Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Developing a human organ-on-a-chip system to investigate and measure the effect of microgravity and radiation in space with a focus on NASH development

B.1.2 Abstract

Ionizing radiation and microgravity are common space stressors that astronauts are exposed to. The full extent of their effects on human physiology after long exposure are questions that need to be fully answered to develop countermeasures before humanity decides to venture beyond low-earth orbit (LEO). Microphysiological systems (MPSs) or organs-on-a-chip have recently gained importance since they allow to model human physiology in vitro in a more reliable manner as compared to 2D culture systems. In 2018, the American National Centre for Advancing Translational Science (NCATS) funded nine projects to develop and bring MPSs to the International Space Station National Laboratory (ISS-NL) and study the effect of space stressors on several tissues. On our search, we observed a gap in the available knowledge, since the liver was not one of the organs that were granted the opportunity. The liver is a complex organ, and space stressors have shown to disturb its normal functioning. One of these distortions is the possible promotion of non-alcoholic fatty liver disease (NAFLD), characterized by steatosis and, later, fibrosis development. In this text, we propose the development of a liver-on-a-chip system that can be induced to fibrosis and steatosis. After development of the model, we will partner with space organizations to bring this system to the ISS for a period of 21 days, where it will be cultured. At the end of the experiment, the system will be brought back to Earth and the effects of space stressors will be characterized by omics analysis, staining and ELISAs with a focus on fibrosis, steatosis, and inflammation.

B.1.3 Layman's summary

Since humanity made history in 1969 when Neil Amstrong and his crew first set foot on the Moon, most space missions have focused on bringing astronauts to low Earth's orbit (LEO). It is only now, in the middle of 2022, that we have sent the Artemis I mission to the moon, with the goal of bringing astronauts on board the Artemis III two years from now. In this context, companies like SpaceX have been making the front pages with their announcements of soon-to-be space missions for Mars exploration and colonization. Earth's atmosphere provides us with protection from high intensity radiation originated in the Sun, but astronauts lack this protection in LEO or beyond. Furthermore, all species on Earth's surface have evolved in a specific set of conditions. Earth has a gravitational effect that pulls us towards its center, with a force that we measure as 1G (one

earth gravity). If humanity aims to further expand in the universe, it is of utmost importance to study the effect that space stressors such as radiation and microgravity will have on human biology. Traditionally, research has been performed taking astronauts as subjects, but the sample size is low and procedures such as biopsies are invasive in nature. Alternatively, rodents have been used as models, but their biological differences to humans impedes the faithful extrapolation of results. Recently, a new technology called "organ-on-a-chip" has been developed. These chips are not electronic chips but are made of glass or other material not toxic to cells that has microscopic channels carved inside them. In these channels, researchers can introduce cells from a specific organ. This chip, with the cells from an organ –for example the liver—can be used to study the response of that organ to certain drugs or stimuli, since it is expected to react on a similar way as the real organ would. When the chip can reliably mimic the behaviour of an organ, we call it a valid "model" of that organ. We propose to send one of these models of a liver to the International Space Station (ISS), where it will be exposed to space stressors, and then bring it back to Earth, where it will be compared to a model that stayed on ground. In this comparison, we will measure the accumulation of fat, formation of scar tissue and inflammation, which are common symptoms of non-alcoholic fatty liver disease (NAFLD). On Earth, this disease is normally related to diet, diabetes, or genetic factors, but it has been suggested that it could happen in space just by exposure to radiation or microgravity. The results that we obtain from this research will open a door to send more models of liver or other organs to space and study the development of different diseases, and potentially find drugs that prevent or attenuate them. This way, we would ensure that the future humans that travel across the universe are protected and can safely reach their destination.

B.1.4 Keywords

Radiation, microgravity, liver, MPS, NASH

B.2 SCIENTIFIC PROPOSAL

B.2.1 Background

On Earth, life forms evolved under a specific set of conditions that includes temperature, gravity, or magnetic shielding from radiation. Leaving Earth is the next logical step for human expansion and is of crucial importance to become an interplanetary species, ensuring the survival of life in case of catastrophic events on the home planet. In missions to low Earth's orbit (LEO) in the International Space Station (ISS) and especially during future interplanetary travels, those conditions and protections are not met. Microgravity, radiation or hypergravity at launch are some of the stressors that may impact astronauts' health when in long term missions or venturing into deep space (White, 2001). Here, shear forces, buoyancy-driven convection, and hydrostatic pressure are all significantly decreased or eliminated. When cells are exposed to microgravity in space, the balance between cell architecture and external forces is disrupted, resulting in alterations at the cellular and subcellular levels (e.g., cytoskeleton, signal transduction, membrane permeability, differentiation, adhesion, migration, proliferation, apoptosis, etc.). Cumulative effects have shown to lead changes in gene expression- (Prasad et al., 2020). Some of these physiological changes resemble certain aging and disease pathologies but reverse sometime after returning to Earth (Low & Giulianotti, 2019). Ionizing radiation can have catastrophic effects on a cellular level, since it can damage cell constituents directly or indirectly by ionizing the water inside of the cell and creating free radicals that damage the DNA. Radiation is particularly harmful to dividing cells, and can cause cell death, mutations and/or cancer. Beyond Earth's magnetosphere, radiation is a mixture of two sources. First, galactic cosmic radiation (GCR) particles come from outside the solar system and possess high kinetic energy. Consequently, they are particularly harmful since they can go through shielding materials, and through nuclear interactions produce secondary particles that travel even further. Second, solar energy particles (SEP), emitted by the sun, are generally found within a lower energy spectrum, and are therefore more easily shielded. Missions within the LEO are only a concern for long-term cancer risk, but deep space missions will encounter a combination of both radiation sources and are potentially more dangerous. Plus, the ISS is orbiting at a high latitude within the LEO, so it may encounter some GCR (Townsend, 2021).

The effects of spaceflight have mostly been studied in muscles, bones, cardiovascular function, or brain, but it also alters energy and lipid metabolism in humans, a task that is carried out by the liver. The liver is a complex organ responsible for maintaining glucose and lipid homeostasis. It also plays a vital role in managing protein metabolism and detoxification by processing amino acids for energy or disposing of waste products in the form of urea (Trefts et al., 2017). Any factor that influences the integrity of the liver will have a significant role in its regenerative and metabolic capacities. Du et al. confirmed that prolonged exposure to microgravity induced significant damage to the liver and triggered hepatocyte apoptosis (F. Du et al., 2015). This negatively affects the normal function of the liver. Moskaleva et al. observed an increase in liver Cytochrome P450 (CYP450) content through mass spectrometry in mice liver after 30 days of space flight and microgravity (Moskaleva et al., 2015). CYP450 is involved in numerous metabolic processes and dysregulations of its isoforms could lead to loss of drug efficiency due to fast hepatic clearance of compounds, for example. On a similar note, other groups investigated the effect of spaceflight on antioxidant sulfur-containing compounds, such as glutathione (GSH). They observed the reduction of liver GSH content (Hollander et al., 1998) and the downregulation of genes related to GSH activity, while gene sets related to oxidative

stress and sulfur metabolism were upregulated. The effects were mitigated to some extent in rats subjected to Earth-like artificial gravity (1G) (Kurosawa et al., 2021).

A multi omics analysis in two mice breeds after stays at the ISS for 21, 27 and 42 days showed a dysregulation in lipid and fatty acid metabolism, which was confirmed by lipid accumulation in the form of Oil Red O positivity in histopathological analysis. Moreover, proteomics showed that apolipoproteins were inhibited. Insulin was shown to be upregulated, glucagon downregulated and carbohydrate metabolism was also increased (Beheshti et al., 2019). Upregulation of insulin may lead to insulin resistance, which, together with lipid accumulation (steatosis) is one of the factors that increase the risk of non-alcoholic fatty liver disease (NAFLD). Steatosis may lead to lipotoxicity, inflammation and finally fibrosis, key components of a secondary stage of NAFLD –non-alcoholic steatohepatitis (NASH). Ultimately, NASH may lead to cirrhosis and hepatocellular carcinoma. NASH-related fibrosis is the accumulation of extracellular matrix components in the liver and formation of scar tissue. It is mediated by the activation of hepatic stellate cells (HSCs). A different group analysed mice livers after space flight. They observed accumulation of lipids in livers and activation of PPARa-mediated pathways via HSC activation, which could possibly lead to early NAFLD (Jonscher et al., 2016).

Radiation seems to have an effect in the development of NAFLD. It causes DNA damage repair in the liver and activates antioxidant mechanisms, even in parenchymal cells (such as hepatocytes) that are non-dividing. Reactive oxygen species (ROS) are induced via hypoxia in the vascular endothelium and contribute to chemokine production that leads to inflammation. Low dose radiation close to that of the ISS (0.5-1 mSv/day) (mSv= milisievert; effective dose) may result in fat accumulation that can lead to in liver cancer (Nakajima et al., 2018).

Originality, innovation, and urgency

The space environment effects in human's tissues while in LEO have been studied and there is some understanding about them. However, little is known about the effects in health or disease development associated to long-term spaceflight. Being aware of the potentially hazardous factors and finding ways to mitigate or being protected from their effects is of critical importance to ensure safe space exploration. As of the time of writing of this proposal, the Artemis I mission is awaiting the green light for launch and perform a reconnaissance flight to the Moon with the goal of bringing a manned flight to the moon in the Artemis III mission in 2024. Moreover, the debate about performing long-distance space travels to Mars has recently gained importance. In the coming years, it will be important to assess if our knowledge about the effects of LEO in human biology are applicable to the lunar environment and beyond.

Traditionally, the effects of space in biology have been studied by taking samples from astronauts and rodents sent to the International Space Station (ISS). However, astronaut sample size is low, a number that is further limited by the number of non-invasive procedures that can be performed. Rodents, on the other hand, are small, inexpensive and have a short lifespan. For these reasons, they have widely been used as animal models to predict the biological response of humans to different stimuli, therapies, or drugs (i.e: pharmacological testing) (Bokhari & Donoviel, n.d.). However, the inherent differences between animals and humans result in poor translation of results. As an example, interspecies variations and their inherent differences to humans result in the failure of 90% of experiments in clinical trials (Ingber, 2020). On the other hand, 2D culture systems are not reliable to model certain organ functions since they lack the tridimensional architecture of organs and therefore the specific cell-cell interactions and gradients. Adapting these cultures to orbit is possible, but due to their inherent disadvantages it is also a waste of time and resources. There is a need for complex culture systems that can reliably mimic the intricate functionality and pathogenesis of tissues without relying on human or animal subjects or simplifying the study to the level of a monolayer cell culture. These systems will open the door for studying the effects of microgravity, radiation, and other spaceflight stressors with larger sample sizes in smaller spaces (Bokhari & Donoviel, n.d.).

Recently, the advances in Tissue Engineering have brought two powerful technologies that could potentially hold the key to reliably mimicking in vivo organ functionality: organoids and organs-on-a-chip. Organoids are 3D culture systems that result from the self-organization of induced pluripotent stem cells (iPSCs), adult progenitor cells, or embryonic stem cells following embryogenesis and organogenesis processes (Ingber, 2020). Organs-on-a-chip (OOCs), also known as tissue chips or microphysiological systems (MPSs). In MPSs, living cells are grown in micrometric chambers with continuous flow conditions that mimic the physiological processes of tissues and organs. These systems can produce a wide range of mechanical deformations that imitate those found in living beings, including physiological levels of fluid shear stress, cyclic strain, and compression. MPSs represent a practical approach and can be configured to focus on the study of one physiological process alone on a specific tissue type or function (Vunjak-Novakovic, Ronaldson-Bouchard, et al., 2021). MPSs allow on-chip assays such as microscopy, -omics, stainings and confocal imaging; and can be coupled with electrical, chemical, or mechanical or optical sensors to incorporate live readouts (Clarke et al., 2021; Zhang et al., 2017).

In 2018, the American National Center for Advancing Translational Sciences (NCATS) partnered with the American National Institute of Health (NIH), the National Institute for Biomedical Imaging (NBIB) and the ISS National Laboratory to study the effect of the unique conditions of the ISS microgravity in MPSs. This way, nine projects were funded under the solicitations RFA-TR-19-019 and RFA-TR-19-001 (RFA-TR-16-019, 2018). These processes and other diseases could be modelled on MPS

platforms to increase our understanding of the onset of diseases on Earth, but also the effect of space stressors on organs. The nine awarded projects aimed to study the effects of microgravity on different systems on the body. Three of them were related to study the dysregulation on the immune system, one was focused on musculoskeletal deconditioning, one for kidney dysfunction, two were destined to study cardiac tissues, one to model the blood brain barrier (BBB) and finally one focused on the epithelial mucosa of the gut (Low & Giulianotti, 2019). While all these studies are important and address different possible complications that might arise in human health on a long-distance spaceflight, the liver is an important organ that is missing from this list. The liver is a complex organ, the biggest organ responsible for metabolism. As stated previously, studies in mice suggest that microgravity and radiation affect the liver negatively, altering the production of enzymes, proteins and promoting symptoms of early NAFLD, such as lipid accumulation, that develops into steatosis and fibrosis. If untreated, this could eventually lead to hepatocellular carcinoma and subsequent death. It is of utmost importance to carefully study the effects of spaceflight in liver function and health, this would shed some light into possible extra protection steps or the use of preventive medicine in astronauts going through long-exposure flights. To our knowledge, this would be the first time that a liver MPS is transported to LEO to study the effects of space stressors on hepatic health.

B.2.2 Approach

This project is planned for a maximum of four years (fig. 1). It will be divided in three phases:

- *Ground operations (Phase I):* Select and optimize a valid liver MPS system. Validate healthy/diseased model.
- *Translation (Phase II):* Work with implementation partners to miniaturize culture system and adapt it too space. Perform training of astronauts and simulated experiments in microgravity.
- *Experiment phase (Phase III):* Culture in ground and space. Fixate cultures and sample media. Perform assays and analyse the data.

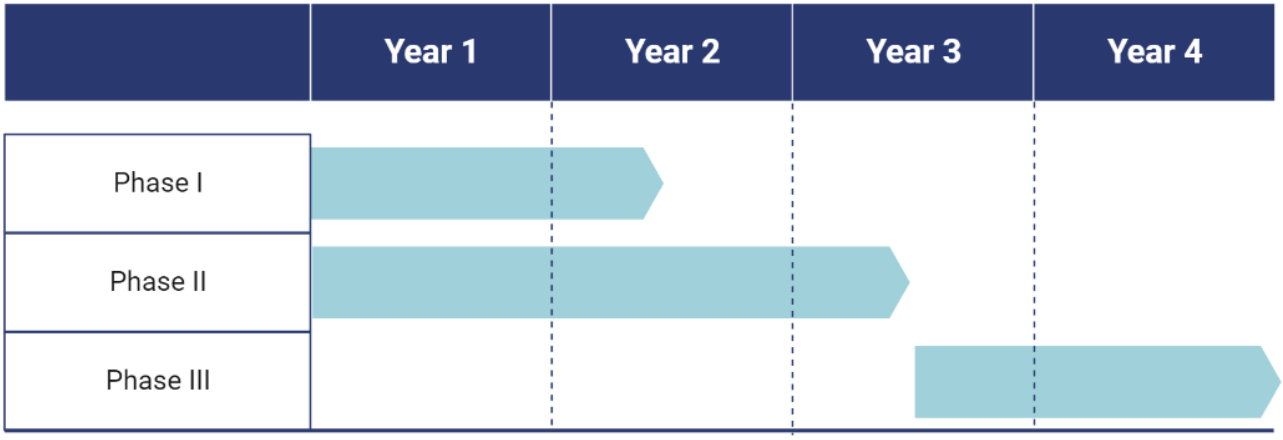

Figure 1. Projected timeline for the study. Phases I and II will take place at the same time since they involve research and negotiations. Phase II will continue after the end of phase I to translate the MPS to space and train astronauts. Phase III will consist of the on-orbit experiment and subsequent analysis.

Phase I. Ground operations

The first phase of the project will consist of selecting the right liver MPS for the project. One key design criterium for this chip is that it should allow for the co-culture of several cell types. MPSs are comprised of one or several microfluidic channels which are in close contact to each other, typically separated by a porous membrane that allows for crosstalk between cells.

The liver is a complex organ, and disease development in the liver involves the interaction of all cell types in the liver lobule. The model should at least include hepatocytes since they are the most important parenchymal cell in the liver, in charge of most of its functions; and HSCs, since, when activated, they are the cell group responsible for the ECM deposition that will result in fibrosis development (Trefts et al., 2017). Nevertheless, the addition of Kupffer cells as the immune component may also be of importance, since they are potentially involved in the amplification of the signalling cascade that results in HSC activation, as well as recruiting of more immune cells in vivo (Kazankov et al., 2019). Finally, adding liver sinusoidal endothelial cells as a side channel acting as liver sinusoids could provide physiological relevance. Liver endothelial cells play an important role in vascularization and crosstalk between cell types (W. Du & Wang, 2022).

Objective 1. Choosing the right platform

A simple microfluidic chip may consist on two channels separated by a porous membrane. The main cell type on the top layer would be the hepatocytes, which would be allowed to settle until they form a monolayer; for the bottom layer, the same procedure would be followed for the LSECs. HSCs could be seeded together with the hepatocytes on the top channel, since they are physiologically found in the Space of Disse, in between the LSECs and the hepatocytes. Kupffer cells are circulating cells in the sinusoids, so in this example they could be seeded on the bottom channel together with the LSECs. Nevertheless, designing a MPS from scratch is a long process. For the scope of this project, and the feasibility of adjusting to the time frame, it may be worth to look at already commercially available liver chip models (Hassan et al., 2020). Emulate (Emulate, n.d.) provides PDMS chips with the possibility of performing co-culture, tri-culture or tetra-culture. Their liver model can be used for toxicity testing, steatosis assays, hepatocellular injury, cholestasis or Kupffer cell depletion. Of importance is the fact that Emulate's chips have already been selected in the past by NCATS to bring blood brain barrier (BBB) model to the ISS (Hinojosa, 2017). Mimetas' OrganoPlate (MIMETAS, n.d.) is a system consisting of plates containing 40, 64 or 96 chips with different topographies of one, two, or three channels that are separated by a phase-guide, which allows direct contact between the ECM matrix in the middle channel and the media or cells on the side channels. Although they have no liver model available yet, the OrganoPlate provides a robust, high throughput platform that could be relied on to build the model. CN Bio's PhysioMimix (CN Bio, n.d.) aims to replicate the physiological environment of the liver in a trans-well, multi-well system, that allows hepatocytes, HSCs and Kupffer cells to be in close contact. InSphero provides spheroids consisting of primary hepatocytes, LSECs and Kupffer cells to recapitulate the microenvironment of the liver (InSphero, n.d.).

Objective 2. Cell sourcing

Another important aspect of this section is to select the adequate cell source. Primary cells are the gold standard for the generation of relevant in vitro cultures. They exhibit physiologically relevant functionality, which makes them the go-to source for pharmacological testing. However, primary cells are difficult to obtain and sometimes hard to culture. Furthermore, donor-to-donor variances pose a challenge in terms of standardization of assays and results. -to source if the goal is to obtain a behaviour and responsiveness as close as possible (Zeilinger et al., 2016). In the case of HSCs and LSECs, most of the commercially available cells are of primary origin. Cell lines, on the other hand, are derived from tumors or from genetically engineered primary cells. Among its advantages, they possess a high proliferation capacity, but they are compromised in terms of functionality. In the case of hepatocytes, two famous hepatoma cell lenes are HepG2 and HepaRG. HepG2 has been used in numerous studies, mostly related to cancer therapies (Samatiwat et al., 2016), but also in studies of hepatic metabolism and drug toxicity. HepaRG cells are a good candidate to substitute primary hepatocytes (PHH) for screening studies for CYP induction (Andersson et al., 2012) and show improved results in comparison to HepG2 in liver injury gene upregulation (Rodrigues et al., 2016). However, they have low drug sensitivity (Gerets et al., 2012). LX-2 cells are immortalized hepatic stellate cells with the SV40 large T antigen and are used for cytokine signalling, retinoid metabolism, and fibrogenesis (Sigma-Aldrich, n.d.) Similarly, LSECs have also been modified with the same technique to obtain cell lines (Innoprot, n.d.). A commonly used substitute for Kupffer cells is the THP-1 cell line. However, data suggests that while they could be valid as a simplified model, their inability to produce cytokines in physiological levels disqualifies them as a reliable candidate for more complex assays or drug screenings (Kazankov et al., 2019). Therefore, it would be of importance to find the right candidate for our model. Finally, induced pluripotent stem cells (iPSCs) have gained relevance in the past years as an ethical alternative to embryonic stem cells. iPSCs-derived hepatocytes can be used for disease modelling or drug testing studies, with results comparable to PHH (Dianat et al., 2013; Takayama et al., 2014).

Objective 3. Development of model and characterization

We will aim to keep cultures alive for at least one month. Media selection will be performed depending on the chip and cell types chosen. Taking the architecture of the chip as in fig. 2, Phase I, it is safe to assume that the hepatocyte lane will be perfused with a media for hepatocytes, while the endothelial cells will be provided with endothelial cell medium. An optimization of the media used will be performed based on manufacturer's instructions of the cells purchased. Similarly, cell seeding density will be optimized to balance physiological relevance with the ability to pick significant differences in healthy vs diseased states. For diseased states, we will expose the cultures with TGFb to simulate HSC activation and subsequent fibrosis into our system. TGFb is the master regulator of HSC activation and is a key player in fibrosis onset (Hellerbrand et al., 1999). For steatosis, we will incubate our system in a high fat medium containing oleic and palmitic acid (Moravcová et al., 2015). Media will be changed every three days and images will be taken under a brightfield or phase contrast microscope to follow the development of the system. On day 30, chips will be fixated in formaldehyde.

Before proceeding onto the next phase, we will perform a characterization of the cells present in our system (table 1). Mature hepatocytes will be stained with anti-HNF4a, anti E-Cadherin and anti-Albumin. HNF4a is a hepatocyte nuclear receptor (Watt et al., 2003), while E-cadherin will confirm the epithelial nature of the selected hepatocytes (van Roy & Berx, 2008). The ability to secrete albumin is a marker of functionality for mature hepatocytes. Kupffer cells are generally stained with anti-CD68, a monocyte transmembrane marker, indicator of Kupffer identity, but not exclusive to it (Ju & Tacke, 2016). For LSECs, we will stain with anti-CD31 and anti-VE-Cadherin, two common endothelial markers (Dietmar Vestweber, 2007; Lertkiatmongkol et al., 2016). Finally, HSCs will be stained with anti-aSMA. For diseased states, aSMA intensity will serve as a marker of HSC activation, while an ELISA procollagen 1 secretion will be employed to further characterize fibrosis state (Acharya et al., n.d.;

Akpolat et al., 2005). Finally, a bodipy staining will be used to assess the ability of the hepatocytes to incorporate lipids (steatosis). We will aim for significant differences in three different experiments (N=3) with eight replicates (n=8) per condition.

Table 1: List of assays

TARGET	*Name*	*Function*
Hepatocyte	Anti-HNF4a; anti-ECadherin; anti-albumin	Hepatocyte nuclear receptor; epithelial cell adhesion molecule;
HSC	Anti-aSMA	Actin isoform
Kupffer	Anti CD68	Monocyte transmembrane protein
LSEC	Anti-CD31; anti-VEcadherin	Endothelial cell adhesion molecules
Steatosis	Bodipy staining	Stains lipids
Fibrosis	Anti-aSMA (same as HSC); Procollagen I ELISA; TGFb ELISA;	Actin isoform; Structural protein; Cytokine, fibrosis regulator;
Inflammation	TNF4a ELISA CellROX (ROS)	Inflammation cytokine; Oxidative stress

Phase II. Negotiations and translation to space

Objective 1. Negotiations

The grants that were made available in 2018 by the NCATS and NASA partnership were the first of these characteristics to bring MPSs to space. Since then, nothing of such a specific nature has been made available to researchers to apply. A first step to translate this research to space would require the negotiation with the principal space agencies, depending on the institute or company where this project would be developed. Some of these agencies are North American Space Agency (NASA), the European Space Agency (ESA), the Russian Space Agency (RSA), the Canadian Space Agency (CSA) or the Japan Aerospace Exploration Agency (JAXA). On the negotiation table, one matter that would be inquired about is the source of funding. At the time of writing of this report, it is possible to apply to the Catalyst Grants Program. This program is being supported by the Translational Research Institute for Space health (TRISH), which is tasked to translate novel approaches to reduce the health risk in space exploration (Catalyst Program, 2021). The scope of this program is larger than the NCATS program and does not specify the technology that needs to be used to achieve its goals. Nevertheless, it provides an invaluable opportunity to obtain the necessary funding to cover the expenses of a study of these characteristics. The provide two tiers of funding –tier 1 projects or proof-of-concept projects, are eligible to obtain up to $150k for a project of a maximum duration of one year, and tier 2 projects (development projects) are eligible for an amount superior to $150k for up to 2 years. Although the duration of our proposal is estimated for four years, concession of a grant of these characteristics would require a re-evaluation of the timeframes dedicated to model development or analysis of results, for example. Upon obtention of funding, negotiations with NASA, for example, and private companies such as SpaceX will proceed.

Objective 2. Selection of Implementation Partners for translation to space

Yeung et al. were one of the groups that were granted the opportunity to bring one of their MPSs to space. In a preliminary report, they addressed one of the main challenges they faced –miniaturizing the support system. Regardless of the size of a chip, to keep the cultures alive and perform assays there are several support systems such as incubators, flow hoods or microscopes that need to be translated as well into a confined, microgravity environment (Yeung et al., 2020). In their commentary, they estimated that whole system including tubing to be around 1350L. To meet spaceflight limitations, that volume had to be reduced to 55L for launch in a powered locker in the SpaceX Dragon and subsequently to 45L for its installation in the ISSNL. Taking this into account, phase II of the experiment will begin by assessing the feasibility of engineering this volume reduction alone or establishing a collaboration with some well-renowned Implementation Partners of the Center for the Advancement of Science in Space (CASIS). They are skilled developers and subject matter experts who can offer a wide variety of products and services to assist in the implementation phase and have shown scientific competence. Implementation Partners offer one-of-a-kind suites of flight-certified gear. Among their services, they provide technical assistance in translating ground-based research objectives to the space environment and the subsequent

development and testing of the hardware and software (RFA-TR-16-019, 2018). They have experience in mission administration, launch, landing and real-time crew operations and planning. Among the possible implementation partners, BioServe provides a Syringe Pump for Organs-on-Chips (SPOC, also known simply as Syringe Pump) that allows for 18 samples per unit and can be customized with other chip types from the ones provided. It allows for media change and sampling in separate casettes, and fixation and preservation with simple crew controls, and temperature control (BioServe, n.d.). They were the team that collaborated with Yeung et al. On a similar note, ScorpioV's BioChip SpaceLab (BCSL) is an alternative that also allows for microfluidics experiments that require multiple reagents such as multiple media formulations, fluorescent probes and indicators for hypoxia, oxidative stress. Cell viability, inflammation…, or RNA/DNA fixatives and preservatives for post flight analysis. It also includes integrated bright-field and fluorescence microscopes (ScorpioV, n.d.)Finally, SpaceTango's CubeLab is a platform with a standard size of 1U (10cm^3), but that can be scaled to 2U, 4U, 6U and 9U to accommodate for different subsystems. Like their competitors, it incorporates manifolds to manage multiple fluids for media exchange, sampling, and fixation, which are designed based on partner-specified flow and pressure parameters. It includes temperature control and can be equipped with a variety of cameras for different imaging capabilities, and a built-in flight computer to provide automated control and near-real time data to investigators (SpaceTango, n.d.). Other implementation partners were reviewed, but although they provide a variety of services and products, they do not list microfluidic-specific systems or specific information about them (Micro Aerospace Solutions, n.d.; Nanoracks, n.d.; RedWireSpace, n.d.).

Objective 3. Training of astronauts

This objective will take place before the start of phase III. Astronauts are highly trained in performing tasks in space and have been performing research on the ISS for many years now. A short training will be given to teach the particularities or our MPS and the system of choice to host the chips while on orbit. We do not expect this step to take a long time, since the main goal is for the system to be as automatic and independent as possible. Astronauts will need to learn the procedure to change the different medium or assay casettes, the rationale behind the experiment and how to repair the modules in case of malfunction.

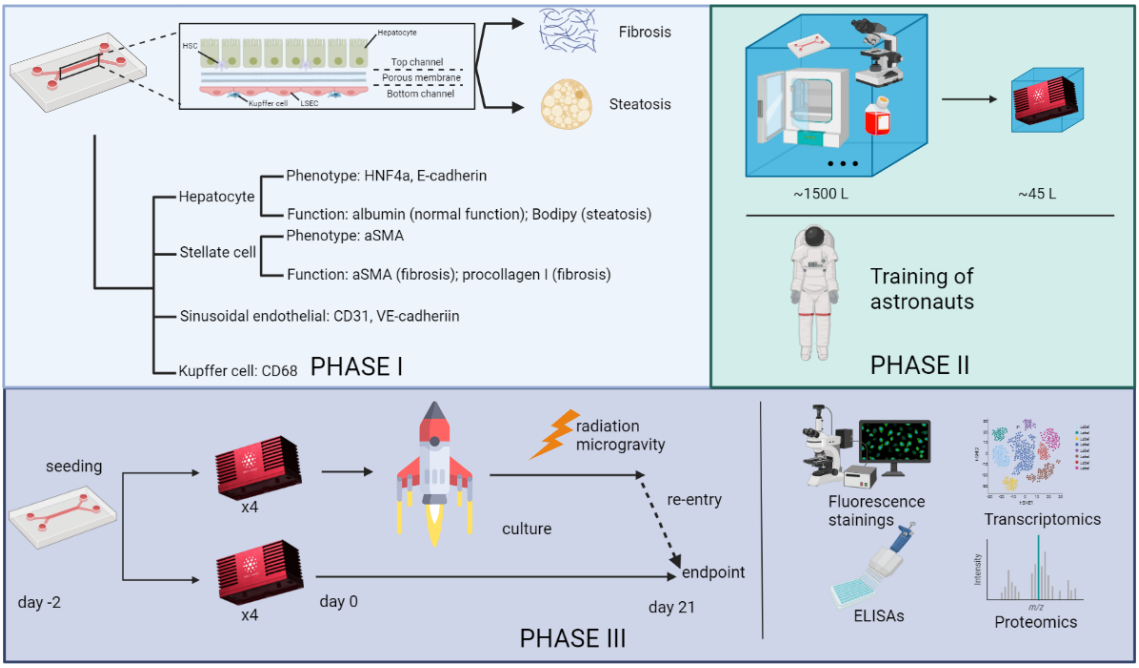

Figure 2. Detailed view of the study plan. Phase I includes the development of a liver model that can be induced to fibrosis and steatosis, and the subsequent characterization. Phase II would take place to reduce the volume to keep the system working to a manageable size for a space shuttle. Phase III will consist of the experiment in ground and space, with the only varying conditions of the exposure to space stressors of the flight (F) chips. A thorough analysis that includes stainings, ELISAs, transcriptomics and proteomics will be performed after day 21, when all the samples are back on Earth.

Phase III. Experiment phase

Objective 1. Flight

Two days before the launch, cells will be seeded in flight (F) and ground (G) modules to allow cells to settle and attach. Four modules will be destined to each. Each module will include at least eight chips. Once in orbit, F and G teams will be coordinated to perform medium changes at the same time every three days. Media from each chip will be collected and frozen in separate compartments. Samples will not be pooled. Pictures will be taken by the incorporated cameras every day. The goal will be to culture the chips for between 14 -30 days, depending on the mission conditions. For three chips per module, Bodipy live probe

will be included in the media, to be able to visualize and measure the ability of hepatocytes to incorporate lipids (steatosis). Images will be taken with fluorescent microscopy, included in each module. On the last day, a CellROX kit will be employed on the last day to measure the oxidative stress present in our system (inflammation assay). At the end of the experiment, F chips will be brought back to ground.

Objective 2. Assays and data analysis

Upon arrival to the lab in ground, F and G chips destined to stainings will be fixated, chips destined to proteomics will be lysed and chips destined to transcriptomics will have their cells extracted. This will be performed following the appropriate instructions. The reasoning behind not fixating in orbit is 1) to prevent negatively affecting the omics analyses and 2) be as faithful as possible to the full trip an astronaut will experience and include re-entry as an additional stressor. From the media samples, we will perform ELISAs to measure procollagen I and TGFb secretion (fibrosis assay), albumin secretion (hepatocyte function) and TNFa secretion (inflammation and fibrosis assay). Three chips of each module will be stained with anti-aSMA (fibrosis assay) to measure their production by stellate cells. Cells will be extracted from the chips and single RNA sequencing will be performed. Transcriptomics and proteomics data will be obtained, and statistically significant genes and proteins of ground vs flight conditions will be studied. For transcriptomics, we will perform single cell RNA requencing (scRNA-seq), which allows the comparison of the transcriptomes of individual cells (Haque et al., 2017). Cells extracted from the chips will be pooled together following the minimum cell number requirement. A similar approach will be followed for proteomics. Cells will be lysed, followed by a protein digestion into peptides. Peptides will then be labelled, and mass spectrometry will be performed to analyze the data (Brun & Couté, 2019).

B.2.3 Feasibility / Risk assessment

A mission with high stakes like this one is associated with an equally high amount of risks. At any point of the timeline, something could fail and risk the outcome of the whole project. A first go/no-go would need to be taken at the end of phase 1. Had we taken the road of developing the cell sourcing and/or the MPS by ourselves; if the liver model is not in place or it does not survive the established 30-day goal, we would have to halt the project. Luckily, the provided literature on already commercially available MPSs alone or liver model MPSs could help us bypass this inconvenience. If this is the road taken, this would also reduce the amount of time spent in model development for phase 1. Phase 2 of the project would involve working with one of NASA's implementation partners to develop the adapted ground-to-space hardware and software and the mission simulation and training of the astronauts in performing the tasks of medium change and tissue fixation. An obstacle in this phase would be that the architecture of the MPS itself cannot be adapted to space limitations for any reason. To address this potential issue before it happens, we will start the negotiations with the potential implementation partners in phase 1. Once we have selected them, we will work closely with them so that phase 2 can start while phase 1 is still in progress and run in parallel to it. This close collaboration will help overcome the potential hardware issues that may arise. When the hardware is in place, the training of the crew will begin. One of the possible risks of phase 3 is that the meteorological conditions or any other unexpected event delay the launch date. Indeed, Yeung et al. reported that their launch time was delayed three times. The SpaceX rocket launched at 2:48 AM EST on May 4th, 8 days later after the scheduled launch window. Launch delays are common, and planning for this possibility is crucial for the success of the mission (Yeung et al., 2020). For this reason, it will be important to have a robust system that can be kept in culture for at least one month. If the delay is for a longer time, we should also have enough material and hadware to perform a second seeding at a different time point. Each module should contain the maximum number of chips possible, and we should send at least four modules to space to have three experiment modules + a backup module in case one of them breaks and is not reparable. Nevertheless, every component of each module will be engineered in such a way that allows for their easy replacement if needed; i.e: casettes for media and fixative reagent, pump, tubing, camera… Finally, should a fatal setback that prevents the mission from starting, some facilities such as the neutron facilities at Colorado State University and Columbia University or the NSRL at Brookhaven National Laboratory specialize in GCR simulations (Vunjak-Novakovic, Brenner, et al., 2021). In this time of 30 days, our chips will be cultured in a simulated microgravity and radiation environment. We will follow the guidelines from NASA and ESA of the maximum radiation that organs can withhold in a period of 30 days (Townsend, 2021).

B.2.4 Scientific (a) and societal (b) impact

We are still taking the first steps in space research and exploration. The insights that we obtain from this or other studies of MPSs beyond Earth will provide invaluable data that will allow us to be more protected when we venture outside of the planet that saw us evolve. In long-term manned missions, astronauts will be exposed to the harsh space environment for periods of time that could dramatically impact their chance of surviving. In this study, we observed a gap in the previous space research, and focused our proposal on the liver. A thorough understanding of the mechanisms that cause or prevent liver injury related to radiation and microgravity will point us in the direction of better medical therapies. These results, along with the results of other organs may also be valuable for the humans that stay on ground, since they will open new pathways in the research related to aging, cancer or immune disorders (Prasad et al., 2020). Finally, the success or failure of this study will provide a

reference and framework for the further investigation of liver-on-chips in space, which will focus either on prevention or treatment of NAFLD or other diseases. Of particular importance is the capacity of the liver for drug clearance and metabolism, which can be disrupted during spaceflight.

B.2.5 Ethical considerations

Commercially available or patient-derived primary cells will be used for the development of the model. The usage of patient cells will require a signed consent by the subject(s).

B.2.6 Literature/references

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