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"DEFINING THE GENETIC DRIVERS OF ADULT-ONSET CHOLESTATIC LIVER DISEASE"

Acronym: FIRST

Version Number: v.2.0

Date: 30/10/2025

Promoter: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via Sforza 28, 20122 Milano, Italia

Coordinating center: SC Medicina Trasfusionale - Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico

Principal Investigator: Dr.ssa Luisa Ronzoni

Luisa Ronzoni

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FLOWCHART

Period	**Visit 1 (T0)**
Enrollment	
Informed consent	**X**
Inclusion/Exclusion criteria	**X**
Study procedures	
Demographic and clinical data collection *	**X**
Blood sample collection*	**X**
Biochemical analysis*	**X**
Genetic analysis°	**X**
Outcomes	
Presence of rare pathogenic variants at WGS analysis	**X**

* standard of care; ° extra standard of care

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ABBREVIATIONS

ALP: alkaline phosphatase

ALT: alanine aminotransferase

ANA: antinuclear antibody

cANCA: cytoplasmatic antineutrophil cytoplasmic antibodies

pANCA: perinuclear antineutrophil cytoplasmic antibodies

AMA: antimitochondrial antibody

AST: aspartate aminotransferase

BMI: body mass index

CE: ethical committe

CI: Informed consent

CRF: case report form

ENA: extractable nuclear antigen

Extra-SOC: extra standard of care

GCP: good clinical practice

GGT: gamma-glutamyltransferase

Hb: hemoglobin

LKM: liver kidney microsome

LPAC: low-phospholipid-associated cholelithiasis

MRC: magnetic resonance cholangiopancreatography

PBC: primary biliary cholangitis

PFIC: progressive familial intra-hepatic cholestasis

PSC: primary sclerosing cholangitis

SOC: standard of care

WGS: Whole Genome Sequencing

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RESPONSIBILITIES (ROLE OF THE PROMOTER AND INVESTIGATORS)

Promoter: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico

Coordinating center: SC Medicina Trasfusionale

Principal Investigator (PI): Dr.ssa Luisa Ronzoni, PI will be responsible for coordinating the study.

Internal collaborators

Center	Participants	Roles and functions
SC Medicina Trasfusionale, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico	Prof. Luca Valenti Dott. Daniele Prati Dott.ssa Giulia Periti Dott.ssa Serena Pelusi Dott.ssa Elena Sinopoli Dott. Lorenzo Miano	Co-investigator: results interpretation Co-investigator: results interpretation Co-investigator: patient enrollment Co-investigator: patient enrollment Co-investigator: sample processing Co-investigator: bioinformatic and statistical analysis

External collaboration

Institution	Units	Roles and functions
Human Technopole	National Facility for Genomics	Whole Genome Sequencing (WGS)

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SUMMARY

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1.BACKGROUND AND RATIONALE

Cholestatic liver diseases are a heterogenous group of conditions, including progressive familial intra-hepatic cholestasis (PFIC), low-phospholipid-associated cholelithiasis (LPAC), primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC), characterized by impaired bile flow at the intra- or extra-hepatic level, leading to fibrosis and end-stage liver disease.

Diagnosis is often challenging due to the heterogeneous and broad overlapping presentations. Reliance upon clinical data, serum chemistries, histological and instrumental assessments (abdominal ultrasound and magnetic resonance cholangiopancreatography [MRC]) is frequently insufficient to make a diagnosis. The diagnostic delay is still more evident in individuals with subtle or atypical phenotypes [1-4].

In this context, genetic analyses through next-generation sequencing technologies have a key role to make an early diagnosis in patients who remained unexplained despite extensive clinical and instrumental evaluations, as demonstrate for other liver diseases [5-7]. The utility of genetic testing has been proven in pediatric cohorts, unrevealing rare damaging variants in genes involved in bile synthesis and secretion contributing to up 25% of unexplained child-onset cholestatic liver disease [8,9] However, despite existing recommendations [1], genetic testing in adults with unexplained cholestasis remains uncommon or targeted to a limited number of genes, mainly involved in PFIC [10,11]. The role of other cholestasis or liver-related genes, as well as the contribution of genetic variants to PSC or PBC pathogenesis is still largely unknown [12-15].

The implementation of genetic testing in the diagnostic algorithm of adult-onset cholestatic disorders will improve diagnostic accuracy, reducing the diagnostic odyssey and allowing to set-up personalized follow-up strategies and to optimize the clinical management of these complex conditions.

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2. STUDY OBJECTIVES

We hypothesize that a subset of patients with unexplained cholestatic liver disease or with atypical presentations harbors rare damaging variants in genes involved in bile homeostasis or related to a wider extent to genetic liver disorders.

2.1 Primary objective:

To evaluate the diagnostic yield of whole genome sequence (WGS) in adult patients with unexplained or atypical cholestatic liver disease. A genetic diagnosis will be defined as the presence of pathogenic variants in genes known to be causative of genetic cholestasis, consistent with the clinical phenotype and mode of inheritance.

2.2 Secondary objectives:

To evaluate the presence of rare damaging variants in liver-related genes and their enrichment at single gene level to get insight into disease pathogenesis.

3. STUDY DESIGN

3.1 Study design and setting

Monocentric, non-pharmacological, no-profit interventional study with genetic analysis.
This study will be conducted under the coordination of the SC Medicina Trasfusionale, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico. Genetic analysis will be performed at Human Technopole Institution.
Whole genome sequencing (WGS) will be performed in 60 adults with unexplained cholestatic liver disease or with atypical presentations.

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As a control cohort, we will take advantage of a well-characterized reference group of healthy individuals (blood donors with medical history, anthropometric, hematological and biochemical evaluation) from the FOGS study (currently n=1025), for whom WGS has already been performed in collaboration with Human Technopole. The genetic data from these subjects are available as pseudo-anonymized data. No additional evaluations or samples will be performed or requested for these subjects (FOGS, promoted by Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, CE Milano Area2, last approval 21/03/2025).

3.2 Inclusion criteria

Cases:

- Adults, aged > 18 years with:

1) persistent or intermittent elevations in serum alkaline phosphatase (ALP) or gamma-glutamyltransferase (GGT) for at least six months not explained following standard diagnostic assessment adhering to the guidelines of the European Association for the Study of the Liver (EASL), or with a positive family history of unexplained cholestasis or hepato-biliary cancer, negative to previous genetic tests (targeted panel for PFIC genes or WES);

2) primary sclerosing cholangitis (PSC) with unusual features: small-duct PSC, non-typical radiological findings according to radiological guidelines on PSC, absence of concomitant inflammatory bowel disease, negative to previous genetic tests (targeted panel for PFIC genes or WES) or who didn't perform previous genetic test;

3) primary biliary cholangitis (PBC) without specific anti-mitochondrial antibodies, negative to previous genetic tests (targeted panel for PFIC genes or WES) or who didn't perform previous genetic test.

- Signature of informed consent

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Controls:

-Blood donors (age 18-65 years) without clinical signs of liver diseases based on the collected clinical parameters: anthropometric (BMI>18 and <25), haematological (Hb, white blood cells, platelets within the reference range), biochemical traits (albumin, bilirubin, AST, ALT, GGT, ALP within the reference range), medical history (negative for chronic or concomitant diseases, including immunological diseases)

3.3 Exclusion criteria

Cases:

Patients who do not possess the above inclusion criteria or have at least one of the following exclusion criteria:

- an already known genetic diagnosis explaining the clinical phenotype
- affected by other causes of liver disease such as viral or autoimmune hepatitis

Controls:

Blood donors with clinical signs of liver diseases

3.4 Withdrawal criteria

Removal of consent of study participation.

4. STUDY DESCRIPTION

4.1 Procedures and timepoints

Patient enrolment will be performed during clinical evaluating as per standard of care at SC Medicina Trasfusionale, Outpatient Clinical Service, Fondazione IRCCS Ca' Granda Ospedale Maggiore

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Policlinico. A informed consent will be obtained prior to performing any study specific procedures. Moreover, a unique code will be assigned to each participant in order to deidentify the data.

After the evaluation of inclusion and exclusion criteria and having obtained patient consent, data about demography, clinical characterization, lifestyle, current medical therapy, and comorbidities will be collected as per standard of care. Clinical report of liver biopsies and imaging data (abdominal ultrasound and magnetic resonance cholangiopancreatography [MRC]), previously performed for clinical indication, will be collected.
Peripheral blood sample will be collected to evaluate biochemical parameters (SOC) such as blood count, albumin, bilirubin, liver indices: AST, ALT, GGT, ALP, immunological profiles (ANA, AMA, ENA, LKM, p-c-ANCA), and for genetic analysis (extra-SOC; 1 EDTA 7ml vial).

Healthy blood donors data (control cohort) has been already collected and will be re-analyzed under the coordination of SC Medicina Trasfusionale, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico. No additional evaluations or samples will be performed or requested for these subjects.

Given the large number of subjects involved (n = 1025), obtaining informed consent would represent a disproportionate effort, potentially making it impossible or seriously compromising the achievement of the research objectives. Therefore, data will be used without consent, in accordance with Article 110 of Legislative Decree 196/2003, as amended by Legislative Decree 101/2018.

Among the reasons qualifying as "organizational impossibility" that justify this choice are both the large sample size, which makes rocontacting patients a disproportionate effort, and the non-profit nature of the study, which limits the financial resources and staff available to support the recontacting process.

In compliance with current legislation, a data protection impact assessment will be prepared and published on the Fondazione's website pursuant to Article 35 of EU Regulation 679/2016, and the

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Italian Data Protection Authority will be notified. Furthermore, the privacy notice on data processing will be made publicly available on the Fondazione's website.

4.2 Sample processing and analysis

For genetic analysis, DNA will be extracted from deidentified peripheral blood samples at the Genomic platform of the Fondazione IRCCS Ca' Granda, and quantified by a Qubit 2.0 analyzer using the Qubit dsDNA BR Assay Kit (Thermo-Fisher). DNA purity will be evaluated using a Nanodrop 1000 spectrophotometer (Thermo-Fisher) and integrity assessed by gel electrophoresis. A rate (concentrations range from 50 to 100 ng/µl in a total volume of 50–100 µl) of DNA will be sent to the National Facility - Human Technopole to perform WGS.

Sequencing and bioinformatic analysis, including annotation and gene-level interpretations and diseases association analysis, will be performed at Human Technopole.

The presence of rare damaging variants, including structural variants, in genes known to be associated with cholestasis, starting from genes described in PFIC or other forms of genetic cholestasis (about 50 genes [15]), will be evaluated. Variants will be filtered according to their frequency in the general population (gnomAD) and their consequence, and classified according to published guidelines [16, 17]. Genetic findings will be integrated with clinical data and a genetic diagnosis will be performed in the presence of pathogenic variants consistent with the clinical phenotype and mode of inheritance. Moreover, we will evaluate the presence of rare damaging variants, including structural ones, in other liver-related genes associated with mitochondrial hepatopathies, metabolic disorders and/or ciliopathy to identify new potential genetic determinants of cholestasis [15]. As a local control cohort, more similar to the genetic background of patients, we will take advantage from the data derived from WGS previously performed in a well-characterized reference group of healthy individuals (blood donors with medical history, anthropometric, hematological and biochemical evaluation), analyzed with the same criteria used for cases. Rare damaging variants in liver-related genes more frequent in

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cases vs local controls could be considered as potential contributors to clinical phenotype. Moreover, the burden of rare damaging variants at single gene level in patient vs controls will get insight into disease pathogenesis.

All the remaining samples not used by Human Technopole for Genomics will be returned and stored at the POLI-MI Biobank, Fondazione IRCCS Ca Granda. These samples could be use, upon specific biobanking consent signature, for research purpose for future studies beyond the end of the present study.

Genetic data will be stored in a secure server of Fondazione IRCCS Ca' Granda, protected through WORM technology, as FastQ and VCF files for 2 and 20 years respectively. Data will be not systematically reanalyzed, unless explicitly requested, in line with international guidelines [18]; in this case, a new informed consent will be required. At the end of the study, data will be submitted in anonymous and aggregate form in public database, such as NCBI GEO e/o European Nucleotide Archive (ENA) and could be used for research purpose for future studies beyond the end of the present study.

5. ENDPOINT

5.1 Primary Endpoint

The prevalence of pathogenic variants in genes known to be involved in genetic cholestasis.

5.2. Secondary Endpoints

The prevalence of rare damaging variants in liver-related genes associated with mitochondrial hepatopathies, metabolic disorders and/or ciliophaties

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6. STUDY TIMELINES

Study starting date: November 2025

Closing of Enrolment: January 2026

Study analysis closing date: July 2026

Study end date and final report: October 2026

7. STATISTICAL ANALYSIS

7.1 Sample size

We plan to include 60 adults with unexplained or atypical cholestasis, according to the clinical criteria described above. For each patient genetic analysis through WGS will be performed. Based on preliminary study [15], assuming that 15% of the patients have pathogenic variants, and 15% of drop-out, the sample size is estimated to detect the expected proportion with 9.8% absolute precision and 95% confidence.

To descriptive aim, we will also evaluate the data derived from WGS previously performed in a well-characterized reference group of healthy individuals (blood donors with medical history, anthropometric, hematological and biochemical evaluation) from the FOGS study (n=1025). Based on preliminary study [15], the sample size is estimated to allow a statical power >95% with an alpha error of 0.05 to detect a five-fold impact of rare variants on cholestatic phenotypes, as calculated using the G* Power software (Universität Dusseldorf).

7.2 Data analysis

Categorical variables will be expressed as frequencies and percentages; continuous variables as mean ±standard deviation or median and interquartile range (IQR), as appropriate. The prevalence of rare damaging variants will be calculated assuming a confidence interval of 95%. Variant frequencies will

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be compared between patients and controls using chi-squared tests adjusted for false discovery rate (FDR). The enrichment in rare variants in cases vs controls will be evaluated by Burden test (SKAT and SKAT-O methods), adjusted for multiplicity of testing. Statistical analyzes will be performed using JMP Pro (SAS Institute) and the latest version of R statistical analysis software (http://www.Rproject. org/).

8. ADVERSE EVENTS

This study does not involve administration of drugs or other substances or invasive clinical practices. Therefore, no adverse events are expected.

During genetic analysis, incidental or secondary findings, defined as results unrelated to the original reason for testing, unintentionally discovered or actively searched for respectively, could be generated. This possibility will be discussed with the patient prior to obtained the informed consent for genetic analysis and the patient will be able to decide whether or not to be informed of these results. Only incidental findings related to serious genetic diseases actionable of early or presymptomatic medical intervention will be considered and refer to the patient, in accordance with recommendations endorsed by the European Society of Human Genetics (ESHG) [18]; secondary findings will be not actively searched for. Geneticists will deliver the final report and provide post-test counselling to the patients.

9. EVALUATION OF RISKS/BENEFITS

By integrating clinical and genomic data, employing a WGS approach in a well-characterized patient cohort, we expect to improve the diagnostic accuracy of these complex rare conditions, reclassifying unexplained cholestatic liver disease and conditions currently diagnosed as immune-mediated. The benefits include the possibility to set-up personalized follow-up strategies, optimizing the clinical

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management, paving the way to a personalized medicine approach. The identification of a genetic diagnosis also allows for screening of first-degree relatives and early treatments or prevention whenever required.

10. STUDY MANAGEMENT

10.1 Data collection and management

The coordinator center has arranged an electronic case report form (eCRF), approved by the ethics committee, where will be registered all the data declared in this protocol. Data required in the CRF are only those necessary to verify the hypothesis of the study. The CRF system is provided by Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico using the RedCap (Research Electronic Data Capture) platform. The REDCap Consortium is composed >1000 institutional partners worldwide (research institutions, universities, etc). The consortium supports a secure web application (REDCap) designed exclusively to support data capture for research studies. The REDCap application enables users to create and manage online databases quickly and securely, and is currently in use for more than 110,000 projects with approximately 150,000 users covering numerous research interest areas across the consortium. Through REDCap, the following will be implemented for this study: a) user-level identification, with specific restrictions based on role in the study; b) real-time data integrity validation and checking; c) de-identification of patients before data export; d) centralized data storage with daily backup within a secure server of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico IT service).

To guarantee data protection, patients will be indicated with a unique code. Only the study investigators will have passwords to access that file. Each process to promote data quality will be guaranteed. Data insertion in the database will be performed in a correct and accurate way according to the source documentation. In case of lack of data, the motivation will be provided. In order to ensure adequate control over the quality of the study, the investigator will allow, if

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required from the regulatory authorities, direct access to all relevant documentation and devote part of his time to discuss the study results.

10.2 Regulatory aspects and ethical considerations

10.2.1 Authority Approval

All the study phases will be conducted in accordance with ICH (International Conference of harmonization)/GCP and all applicable law, including the Declaration of Helsinki of June 1964, modified by the last World Medical Association General Assembly (Helsinki, Finland, 2024). The PI will obtain the ethical board approval before the trial commencement.

10.2.2 Ethical Committee Approval

Principal Investigator will make sure that the study protocol had obtained the local ethical board approval before the study commencement.

The local ethical board must verify and approve the informed consent module before the study enrollment. Whenever it is necessary to modify the protocol and/or the informed consent along the study, the PI will guarantee and perform the protocol revision and approval according to the Ethical board requirement. The contents of the above-mentioned changes to the protocol will be included only after the ethical board approval.

10.2.3 Informed Consent

The PI or other personnel in charge must inform the patients about all aspects the procedures involved by the study. The local PIs and sub-investigators will collect the written informed consent to the study participation.

The process for obtaining informed consent must be in agreement with the current rules. The PIs or sub-investigators and the patient must sign and date the consent before starting any procedure involved in the study. The PIs and other personnel involved in the study must not force in any way

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or influence the choice of participating or continuing to participate.

The choice of participating to the study must be completely free. The PIs must underline during the consent collection process that the subject can decline the participation at any time without any penalty or loss of benefits for which they have the right. The written or oral information concerning the study – including the informed consent module – must not contain any language expression that will oblige the subject to neglect their legal rights or relieve PIs or the Institution from any responsibility because of negligence.

The consent form signed by patients will include the consent for data processing and for the participation to the study, as well as, for the storage of biological samples (biobanking) and genetic data and their use for future studies.

With regard to the control group, as already specified in the protocol, consent will likewise not be obtained, in line with Article 110 of Legislative Decree 196/2003, as amended by Legislative Decree 101/2018. The reasons underlying this choice are related to "organizational impossibility," whereby recontacting patients would represent a disproportionate effort, potentially hindering or even preventing the achievement of the research objectives.

10.3 Investigators' responsibilities

According to the currently applicable rules, the PI will send reports about the study enrollment and progression to the ethical board every year and will notify the end of the study. The year report and the communication of the study end are the investigator's responsibilities.

10.4 Monitoring

According to the currently applicable rules of good clinical practice, monitoring visits will be performed on line and/or in person in each participating centre. The duration, nature and frequency of the visits will depend on the actual recruitment rate of each centre, the amount and quality of

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documents and adherence to the protocol.

Through the monitoring visit, the monitor will:

- Check and evaluate the progression of the study
- Examine the data collected
- Verify the source documents
- Identify problems and find solutions

The objectives of the monitoring activity will be verifying that:

- Patients' rights and wellbeing is respected
- The data are accurate, complete and verifiable by the source documents
- The study follows the protocol and eventual approved amendments, GCP and applicable rules.

The investigator must:

- Provide access to all the documents related to the patients to the monitor
- Dedicate portion of his/her own time and staff to discuss the results of the monitoring visit and any other possible aspects related to the study.

The monitor must contact the center before the beginning of the study to discuss the protocol and data collection with the staff.

10.5 Quality of the study assurance

As promoter, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico could apply a quality control to the study. In this case, the PI must allow the monitor to verify directly to all the study documents and to spend his/her time and staff to discuss the monitoring results and other aspects of the study.

Moreover, the Regulatory Authorities can perform inspections. In this case, the PI must allow the authorities to directly verify all the documents related to the study and spend the residual volume of his/her time and staff to allow the inspector to discuss the monitoring and eventually other aspects of the study.

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10.6 Study Conclusion

At the study conclusion, the monitor and the PI must:

- review all the documents related to the study
- reconcile the data
- harmonize all the clarification reports.

10.7 Document archiving

According to the current national rules, the PI must keep a copy of all the study documents and store it in a dry and safe place after the study end.

10.8 Dissemination of information regarding the scientific results

10.8.1 Confidentiality

The PI or other personnel involved in the study must deal with all the information related to the study (including the protocol, data and documents produced during the study), and must not use the study information, study data or reports for any purpose different from those described by the protocol. The above-mentioned restrictions are not applicable to

1) information that will become available in public, not because of negligence of the PI and his/her personnel.

2) information that requires communication to the ethical board only because of evaluating the protocol

3) information that are communicated with the purpose of getting adequate medical care for the study subject.

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10.8.2 Publications and intellectual property

- ***Monocentric study with collaboration***

Publications:

As the Sponsor, Fondazione will ensure the dissemination and publication of the study results, even in the event of negative results, without any restrictions and ensuring the collaborating center's visibility proportionate to its actual participation. Each scientific journal or publication containing the data and study results must indicate the role and participation of the collaborating center and of Fondazione, according to their actual contribution to the study and their role. The data may be published in aggregate form or otherwise anonymized, to avoid the identification of the subject to whom the data refers.

Intellectual property:

The Parties acknowledge that, in conducting the collaboration within the scope of the study, data, information, know-how, and inventions (whether patentable or not) owned by each party may be used and shared. Each party retains exclusive ownership of the data, information, know-how, and inventions, even if it grants the other a non-exclusive, free right of access and use, solely for the purposes of carrying out the activities covered by the study and limited to the duration of the study. It is understood that this right of use does not include the right to sublicense to third parties.
In accordance with applicable law, the data and results generated within the scope of the Study will be the property of the Sponsor, unless otherwise agreed between the Sponsor and the collaborating center(s).

11. INDEMNITY AND COMPENSATION IN THE EVENT OF DAMAGE

In the event of unwanted events or any damages that may derive from participation in the research,

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the Insurance Policy of our Institute is also extended to cover the subjects participating in the research projects.

12. CONFLICT OF INTEREST DISCLOSURES

The PI and collaborators of the study have no conflict of interest to disclose.

13. FUNDING

Samples analysis and related data will be carried out free of charge at the HUMAN TECHNOPOLE NATIONAL FACILITY, as foreseen in the "HUMAN TECHNOPOLE NATIONAL FACILITY FOR GENOMICS CALL FOR ACCESS 25-G-ROUND1 (project code ID 2166994)"

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