2019).
20. Leeming, D. J. *et al.* PRO-C3 in Combination With Clinical Parameters is Associated With Severity of Histological Features of Nonalcoholic Steatohepatitis and Fibrosis. *AASLD LiverLearning®* 195639 (2017).
21. Leeming, D. J. *et al.* Estimation of serum “true collagen type III formation” (Pro-C3) levels as a

marker of non-alcoholic steatohepatitis in a prospective cohort. *J. Hepatol.* **66,** S154 (2017).
22. Leeming, D. J. *et al.* Plasma collagen type III (PRO-C3) levels associate with severity of histological features of non-alcoholic steatohepatitis and fibrosis within the screening population from the CENTAUR study. *NASH Biomarkers Work.* (2017).
23. Bril, F. *et al.* Performance of Plasma Biomarkers and Diagnostic Panels for Nonalcoholic Steatohepatitis and Advanced Fibrosis in Patients With Type 2 Diabetes. *Diabetes Care* (2019). doi:10.2337/dc19-1071
24. Bril, F. *et al.* Use of Plasma Fragments of Propeptides of Type III, V, and VI Procollagen for the Detection of Liver Fibrosis in Type 2 Diabetes. *Diabetes Care* dc182578 (2019). doi:10.2337/dc18-2578
25. EMA. *Reflection paper on regulatory requirements for the development of medicinal products for chronic non-infectious liver diseases (PBC, PSC, NASH)*. (2018).
26. Martinez-Hernandez, A. The hepatic extracellular matrix. I. Electron immunohistochemical studies in normal rat liver. *Lab. Invest.* **51,** 57–74 (1984).
27. Orasan, O. H., Ciulei, G., Cozma, A., Sava, M. & Dumitrascu, D. L. Hyaluronic acid as a biomarker of fibrosis in chronic liver diseases of different etiologies. *Med. Pharm. Reports* **89,** 24–31 (2016).
28. Bataller, R. & Brenner, D. A. Liver fibrosis. *J. Clin. Invest.* **115,** 209–218 (2005).
29. Gressner, A. M. & Weiskirchen, R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J. Cell. Mol. Med.* **10,** 76–99 (2006).
30. Karsdal, M. A. *et al.* The good and the bad collagens of fibrosis – Their role in signaling and organ function. *Adv. Drug Deliv. Rev.* **121,** 43–56 (2017).
31. Rojkind, M. *et al.* Collagen Types in Normal and Cirrhotic Liver. *Gastroenterology* **76,** 710–719 (1976).
32. Higashi, T., Friedman, S. L. & Hoshida, Y. Hepatic stellate cells as key target in liver fibrosis. *Adv. Drug Deliv. Rev.* **121,** 27–42 (2017).
33. Hansen, J. F. *et al.* PRO-C3: a new and more precise collagen marker for liver fibrosis in patients with chronic hepatitis C. *Scand. J. Gastroenterol.* **53,** 83–87 (2018).
34. Nielsen, M. *et al.* Plasma Pro-C3 (N-terminal type III collagen propeptide) predicts fibrosis progression in patients with chronic hepatitis C. *Liver Int.* **3,** 1–9 (2014).
35. Leeming, D. J. *et al.* True Collagen Type III Formation (Pro-C3) is Predictive of Outcome in HCV Patients with Advanced Liver Fibrosis with in the Trent Study. *J. Hepatol.* **64,** S719–S720 (2016).
36. Mueller, S. & Sandrin, L. Liver stiffness: a novel parameter for the diagnosis of liver disease. *Hepat. Med.* **2,** 49–67 (2010).
37. Eddowes, P. J. *et al.* Accuracy of FibroScan Controlled Attenuation Parameter and Liver Stiffness Measurement in Assessing Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* **156,** 1717–1730 (2019).
38. Daniels, S. J. *et al.* ADAPT: An Algorithm Incorporating PRO-C3 Accurately Identifies Patients With NAFLD and Advanced Fibrosis. *Hepatology* **69,** 1075–1086 (2019).
39. Boyle, M. *et al.* Development and Validation of the Collagen Neo-Epitope Biomarker Pro-C3 "FIB-C3 Score" for Detection and Staging of Advanced Non-Alcoholic Fatty Liver Disease in a Large International Multi-Centre Patient Cohort. *AASLD LiverLearning®* 201278 (2017).