ALLEGATO B

Bando 2020-21 - Programma 5 per mille anno 2018-2019
Investigator Grant (IG)

TRANSLATIONAL RESEARCH

LILT will support research projects in the field of cancer aimed at improving cancer diagnosis and treatment. Particularly considered will be those translational research projects that promise short-medium term effects in clinical practice, concerning new diagnostic methodologies and new therapies. Multicentric studies with national coordination, aimed at validating new diagnostic methods, diagnostic, prognostic and predictive tumor markers, able to improve the clinical management of cancer patients are potentially eligible for funding. Specific research projects on new oncological therapeutic approaches are also eligible for LILT funding as IG. For this type of grants it is necessary to demonstrate solid preliminary experimental data supported by a rigorous biological rationale.

1. Principal investigator's full name and qualification:
   Silvia Soddu, MD, PhD
   Head of Department Unit "Cellular Networks and Molecular Therapeutic Targets"

(The CV in European format with list of publications, IF, and H-index is presented as a separate file in the e.mail)

2. Proposal title: **HIPK2 as a prognostic biomarker for liver fibrosis: evidence and underlying mechanisms**

3. Primary area of Relevance: Hepatobiliary patho-physiology

4. Relevance for the National Health System: Identification of novel biomarkers for liver fibrosis progression

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Direttore Generale
Dott. Francesco Ripa di Meana

9. Place and date Roma 14/01/2021

SELF EVALUATION FORM

1. Investigator's full name (PI): Silvia Soddu
2. Total papers: 124 IF: 761.5
3. Total papers (last 10 years): 40 IF: 223.2
4. Total Papers as first/last author or corresponding author: 41
5. Total H-index 39 (Scopus), 40 (WOS)

PROPOSAL MAIN BODY

1. Proposal title: HIPK2 as a prognostic biomarker for liver fibrosis: evidence and underlying mechanisms

2. Abstract

Liver fibrosis is a major cause of morbidity and mortality from chronic liver diseases (CLDs). The development of fibrosis is a common pathological consequence of a variety of chronic stimuli, including viral, autoimmune, drug- and alcoholic-induced, cholestatic and metabolic diseases. Fibrosis is the first step toward the progression to cirrhosis and eventually to hepatocellular carcinoma (HCC). Currently, there are no non-invasive, cost-effective, and clear biomarkers able to predict either progression or effective therapies for their prevention.

Fibrosis is characterized by the excessive synthesis, deposition, and contraction of extracellular matrix (ECM) components produced by myofibroblasts. In the pathogenesis of liver fibrosis, a key role is played by the hepatic stellate cells (HSCs) that are the main source of ECM. Following chronic liver injury, quiescent HSCs are exposed to pro-fibrogenic factors and activated toward a myofibroblast phenotype. Therefore, inhibition of HSC activation is a possible target to attenuate liver fibrosis.

HIPK2 is an evolutionarily conserved kinase which acts as a co-regulator of several transcription factors and modulates different cellular processes such as proliferation, morphogenesis, and cell death. HIPK2 contributes to numerous signaling pathways, including those primarily involved in fibrosis, such as TGF-β/SMAD, WNT, and Galectin3. A direct role for HIPK2 in fibrosis was first identified in a model for human HIV-associated nephropathy through a systems biology approach that takes into account protein–protein and protein–DNA interactions. Following this initial study in kidney fibrosis, HIPK2 has been identified as a regulator of signaling pathways activated in lung fibrosis, fibrotic keloid formation, and pro-fibrogenic response of HSCs in liver fibrosis. In addition, HIPK2 has been proposed as a new drug target for anti-fibrosis therapy.

A previous unbiased, broad-spectrum study has detected circular RNAs (circRNAs) from the *HIPK2* gene (circHIPK2) among the most abundant circRNAs. Together with long non-coding- and micro-RNAs (lncRNA and miRNA), circRNAs are a novel class of naturally occurring non-coding RNAs (ncRNAs). Due to the greater abundance and stability compared to miRNAs and lncRNAs, circRNAs are emerging as biomarkers in both solid and liquid biopsies. Thus, taken together, these observations open the possibility of testing circHIPK2 as a potential prognostic biomarker for liver fibrosis.

Here, we propose to investigate the role of HIPK2 in the pathogenesis of liver fibrosis and evaluate whether circHIPK2 can be employed as a new prognostic biomarker in cirrhosis progression and/or HCC development of patients with liver fibrosis.

This proposal has a high likelihood of identifying a novel, non-invasive prognostic biomarker for liver fibrosis progression.

3. Introduction

Liver Fibrosis - CLDs represent an important global public health problem. They are highly prevalent and silent, related to different, sometimes associated causes of fibrosis, which include virus-induced CLDs, alcoholic liver disease (ALD), and non-alcoholic steatohepatitis (NASH) [Worner and Lieber, 1985]. NASH is closely related to the triple epidemic of obesity, pre-diabetes, and diabetes and its contribution to the causes of CLDs is expected to increase in the next decade [Marcellin and Kutala, 2018]. Necroinflammation and liver fibrosis due to the excessive deposition of ECM are the key mechanism in the progression of CLDs and the first step toward cirrhosis and eventually to HCC [Bataller and Brenner, 2005]. Activation of HSCs is a key event in the pathogenesis of liver fibrosis. HSCs are fat-storing quiescent cells which undergo a process of activation following chronic liver injury, leading to a myofibroblastic phenotype, finally resulting in the excess production and deposition of ECM [Geerts, 2001]. Therefore, inhibition of the activation of HSCs is thought to be a possible target to attenuate liver fibrosis.

HIPK2 - Homeodomain-Interacting Protein Kinase 2 (HIPK2) is a tyrosine-regulated serine/threonine kinase [Saul et al, 2013; Siepi et al., 2013] originally identified as co-repressor for transcription factors. In the past two decades, we and others have shown that HIPK2 interacts with a variety of proteins involved in cellular stress response, morphogenesis, and proliferation [Blaquiere and Verheyen, 2017]. HIPK2 participates in several signaling pathways, being involved in TP53-dependent and -independent apoptosis [D'Orazi et al, 2002; Hofmann et al, 2002; Zhang et al, 2003], in the WNT pathway, a major driving force for proliferation, in the TGF-β/BMP/SMAD pathway [Kanei-Ishii et al, 2004], a key inducer of invasion and epithelial to mesenchymal transition, and in the fission machinery required for abscission, the final step of cell division [Rinaldo et al, 2012; Monteonofrio et al, 2019]. These HIPK2 activities and their alterations have been mainly involved in tumorigenesis [D'Orazi et al, 2012].

HIPK2 in fibrosis - Accumulating evidence indicates that HIPK2 plays a role in the development of tissue fibrosis [Saul and Schmitz, 2013]. A systematic approach has identified HIPK2 as a key regulator of kidney fibrosis [Jin et al, 2012] and a potential target for anti-fibrosis therapies [Nugent et al, 2015]. Three different animal models for kidney fibrosis have been employed to identify HIPK2 as a driver of this disease: HIV-associated nephropathy, unilateral ureteral obstruction, and folic acid-induced renal fibrosis. Direct evidence for a causative role of HIPK2 in kidney fibrosis was revealed by knocking-out the *Hipk2* gene (HIPK2-KO) in transgenic mice that express the HIV transgene. While the mice carrying the HIV transgene develop tubulointerstitial injury and fibrosis, the HIV/HIPK2-KO mice were largely protected from the disease [Jin et al, 2012]. Other recent studies implicated dysregulated HIPK2 levels in idiopathic pulmonary fibrosis [Ricci et al, 2013] and fibrotic keloid formation [Zhao et al, 2017]. More recently, it has been shown that HIPK2 may function as a novel regulator to modulate HSC activation, potentially by inhibiting the TGF-β1/Smad3 signaling pathway [He et al, 2017], suggesting that HIPK2 contributes also to liver fibrosis.

HIPK2 circular RNA - circRNAs are a novel class of naturally occurring ncRNAs, differentially generated by backsplicing of exons from a single pre-mRNA. The 5' and 3' ends are joined together, forming a covalent closed loop [Jeck et al, 2013]. circRNAs were originally considered by-products of splicing errors; however, emerging evidence indicates that exon circularization correlates with exon skipping, suggesting novel mechanisms involved in the splicing of linear and circular isoforms of the same gene [Gao et al, 2019; Kelly et al, 2015]. circRNAs are abundant, highly stable, evolutionarily conserved and exhibit cell-specific expression patterns [Jeck et al, 2013]. circRNAs are known to play important roles as miRNA sponges and to interact with RNA-associated proteins, forming RNA-protein complexes that regulate gene transcription [Greene et al, 2017; Yu and Kuo, 2019; Du et al, 2017]. Importantly, recent studies have shown the involvement of circRNAs in the pathogenesis of many diseases, including cancer and fibrosis

[Kristensen et al, 2018; Cao et al, 2017]. Due to their abundance and stability, circRNAs may act as better biomarkers than linear RNAs. In addition, circRNAs are enriched in body fluids including blood, saliva, and urine. Taken together all these features have opened the possibility of using circulating circRNAs as biomarker for liquid biopsy [Greene et al, 2017; Ojha et al, 2018; Zhang et al, 2018]. An unbiased, broad-spectrum study has shown that genes of the HIPK family produce abundant circRNAs [Jeck et al, 2013; Zheng et al, 2016]. circRNAs for the HIPK2 and HIPK3 genes (circHIPK2 and circHIPK3) are generated from the second exon of each gene, respectively [Jeck et al, 2013, Li et al, 2018]. Several reports have shown the involvement of both circHIPK2 and circHIPK3 in the physio-pathogenesis of different types of human cancers, including liver cancer [Chen et al, 2018] and, most relevant for this project proposal, circHIPK2 has been found to promote ER stress in human pulmonary fibroblasts [Cao et al, 2017].

4. Background and rationale

In the clinical practice, liver biopsy associated with several scoring systems for risk stratification are currently used for accurate staging of the degree of liver injury and specific treatment decisions. However, liver biopsy is an invasive procedure that carries the risk of morbidity and mortality, and its prognostic value is still limited. In the past decade, several non-invasive tests for liver fibrosis, such as the Enhanced Liver Fibrosis test, the FibroTest, the FibroMAX, and transient elastography FibroScan® have been developed. However, their diagnostic accuracy, cost-effectiveness, and effect on patient outcomes are still debated. Thus, identification of new candidate prognostic biomarkers is a major objective in the current research.
Accumulating evidence supports the contribution of HIPK2 in tissue fibrosis and identifies circHIPK2 as a promising biomarker in both solid and liquid biopsies. We have performed seminal and largely cited studies on the p53 activator HIPK2 [D'Orazi et al. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat Cell Biol 2002] and have been interested for a long time in studying the HIPK2 role in apoptosis, DNA damage response, cell proliferation, and cytokinesis (see "Ten selected relevant publications by the research group" section). Here, we propose to employ our large body of HIPK2 tools and experience to investigate the role of HIPK2 in the pathogenesis of liver fibrosis and evaluate whether circHIPK2 can be employed as a new prognostic biomarker in cirrhosis progression and/or HCC development of patients with liver fibrosis.

5. Experimental design

***Task 1*- Evaluate the contribution of HIPK2 in liver fibrosis by generating liver-specific HIPK2 knockout mice**

To evaluate whether HIPK2 contributes to liver fibrosis, we will generate liver-specific HIPK2 knockout (HIPK2-LKO) mice and compare them with the wild-type HIPK2 controls (HIPK2-WT) before and after induction of liver fibrosis. HIPK2-LKO mice (*i.e.*, liver-specific HIPK2$^{LOX/LOX}$ mice) will be obtained by crossing our HIPK2$^{CKO/CKO}$ mice (*i.e.*, our HIPK2-WT mice) (see "Work carried out and preliminary results" section) with a mouse carrying the Cre recombinase under the control of a liver-specific promoter. In particular, we will employ the Albumin-Cre mice, commercially available at Jackson Laboratories (B6.Cg-Speer6-ps1<Tg(Alb-cre)21Mgn>/J), that are transgenic mice expressing Cre recombinase under the serum albumin gene promoter control. Albumin promoter has a very high activity in liver cells, therefore Albumin-Cre mice are considered to be useful transgenic mice for deletion of loxP-flanked genes in the liver [Yakar et al, 1999; Michelotti et al, 2013; Lemaigre 2015]. The occurrence of allele rearrangement will be verified by specific PCR and HIPK2 depletion will be evaluated by RT-PCR.

Total time frame: months: 1-6

Next, we will analyze the effect of HIPK2 knockout in liver fibrosis by employing two well-

established approaches: 1) a chemical approach, by intraperitoneal injection of carbon tetrachloride (CCl_4) [Domenicali et al, 2009] that is metabolized in the liver and converted to a highly reactive tri-chloromethyl (CCl_3) radical, ultimately leading to hepatotoxic damage, inflammation and fibrosis mimicking advanced CLD [Weber et al, 2003]; 2) a diet approach, by feeding mice with a methionine- and choline-deficient diet (MCD) that is high in sucrose (40%) and provides a moderate amount of fat (10%), but being deficient in methionine and choline results in a reduced clearance of triglyceride and lipids accumulate in the liver, mimicking the non-alcoholic fatty liver disease [Itagaki et al. 2013].

To evaluate whether HIPK2 contributes to liver fibrosis, we will analyze liver tissues obtained from HIPK2-WT, HIPK2-LKO homozygous and heterozygous mice treated with or without CCl_4 or MCD. Together with standard liver parameters including size, shape, and color, we will evaluate structural changes of hepatic tissue, hyperplasia of collagen fibers, and ECM deposition. The expression levels of pro-collagen 1α1, collagen I, collagen III, TGF-β1, HSP74 and Galectin-3 [Van de Bovenkamp et al, 2007] will be evaluated by immunohistochemistry (IHC), Western blotting and/or by RT-qPCR. *Total time frame: months: 7-12*

At this point, we cannot envisage whether we will find a different contribution of HIPK2 in liver fibrosis caused by different harms. In the case we will find significant differences between CCl_4 and MCD treated mice, additional mouse model for CLDs are available [Nevzorova et al. 2020] and might be employed to better define the role of HIPK2 in their pathogenesis. However, these models, such as those mimicking ALD and NASH, require longer term (one additional year).

Total time frame: months: 13-24

***Task 2* – Verify whether circHIPK2 can be used as a prognostic biomarker in liver fibrosis progression**

To begin evaluating the potential use of circHIPK2 as biomarker in liver fibrosis, expression level of this ncRNA will be evaluated in freshly isolated HSCs from the HIPK2-WT mice with and without fibrosis induced by CCl_4 and MCD, as reported in Task 1. HIPK2-LKO mice will be used as negative control. We will also investigate the relationship between circHIPK2 and fibrosis using two different model of in vitro liver fibrosis. 1) We will obtained freshly isolated HSCs from both HIPK2-WT and HIPK2-LKO mice. Real Time-qPCR will be performed in quiescent and activated HSCs obtained by culturing them on uncoated plastic dishes for two days (*i.e.*, quiescent state) and seven days (*i.e.*, activated state) post-isolation [Guimaraes et al, 2010; Thoen et al, 2011]. 2) We will stimulate a fibrosis-like phenotype by treating the human HSC line (LX-2) with TGF-beta1 [He et al, 2017] and both circHIPK2 and linear-HIPK2 levels will be evaluated by Real Time-qPCR. Activation and fibrosis-like phenotype will be confirmed by standard procedures (see "Further details on the overall methods that will be used in this project" section).

Total time frame: months: 1-12

In parallel with the mouse and cell-culture experiments, we will evaluate the expression level of circHIPK2 in a bio-banked collection of 157 liver biopsies previously scored with both Knodell and METAVIR fibrosis systems and analyzed by IHC for expression of collagens, cytokeratins, and galectins at the Sant'Andrea Hospital (see "Work carried out and preliminary results" section). Using a validated, custom-made BaseScope™ probe for human circHIPK2 (Laura Monteonofrio, unpublished data), we will be perform a highly sensitive in situ hybridization assay. Formalin-fixed and paraffine-embedded (FFPE) tissues will be used to detect circRNAs at single-cell level with a spatial and morphological resolution. Expression levels of linear HIPK2 mRNAs will be also analyzed in the same cohort. Together with this newly in situ hybridization, expression of both circHIPK2 and linear HIPK2 mRNAs will be evaluated isolating total RNA from FFPE tissue to perform Real Time-qPCR. Overall, detection and localization of circHIPK2 in liver biopsies, together with its relative expression in different stage of fibrosis progression, will contribute to

elucidate circHIPK2 function especially given the fact that it could be used as clinical biomarkers.

Total time frame: months: 6-12

6. Further details on the overall methods that will be used in this project

All biochemical, molecular, and functional analyses related to cell cultures will be carried out as described in the PI's bibliography. IHC will be performed in collaboration with Sant'Andrea Hospital, using commercially available Abs in an automated autostainer (Bond III, Leica). All slides will be analyzed and scored blindly by the pathologist following the Histological Grading and Staging of Chronic Hepatitis for Fibrosis [Li et al 2012]. Specific PCR analyses of genomic DNA from the mouse livers will be performed to reveal the expected LOX recombination products. Isolation of primary HSCs will be performed as described in [Guimaraes et al 2010], expression level of α-SMA will be evaluate to confirm HSCs activation. RT-qPCR will be performed using validated and commercially available primers to detect both circHIPK2 and linear-HIPK2 using a Quantum 6-Applied biosystem. Basescope assay (ACDbio) will be performed on FFPE samples. 1 µm-thick slides from our cohort will be used to perform the in situ hybridization assay following manufacturing instruction. Probe targeting common housekeeping gene will be used as positive control. Probe targeting bacterial gene dapB will be used as negative control.

7. Work carried out and preliminary results

Related to Task 1 We have previously obtained from the EUCOMM program (IKMC project), C57BL/6 mice carrying an HIPK2 knockout-first (KOF) allele cassette with conditional potential [Skarnes et al, 2011]. Part of this cassette is flanked by FRT sites and removable by FLP recombinase activity (**Scheme 1**), thus, we have generated mice carrying HIPK2 conditional knockout (CKO) allele by crossing HIPK2 KOF mice with transgenic mice ubiquitously expressing the FLP recombinase. Since exons 3 and 4 of HIPK2 are flanked by LoxP sites, a further activity of CRE recombinase is required for excision of these critical HIPK2 exons and the generation of ubiquitous or tissue-specific HIPK2 knockout ($HIPK2^{LOX/LOX}$) mice. We have already obtained pancreas-specific $HIPK2^{LOX/LOX}$ mice (HIPK2-PKO) in an independent study (Davide Valente, unpublished results), by crossing the $HIPK2^{CKO/CKO}$ mice with the C57BL/6 Pdx1-Cre mice which carry the Cre recombinase under the control of the pancreas-specific promoter, Pdx1 [Hingorani et al, 2003].

Related to Task 2 In collaboration with Dr. Paola Begini at the Sant'Andrea Hospital, we have analyzed a biobanked collection of 157 liver biopsies which includes 73 hepatitis, 60 cirrhosis, and 24 HCC. All patients have been analyzed and scored blindly and independently using both the histologic activity index (HAI/Knodell model) and the METAVIR system. The presence of HBV and HCV have been determined by IHC and RT-PCR, respectively (**Table 1**). In addition, IHC was performed for cytokeratines (7, 8, 18, 19) and for Galectin-3, a key player of HCSs activation [Maeda et al, 2004; Guimaraes et al, 2010; Jiang et al, 2011] and a target of HIPK2 [Cecchinelli et al, 2006].

Scheme 1

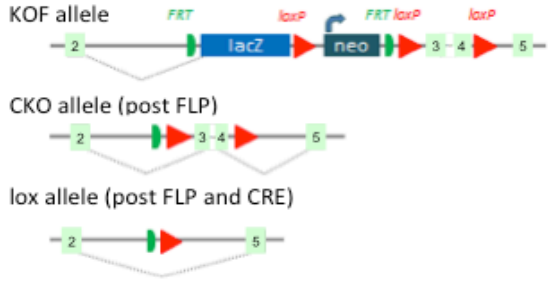

Table 1

	HBV	HCV	HIV Co-infection		Alcohol Abuse	Not known	Tot
			HBV	HCV			
Mild hepatitis	4	18	1				23
Moderate hepatitis	3	23	2	7			35
Severe hepatitis	1	7	1	5	1*		15
Cirrhosis	2	32	2**	3	5	16	60
HCC	1	8	1		4***	10	24

*HCV and Alcohol abuse

** One of them is also related to alcohol abuse

*** Two of them are HCV and alcohol abuse

8. Expected results and relevant corresponding milestones

Identify the causal role of HIPK2 in liver fibrosis. This will be obtained both in vitro, in mouse and human HSCs, and in vivo, in CCl_4- and MCD-induced liver fibrosis in HIPK2-LKO mice;
Set up of circHIPK2-specific Basescope assay to be performed on FFPE samples for a spatial and morphological resolution at single-cell level;
Identify circHIPK2 as a novel biomarker for liver fibrosis progression.
Overall, these results might open the possibility of testing circHIPK2 as a prognostic biomarker of CLDs by liquid biopsies

9. References and relevant publications by the research group, already available

References

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Ten selected relevant publications by the research group

1. Gatti V, Ferrara M, Virdia I, Matteoni S, Monteonofrio L, di Martino S, Diodoro MG, Di Rocco G, Rinaldo C, **Soddu S**. An Alternative Splice Variant of HIPK2 with Intron Retention Contributes to Cytokinesis. Cells. 2020 Feb 20;9(2). pii: E484.
2. Monteonofrio L, Valente D, Rinaldo C, **Soddu S**. Extrachromosomal Histone H2B Contributes to the Formation of the Abscission Site for Cell Division. Cells. 2019 Nov 5;8(11). pii: E1391.
3. Monteonofrio L, Valente D, Ferrara M, Camerini S, Miscione R, Crescenzi M, Rinaldo C, **Soddu S**. HIPK2 and extrachromosomal histone H2B are separately recruited by Aurora-B for cytokinesis. Oncogene 2018 Jun;37(26):3562-3574.
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PERSONNEL INVOLVED IN THE RESEARCH

Name and date of birth	Role on Project	Fellowship required	Effort on project (%)	Present position
Silvia Soddu 26/02/1961	PI	No	40	Head of unit
Laura Monteonofrio 01/09/1985	circHIPK2 study	No	50	Researcher
Davide Valente 08/05/1984	mouse model	No	60	Researcher
Giulia Calconi 18/04/1989	circHIPK2 study	Yes	100	Fellow
Alessia Garufi 24/10/1981	Biochemistry	No	50	Senior post-doc
Paola Begini 23/03/1981	Hepatologist	No	20	Hepatologist
Antonello Cardone 21/03/1991	Pathologist	No	30	Pathologist
Maria Pia Gentileschi 11/08/1964	Cell biology	No	30	Technician

DESCRIPTION OF THE WORK FOR EVERY UNIT OF PERSONNEL

1. Silvia Soddu: the PI will coordinate the experimental work performed by the different units of personnel
2. Laura Monteonofrio: she will analyze the circHIPK2 in the samples from tissue biobank and on mouse livers
3. Davide Valente: he will generate and characterize the liver-specific HIPK2 knockout mouse model
4. Giulia Calconi: she will support Laura Monteonofrio and Davide Valente in the characterization of HIPK2 role and circHIPK2 expression in the mouse model
5. Alessia Garufi: she will perform the biochemical experiments required in the two tasks
6. Paola Begini: she will support the study with all the required clinical information and expertise
7. Antonello Cardone: he will perform the histochemical analyses on the samples from tissue biobank and on mouse livers
8. Maria Pia Gentileschi: will support the work of the other personnel by cell culturing, laboratory organization and solution supply

Budget Form /year

1. research costs:		€ 16,000
2. Instruments		-
3. Indirect costs		-
4.	**Sub-total**	**€ 16,000**
5. Overheads:		€ 4,000
6. Fellowships:		€ 20,000
7.	**Total**	**€ 40,000**

Justifications

Itemized research costs: the proposed costs will be used to purchase *i*) the mouse model B6.Cg-Speer6-ps1Tg(Alb-cre)21Mgn from Jackson laboratory, *ii*) the human hepatic stellate cell line LX-2, *iii*) the Basescope™ probes, *iv*) reagents for circHIPK2 analysis, *v*) to fund one fellowship.

EXISTING/PENDING SUPPORT

2018-2021 Italian Ministry of Health Grant “Hipk2 as a prognostic biomarker in stage I and stage II colorectal cancer: validation and underlying mechanisms”. PI: Silvia Soddu

SUGGESTED REVIEWERS (MAX 3)

1. Salvatore Sciacchitano; salvatore.sciacchitano@fondazioneniccolocusano.it
2. M. Lienhard Schmitz, lienhard.schmitz@biochemie.med.uni-giessen.de
3. Alessandra Marchetti, alessandra.marchetti@uniroma1.it

BIOETHICAL REQUIREMENT

1. Human experimentation NOT – please provide clearance from the competent ethical committee as addendum A

2. Animal experimentation YES – please include a statement as addendum B specifying which regulations the proposed research meets

Declaration
I shall confirm to the Declaration of Helsinki in its latest version.
I shall also apply the Bioethics Convention of the Council of Europe.
In implementing the proposed research, I shall adhere most strictly to all existing ethical and safety provisions applicable.
Before start of the research, I shall obtain clearance from the competent ethical committee in case of involvement of human subjects in the research and /or in case of other ethical implications.
I shall conform with all regulations protecting the animals used for research purpose.

Date: 14/01/2021 Name of PI Silvia Soddu Signature ..... Silvia Soddu

Principal investigator's signature ..... Silvia Soddu .....

Authorized Administrative Official's signature.....

ISTITUTI FISIOTERAPICI OSPITALIERI
Direttore Generale
Dott. Francesco Ripa di Meana

Date 14/01/2021

Si autorizza al trattamento dei dati ai sensi dell'articolo 5 del Regolamento (UE) 2016/679

ADDENDUM B

The presented proposal includes the use of animal experimentation. In particular, HIPK2$^{CKO/CKO}$ mice, alb-Cre and HIPK2$^{LOX/LOX}$ mice will be used as described in Task 1. Ministry of Health approval for animals and procedures presented in this proposal will be requested. To note, experimentation on HIPK2$^{CKO/CKO}$ at IRE-Animal housing facility has already been approved by the Ministry of Health (Allegato 6, D. lgs 26/2014) for different research project. Given that, we plan to obtain the complete approval within a few months.

W HIPK2-LKO e are aware and informed about the guidelines established in article 31 of Italian law D. Lgs. 26/2014 regarding animal experimentation and related ethical issues. To this end, all the animal experimentations presented will be conducted pursuing the principle of 3Rs: Replacement, Reduction and Refinement. Replacement criterion is to ensure that whenever possible other methods should be used and the animal use is implemented only in the absence of other suitable ways of experimentation. Reduction criterion indicates that the number of animals used in this experiments must be kept as low as possible. Refinement criterion relates to devising methods, which are not painful or make the animals distressed.

HIPK2$^{CKO/CKO}$ mice, Alb-Cre mice, and HIPK2-LKO mice will be bred at IRE animal housing facility and will be monitored periodically by group members, animal technicians and department veterinarian.
The aim of this proposal requires the use of liver fibrosis murine model. Liver fibrosis will be obtained by intraperitoneal injection of CCl_4 or by MDC diet feeding. Treated animals will be monitored daily. Body condition scoring will be regularly carried out to evaluate the overall health. In case of illness, suffering animals will be euthanized as soon as possible using appropriate ethically approved methods, such as CO_2 and/or cervical dislocation.

To pursue 3Rs principles, we will obtain liver HSCs derived by HIPK2-LKO and HIPK2-WT mice in order to conduct in vitro experiments (Replacement). Moreover, we will performed statistical power calculation to establish the minimum number of mice required to obtain significant results (Reduce). Painful and stressful procedures will be carried in general anaesthesia condition (Refinement).

Principal investigator's signature

Date 14/01/2021