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Targeting the Epigenetic Regulators Suv420h1/2 in hepatocytes to treat nonalcoholic fatty liver disease

Acronym: TERS

Project code: RF-2021-12373889

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Study Sponsor: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico,
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Principal Investigator: Prof. Luca Valenti

FLOWCHART

Activities	Months	
	T (1) Enrolment	T(2) Follow-up
Enrollment: exclusion criteria check	X	
Informed consent	X	
Clinical evaluation	X	X
Intervention: Isolation, generation and characterization of organoids	X	
Data analysis	X	X

T (1)=basal or routine follow-up visits (REASON/PERSPECTIVE)

LIST OF ABBREVIATIONS

A1AT: Alpha-1 antitrypsin
AFP: Alpha fetoprotein
Alpha-SMA: Alpha smooth muscle actin
ASOs: Antisense oligonucleotides
CI: Informed Consent
CRF: Case Report Form
GCP: Good Clinica Practice
GWAS: Genome Wide Association Studies
HLA: Human liver assembloids
HLO: Human liver organoids
HNF4: Hepatocyte nuclear factor 4
HSC: Hepatic stellate cells
IEC: Independent Ethics Committee
IF: Immunofluorescence
IHC: Immunohistochemistry
KC: Kupffer cells
KMT5B/C: Lysine methyltransferase 5B/C
LSEC: Liver sinusoidal endothelial cells
MAF: Minor allele frequency
MAFLD: Metabolic associated fatty liver disease

MRI-PDFF: Magnetic resonance imaging proton density fat fraction
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
NPC: Non-parenchymal cells

RESPONSABILITIES (role of promotor and collaborators)

The Promoter of the study is Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico. The Coordinating Center will be the SC Medicina Trasfusionale unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico. The PI of the study will be Prof. Luca Valenti, responsible for the patients enrollment and coordination of the project.

Unità operativa	***Nome partecipante***	***Ruolo e funzioni nello studio***
SC Medicina Trasfusionale, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico	*Prof. Luca Valenti (PI)* *Dr. Daniele Prati* *Dr. Alessandro Cherubini* *Dr. Serena Pelusi*	*Sample/data recruitment and characterization;* *Isolation and generation of organoids, spheroids and tissues;* *Characterization of organoids by gene and protein expression;* *Data analysis* *Study coordination*

Internal Collaborations

Unità operativa	***Nome partecipante***	***Ruolo e funzioni nello studio***
SC Chirurgia Generale E Trapianti Di Fegato, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico	*Dr. Daniele Dondossola*	*Referral of eligible patients and characterization of samples (as part of the REASON study)*

External Collaborations (biological samples analysis, data analysis, diagnostic procedures)

Istituzione	***Unità operativa***	***Nome partecipante***	***Ruolo e funzioni nello studio***
IRCCS Ospedale San Raffaele	*Division of Genetics and Cell Biology*	*Dr.ssa Laura Silvestri*	*Preclinical and experimental models in mice;* *Evaluation of the impact*

			of therapy with antisense oligonucleotides in pre-clinical models.
Università Magna Graecia Catanzaro	*Dipartimento di Scienze Mediche e Chirurgiche*	*Prof. Stefano Romeo*	*Transcriptomic, lipidomic and genetic analyses in clinical (in collaboration with Milan) and genetic analyses in population-based cohorts (UK Bi-obank).*

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SUMMARY

1. BACKGROUND

1.1 Overall Summary

Nonalcoholic fatty liver disease (NAFLD) is globally the leading cause of liver disease and frequently progresses to cirrhosis and liver cancer. The identification of effective drugs is the main unmet clinical need. Changes in liver histones methylation accompanies the development and progression of NAFLD. Our preliminary data demonstrate that inactivation of the methyltransferases SUV420H1/2 in hepatocytes protects mice against NAFLD. In this project we propose to examine the relevance of these findings by evaluating the impact of genetic deletion of hepatic SUV420H1/2 in mice fed a steatogenic diet. To further evaluate the potential for clinical translation of these results, we will next 1) evaluate the expression of SUV420H1/2 in human liver transcriptomic data and 2) analyze the impact of genetic variations on disease outcomes in population-based cohorts; 3) test an innovative therapeutic approach based on hepatocyte-targeted antisense oligonucleotides downregulating SUV420H1/2 in human liver organoids/assembloids.

1.2 Background and State of Art

Nonalcoholic fatty liver disease (NAFLD), the leading cause of liver damage worldwide, is characterized by liver fat accumulation and association with insulin resistance, and frequently progresses to steatohepatitis (NASH), ultimately leading to cirrhosis and liver cancer[1-3]. NAFLD/NASH is becoming globally a leading cause of liver-related morbidity and mortality and by altering liver function contributes more widely to the burden of cardiometabolic, renal and neoplastic diseases [4,5]. In the presence of concomitant hepatotoxic factors such as in particular at-risk alcohol intake and some medications (defined in this case as metabolic dysfunction associated fatty liver disease or MAFLD) synergizes with them representing a major driver of liver disease progression [6]. However, for those with advanced disease or who cannot successfully change their diet and lifestyle, no effective treatment is yet available to prevent or treat this condition, which is therefore projected to become a major public health threat in the next decade. Although understanding the mechanisms of NAFLD development and progression is essential for its prevention/treatment, molecular players involved in its progression are poorly defined. Recently, an epigenetic component is recognized in this disorder and the histone methyltransferases SUV420H1/2 are promising candidates for this function. Multiple evidences connect lipid and iron metabolism in the hepatocyte: 1) Genome Wide Association Studies (GWAS) in human populations showed an overlap of loci affecting iron and lipid metabolism, and excess iron in hepatocytes favors dyslipidemia and NAFLD [7]; 2) upregulation of the liver hormone hepcidin, under the control by the BMP-SMAD pathway, is protective against NAFLD-NASH [8,9]. Interestingly, a GWAS performed in mouse strains kept on high-iron diet identified a shared association between liver iron and triglyceride levels at a region of chromosome 7 encompassing the histone

methyltransferase Suv420h2. Our preliminary data show that mice with Suv420h inactivation in adipose tissue are resistant to diet-induced liver steatosis due to increased PPAR signaling. Since mice with liver BMP-SMAD pathway upregulation showed Suv420h downregulation, we hypothesize that the protective effect of increased hepatocyte BMP-SMAD signaling on NAFLD development is due to Suv420h. In agreement, Suv420h inactivation in hepatocytes counteracts diet-induced NAFLD, as highlighted by our preliminary results.

The present study is part of the RF project (Ricerca Finalizzata) – Project code: **RF-2021-12373889** funded by the grant call of the Ministery of Health (2020-2021). In the attached project we proposed 3 different aims:
1) To characterize the disease progression in *Suv420h1/2*-liver conditional KO mice and to identify the molecular pathways/genes involved in the protection against NAFLD-NASH development.
2) To examine the impact of SUV420H1/2 genetic, epigenetic and transcriptional variability on clinical outcomes for the identification of novel biomarkers of NAFLD-NASH.
3) To propose and test an innovative therapeutic approach based on hepatocyte-targeted antisense oligonucleotides (ASOs) against Suv420h1/2 in preclinical models in mice and in human liver organoids and assembloids.

The present clinical study protocol will focus on the clinical aspects and procedures that will regard an intervention on data and sample of clinical cohorts. Further details about the analyses conducted in animal models, for which a specific Authorization has been requested, can be found in the attached project.

2. PURPOSE AND AIMS

As previously reported the general aim of the project is to study the cellular and molecular mechanisms of correlation between the SUV420H1/H2 methyltransferase and the progression of liver damage in mice (San Raffaele Institute) and to confirm these findings in human samples and *in vitro* 3D liver models.

2.1 Primary Aim

A) To identify the main genes and pathways differentially expressed and the main factors associated with SUV420H1/H2 expression by comparing the transcriptomic and lipidomic profile of individuals with low hepatic SUV420H1/H2 mRNA expression levels (lowest expression quartile) to that from individuals with high expression levels (top quartile).

In order to evaluate the role of SUV420H1/H2 in the regulation of gene expression in NAFLD patients, we will exploit already available transcriptomic and lipidomic data by analyzing the gene expression databases deriving from ongoing studies conducted at the Fondazione and by the PI of the Catanzaro collaborating centre. Data have been generated from liver and visceral adipose biopsy of obese individuals at high risk of developing NASH (PERSPECTIVE and REASON studies at Fondazione, and MAFALDA Study, coordinated by prof Stefano Romeo, PI of the Università Magna Graecia Catanzaro).

2.2 Secondary Aims

B) To evaluate whether there is a causal association between SUV420H1/2 activity and NAFLD.

In order to address this aim, we will examine the impact of both common naturally occurring genetic variants in the *SUV420H1/2* genes (minor allele frequency (MAF) >0.01), rare (MAF< 0.01) on the risk of developing liver disease. This will be achieved by analyzing in pseudo-anonymised and aggregated form the genetic databases deriving from previously characterized human cohorts comprising patients with severe NAFLD and controls in NGS studies conducted at the Fondazione (PERSPECTIVE study) and by analyzing the public population-based UK Biobank cohort (N>200,000 with complete genomic data available), for which the PI of the Catanzaro group has approved authorisation.

C) To validate the protective effect of SUV420H1-H2 downregulation in human cells and to propose an innovative therapeutic approach based on hepatocyte-targeted antisense oligonucleotides (ASOs) by evaluating the intracellular fat reduction and the fibrosis and inflammation regulation.

In order to fulfil this aim, we will take advantage of human liver organoids (HLO) and assembloids (HLA) isolated from surgical specimens from an ongoing study at the Fondazione (REASON study).

3. STUDY DESIGN

3.1 Study design

Interventional, genetic, biological, non-pharmacological and multicentric.

3.2 Inclusion Criteria

We will analyse data and samples from subjects with the following criteria:

- Subjects aged>18;
- Subjects who have already given their consent to genetic analysis and whose samples and data have already been collected as part of the PERSPECTIVE, REASON and MAFALDA studies;
- Subjects who have given their consent to participate in this study.

In particular, subjects with the following characteristics were included respectively:

- in the PERSPECTIVE study:

1. Diagnosis of NAFLD
2. Age between 45 and 75 years old
3. Any of the following criteria:
   a. F3-F4 fibrosis, determined histologically, or by non-invasive techniques, or evidence of cirrhosis deriving from biochemical tests or imaging methods;
   b. Family history of related first-degree primary liver cancer, or carrier status of rare mutations associated with the development of HCC (such as mutations in *APOB* and *TERT*)
   c. Male patient with type 2 diabetes or obesity carrying at least three genetic variants in *PNPLA3*, *TM6SF2*, *MBOAT7*.

- in the REASON study:

Patients aged>18, who have given their consent to participate in the study, who underwent the following procedures:

- liver biopsy for suspected non-alcoholic steatohepatitis (NASH) at the time of diagnosis;
- liver resection for hepatocarcinoma, other liver lesions (including secondaries from other neoplasms and benign focal lesions, which will allow obtaining healthy starting liver tissue), biopsies of whole liver explants obtained at the time of liver transplantation AND cholecystectomies.

- In the MAFALDA study:

Patients undergoing bariatric surgery for grade 3 obesity (BMI ≥40 Kg/m2) or grade 2 obesity plus:

- metabolic comorbidities (uncontrolled hypertension, diabetes, dyslipidemia);
- lack of contraindication to surgery (e.g. advanced liver disease with portal hypertension);
- willingness to sign an informed consent.

3.3 Exclusion Criteria

Individuals not reporting one of the inclusion criteria listed above or reporting at-risk alcohol intake (>30/20 g/day in M/F), viral and autoimmune hepatitis or other causes of liver disease will be excluded.

4. STUDY PROTOCOL, PROCEDURES AND METHODS

4.1 Procedures

An informed consent to the use of previously collected data and samples, to carry out new specific analysis related to this study, will be signed by the study participants during the basal or routine follow-up visits already planned by the normal clinical practice.

Patients enrolled in the REASON study will be asked their consent to the use of the biological samples already collected in the context of the study, to conduct the experiments and analysis related to this specific study.

For patients in the PERSPECTIVE study, anamnestic data, family history, lifestyle, demographic and anthropometric parameters, and clinical data (Glucose, HbA1c, Insulin, Tot. Chol, HDL, TGL, AST, ALT, GGT, Ferritin, Albumin, PLTs, HBcAb, AFP) will be collected during the basal or routine annual follow-up visit, as normal clinical practice

All raw data and biological samples are available respectively from the ongoing PERSPECTIVE and REASON studies (Fondazione IRCCS Ca Granda Ospedale Maggiore Policlinico - Milano) and MAFALDA and UK Biobank cohorts (Prof. Romeo, Università della Magna Grecia Catanzaro).

4.2 Analysis

Analyses related to aim A

As previously reported, in order to address the aim A the transcriptomic and lipidomic profile of SUV420H1/2 will be analyzed in ongoing studies (PERSPECTIVE and REASON) and characterized cohort of ~500 individuals at high risk of NASH who underwent liver and visceral adipose tissue biopsy during metabolic surgery[10] (Transcriptomic cohort, n= 250 individuals in whom liver and visceral adipose are paired from the same individual; MAFALDA cohort, a cross-sectional study comprising a total of 264 severely obese adult participants (BMI > 40 kg/m2) undergoing bariatric surgery enrolled at the Campus Bio-Medico University of Rome (study PI: Prof. Romeo).
The main genes and pathways differentially expressed and the main factors associated with SUV420H1/H2 expression will be identified as previously described [10]. Lipidomic profile will be analyzed as previously described [11]. Specifically, we will compare transcriptomic and lipidomic profile from individuals with low hepatic SUV420H1/H2 mRNA expression levels (≤25 centile) to that from individuals with high expression levels (≥75 centile). Through Gene Set Enrichment Analysis (GSEA)

and Ingenuity Pathway Analysis (IPA), the pathways differentially expressed and the main factors associated with SUV420H1/H2 expression will be identified.
Then, genes significantly regulated in the two groups (high versus low SUV420H1/2 expression) will be confirmed at protein level (Western Blotting and immunohistochemistry assays). Finally, a CHIP-Seq analysis will be performed in a subset of liver samples (n=25 from PERSPECTIVE and REASON Studies) to evaluate the direct involvement of histone methylation regulation on expression modulation of the main SUV420H1/H2 targets. This aim will be conducted in collaboration between the Milan and Catanzaro Units.

Analyses related to aim B

To support a causal association between impairment of SUV420H1/2 activity and the risk of NAFLD, we will examine the impact of both common (MAF >0.01), rare (MAF <0.01) variants altering *SUV420H1* (also known as *KMT5B*) and *SUV420H2* (also known as *KMT5C*) genes expression and predicted protein activity and those of genes differentially expressed on the risk of NAFLD-NASH and liver-related and metabolic outcomes in two cohorts we previously exploited to uncover NAFLD genetics: patients with severe NAFLD (that is with advanced fibrosis and/or hepatocellular carcinoma) and controls (patients from the PERSPECTIVE cohort not carrying the variants)[11] and the population-based UK Biobank cohort (n>350,000).
The common variant analyses will be done by assuming an additive genetic model and using a binary logistic (for categorical traits) or a liner regression model (for linear traits) adjusting for age, gender and body mass index. The rare variant analyses will be conducted by using two models: a) in nonsense genetic variation (splicing, frameshift, premature stop codon) burden test will be used and b) missense (nonsynonymous) SKAT-O test will be used assuming bidirectionality of the effect association with the genetic variants.
We will consider as outcomes liver-related traits (cirrhosis, hepatocellular carcinoma, severe liver disease, hepatic fat content measured by magnetic resonance imaging proton density fat fraction (MRI-PDFF) and liver enzymes) and metabolic traits (adiposity, diabetes, circulating lipids). We will assess the interaction between genetic traits and environmental triggers (adiposity and insulin resistance) [12]. All these variables are regularly collected for clinical practice except for the MRI-PDFF which is however available for the participants in the UKBB cohort study. This aim will be conducted in collaboration between the Milan and Catanzaro Units.

Analyses related to aim C

As previously reported, to validate the protective effect of SUV420H1-H2 downregulation in human cells we will take advantage of human liver organoids (HLO) isolated from surgical specimens from

an ongoing study at the Fondazione (REASON).
In the contest of the REASON study, in collaboration with Unità dei Trapianti di Fegato of IRCCS Ca' Granda Foundation, we collect liver samples starting from waste tissue of patients who underwent cholecystectomy, both intra-tumoral and extra-tumoral hepatocellular carcinoma resection, or from transplanted liver.
Liver samples are collected in perfusion solution at 4°C and processed to generate liver organoids (HLO) and assembloids (HLA) as soon as possible. We will exploit already available data generated by previously collected samples and collect new samples, after informed consent.
HLO will be generated, expanded and differentiated as previously described [13] and reported in the attached project.
To generate HLA, single HLO will be combined with NPC (physiologic ratio of 30:8:6:5 HC:HSC:KC:LSEC) [14] in a small drop of extracellular matrix and cultured in a spinning bioreactor (95 rpm) as previously reported [15]. HLA structure will be analyzed by IHC/IF and compared to the starting liver tissue.
To induce NASH *in vitro*, both HLO and HLA will be treated for 7 days with a steatogenic insults: high-fat (palmitic and oleic acid 300 mcM), high cholesterol (50 mcM), high fructose (10 mM d-fructose) and high-insulin (300 nM) or a mix of them. Concentrations will be optimized to maximize the impact avoiding excessive lipotoxicity and cell death.
ASO-mediated SUV420H1/H2 knock-down will be performed via transfection-free uptake of SUV-specific or scramble ASO in HLO/A culture medium for 6 days. To confirm the ASO- mediated SUV420H1/H2 knock-down at the RNA and protein level we will perform respectively a quantitative real time PCR and a Western blot analysis as detailed in the attached project.
Finally, to measure the impact of SUV420H1/H2 reduction in fatty liver disease pathophysiology, HLO/A will be exposed for long period to the most effective steatogenic environment (palmitic and oleic acid both 300 µM), and genes involved in lipid metabolism (identified in aim A) will be analysed by gene expression by qRT-PCR as previously described. Furthermore, intracellular fat reduction will be detected by BODIPY™ 493/503 staining and by using a quantitative Triglyceride Assay Kit (Abcam), TNFalpha mRNA expression will be evaluated by in situ hybridization assays, type 1 collagen will be quantified by immunofluorescence assays (as detailed in the project). This aim will be conducted by the Milan Unit.

5.ENDPOINTS

5.1 Primary endpoint

A) The primary endpoint of this study is the identification of biological pathways differentially expressed in NAFLD individuals, a complementary analysis for replicating the findings of the main aim

of the RF project (which is a Theory Enhancing, mostly basic study project).

5.2 Secondary endpoints

B) The identification of a correlation between genotype influencing the SUV420H1/2 pathway and NAFLD phenotype.

C) Reduction of intracellular fat accumulation, collagen accumulation and inflammation in terms of TNFalpha mRNA expression levels.

In case of positive results from SUV420H1/H2 downregulation by antisense technology, both in mouse models than in human liver organoids and assembloids, we will submit a patent request for the human anti-SUV420H1-H2 ASO for the treatment of steatohepatitis (Fondazione IRCCS Ca' Granda and San Raffaele Institute).

6. PROJECT TIMELINE

The clinical cohorts for transcriptomic and genomic analysis are being recruited and data are stored in consolidated database available for gene expression and genomic studies. Initial analyses have already led to peer-reviewed publications in international journals [10, 12, 16].

Study start: March 2023
Enrolment and data collection start: March 2023
Enrolment and data collection closure: February 2025
End of study: February 2026

6.1 Gantt Chart

Aims	Activities	Months					
		01-06	07-12	13-18	19-24	25-30	31-36
A	**Characterize the disease progression in Suv420h1/2 liver conditional KO mice and HLO**						
	Identification of the main genes and pathways associated with SUV420H1/2 expression in mice (HSR Institute)						
	Human Transriptomic data analysis and identification of the SUV420H1-H2 gene signature						
	Evaluation of human lipidomic fingerprint associated with different levels of SUV420H1-H2 expression						

	ChIP-seq analysis						
B	**Identification of novel biomarkers of NAFLD-NASH**						
	Genomic data analysis to evaluate the impact of rare and common variants altering SUV420H1-H2 pathway expression on NAFLD progression						
C	**Innovative therapeutic approach in preclinical models and in human liver organoids and assembloids**						
	Impact of Suv420h1-h2 downregulation on hepatic fat accumulation, inflammation, fibrosis and targets engagement in mice after 10 weeks of the FPC diet						
	ASOs downregulate SUV420H1/2 specifically in human hepatocytes						
	SUV420H1/H2 target genes modulation						
	Parenchymal and NPC (LSEC, KC, and HSC) co-colture in 3D structures						
	ASO beneficial impact on intracellular fat accumulation and on the expression of genes involved in the induction of inflammation and fibrogenesis in steatogenic HLO						
	ASO beneficial impact on intracellular fat accumulation and on the expression of genes involved in the induction of inflammation and fibrogenesis in steatogenic HLA						

7. STATISTICAL ANALYSIS

7.1 Sample size

Considering the large number of individuals with severe liver disease in the NAFLD PERSPECTIVE and MAFALDA cohorts (n= 500) and the large size of UKBB population-based cohort (n=350,000 for common variants and n=200,000 for rare variants), for variants with a minor allele frequency (MAF) >0.01, we estimated that the study has a power >80% to detect six-fold difference in the risk of severe NAFLD in the PERSPECTIVE/MAFALDA, a 1.8% difference in the NAFLD prevalence (UK Biobank), and 4.4% difference in the liver fat content as measured by PDFF (UK Biobank) between carriers and non–carriers (Aim A and B).

The high number of HLOs (>10^3), HSCs (>10^5), KC (>10^6) and LSEC (>10^5) isolated from a single donor and the high efficiency of isolation, will confer >80% power to detect and analyze distinct cell subsets representing >1% of overall liver cell population. The variable monitored to define the group size is the level of intracellular fat reduction in treated or untreated HLO or HLA with SUV420H1-H2-GalNAc-ASO (Aim C). Then, 9 HLO or HLA will be analysed to determine an effect size of 1.5 between the two groups, with a power of 80% and an alpha error of 0.05 (effect size = 1.5). This sample size is determined by Student's t-test to compare the means of two independent

groups, using the G* Power software. 1-3: Student's t test (for 2 groups) or ANOVA (for multiple comparisons) will be used. Statistical significance will be considered for P<.05.

7.2 Statistical analysis plan

Statistical analyses will be carried out using both JMP (SA, Cary, NC) and R (http://www.Rproject.org/) softwares. For descriptive statistics, continuous variables will be shown as mean and SD. Categorical variables will be tested by chisquare test and presented as number and proportion. In all transcriptome-wide unsupervised analysis and GSEA, p values will be corrected for multiplicity by Benjamini-Hochberg false discovery rate method, and adjusted p values 0.01) analyses will be performed assuming an additive genetic model and using a binary logistic (for categorical traits), liner regression (continuous traits), or ordinal regression (ordinal traits) model as appropriate, adjusted for confounding factors including age, gender, and body mass index. The rare variant (MAF >0.01) analyses will be performed assuming an additive genetic model and using a binary logistic (for categorical traits), liner regression (continuous traits), or ordinal regression (ordinal traits) model as appropriate, adjusted for confounding factors including age, gender, and body mass index. The rare variant (MAF <0.01) analyses will be performed using burden, sequence kernel association test (SKAT) and SKAT-O gene-based tests as implemented in SAIGE-GENE to identify coding variants associated with PDFF used as a linear trait, alternatively with cirrhosis or chronic liver disease (defined by using ICD-10 codes), as categorical traits in the analyses.
Interaction between genetic traits and environmental triggers in determining liver disease will be assessed by including the interaction term into each statistical model.

8. ADVERSE EVENTS

The project does not include the administration of drugs or other substances or invasive clinical practices. Therefore, no adverse events are expected.

9. RISK / BENEFIT EVALUATION

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, to resolve the temporal order of the events that regulate its evolutionary trajectory, to understand progression, characterization and improve the therapeutic management of patients.

10. PROJECT MANAGEMENT

10.1 Regulatory and Ethics

This study will be conducted by Good Clinical Practice (GCP) rules; in accordance with the ethical

principles that have their origin in the Declaration of Helsinki and with the respect to the European clinical practice, in compliance with all international guidelines and national law regulation in Italy.
The protocol and the informed consent document must be submitted to the Independent Ethics Committee (IEC) for review and will receive IEC approval/favourable opinion before initiation of the study. During the study, any amendments to the protocol must also be approved by IEC, before their implementation. A progress report is sent to the IEC at least annually, and a summary of the study's outcome is sent at the end of the study.

10.1.1 Patient information

Participants enrolled will be required to consent to the deidentified use of personal data for the study. Information form and the module for the acquisition of informed consent for the handling of sensitive data will be given to the patient.
The investigator will fulfil the current regulations for research and documentation of informed consent, the standards of Good Clinical Practice and the ethical principles derived from the Declaration of Helsinki. The approval by the Ethics Committee will be required whether an update of the informed consent form will be needed during the study.
According to the recommendations of the Declaration of Helsinki and local regulations, each patient will be adequately informed about the aims, methods, expected benefits, potential risks and problems related to the study. Moreover, patients will be informed of their right to refuse consent to the use of their sensitive data or to withdraw it at any time, without having any effect on their medical care.
The patient will have all the time necessary for the evaluation of the information received before providing their informed consent to the use of sensitive data. The investigator will have to obtain spontaneous informed consent in writing by the patient, before using them in any way for the study. The written consent to the handling of sensitive data must be subscribed by the date and signature of the patient and by the investigator's or his representative's ones.
The investigator has to give to the patient a signed copy of informed consent; the original form will be retained with the other documents of the study protocol; the module for the acquisition of informed consent to the treatment of sensitive data will be attached to the clinic folder. The collaborator will be appointed to review the original forms of all patients' informed consent.

10.3 Confidentiality

According to the ICH guidelines for the Good Clinical Practice, the monitoring team must check the CRF entries against source documents. The personnel bound by professional secret must maintain the confidentiality of all personal identity or personal medical information (according to the confidentiality and personal data protection rules). The confidentiality of records that could identify sub-

jects should be protected, only initials of the name and the first name will be registered with an inclusion coded number for the study (no name nor address nor identifying data).

10.3.1 Publications

The Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico is the only owner of the data. Communications, reports, and publication of the results of the study will be under the responsibility of the principal investigator of the study. A summary of the results of the study will written and provided on request of the participating patients

10.4 Intellectual property rights of study results

The Promoter of the study and the Participating Centres acknowledge and accept that during the implementation of the Project, to the extent strictly necessary to the performance of the Project, know-how, technical material and/or goods protected by industrial and/or intellectual property rights or susceptible to protection, developed prior to the commencement of the Project by the Promoter and the Participating Centres, shall be and shall remain their exclusive ownership. Nothing in this protocol shall be construed as a grant of rights under such intellectual property.
The results arising from the research activities will be jointly owned by the Promoter of the study and the Participating Centres in proportion to their respective contribution, being understood that the provision of biological samples underlying the research project, related clinical information and related medical know-how by the Study Promoter will be considered as an essential contribution.
In cases of innovative results, which are subject to patent protection (or similar rights) and/or economic exploitation, ownership of such Invention shall be owned based on inventorship contribution. In case of joint ownership, the Promoter of the study and the Participating Centres will regulate, in fair and good condition, the protection and the exploitation of the results.

11. INSURANCE

No additional blood sampling, biological sampling or clinical data will be required in addition to those already collected during the regular clinical practice or ongoing studies. The internal institutional insurance policy will cover for any undesirable effects due to the participation in the study.

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