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MSC beneficial effects and limitations, and MSC-derived extracellular vesicles as a new cell-free therapy for tissue regeneration in irradiated condition

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ABSTRACT

The unique immunomodulatory and anti-inflammatory properties of mesenchymal stem cells (MSCs) make them an invaluable cell type to contribute to the reparation of tissues and organs affected by high exposure to ionising radiation. Relevant experience has been obtained using MSCs in the medical treatment of overexposed persons from radiation accidents worldwide. Although MSCs have provided substantial clinical evidence of their therapeutic efficacy in local radiation injuries, in combination with other treatments, there are still challenges to overcome regarding authorizations, production, conditions of use and applicability as a medical countermeasure. The success of MSC-based therapy was established based on the premise that transplanted cells home in to and engraft within injured tissues and subsequently differentiate to replace injured cells. However, subsequent work has affirmed that the paracrine effect is mediated by secreted extracellular vesicles, particularly exosomes in several models of injury such as myocardial infarction, kidney injury and skeletal muscle repair. This paracrine hypothesis also suggests that certain safety concerns related to stem cell transplantation, could be readily overcome using stem cell-derived extracellular vesicles in the absence of the stem cells of origin. Advances have already been made towards the translation of MSC-derived extracellular vesicle in regenerative therapy. Moreover, according to preclinical studies, MSC-derived extracellular vesicles may have a promising role in the medical management of radiation injuries. Current and future perspectives on these cell-free therapies applied to treat overexposed persons to ionising radiation are part of this opinion paper.

1. Introduction

The wound healing process can be altered when tissues absorb moderate to high doses of ionising radiation resulting in fibrosis and, eventually, necrosis of the irradiated volume. Thus, the severity of the organ dysfunction is shaped by the affected volume and absorbed dose received by organs. Regenerative therapy offers a long-term approach for optimum radio-curability and patient quality of life. Stem cell therapy has piqued interest because it differs from traditional therapies in that it aims to address the root cause of the disease by focusing on tissue repair and regeneration (Desgres and Menasché, 2019).

Therapeutic use of stem cells was initiated 60 years ago with the success of the first transplant of hematopoietic stem cells from bone marrow and cord blood in the treatment of aplasia, leukaemia and hematologic genetic diseases. Development was pursued with mesenchymal stem cells (MSCs) produced in low quantities by the bone

marrow and other tissues, mainly the fat tissue. MSCs have shown effectiveness in clinical studies for treating various pathologies, including recurring ulcers in rheumatoid arthritis, Crohn's disease, graft vs. host disease (GvHD), and eczema. Furthermore, MSCs were shown to promote wound healing in preclinical models and clinical studies of skin burns, diabetic skin ulcer (Bailey et al., 2021) and radiation injuries (Rodgers and Jadhav, 2018). Regardless of its therapeutic potential, a number of limitations were raised for MSC-based therapy, that include the predominant use of autologous adult stem cells, the *in vitro* expansion in compliance with good manufacturing procedures (GMP) and the limited amount of cells generated, which preclude their use in mass accident situation, the risk of functional impairment of the stem cells, as well as ectopic tissue formation and costs issues. Alternatively, MSCs derived from human induced pluripotent stem cells (hiPSCs), would offer the possibility to circumvent some of these issues. In particular, hiPSCs can be virtually unlimitedly expanded in order to generate high

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yields of iPSC-MSCs and their therapeutic potential is not affected by donor's age, compared to adult stem cells. However, generalization of iPSC-MSC therapy requires large efforts in reducing the risk of tumorigenesis associated with the use of oncogenic transgene for the generation of iPSCs, together with technical issues regarding the efficiency of iPSC generation, the viral integration of transgenes. standardisation of mass production and controlled cost of manufacturing to keep it within reasonable limits (Andrews et al., 2017; Stacey and Healy, 2021).

Although the transplanted MSCs were originally thought to exert their beneficial effect through successful homing and engraftment into injured tissue and their subsequent differentiation to replace dead cells, it was soon shown that the paracrine activity of MSCs was the main contributor to their beneficial outcomes. In particular, conditioned media from hypoxic MSCs was shown to reduce infarct size and improve heart function (Gnecchi et al., 2005; Gnecchi et al., 2006). It was later demonstrated that this paracrine effect was mediated by secreted extracellular vesicles (EVs) (Bruno et al., 2009; Lai et al., 2010), in various preclinical models of myocardial infarction, kidney injury and skeletal muscle repair. In addition, it was shown that EV beneficial effect was dependent on the transfer of their molecular cargo, comprising growth factors, proteins, lipids, as well as nucleic acids, including mRNAs, and non-coding RNAs (Biancone et al., 2012; Ratajczak et al., 2012). Owing to this paracrine effect, some safety concerns associated with transplantation of stem cells, such as aberrant proliferation or differentiation, or vascular occlusion, could be easily circumvented by using stem cell-derived EVs without the stem cells of origin. Thus, MSC-derived EVs constitute a promising therapeutic solution for leveraging the benefits of MSC-based therapies while still providing an "off-the-shelf" treatment option Bailey et al. (2021).

2. Strength and achievements of MSC therapy

A better understanding of the pathophysiological mechanism of radiation injuries, through preclinical studies and advances in the clinical management of irradiated patients, has provided a number of therapeutic options such as surgical techniques (e.g. skin graft, flaps and others from the plastic surgery), or pharmacological products, including corticosteroids, antibiotherapy and anti-inflammatory drugs. More sophisticated measures such as hyperbaric oxygen therapy and MSC therapy based on the severity of the lesion, the volume of irradiated tissue and availability of resources, have shown interesting perspectives for the medical support these last decades (International Atomic Energy Agency 2019; International Atomic Energy Agency 2020).

MSCs have been successfully extracted from various tissues including the bone marrow, fat, and synovium, and are known to differentiate into multiple cell lineages according to specific application needs. Furthermore, owing to their immunological properties, MSCs are relatively preserved from immune surveillance and do not lead to graft rejection after transplantation (Han et al., 2019). Immunomodulatory and paracrine pathways have been identified as primarily action mechanisms of MSCs, migrating to inflamed sites to secrete bioactive molecules. Although the exact mechanisms of this homing property is not entirely understood, MSC tropism into injury sites makes them ideal vehicles for targeted therapy (Lin et al., 2019).

However, from our experience, their clinical application has been strictly performed in autologous administration to avoid any risk of graft rejection as a safety procedure (Ji et al., 2016). Additionally, the pain relief after systemic or local MSC administration is remarkable (Ji et al., 2016; International Atomic Energy Agency 2018; International Atomic Energy Agency 2019).

Searching for terms "Mesenchymal stromal/stem cells" revealed more than 1100 clinical trials registered on the NIH clinical trial database (<https://clinicaltrials.gov/> as of Nov 4, 2022), with half of the trials for musculoskeletal diseases, central nervous system injury, respiratory tract diseases and wound healing. Most of these trials (75%) are in phase 1/2 to evaluate safety and efficacy. In France, Advanced Therapy

Medicinal Products (ATMPs) market authorization was obtained for stem cell use by the Percy hospital (Clamart, France) with the IRSN's support for the treatment of radiological burns. For 15 years, 12 patients already received this treatment and were included in a strict follow-up (Reyes et al., 2016; International Atomic Energy Agency 2018; International Atomic Energy Agency 2020). Furthermore, a phase 2 clinical trial was initiated by the Saint Antoine hospital with IRSN's support for the treatment of Pelvic Radiation Disease (NCT02814864).

3. Weakness and long-standing problems in MSC therapy

In spite of the strengths and perspective in the use of MSCs, the production of GMP-grade human MSCs requires an accredited stem cell laboratory and national regulations. The possibility to have a large amount of cell production and cell banking remains still challenging and at very high costs. Another aspect to be considered is the bone marrow collection, which requires an invasive medical procedure for harvesting (iliac crest/sternum aspiration), that may be accompanied by pain and not exempt from complications and other risks (infection), together with elevated hospitalization costs (Mastrolia et al., 2019).

In addition, the time duration of the culture and its quality controls ranges from 7 to 14 days, which requires that the treatment be planned in advance and the MSC administration will be delayed for several weeks. Indeed, MSC precursors account for less than 0.01% of the overall bone marrow cell population, therefore requiring to harvest a large amount of raw material, along with extensive ex vivo culture in order to obtain sufficient cell numbers for clinical applications in humans (Mastrolia et al., 2019). If MSCs are frozen, an additional culture step is required after cell thawing for obtaining an optimal stem cell product (Parekkadan and Milwid, 2010). In the case of autologous use of MSCs, a weakness lies in the variation of the efficiency of the stem cell product depending on the donor. MSCs obtained from bone marrow have a longer doubling time compared with such cells from other tissue sources (Peng et al., 2008).

Furthermore, MSC aging or other comorbidity factors such as diabetes or hypertension have an important impact on the effectiveness of bone marrow cells, influencing their number, maximal lifespan and differentiation potential compared to other types of stem cells (Mastrolia et al., 2019). Therefore, if MSCs demonstrate interesting properties for tissue repair, tissue reconstruction is not possible. In order to counteract the issues of stem cell survival after injection, an important research these last decades has been developed on the bio-engineering strategy, which aims to combine bio-compatible material with MSC, especially in bone and cartilage reconstruction applications (Blery et al., 2014).

4. Recent innovation by using MSC-derived extracellular vesicles and upcoming challenges

EVs are nano-sized vesicles secreted by most cell types, including MSCs, and are traditionally classified considering their origins and sizes (Casado-Díaz et al., 2020). They are enclosed by a lipid bilayer containing various proteins and receptors, which envelopes a diverse array of proteins, nucleic acids, chemicals, and structural molecules derived from the cell of origin, the nature of which depends on the cellular source, state, and environmental conditions (They et al., 2009; Raposo and Stoorvogel, 2013; Yanez-Mo et al., 2015). Two main EV categories can be distinguished, with small EVs (~30-150 nm), also called exosomes, released from endosomal-derived multivesicular bodies upon fusion with plasma membrane, which contain endosome-associated proteins such as CD63, CD9, ALIX (Harding et al., 2013; Raposo and Stoorvogel, 2013), and large EVs (~150-1000 nm), previously known as microparticles or microvesicles, that are formed by blebbing of the plasma membrane and expose phosphatidylserine at their surface (Bailey et al., 2021). The physiological roles of EVs depend on their cargos and their capacity to transfer proteins, nucleic acids, and other

molecules into the receptor cells. EVs contribute to the regulation of numerous biological processes, including stem cell maintenance, angiogenesis, tissue repair and immune modulation (Casado-Díaz et al., 2020). More precisely, the biological effects of large EVs / microvesicles have been associated with irradiation- and other stress-induced vascular dysfunction, with a role in the regulation of coagulation, inflammation, and angiogenesis processes (Flamant and Tamarat, 2016). In consequence, the clinical use of large EVs has been essentially investigated as potential biomarkers in various pathological conditions, including cardiovascular diseases (Chironi et al., 2009; Flamant and Tamarat, 2016). Furthermore, we recently showed that circulating large EVs, isolated from a cohort of patients overexposed to medical irradiation during their radiotherapy, could be used as potential biomarkers associated with the severity grade of late occurring radiation proctitis complications (Ribault et al., 2023).

On the other hand, small EVs / exosomes have been extensively studied as both promising biomarkers and therapeutics. Indeed, small EVs released in most biological fluids including blood, urine, saliva or breast milk, have been suggested as relevant biomarkers for the diagnosis, prognosis, and response to treatment in various cancers and cardiovascular diseases (see for review (Makler and Asghar, 2020; Burtenshaw et al., 2022)). Besides, the therapeutical use of small EVs derived from MSCs (thereafter named MSC-EVs) has first emerged to address the insufficient responses to myocardial damage typically achievable by endogenous mechanisms. Initial studies established that human embryonic-derived MSC-EVs could reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury (Lai et al., 2010). In addition, the therapeutic efficacy of MSC-EVs over their cells of origin was highlighted in a comparison study using amniotic fluid MSCs and their derived EVs for the treatment of osteoarthritis. MSC-EV-treated defects showed superior pain tolerance level and improved histological scores than the MSC-treated defects (Zavatti et al., 2020).

Evidence suggests that MSC-EVs display angiogenic, immunomodulatory, and therapeutic properties similar to MSCs, thereby accounting for much of the paracrine effects of MSCs (El Andaloussi et al., 2013). Specifically, a recent meta-analysis of studies investigating the effect of MSC-EVs in preclinical models of diabetic wounds demonstrated that angiogenesis and inflammation are among the physiological processes most commonly targeted by MSC-EVs (Bailey et al., 2021). Human umbilical cord derived MSC-EVs have been reported to intensively promote the healing of second-degree burn wounds in vivo, mediated mainly by the activation of Wnt/ β -catenin signalling pathway and subsequent increased dermal fibroblasts proliferation, angiogenesis, and reduced skin cell apoptosis (Zhang et al., 2015). Wound healing and suppressed scar formation have been facilitated by inhibiting myofibroblast differentiation at the site of skin defects by treating with MSC-EVs from human umbilical cord (Fang et al., 2016). Human MSC-EVs were showed to promote myogenesis and angiogenesis in vitro and to enhanced muscle regeneration (Nakamura et al., 2015). Moreover, it was noted that small EVs derived from amniotic fluid MSCs contain a spectrum of proteins and miRNAs capable of regulating inflammation and angiogenesis which, in turn, underpin skeletal muscle regeneration (Mellows et al., 2017). In addition, human adipose derived MSC-EVs have been shown to prevent muscle damage in a mouse model of critical hindlimb ischemia, mainly through neuregulin 1 protein (NRG1)-mediated signals playing a crucial role in angiogenesis, prevention of inflammation, and muscle protection (Figliolini et al., 2020). The therapeutic use of EVs secreted by various cell types and in particular stem/progenitor cells presents a significant advantage compared to corresponding parental cells, such as less complex production and storage conditions (Ciferri et al., 2021) (Table 1).

On a cellular level, EVs interact with the plasma membrane through a variety of endogenous ligand/receptor interactions (van Dongen et al., 2016), which may be advantageous for cellular uptake and delivery of therapeutics with intracellular targets. On an intracellular level, EVs seem to be mainly internalized via endocytosis and to use endogenous

Table 1

Advantages and disadvantages/challenges of MSC-EVs over MSCs for clinical applications. Adapted from (Gowen et al., 2020; Lee et al., 2020; Parfejevs et al., 2020).

	Advantages	Disadvantages/Challenges
MSCs	Production of GLP grade human cells	Autologous use
	High proliferation ability	Limited number of cells
	Multilineage differentiation	Invasive harvesting procedure
	Minimal risk of immune issues	Duration of culture time
MSC-EVs	Accumulated experimental and clinical results	Efficiency decrease with age and comorbidity
		Banking
		Risk of transmitting infection or genetic diseases
		Risk of ectopic tumor formation
	Allogeneic use	No large scale, standardized production and isolation methods
	Stable upon freezing and thawing	No rapid and accurate quantification and characterization procedures
	Good safety profile	Lack of standardized modification methods
	Low immunogenicity and non-cytotoxic	Limited research on MSC-EVs content characterization
	Good drug delivery vehicle	Rapid clearance from blood after administration in vivo
	No ectopic tumor formation	Safety profiles to determine the optimal clinical dosage and possible toxicities upon repeated administration.
No entrapment in lung microvasculature	Pharmacokinetics, targeting and transfer mechanisms of EV to the target sites	
Cross biological barriers		
No particular ethical issues		

mechanisms for the delivery of cargo, including functional RNAs, to the cytosol. Therefore, EVs most likely constitute potent mediators for drug delivery through efficient crossing of lipid bilayers, releasing their content and inducing recipient cell response. Nonetheless, EV-mediated delivery remains to be improved by exploring the mechanisms governing EV fusion and cargo release, as well as clearance kinetics of specific EV types.

In addition, the use of EVs as drug delivery systems requires to determine a strategy for efficient cargo loading, whether it is performed after EV isolation or during EV biogenesis (Vader et al., 2016). Exogenous loading of EVs has been achieved using different methods, including simple incubation (Zhuang et al., 2011; Haney et al., 2015), electroporation (Alvarez-Erviti et al., 2011), extrusion and freeze-thawing (Haney et al., 2015), or sonication (Kim et al., 2016), although it could result in the aggregation of EVs or their cargo, and modify their physicochemical properties (Kooijmans et al., 2013). Alternatively, cells can be loaded with specific cargo via direct RNA transfection (Mathiyalagan and Sahoo, 2017) or co-incubation with cytostatic drugs (Pascucci et al., 2014), after which EVs follow their endogenous biogenesis before being secreted. Another method consists in engineering cells to stably express the RNA or protein drug of interest, in combination with approaches to increase active EV loading through fusion or interaction of the cargo molecule with naturally EV-enriched components.

Alternatively, MSC-EVs could be modified by priming/preconditioning their cells of origin, by using chemicals, cytokines or growth factors, in order to potentiate their functional activity. Human MSC-EVs modified with dimethylxaloylglycine further stimulated angiogenesis through the Akt/mTOR pathway to enhance bone healing (Liang et al., 2019). The efficacy of adipose derived MSC-EVs towards bone regeneration was also enhanced by preconditioning the MSCs with tumor necrosis factor-alpha (TNF- α), as was evidenced by increased proliferation and osteogenic differentiation of osteoblastic cells in vitro (Lu et al., 2017).

IRSN group proposed for the first time MSC-EVs as an optimal MCM for radiological and nuclear emergencies with particular advantage in

case of mass casualties (Flamant and Tamarat, 2016), so that supplies could be added to a national stockpile (Forsberg et al., 2020). Our group brought the first proof of concept of MSC-EVs therapeutic potential in models of radiation-induced ulceration of the gastro-intestinal tract (Accarie et al., 2020) and localized radiation injury (Loizard et al., 2023). EVs could be developed as radiation MCMs due to their fast and targeted action, although multiple doses of EVs might be necessary to enhance their efficacy. On the other hand, since acute radiation syndrome involves multi-organ failure, and single cell-source derived EVs with specific signalling might not yield a successful recovery, a combination of EVs derived from multiple cell sources (e.g. stem, immune, differentiated, or reprogrammed cells) might be necessary for a broader range of efficacy. In particular, it has been already published the potential of applying EVs derived from mesenchymal stromal/stem cells, endothelial cells, and macrophages for promoting repair and regeneration of organs after radiation injury (Nanduri et al., 2021).

5. Clinical applications of MSC-EVs

The first demonstration of the clinical benefit of MSC-EV therapy involved one patient with severe therapy-refractory GvHD. MSC-EVs obtained from four different bone marrow donors led to the rapid and stable improvement in clinical GvHD symptoms for up to 4 months after treatment (Kordelas et al., 2014). A limited number of clinical applications have since been reported, with the main goal of assessing safety and absence of adverse effects following MSC-EV administration. Although the number of individuals treated have generally been low in these studies no adverse effects have been attributed to the EV treatments used. A randomized, placebo-controlled, phase 2/3 clinical pilot study investigated the safety and therapeutic efficacy of intravenous administration of human cord blood-derived EVs in inhibiting the progression of grade III and IV chronic kidney disease. Outcomes showed that MSC-EV administration was safe, modulated the inflammatory response, and contributed to improve overall kidney function in patients (Nassar et al., 2016). In addition, an ongoing phase 1 clinical trial (NCT03437759) aims to assess the safety and efficacy of intravitreal injection of human cord blood-derived MSC-EVs for the treatment of large and longstanding idiopathic macular holes (Zhang et al., 2018). Closure of macular holes was reported in 4/5 patients treated with MSC-EV injection associated with improved visual acuity. Moreover, interrogation of clinicaltrials.gov with the terms “MSC-EV” or “MSC-exosomes” and therapy (search performed Nov 4, 2022) resulted in approx. 30 clinical trials with either completed, ongoing or not-yet-recruiting status. A large proportion of these trials aims at safety and tolerability studies of MSC-EV in patients with severe SARS-CoV-2 associated pneumonia or acute respiratory distress syndrome (ARDS), either through aerosol inhalation (NCT04276987; NCT04491240) or intravenous injection (NCT05125562). Notably, a number of studies sponsored by Direct Biologics, LLC investigate the efficacy of an MSC-EV product (ExoFlo) in mild/moderate, severe, or critically ill COVID-19 ARDS patients including a currently recruiting phase III study (NCT05354141). Two upcoming phase I safety trials are also planned to investigate the ExoFlo product for the treatment of ulcerative colitis and Crohn’s disease (NCT05176366, NCT05130983). Other MSC-EV trials involve safety studies for topical application in psoriasis (Paracrine Therapeutics Dermatology Pte, Ltd, Singapore, NCT05523011), burn wounds and dystrophic epidermolysis bullosa (Aegle Therapeutics, US, NCT05078385; NCT04173650).

In summary, the future of EVs is promising, upcoming challenges to be considered in the future are: (i) re-enforcing the preclinical demonstration of the safety, and therapeutic efficacy of MSC-EVs as an innovative MCM, preclinical investigation in a large animal model will contribute to the demonstration, (ii) standardizing MSC-EVs culture process for clinical transfer in a large scale with this new treatment option.

6. Necessary conditions before clinical transfer for mass casualties

According to the preclinical and clinical studies, it clearly indicates that research on EVs suffers from limitations. A number of technical points remain to be urgently solved:

The heterogeneity of EV subpopulations makes it difficult to purify a single EV population (Kowal et al., 2016). Reproducibility in the generation of thoroughly characterized EVs of interest is essential. In addition, clinical translation requires the development of GMP grade methodologies for EV preparation. More importantly, the development of EV-based radiation MCM calls for standardization of the quality of cell source, cell culture, and method of EV preparation for reproducible use across respective fields, associated with appropriate *in vitro* and *in vivo* functional validation assays (Thery et al., 2018). Guidelines regarding minimal information for studies of extracellular vesicles have been proposed by the International Society for Extracellular Vesicles (ISEV), which define protocols and steps to follow in order to document specific EV-associated functional activities (Thery et al., 2018).

A limited number of clinical studies have documented the safety of EV treatments, and generally concluded on the absence of adverse effects, despite the low number of individuals being treated (Dai et al., 2008; Qi et al., 2016; Haga et al., 2017; Zhu et al., 2017). Complementary studies are necessary to ensure the safety of each specific application for EVs. For example, current therapeutic EVs are produced from immortalized cell lines, with a non-negligible risk of carrying oncogenic material, that may lead to cell transformation following repeated systemic EV treatments.

Clinical transfer of EVs requires the upscaling of EV production to industrial levels, for which new approaches regarding cell culture condition, EV isolation and purification, need to be identified and validated in order to provide large amounts of EVs necessary for clinical development programs and market supply (Elsharkasy et al., 2020). Further attention should also be paid to the storage of EVs, which requires a suitable container system, with specific buffer and temperature conditions in order to maintain EV stability. In that matter, storage of lyophilized or freeze-dried EVs at -80°C constitutes a suitable option for increased stability and shelf life of EVs (Witwer et al., 2013; Bosch et al., 2016; Charoenviriyakul et al., 2018). Similarly, the surface quality, plastics and coatings of the storage container, should be tested before use, since it was shown that certain surfaces can modify EV characteristics (Lener et al., 2015). However, storage conditions may need to be optimized for EVs of different cell sources or EV subpopulations, associated with functional validation for translational research.

All steps from sample collection, EV purification and storage conditions are interconnected and can impact the outcome of any EV research and clinical transfer. Although some issues regarding isolation, storage and GMP production of EVs have been solved over the last few years, many challenges remain to fully embark EVs on clinical translation, including large-scale production and improved storage conditions of therapeutically relevant EVs.

7. Conclusion

It is undeniable that the significance of MSC-EV efficiency provides a promising option in case of a radiological or nuclear event to address the critical needs related to the medical management of mass casualties. EV cell-free therapy presents numerous advantages over cell-based therapy, including convenient storage or transportation, increased shelf-life, avoiding immune rejection, absence of ethical concerns. Interestingly, exploring the specific effects of microenvironmental conditions on EV function will greatly improve their therapeutic potential in regenerative medicine. There is no doubt that EV therapy will have a broad clinical application prospect in the medical management of mass casualties, even though large scale GMP-grade EV-based pharmaceuticals and

subsequent clinical trials demand the resolution of several technological and mechanistic issues.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Author contributions statement

R. Tamarat, S. Flamant, C Loinard: manuscript writing and final approval of the manuscript.

R. Tamarat and S. Flamant: Writing - review and editing

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