"Human Liver Organoids as a Model to Study the role of the I148M variant of the gene *PNPLA3* in the development of Non-Alcoholic Steatohepatitis (NASH)"

Acronym: REASON

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FLOWCHARTS

	Screening	Intervention
Period	(-t1)	(t1)
Enlistment		
informed consent	-	
inclusion/exclusion criteria	-	
Administration of the intervention		
Biological sample collection		-

LIST OF ABBREVIATIONS

ADH: alcohol dehydrogenase ARAT:
retinol acyltransferase GWAS: genome-
wide association study HSCs: Hepatic
stellate cells
LRAT: lecithin retinol acyl-transferase
MMP13: matrix metalloprotease 13
NAFLD: nonalcoholic fatty liver disease
NASH: nonalcoholic steatohepatitis
PNPLA3: Patatin-like phospholipase domain-containing protein 3
RA: retinoic acid
RALDH: retinaldehydedehydrogenase-- TGFβ1:
transforming growth factor β1 TIMP: tissue
inhibitor matrix metalloproteases
-SMA: α-smooth muscle actin

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1. INTRODUCTION

1.1 Background and rationale

Nonalcoholic fatty liver disease (NAFLD), defined as the accumulation of fat in the liver not explained by risky alcohol consumption, is the leading cause of liver damage and occurs in approximately one-third of the population (25–30%). NAFLD encompasses a broad spectrum of conditions ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). NASH is defined histologically by the coexistence of steatosis, lobular inflammation, and hepatocellular damage and is frequently complicated by the development of fibrosis. NASH occurs in 20–30% of patients with NAFLD and may progress to cirrhosis and hepatocellular carcinoma (HCC) (1). Recent advances in genetics have highlighted that genetic factors play a key role in the susceptibility, severity, and long-term prognosis of the disease (2). In particular, in a genome-wide association study (GWAS) conducted on a multiethnic population, the gene was identified PNPLA3 (Patatin-likephospholipase domain-containingprotein 3),located on chromosome 22, as the major genetic factor that strongly associates with intrahepatic fat content, independently of body mass and insulin resistance (3). In particular, the polymorphism in the genePNPLA3 (rs738409, C>G), which determines an amino acid substitution from isoleucine to methionine in position 148 of the protein (I148M), currently represents the most important genetic predictor of steatosis, steatohepatitis and progression of liver damage and strongly increases the risk of developing hepatocellular carcinoma (4, 5).PNPLA3encodes a protein, also called adiponutrin, located at the level of the endoplasmic reticulum and on the surface of lipid droplets, with an important enzymatic function (triacylglycerol lipase), involved in the hydrolysis of triglycerides in hepatocytes and adipocytes and in the catabolism of retinol esters in stellate cells. The presence of the amino acid methionine in position 148 would lead to a loss of function of the protein with accumulation at the surface of lipid droplets and reduced remodeling of triglycerides (6). Furthermore, it has been demonstrated that PNPLA3, following induction by TGF--, regulates the metabolism of retinol in stellate cells (retinyl-esterase activity) determining the release of retinol ex vivoandin vitro (7). The risk variant 148M instead seems to prevent the release of retinol. Furthermore, it has been observed that the progression of chronic liver diseases related to steatosis is associated with cellular senescence and therefore patients carrying the risk variant (148M) show an up-regulation of cellular senescence andpathwaysfibrogenic.

These preclinical studies suggestPNPLA3as a new therapeutic target, especially with regard to the possibility of reducing its expression in the mutated variant. Therefore, screening based on the determination of the I148M variant status ofPNPLA3could be fundamental for designing future precision medicine approaches. Although the I148M variant ofPNPLA3causes progression of liver damage to advanced fibrosis and hepatocellular carcinoma (1), the phenotypic expression is highly variable and depends on the coexistence of other genetic factors and environmental factors. Furthermore, the relative contribution of the I148M variant carrier state on the steatohepatitis phenotype in

hepatocytes vs. stellate cells is not yet clear. The study of new molecular pharmacological approaches that aim at regulating the expression and activity of PNPLA3 for the prevention and treatment of liver diseases is however still difficult, due to both the limitations of experimental models in animals (cost, suffering, different regulation of lipid metabolism and PNPLA3 compared to humans) and the difficulty of reproducing NASH in modelsin vitro.
The current modelsin vitro,based on liver cell lines, are in fact unable to reproduce the intrinsic cellular heterogeneity, the three-dimensional architecture of the tissue and the cell-cell interactions. These characteristics are fundamental in the study of the processes that can lead to the onset of steatosis, progressing to a fibrotic, cirrhotic and tumorous state following an external insult. Organoids, three-dimensional (3D) cellular structures, generated both from induced pluripotent stem cells and from tissue-resident adult stem cells, are recently establishing themselves as a new culture model to overcome the limitations of traditional cell culture systems, emerging as powerful tools for the study of human diseases (8, 9). This is possible because they are able to stably preserve the genetic information of the autologous tissue and mimic the physiological and pathological state of the tissue of interest. Despite the cellular heterogeneity exhibited by organoids, these structures present some limitations such as the low capacity to mimic the architecture of the mature organ of interest due to the absence of cells that compose the surrounding environment (10, 11). This limitation can be overcome by creating 3D cultures, defined as “assembloids”, composed of the cells that constitute the organoids in co-culture with cells of the surrounding environment (stromal, stellate, endothelial cells, etc.), capable of recreating a more precise architecture of the starting tissue, represented by the epithelial component surrounded by its stroma (12, 13). These structures will be the basis for studying the molecular mechanisms that underlie the onset of steatosis and its progression to a fibrotic condition, due to the activation of stellate cells, following the damage suffered by hepatocytes.

2. OBJECTIVE OF THE EXPERIMENT

The aim of this project is to study the cellular and molecular mechanisms through which the variant at risk ofPNPLA3 (148M) accelerates the progression of liver damage in models based on the development of liver organoids and assemblyloids, and to test in these the efficacy of new molecular pharmacological approaches aimed at silencing the expression of the mutated protein variant in improving the pathological phenotype. Our hypothesis is that the loss of function ofPNPLA3in hepatocytes and hepatic stellate cells may represent a link between accumulation of hepatic fat, development of lipotoxicity and inflammation, fibrosis and finally liver cancer. The 148M variant could determine a persistent and uncontrolled activation of stellate cells (HSCs) due to an altered ability to release retinol. Activated HSCs would then undergo premature senescence with consequent release of pro-inflammatory and carcinogenic mediators. Silencing of PNPLA3 by using antisense RNA would therefore lead to a greater improvement of the

hepatocellular damage specifically in models derived from cells carrying the risk variant.

2.1 Primary objective

The primary objective of the study conducted at the Foundation is to generate and characterize three-dimensional models, called “assembloids”, composed of the main liver cell populations (in particular from the coculture of organoids derived from clinical samples together with stellate cells responsible for fibrogenesis). These models will be used to mimic the early stages of steatohepatitis onset in conditions of altered lipid metabolism (induced by exposure to the main environmental determinants of this condition: excess fatty acids, fructose, cholesterol) in the presence or absence of the I148M mutation ofPNPLA3. Other genetic variants will also be evaluated.

2.2 Secondary objectives

1. Identification of novel non-invasive biomarkers of pathological activation of human stellate cells and progression of liver damage to be subsequently validated in clinical series for future use in clinical management for individual risk stratification.

2. Study of epigenetic factors underlying the onset of non-alcoholic steatohepatitis and its progression to fibrosis, cirrhosis and HCC.

3. Evaluation of the impact of antisense oligonucleotides directed against PNPLA3 on the severity of the “steatohepatitic” phenotype (lipid accumulation, lipotoxicity and inflammation and fibrogenesis) in assembloids.

3. STUDY DESIGN

3.1 Study design

Genetic interventional, with the collection of biological, non-pharmacological, monocentric material.

RESPONSIBILITY (role of the promoter and collaborators)

Operational unit	Participant Name	Role and functions in the study
Department of Transfusion Medicine and Hematology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico	Dr. Daniele Prati Prof. Luca Valenti (principal investigator)	Patient recruitment and sample characterization; Isolation and generation of organoids, spheroids and tissues; Characterization of organoids by gene and protein expression Data analysis

Internal collaborations:

Operational unit	Participant Name	Role and functions in the study
UOC General Surgery and Liver Transplants, IRCCS Foundation Ca' Granda Hospital Maggiore Polyclinic	Professor Giorgio Rossi, Dr. Daniele Dondossola	Reporting of eligible patients and characterization of samples
Scientific Direction IRCCS Foundation Ca' Granda Hospital Maggiore Polyclinic	Dr. Stefano Gatti	Sample characterization and support in cell isolation protocols

External body that will handle the analysis of the biological sample:

Operational unit	Participant Name	Role and functions in the study
Institute for Liver and Digestive Health, Division of Medicine, University College London	Prof. Krista Rombouts	Immunohistochemical and biomarker analyses on liver histological samples

3.2 Inclusion criteria

Patients of legal age who have given consent to participate in the study will be included and will be listed for the following procedures:

- liver biopsy for suspected nonalcoholic steatohepatitis (NASH) at the time of diagnosis;
- liver resection for hepatocellular carcinoma or other liver lesions (including secondary lesions from other neoplasms and benign focal lesions, which will allow obtaining starting liver tissue

healthy);
- post-transplant healthy liver biopsies;
- cholecystectomies.

You will also be required:

- willingness to sign informed consent for the study
- availability of DNA sample for genetic analysis and clinical data,
- blood sampling for genetic and epigenetic analysis and analysis of non-coding RNAs (lncRNAs, miRNAs and circRNAs).

3.3 Exclusion criteria

Patients with the following will be excluded:

- positivity for chronic viral hepatitis (HCV-RNA and/or HBsAg);
- positivity to other liver diseases such as autoimmune and viral hepatitis (hepatitis B and C), hereditary hemochromatosis, alpha-1-antitrypsin deficiency, Wilson's disease.

4. PROCEDURES RELATING TO THE STUDY

4.1 Intervention

In collaboration with the Liver Transplant Unit of the Foundation, samples of discarded tissue from patients undergoing liver resection will be collected (both intra-tumoral and extra-tumoral tissues in the case of hepatocarcinoma) or taken from the whole liver post-transplant. This procedure will not entail any additional risk for the patient compared to the usual routine, nor will it reduce the availability of material for standard pathological analyses.
These samples will allow us to isolate cells (ovalocytes – liver progenitor cells) to generate organoids from normal liver tissue (cholecystectomy) and suspected NASH.
We plan to collect up to 20 independent samples per tissue type. The groups considered will be:

- organoids, cultures of sinusoidal stellate cells isolated from 20 patients with CC genotype (I148I)
- organoids, cultures of sinusoidal stellate cells isolated from 20 patients with CG genotype (I148M)
- organoids, cultures of sinusoidal stellate cells isolated from 20 patients with GG genotype

(M148M)

A blood sample (approximately 7 cc) will also be taken, in addition to those already scheduled during regular clinical checks, at the time of surgery or liver biopsy.

It is planned to share a portion of the liver biopsy samples for immunohistochemical analysis to be carried out at the Division of Medicine, Institute for Liver and Digestive Health, University College London, in collaboration with Prof. Krista Rombouts, in order to further evaluate the expression of PNPLA3 associated with liver disease, subject to the preparation of an appropriate MTA.

4.2 Isolation and culture of organoids

Biopsy fragments or fragments obtained from surgical resections will be transported in Celsior perfusion solution at 4°C and will be processed at the Translational Medicine laboratory, UOC Transfusion Center of the Foundation within 24 hours of collection to isolate liver organoids. The tissues will be mechanically fragmented into small pieces avoiding reducing them to single cells; this will increase the efficiency of organoid formation. Then the fragments will be further subjected to enzymatic digestion with a solution containing Collagenase and DNase at 37°C. The cell clusters obtained from enzymatic digestion will be included inreducedgrowthfactorMatrigel. Once the Matrigel has polymerized, the complete culture medium for liver organoids will be added: Advanced DMEM/F12 supplemented with 1% N2 and 1% B27 (both from GIBCO), 1.25 mM N-Acetylcysteine (Sigma), 10 nM Leu-Gastrin (Sigma), 50 ng/ml EGF (Peprotech), 1 -g/ml RSPO1 (Peprotech), 100 ng/ml FGF10 (Peprotech), 25 ng/ml HGF (Peprotech), 10 mMNicotinamide (Sigma), 5 -M A83.01 (Peprotech) and 10 -M Forskolin (Peprotech). In order to increase the efficiency of organoid isolation, 25 ng/ml Noggin (Peprotech), 100 ng/ml Wnt3a (peprotech) and 10 -M Rock inhibitor Y27632 (Peprotech) will be added to the medium for the first few days. The organoids will then be differentiated towards a hepatocyte phenotype.

4.3 Isolation and culture of sinusoidal stellate cells

A part of the biopsy fragments or those obtained from surgical resections, once digested to obtain the epithelial cells that will form the organoids, will be processed to obtain hepatic stellate cells (HSC) and sinusoidal cells (LSEC). The latter will be isolated by exploiting their low density, due to the presence of lipid droplets that characterize them. The stellate cells will be cultured on plastic and we will characterize their activation state and the purity of the cell population, by flow cytometry and quantitative Real Time PCR (RT-qPCR), evaluating the presence of the

retinol and specific cellular markers (15). Sinusoidal cells will be cultured on plastic pre-treated with a fibronectin coating and grown in the presence of the growth factor VEGF.

4.4 Generation of assemblyloids

In order to obtain the assemblyloids, both the organoids and the stellate and sinusoidal cells will be dissociated into single cells by enzymatic digestion using trypsin. The cells will then be included in reducedgrowthfactorMatrigel in different ratios in order to mimic the physiological conditions present in the liver tissue. Once the Matrigel has polymerized, the culture medium appropriately formulated to allow the survival of both hepatocytes, ductal cells and stellate cells will be added: Advanced DMEM/F12 supplemented with 1% N2 and 1% B27 (both from GIBCO), 1.25 mM N-Acetylcysteine (Sigma), 10 nM Leu-Gastrin (Sigma), 50 ng/ml EGF (Peprotech), 25 ng/ml HGF (Peprotech) and 2% FBS (GIBCO).

4.5 Treatmentin vitro

It has recently been shown that the addition of oleate and palmitate to the culture medium is able to increase the formation of lipid droplets in hepatocytes and primary stellate cells, simultaneously causing a down-regulation of the expression ofPNPLA3.Conversely, in the absence of retinol and palmitate, a reduction in intracellular lipid content and an increase in the expression ofPNPLA3 (7).
The generated assembloids will be treated for 36 or 120 hours with oleic acid and palmitic acid (300 µM both) (Sigma Aldrich, St Louis, MO). Treatments with the addition of cholesterol, fructose, insulin and low concentrations of ethyl alcohol will also be tested to mimic a condition of dysmetabolism, unbalanced diet and the main environmental determinants of liver disease. This experiment will allow us to evaluate differences in the accumulation and subsequent release of lipid droplets in cells with different genotypes ofPNPLA3.After 36 hours, the lipid droplet content will be visualized by OilRed O histochemical staining. An analysis of stellate cell activation will also be performed after 36 hours. Instead, after 120 hours, the assembloids will be partly fixed in formaldehyde to analyze morphology, biomarkers and fibrotic status and partly frozen in liquid nitrogen for further gene expression analysis.
PNPLA3 will be silenced using antisense RNA (16)

4.6 Genetic Analysis

DNA will be extracted by phenol-chloroform from peripheral blood and the I148M variant

PNPLA3(rs738409) will be determined by TaqMan assay on ABI 7500 Fast apparatus (Life Technologies, Carlsbad, CA, USA) (17).

4.7 RNA isolation

RNA will be extracted from cultured organoids using Trizol reagent (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. 1 µg of total RNA will be reverse transcribed using the VILO kitrandom hexamerssynthesissystem (Life Technologies, Carlsbad, CA). Gene expression will be assessed by quantitative real-time PCR (RT-qPCR) using an ABI 7500 Fast thermal cycler (Life Technologies, Carlsbad, CA) and SYBR Green chemistry (Fast SYBR Green Master Mix; Life Technologies, CA). All reactions will be performed in triplicate.

4.8 Characterization of the activation state of human hepatic stellate cells

Quiescent hepatic stellate cells accumulate 80% of retinol as retinyl palmitate in lipid droplets in the cytoplasm, and can release it depending on the extracellular retinol state (18). However, following liver injury, stellate cells can become activated, proliferate, lose retinol droplets, and secrete extracellular matrix (ECM), becoming myofibroblast-like cells. In this activated state, they may be responsible for the progression of liver disease to fibrosis (19).
In stellate cells isolated from patients we will evaluate the expression of genes involved in fibrogenic processes, such ascollagen-1, α-smoothmuscleactin (-SMA),transforminggrowthfactor (TGF) β1, tissueinhibitormatrixmetalloproteases (TIMP) andmatrixmetalloprotease (MMP) 13 in order to investigate its activation status and evaluate any differences determined by the variants of PNPLA3.

However, stellate cells do not release retinol or its metabolites only during fibrogenic processes, but also during regenerative processes, such as after partial hepatectomy (20). In fact, these cells possess enzymes for the storage and metabolism of retinol. Retinol acquired from outside is preferentially stored as retinyl esters of retinol by an enzyme lecithin retinol acyl-transferase (LRAT) using acyl-CoA, or fromretinol acyltransferase (ARAT) in the case of quiescent cells (21). Retinol can then be metabolised into retinaldehyde by alcohol dehydrogenase (ADH) and subsequently be converted to retinoic acid (RA) by retinaldehyde dehydrogenase (RALDH) (22).
To better understand retinol metabolism in stellate cells isolated from patients with different PNPLA3 genotypes, we will investigate the expression of genes such as LRAT, ARAT, ADH and RALDH, involved in retinol storage and release.
Furthermore, depending on their activation state, stellate cells can regulate the progression of liver disease also by supporting inflammatory processes, producing cytokines, chemokines and/or

by directly interacting with T cells, natural killer (NK) and NKT cells (23). Therefore, we will also investigate the expression of these genes that contribute negatively or positively to the resolution of liver damage in assembloids.

5. ENDPOINTS

5.1 Primary Endpoints

1. Isolation of epithelial cells in order to generate models of the main populations of liver cells in a three-dimensional culture environment, called organoids, and molecular characterization of the generated models. Isolation of stellate cells and their phenotypic and molecular characterization. Generation of assemblyloids that mimic the hepatic tissue architecture and its phenotypic and molecular characterization to study the onset and development of steatohepatitis.

2. Evaluation of the impact of the I148M variant (evaluation of possible combinations both in epithelial cells - hepatocytes and in stellate cells) on the steatohepatitic phenotype in human liver assembloids, with particular reference to:
   - Lipid accumulation in hepatocytes (quantitative and qualitative analysis)
   - Lipotoxicity and expression of inflammatory markers (gene expression)
   - Activation of fibrogenesis (assessment of the activation state of stellate cells, retinol metabolism and extracellular matrix deposition)

   Steatohepatitis will be defined in the simultaneous presence of all these alterations in the assemblage.

3. Analysis of variants of the TM6SF2, MBOAT7 and GCKR genes, previously correlated with the development of non-alcoholic steatohepatitis (14).

Secondary Endpoints

- Knowledge of the behavioral and morphological differences between physiological and NASH hepatocytes and stellate cells.

- Detection of new biomarkers predictive of the development and progression of the disease.

a. - Analysis of epigenetic differences in physiological conditions, onset and progression of NASH.

- Knowledge of the molecular mechanisms underlying cellular-level observations using "omics" scale assessments and CRISPR-Cas9 genetic engineering.

- Analysis of the impact of PNPLA3 silencing on the steatohepatic phenotype of human liver assembloids, stratified by I148M variant carrier status in epithelial cells vs. stellate cells.

6. DURATION / TIMELINE OF THE STUDY

Study start month and year: 07/2021

Enrollment close month and year: 07/2024

Study end month and year: 07/2026

The study will last 60 months. In the first three years we expect to enroll approximately 60 subjects in accordance with the inclusion criteria.

PROJECT GANTT

Intervention	Period (months)			
	1-12	13-24	25-36	37-60
Enlistment	-	-	- -	
Informed consent	-	-	-	
Criteria of inclusion/exclusion	-	-	-	
Generation of organoids	-	-	-	
Expression Evaluation Genetics in organoids hepatic	- -	- -	- -	
Evaluation of specifications liver proteins in liver organoids	- -	- -	- -	
Differentiation to hepatocytes of the	-	- -	- -	-

organoids				
Isolation and characterization of star cells	- -	- -	- -	-
Generation of assemblyloids	-	- -	- -	- -
In vitro treatment with fatty acids, cholesterol and sugars, etc.	-	- -	- -	- -
Therapeutic silencing PNPLA3 in assemblyloids	-	-	-	- -
Correlation analysis data		-	-	-

7.STATISTICAL ANALYSIS

7.1 Sample size and data analysis

The sample size was estimated in view of achieving the two primary outcomes of the project.
Previously published works (24, 25, 26, 27) have shown that the generation efficiency of liver organoids is approximately 100% starting from surgical resections and 33% from biopsies (24, 25).
Considering these estimates, in order to isolate at least 20 independent organoid cultures of each type of condition of interest in our study, we estimate to recruit at least 30 donors for each genetic variant. The intent is to obtain replicates for the analyses with a sufficient representation of the different stages of liver damage (normal liver, inflammation/fibrosis and hepatocellular carcinoma) and to be able to map the impact of the main most common inherited genetic variants (present in at least 20% of individuals) in the population.
For the first primary outcome, the primary analyses will be conducted from single-individual cell cultures. Given the descriptive nature of mapping cellular phenotypes and gene expression, and the large number of organoids (>10^3) and cells (>10^9) from a single individual, we will have sufficient statistical power (>80%) to identify and analyze specific cellular subsets representing >1% of the overall population.
As regards the second primary outcome, although there are important limitations in the estimate inherent to the type of project and linked to the uncertainty regarding the final characteristics of the model that will be developed during the project, we expect to observe a progressive increase in the

presence and severity of the steatohepatitis phenotype in organoids with increased number of PNPLA3 I148M variants. In particular, we estimated that we could reach a statistical power of 95% (alpha 0.05, two-tailed test) to observe a frequency of steatohepatitis phenotype of 75% vs. 25% in assemblyloids with extreme PNPLA3 genotypes, homozygous for the at-risk variant vs. wild-type (20 cases vs. 20 controls), which is consistent with the increased risk observed in clinical cohorts (28).
Statistical analyses will be performed using JMP (SA, Cary, NC) and R (https://www.r-project.org) software and statistical comparisons will be made using the one-way ANOVA or chi-square test, where appropriate. P values < 0.05 will be considered statistically significant.

8. ADVERSE EVENTS

The project does not involve the administration of drugs or other substances or ad hoc invasive interventions outside of normal clinical practices. Therefore, no adverse events are expected.

9. RISK/BENEFIT ASSESSMENT

The study does not foresee an immediate benefit for patients, but the results of this trial will have the potential to lead to a decoding of the mechanisms underlying the development of NASH, resolve the temporal order of events that regulate its evolutionary trajectory, understand its progression, characterization and improve the therapeutic management of patients.

10. STUDIO MANAGEMENT

10.1Data collection and management

Each participant will be assigned a unique code at enrollment. The file that associates the participant code with the participant identification data will be stored separately on a password-protected computer. The study database will be password-protected and uploaded to a password-protected computer and accessible only to study personnel designated by the principal investigator. De-identification of the data will be done in such a way that researchers accessing the database will not be able to trace the identity of the subjects in any way. Only local investigators will be able to trace the identity of the enrolled subjects.

10.2Regulatory aspects and ethical considerations

10.2.1 Approval by the Competent Authority

In accordance with applicable regulations, the principal investigator must obtain approval from the appropriate Competent Authority prior to initiating the clinical study.
This study will be conducted in accordance with the ICH/GCP (International Conference of Harmonization/GoodClinicalPractice) and all applicable laws, including the Helsinki Declaration of June 1964, as amended by the last World Medical Association General Assembly in Seoul, 2008.

10.2.2 Ethics Committee Approval

The investigator must ensure that the protocol has been reviewed and approved by the local independent Ethics Committee (EC) before starting the study.
The CE must also review and approve the informed consent (IC) form and all written information received from the patient prior to enrollment in the study.
If it is necessary to modify the protocol and/or the IC during the study, the investigator will be the guarantor and therefore the person responsible for ensuring the review and approval of such modified document as requested by the CE.

The content of these changes will be implemented only after the CE has approved them. Until then, it will be necessary to refer to the previous version of the document already approved.

10.2.3 Informed consent (IC)

The investigator or other personnel designated by him have the task of informing people about all aspects and procedures of the study.
The process for obtaining informed consent must comply with applicable regulatory procedures. The investigator (or designated staff member) and the subject must date and sign the informed consent form prior to the patient initiating any study-related procedures. The subject will receive a copy of the IC dated and signed by both parties; the original copy will be retained in designated study archives. Neither the investigator nor designated staff member should in any way coerce or influence a subject to participate or continue to participate in the study. A subject's decision to participate in the study must be completely voluntary. The investigator and designated staff member should emphasize to the subject that he or she may withdraw consent at any time without penalty or loss of any benefits to which the subject may be entitled.
The isolated biological material may be used for research purposes only following the informed consent expressed by the recipient of the transplanted organ..Written or oral information relating to the study, including the written consent form, must not contain any linguistic expressions that

forces the subject to waive (even apparently) his or her legal rights, or that would exonerate the investigator, agency, or sponsor from liability for negligence.

10.3Duties of the experimenter

In accordance with applicable local regulations, the investigator must submit periodic reports regarding the progress of the study at his/her site to the CE and notify the CE of the study closure. Periodic reports and closure notification are part of the investigator's responsibilities.

10.4 Study monitoring

In accordance with applicable regulations and good clinical practice (GCP), the monitor must visit or contact the center periodically. The duration, nature and frequency of such visits / contacts depend on the recruitment frequency, the quality of the documents held by the center and their adherence to the protocol.

Through these contacts, the monitor must:

• monitor and evaluate the progress of the study

• examine the collected data

• conduct source document verification

• identify each problem and related solutions

The purposes of the monitoring activity are to verify that:

• the rights and well-being of the subject are respected

• the study data are accurate, complete and verifiable from the original documents

• the study is conducted in accordance with the protocol and any approved amendments, GCP and applicable regulations

The experimenter must:

• give the monitor direct access to all relevant documentation
• dedicate part of his time and his staff to the monitor to discuss the monitoring results and any other possible aspects.

The monitor must also contact the center prior to the start of the study to discuss the protocol and data collection procedures with the staff.

10.5Study quality assurance

As the Sponsor, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico may, at its discretion, carry out a quality control of the study. In this case, the investigator must allow the monitor direct access to all relevant documentation and dedicate part of his/her time and personnel to the reviewer to discuss the results of the monitoring and any other aspects of the study.
In addition, Regulatory Authorities may conduct inspections. In this case, the investigator must allow the inspector direct access to all relevant documentation, and dedicate part of his time and personnel to the inspector to discuss the monitoring results and any other aspects of the study.

10.6 Closing of the study

At the time of study closure, the monitor and the experimenter must activate a series of procedures:

• review all study documentation

• reconcile study data

• reconcile all clarifying reports.

10.7 Document storage

In accordance with current national regulations, the investigator must keep a copy of all documentation and store it in a dry and safe place after the study has been closed.

10.8 Disclosure of information regarding scientific discovery

10.8.1 Confidentiality

The investigator and other personnel involved in the study must handle all information relating to the study (including the protocol, data obtained and all documentation generated during the study) and must not use such information, data or reports for purposes other than those described in the

protocol.

These restrictions do not apply to:

1) information that becomes publicly available, not due to negligence on the part of the investigator or his staff;

2) information that requires confidential disclosure to CE for the sole purpose of evaluating the study;

3) information that must be disclosed in order to obtain appropriate medical care for a study subject.

10.8.2 Publications

The Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico is the sole owner of the data.

10.9 Intellectual property rights on the results of the study

The Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico is the sole owner of the intellectual property rights on the study.

11. INDEMNITY AND COMPENSATION IN CASE OF DAMAGES

In case of unwanted events or any damages that may arise from participation in research, our Institute's Insurance Policy also extends to cover subjects participating in research projects.

12. FINANCIAL AGREEMENTS

The costs of the study procedures exceeding normal clinical practice will be entirely covered by funds deriving from the Horizon2020 – Europa EU funding, Grant H2020-ICT-2020-2 Project Number 101016726

The costs of immunohistochemical analyses of liver histology samples to be carried out at the Institute for Liver and Digestive Health, University College London, will be covered in full by the Institute for Liver and Digestive Health

13. DISCLOSURE ON CONFLICTS OF INTEREST

The experimenters declare no conflicts of interest.

14. REFERENCES

1- Dongiovanni P. et al. 'Genetic Predisposition in NAFLD and NASH: Impact on Severity of Liver Disease and Response to Treatment'. Curr Pharm Des, 2013
2- Wang WY et al. 'Genome-wide association studies: theoretical and practical concerns'. Nat Rev Genet, 2005
3- Romeo S. et al. 'Genetic variation in PNPLA3 confers susceptibility to non-alcoholic fatty liver disease'. Net Genet, 2008
4- Valenti L. et al. 'PNPLA3 I148M Polymorphism, Clinical Presentation, and Survival in Patients with Hepatocellular Carcinoma'. PLoS One, 2013
5- Trepo E. et al. 'PNPLA3 (rs738409 C>G) is a common risk variant associated with hepatocellular carcinoma in alcoholic cirrhosis'. Hepatology, 2012
6- Luukkonen PK et al. 'Human PNPLA3-I148M variant increases hepatic retention of polyunsaturated fatty acids'. JCI Insights, 2019
7- Pirazzi C. et al. 'Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro'. J Hepatol, 2012
8- Clevers H. 'Modeling Development and Disease with Organoids'. Cell, 2016
9- Fatehullah A. et al. 'Organoids as an in vitro model of human development and disease'. Nat Cell Biol, 2016
10- Kim J. et al. 'Human organoids: model systems for human biology and medicine'. Nat Rev Mol Cell Biol, 2020
11- Rossi G. et al. 'Progress and potential in organoid research'. Nat Rev Genet, 2018
12- Kim E. et al. 'Creation of bladder assembloids mimicking tissue regeneration and cancer'. Natures, 2020
13- Vogt N. 'Assembloids'. Nat Methods, 2021
14- Eslam M. et al. 'Genetics and epigenetics of NAFLD and NASH: Clinical impact'. J Hepatol, 2018
15- Dongiovanni P. et al. 'Insulin resistance promotes Lysyl Oxidase Like 2 induction and fibrosis accumulation in non-alcoholic fatty liver disease'. Clin Sci (Lond), 2017
16- Lindén D. et al. 'Pnpla3 silencing with antisense oligonucleotides ameliorates nonalcoholic steatohepatitis and fibrosis in Pnpla3 I148M knock-in mice'. MolMetab, 2019

17- Valenti L. et al. 'Homozygosity for the patatin-like phospholipase-3/adiponutrin I148Mpolymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease'. Hepatology, 2010
18- Higashi N. et al. 'Distribution of vitamin A-storing lipid droplets in hepatic stellate cells in liver lobules—a comparative study'. Anat Rec ADiscovMol Cell EvolBiol, 2003
19- Mederacke I. et al. 'Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology'. Nat Commun, 2013
20- Kordes C. et al. 'Hepatic stellate cells contribute to progenitor cells and liver regeneration'. J Clin Invest, 2014
21- Blomhoff R. et al. 'Overview of retinoid metabolism and function'. J Neurobiol, 2006
22- Duester G. et al. 'Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid'. ChemBiol Interact, 2003 23- Maher JJ 'Interactions between hepatic stellate cells and the immune system'. Sowing Liver Dis, 2001
24- Huch M. et al. 'Long-term culture of genome-stable bipotent stem cells from adult human liver'. Cell, 2015
25- Broutier, L. et al 'Human primary liver cancer–derived organoid cultures for disease modeling and drug screening'. Nat Med, 2017.
26- Nuciforo S. et al 'Organoid Models of Human Liver Cancers Derived from Tumor Needle Biopsies'. Cell Rep, 2018
27- Sorrentino G. et al. 'Mechano-modulatory synthetic niches for liver organoid derivation'. Nat Common, 2020
28- Trépo E. and Valenti L. 'Update on NAFLD genetics: From new variants to the clinic'. J Hepatol, 2020