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Title:Study for the Evaluation of Risk of hEpatocellular carcinoma in NonAlcoholic fatty liver

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SUMMARY

Background and rationale:Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality. Non-alcoholic fatty liver disease (NAFLD), i.e. the accumulation of fat in the liver to an extent greater than 5% of the organ's weight in the absence of alcohol abuse, will become the main cause of HCC in Western countries by 2025 and represents one of the leading causes of liver disease in the world. While there is new evidence demonstrating that steatosis directly promotes liver carcinogenesis, the progression of liver disease to cirrhosis and HCC is more frequent in patients with non-alcoholic steatohepatitis (NASH), a condition characterized by active inflammation and fibrosis. However, NAFLD is often asymptomatic so many individuals are unaware that they have a progressive form of liver disease. Furthermore, the high prevalence of NAFLD, the incidence of HCC in patients without advanced fibrosis, and the lack of disease awareness, together make classical screening strategies for the diagnosis of HCC ineffective. As a result, most patients who develop NAFLD-HCC are diagnosed at an advanced stage of the disease, when curative treatments are no longer possible. This makes the development of new non-invasive biomarkers capable of stratifying the risk of HCC development in patients with NAFLD a public health priority.

Our group has previously contributed to demonstrating that some common genetic factors (I148M variants in PNPLA3, E167K in TM6SF2, rs641738 C>T polymorphism in the MBOAT7 locus) influence disease progression in NAFLD towards HCC. Furthermore, rare mutations that affect the function of proteins encoded by the hTERT and APOB genes predispose to steatosis and HCC.

Purpose of the study:The aim of the proposed study is to quantify the impact of genetic risk factors for the development of primary liver cancer in patients with non-alcoholic fatty liver disease (NAFLD-HCC) and their interaction with acquired risk factors, on the incidence of disease in a prospective cohort of NAFLD patients aged 45 to 75 years ed

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at risk of HCC, selected for: (1) presence of advanced liver fibrosis, (2) carrier status of rare genetic mutations known to determine a strong increase in the risk of HCC, (3) carriers of at least three common genetic factors predisposing the disease and acquired risk factors (diabetes and/or obesity). The study aims to develop a diagnostic score capable of predicting the incidence of HCC and identifying those patients for whom clinical screening is cost-effective.

Study design:Multicenter, spontaneous, non-pharmacological, biological study

Experimental plan and methods:The study will be divided into three phases:

1. In thefirst phase the impact of a score based on the evaluation of common genetic variants in genes predisposing to the development of NAFLD-HCC (PNPLA3, TM6SF2, and MBOAT7), and rare mutations determining high risk of NAFLD-HCC, e.g. in genes involved in telomere shortening (TERT) and lipid metabolism (APOB) on the risk of developing HCC and on survival, in the entire patient cohort and in the individual groups listed above.

2. In thesecond phase we will use next generation sequencing techniques (whole exome / genome sequencing) to identify new genetic risk variants for the development of HCC.

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RATIONAL

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor and the second leading cause of solid malignancy mortality worldwide [1]. Non-alcoholic fatty liver disease (NAFLD), i.e. hepatic fat accumulation greater than 5% not explained by alcohol abuse, is set to become the leading cause of HCC in Western countries by 2025 [1,2,3,4] . NAFLD is very frequently caused by insulin resistance due to unhealthy lifestyles. Due to the epidemic of obesity and type 2 diabetes, NAFLD affects a third of the planet's inhabitants. NAFLD-HCC frequently develops even in individuals without advanced liver fibrosis suggesting that steatosis directly promotes liver carcinogenesis [5,6,7,8]. However, the progression of liver disease to cirrhosis and hepatocellular carcinoma is more frequent in the subgroup of subjects affected by non-alcoholic steatohepatitis (NASH), a condition characterized by active inflammation and fibrosis [9]. Risk factors for NAFLD progression include advanced age, obesity, severe insulin resistance, and hypertension. Consistently, NAFLD-HCC patients are most commonly adult males, with type 2 diabetes (T2D) and meet criteria for at least one of the features of metabolic syndrome. Several epidemiological studies have established the association between overweight and obesity, which are considered the major determinants of insulin resistance and NAFLD, and a higher risk of developing HCC (17% and 89% respectively, when compared with normal weight subjects) [10,11]. T2D has also been independently linked to the onset of HCC in large epidemiological studies, in which it was found that among men with T2D the risk of developing HCC was doubled [12,13]. On the one hand, these data suggest that obesity and T2D are major epidemiological determinants of HCC incidence in Western countries. On the other hand, most individuals with NAFLD are completely unaware that they have progressive liver disease. Consequently, due to the high prevalence of NAFLD, the occurrence of HCC in patients without advanced fibrosis, and the lack of disease awareness, many of the patients who develop NAFLD-HCC are diagnosed at advanced stages, when curative treatments are no longer available. possible [14,15]. This makes the development of new non-invasive biomarkers capable of stratifying the risk of developing HCC in patients with NAFLD a healthcare priority.

The mechanisms linking NAFLD to the progression of liver disease to HCC have not yet been identified. In any case, in obesity and diabetes different pathways could be activated that favor a pro-carcinogenic environment, distinguishing the pathogenesis of NAFLD-HCC from those of HCC from other etiologies [1,3,16,17]. First, increased cancer risk is associated with a low degree of chronic inflammation, a typical manifestation of obesity and metabolic syndrome. In fact, the expansion of adipose tissue promotes the release of proinflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 6 (IL6), both potent activators of key oncogenic signaling pathways [18]. Furthermore, obesity impairs the release of

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adipokines by reducing the level of those with anti-inflammatory effects, such as adiponectin, and increasing the levels of those with pro-inflammatory and fibrogenic effects, such as leptin [19-20]. Overall, the factors listed above jointly induce hyperinsulinemia, with a consequent increase in the bioavailability of insulin like growth factor-1 (IGF1) which in turn promotes cell proliferation and inhibits apoptosis [21]. The activation of hepatic stellate cells (HSCs) is also an important step in the development of HCC on cirrhosis, however these cells not only secrete collagen which causes liver fibrosis, but can also produce several growth factors which stimulate oncogenic pathways which contribute to the expansion of neoplastic clones [22].

Genetic factors have been shown to influence the progression of NAFLD, and family history remains the main risk factor for the development of HCC [9,23]. The common genetic polymorphism rs738409 C>G encoding the I148M variant of Patatin-like phospholipase domain-containing protein 3 (PNPLA3 or adiponutrin) has been recognized as the major common genetic determinant of hepatic fat content and NAFLD progression [24 ,25,26,27,28]. The mechanism is related to the accumulation of the mutated protein [29, which interferes with the remodeling of lipid droplets in hepatocytes [27,30,31], and with the release of retinol from HSCs [32,33]. The PNPLA3 variant predicts the development of HCC in NAFLD patients of European origin [34]. This evidence suggests that this genetic risk factor may be useful for selecting high-risk individuals for screening [34,35,36], but has too low sensitivity to be used alone as a prognostic marker [37]. The rs58542926 E167K variant in Transmembrane 6 superfamily member 2 (TM6SF2) also predisposes to the progression of NAFLD by altering the secretion of VLDL (very low-density lipoproteins) [38,39,40], but its direct role in the predisposition to HCC is debated [39,40,41]. More recently, it has been demonstrated that the rs641738 C>T sequence variant in the Membrane bound O-acyltranferase domain containing 7/ Transmembrane channel like 4 (MBOAT7/TMC4) locus, involved in phospholipid remodelling, predisposes to the development of cirrhosis in individuals heavy drinkers [42], and the development and progression of NAFLD in individuals of European origin [43]. We recently reported in a cross-sectional study that the rs641738 variant is also associated with the risk of HCC in patients with NAFLD (Donati and Dongiovanni, Scientific Reports 2017, forthcoming)

Furthermore, rare germ line mutations in genes involved in the development of Mendelian liver diseases or familial cancer, which severely affect the function of the encoded proteins, can predispose to NAFLD-HCC (our data being published, deriving from a project previously approved and financed by IRCCS Foundation). For example, mutations in Telomerase reverse transcriptase (hTERT),they can predispose to a wide spectrum of familial liver diseases characterized by steatosis [44] and possible evolution to cirrhosis and HCC [45,46]. In fact, mutations in Apolipotrotein B (APOB) can explain some familial cases through the predisposition towards the development of severe steatosis caused by hepatocyte retention of lipids [47].

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PURPOSE OF THE STUDY

The overall aim of the study is to quantify the impact of genetic risk factors for the development of NAFLD-HCC, and their interaction with acquired risk factors, on the incidence of the disease in a prospective cohort of at-risk patients. The main aim will therefore be to:

1. Validate a score capable of predicting NAFLD-HCC and select patients for whom screening is cost-effective.

This presupposes (secondary purposes):

2. Validation of inclusion criteria capable of identifying individuals at risk of NAFLD-HCC among NAFLD patients in follow-up.
3. Identification of the impact of individual genetic variants on the risk of HCC (both on the entire cohort and in patients stratified according to the enrollment criteria).

EXPERIMENTAL PLAN

Study design

Spontaneous, non-pharmacological, biological study.

Patients and methods

To maximize the power of the study, we will select 500 NAFLD patients aged 45 to 75 years, at high risk of HCC and selected based on the following criteria:

- Presence of advanced liver fibrosis, stage F3-F4 (Criterion 1);
- Subjects with family history and/or carriers of rare genetic mutations strongly predisposing to HCC (Criterion 2);
- Subjects with strong acquired risk factors (diabetes or obesity) carriers of at least three common genetic variants at risk for the disease (Criterion 3).

We aim to identify the highest possible number of patients who will develop HCC while screening the smallest number of patients with NAFLD. To test this hypothesis, we will register at the participating centers the number of HCC cases in follow-up whose patients do not meet these criteria. Older patients will be excluded because the diagnosis of HCC in the elderly with high comorbidities may not offer benefits regarding survival and quality of life.

Evaluation of candidate common risk variants in PNPLA3, TM6SF2, MBOAT7 will be offered to participating centers for patient screening. The results of genetic tests will be

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immediately recorded and analyzed in the first phase of the study. Furthermore, we will also consider a group of rare variants, which have been identified as associated with NAFLD-HCC in ongoing studies in cross-sectional cohorts (by targeted resequencing of candidate genes).

Inclusion and exclusion criteria

They will comeincluded in the study subjects with the following characteristics:

1. Diagnosis of NAFLD or cryptogenic liver disease, allowing a more liberal alcohol intake limit (<60/40 g/day in M/F), so as to also include subjects with a moderate alcoholic component of liver disease, an important factor given the high epidemiological burden of this group
2. Age between 45 and 75 years
3. Any of the following criteria:
    to. F3-F4 fibrosis, determined histologically, or by non-invasive techniques (stiffness > 7.9 kPa at Fibroscan and positivity at the NAFLD fibrosis score or at APRI or at FIB4), or evidence of cirrhosis deriving from biochemical tests or imaging methods;
    b. Family history of primary liver cancer in first degree parentage, 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.
4. Willingness to sign the informed consent.

They will be insteadexcluded from the study patients with:

1. Alcohol intake >60/40 g/day in M/F
2. Chronic viral or autoimmune hepatitis
3. Any previously diagnosed genetic liver disease associated with increased risk of HCC (such as hereditary hemochromatosis, Wilson's disease, Alpha-1 Antitrypsin deficiency)
4. Use of drugs known to induce steatosis and liver disease
5. HCC diagnosed before the study start date.
6. Other pathological conditions with a prognosis of less than two years.

Enlistment

We expect to enroll 500 patients with NAFLD who meet the study criteria from January 2018 to January 2020. The study follow-up will continue until January 2024. The analyzes relating to the first and second phases of the study will continue until December 2025.

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Recruitment and follow-up

At the baseline, the objective examination will be carried out and the anamnestic data, family history, eating habits and lifestyle, and the demographic and anthropometric parameters of each patient recruited according to the criteria listed above will be collected.
After signing the informed consent, blood samples will be taken for liver function, genetic and biomarker studies as per attachment 1. The presence of steatosis and/or nodules will also be assessed by ultrasound.

Every 6 months the patient's status will be checked (alive/dead, possible cause of death; presence of HCC; onset of hepatic decompensation) and the number of HCC cases will be recorded in each centre; patients in follow-up for non-NAFLD were recruited into the study because they did not meet the enrollment criteria. Clinical characteristics at diagnosis and DNA samples from these cases will be collected to compare characteristics with those of incident cases included in the study.
Lifestyle and eating habits will be re-evaluated annually, while, where available, the presence/ progression of fibrosis will be monitored every two years using Fibroscan.

The diagnosis of HCC must be made according to the EASL criteria. In patients with cirrhosis, as per recognized clinical practice, a six-monthly or quarterly ultrasound screening will be performed in the presence of focal lesions. Limited to patients at risk, but not cirrhotic, annual ultrasonographic screening will be acceptable.

Participating centers (confirmed):

1. Gastroenterology and Hepatology, Policlinico, Milan (prof. P. Lampertico)
2. Internal Medicine, University of Udine (dr. G. Soardo)
3. Gastroenterology, University of Turin (prof. E. Bugianesi)
4. Gastroenterology, Humanitas University (prof. A. Aghemo)
5. Gastroenterology, Palermo Polyclinic (dr. S. Petta)
6. Internal Medicine, University of Bologna (prof. F. Piscaglia)
7. Internal Medicine, Catholic University of Rome (dr. L. Miele)

Genetic analyses

Genetic screening will be carried out on DNA extracted from peripheral blood at the extraction platform of the IRCCS Cà Granda Foundation.

In the first phase of the study, the entire cohort will be genotyped for the following polymorphisms rs738409 C>G (PNPLA3 I148M), rs58542926 C>T (TM6SF2 E167K), rs1260326 C>T (GCKR P446L)

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and rs641738 C>T in the MBOAT7 locus, using Taqman SNP genotyping assays (Life Technologies, Carlsbad, CA), as previously described [40,43].
Additionally, patients will be investigated for the presence of rare mutations in the hTERT and APOB genes by resequencing the entire protein coding sequence.

Exome sequencing and analysis of resulting data

In the second phase (study of rare genetic variants possibly associated with NAFLD-HCC and the discovery of new non-coding risk variants), DNA samples will be extracted from peripheral blood by the automatic platform of the IRCCS Ca' Granda Foundation. Quality control will be carried out by evaluating the absorbance ratio 260/280 nM and by gel electrophoresis. DNA library enrichment for exome sequencing will be performed using SureSelect Human All Exon v6 (Agilent). Sequencing will subsequently be performed on the HiSeq 4000 platform (Illumina). This procedure will guarantee an average coverage of 70-80X of the target region.

Raw data will be analyzed using standard bioinformatics pipelines, including alignment, variant and function annotation. Functional mutation analysis will focus on data integration by performing different analyzes to comprehensively characterize variants in known genes associated with liver disease, as well as to search for new genes and genetic risk factors potentially involved in progression to HCC.
Multiple available resources will be used such as frequency data of genetic variants (ExAC, ESP, 1000Genomes), pathogenic variants (ClinVar) and predictorsin-silicoof damage (SIFT, PolyPhen, CADD) to prioritize variants and candidate genes involved in predisposition to liver disease.

STATISTIC ANALYSIS

Time to progression to HCC will be assessed by Kaplan-Meier estimates of cumulative incidence rates. The survival distribution between genotypes will be compared using Cox proportional hazard models. Hazard ratios and P-values will be adjusted for confounding factors. In the analysis, risk genotypes will be encoded using additive genetic models.

We will evaluate the impact of common genetic risk variants in PNPLA3, TM6SF2 and MBOAT7 and of mutations in genes involved in telomere shortening (e.g. TERT, Donati Cancer Medicine 2017; forthcoming) and lipid metabolism (e.g. APOB, data in progress) on the risk of HCC, using Cox regression models adjusted for classic risk factors for HCC (age, sex, BMI, type 2 diabetes, advanced fibrosis, smoking, alcohol intake) .

As a secondary outcome, we will also evaluate the interaction between genetic and acquired risk factors in the pathogenesis of HCC. As the main purpose of the study, we will develop a score

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of combined risk to predict HCC incidence, which will be cross-validated within the cohort.

The analyzes will be conducted both in the entire cohort and in the groups of patients stratified according to the enrollment criterion.
Statistical analyzes will be performed using statistical analysis software version 3.3.2 (http://www.R-project.org/). P-values <0.05 (two-tailed test) will be considered statistically significant.

ESTIMATION OF THE POWER OF THE STUDY

Under the assumption based on previous literature that we will observe an HCC incidence rate of 1.5% across the cohort over a mean follow-up of 5 years, and that based on recently generated data in a cross-sectional cohort (Donati, Scientific Reports 2017 and Cancer Medicine 2017; currently being published) we will be able to identify at least 30% of individuals with at least a tripled risk of HCC, for a type I error rate of 5%, we will have to enroll 375 patients to have statistical power of 80% to detect a significant association of the genetic score with HCC risk in the first phase of the study. However, we estimated the need to increase the number by 30% to account for patients potentially enrolled due to the occurrence of other major clinical events before the onset of HCC (death, cancer, major cardiovascular events and hepatic decompensation) and a small group lost to follow-up. Therefore, we intend to enroll 500 patients to complete the study.

COSTS

No additional costs are foreseen for the UOC and the Foundation as the data collected for the study are those expected in the normal patient care path. The blood sample to perform genetic analyzes will be obtained during a regular routine clinical check-up.
The costs of the study will be partially covered by the funds of the Ricerca Corrente Fondazione IRCCS Ca' Granda INGM and by the myFirst AIRC grant 2016 (n. 16888), LITMUS IMI2 Funding.
For the realization of this project, specific requests for funding are being submitted to research funds such as Young Investigator's Awards, Italian Association of Cancer Research - AIRC, Ministry of Health.).

EXPECTED RESULTS AND IMPACT ON THE HEALTH SYSTEM

In the first phase of the study we expect to define whether a genetic HCC risk score is able to accurately predict the onset of NAFLD-HCC in a high-risk cohort. This “Precision Medicine” approach would have an important clinical impact, allowing the identification of subjects for whom ultrasound screening for HCC could be cost-effective with potential clinical advantages for patients and savings for the healthcare service.

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