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DOCTORAL THESIS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY
XXXIV CYCLE

Coordinator: Prof. Lorenza Trabalzini

PHARMACOLOGICAL MODULATION OF ENDOTHELIAL FUNCTION
DURING TISSUE REMODELING IN PHYSIO-PATHOLOGICAL
CONDITIONS

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Supervisor:

Prof.ssa Lucia Morbidelli

PhD candidate:

Dott. Shirley Genah

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Abstract

Vascular endothelium plays a pivotal role in the maintenance of many biological functions, including angiogenesis, defined as the formation of new capillaries from pre-existing vasculature. The process is aimed at supplying nutrients and oxygenation to growing or healing tissues following injury and, in the context of wound healing, endothelial cells lining the inner surface of blood vessels, are continuously engaged in a crosstalk with other cell types, immunity cells or fibroblasts, to assure a correct progression in the process of regeneration. Disruption of endothelial functions, which can occur for several reasons, including systemic administration of certain dugs or hyperglycemia, leads to a variety of pathological cardiovascular consequences which are commonly featured by a dysregulation in gaseous transmitters production such as nitric oxide (NO) and hydrogen sulfide (H_2S). Experimental data reported in literature proved exogenous H_2S-donors ability to recover several pathological conditions associated with EC dysfunction, such as hypertension or diabetes. However, endothelial dysfunction and associated inflammation continue to be one of the main causes of morbidity and mortality all over the world. Therefore, new therapeutical approaches are needed to prevent endothelial dysfunction or revert cardiovascular disorders, and sulfur compounds with natural origin represent helpful pharmaceutical/nutraceutical tools to be used in therapy or as a template for the ideation of advanced H_2S-donor molecules with improved pharmacodynamic and/or pharmacokinetic properties. The arguments of this dissertation concern the stromal components of tissues in particular vascular endothelium and fibroblasts and their reciprocal interaction, in order to define new therapeutic strategies for diseases accompanied by exacerbated inflammation and endothelial dysfunction.

The first aim of this thesis was to investigate the pro-angiogenic effect of erucin, a natural isothiocyanate with "smart" H_2S-releasing properties, particularly abundant in the edible cruciferous plant *Eruca sativa*. In this study we characterized the pro-angiogenic effect of erucin on endothelial cells (HUVEC) by using different functional *in vitro* assays aimed at evaluating cell migration and ability to organize in a capillary network on basement membrane matrix (Matrigel). A special focus was paid to the molecular mechanisms involved in endothelial cell response to the compound by investigating the early activation of enzymes involved in angiogenesis, such as eNOS, ERK1/2, Akt. Secondly, we demonstrated erucin ability, alone or in combination with VEGF, to protect endothelial cells from high glucose-induced damage and recover impaired functional responses to physiological levels. In the second part of this topic we started to analyze the activity of the isothiocyanate in the context of wound healing by assessing *in vitro* its pro-migratory, pro-survival effect on dermal fibroblasts (NHDF) and keratinocytes (HaCaT). Lastly, a preliminary study using indirect co-cultures on NHDF and HUVEC, was carried out in order to investigate erucin ability to promote and sustain endothelial-stromal crosstalk, a fundamental step in wound healing.

The second part of my thesis addressed the role of activated fibroblasts in the context of impaired wound healing characterized by eccessive inflammation, which underlies several pathologic conditions ranging from healing delay (e.g., chronic ulcers) to fibrosis. The aim of the second topic was to characterize the cellular and molecular events associated with the anti-inflammatory activity of photobiomodulation therapy (PBMT) on human dermal fibroblasts exposed to a mix of inflammatory cytokines followed by laser treatment. Results demonstrated laser ability to revert fibroblast inflammatory phenotype by reducing to basal levels pro-angiogenic factors, as VEGF, and inducible inflammatory key enzymatic pathways, as iNOS and COX-2/mPGES-1/PGE2, by retaining NF-kB transcription factor in a cytoplasmic localization. These molecular changes are accompanied by a shift in cell morphology attributed to a re-distribution of fundamental cytoskeletal proteins (Tubulin, F-actin, and α-SMA) to basal localization following laser treatments.

In the third and final topic of this dissertation we discussed the importance of assuring endothelial safety during drug development. The cardiovascular system has proven to be particularly sensitive to a large variety of drugs, especially chemotherapeutic agents, which can promote or accelerate the onset of relevant cardiovascular diseases by impairing vascular integrity and tone. Recently, carbonic anhydrase IX (CA-IX), emerged as a promising new anticancer target for the treatment of solid hypoxic tumors and many efforts have been made to develop selective inhibitors for biomedical applications. In the last project presented, the safety profile of two CA-IX inhibitors, SLC-0111 and AA-06-05 on human endothelial cells was assessed.

Preface: Report on the activities carried out during Ph.D.

During my Ph.D. fellowship (XXXIV cycle) at the School of Biochemistry and Molecular Biology, I performed my experimental project in the laboratory of Angiogenesis, Pharmacogenetics and Pharmacogenomics, in the Department of Life Sciences at the University of Siena under the supervision of Prof. Lucia Morbidelli. Over these three years I was engaged in several research projects that can be grouped into three main macro-areas, each one of them representing a section of this thesis.

In the first topic I carried out the characterization of erucin activity, a natural isothiocyanate with H_2S-releasing properties, on angiogenesis *in vitro*, with a special focus on the molecular mechanisms involved in endothelial cell response to the compound. An investigation of erucin ability to recover endothelial dysfunction induced by hyperglycemic conditions followed. Lastly, in a context of wound healing, I analyzed the effect of the isothiocyanate on human dermal fibroblasts and keratinocytes, and demonstrated erucin ability to promote and sustain endothelial-stromal crosstalk.

In parallel, in collaboration with the University of Florence, we investigated the anti-inflammatory effect of near infrared (NIR) laser radiation (o exposure) as a therapeutical approach to extinguish and control inflammation in impaired and chronic tissue healing, in an *in vitro* model of dermal fibroblasts activated by a mix of cytokines (IL-1β and TNF-α) (Topic 2). In relation to wound healing, we also analyzed the influence on endothelial cells by fibroblasts exposed to unloading conditions using a Rotating Wall Vessel device to simulate microgravity. The project is in collaboration with the University of Florence and funded by the European Space Agency (ESA) in the frame of the MAP Project "WHISPER— Wound Healing in Space: problems and Perspectives for tissue Regeneration and engineering". However experiments are still running and results are not reported in this thesis.

In the final topic of this elaborate we outlined the importance of assuring endothelial safety during drug development, especially chemotherapeutics. In this last study we assessed and compared the safety profile of two carbonic anhydrase IX (CA-IX) inhibitors, SLC-0111 and AA-06-05 (developed by prof. C.T. Supuran lab) on cultured human endothelial cells. (Topic 3)

Referring to safety studies on endothelium, during this Ph.D. program I also collaborated with the Colombian University of Bogotà "La Sabana", to investigate *in vitro* the biocompatibility on human cells of gelatin-based nanofibers (NFs) and nanoemulsions (NEs) loaded with high oleic palm oil (HOPO), considered for food industry. Specifically, taking into account the intended employment for edible applications and therefore an oral route of administration, toxicological assessment was carried out on fibroblasts of the gastrointestinal tract (CCD-18Co) and endothelial cells (HUVEC), as the main barrier for nanostructures systemic distribution in the organism. Results obtained in this work confirm

the non-toxicity of NEs and NFs *in vitro* and these data, although not included in the thesis, could contribute to the development of normative rules for nanostructures on foodstuffs. Finally, in these three years, I have contributed as co-author to the writing of three reviews which I have partially used for the introductory chapters of the following dissertation. The three contributions are available for reading in the supplementary materials of this thesis.

List of publications

Genah S., Angeli A., Supuran C.T., Morbidelli L. Effect of Carbonic Anhydrase IX inhibitors on human endothelial cell survival. Pharmacol Res. 2020 Sep;159:104964. doi: 10.1016/j.phrs.2020.104964. Epub 2020 May 30. PMID: 32485281.

Genah S.[#], Cialdai F.[#], Ciccone V., Sereni E., Morbidelli L., Monici M. Effect of NIR Laser Therapy by MLS-MiS Source on Fibroblast Activation by Inflammatory Cytokines in Relation to Wound Healing. Biomedicines. 2021 Mar 16;9(3):307. doi: 10.3390/biomedicines9030307. PMID: 33809724; PMCID: PMC8002295. ([#] = equal contribution)

Ciccone V., **Genah S.**, Morbidelli L. Endothelium as a Source and Target of H2S to Improve Its Trophism and Function. Antioxidants (Basel). 2021 Mar 19;10(3):486. doi: 10.3390/antiox10030486. PMID: 33808872; PMCID: PMC8003673.

Morbidelli L., **Genah S.**, Cialdai F. Effect of Microgravity on Endothelial Cell Function, Angiogenesis, and Vessel Remodeling During Wound Healing. Front Bioeng Biotechnol. 2021 Sep 22;9:720091. doi: 10.3389/fbioe.2021.720091. PMID: 34631676; PMCID: PMC8493071.

Genah S., Monici M., Morbidelli L. The Effect of Space Travel on Bone Metabolism: Considerations on Today's Major Challenges and Advances in Pharmacology. Int J Mol Sci. 2021 Apr 27;22(9):4585. doi: 10.3390/ijms22094585. PMID: 33925533; PMCID: PMC8123809.

Participation to congresses

<u>National conferences</u>

39° National SIF Congress. Florence, 20th - 23th November 2019.

    Poster: "Pharmacological studies for treatment of space-related disorders". **Genah S.**, Ciccone V., Morbidelli L., Cialdai F., Monici M.

19° National SITOX Congress. Bologna, 11th - 12th February 2020.

    Poster: "Efficacy and safety studies of two Carbonic Anhydrase IX inhibitors on tumor and endothelial cells". **Genah S.**, Filippelli A., Ciccone V., Angeli A., Supuran C.T., Morbidelli L.

40° National SIF Virtual Congress. 9th - 13th March 2021.

- Poster 1: "Characterization of Carbonic Anhydrase IX inhibitors on human endothelial cells". **Genah S.**, Angeli A., Supuran C.T., Morbidelli L.

- Poster 2: "In vitro biocompatibility studies of nanostructures (nanoemulsions and nanofibers) for edible application". **Genah S.**, Ricaurte L., Quintanilla-Carvajal M.X., Donnini S.

1st Joint meeting on Natural Products Pharmacology - SIF - SIPHAR - IMGNPP. Naples, 24th - 26th February 2022.

Poster "Characterization of Erucin pro-angiogenic effect on endothelial cells. **Genah S.**, Martelli A., Pagnott E., Pecchioni N., Calderone V, Morbidelli L.

International conferences

26th European Low Gravity Research Association Biennial Symposium and General Assembly. Granada, 24th – 27th September 2019.

Poster: "Pharmacological approach to space related disorders: set-up of co-culture system for the study of wound healing in microgravity". L. Morbidelli, **S. Genah**, V. Ciccone, F. Cialdai, M. Monici

3rd ESA Topical Team meeting "Tissue healing in Space: Techniques for promoting and monitoring tissue repair and regeneration". ESTEC, Netherlands, 11th June 2019

Poster: "How gravity affects endothelial-stromal cell interaction for angiogenesis outcome in wound healing". L. Morbidelli,V. Ciccone, **S. Genah**,, F. Cialdai, M. Monici

8° European Virtual Congress of Pharmacology (EPHAR). 6th - 8th December 2021.

Poster: "Erucin, a natural isothiocyanate with H_2S-releasing property, exhibits pro-angiogenic effect on endothelial cells". **Genah S.**, Martelli A.,, Pagnotta E., Pecchioni N., Calderone V., Morbidelli L.

1. Introduction

1.1. Endothelial cells and angiogenesis

The vascular endothelium consists of a single layer of squamous endothelial cells (ECs) lining the inner surface of blood vessels. The adult human body contains 10-60 trillion of ECs that cover a vast surface area (Rafii et al., 2016). ECs, arranged in a single layer oriented on the longitudinal axis of the vessel, assume a flattened shape and lay side by side with each other to form a complete monolayer. Structurally, the apical domain is in direct contact with blood or lymph, while the basolateral domain anchors to the basal lamina, which connects ECs to the underlying tissues (Rafii et al., 2016; Ciccone et al., 2021). The main role for vascular endothelium is to regulate the exchange of fluids and solutes between the lumen and surrounding tissues. Moreover, ECs affect smooth muscle cells and blood components as platelets, exerting paracrine and endocrine functions on them (Michielis, 2003; Triggle et al., 2012). As regulators of blood homeostasis, ECs play a fundamental role in the maintenance of blood fluidity, while they initiate blood clotting and/or consequent fibrinolysis when necessary (Michielis, 2003; Triggle et al., 2012). Endothelial cells are the principal actors in *de novo* vessel formation during embryonic neovascularization (vasculogenesis), differentiation and maturation (arteriogenesis), and remodeling during the development of new capillaries from the preexisting vasculature (angiogenesis) (Carmeliet et al., 2000; Potente et al., 2011).

Activation of physiological angiogenesis is a well-orchestrated multistep process that involves a complex cascade of molecular and cellular events. The first step of the angiogenic process starts with the binding of growth factors to their receptors present on the surface of specific endothelial cells, called "tip cells", belonging to the pre-existing blood vessels. ECs, in response to angiogenic stimuli, convert from a quiescent to an active phenotype characterized by a high mitotic index and increased capacity for migration; this binding activates a cascade of intracellular signaling pathways and the tip cell starts to secrete proteolytic enzymes (metalloproteinases, MMPs) that degrade the basement membrane, allowing them migrate and guide the developing vessel generated by proliferation and elongation of "stalk cells". ECs orient their migration thanks to the presence of adhesion molecules such as integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$) and by migrating they lead to the formation of "sprouts" into the stroma, small tubular channels which then interconnect among them (Reinke and Sorg, 2012). Subsequently, the new vessels mature through further stabilization of their vessel wall, by recruiting smooth muscle cells and pericytes until the initial blood flow completes the angiogenesis process (Carmeliet, 2000 and 2003; Potente et al., 2011; Francavilla et al., 2009) (Figure 1.1).
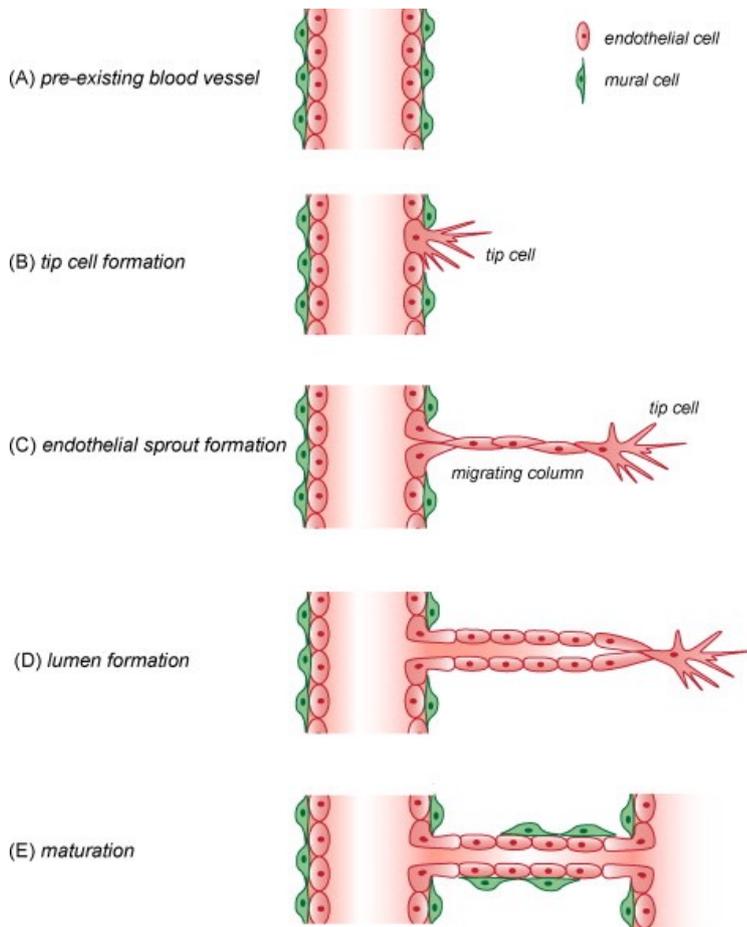
(A) pre-existing blood vessel

endothelial cell
mural cell

(B) tip cell formation

tip cell

(C) endothelial sprout formation

tip cell

migrating column

(D) lumen formation

(E) maturation

Figure 1.1 Schematic representation of the angiogenic process. Angiogenesis starts from pre-existing vessels (A), with the conversion of a previously quiescent EC into a tip cell (B). The latter forms filopodia that probe the surrounding environment which is then invaded by the endothelial sprout, where the tip cell is followed by the migrating column, formed by proliferating ECs (C). During endothelial sprouting, vacuoles forming within stalk cells merge and give rise to the vascular lumen (D). Upon formation of the new vessel, this undergoes stabilization and maturation, a process mainly mediated by intercellular adhesion and pericytes (or mural cells) coverage (E) (Francavilla et al., 2009).

In vivo, angiogenesis appears to occur by two mechanisms, namely non-sprouting (intussusceptive) and sprouting angiogenesis (Ribatti and Crivellato, 2012). During intussusception, endothelial protrusions of opposing capillary walls extend towards each other and fuse creating an interendothelial contact (Burri and Djonov 2002); sprouting angiogenesis is however the major form of neovascular growth.

The development of blood vessels, which is a requirement for growth and regeneration, depends on a highly structured communication of ECs with their surrounding tissue. In vivo vascularization is based on complex cell-matrix and cell-cell interactions, where the ECM seems to play a pivotal role (Neve et al., 2014; Mongiat et al., 2016; Tracy et al., 2016).

The principal stimulus for angiogenesis occurrence is usually a lack of nutrients and oxygen, which characterize the hypoxic condition arising for example during tissue growth or following tissue injury and impaired blood flow (Ahluwalia and Tarnawski, 2012). Hypoxia is indeed able to promote hypoxia inducible factor1-α (HIF1-α) up-regulation at nuclear level, which is responsible for angiogenic factor overexpression and vasoactive molecule up-regulation. Under the action of the angiogenic factors, acting in a consecutive, concerted, or synergistic manner, ECs undergo receptor activation and modification of intracellular pathways and cytoskeleton structure, leading to several angiogenic outcomes (Ahluwalia and Tarnawski, 2012; Lamalice et al., 2007; Muñoz-Chápuli et al., 2004).

11

A large plethora of agiogenesis inducers have been characterized, including VEGF (vascular endothelial growth factor), FGF-1 and 2 (fibroblast growth factor 1 and 2), PDGF (platelet-derived growth factor), TGF-α and β (transforming growth factors alpha and beta), angiopoietins (Ang), and other molecular regulators as nitric oxide (NO), prostanoids, angiogenin, chemokines and cytokines (Ucuzian et al., 2010; Ferrara et al., 2003; Reinke and Sorg, 2012; Folkman and Klagsburn 1987). Angiogenic growth factors and cytokines are produced by different types of cells including endothelial cells, fibroblasts, smooth muscle cells, platelets, inflammatory cells, and cancer cells (Ucuzian et al., 2010; Li et al., 2003).

1.1.1. Signaling pathways involved in angiogenesis

ECs express a large number of receptors that make them responsive to several growth factors and cytokines involved in the promotion of angiogenesis, but the most important and specific for their action is VEGFR (Hofer and Schweighofer, 2007). In particular, the binding of VEGF-A, secreted by cells in the hypoxic environment, to VEGFR-2 is the major way by which angiogenesis is promoted (Olsson et al., 2006). Specifically, like other tyrosine kinase receptors, VEGFR-2 undergoes ligand induced dimerization and oligomerization, which activates its tyrosine kinase activity, resulting in auto- and trans-phosphorylation on specific tyrosine residues in the cytoplasmic domain, ultimately leading to the transduction of different signals involved in proliferation and survival (Figure 1.2), degradation of extracellular matrix, migration and differentiation of ECs (Muñoz-Chápuli et al., 2004). In this thesis the transduction of signals involved mainly in proliferation, survival and migration will be described more in depth.

Transduction of proliferation signals: the endothelium is mostly quiescent, only a 0.01% of all the ECs of a healthy adult are dividing at any given moment (Muñoz-Chápuli et al., 2004). However, in response to angiogenic stimulation, endothelial cells enter into an actively proliferative state. The main VEGFR-2 (but also FGFR-2) induced proliferative pathway is mediated by the triggering of a phosphorylation cascade that successively activates MEK1/2 and the ERK kinases, and PI-3K/Akt/eNOS pathway (Figure 1.2) (Simons et al., 2016; Lamalice et al., 2007).

Transduction of survival signals: during migration, activated ECs degrading the extracellular matrix should reinforce the mechanisms of apoptosis inhibition to avoid the risk of anoikis (apoptosis induced by lack of adhesion to the substrate)(Frisch and Ruoslahti, 1997), for that reason signals inducing endothelial cell migration must also promote cell survival (Stupack et al., 2001). ECs repress apoptosis program through PI-3K/Akt/eNOS pathway triggered by both VEGFR-2 activation and ligated integrins. Specifically, Akt plays a crucial role for apoptosis prevention by stimulating the expression of survivin and Bcl-2, activation of NF-kB and by inhibiting apoptogenic proteins (eg. Bad, Bax,

Caspase-9, GSK3) engaged by unligated integrins (Figure 1.2) (Abeyrathna and Su, 2015; Muñoz-Chápuli et al., 2004).
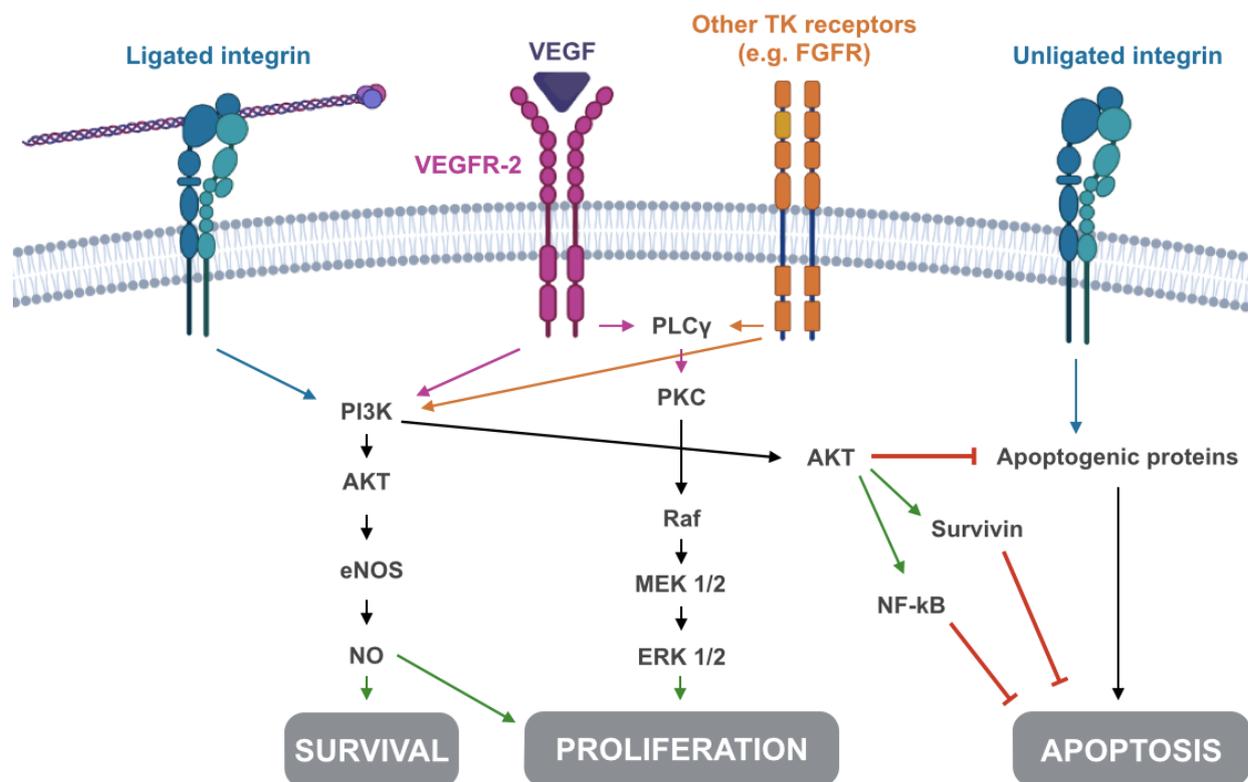
Figure 1.2. Molecular pathways mediating EC survival and proliferation. Modified from Muñoz-Chápuli et al., 2004. Bio-render platform was used for graphic re-design.

Transduction of migration signals: For the realization of the angiogenic process, migration of ECs is essential and it can be achieved through three main mechanisms such as chemotaxis, haptotaxis and mechanotaxis (Michelis, 2014; Morbidelli et al., 2021). In haptotaxis, migration occurs thanks to a gradient generated by immobilized proteins in the extracellular matrix (ECM); in mechanotaxis cell migration is induced by a mechanical stimulus (e.g. blood flow) which gives direction to the movement by influencing the polarization of the cytoskeleton; finally, in chemotaxis, migration takes place in the presence of a gradient generated by a chemo-attracting substance, such as VEGF and/or FGF-2 (Michelis, 2014; Lamalice et al., 2007). The signaling pathways leading to EC migration are today the best characterized (Figure 1.3) and converge on three principal outcomes: actin polymerization and stress fiber formation, mainly regulated by p38 mitogen activated protein kinase (p38 MAPK); focal adhesion turnover, orchestrated by focal adhesion kinase (FAK); and ECs permeability modification mediated by the release of NO derived from PI-3K/Akt/eNOS pathway.

FAK activation, via Hsp90 (heat shock protein 90)/RhoA/ROCK (RhoA-associated kinase), is important for the association of signaling molecules and actin-anchoring proteins, such as talin, vinculin, paxillin, which link actin microfilament network to adhesive integrins and enable focal adhesion turnover. The activation of small GTPases (Cdc42, Rac1, RhoA) is essential for the formation of specific cellular structures, involved in EC motility: Cdc42 activation results in filopodia formation, structures acting as cell "sensors" of extracellular environment; Rac1 involvement leads to lamellipodia formation, protrusions at the leading edge of migrating cells; finally, RhoA is implicated in focal adesion assembly and stress fiber formation, necessary for cell anchoring to ECM and cell forward movement (Yang et al., 2017; Lamalice et al., 2007).
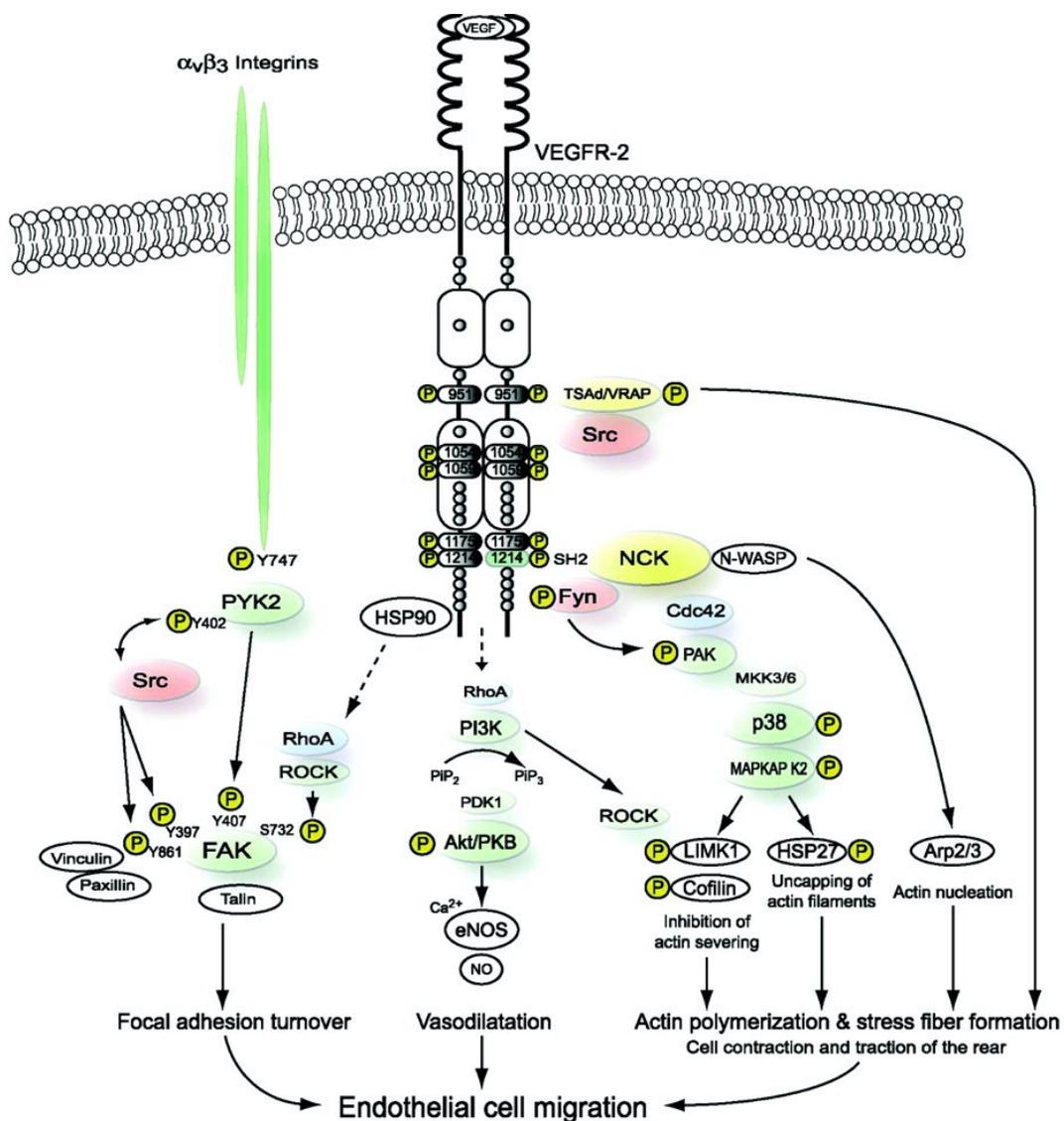

Figure 1.3. Molecular pathways mediating EC migration. VEGF binding to VEGFR-2 and engagement of integrins on endothelial cell membrane activate intracellular signaling that converge on cytoskeleton modifications, mediated by different kinases and adaptors proteins, responsible for endothelial cell migration during angiogenesis (Lamalice et al., 2007).

Intermediate messengers are up-regulated during angiogenic cell activation as gaseous transmitters nitric oxide (NO) and hydrogen sulfide (H$_2$S). PI-3K/Akt-activated eNOS produces NO, which is a major regulator of EC migration and angiogenesis, by inducing expansion of EC surface, after vasodilatation, associated with a more proper response of endothelium to angiogenic and pro-migratory stimuli (Dimmeler et al., 2000; Morbidelli, 2016a). In addition, a particular attention has been put on H$_2$S, whose role in the process of angiogenesis is discussed further on in Topic 1, paragraph 2.1.2 and 3.

Among the various angiogenic mediators, NO is particularly relevant. Nitric oxide is an endogenous gaseous transmitter involved in fundamental physiological processes, aside of angiogenesis and vasodilation, such as inflammation, neurotransmission and platelet aggregation inhibition (Förstermann and Sessa, 2012; Panthi and Gautam, 2017). NO is generated from L-arginine and oxygen in a reaction mediated by nitric oxide synthase (NOS). In mammal cells, three types of NOS can be characterized: two isoforms, neuronal NOS (nNOS or NOS 1) and endothelial NOS (eNOS or NOS 3), are constitutively expressed and regulate neuronal and vascular functions, respectively; the isoform NOS-2, also known as inducible NOS (i-NOS), is instead transcriptionally up-regulated following inflammatory stimuli (e.g. cytokines) (Förstermann and Sessa, 2012). During inflammation NO is generated at high concentrations and is mainly employed as cytotoxic agent through the production of nitrogen free radicals, such as peroxynitrite (ONOO$^-$), highly reactive and responsible for damage to infective microorganisms, tissues or other cells. NO is also a modulator of apoptosis in immune cells (Sharma et al., 2007). Besides ECs, many other cell type are able to up-regulate NOS-2, included fibroblasts, epithelial cells, keratinocytes, macrophages, neutrophils and glial cells (Förstermann and Sessa, 2012).
Abnormal NO signalling is therefore associated to a variety of disorders, ranging from neurodegenerative pathologies (e.g. multiple sclerosis, Alzheimer's, and Parkinson's diseases) (Zhou et al., 2009; Steinert et al., 2010), to alteration of immune response (such as septic shock, non-specific allograft rejection, tissue damage, inflammatory neurodegeneration) (MacMicking et al., 1995; Lange, 2009; Brown and Bal-Price, 2009) and endothelial dysfunction leading to relevant cardiovascular diseases.

1.1.2. Endothelial dysfunction and hyperglycemia

Since endothelium plays a fundamental role in the maintenance of functional vascular and lymphatic system, alteration of ECs function is associated with the pathogenesis of a broad variety of human diseases, causing damage to other tissues as well. Endothelial dysfunction is defined as a systemic condition in which the endothelium loses its physiological properties, including the control of vasodilation, angiogenesis/neovascularization, fibrinolysis and platelet aggregation (Daiber et al., 2017; Ciccone et al., 2021). Alterations in endothelial function are characterized by a reduction in local endogenous synthesis of vasodilating mediators such as H$_2$S and NO (Rajendran et al., 2013), oxidative stress,

elevated levels of inflammatory and coagulation markers, and increased EC apoptosis (Daiber et al., 2009). It is widely assumed that endothelial dysfunction is crucial for the development of a plethora of diseases, especially cardiovascular diseases (CVD) such as atherothrombosis, diabetes, sepsis, pulmonary hypertension, microangiopathies associated with neurodegenerative diseases, liver steatosis and cancer metastasis (Morbidelli et al., 2016). Endothelial dysfunction risk factors are represented by pathological states such as hypertension, and hyperlipidemia, and improper lifestyles such as high-fat diets, tobacco and alcohol consumption, and physical inactivity (Daiber et al., 2017; Donato et al., 2018: Ciccone et al., 2021). Among the major risk factors of endothelial damage we also find pathological states characterized by altered blood glucose concentrations such as hyperglycemia (excessive levels of blood glucose) typical of diabetes (Donato et al., 2018; Daiber et al., 2017).

Diabetes is hallmarked by hyperglycemia and contributes to EC dysfunction which arises early and plays a key role in the pathogenesis of diabetes-associated micro- and macrovasculopathies. Indeed, in diabetic patients CVDs are more prevalent than in the non-diabetic population, furthermore the classic risk factors for CVD are present in a much more severe form (Funk et al., 2012; Bertoluci and Rocha, 2017). Diabetes diagnostic criteria are based on plasma glucose level and glycemic control represents the foundation of diabetes therapy, although it provides little protection against CVDs in patients with long-standing hyperglycemia (Funk et al., 2012). According to the WHO (World Health Organization), the physiological fasting values of blood glucose are between 70 mg/dL (3.9 mmol/L) and 100 mg/dL (5.6 mmol/L); we therefore refer to a condition of hyperglycemia when the blood glucose concentration overcomes these physiological values, and to a condition of diabetes when the glucose exceed 126 mg/dl (> 7 mM/L).

EC dysfunction induced by chronic exposures of the endothelium to hyperglycemia is mainly mediated by the formation of cellular reactive oxygen species (ROS). Glucose auto-oxidation and mitochondrial superoxide production are likely to be the initial contributors to ROS-mediated dysfunction elicited by hyperglycemia (Giacco and Brownlee, 2010; Wolff et al., 1991; Funk et al., 2012). Normally, ROS produced by the oxidation of glucose during glycolysis and mitochondrial respiration, are kept under control by the cell antioxidant defenses, such as the activation of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase and thioredoxin (Gelain, 2009). However, in hyperglycemic conditions, these control systems tend to become saturated and ROS accumulate in the cell leading also to a disruption of the mitochondrial electron transport chain, and therefore to further promote oxidative stress (Funk et al., 2012; Giacco and Brownlee, 2010). ROS are responsible for several downstream consequences: they lead to an activation of NF-κB which drives the expression of pro-inflammatory and pro-thrombotic genes (Gloire et al., 2006); reduce endothelial barrier function by contributing to lipoprotein deposition and subsequent oxidative modification of LDL particles in the vessel wall (Patel et al., 2000;

Galle et al., 2006); sustain EC dysfunction by directly scavenging NO or by eNOS uncoupling (Landmesser et al., 2003). Thus, ROS reduce endothelial cell NO production while activating both direct (NF-κB activation) and indirect mediators (LDL oxidation) responsible for EC activation (Funk et al., 2012). Oxidative stress induced by hyperglycemia persists in the cells long after glucose levels are normalized and this phenomenon is known as "glucose memory" (Schisano et al., 2011).

Besides NO, insufficient H_2S production has been associated with EC dysfunction in both diabetic patients and animal models (Sun et al., 2020; Ciccone et al., 2021). Considering the crucial role of this two gaseous transmitters in the maintenance of vascular homeostasis, ROS scavenging, reduction of inflammation and in the process of angiogenesis, a deregulation in the enzymes producing NO and H_2S can lead to a serious alteration of ECs functional responses (for a more detailed discussion about H_2S role in cardiovascular system and angiogenesis, see Topic 1, paragraphs 2.1.2 and 3). Multiple *in vitro* and *in vivo* studies have found that preventing ROS production by enhancing the cell antioxidant systems, targeting mitochondria and reducing chronic inflammation limits the capacity of hyperglycemia to promote endothelial dysfunction and activation; a list of pharmacological and non-pharmacological countermeasures is reviewed in reference (Teodoro et al., 2019). However, the majority of these pharmacological interventions have failed to show consistent reduction in cardiovascular mortality, despite providing a good glycemic control, leaving this clinical condition a still open concern that urges to be addressed.

Recently, results from the ADDENDA-BHS2 clinical trial (Sposito et al., 2021) demonstrated that dapagliflozin treatment improves macro- and microvascular endothelial function in diabetic individuals presenting with atherosclerotic disease, attributing the effect to an increase in NO production. As discussed above, NO is not the only gaseous transmitter that undergoes down-regulation during high glucose induced EC dysfunction, although maybe the best characterized to date in terms of synthesis and effects on cardiovascular system. In the last decade, H_2S has emerged as the third gaso-transmitter regulating many biological processes such as ECs homeostasis, vascular tone, angiogenesis and wound healing (Ciccone et al., 2021; Cheng and Kishore, 2020). Indeed, insufficient production of H_2S plays a crucial role in impaired microvascular endothelial function and angiogenesis *in vivo* (Cheng et al., 2018), and recent studies demonstrated that up-regulation of H_2S biosynthesis is able to rescue endothelial dysfunction in diabetic animal models (Cheng et al., 2016). In this scenario, pathways involved in the up-regulation and release of hydrogen sulphide may represent a valuable target for the development of therapeutical countermeasures aimed at restoring EC function in hyperglycemic or diabetic conditions.

1.2 Wound and fracture healing

(modified from recent reviews: Morbidelli et al, 2021 and Genah et al., 2021)

The skin is the largest organ by surface area in the human body. Its structure is highly organized with different cell types (epithelial, stromal, and endothelial cells-ECs), which finely cooperate in order to guarantee a constant functioning and structural homeostasis of the organ. Indeed, the skin is the first defensive barrier of our body that protects internal tissues from microbial infections, mechanical damages, UV radiations, dangerous substances, and high temperatures. Therefore, the maintenance of its integrity is fundamental for our survival (Sorg et al., 2017; Rodrigues et al., 2019).

Wound healing involves a coordinated interaction of cells, proteins, growth factors, small molecules, proteases, and extracellular matrix (ECM) components aiming at restoring tissue morphology and functioning. The network of communications between stromal, endothelial, and immune cells is the key for determining the course of healing and recovery of tissue function and features (Rodrigues et al., 2019). Skin repair process can be divided into sequential phases that require different kinetics: coagulation and hemostasis, starting immediately after injury; inflammation, shortly after, lasting few days; proliferation, occurring in several days; wound remodeling, lasting days or even many months (Figure 1.4) (Reinke and Sorg, 2012; Velnar and Gradisnik, 2018). Although different growth factors, cytokines, and predominant cell types characterize each phase at different times, a considerable amount of overlap can occur (Sorg et al., 2017; Rodrigues et al., 2019).

Phase 1: The first response to a wound is constriction of the injured blood vessels, accompanied by activation of platelets and coagulation to form a fibrin clot to stop blood flow and provide a scaffold for incoming inflammatory cells. Indeed, in response to a mechanical injury, coagulation and hemostasis activate to prevent exsanguination and form a supportive matrix (including fibrin, fibronectin, vitronectin, and thrombospondins) that represents a substrate for invading cells, required later during the process. Upon injury, the microvasculature is disrupted leading to fluid accumulation, inflammation, and the development of hypoxia (Veith et al., 2019). At this stage, platelet-derived chemotactic factors released by platelet α-granules recruit leukocytes and monocytes to the area of injury to start the inflammatory phase finalized to tissue debris removal and bacteria killing (Etulain, 2018; Ridiandries et al., 2018).

Phase 2: Leukocytes produce and release chemokines and cytokines (interleukin IL-1α, -1β, -6 and tumor necrosis factor-alfa, TNF-α), which, together with ROS release, amplify the inflammatory response (Reinke and Sorg, 2012; Veith et al., 2019). This second phase, often associated with edema, erythema, heat, and pain, aims to prepare the wound bed for the growth of new tissue. However, inflammation can become problematic if it is prolonged or excessive.
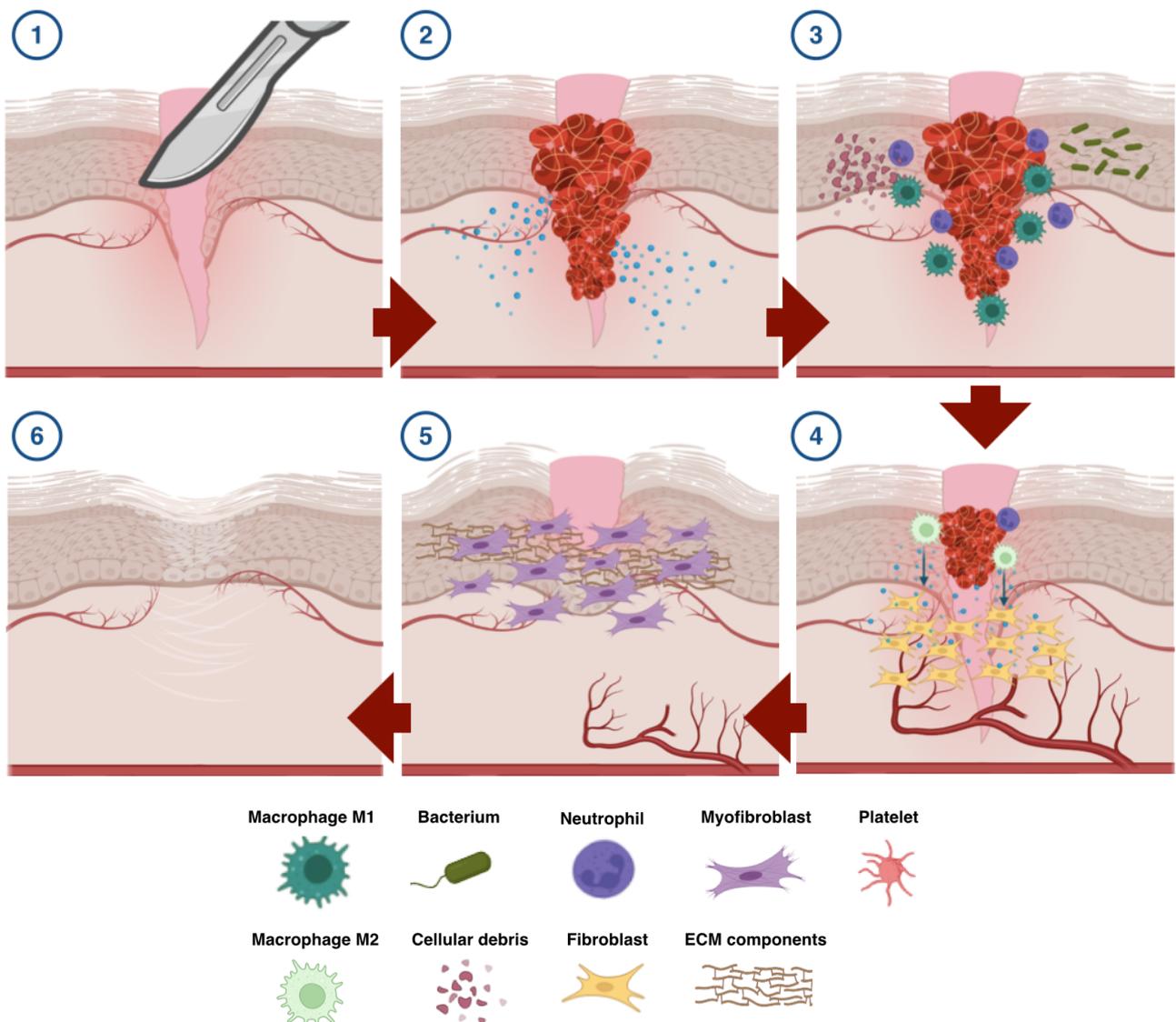
Figure 1.4: Phases of physiological wound healing (image modified from Morbidelli et al., 2021). Phases of physiological wound healing: 1)Wound occurrence; 2) Coagulation; 3) Inflammation; 4) Proliferation; 5) Remodeling; 6) Wound closure and scar formation. Bio-render platform was used for graphic design.

Phase 3: Once the lesion is cleaned out, the wound enters the third proliferative phase, where the aim is to fill and contract the wound edges and cover the gap. The proliferative phase is characterized by granulation tissue formation, collagen deposition, angiogenesis, and reepithelialization. Granulation tissue consists of layers of fibroblastic cells separated by a collagenous extracellular matrix containing capillary buds and inflammatory cells. The recruited neutrophils and macrophages release growth factors, such as TGF-β, FGF-2, PDGF, and VEGF, responsible for activating resident ECs toward an angiogenic phenotype. Neovascularization, regulated predominantly by hypoxia-induced VEGF released by macrophages and fibroblasts, is needed to deliver nutrients and maintain the granulation tissue bed (Tonnesen et al., 2000, Li et al., 2003; Reinke and Sorg, 2012). Additionally, soluble factors induce endothelial precursor cells (EPC) recruitment in the granulation tissue and promote fibroblast proliferation and migration with changes in the ECM

architecture. In this intermediate phase, macrophages continue to supply growth factors, promoting angiogenesis and stimulating resident fibroblasts to invade the wound and proliferate and remodel ECM through the synthesis of collagen, fibronectin, laminin, and metalloproteases, providing strength and elements to the injured tissue (Gurtner et al., 2008; Darby et al., 2014; Etulain, 2018; Ridiandries et al., 2018; Rodrigues et al., 2019). During healing of the tissue defect, the edges of the wound are progressively brought together by the retraction of granulation tissue. This is due to the effect of TGF-β1 on fibroblasts which are induced to differentiate in myofibroblasts, contractile cells with stress fibers containing α-smooth muscle actin (α-SMA). This phenomenon, called wound contraction, is of great clinical importance in reducing the size of the wound. Reepithelialization simultaneously occurs and involves the proliferation of both unipotent epidermal stem cells from the basal layer and dedifferentiation of terminally differentiated epidermal cells (Gurtner et al., 2008; Rodrigues et al., 2019).

Phase 4: The final stage of the repair process, the remodeling or maturation phase, is characterized by the transition from granulation tissue to scar formation and maturation. Wound remodeling takes place when components of ECM undergo changes (e.g., replacement of collagen III by stronger collagen I) and myofibroblasts reduce the scar surface (Tomasek et al., 2002; Darby et al., 2014). The process concludes with a decrease in cell density and a gradual arrest of angiogenesis, involving cell apoptosis and release of antiangiogenic factors (Reinke and Sorg, 2012; Sorg et al., 2018).

When all these steps proceed in a regular and coordinated manner, a morphological and functional recovery of the injured tissue is obtained. However, if one or more of these mechanisms is impaired or delayed, healing is not guaranteed, and ulcer can occur with the risk of infections and chronic damage.

Similarly to skin repair, also the bone healing process can be divided into the same steps, and a communication network between stromal, endothelial, bone and immune cells is very important in determining the course of healing and recovery of tissue function (Schindeler et al., 2008; Deschaseaux et al., 2009). During inflammation, macrophages release growth factors and cytokines signals to recruit mesenchymal stem cells (MSCs), which proliferate and differentiate into osteoprogenitor cells and then into osteoblasts and osteoclasts to form and remodel newly formed bone tissue (Schindeler et al., 2008; Deschaseaux et al., 2009). Much evidence in the literature highlights the pivotal role of blood vessels in the process of bone repair and osteogenesis, which indicates intimate molecular crosstalk between endothelial cells and osteoblasts (Saran et al., 2014; Peng et al., 2017). Angiogenesis is required at different steps and, among the key roles played in the process, it furnishes oxygen and nutrient supply for the regenerating tissue, while ECs secrete osteogenic growth factors (Brandi and Collin-Osdoby, 2006) to promote osteogenesis and osteoblast differentiation from their precursors. Newly formed blood vessels ensure steady transport of circulating osteoclast and osteoblast precursors to remodeling sites, regulating,

therefore, osteoprogenitor cell invasion (Brandi and Collin-Osdoby, 2006; Maes et al., 2010). The human skeleton has remarkable regenerative properties, being one of the few structures in the body that can heal by recreating its normal cellular composition, orientation and mechanical strength. When the healing process of a fractured bone fails owing to inadequate immobilization, failed surgical intervention, insufficient biological response or infection, the outcome after a prolonged period of non-healing is defined as non-union (Wildermann et al., 2021).

1.2.1. Physical-chemical factors controlling wound and fracture healing

Tissue (bone or skin) repair is strictly regulated by a multitude of biochemical and physical factors, including gravitational/mechanical forces acting at cellular and tissue level. Interruption, failure, or alteration in one or more phases of the repair process can lead to the formation of non-healing chronic wounds or non-union fractures, accompanied by pain and inflammation (Guo and DiPietro, 2010; Wildermann et al., 2021). In general terms, the factors that influence repair can be categorized into local and systemic. Local factors are those directly influencing the characteristics of the wound itself like oxygenation/hypoxia, infection occurrence, vascular insufficiency, or presence of foreign bodies. On the other hand, systemic factors are the overall health or disease state of the individual affecting his or her ability to heal, such as genetic factors, age and gender, stress, nutrition, alcoholism and smoking, immunocompromised conditions, and co-morbidities (as diabetes) (Guo and DiPietro, 2010; Wildermann et al., 2021). Several factors related to a patient's conditions cannot be changed, however local factors can be controlled and improved in order to obtain the best therapeutic result. Among the exogenous factors, there are mechanical stressors as pressure, vibrations, and loading. Although the effect of mechanotransduction still needs to be further characterized, ECs (but also other cell lines involved in bone and skin repair) are particularly sensitive to changes in gravitational forces as they undergo important cytoskeletal remodeling and show impairment in proliferation and survival (Maier et al., 2015; Li et al., 2018).

1.2.2. Diabetic wound healing

One of the major complications of DM is the development of chronic distal ulcers. Diabetic individuals often are unable to fight infections due to defective immune responses, thus, even small scrapes can shift to open, infected sores. The most affected part of the body are feet and foot ulcers are typical hallmark of diabetes (Greenhalgh, 2003). Impaired healing in diabetes is the result of a complex pathophysiology. Hyperglycemia correlates with microvascular and angiogenic dysfunction in the wound bed which can chronicize and, in worse cases, result in necrosis caused by insufficient oxygenation and nutrient supply to the injured tissue (Kolluru et al., 2012). However, neovascularization is not the only process to be compromised in diabetic wound healing, which is also characterized by a persistence of the inflammatory conditions and nerve damage which results in lack of peripheral sensory

function and in a diminished capacity to notice cuts, blisters, or ulcers (Spampinato et al., 2020; Kolluru et al., 2012). Specifically, during the inflammatory phase, infiltrating macrophages and neutrophils release cytokines, such as IL-1β and TNF-α, whose levels are elevated not only during the initial acute repair phase, but remain at high concentrations for prolonged time (Wetzler et al., 2000; Spampinato et al., 2020). This chronicization of the inflammatory phase is also due to the inability of macrophages to perform polarization transition from M1 to M2 phenotype, and therefore release all of those factors implicated in tissue repair progression, such as VEGF-A for angiogenesis, or growth factors for fibroblast and other cell type proliferation (Louiselle et al., 2021; Spampinato et al., 2020). Macrophages isolated from wounds of diabetic mice showed significant impairment in efferocytosis (efficient dead cell clearance) (Khanna et al., 2010) and phagocytosis of microorganisms infiltrated in the wound, thus favoring pathogen colonization and infection of the diabetic wound bed (Pavlou et al., 2018). Diabetic patients have a 15–25% lifetime risk of developing diabetic foot ulcers, of which 40–80% become so severely infected it infiltrates the bone, leading to osteomyelitis (Giurato et al., 2017). A high number of cases of foot ulcerations require hospitalization and surgical intervention with amputation of the interested body part. This has negative consequences for both the patient and the medical system and considering the growing prevalence of diabetes, it will be a significant medical, social, and economic burden in the near future (Raghav et al., 2018).

2. Topic 1: Characterization of erucin, a natural isothiocyanate with H$_2$S-releasing properties, in the context of angiogenesis and wound healing

2.1. Preface

Plants have always been considered a valuable source of bioactive molecules with beneficial activity for human health. Today phytochemicals found in certain edible vegetables are providing new principles with therapeutical and/or preventive potential against various pathological conditions.

2.1.1. Erucin as a smart hydrogen sulphide donor

Erucin (ERU; 4-(methylthio) butyl isothiocyanate) is a natural isothiocyanate particularly abundant in *Eruca sativa* Mill. (rocket salad), an edible cruciferous plant belonging to the family of Brassicaceae (Melchini and Traka, 2010). These plants represent not only a great source of macro- and micronutrients necessary for sustenance, but also one of the most valuable sources of phytochemicals such as glucosinolates (GLs), phenols and carotenoids (Villatoro-Pulido et al, 2013). GLs, like glucoerucin, are hydrophilic molecules, stable and inactive, particularly abundant in vacuoles of plant cell and released following any act that leads to a loss of cell integrity such as grinding, cutting or chewing. Once released from the vacuole, the GLs become the substrate of myrosinase, a β-thioglucosidase which, under physiological conditions, is confined into the cytoplasm, thus resulting physically separated from its substrate (Grubb and Abel, 2006). However after plant biotic or abiotic lesions, GLs and myrosinase interact and the enzyme can hydrolyzes glucosinolates to biologically active metabolites such as isothiocyanates (ITCs), which are sulfur molecules whose characteristic functional group is "-N = C = S" (Zhang et al, 1994).

Therefore, erucin is obtained from the precursor glucoerucin by myrosinase, but also as the product of biotransformation in the human body of sulforaphane (SFN; 4-(methylsulfinyl) butyl isothiocyanate), the most studied isothiocyanate present in Brassicaceae vegetables, such as broccoli (Melchini and Traka, 2010) (Figure 2.1).

Isothiocyanates (ITCs) have elicited interest of the scientific research due to fact that they exert beneficial effects on human health. ITCs bioactivity is attributed to the property of these molecules, to release hydrogen sulphide (H$_2$S) slowly and steadily, mimicking the physiological concentration of the endogenous gaseous transmitter (Martelli et al., 2020a). With regard to erucin, the ability of this isothiocyanate to release H$_2$S is dependent on the presence of thiols (R-SH) and, indeed, it occurs preferentially in the cellular cytoplasm, a microenvironment characterized by high concentrations of organic thiols, cysteine (30-200 µM), and glutathione (1-10 mM) (Ciccone et al., 2021).
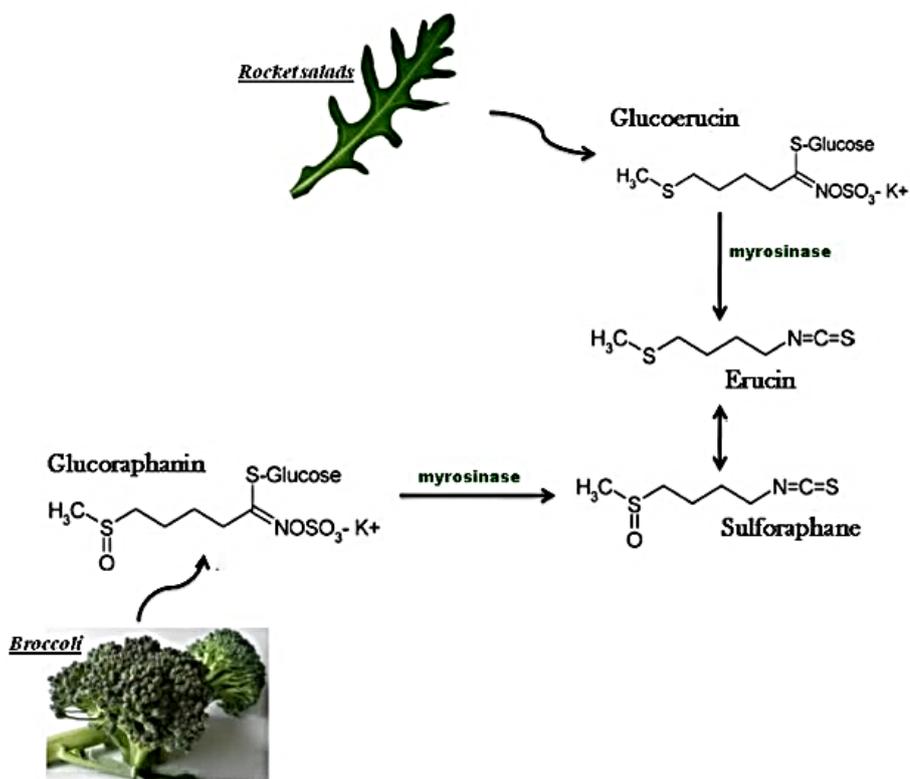
Figure 2.1. Erucin enzymatic production. Erucin is obtained both from glucoerucin, a glucosinolate found at high levels in *Eruca sativa* Mill, via myrosinase enzymatic hydrolysis reaction, and from *in vivo* reduction of sulforaphane, derived from broccoli, *Brassica oleracea* L. (Melchini and Traka, 2010).

Isothiocyanates in general represent a source of different beneficial biological effects on human health, the most investigated are related to their chemo-preventive and anti-cancer properties (Citi et al., 2019; Dinkova-Kostova and Kostov, 2012; Melchini and Traka, 2010). Several studies demonstrated their anti-proliferative and pro-apoptotic effects on cancer cells, but also a general anti-inflammatory and antioxidant activity (Martelli et al., 2020a; Wagner et al., 2013). Recently, other ITCs effects have also been highlighted for the cardiovascular system, where ITCs exhibit vasorelaxing and antihypertensive activity, and a protective effect against endothelial dysfunction (Martelli et al., 2020 a; Martelli et al., 2020 b).

Among ITCs, erucin has been object of pharmacological investigation, thanks to its favorable characteristics to release hydrogen sulfide in a slow, controlled and long-lasting manner and because of its presence in edible plants, such as rocket salad, which is widely used in human nutrition (Martelli et al, 2020 b). Moreover, different studies demonstrated that erucin is well absorbed after oral administration (Clarke et al., 2011; Platz et al., 2015). Erucin has been shown to possess anticancer, and anti-diabetic properties and to exert an antioxidant, vasorelaxing and antihypertensive activity on the cardiovascular system (Citi et al., 2019; Martelli et al., 2020 b).

2.1.2. H_2S as gaseous transmitter in the cardiovascular system

When we talk about endogenous gaseous transmitters we consider nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S), which are still the subject of many studies. In recent decades these molecules assumed increasing importance in pharmacological research due to the contribution in the modulation of both physiological and pathological functions within the cells of the human body. Endogenous gaseous transmitters are small molecules characterized by a short half-life and a highly regulated synthesis, due to the toxicity of their possible accumulation in high concentrations within the cell. These molecules are able to diffuse through cell membranes and act on specific molecular targets, thus exerting their influence on certain biological functions (Wang, 2002).

For many years, hydrogen sulfide was considered exclusively as a toxic gas. Scientific literature is unanimous in recognizing its toxicity; indeed, at high concentrations (> 700 ppm, or parts per million) H_2S can cause death within a very short time. Over the years, and thanks to several studies addressed in this regard, it has been understood that H_2S is actually very important for the physiology of the organism, as suggested by the fact that it is produced by human (and mammalian more generally) cells (Swaroop et al., 1992). It is known that hydrogen sulphide participates in the regulation of the cardiovascular and central nervous system, besides renal, reproductive, respiratory and digestive systems (Cao et al., 2019; Ciccone et al., 2021). The exact plasma concentration of endogenous H_2S has long been a matter of debate, but taken together data suggest that this gaseous transmitter is produced in the range that goes from sub-micromolar to nanomolar concentration range, via both enzymatic and non-enzymatic synthesis pathways (Cao et al., 2019). In the non-enzymatic pathway H_2S is produced through the reduction of thiol-containing molecules (Cao et al., 2019).

In the enzymatic pathway of endogenous H_2S synthesis, hydrogen sulphide is produced starting from cysteine and homocysteine. Among the enzymes involved in this process we find cystathionine β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST). CSE is only present in the cytoplasm, while CBS and 3-MST both have a cytosolic and mitochondrial form (Dilek et al., 2020; Ciccone et al., 2021). For details on H_2S endogenous biosynthesis pathways by CSE, CBS and 3-MST in mammalian cells, see Figure 2.2. These three enzymes are differentially expressed within the body; CSE, however, is the most widely distributed in human tissues with a predominant localization in the cardiovascular and respiratory system (Hosoki Rumiko et al., 1997), it is also commonly found in kidneys, uterus, liver and pancreatic tissues (Dilek et al., 2020; Cao Xu, et al., 2019).
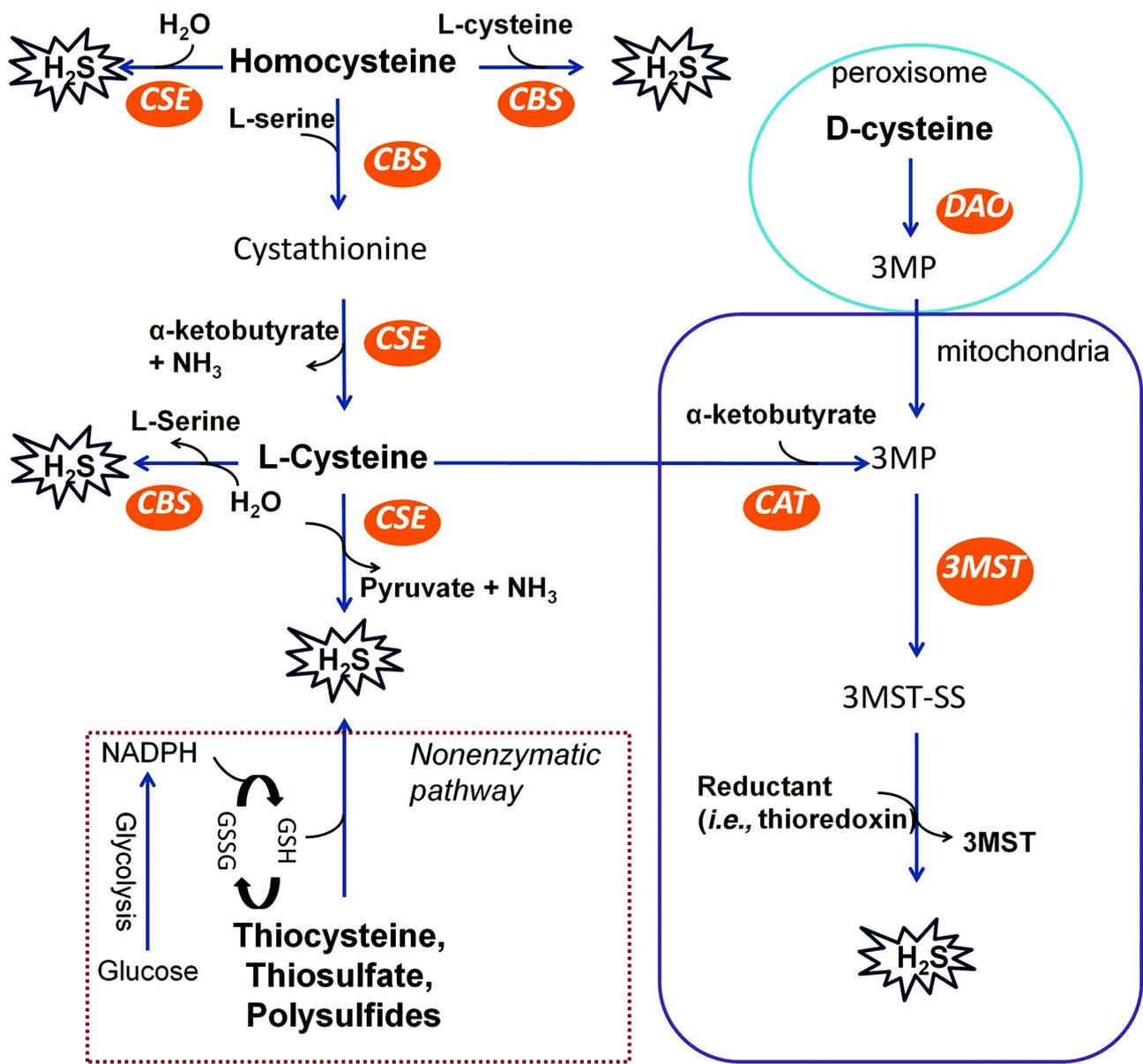
Figure 2.2. Endogenous H₂S production pathways. There are four enzymatic pathways for the biosynthesis of H₂S, including CBS, CSE, 3MST coupled with cysteine aminotransferase (CAT), and 3MST coupled with d-amino acid oxidase (DAO). The first three pathways utilize l-cysteine as a main precursor of H₂S, whereas peroxisome-located DAO (blue circle) can catalyze d-cysteine into 3-mercaptopyruvate (3MP), which can be further transformed into H₂S by 3MST. This d-cysteine pathway may exclusively exist in brain and kidney. In addition, CBS and CSE may generate H₂S in the cytosol whereas 3MST mainly resides and synthesizes H₂S in mitochondria (purple rectangle). A small portion of endogenous H₂S is derived via nonenzymatic reduction: in the presence of reducing equivalents such as nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH), reactive sulfur species in persulfides, thiosulfate, and polysulfides are reduced into H₂S and other metabolites (Cao et al., 2019).

In the cardiovascular system, CSE is located in vascular smooth muscle cells and is found mainly in ECs; as a matter of fact, the concentration of hydrogen sulphide in the vascular tissues is around 100 times greater than in other tissues (Levitt et al., 2011), suggesting a crucial role in vascular homeostasis, endothelial function, and trophism (Yu et al., 2014). Indeed, CSE knockout mice exhibit phenotypic alterations mainly affecting the

cardiovascular system, with hypertension and endothelial dysfunction. Furthermore, altered levels of H_2S in humans are commonly associated with cardiovascular diseases, such as hypertension and diabetes (Ciccone et al., 2021). Since the discovery of H_2S as an endogenous gaseous transmitter, many researchers have tried to characterize its biological profile especially in the context of the circulatory system (Figure 2.3).
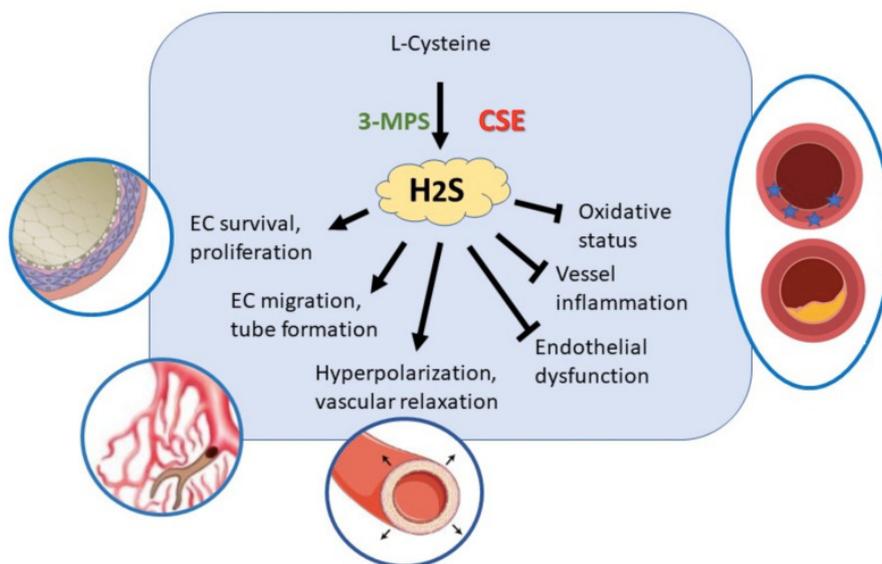

Figure 2.3. Main activities of H_2S on cardiovascular system. Hydrogen sulfide acts on vascular ECs through both an autocrine and paracrine mode of action. On the right, the inhibitory effects of hydrogen sulphide are shown in the context of pathological situations such as endothelial dysfunction, oxidative stress and inflammation; on the left, the stimulatory effects of H_2S described in the context of vascular tone and control of angiogenesis (Ciccone et al., 2021).

The protective effect that hydrogen sulphide performs on vascular tissues passes through both an autocrine and paracrine modality and plays a key role in the regulation of vasodilation, apoptosis, inflammation, angiogenesis and oxidative stress (Wang et al., 2015). H_2S has been shown to exert its biological effect by acting as a post-translational modifier, through S-sulfhydration on reactive cysteine residues (-SH) to form a persulfide group (-SSH) on target proteins, modifying both their structure that activity (Paul and Snyder, 2015). In addition to being considered as an important blood pressure regulator, hydrogen sulfide possesses anti-inflammatory and antioxidant properties (Kimura and Kimura, 2004; Martelli et al., 2021).

2.1.3. Hydrogen sulphide in angiogenesis

Scientific literature is unanimous in considering hydrogen sulphide as a regulator of the angiogenic process; in fact, its positive effect on the formation of new blood vessels derives from H_2S ability to enhance ECs proliferation, migration and formation of tubular structures *in vitro* (Wang et al., 2010). It is important to underline that this effect has also been proven through *in vivo* in studies conducted on mice and on models of chicken

chorioallantoic membrane (Papapetropoulos et al., 2009). Furthermore, there is also evidence of angiogenesis regulation by exogenous H_2S-donors in rat models where the administration of sodium hydrosulfide, NaHS, (50 µM/Kg) has been shown to increase plasma levels of the pro-angiogenic factor VEGF (Wang et al., 2010). On the other hand, *in vitro* studies highlighted how CSE over-expression in ECs elicits angiogenesis (Coletta et al., 2012), while, on the contrary, CSE silencing, both *in vitro* and *in vivo*, leads to an inhibition of the angiogenic mechanism (Papapetropoulos et al., 2009).

Researchers have investigated the molecular mechanisms by which hydrogen sulfide mediates its pro-angiogenic effect, concluding that H_2S activates numerous intracellular signaling pathways involved in angiogenesis, especially those that intervene during EC migration. Currently, the direct molecular targets of H_2S in the context of angiogenesis remains to be elucidated, although mass spectrometry studies and further investigations have revealed that disulfide bond between Cys1045 and Cys1024 residues in the intracellular kinase domain of VEGFR- 2, acts as a molecular target for hydrogen sulfide to regulate the functionality of this receptor (Tao et al., 2013). Indeed, it has been observed that H_2S in aqueous solution dissociates leading to the formation of the HS^- anion which causes the rupture of the VEGFR-2 inhibitory disulfide bond, inducing a conformational change towards the active and competent form for the signal transduction inside of the cell (Tao et al., 2013). Therefore, H_2S plays a key role especially in the VEGFR-mediated signaling pathway related to the migration of endothelial cells during angiogenesis.

In vitro experiments, conducted with ECs stimulated with H_2S-releasing molecules, have shown the activation, by phosphorylation, of specific molecular pathways including that of the Akt protein, ERK1/2 (kinase-1 and 2 regulated by extracellular signals) and p38/MAPK (p38 mitogen-activated protein kinase) (Papapetropoulos et al., 2009). The effect of hydrogen sulphide on eNOS isoform (endothelial NO synthase) activation has also been demonstrated, resulting in an increase in NO levels (Predmore et al., 2011): the latter and H_2S have demonstrated to synergistically regulate not only the vascular tone by inducing vasodilation, but also the process of angiogenesis (Coletta et al., 2012).

2.1.4. Hydrogen sulphide in wound healing

In literature there are clear evidences that link several skin diseases to the alteration of gaseous transmitters production, especially NO and H_2S. This evidences lay the foundations for hypothesizing a role of these molecules in maintenance of skin integrity (Pinto et al., 2022). Indeed, pathological conditions like psoriasis, skin cancer, atopic dermatitis, lupus erythematosus and irritant or allergic contact dermatitis, are commonly associated with deficiencies in NO and/or H_2S levels in the wound bed (Alder and Friedman, 2015; Coavoy-Sánchez et al., 2020). Although the contribution of hydrogen sulfide in each of the different stages of wound healing still needs further characterization, H_2S role in the whole process is today well established and many therapeutical approaches for treatment of tissue

healing defects, are basing their strategy on H_2S-releasing molecules (Nazarnezhada et al., 2020; Zhao et al., 2020). Indeed, *in vivo* studies demonstrated that topical applications of exogenous hydrogen sulfide is able to accelerate the healing of skin ulcers in rats (Wallace et al., 2007).

Among the main effects exerted by hydrogen sulfide in tissue repair we find the promotion of angiogenesis, a key step in the proliferative phase for the formation of granulation tissue, reduction of both inflammation and oxidative stress, and enhancement of cell migration and proliferation in both skin fibroblasts and ketatinocytes (Xu et al., 2019; Zhao et al., 2017; Zhao et al., 2020; Yang et al., 2020). These effects are consolidated in many studies showing how, in diabetic mice, H_2S improves angiogenesis in wound healing by promoting transcription of VEGF, epidermal growth factor (EGF), HIF-1α and eNOS, and by up-regulating VEGF and PDGF proteins and receptor phosphorylation (Xu et al., 2019; Wang et al., 2015). Hydrogen sulfide has also been shown to accelerate wound healing in mice by promoting granulation tissue formation and by increasing levels of anti-inflammatory molecules (Zhao et al., 2017). Among the anti-inflammatory effects it was documented ability of exogenous H_2S to inhibit neutrophil extracellular traps (NET) release-coupled neutrophil death (NETosis) in db/db mice (Yang et al., 2019) and promote, both *in vitro* and *in vivo*, the polarization of macrophages from inflammatory M1 to pro-reparative M2 phenotype (Wu et al., 2019), thus promoting a proper remodeling phase and favoring the extinction of inflammation (Sindrilaru et al., 2011).

Recent literature also report H_2S involvement in later stages of wound healing, during re-epithelialization, by observing an up-regulation of CSE in differentiating keratinocytes of the newly formed epidermis and the capability of exogenously applied H_2S to trigger expression of the differentiation marker keratin 10 (Goren et al., 2019).

2.2. Aim of the study

Erucin is a natural isothiocyanate with "smart" H_2S-releasing properties, particularly abundant in *Eruca sativa*, an edible cruciferous plant. Many studies in literature highlighted the importance of H_2S, as endogenous gasotransmitter, in many biological processes including angiogenesis. In this context, an investigation about erucin effects on angiogenesis appears an interesting perspective, considering its property to deliver and release H_2S inside cells. In this study we characterized *in vitro* the effect of erucin on cultured endothelial cells (HUVEC) in different functional assays, with a special focus on the molecular mechanisms involved in endothelial cell response to the compound, and erucin ability to recover endothelial dysfunction induced by the presence of high glucose. In the second part of this topic we started to investigate the activity of the isothiocyanate in the context of wound healing by assessing *in vitro* its pro-migratory, pro-survival effect on dermal fibroblasts (NHDF) and keratinocytes (HaCaT). Lastly, a preliminar study using indirect co-coltures on NHDF and HUVEC, was carried out in order to investigate erucin ability to promote and sustain endothelial-stromal crosstalk, a fundamental step in wound healing.

2.3. Materials and methods

2.3.1. Reagents

Erucin (ERU) was produced by myrosinase-catalyzed hydrolysis of glucoerucin isolated from *Eruca sativa* Mill. seeds according to reference [Citi et al., 2019]. Erucin was dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany) to a concentration of 10^{-2} M, and stored at -20°C. For stimulations, the compound was freshly diluted in the appropriate culture medium.

Vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN, USA) was used diluted into culture medium to a final concentration of 25ng/mL. eNOS inhibitor N(G)-Nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, St. Louis, MO, USA) was diluted into culture medium to a final concentration of 200μM. An aqueous solution of d-(+)-Glucose (45% w/w; Merck KGaA, Darmstadt, Germany) was diluted before each experiment in culture medium up to a final concentration of 30 mM. CelLytic™ MT Cell Lysis Reagent was obtained from Life Technologies (Carlsbad, CA, USA). MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich (CAS # M 2128), while Matrigel (CAS # 354234) was purchased from Corning and stored at -20 ° C.

For Western Blots, protein detection was obtained with the following antibodies diluted 1:1000 in 1% (wt/v) milk: anti-total and phosphorylated Akt (MW: 60 kDa; Cell Signaling, Danvers, Massachusetts, US; #9272 and #9271, respectively); anti-total and phosphorylated eNOS (MW: 140 KDa; BD Tecnology, Franklin Lakes, New Jersey, US #610297 and Cell Signaling #9570, respectively); anti-total and phosphorylated ERK1/2 (MW: 42/44 kDa; Cell Signaling; #9102 and #9101, respectively); anti-VEGF (MW: 24 KDa; Cell Signaling; #9943); anti-FGF2 (MW: 16 KDa; Cell Signaling; #3196) and anti-GAPDH (MW: 37 KDa; Cell Signaling; #2118).

2.3.2. Cell cultures

The experiments were performed on human umbilical vein endothelial cells (HUVEC, Lonza, Basel, Switzerland), immortalized human keratinocytes (HaCaT, Voden Medical, Meda, MB, Italy) and normal human dermal fibroblasts (NHDF, Lonza, Verviers, Belgium). HaCaT cells were grown in Dulbecco's modified Eagle's medium (DMEM 4500 mg/L, Euroclone, Milan, Italy) supplemented with 10% of fetal bovine serum (FBS, Euroclone, Milan, Italy). NHDF were grown in fibroblast growth medium (FGM-2, Lonza, Basel, Switzerland) supplemented with 10% of FBS (Euroclone). HUVEC were grown in endothelial growth medium (EGM-2, Lonza, Basel, Switzerland) added with 10% of FBS (Hyclone, Celbio, Milan, Italy). Each medium was completed with 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA). Cells were cultured in 10 cm diameter Petri dishes up to a confluent state, in a humidified incubator with 5% CO_2. For HUVEC, plates were previously coated with gelatin derived

from bovine skin (Sigma-Aldrich, St. Louis, Missouri, US). Cells were expanded by splitting 1:6 twice a week for HaCaT, while 1:3 twice a week for NHDF and HUVEC. For all experiments, HaCaT were used until the passage 31, NHDF until passage 7, and HUVEC until passage 6. For fibroblasts and endothelial cells, stimulations were performed in basal medium, respectively FBM-2 and EBM-2 (Lonza, Basel, Switzerland) supplemented with 0.1 % or 5% FBS.

2.3.3. Evaluation of cell viability

To evaluate the effect of erucin on the survival of each cell line, the colorimetric quantitative assay using MTT was performed following the protocol described in reference (Genah et al., 2020). HUVEC, NHDF and HaCaT cells were seeded at the cell density of 4'000 cells/100 µl in a 96-well plate in the appropriate medium supplemented with 10% FBS. After 24 h of incubation to allow cell adherence, cells were stimulated for 48 h in basal medium, or DMEM for HaCaT, supplemented with 0.1 or 5% FBS. After two days, medium was removed and cells were incubated for 4 h with fresh medium containing 1.2 mM of MTT. After solubilization of the resulting formazan derivate in DMSO, absorbance was measured with a micro-plate absorbance reader (Infinite 200 Pro, Tecan Life Sciences, Switzerland) at 540 nm. Data are reported as relative absorbance/well.

2.3.4. *In vitro* cell migration by scratch assay

HUVEC, NHDF and HaCaT migration assay was conducted according to the protocol described by Dr. Terzuoli et al. (Terzuoli et al., 2015). Briefly, cells were seeded into 24-well plate (80'000 cells/well) and, after reaching confluence, the monolayer was scraped using a 200 µl sterile tip. The scratch generated was gently rinsed with DPBS to remove debris and cells were stimulated in basal medium with 1 or 5% serum for 24 h in the presence of the antimitotic ARA-C (25 ug/mL) (Sigma-Aldrich, St. Louis, MO). Scratch images were acquired after scratch induction (T0) and at different time points. Images of the scratched area were acquired to analyze cell migration under an inverted microscope at 10 X magnification. The results, expressed as the percentage of wound area closed with respect to initial time (T0), were analyzed using Image J 1.48v software (U.S. National Institutes of Health, Bethesda, MD, USA).

2.3.5. *In vitro* angiogenesis model on Matrigel

In order to assess erucin ability to promote angiogenesis and increase ECs ability to organize in capillary-like structures, an *in vitro* angiogenesis assay was performed following the protocol described in reference (Terzuoli et al., 2014). HUVEC were seeded at a cell density of 40'000 cells/well in basal medium supplemented with appropriate stimuli on a 48-well plate previously coated with 100 µL of basement membrane matrix (Matrigel; Becton Dickinson, Waltham, MA, USA). After 4 h of incubation at 37°C, ECs were photographed and network formation was measured by means of the number of tubular

structures formed and branching points per well (Nikon Eclipse E400 and camera Nikon DS-5MC).

2.3.6. Western blot analysis

Western blot was performed on cell lysates of sub-confluent HUVEC, NHDF and HaCaT cells seeded in 60 mm-diameter Petri dishes (350'000 cells/dish). Following protocol described in reference (Genah et al., 2021), at the end of stimulations, cells were washed twice with cold PBS and then lysed in 60 μL of CelLytic^{TM} MT Cell Lysis Reagent supplemented with 2 mM Na3VO4 and 1X Protease inhibitor cocktail for mammalian cells (Sigma-Aldrich). After protein extraction and quantification, performed with Bradford method, 50 μg of protein/sample were subjected to electrophoresis in 4–12% Bis-Tris precast Gels (Life Technologies, Carlsbad, CA, USA). Separated proteins were then blotted onto nitrocellulose membranes, blocked for 1 h in a PBS–0.05% Tween solution supplemented with 5% (wt/vol) of Blotting-Grade Blocker (Sigma-Aldrich) at room temperature, and then incubated overnight at 4°C with the primary antibodies listed in paragraph 2.3.1 properly diluted in PBS–0.05% Tween solution with 1% (wt/vol) of Blotting-Grade Blocker. The membranes were finally incubated with SuperSignalTM West Pico PLUS chemiluminescent Substrate (Thermo Fisher Scientific), and the immunoreaction was revealed by ImageQuant LAS 4000 chemiluminescence system (GE Healthcare, Chicago, IL, USA). Immunoblots were analyzed by densitometry using Fiji ImageJ software. Data are analyzed as ratio of the arbitrary densitometry unit (A.D.U.) of target protein respect to the reference GAPDH (or phosphorylated respect to the total form).

2.3.7. Fibroblast-endothelium co-colture

The transwell co-cultivation apparatus involves the reconstruction of a microenvironment in which endothelial cells and fibroblasts are separated by a polycarbonate membrane to evaluate cell-cell interactions. Co-cultivation models were set up as follows. NHDF (5'000 cells/500 μL) were plated on the bottom of 24 multi-plates in FGM-2 with 10% FBS. HUVEC were seeded separately in EGM-2 with 10% FBS at density of 20'000 cells/250 μL on the top of polycarbonate membrane, pre-coated with gelatin, with 0.4 μm pores. After 24 h of incubation to ensure cell adherence, ECs grown on transwell were transferred over fibroblasts for 48 h of co-culture in EBM-2 medium (without growth factors) supplemented with 0.1% FBS and stimulated with erucin. NHDF on the bottom of the wells were then fixed, stained and randomly counted at 20X original magnification in 5 fields as previously described (Ciccone et al., 2018). Parallel proliferation experiments were performed exposing NHDF to media (EBM-2 with 0.1% serum) conditioned by HUVEC in the absence or presence of erucin for 48 h.

2.3.8. Statistical analysis

Each experimental condition was run in triplicate and data are reported are the mean ± SD of at least three separate independent experiments, unless differently indicated. Statistical analysis was performed using Student's t test. A value of $p < 0.05$ was considered statistically significant.

2.4. Results

2.4.1. Erucin promotes and accelerates HUVEC migration *in vitro* but does not affect cell viability

The effect of the compound on ECs viability was initially tested by means of an MTT assay. To this end, HUVEC were seeded in a 96-well plate and, after 24 h of incubation to allow cell adherence, cells were challenged for 48 h with 10-fold erucin serial dilutions from 300 to 3 nM in basal medium EBM-2 supplemented with 0.1 or 5% FBS. As it can be observed from the graphs in Figure 2.4-A, in our experimental conditions, the isothiocyanate does not seem to influence HUVEC viability at any of the tested concentrations.
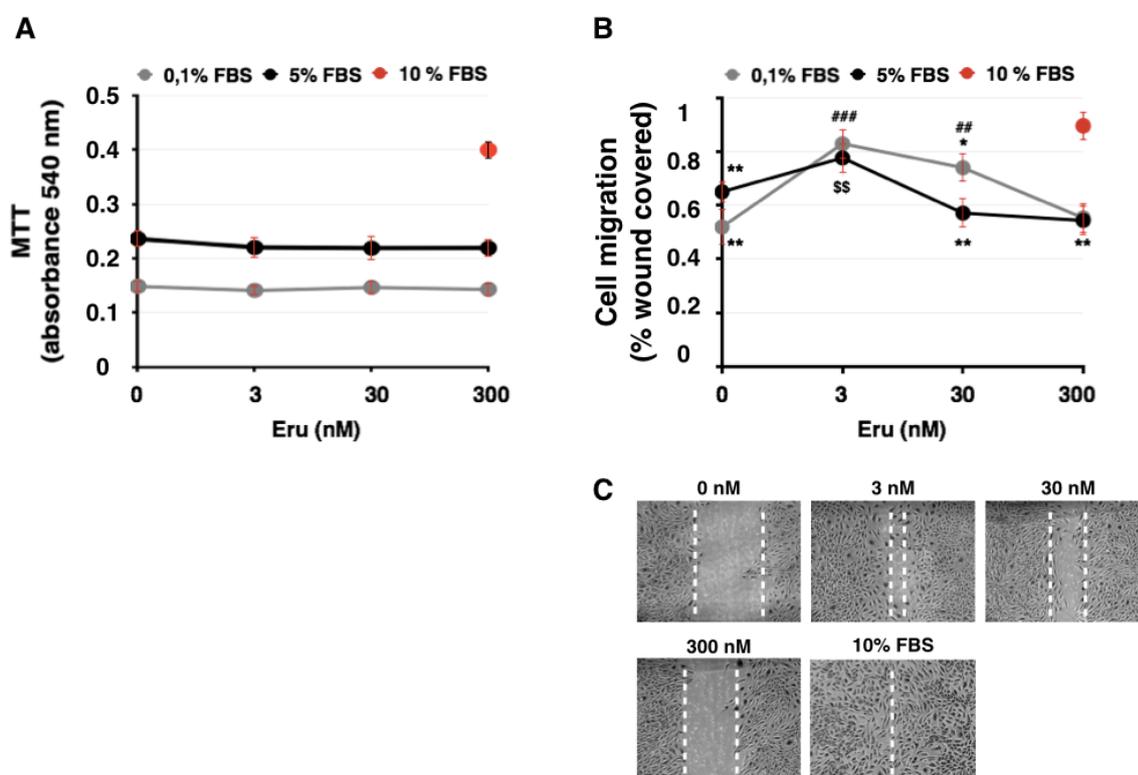

Figure 2.4. Erucin at nanomolar concentrations does not influence cell viability but significantly affect cell migration. A) HUVEC viability analyzed by MTT assay with 10-fold erucin (Eru) serial dilutions at 48 h in presence of low and high serum (0.1% and 5% FBS). Data represent means +/- SD of at least 2 independent experiments. **B)** Cell migration assay with 10-fold erucin dilutions at 18 h in presence of high and low serum. Data represent means +/- SD of 5 independent experiments. P values of *p < 0.05; **p < 0.01, ***p < 0.001 vs positive control (10% FBS); ##p < 0.01, ###p < 0.001 vs not stimulated in low serum (0.1% FBS); $$p < 0.01 vs not stimulated in high serum (5% FBS), were considered statistically significant. C) Representative micrographs (10X original magnification) of the scratched areas covered by HUVEC migration at 18h.

Subsequently, the ability of the compound to promote cell migration was evaluated, as an essential aspect in the angiogenesis process. The aim was to investigate the effective concentration of the natural isothiocyanate that most closely approximated the positive control, therefore EBM-2 at 10% of FBS. For this purpose, HUVEC were seeded into 24-well

plate (80'000 cells/well) and after 48 h monolayers were scraped using a 200 μl sterile tip and stimulated in basal medium with the same erucin concentrations tested in MTT, both in low and high serum for 18 h in the presence of the antimitotic ARA-C (25 ug/mL). When HUVEC are exposed to erucin, cell migration increases in a concentration-dependent manner (Figure 2.4-B). At the effective concentration of 3 nM, the highest migration rate is reached by the cells in both low and high serum conditions; indeed, the percentage of scratched area covered by HUVEC appears to be around 80%, with respect to time 0, in which a migration of about 50-60% is observed in the absence of stimuli.

Following this initial result, a second scratch protocol was set up and the entity of the wounded area covered by HUVEC migration, under erucin stimulation with the effective concentration of 3 nM, was monitored at consecutive time points of 4-6-8-10-24 h and in different serum concentrations 0.1%, 5% and 10%.

Figure 2.5. The effective concentration of 3 nM accelerates scratch closure compared to untreated conditions. HUVEC migration, under erucin stimulation, was monitored at consecutive time points of 4-6-8-10-24 h and in different serum concentrations 0.1%, 5% and 10%. Data represent means +/- SD of 3 independent experiments. P values of *p < 0.05, **p < 0.01, ***p < 0.001 vs not stimulated, were considered statistically significant.

As reported in the concentration-response curves shown in Figure 2.5, HUVEC treatment with erucin, in all serum conditions tested, accelerates cell migration when compared with untreated cells. By analyzing the trend of the graphs, it is possible to observe a significant difference in erucin pro-migratory effect already in the initial phase of the process, starting

from 6 h of stimulation (for the conditions of 0.1 and 5% FBS), or 4 h in high serum conditions (10% FBS). Remarkably, after only 10 h of treatment with the natural isothiocyanate, the scratch results to be healed in conditions of high serum (10% of FBS) and the percentage of the area occupied by HUVEC that exceeds 95% compared to the corresponding untreated cells, in which ECs migration covers 70% of the scratched area. Although the pro-migratory effect is particularly marked in high serum conditions, erucin is able to significantly speed up scratch closure also in lower percentages of FBS tested.

2.4.2. Erucin promotes *in vitro* angiogenesis and increases ECs ability to organize in capillary-like structures

After demonstrating erucin ability to favor cell migration in HUVEC at the effective concentration of 3 nM, the pro-angiogenic effect of the isothiocyanate was further investigated by analyzing its ability to promote the formation of capillary-like structures by ECs.

To this purpose HUVEC were seeded at a cell density of 40'000 cells/500 µL in basal medium containing 3 nM of erucin on a 48-well plate previously coated with 100 µL/well of basement membrane matrix (Matrigel). After 4 h of incubation at 37°C, endothelial cells were photographed and network formation was measured by means of the number of tubular structures formed.

From graphs in Figure 2.6 we can observe, in agreement with previous results, that erucin positively modulates ECs ability to organize in capillary-like network, both in low and high serum concentrations. Indeed, the compound tested leads to the formation of a greater number of tubes (circular closed structures) compared to the respective non-stimulated basal conditions.

Figure 2.6. Erucin positively modulates ECs ability to organize in capillary-like structures. A) Tube formation assay with or without erucin at the effective concentration of 3 nM, in presence of high and low serum, after 4 h of incubation. ***p < 0.001 ERU vs NS in high serum (5% FBS); ###p < 0.001 ERU vs NS in low serum (0.1% FBS). Data represent means +/- SD of 3 independent experiments. B) Representative micrographs of tubes formed after 4 h of incubation in Matrigel with erucin.

2.4.3. Erucin effect on angiogenesis relies on the early activation of e-NOS - ERK1/2 - Akt pathway and late expression of VEGF and FGF-2 growth factors

As discussed above, the migration of endothelial cells is an essential event during angiogenesis and is associated with the activation of the complex intracellular signaling pathways triggered by the binding of growth factors, such as VEGF, to its receptors. A number of studies in literature have reported how stimulation mediated by H_2S, both endogenous and exogenous, leads to the activation of key enzymes in the process of migration and cell viability that are part of the downstream signaling cascade of VEGF-R (Tao et al., 2013). Considering all this, a further investigation was conducted on the molecular pathways activated in the early response to erucin in ECs during cell migration, by analyzing molecular markers such as eNOS, ERK1/2 and Akt, in their phosphorylated from respective total forms.

To evaluate the activation of proteins involved in EC migration, HUVEC were starved for 18 h and then treated for 5 min with erucin, diluted in EBM-2 0.1% FBS at a final concentration of 3 nM. VEGF 25 ng/ml was used as a positive control for pathway activation, alone or in combination with erucin.

Figure 2.7. Erucin effect on angiogenesis relies on the early activation of eNOS - ERK1/2 - Akt pathway and on the late expression of VEGF and FGF-2 growth factors. A-B) Whole cell lysates were collected after 5 minutes of exposure to 3 nM of erucin. VEGF was used as positive control for molecular pathway activation. A) Western blot was performed to assess protein activation by phosphorylation of eNOS, Akt and ERK1/2. Results, expressed as arbitrary density units (A.D.U.), were normalized to the respective total forms. Data represent means +/- SD of 2 independent experiments. Values of *p<0.05, **p<0.01, ***p<0.001 eNOS

phosphorylation vs basal condition; #p<0.05, ##p<0.01, ###p<0.001 ERK1/2 phosphorylation vs basal condition; $p<0.01, $$p<0.05 Akt phosphorylation vs basal condition, were considered statistically significant. C-D) Cell lysates were collected after 24 hours of exposure to 3 nM of erucin, VEGF and FGF-2 protein abundance was assessed by Western blot (C). Results, expressed as arbitrary density units (A.D.U.), were normalized to GAPDH. Data (D) represent means +/- SD of one independent experiments run twice. **p<0.01 VEGF expression after erucin stimulation vs not stimulated (NS); ##p<0.01 FGF-2 expression after erucin stimulation vs NS.
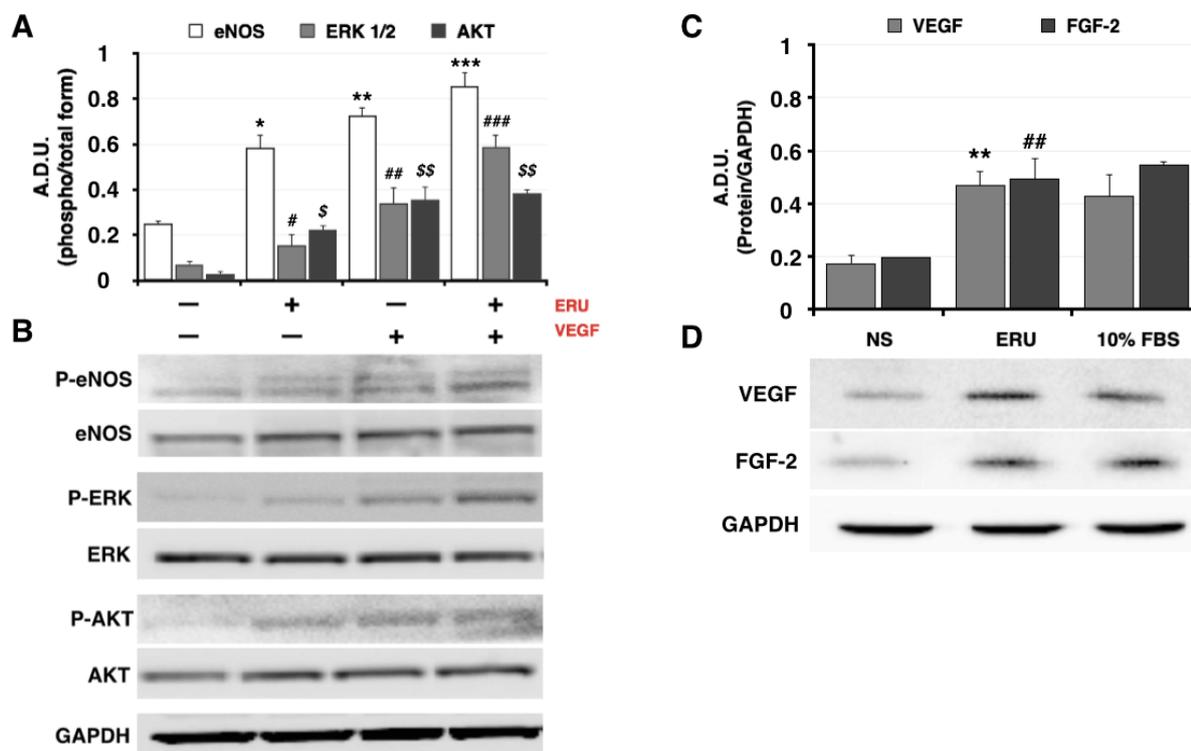
From the blots in Figure 2.7-A and B we can observe, compared to basal condition, a significant increase in the activation of all the three markers analyzed after stimulation with erucin. Phosphorylation of eNOS and ERK1/2 increases with the addition of VEGF, and ECs co-stimulation with both erucin and VEGF leads to an enhanced effect in the activation of the two enzymes. Although erucin alone leads to an increase in Akt phosphorylation with a reduced effect respect to VEGF alone, Akt does not appear to be further modulated following co-stimulation with both erucin and VEGF.

The results described above explain the molecular mechanism underlying the early response to erucin on HUVEC migration. Previous studies demonstrated that intracellular erucin-mediated H_2S release kinetics, measured with the fluorescent dye WSP-1, implies a slow and progressive release of hydrogen sulfide which, however, reaches a plateau after 40 minutes (Martelli et al., 2021). No information is available at longer stimulation times, but given the velocity in reaching a plateau state, it can be inferred that without a continuous supplementation of the stimulus, H_2S released by erucin will tend to extinguish over several hours. However, the effect of the compound, in our experimental conditions, is also observable at late stimulation times, up to 24 hours. Considering that, we hypothesized that ECs stimulated by the isothiocyanate, would activate the expression of factors that promote a positive feedback on the process of angiogenesis. In this regard we investigated the expression of two crucial growth factors in this process, namely VEGF and FGF-2.
To this end, HUVEC were seeded in 60-mm diameter Petri dishes (350'000 cell/dish) and after reaching a sub-confluent state, cells were stimulated for 24 h with erucin 3 nM. Interestingly, (Figure 2.7-C and D) both growth factors seem to be up-regulated following erucin stimulus and protein quantifications can be compared with those obtained in positive control (10% FBS).

Many evidences in literature report a link between NO and H_2S in the endothelium, explain how the two influence each other and how a correct balance of the two gas-transmitters is fundamental for the maintenance and control of vascular physiological functions. A number of studies demonstrated that H_2S promotes angiogenesis (Cai et al., 2007; Papapetropoulos et al., 2009; Tao et al., 2015) through the increase of eNOS phosphorylation in endothelial cells with a subsequent increased NO production (Coletta et al., 2012; Predmore et al., 2011). In this thesis, we confirmed that eNOS is activated within 5 minutes following erucin exposure of HUVEC during cell migration. Considering the above, we therefore

hypothesized the involvement of this enzyme in the first cellular response to the substance. To asses the need of this enzyme in the response to the isothiocyanate, we performed a further scratch assay, using a selective inhibitor for eNOS (L-NAME), alone or in combination with erucin. For this experiment, HUVEC were first pre-treated for 30 minutes with L-NAME (200 μM), and subsequently cells were scratched and stimulated with erucin for 8 hours.
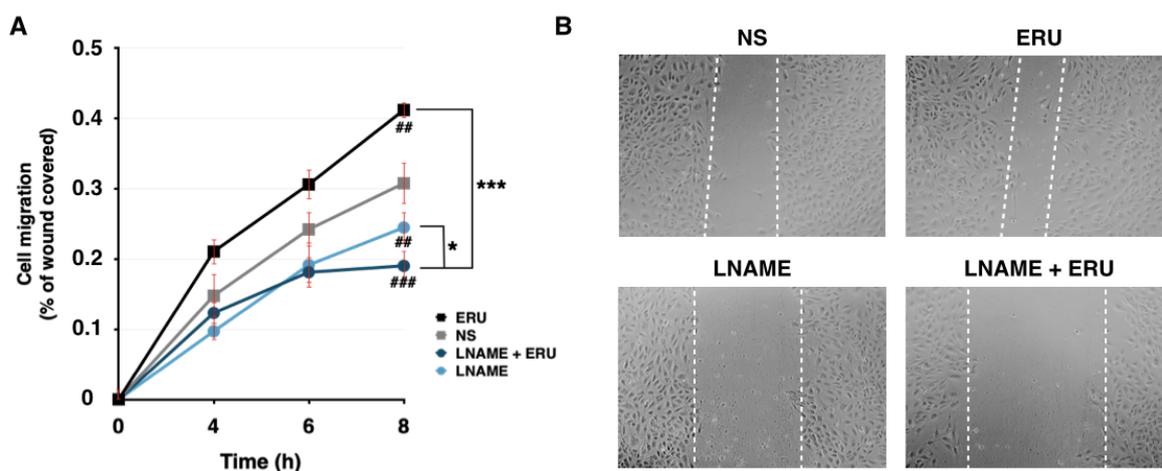

Figure 2.8. eNOS activation is essential for erucin-mediated migration. Cell migration assay performed in low serum using the molecular eNOS inhibitor, L-NAME. In tested conditions, HUVEC were pre-incubated for 30 mins with L-NAME, and after the scratch, cells were treated or not with erucin 3nM. A) Scratched area closure was evaluated at consecutive times of 4, 6 and 8 hours. Results highlight the pivotal role of this enzyme in the immediate response of the cell to the compound. Data represent means +/- SD of 2 independent experiments. *p < 0.05; **p < 0.01 were considered statistically significant. B) Representative micrographs of the scratched area at 8 h.

The results in Figure 2.8 show how eNOS inhibition by L-NAME, significantly reduces HUVEC migration at 8 h compared to untreated baseline control. This impairment is even more evident in the presence of the stimulus given by erucin, underlining the importance of eNOS in cell migration in conditions of non-stimulation, but especially its role in the early response to the compound.

2.4.4. Combined effect of erucin and VEGF on HUVEC vitality and migration

In the previous paragraph we demonstrated that H_2S released by erucin promotes angiogenesis *in vitro* by targeting enzymes (eNOS, Akt and ERK1/2) involved in the signaling pathway mediated by VEGFR. Western blots also highlighted that co-stimulation with both VEGF and erucin leads to a higher activation, by phosphorylation, of the enzymes compared to single treatments. In this scenario we found it interesting to evaluate the hypothetical enhanced effect of VEGF and erucin, not only at the molecular level, but also on HUVEC viability and migratory capacity.

This combined effect was initially investigated in relation to cell viability by MTT assay; HUVEC were seeded in 96-well plates and after 24 h cells were treated for additionally 48 h with erucin 3 nM and VEGF 25 ng/mL, both in single stimulation and in combination. From the graph in Figure 8-A it can be observed that, again, erucin alone is unable to promote an increase in cell viability compared to the baseline control. VEGF, as expected, increases cell survival but its combination with the isothiocyanate does not seem to enhance the response.
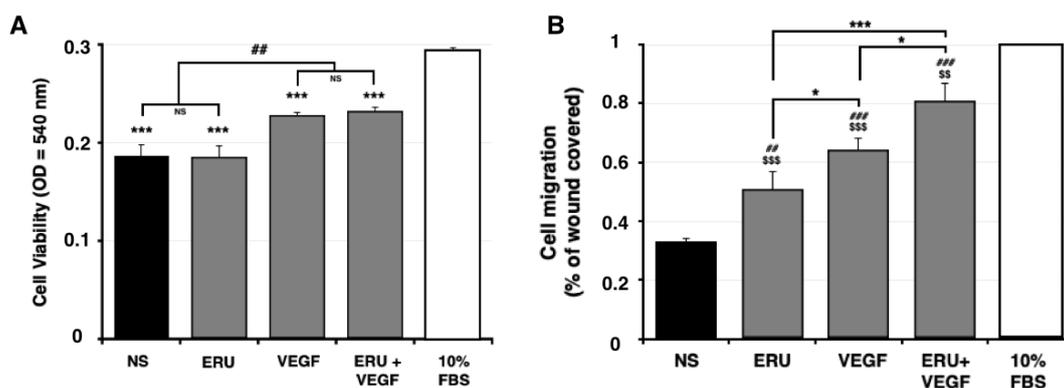

Figure 2.9. Effect of erucin and VEGF co-stimulation on ECs viability and migration. A) VEGF increases cell viability compared to erucin. HUVEC viability was analyzed by MTT assay after 48 h of stimulation with erucin 3 nM and VEGF, 25 ng/mL alone or in combination, in presence of low serum (0.1% FBS). Data represent means +/- SD of at 3 independent experiments conducted 4 replicates. P values of **$p<0.01$, ***$p<0.001$ between experimental conditions, were considered statistically significant. B) HUVEC co-stimulation leads to an enhanced effect on cell migration. Once confluence was reached, endothelial monolayers were scratched and cells were stimulated for 24 h with 3 nM of erucin and 25 ng/mL of VEGF, either separately or in combination. Graphs show the percentage of the scratched areas covered by HUVEC migration at 24 h. Data represent means +/- SD of 5 independent experiments conducted in triplicate. *$p<0.05$, ***$p<0.001$ between experimental conditions; ##$p<0.01$, ###$p<0.001$ vs not stimulated (NS) and \$\$ $p<0.01$, \$\$\$ $p<0.001$ vs positive control (10% FBS), were considered statistically significant.

Then, we investigated the phenomenon in relation to EC migration by scratch assay. On confluent HUVEC monolayers, seeded in a 24-well multi-well plate (80'000 cells/well), scratches were induced and cells were stimulated for 24 h with erucin and VEGF, alone or in combination. As showed in the graph of Figure 2.9-B, ECs treatment with the H₂S-donor leads to a percentage of scratch closure significantly lower than that obtained with VEGF alone. On the other hand, simultaneous stimulation produced an enhanced pro-migratory effect on HUVEC; indeed the combined use of erucin and VEGF leads to a wound closure of approximately 80%, a much higher percentage than that obtained with their use as single stimuli.

2.4.5. Erucin, alone or in combination with VEGF, recovers HG-induced impairment of EC viability and functional responses

In a recently published work, it has been shown that erucin, at a concentration of 3 µM (which is 1×10^3 times higher than the one used in this study), is able to recover endothelial damage associated with the presence of a high concentration of glucose, restoring cell viability and reducing both inflammation and oxidative stress (Martelli et al., 2021). On the basis of these evidences and results obtained in previous chapters, related to the pro-angiogenic activity of erucin at nanomolar concentrations, we investigated the ability of the isothiocyanate 3 nM to restore HUVEC vitality and functional responses compromised by hyperglycemic conditions.

To this end, an MTT assay was initially conducted to test the H₂S-donor ability to restore the reduction in cell viability due to the presence of glucose at a concentration of 30 mM. In this experiment HUVECs were seeded at 4'000 cells/100 µl in a 96-well plate and the following day medium was removed and, in some conditions, cells were pre-treated for 1 h with the effective concentration of erucin 3 nM (P^{+ERU}); in the other conditions cells did not undergo pretreatment. Afterwards, in all wells of the experiment, medium was replaced with fresh EBM-2 supplemented with 0.1% FBS and glucose 30 mM in the absence or presence of erucin 3 nM for additionally 24 h.
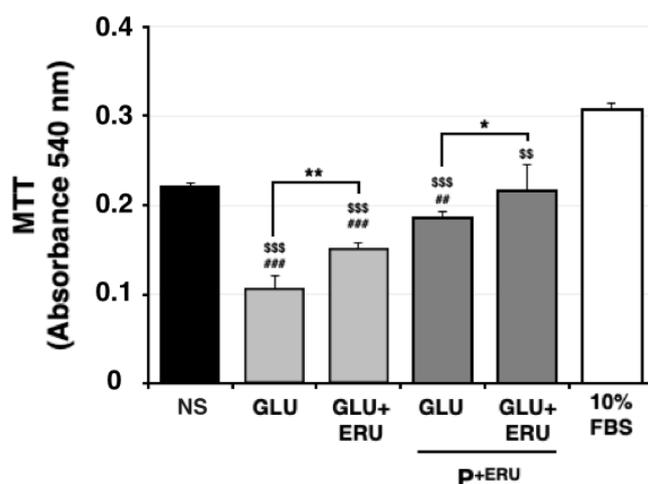
Figure 2.10. Erucin prevents HG-induced reduction in cell viability. Graphs show HUVEC viability exposed to high levels of glucose (30 mM) in the presence or absence of erucin 3 nM. Pre-incubation with erucin for 1 h (P^{+ERU}) prevents a dramatic reduction in cell viability and addition of the isothiocyanate, both in pre-incubation and together with glucose, restores viability values to basal levels (NS). Graphs represent means +/- SD of 3 independent experiments. In statistical analysis, values of *$p < 0.05$, **$p < 0.01$ between treatments; ##$p < 0.01$, ###$p < 0.001$ vs not stimulated (NS); and \$\$$p < 0.01$, \$\$\$$p < 0.001$ vs Positive control (10% FBS), were considered statistically significant.

As represented by the graphs in Figure 2.10, the co-treatment of HUVEC with glucose and erucin, in the absence of a pre-treatment, leads to a significant recovery of cell viability,

compared to the treatment with glucose alone, during which, instead, a remarkable reduction in absorbance can be observed (of about 50% compared to basal levels). However, only the addition of erucin, both in pre-incubation (P^{+ERU}) and, subsequently, as a co-treatment with glucose, restores the impairment to basal levels of cell viability and can be compared with the non-stimulated control (NS).

Following the above results, in the context of cell migration, the protective effect of erucin alone, or combined with VEGF, on ECs in hyperglycemic conditions was evaluated by scratch assay. To this end, HUVEC were starved overnight in EBM-2 with 0.1% FBS and then pre-treated with glucose 30 mM for 24 h. Subsequently, endothelial monolayers were scratched and erucin 3 nM, alone or combined with VEGF, were added in each well for 24 h.
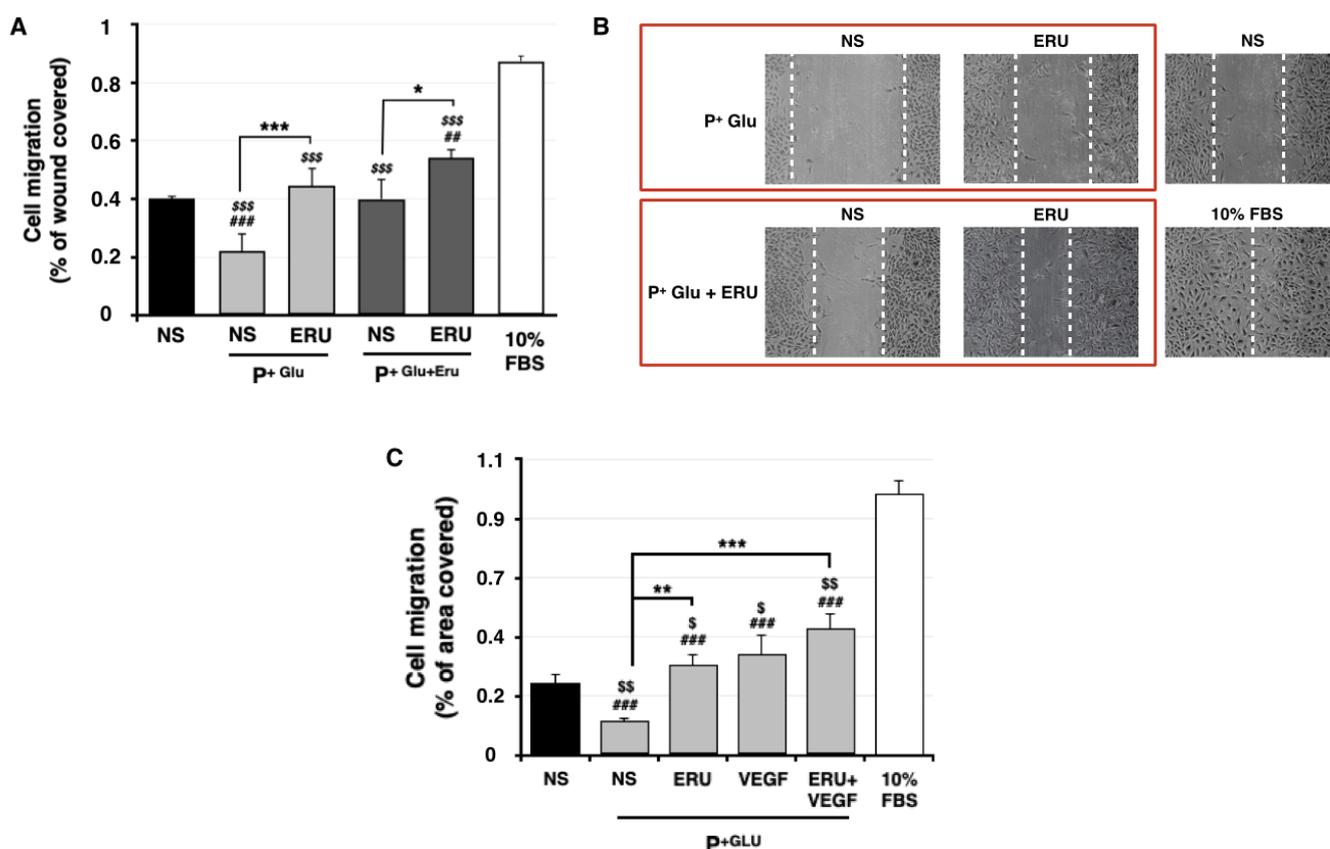
Figure 2.11. Erucin, singularly or combined with VEGF, recovers HG-induced impairment of cell migration. A) Erucin recovers high glucose-induced impairment of cell migration. Endothelial cells were starved overnight and then pre-incubated with high glucose (30 mM) for 24 h in presence or absence of erucin 3 nM. After that, HUVEC monolayers were scratched and cells were stimulated with or without erucin. B) Micrographs (10X) of the scratched areas covered by HUVEC after 24 h. C) Erucin and VEGF co-stimulation promote endothelial cell migration in conditions hyperglycemia. HUVEC were starved overnight and then pre-treated with glucose for 24 h, subsequently scratches were generated and cells were stimulated with erucin and VEGF, alone or in combination. Both graphs A) and C) represent means +/- SD of 3 experiments conducted in triplicate. In statistical analysis, values of *p < 0.05, ***p < 0.001 between treatments; ##p < 0.01, ###p < 0.001 vs not treated (NT); and $$$p < 0.001 vs positive control (10% FBS), were considered statistically significant.

Pre-treatment with 30 mM of glucose (P^{+GLU}) (Figure 2.11-A and B) exerts a strong inhibitory effect on HUVEC migration, leading to a percentage of scratch closure which is approximately 50% lower than basal control (NS). Stimulation with erucin 3 nM has been shown to restore the migratory capacity of ECs to basal levels following high glucose damage. When the isothiocyanate is added both in the pretreatment with 30 mM glucose (P$^{+GLU+ERU}$) and during stimulation, it leads to values of wound closure that overtake those of the basal condition (NS) by 30%. So erucin, in the context of high glucose endothelial dysfunction, not only recovers ECs migratory capacity, but also exerts its pro-migratory effect in hyperglycemic conditions. Stimulation of ECs with erucin and VEGF individually in high glucose conditions (Figure 2.11-C), restores migration values to physiological levels. However, although no significant difference between single treatments can be observed, co-stimulation with both compounds promotes wound closure that exceed baseline migration levels by 40%.

After demonstrating the ability of erucin to restore and preserve both EC vitality and migration in high glucose conditions, we verified whether the H$_2$S-donor was able to promote the formation of capillary-like structures in hyperglycemic conditions by performing a tube formation assay on Matrigel. To this purpose HUVEC were grown to sub-confluence in 60 mm-diameter Petri dishes and then treated for 24 h with glucose 30 mM in EBM-2 supplemented with 0.1% of FBS, in absence or presence of erucin 3 nM. Subsequently cells were detached, counted and seeded on Matrigel in basal medium with low serum for 4 h of incubation, at the end of which network formation analyzed by means of the number of tubular structures formed and branching points per well.
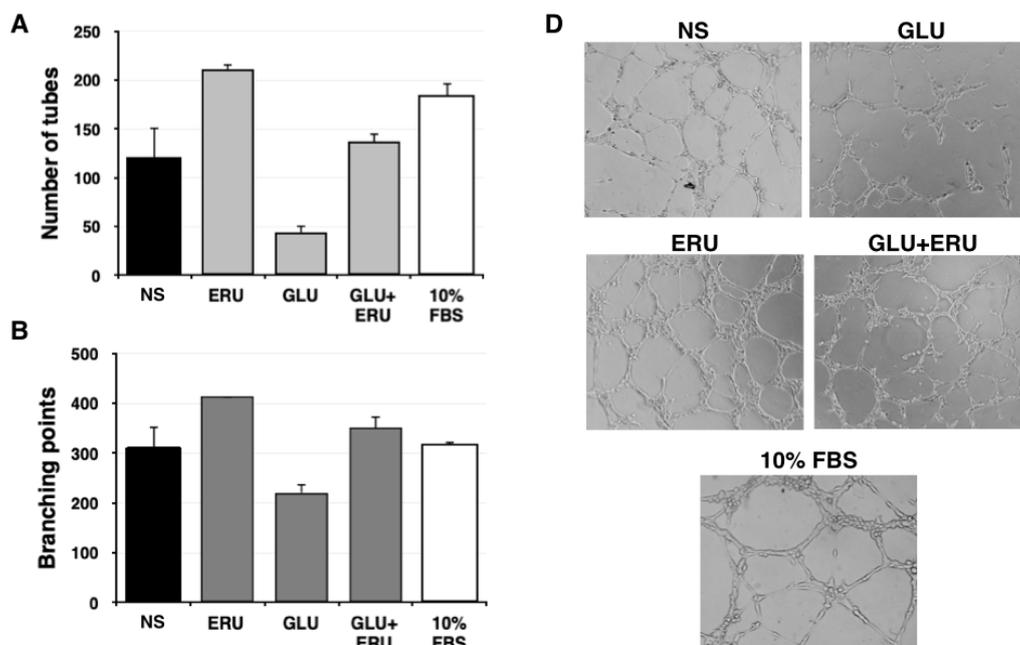

Figure 2.12. Erucin fully recovers HUVEC ability to form capillary-like structures in the presence of high concentrations of glucose. Adherent HUVEC were pre-treated with glucose, erucin, or the combination of

both, in presence of low serum. After 24 h cells were detached, counted and seeded a Matrigel-coated 48-well plate, in medium with low serum, at a density of 40'000 cell/well. HUVEC ability to organize in capillary like structures was monitored after 4 h using an inverted microscope (20X magnification). Data shown in graphs A-B) represent means +/- SD of 1 independent experiment and network structure was characterized by the number of tubes and branching points per well. D) Representative micrographs of the network formed after 4 h of incubation on Matrigel.

In our experimental conditions (Figure 2.12) erucin seems to fully recover ECs ability to form capillary like-structures in hyperglycemic conditions (GLU+ERU); as a matter of fact, the network formed can be compared to the one obtained without treatments (NS) in terms of number of tubes formed and branching points per well. From the images shown in Figure 2.12 it is also re-confirmed erucin ability to induce a more complex network of tubular structures, in normoglycemic conditions, compared to positive controls (10% FBS); indeed the mesh obtained from erucin stimulation is characterized by a significant increase in the number of tubes and branching points.

2.4.6. Erucin affect HaCaT, but not NHDF, viability and migration *in vitro*

Angiogenesis is a fundamental process in the context of wound healing to which it contributes, in the proliferative phase, by sustaining the formation of granulation tissue together with fibroblasts. The proliferative phase starts with the recruitment of fibroblasts and endothelial cells; new collagen, fibronectin and other matrix molecules are deposited by fibroblasts, and endothelial cells perform angiogenesis to support the new tissue and restore the wound integrity (Li et al., 2003; Reinke and Sorg, 2012). Granulation tissue develops in the last step of the proliferative phase, characterized by a high density of fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles. Epithelial cells and keratinocytes also proliferate and migrate over the provisional matrix of the underlying granulation tissue, eventually closing the wound (Werner et al., 2007).

In order to contextualize erucin pro-angiogenic effect in tissue repair, it is important to investigate the action of the isothiocyanate on other cell lines involved in this process, such as fibroblasts and keratinocytes. As a first approach we considered cell vitality by performing an MTT assay: NHDF and HaCaT cells were seeded at the cell density of 4'000 cells/100 µl in a 96-well plate in the appropriate medium supplemented with 10% FBS. After 24 h of incubation to allow cell adherence, cells were stimulated for 48 h with erucin diluted in basal medium FBM-2 for NHDF, or DMEM for HaCaT, supplemented with 0.1% FBS. Specifically, cells were challenged with 10-fold erucin serial dilutions from 300 to 3 nM.

By analyzing the concentration-response curves shown in Figure 2.13-A and D, it can be observed that, while fibroblasts do not seem to be affected by the presence of erucin, as similarly noted with HUVEC, keratinocytes show an increase in viability at the concentration of 3 nM, the same used in this work for its pro-angiogenic effect.
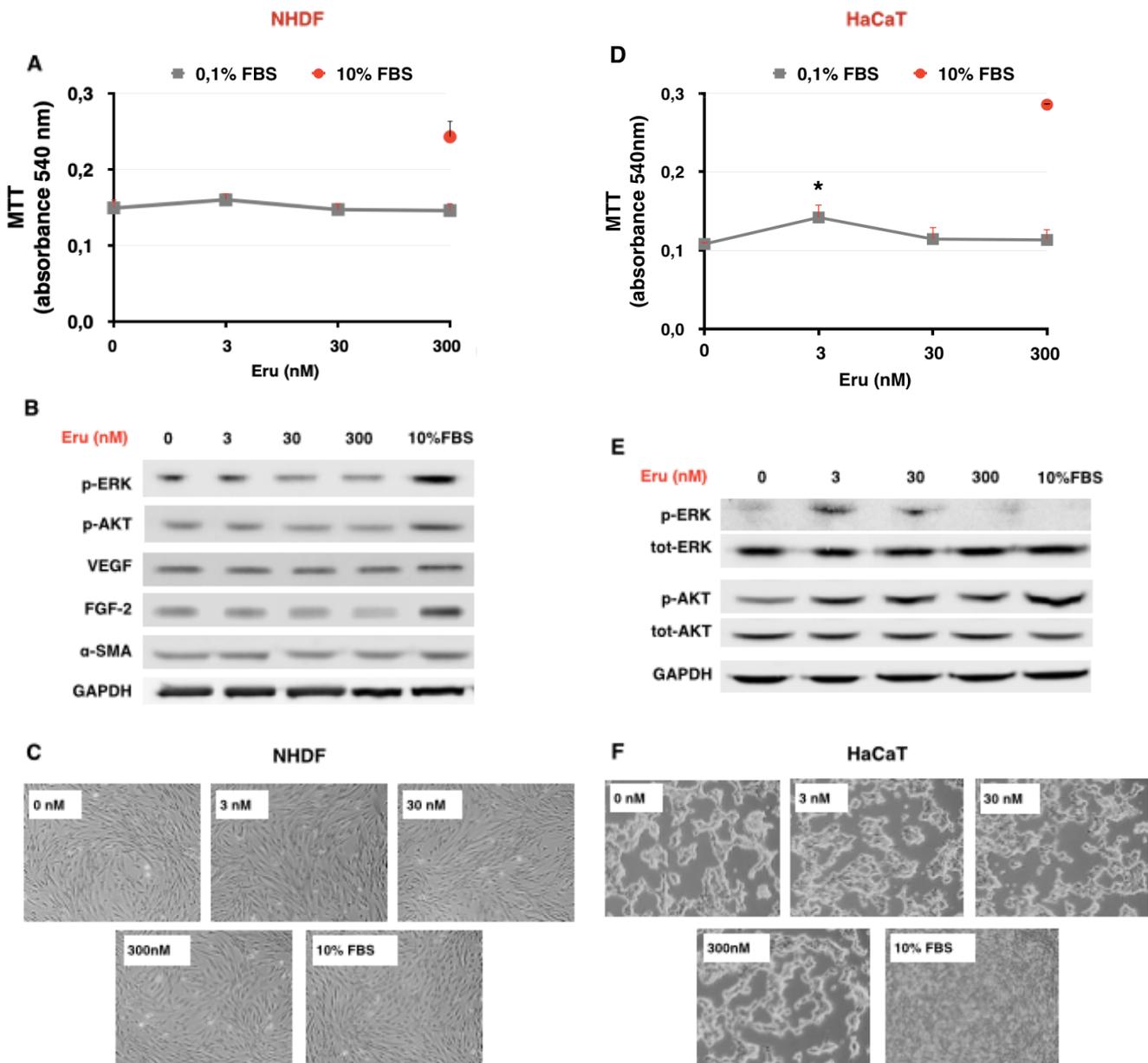
Figure 2.13. Differential effects of erucin on HaCaT and NHDF. Fibroblasts (A) and keratinocytes (D) were seeded at a cell density of 4'000 cells/well and then stimulated for 48 h with erucin serial dilutions in order to assess cell viability by MTT assay. Both graphs A and D represent means +/- SD of 3 independent experiments conducted in triplicate. Values of *p < 0.05, was considered statistically significant.

B and E) HaCat and NHDF were seeded on 60 mm-diameter Petri dishes at a cell density of 350'000 cells/dish and, after reaching a sub-confluence state, cells were stimulated with 10-fold serial dilutions of the compound. After 24 h, whole cell lysates were collected and western blot was performed to assess protein activation by phosphorylation of Akt and ERK1/2 in both cell lines, or protein abundance of VEGF, FGF-2 and α-SMA in fibroblasts. Before cell lysis and protein extraction, micrographs of stimulated NHDF (C) and HaCaT (D) were acquired. Data of panels B, C, E and F, are representative of a single experiment.

Both trends were subsequently confirmed by molecular analyses obtained by western blot (Figure 2.13-B and E) on the survival markers, ERK1/2 and Akt, after stimulation of the

two cell lines with erucin 3 nM for 24 h. Again, the H$_2$S-donor does not seem to affect fibroblast vitality, as phosphorylation of ERK and Akt is not modulated, nor promote angiogenesis by the up-regulation of VEGF or FGF-2, and it also does not seem to favor an increase in alpha-SMA, a marker of myofibroblast transition. On the other hand, in keratinocytes both pro-survival markers are activated following long-term stimulation with erucin, in agreement with data obtained by MTT. These findings are in agreement with micrographs acquired of NHDF and HaCaT following stimulation with the isothiocyanate for 24 h in low serum (0.1% FBS - Figures 2.13-C and F). Images do not highlight any changes in NHDF shape or numerosity (2.13-C); indeed, at all erucin concentrations tested, cell densities are maintained unvaried. For keratinocytes (2.13-F), instead, an increase in size of cell aggregates can be observed at 3 and 30 nM of erucin, suggesting a pro-proliferative effect which, however, still needs confirmation by appropriate proliferation measurements.

These preliminary results were subsequently investigated in the context of cell migration, which is fundamental in the final stages of tissue healing leading to wound edges closure and re-epithelialization. Also in this case the two cell lines, NHDF and HaCaT, were brought to confluence in a 24-well plate and subsequently the monolayers were scratched and stimulated with serial dilutions of the H$_2$S-donor in basal medium, or DMEM for HaCaT, containing low serum (0.1% FBS).
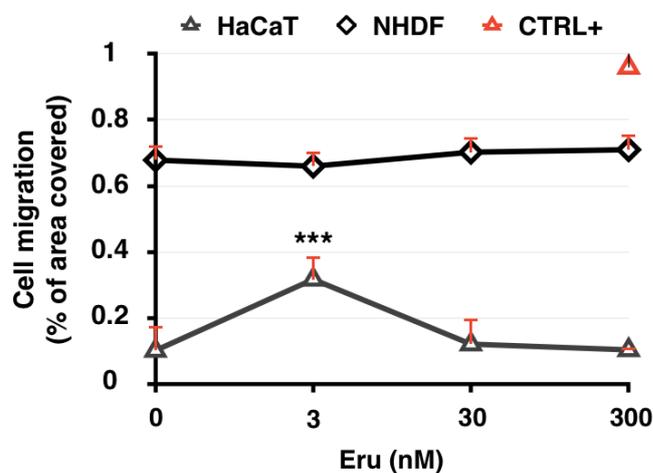
Figure 2.14. Erucin promotes HaCat, but not NHDF, migration. Both cell lines were brought to confluence and monolayers were scratched with a 200 µl sterile tip. Subsequently cells were stimulated for 24 h, with 10-fold erucin serial dilutions in medium containing 0.1% FBS. For both HaCaT and NHDF stimulation with 10% FBS leads to a complete closure of scratched area and represents our positive control (CTRL+). Preliminary data shown in the graph represent means +/- SD of 2 independent experiment, each condition was tested in 4 replicates. Values of ***$p < 0.001$, was considered statistically significant.

Interestingly, fibroblasts do not appear to migrate in response to erucin stimulation, not even at the effective concentration of 3 nM examined in this experimental work (Figure

2.14). On the other hand, keratinocytes respond positively to nanomolar concentrations of the H₂S-donor, albeit with very poor performance; indeed, although erucin 3 nM clearly increases migration compared to the basal level after 24 h of stimulation, the percentage of scratch covered does not even reach 50%. The baseline condition also shows a very poor migration rate.

In conclusion, despite the isothiocyanate shows pro-angiogenic activity on ECs and promotes the vitality and migration of keratinocytes, it does not appear to exert any effect on dermal fibroblasts in the experimental conditions tested.

2.4.7. Erucin indirectly influences the proliferation of NHDF through its action on endothelial cells

In section 2.4.3 we reported that ECs treatment with erucin leads to the intracellular up-regulation, and presumably secretion, of key growth factors such as VEGF and FGF-2 (Figure 2.7-C and D) which are thought to promote a positive feedback and support the angiogenesis process over a prolonged time. However FGF-2, in the context of wound healing, is also a crucial signaling molecule for fibroblasts, both for the maintenance of vitality and in the proliferation process (Alhajj et al., 2021). For this reason we hypothesized that *in vitro* HUVEC stimulation with the isothiocyanate could support both the generation of new blood vessels and fibroblast proliferation, two fundamental events in the formation of granulation tissue. To demonstrate this hypothesis, indirect co-cultures were set-up between HUVEC and NHDF aimed at evaluating the proliferation of the latter, under the effect of the ECs stimulated by erucin. The first proliferation assay involved the stimulation of fibroblasts with medium conditioned by HUVEC treated with erucin for 48 h; the second experiment involved joint cultures, through the use of transwell (for protocol see section 2.3.7).
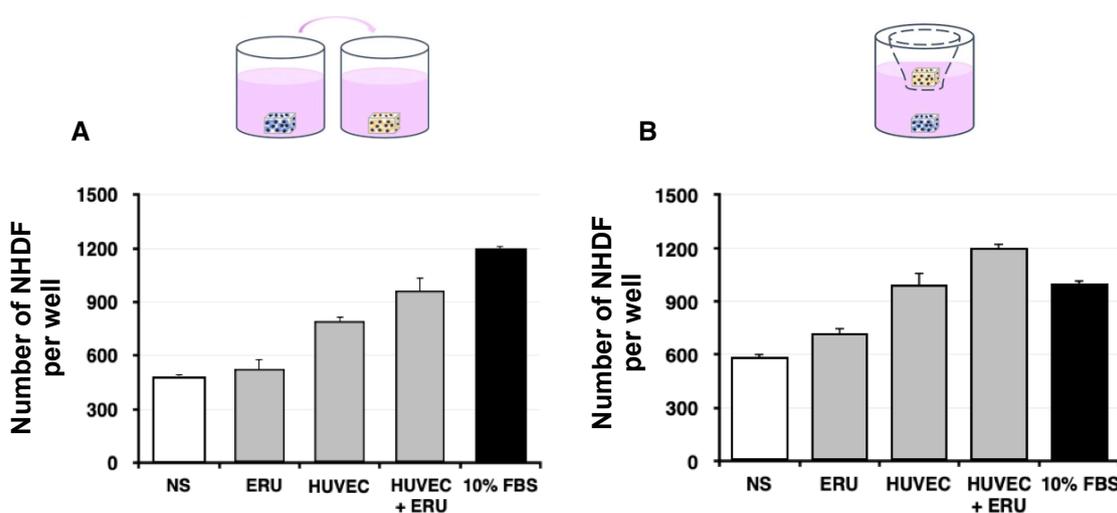
Figure 2.15. HUVEC stimulated with erucin positively influence fibroblast proliferation. In the first experiment (A) NHDF were seeded in a 96-well and the following day, fibroblasts were treated for 48 h with medium (EBM-2 with 0.1% serum) conditioned by HUVEC under stimulation with 3 nM erucin for 48 h. After two days, cells were fixed, stained and counted (5 fields per well). B) NHDF were co-cultured with HUVEC

using transwell for 48 h (0.1% FBS) in presence of erucin 3 nM; fibroblasts were then fixed, stained and counted (5 random fields per well). Data shown in graphs A and B derive from a single experiment in which every condition was tested in at least three replicates. No statistical analysis was performed.

From both types of assays it can be deduced how HUVEC considerably influence fibroblast proliferation when stimulated by erucin. The effect is even more pronounced when both cell lines are co-cultured (Figure 2.15-B) using a transwell system, presumably because in this case the growth factors released in the medium directly affect the underlying NHDF, while in the experiment shown in Figure 2.15-A conditioned medium underwent a freezing process for conservation, which led to a partial degradation of the factors contained.

In conclusion, it can be discussed that erucin, despite its limit of action on dermal fibroblasts, can still act on NHDF proliferation by an indirect effect that passes through endothelial cells, and therefore is able, in the context of wound healing, to promote a stromal-endothelial crosstalk and sustain the proliferative phase also considering its direct pro-angiogenic properties.

2.5. Discussion

The interesting physiological and pharmacological properties of the gaseous transmitter H_2S have been the subject of intense studies over the last few years, and today the profile characterization of novel H_2S-releasing molecules is needed as a preventive and therapeutic strategy for the treatment of diseases affecting the cardiovascular system (Yang and Wang, 2015). In this context, it is evident from literature that hydrogen sulfide released by natural ITCs is characterized by a slow, controlled and constant kinetics and therefore is capable of mimicking the various physiological effects of endogenous H_2S (Martelli et al., 2020a; Citi et al., 2014). Erucin has shown favorable characteristics, especially by virtue of its presence in an edible plant without toxicity, such as rocket salad, which is constantly used in human nutrition. The pharmacological effects of erucin, including an already known antihypertensive and vasodilatory action (Martelli et al., 2020b), led researchers to investigate the effects of this molecule on the cardiovascular system, with the aim of being able to expand its field of application.

Considering that there is currently no scientific evidence that indicates erucin as a compound with a pro-angiogenic properties, in this work we have decided to investigate this aspect, both in physiological and pathological conditions of hyperglycemia, which is known to induce endothelial dysfunction. Our results demonstrate that erucin, at the effective concentration of 3 nM, positively modulates HUVEC angiogenic functional responses *in vitro*, such as the ability to migrate and organize in tubular and complex structures, through the early activation of eNOS - ERK1/2 - Akt pathway. Finally, erucin alone or in combination with VEGF, shows the ability to protect endothelial cells from high glucose-induced damage and recover impaired functional responses to physiological levels. These data shed new light on the use of erucin for the promotion of physiological angiogenesis and recovery of endothelial dysfunction induced by the presence of high concentrations of glucose, typical of patients affected by diabetes.

Angiogenesis is a fundamental process in the context of wound healing to which it contributes, in the proliferative phase, by sustaining the formation of granulation tissue to support the new tissue and restore the wound integrity (Sorg et al.,2018). Considering the pro-angiogenic action of erucin highlighted in this work, and considering the many evidences that underline the importance of H_2S, not only in the process of angiogenesis, but also in the various phases of wound healing (as discussed in chapter 1.4.) by directly acting on different cell lines involved in this process, in the final phase of this experimental work we began to investigate the properties of the isothiocyanate in the context of tissue regeneration by combining multiple types of cells (namely keratinocytes and fibroblasts). From preliminary data obtained in this thesis we can conclude that erucin exerts pro-survival and pro-migratory effects on keratinocytes in a concentration dependent manner by up-regulating Akt and ERK1/2. In accordance with pro-angiogenic data, also in this context the effective concentration of erucin 3 nM seems to elicit optimal responses in HaCaT cells. On the other hand, fibroblast vitality and migration are not directly affected by

the compound at any concentration tested. However, in a model of indirect co-cultures, HUVEC stimulation with erucin promotes the release of growth factors that positively modulate NHDF proliferation. Therefore erucin is able, in the context of wound healing, to promote a crosstalk between ECs and fibroblasts. Further studies will be addressed at validating the preliminary data shown in this thesis. However, based on these initial findings, we can start to consider erucin as a therapeutic perspective for pathological conditions due to defects related to wound healing.

3. Topic 2: Therapeutical control of inflammation in impaired tissue healing

3.1. Macrophage-fibroblast crosstalk in normal and chronic wound healing

Chronic wounds are characterized by a disrupted repair process that solves within longer time than normal healing, which usually takes between 4 to 6 weeks. In this condition, injured tissue fails to progress through the orderly stages of healing, remaining in a prolonged and intensified inflammatory state which cause pathologic conditions ranging from healing delay (e.g., chronic ulcers) to fibrosis (Qing, 2017).

Inflammation is regulated by a plethora of cell populations, biochemical, and physical factors, but it is widely recognized that the cross-talk between macrophages and fibroblasts, and their ability to assume different phenotypes play a crucial role in determining not only the evolution of inflammation, but also the subsequent stages of the healing process (Glaros, 2009). In normal healing, activated macrophages induce the stimulation of resident fibroblasts via production of transforming growth TGF-β, TNF-α, IL-1β, and other inflammatory mediators (Wynn and Vannella, 2016; Zhao et al., 2016). In turn, activated fibroblasts modulate the recruitment and behavior of immune cells through an NF-κB dependent release of cytokines, vasoactive and pro-angiogenic factors, as VEGF, NO and prostanoids (e.g. prostaglandin E2). Furthermore, activated fibroblasts regulate tissue remodeling by combining their ability to synthesize ECM components and that of assuming contractile properties (Hinz, 2016; Zhao et al., 2016) (Figure 3.1).
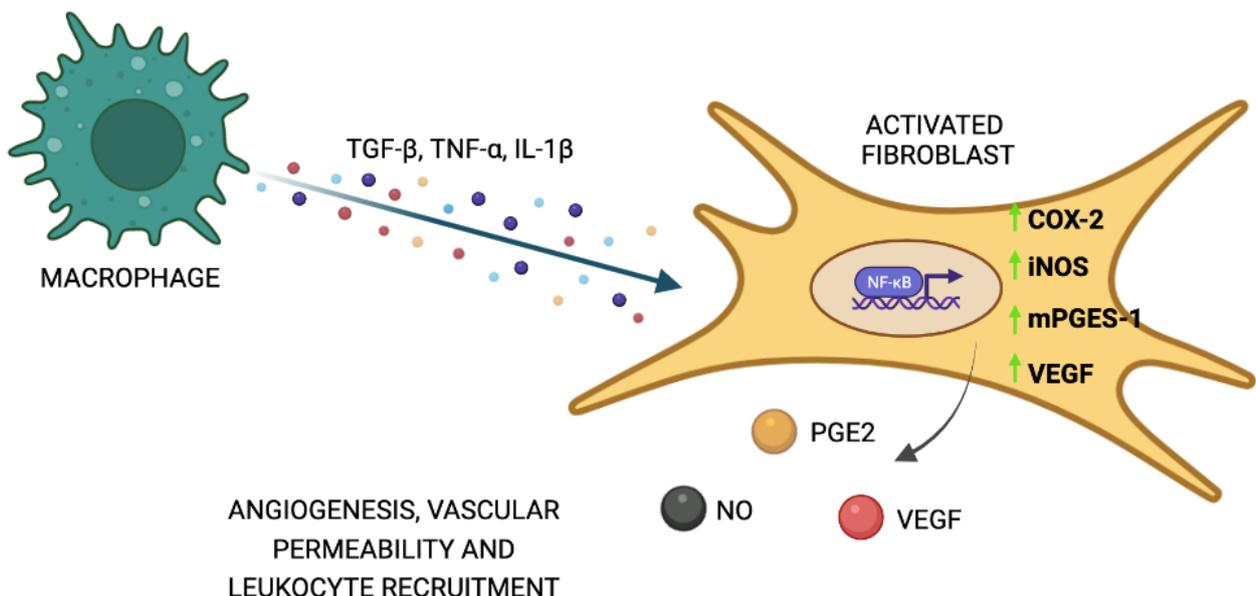

Figure 3.1. Schematization of macrophage-fibroblast crosstalk in inflammation. Illustration realized with Bio Render platform.

Inflammation dysregulation and persistence of macrophage-fibroblast activation state, with excessive production of pro-inflammatory agents, ECM components and vasoactive factors

by fibroblasts, leads to altered repair processes which, at the tissue level, is characterized by redness, heat, edema, pain, and can lead to fibrosis (Hinz, 2016; Qing, 2017; Zhao et al., 2016). Thus, eliminating or controlling the excessive inflammation can represent a turning point in the transition to the following stages of healing and can effectively resolve many chronic wounds.

3.1.2. Standard and adjuvant therapies for chronic wound management

The standard practices in chronic wound management include surgical debridement (removal of all necrotic and devitalized tissue), dressings to facilitate a moist wound environment and exudate control, wound off-loading (relieving pressure and shear stress, especially for feet ulcers) and resolution of bacterial infection with topical or systemic antibiotic administration (Everett and Mathioudakis, 2018). Other therapeutical practices aim at terminating the excessive and chronic inflammation by employing steroidal or non-steroidal anti-inflammatory drugs. However, side effects, or even opposite effects on wound healing, such as hypertrophic scarring, contraction, and necrosis, can limit their employment, especially considering their long-term use, raising the necessity for alternative countermeasures (Dreifke et al., 2015).

Alongside to standard practices, adjuvant therapies have been implemented in recent years to reduce inflammation and accelerate wound healing such as ultrasounds, laser therapy, and other forms of photobiomodulation (Dreifke et al., 2015; Nesi-Reis et al., 2018; Priyadarshini et al., 2018; Micheli et al., 2019). Specifically, photobiomodulation therapy (PBMT), is one of the most widely applied to manage many different diseases characterized by acute or chronic inflammation. The benefits of PBMT in terms of anti-inflammatory (Hamblin, 2017), anti-pain (Tantawy et al., 2019; Micheli et al., 2017), and anti-edema properties are widely documented in literature (Enwemeka et al.,2004). Moreover, PBMT enhances cell energy metabolism and promotes anabolic and repair processes (Enwemeka et al., 2004). Further, PBMT has been shown to stimulate angiogenesis and collagen remodeling (de Medeiros et al., 2017) and is a safe, non-invasive, therapy well accepted by patients, due also to the short duration of application.

3.3. Aim of the study

In the following paper, an *in vitro* model of fibroblast activation via stimulation with the pro-inflammatory cytokines IL-1β and TNF-α has been proposed and used to test the anti-inflammatory effect of a dual wavelength NIR laser source, widely used in clinics to promote healing and reduce inflammation and pain. The anti-inflammatory effect of near infrared laser radiation has been already reported in a number of studies but, to the best of our knowledge, it is the first time that it is evaluated and confirmed in an *in vitro* model of dermal fibroblasts activated by a mix of cytokines (IL-1β and TNF-α). Results reported in this paper demonstrate the effectiveness of PBMT by a dual wavelength NIR laser source (MLS-MiS) in modulating the inflammatory response and favoring cell return to the basal physiological state.

3.4. Paper: "Effect of NIR Laser Therapy by MLS-MiS Source on Fibroblast Activation by Inflammatory Cytokines in Relation to Wound Healing"

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Article

Effect of NIR Laser Therapy by MLS-MiS Source on Fibroblast Activation by Inflammatory Cytokines in Relation to Wound Healing

Shirley Genah [1,†]**, Francesca Cialdai** [2,†]**, Valerio Ciccone** [1]**, Elettra Sereni** [2]**, Lucia Morbidelli** [1,‡]
and Monica Monici [2,*,‡]

1   Department of Life Sciences, University of Siena, I-53100 Siena, Italy; shirley.genah@student.unisi.it (S.G.);
    ciccone3@student.unisi.it (V.C.); lucia.morbidelli@unisi.it (L.M.)
2   ASA Campus Joint Laboratory, ASA Research Division & Department of Experimental and Clinical
    Biomedical Sciences "Mario Serio", University of Florence, I-50139 Florence, Italy;
    francesca.cialdai@unifi.it (F.C.); elettra.sereni@unifi.it (E.S.)
*   Correspondence: monica.monici@unifi.it; Tel.: +39-0552758366
†   These authors contributed equally to this work.
‡   These authors contributed equally to this work.

Abstract: The fine control of inflammation following injury avoids fibrotic scars or impaired wounds. Due to side effects by anti-inflammatory drugs, the research is continuously active to define alternative therapies. Among them, physical countermeasures such as photobiomodulation therapy (PBMT) are considered effective and safe. To study the cellular and molecular events associated with the anti-inflammatory activity of PBMT by a dual-wavelength NIR laser source, human dermal fibroblasts were exposed to a mix of inflammatory cytokines (IL-1β and TNF-α) followed by laser treatment once a day for three days. Inducible inflammatory key enzymatic pathways, as iNOS and COX-2/mPGES-1/PGE2, were upregulated by the cytokine mix while PBMT reverted their levels and activities. The same behavior was observed with the proangiogenic factor vascular endothelial growth factor (VEGF), involved in neovascularization of granulation tissue. From a molecular point of view, PBMT retained NF-kB cytoplasmatic localization. According to a change in cell morphology, differences in expression and distribution of fundamental cytoskeletal proteins were observed following treatments. Tubulin, F-actin, and α-SMA changed their organization upon cytokine stimulation, while PBMT reestablished the basal localization. Cytoskeletal rearrangements occurring after inflammatory stimuli were correlated with reorganization of membrane α5β1 and fibronectin network as well as with their upregulation, while PBMT induced significant downregulation. Similar changes were observed for collagen I and the gelatinolytic enzyme MMP-1. In conclusion, the present study demonstrates that the proposed NIR laser therapy is effective in controlling fibroblast activation induced by IL-1β and TNF-α, likely responsible for a deleterious effect of persistent inflammation.

Keywords: wound healing; NIR laser radiation; inflammation; fibroblasts; photobiomodulation

1. Introduction

Any injury or infection triggers an inflammatory reaction via cytokines deriving from platelet degranulation and pathogen-associated molecular patterns. Moreover, in both cases, damaged cells release reactive oxygen species and non-specific factors which contribute to activate the inflammatory response in cells of the innate immune system, fibroblasts, and epithelial and endothelial cells [1]. The induction of the inflammatory response triggers a cascade of events mediated by recruitment, proliferation, and activation of several cell populations, primarily immune and stromal cells, as well as further release of cytokines, vasoactive factors, and growth factors that all together contribute to the repair process [2,3].

Therefore, the correct progression of any acutely occurring inflammatory reaction is a key factor in the path leading to successful healing, which consists in repair/regeneration of damaged tissues and function recovery. However, the occurrence of alterations in the finely-tuned regulation of inflammation can cause pathologic conditions ranging from healing delay (e.g., chronic ulcers) to fibrosis. Moreover, conditions of tissue stress or altered function can induce an adaptive response, known as parainflammation or low grade chronic inflammation, which is an intermediate condition between basal homeostasis and acute inflammation, and is associated with serious diseases, including obesity, diabetes, atherosclerosis, asthma, and neurodegenerative diseases [4].

Inflammation is regulated by a plethora of cell populations, biochemical, and physical factors, but it is widely recognized that the cross-talk between macrophages and fibroblasts, and their ability to assume different phenotypes play a crucial role in determining not only the evolution of inflammation, but also the subsequent stages of the healing process.

During inflammation, macrophages shift from a pro-inflammatory phenotype (the so called M1), characterized by massive production of pro-inflammatory molecules, to an anti-inflammatory phenotype (the so called M2), which secretes suppressors of cytokine signaling, passing through intermediate phenotypes [3,5–7].

In response to pro-inflammatory mediators, resident fibroblasts or circulating fibrocytes become the protagonists of the stromal activation and transdifferentiate in myofibroblasts, their activated counterpart. Many pro-inflammatory mediators are implicated in fibroblast activation, migration, proliferation, and transdifferentiation, including the cytokines tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and the growth factors platelet derived growth factor (PDGF) and fibroblast growth factors (FGFs). Activated fibroblasts and other mesenchymal cells engage a crosstalk, which also reinforces the local immune response due to the induction of vasodilation throught production of nitric oxide (NO) and prostanoids, and stimulates angiogenesis via vascular endothelial growth factor (VEGF) production [8,9].

In a normal evolution of the process, the turning off of the inflammatory response, mediated by the shift of the macrophage phenotype from M1 to M2, opens the way to the remodeling phase, which is dominated by fibroblasts through the production of extracellular matrix (ECM) proteins and matrix metalloproteinases (MMPs) [10]. A well-timed resolution of inflammation is crucial for successful restoration of tissue architecture and function, while persistence of macrophage-fibroblast activation state, with excessive production of pro-inflammatory agents by fibroblasts and further recruitment of immune cells, leads to altered repair processes, from chronic wounds to fibrosis and scarring [11,12].

In summary, activated macrophages induce the stimulation of fibroblasts via production of transforming growth factor-β (TGF-β), TNF-α, IL-1, and other cytokines. In turn, activated fibroblasts can modulate the recruitment and behavior of immune cells via release of cytokines and vasoactive factors as NO and prostanoids. Activated fibroblasts, or myofibroblasts, regulate tissue remodeling by combining their ability to synthesize ECM proteins and that of assuming contractile properties [13,14]. Contractile activity of myofibroblasts increases ECM stiffness. In turn, ECM stiffness is, together with TGF-β1, among the most important factors in inducing myofibroblast differentiation and persistence. Therefore, inflammation dysregulation can generate a feed-forward loop with detrimental effects [15]. Therefore, the control of inflammation and fibroblast activation is crucial to obtain satisfactory morpho-functional recovery and avoid defective healing.

Whatever the cause of inflammation (wound, trauma, infection), at the tissue level it is characterized by redness, heat, oedema, pain, and loss of function.

In current clinical practice, a series of steroidal and nonsteroidal anti-inflammatory drugs can be used to control inflammation and the associated oedema and pain [16]. However, side effects, or even opposite effects on wound healing and other conditions inducing inflammation, limit their use, especially considering long-term therapy, raising the need for alternative countermeasures [16]. Moreover, anti-inflammatory strategies focused on a specific target (e.g., TNF-α) did not produce the desired results [17]. Several

physical therapies and devices aimed to favor the healing process through the control of inflammation and fibroblast behavior have been proposed, such as ultrasound, laser therapy, electrical stimulation, and vacuum-assisted closure [16,18,19]. Studies aimed at elucidating the effectiveness of these therapies in controlling inflammation and the deriving fibroblast activation might strengthen their use.

Laser therapy, currently called photobiomodulation therapy (PBMT), is one of the most widely applied to manage many different diseases characterized by acute or chronic inflammation. The benefits of PBMT in terms of anti-inflammatory [20], anti-pain [21–23], and anti-oedema [24] properties are widely documented in literature. Moreover, PBMT enhances cell energy metabolism and promotes anabolic and repair processes [25]. Further, PBMT has been shown to stimulate angiogenesis and collagen remodeling [16,26].

PBMT, being safe, non-invasive, and non-time-consuming (short-duration application) is also well accepted by patients.

Over the last years, new molecular insights into the action mechanisms of PBMT have been obtained. In particular, it has been demonstrated that in chronic inflammatory conditions, such as those related to periodontal diseases and osteoarthritis, PBMT is effective in reducing the expression of pro-inflammatory genes (TNF-α, IL-1β, IL-6, IL-8) through the downregulation of NF-kB signaling pathway via cAMP increase [27].

Another PBMT effect, which can be relevant in the evolution and outcome of the inflammatory response, is to induce a decrease in matrix metalloproteinases (MMPs) expression, as it has been recently demonstrated in an in vitro model of osteoarthritis [28]. MMPs are an important family of proteinases, able to degrade extracellular matrix components and covering a broad range of tasks in inflammation, acquired immunity, defense from injury and repair. MMPs are always present in acute and chronic, physiological and pathological inflammatory processes, and experimental evidence suggests that they can protect against or contribute to pathological evolution of inflammation [29,30].

Despite the abundant literature on the ability of PBMT to control inflammation, promote healing mechanisms, and counteract scarring, the effects that laser emissions commonly used in PBMT exert on fibroblasts activated by a strong and persistent inflammatory stimulation have not been clearly defined. In fact, for the most part, studies used in vitro models of fibroblasts in the basal state.

Therefore, the present study was aimed at investigating the effect of PBMT on activated fibroblasts. An in vitro model of fibroblasts, activated by exposure to inflammatory stimuli, was characterized for morphological features, canonical inflammatory and vasoactive cascades (inducible NO synthase and cyclooxygenase (COX)/prostaglandin synthase enzymes), and outcomes on angiogenesis and ECM remodeling. Then, the effectiveness and underlying molecular mechanisms of a high power, dual wavelength NIR laser source in reducing fibroblast inflammatory phenotype was investigated.

2. Materials and Methods

2.1. Cell Cultures

Normal human dermal fibroblasts (NHDF) were purchased from Lonza (Verviers, Belgium) and grown in Fibroblast Growth Basal Medium (FBS; Lonza, Basel, Switzerland) containing 10% Fetal Bovine Serum (FBS; Hyclone, Euroclone, Milan, Italy), 2 mM glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Merck KGaA, Darmstadt, Germany). Cells were cultured at 37 °C with 5% CO_2 in Petri dishes and were split 1:3 twice a week until passage 10.

2.2. In Vitro Model of Inflammation

Cells (1 $\times$ 10^4) were seeded in 24-multiwell plates and allowed to adhere (when immunofluorescence analysis were planned, cells were seeded on 13 mm diameter glass coverslips placed inside the 24-multiwell plates). After 24 h, complete culture medium was replaced by fresh complete culture medium supplemented with a mix of IL-1β (10 ng/mL; #201-LB/CF R&D System, Minneapolis, MN, USA) and TNF-α (10 ng/mL; #201-LB/CF and

#410MT, R&D System, Minneapolis, MN, USA). Cells were maintained with the cytokines mix for 48 h. Control samples were treated in the same way, omitting the cytokines mix.

2.3. Laser Treatment

At the end of the 48 h of stimulation with cytokines mix, the medium of all samples was replaced by a fresh complete culture medium. Then, samples which had been previously stimulated with the cytokines mix were divided into two groups: A "treated group", that received laser irradiation and an "untreated group" that was not laser irradiated. Laser treatment was performed with a Multiwave Locked System laser (MLS-MiS, ASA S.r.l., Vicenza, Italy) widely used in clinics. It is a class IV, NIR laser with two synchronized sources (laser diodes): The first one is a pulsed laser diode emitting at 905 nm wavelength, with peak power from 140 W $\pm$ 20% to 1 kW $\pm$ 20% and pulse frequency varying in the range 1–2000 Hz; the second laser diode emits at an 808 nm wavelength and can operate in continuous (max power 6 W $\pm$ 20%) or frequent (repetition rate 1–2000 Hz, 50% duty cycle) mode. The two laser beams work simultaneously and synchronously, and the propagation axes are coincident.

For laser exposure, only 6 wells of 24-well plates contained cells. The wells surrounding those with cells were filled with black cardboard to avoid light diffusion and reflection. The exposure was performed by placing the plate inside a holder, which allows the positioning of the laser handpiece at a 1.5 cm distance from the bottom of the wells, so that the spot of the two laser beams, impinging perpendicular to the sample surface, had the same diameter as a well (13 mm). Cells were irradiated for 10 sec with the following parameters: 10 Hz repetition rate; 50% int (mean power 1840 mW); peak power 1 kW $\pm$ 20%, fluence 5.19 J/cm^2. All treatments were performed under laminar flow hood at room temperature. The samples belonging to the untreated group were prepared and kept under the same conditions used for the exposed samples, except for laser irradiation.

2.4. Experiment Design

The following samples were prepared, analyzed, and compared:

(i)   samples stimulated with a mix of IL-1β and TNF-α for 48 h and then exposed to 3 laser treatments, repeated once a day, for 3 consecutive days under sterile conditions (CYKs + LASER Group);

(ii)   samples stimulated with a mix of IL-1β and TNF-α for 48 h and not exposed to laser treatments CYKs Group);

(iii)   samples not stimulated with a mix of IL-1β and TNF-α for 48 h and not exposed to laser treatments (CTRL Group).

For immunofluorescence analysis, an additional experimental group was included, namely cells exposed to laser treatment alone (LASER Group).

Six hours after the third laser treatment, all samples were prepared for the subsequent analysis described in the following paragraphs.

2.5. Immunofluorescence Analysis

Cells grown on glass coverslips and treated as previously described, were fixed for 5 min with ice cold acetone. Unspecific binding sites were blocked with PBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Then, cells were incubated overnight at 4 °C with specific anti-NF-kB (1:50; #sc-372, Santa Cruz, Dallas, TX, USA), anti-cyclooxygenase-2 (COX-2) (1:100; #TA313292, Origene, Rockville, MD, USA), anti-VEGF (1:50; #sc-57496, Santa Cruz), anti-α actin (1:100; #MAB1501X, Millipore, Billerica, MA, USA), anti α-smooth muscle actin (α-SMA) (1:100; #CBL171, Chemicon® by Thermo Fisher Scientific, Waltham, MA, USA), anti-tubulin (1:100; #05-829, Millipore, Billerica, MA, USA), anti-collagen I (1:100; #MAB3391, Millipore, Billerica, MA, USA), anti-fibronectin (FN) (1:100; #MAB1926-I, Millipore, Billerica, MA, USA), anti-MMP-1 (1:100; #MAB13439, Millipore, Billerica, MA, USA), and anti-α5β1 integrin (1:100; #MAB1999, Millipore, Billerica, MA, USA) primary antibodies properly diluted in

PBS with 0.5% BSA. After washing three times with PBS-0.5% BSA, samples were then incubated for 1 h at 4 °C in the dark with: Alexa Fluor 555™ conjugated secondary antibodies [specifically: Anti-mouse IgG (#A-21422, Invitrogen™ by Thermo Fisher Scientific) for anti-NF-kB and anti-VEGF antibodies and anti-rabbit IgG (#A-21428, Invitrogen™ by Thermo Fisher Scientific) for anti-COX-2 antibody] and fluorescein isothiocyanate (FITC) conjugated specific secondary antibody [specifically: Anti-mouse IgG (#AP124F, Millipore) for anti α-SMA, anti-tubulin, anti-collagen I, anti-fibronectin, anti-MMP-1, anti-α5β1 integrin primary antibodies]. All secondary antibodies were diluted 1:200 in PBS with 0.5% BSA. Cells incubated with anti-α actin antibody did not need incubation with the secondary antibody since a mouse anti-actin Alexa Fluor® 488 conjugated was used. Again, samples were washed three times and then mounted on glass slides using Fluoromount™ aqueous mounting medium (Sigma-Aldrich St. Louis, MO, USA) [31]. In samples of incubated anti-NF-kB, anti-COX-2, and anti-VEGF, before mounting, nuclei were marked with DAPI (#D9542, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:5000 in PBS with 0.5% BSA for 30 min at room temperature. The fluorescent signal of samples stained with anti-NF-kB, anti-COX-2, and anti-VEGF antibodies was acquired using a Leica TCS SP5 laser scanning confocal microscope (Leica, Wetzlar, Germany). All other samples were evaluated by an epifluorescence microscope (Nikon, Florence, Italy) at 100x magnification and imaged by a HiRes IV digital CCD camera (DTA, Pisa, Italy). Based on the CCD images, a relative immunofluorescence quantification was carried out by image analysis routines (ImageJ 1.53 analysis software, National Institutes of Health, Bethesda, MD, USA) for samples stained with anti-collagen I and anti-α5β1 integrin antibodies. After appropriate thresholding to eliminate background signal and creation of a proper image mask, a pixel intensity histogram was acquired.

2.6. Western Blot

Cells derived from the different experimental conditions, were detached from 24 multi-well plates, collected in 15 mL tubes, and lysed with CelLytic™ MT Cell Lysis Reagent supplemented with 2 mM Na_3VO_4 and 1X Protease inhibitor cocktail for mammalian cells (Sigma-Aldrich). Cell lysates were centrifuged at $16000\times g$ for 20 min at 4 °C, and the supernatants were then collected. Protein concentration was determined using the Bradford protein assay (Sigma-Aldrich). Electrophoresis with equal amounts of proteins (50 μg) was carried out in NuPAGE™ 4–12% Bis-Tris precast Gels (Thermo Fisher Scientific) as previously reported [32].

Proteins were transferred onto nitrocellulose membranes, blocked for 1 h in a PBS–0.05% Tween solution (Sigma-Aldrich) supplemented with 5% (wt/vol) of Blotting-Grade Blocker (Bio-Rad, Hercules, CA, USA). Membranes were then incubated overnight at 4 °C with the primary antibodies properly diluted in PBS–0.05% Tween solution supplemented with 1% (wt/vol) of Blotting-Grade Blocker: anti-inducible NO synthase (iNOS) (1:250; #sc-7271, Santa Cruz), anti-COX-2 (1:1000; #160106, Cayman Chemical, Ann Arbor, MI, USA), and anti-microsomal prostaglandin E synthase-1 (mPGES-1) (1:500; #160140, Cayman Chemical). Immunoblots were washed three times with PBS–0.05% Tween solution and then incubated for 1 h with the respective species-specific secondary antibody conjugated with horseradish peroxidase HRP (Promega, Madison, Wisconsin, US) diluted 1:2500 in PBS–0.05% Tween solution. The membranes were finally incubated with SuperSignal™ West Pico PLUS chemiluminescent Substrate (Thermo Fisher Scientific), and the immunoreaction was revealed by ImageQuant LAS 4000 chemiluminescence system (GE Healthcare, Chicago, IL, USA). Results were normalized to those obtained by using an antibody against β-Actin (#A5441, Sigma-Aldrich) diluted 1:10,000 in PBS–0.05% Tween solution.

Immunoblots were analyzed by densitometry using Image J software, and the results, expressed as arbitrary density units (A.D.U.), were normalized to β-Actin.

2.7. Immunoassays for Prostaglandin E-2 and VEGF Quantification

Conditioned media were collected at the end of the experiment, frozen, and stored at −80 °C until use. Prostaglandin E-2 (PGE-2) and VEGF levels were measured using ELISA kit: Prostaglandin E$_2$ ELISA kit-Monoclonal (Cayman Chemical, Ann Arbor, Michigan, US) and VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA), respectively, following the manufacturer's instructions. Dosing of each sample was performed in double, and PGE-2 and VEGF levels were expressed as (pg/mL).

2.8. Statistics

Three different experiments were carried out in triplicate. Data are reported as means ± SD. Statistical significance was determined using two-sided Student's *t* test. A *p* value lower than 0.05 was considered statistically significant. For immunofluorescence analysis, at least 30 cells per slide were scored in 10 random fields/slide.

3. Results

3.1. Set up of an "In Vitro" Inflammatory Model in Fibroblasts Cultures

The human dermal fibroblasts NHDF have been treated with a mix of cytokines (IL-1β and TNF-α, each at 10 ng/mL) for 24 h and 48 h, then the occurrence of inflammatory features depending on the exposure time has been evaluated.

Microsomal PGE synthase-1 (mPGES-1), the pivotal inducible enzyme of the prostanoid inflammatory pathway, was evaluated by western blot after 24 h and 48 h of stimulation. A consistent rise in mPGES-1 was observed after both 24 h and 48 h, the increase being more evident at a longer time of exposure (Figure 1, upper panel, 0.8 ± 0.2 and 4.8 ± 0.9 fold increase of mPGES-1 expression in the presence of cytokines with respect to control, at 24 h and 48 h, respectively). The up-regulation of the mPGES-1 enzyme generated a significant increase in the final product, prostaglandin E2 (PGE-2), released by fibroblasts in the conditioned medium, documenting an activation of the enzymatic cascade (Figure 1, lower panel). Based on these results, the stimulation time of 48 h was chosen for further experiments.

3.2. Effect of Laser Treatment on Inflammatory Phenotype in Fibroblasts

3.2.1. Expression of Inflammatory Markers

In order to evaluate whether laser treatment could affect the inflammatory model described above, samples, after the stimulation with cytokine mix, were exposed to laser radiation according the following experimental protocol: NHDFs were treated with cytokine mix (IL-1β and TNF-α, each at 10 ng/mL) for 48 h; then, culture medium was replaced by fresh culture medium, and samples were divided into the following groups: (i) CYKs + LASER Group—samples previously stimulated with the cytokine mix and then exposed to laser treatment (3 treatments, repeated once a day, for 3 consecutive days); (ii) CYKs Group—samples previously stimulated with the cytokine mix and not exposed to laser treatment; (iii) CTRL Group-samples not stimulated with the cytokine mix and not exposed to laser treatment. Six hours after the third laser treatment (T = 126 h), all the samples were recovered and iNOS, COX-2 and mPGES-1 protein expression was evaluated by western blotting.

Following the exposure to inflammatory cytokines, a significant up-regulation of the inflammatory enzymes was observed (Figure 2). The group stimulated with cytokines and then treated with lasers showed a strong decrease in inflammatory enzyme expression compared to the group stimulated only with cytokines. For iNOS and COX-2, the decrease reached statistical significance (Figure 2).
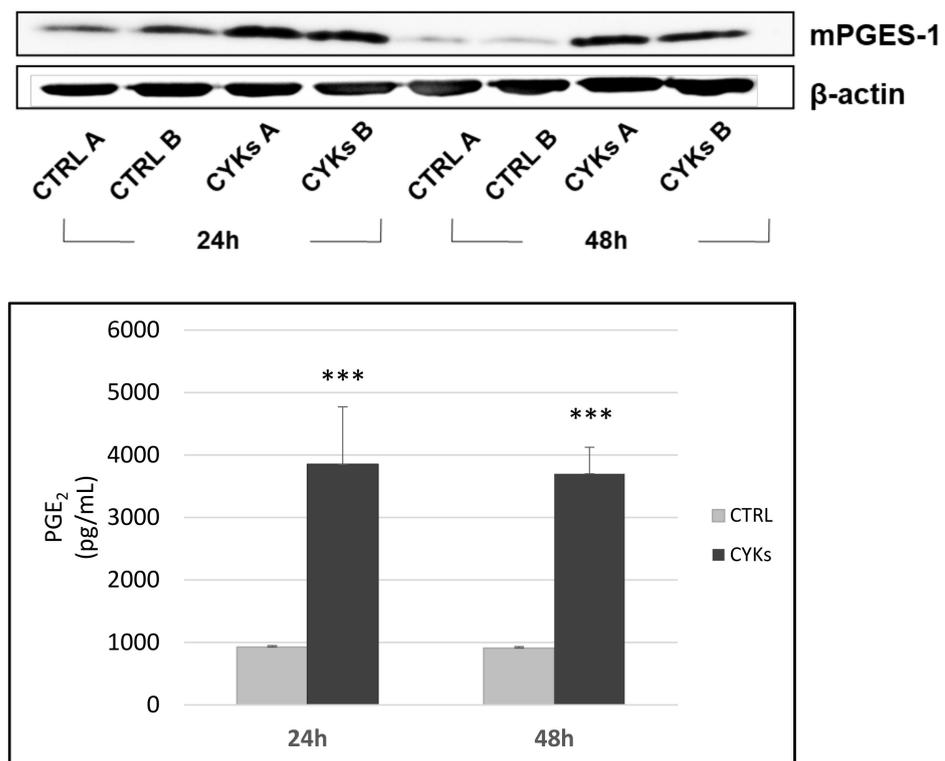
Figure 1. Development of an inflammation model on normal human dermal fibroblast (NHDF) cells. Fibroblasts were treated with IL-1β (10 ng/mL) + TNF-α (10 ng/mL) for 24 h and 48 h. Whole cell lysates were collected to assess mPGES-1 expression by Western blot (upper panel). Samples A and B represent intra-experimental duplicates. The measurement of PGE-2 performed by immunoenzymatic assay is reported (lower panel). At both times, there is an upregulation of the prostanoid system. Data represent means +/− SD (*n* = 3) *** *p* < 0.001 CYKs group vs. CTRL group.

To validate data obtained by Western blot, the main product of prostanoid enzymatic cascade, PGE-2, was measured in NHDF conditioned media recovered from the samples 6 h after the third laser treatment (T = 126 h). The cytokine mix-stimulated fibroblasts showed a significant increase in PGE-2 released in the medium in comparison with control samples (209 pg/mL in basal condition and 11000 pg/mL after 48 h of stimulation with the inflammatory mix). Although the resulting data were not significant, laser exposure reduced PGE-2 levels with a clear trend towards damping of the prostanoid pathway (Figure 3, upper panel).

Additionally, conditioned media were assessed for the release of the angiogenic factor VEGF, involved in neovascularization and granulation tissue formation. While inflammatory stimuli significantly increased VEGF levels, laser exposure strongly reduced VEGF availability in the medium, being the levels well below the basal, unstimulated condition (Figure 3, lower panel).

Ultimately, the modulation of the inflammatory response at the cellular level was evaluated through confocal microscopy. Inflammation is a protective response characterized by a series of reactions, such as vasodilation and recruitment of immune cells to the site of injury. NF-κB is an inducible transcription factor, responsible for the activation of genes involved in this process, including COX-2 and VEGF [33]. The localization of the nuclear transcription factor NF-κB and the intensity of the fluorescent signal given by the expression of its downstream genes COX-2 and VEGF were analyzed in the samples described above. In control samples, the transcription factor seemed to remain outside the nucleus, since the fluorescent signal was mainly cytoplasmic (Figure 4a). NHDF stimulation with IL-1β and TNF-α induced a consistent increase in the expression of NF-κB, as evidenced by a higher fluorescence intensity. Furthermore, in many cells, a change in the localization was

observed, with accumulation of the signal at the nuclear level (Figure 4b; white arrows). In samples stimulated with the cytokine mix and then treated with laser, a clear decrease in intensity of the signal linked to the transcription factor was observed, although some cells with NF-κB located in the nucleus (Figure 4c; white arrows) were still present. In cells treated with laser alone, the presence of some NF-κB punctuation at nuclear level was observed (Figure 4d).
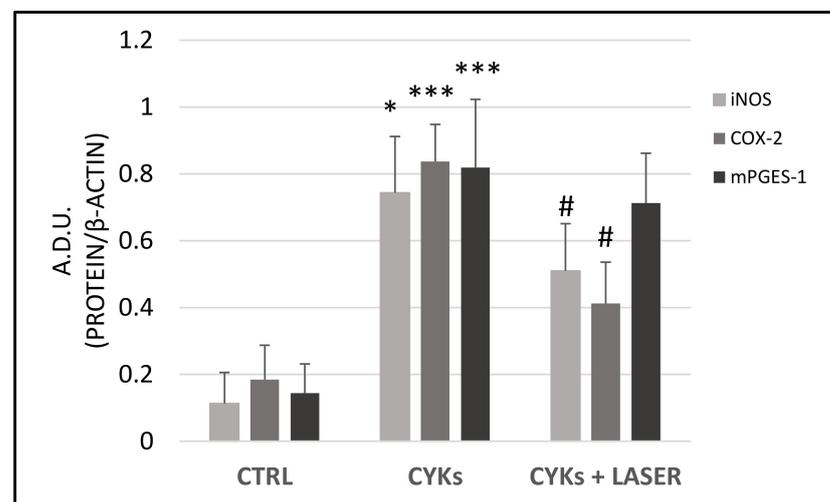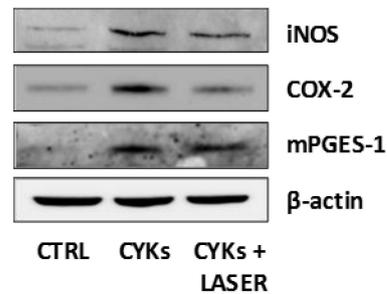
Figure 2. Effect of laser treatment on NDHF cells stimulated with pro-inflammatory cytokines. Fibroblasts were treated with IL-1β and TNF-α, each at 10 ng/mL for 48 h, then culture medium was replaced by fresh culture medium and samples divided into 3 groups: CYKs + Laser—samples stimulated with cytokine mix and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days); CYKs—samples stimulated with cytokine mix and not exposed to laser treatment; CTRL—samples not stimulated and not exposed to laser treatment. Six hours after the third laser treatment, whole cell lysates of all samples were collected, and Western blot was performed to assess protein abundance of iNOS, COX-2, and mPGES-1 (upper panel). Immunoblots were analyzed by densitometry and the results, expressed as arbitrary density units (A.D.U.), were normalized to β-Actin (lower panel). Data represent means $+/-$ SD ($n = 2$) * $p < 0.05$ and *** $p < 0.001$ CYKs group vs. CTRL group, # $p < 0.05$ CYKs + Laser group vs. CYKs group.

A similar trend was described for COX-2. The enzyme expression resulted strongly enhanced by the cytokine mix (Figure 4f) in comparison with unstimulated and laser alone controls (Figure 4e,h), where the fluorescence signal was weak and located in the cytoplasm. In samples stimulated with the cytokine mix and then treated with laser, the signal was similar to that observed in control (Figure 4g). However, in the last condition, mixed cell populations were noticed, some still over-expressing the enzyme and others returned to control levels. Similarly, the VEGF signal also followed a modulation superimposable to that of COX-2, presumably dictated by the transcription factor NF-kB. VEGF labelling, not affected by laser alone (Figure 4k), increased by stimulation with cytokines mix (Figure 4j).

Following laser treatment, the cytokine-induced VEGF intensity completely returned to the basal levels (Figure 4l), confirming the data obtained by protein dosage carried out on conditioned media, as previously illustrated (Figure 3, lower panel).
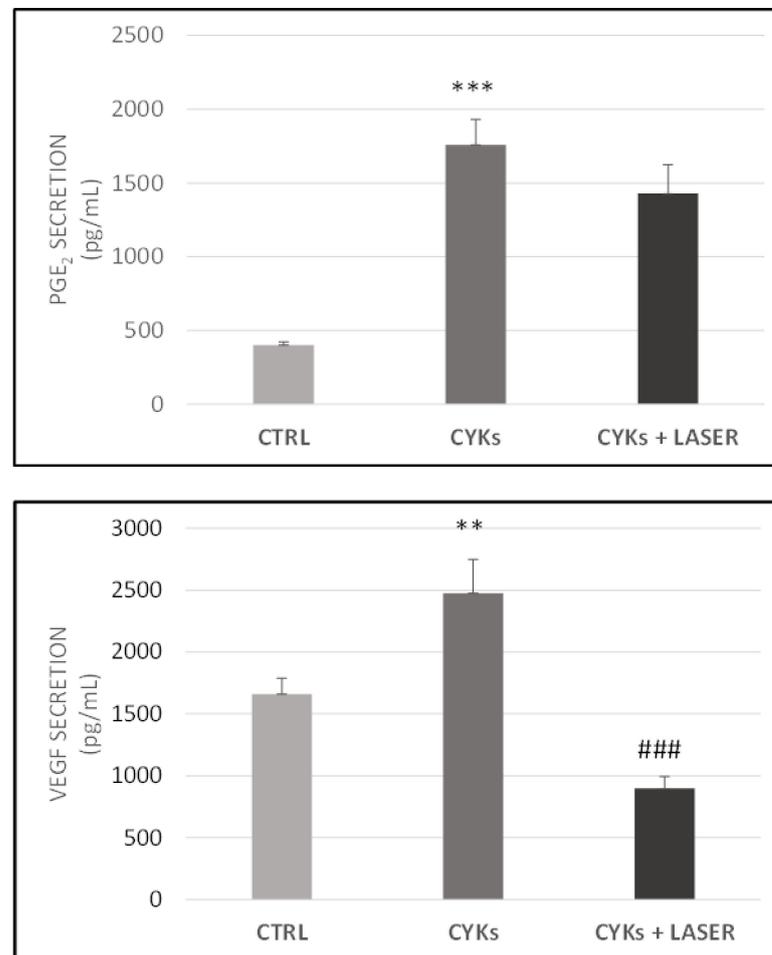
Figure 3. Modulation of PGE-2 and vascular endothelial growth factor (VEGF) release in NHDF cells exposed to pro-inflammatory cytokines and laser treatment. Fibroblasts were treated with IL-1β and TNF-α, each at 10 ng/mL for 48 h, then the culture medium was replaced by fresh culture medium and samples divided into 3 groups: CYKs + Laser—samples stimulated with cytokine mix and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days); CYKs—samples stimulated with cytokine mix and not exposed to laser treatment; CTRL—samples not stimulated and not exposed to laser treatment. Six hours after the third laser treatment, all samples were recovered, and PGE-2 (upper panel) and VEGF (lower panel) levels were evaluated in conditioned media using specific ELISA kits. Dosing of each condition was performed in double, and quantification is expressed as pg/mL. Data represent means $+/-$ SD (n = 2) ** $p < 0.01$ and *** $p < 0.001$ CYKs group vs. CTRL group, ### $p < 0.001$ CYKs + Laser group vs. CYKs group.

3.2.2. Morphology and Cytoskeleton Organization

The stimulation with the mix of IL-1β and TNF-α produced a marked change in cell morphology. The organization of the microtubules, which control cell architecture, changed as well. In the control samples, fibroblasts were generally star-shaped and spread on the substrate. The specific labeling for tubulin showed the well-known radial distribution of microtubules which branch off from a nucleation center (Figure 5a), usually anchored at the centrosome and the Golgi apparatus [34]. In the stimulated samples, the cells appeared spindle-shaped, elongated, with a dense, longitudinal microtubule network [34], where it was difficult to distinguish a nucleation center (Figure 5b). In the samples first stimulated

and then treated with laser, fibroblasts regained a shape similar to the controls, with a clearly distinguishable microtubule nucleation center and radially organized microtubules (Figure 5c).

Figure 4. Laser treatment reduces inflammatory response in NHDF cells by limiting NF-κB translocation into the nucleus and down-regulating COX-2 and VEGF expression. Confocal analysis of NF-κB (panels (**a–d**)), COX-2 (panels (**e–h**)) and VEGF (panels (**i–l**)) expression and localization (magnification 63×) evaluated by immunofluorescence on NHDF in basal conditions (C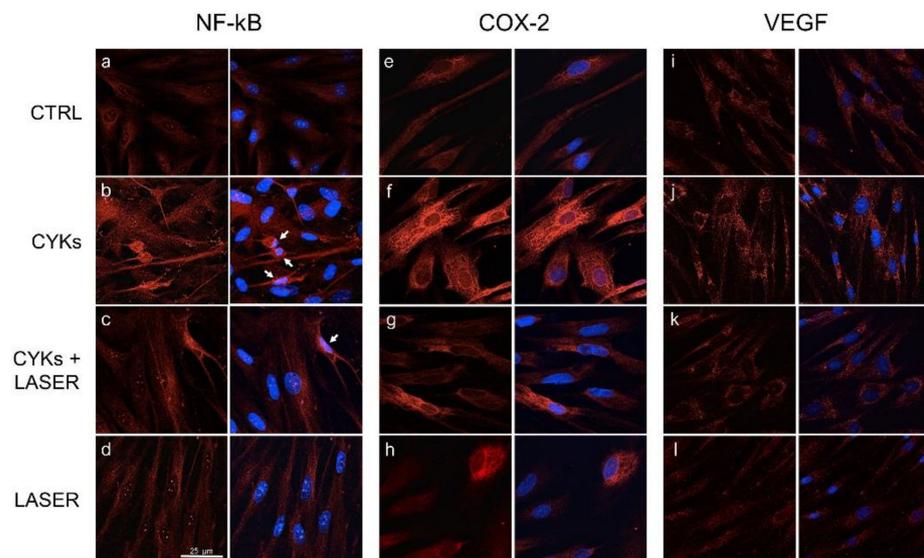TRL; panels (**a,e,i**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panels (**b,f,j**)), stimulated with IL-1β and TNF-α for 48 h, and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + LASER; panels (**c,g,k**)) and exposed to laser alone (LASER; panels (**d,h,l**)). For each series, the left panels show the protein of interest in red, while DAPI staining (blue) was merged on the right panels. White arrows indicate cells with nuclear localization of NF-kB. Bar = 25 μm.

As regards actin distribution and organization, control fibroblasts showed a perinuclear area rich of G-actin, a network of very thin microfilaments distributed in the cell cytoplasm, and a thin actin layer placed close to the plasma membrane (Figure 5d). In the stimulated fibroblasts, F-actin was predominant, with microfilaments arranged in parallel and thicker in comparison with those observed in control cells (Figure 5e). As already noted for tubulin, also in the case of actin, the stimulated cell samples which were then exposed to laser radiation recovered a condition similar to the control cells, with G-actin thickened in the perinuclear area, few very thin microfilaments and a thin actin layer close to the cell membrane (Figure 5f).

Alpha-smooth muscle actin (α-SMA) is the actin isoform that predominates within smooth-muscle cells. Its expression generally increases in the transition fibroblast-myofibroblast. In fact, myofibroblasts acquire a contractile phenotype, which is responsible for merging the wound edges in the healing process. Therefore, α-SMA is considered a marker of myofibroblast differentiation. In control samples, α-SMA staining revealed some stress fibers, which completely disappeared in fibroblasts stimulated with IL-1β and TNF-α, where the fluorescence signal coincided with the nucleus and was detectable only in the nuclear area (Figure 5g,h). The samples exposed to laser radiation after the cytokine mix stimulation showed an intermediate situation. The signal in the nuclear area was still detectable, but fibers organized in parallel appeared (Figure 5i).
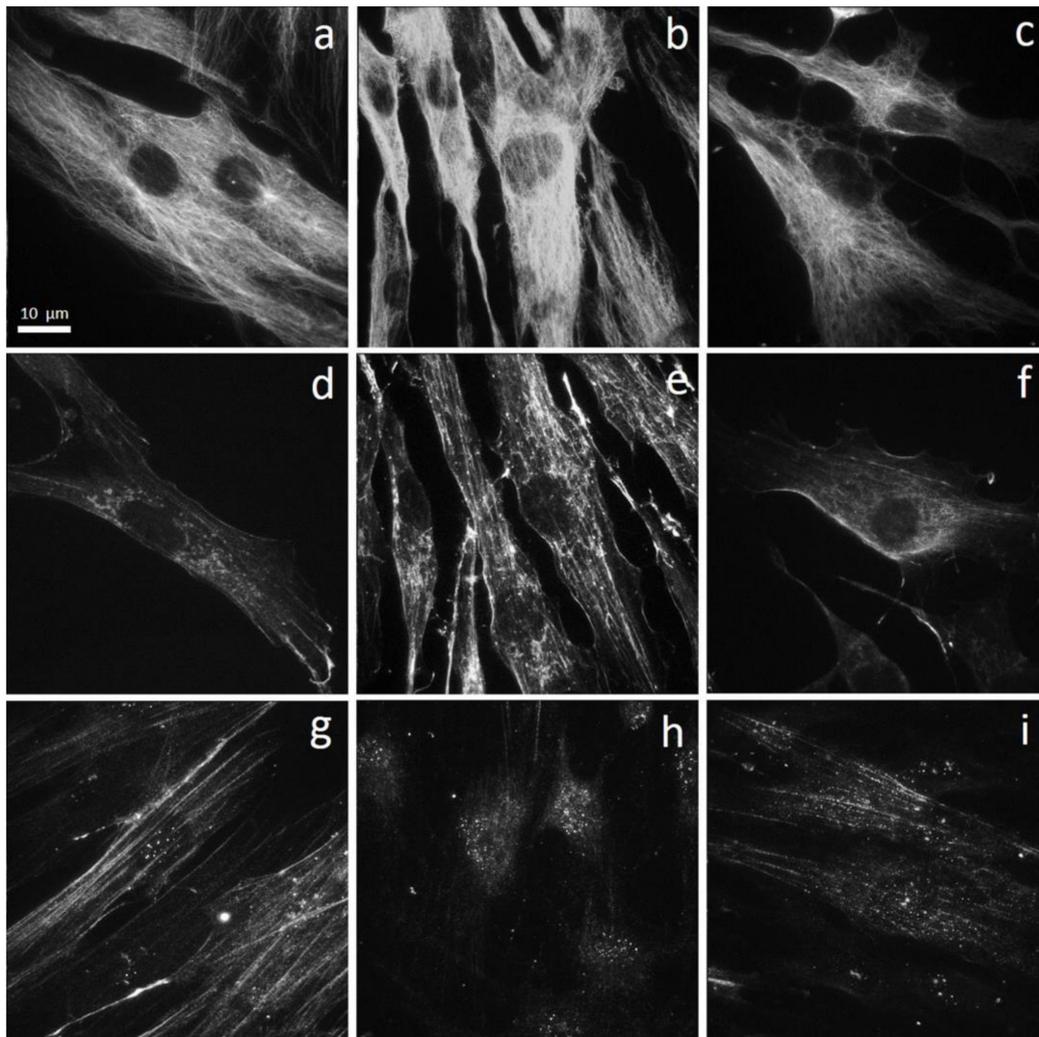
Figure 5. Effect of laser treatment on tubulin, α-actin, α-SMA expression, and distribution. Microscopy analysis of tubulin (**a-c**), α-actin (**d–f**), and α-SMA (**g–i**) expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panels (**a,d,g**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panels (**b,e,h**)), stimulated with IL-1β and TNF-α for 48 h, and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panels (**c,f,i**)). Bar = 10 μm.

3.2.3. Extracellular Matrix Proteins and Membrane Integrin

Integrins are cell surface receptors which control various cellular functions. Integrin receptors connect the cell cytoskeleton with the ECM proteins, thus being involved in signaling changes of the extracellular microenvironment and leading to cellular responses. In particular, α5β1 integrin is a fibronectin receptor and has a well-defined role in cell adhesion, migration, and matrix formation, which are functions of crucial importance in physiological and pathological processes such as wound healing and fibrosis. In the control samples, α5β1 clusters were located at focal adhesion points mostly in the perinuclear area, along cellular protrusions, and at their ends, generally arranged parallel to the major axis of the cells (Figure 6a). In the samples stimulated with the cytokine mix, the expression of the integrin significantly increased (Figure 6b). In these samples, half of the cells still retained a morphology similar to the control (star-shaped and spread), but showed a higher density of integrin clusters with centripetal distribution in the perinuclear area. In the other half of the cells, characterized by spindle-shaped and elongated morphology, the α5β1 clusters became point-like, smaller, distributed in the perinuclear area, and at lateral intercellular surfaces forming cell–cell contact points (Figure 6b). After laser treatment,

fibroblasts appeared similar to the controls, both for the signal intensity and distribution of α5β1 clusters (Figure 6c).

Figure 6. Effect of laser treatment on α5β1 expression and distribution. Microscopy analysis of α5β1 expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 μm. The histogram reports the mean pixel intensity, acquired by ImageJ software after appropriate thresholding and subsequent image masking (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group ($n = 3$).

Through its interaction with different cell types, cytokines, and other ECM molecules, and facilitating collagen fibrogenesis by scaffolding action, fibronectin plays a preeminent role in both wound healing and scarring [35,36]. Similarly to its receptor α5β1, fibronectin significantly increased in fibroblast cultures stimulated with IL-1β and TNF-α, when compared to unstimulated controls, and formed a dense extracellular network of fibrils (Figure 7a,b). After laser treatment, fibronectin expression returned to the basal levels observed in control cells with evident reduction of extracellular fibrils (Figure 7c,d).
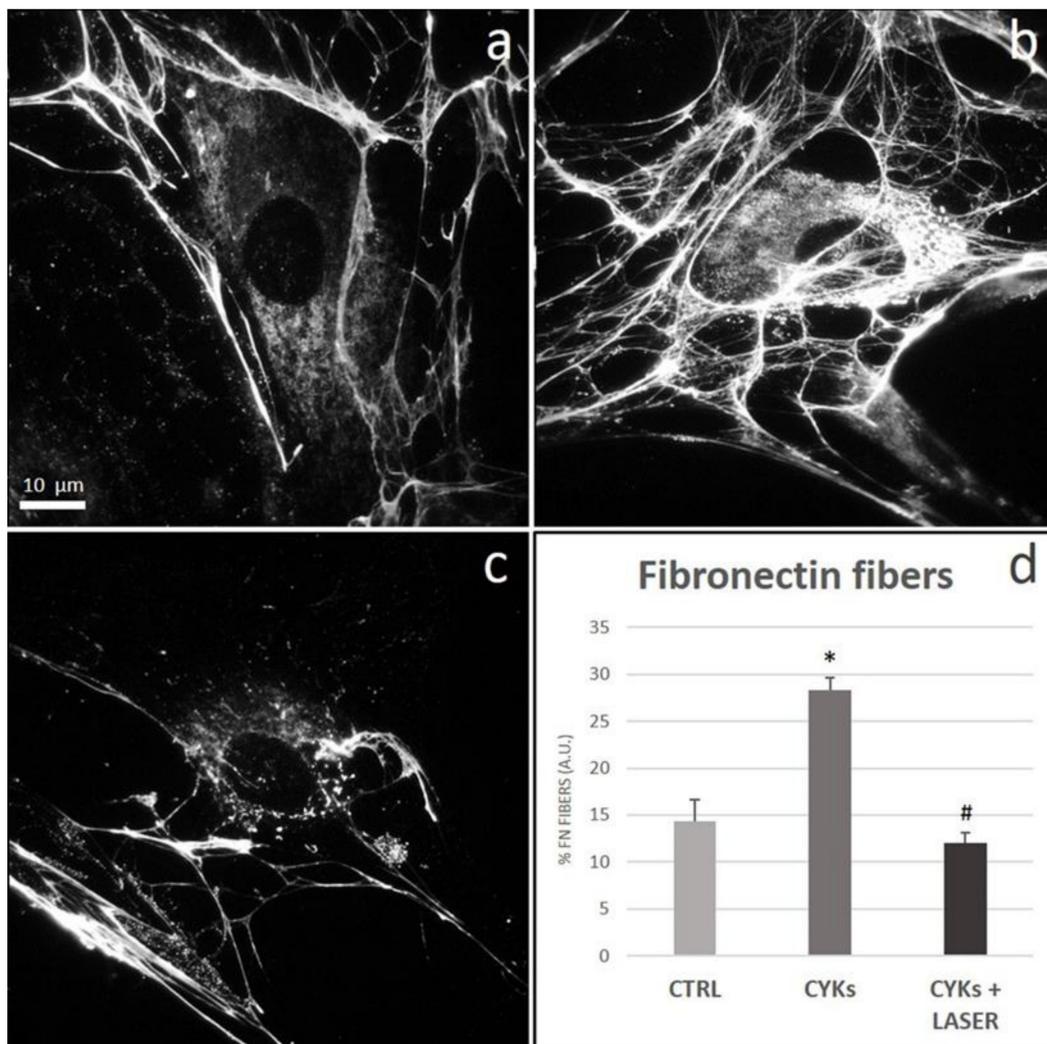
Figure 7. Effect of laser treatment on fibronectin expression and organization. Microscopy analysis of fibronectin expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 μm. The histogram reports the % of the surface area with fibers, acquired by ImageJ software after appropriate thresholding to only include the stained fibers (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group ($n = 3$).

In addition, the synthesis of collagen I, one of the most abundant ECM components, was significantly enhanced by the exposure to the cytokine mix in comparison with control unstimulated cells (Figure 8a,b). Interestingly, stimulated fibroblasts showed an intracellular accumulation of collagen I, apparently in the endoplasmic reticulum and/or Golgi apparatus, while the protein was not released in the extracellular environment (Figure 8b). Cytokine-mix stimulated fibroblasts exposed to the laser treatment revealed a collagen I signal similar to that observed in the control, both for distribution and fluorescence intensity (Figure 8c,d).

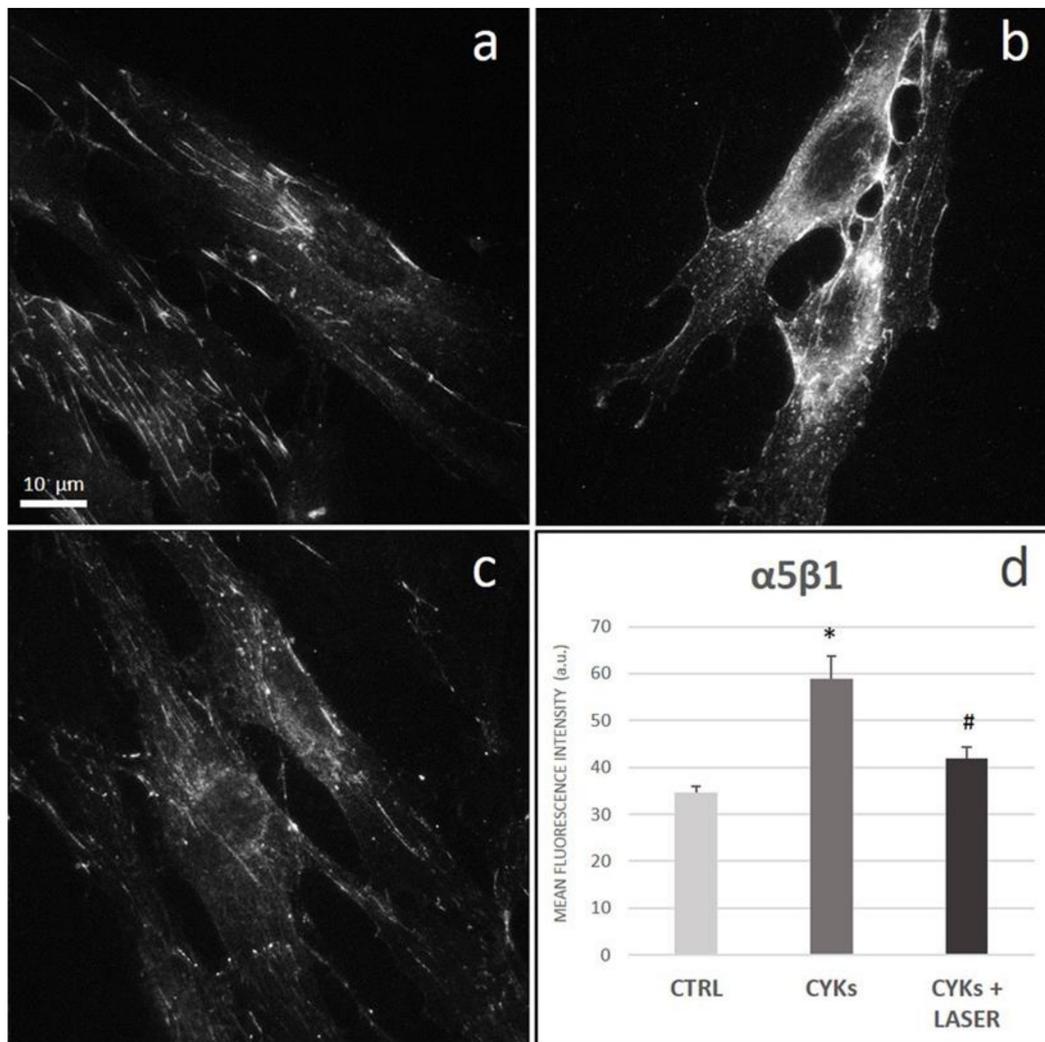**Figure 8.** Effect of laser treatment on Collagen I expression and distribution. Microscopy analysis of Collagen I expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 μm. The histogram reports the mean pixel intensity, acquired by ImageJ software after appropriate thresholding and subsequent image masking (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group (n = 3).

Matrix metalloproteinases (MMPs) are endopeptidases that can degrade the ECM proteins. They have important roles in fundamental physiological processes, such as embryonic development, morphogenesis, and tissue remodeling, and are involved in a number of diseases. MMPs are present in both acute and chronic wounds, where they regulate ECM degradation/deposition that is essential for wound healing. The excess protease activity can lead to chronic nonhealing wounds [37].

Specifically, MMP-1 is able to degrade collagen types I, II, and III. Similarly to fibronectin and collagen, also MMP-1 significantly increased in fibroblasts stimulated with the cytokine mix (Figure 9b), compared to non-stimulated controls (Figure 9a). Laser treatment counteracted the effect of the cytokine mix and reported MMP-1 expression to a level comparable to that found in the basal state (Figure 9c).
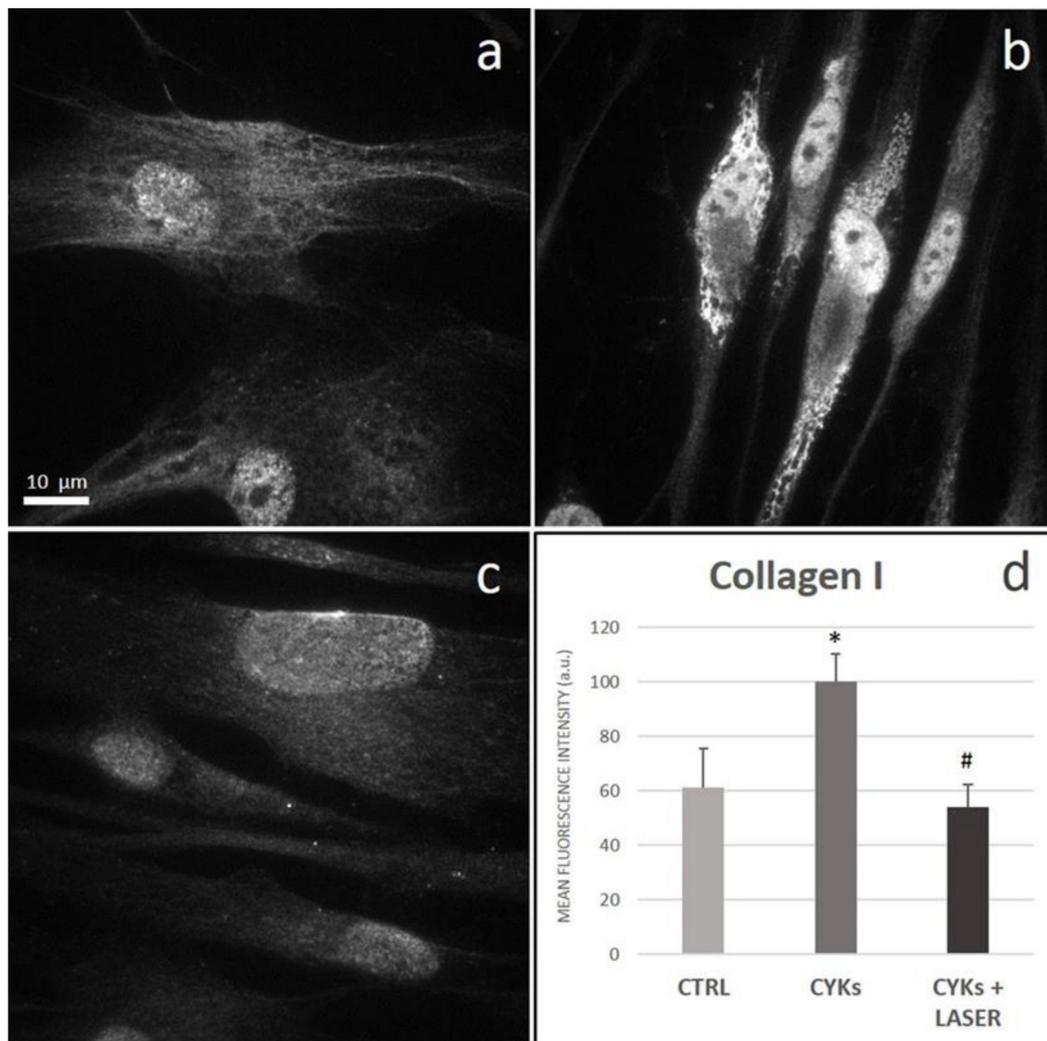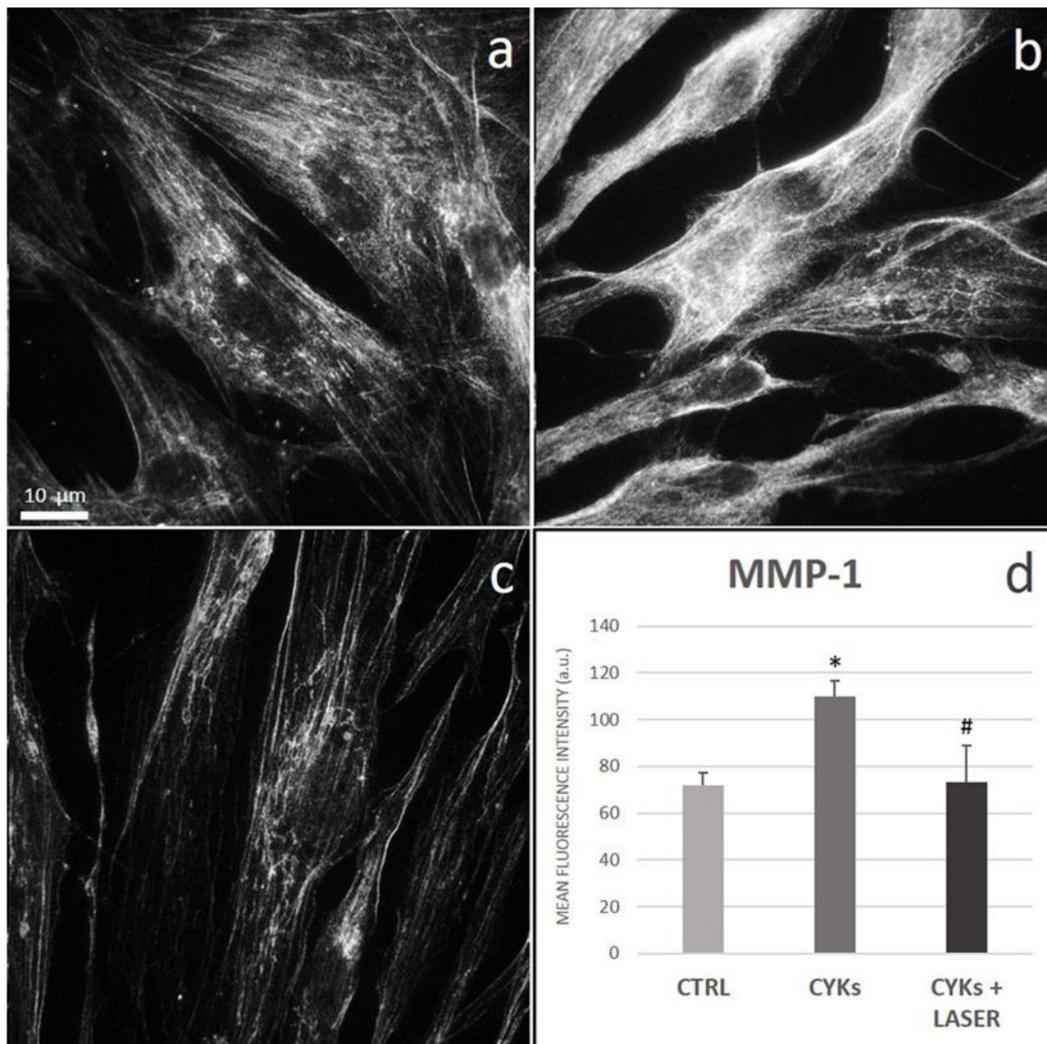
Figure 9. Effect of laser treatment on MMP-1 expression and distribution. Microscopy analysis of MMP-1 expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 μm. The histogram reports the mean pixel intensity, acquired by ImageJ software after appropriate thresholding and subsequent image masking (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group ($n = 3$).

4. Discussion

The cytokines IL-1β and TNF-α have already been used at different concentrations, individually or in association, to stimulate an inflammatory response in various cell types, dermal fibroblasts included [28,38,39]. In this study, IL-1β and TNF-α have been used jointly to induce a pro-inflammatory phenotype in dermal fibroblasts, with the aim to investigate if PBMT delivered via a dual-wavelength NIR laser system (MLS-MiS) was effective in counteracting cell inflammatory response and modulating fibroblast functions involved in stromal activation, wound healing, and its alterations, which can lead to chronic ulcers or fibrosis. Preliminary experiments performed to define the protocol for preparing the in vitro model of inflammation in dermal fibroblast cultures showed that both 24 h and 48 h exposure to IL-1β and TNF-α produced a significant increase in the inducible enzyme mPGES-1 and in the release of its product PGE-2. The increase in mPGES-1 was higher after 48 h, while the increase in PGE-2 was similar at 24 h and 48 h. Therefore, a stimulation time of 48 h was chosen for the subsequent experiments in which non-stimulated controls, samples stimulated with IL-1β and TNF-α, and samples exposed

to laser radiation after the stimulation with the inflammatory cytokines were compared for their morphology, inflammatory profile, and expression of molecules involved in ECM remodeling. In stimulated samples, the inflammatory signals iNOS, COX-2, and mPGES-1 significantly increased in comparison with non-stimulated controls, in agreement with data reported in literature [40] and supporting the validity of the inflammatory model used. Samples stimulated and then treated with PBMT showed a significant decrease in iNOS and COX-2, compared to the stimulated but non-laser irradiated samples. The mPGES-1 level and that of the final product PGE-2 decreased, but not significantly, suggesting a multimodal action of PBMT, which could act at different cellular levels (gene transcription, protein expression, and localization), as demonstrated by the reported results.

Modulation of the three mediators mentioned above is closely related to their upstream activator NF-κB, an inducible transcription factor which is activated upon binding of pro-inflammatory cytokines, such as TNF-α, to their membrane receptors. In basal conditions, NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins. Following inflammatory stimuli, this protein moves to the nucleus, binds to specific elements on DNA, and recruits cofactors for the transcription of target genes *iNOS, COX-2*, and *mPGES-1* [41]. In the presence of inflammatory stimuli, the co-localization of the transcription factor NF-κB within the nucleus, observed by immunofluorescence, correlates with an increased expression of iNOS, COX-2, and mPGES-1 at the cytoplasmic level and a consequent increase of PGE-2 released in the extracellular medium. Following laser treatment, these values are significantly reduced, demonstrating the effectiveness of the laser source and treatment parameters used in counteracting the inflammatory response.

The anti-inflammatory properties of the NIR source used had already been highlighted by a proteomics study on laser-irradiated myoblasts, in which a marked increase in NLRP10, a strong inhibitor of the inflammasome, and in turn of IL-1β and interleukin-18 (IL-18) release, was observed [25]. These data are in agreement with previous studies showing the effectiveness of red and NIR radiation in reducing the inflammatory signals both in fibroblast cultures and at the wound level [42,43].

As previously mentioned, inflammation is a protective response characterized by a series of reactions modulated by the master regulator NF-kB, whose gene targets are involved both in the recruitment of immune cells to the site of injury and in vasodilation [33]. In this study, fibroblast production of VEGF at the cytoplasmic level and its secretion in the extracellular milieu were therefore analyzed. Compared to untreated controls, IL-1β and TNF-α stimulation of fibroblasts induced an increase in VEGF production and release in culture medium, that further confirms the validity of the model used for the present study. The proinflammatory cytokines-induced enhancement in VEGF levels is widely documented in vitro [44], and it occurs in vivo in chronic inflammatory diseases as well as in acute inflammatory response to infections and injuries. VEGF is produced by the most part of cell populations involved in wound healing, as platelets, immune cells (neutrophils and macrophages), fibroblasts, and endothelial cells, and reaches the maximum concentration during the proliferative phase. In the wound, VEGF promotes angiogenesis [45] and influences re-epithelialization and collagen deposition through stimulation of keratinocytes and fibroblasts [46]. However, if in a proper inflammatory response VEGF upregulation is needed to promote angiogenesis, excessive or persistent inflammation and VEGF production can lead to fibrosis and should be controlled. Laser treatment subsequent to IL-1β and TNF-α stimulation abolished the cytokine-mediated VEGF increase and brought VEGF levels back to values even lower than those seen in unstimulated controls. In literature, a modulation of VEGF expression following irradiation with red-NIR wavelengths has been described, the final effects depending on irradiation parameters and experimental models used [42,47,48]. The decrease observed in the present study, irradiating activated fibroblasts with the source and parameters described, further supports the strong anti-inflammatory action of the proposed laser treatment. At the same time, the results on cells exposed to laser alone, which did not affect markers of fibroblast activation, substantiated the safety of laser irradiation on quiescent unstimulated cells.

IL-1β and TNF-α treatment induced also noticeable morphological changes with cytoskeletal rearrangements in the network of microtubules and actin microfilaments.

Microtubules form a scaffold which controls cell shape, intracellular transport, signaling, and organelle positioning. Microtubules are stiff and intrinsically polarized structures built of directionally aligned αβ-tubulin dimers. Their "minus" end is anchored at so called microtubule-organizing centers, whereas the "plus" ends can extend or shrink and interact with different intracellular structures [49]. Cells able to readily reorient their polarity axis, such as fibroblasts, generally present a radially organized microtubule array, whose changes are mutually related to cell polarity and can mechanically contribute to cell asymmetry by promoting cell elongation [34]. The results of the present study show that, following cell activation by cytokines, changes in microtubule density and orientation occurred and probably contributed to the observed cell elongation.

Following IL-1β and TNF-α stimulation, the actin filament network changed as well. Density and thickness of actin filaments increased, while their distribution underwent a rearrangement, giving rise to an array of filaments aligned parallel to the major cell axis. Considering that microtubules are connected to the layer of actin filaments close to the cell membrane through a complex of adaptor proteins often associated with focal adhesions, the changes in microtubule and actin filament networks are probably interrelated, and the rearrangement in α5β1 membrane integrin distribution observed in cytokine-stimulated cells further support this hypothesis. The inhibition of the inflammatory response due to laser treatment led to a partial recovery of the basal cytoskeleton organization in fibroblasts irradiated after cytokine stimulation. Cytoskeleton changes connected with effects produced by NIR laser irradiation have been previously described in different cell models [25,50] and depend on cell type, cell status, parameters, and sources used.

α-SMA is one of the six actin isoforms. Together with β- and γ-actin isoforms, α-SMA is expressed in some fibroblast/myofibroblast subpopulations in the basal state, where it has a cytoplasmic localization and participates in stress fiber formation. α-SMA is strongly induced by mechanical stress and TGF-β1 in activated myofibroblasts [51], therefore it is generally considered a marker of fibroblast-myofibroblast transdifferentiation. In the present study, dermal fibroblasts stimulated with IL-1β and TNF-α showed α-SMA expression seemingly concentrated in the nucleus, while cytoplasmic stress fibers, to some extent present in unstimulated cells, completely disappeared in the stimulated ones. These results are consistent with in-depth investigations on α-SMA distribution and roles carried out in the last two decades. It has been demonstrated that the apparent nuclear localization is due to deep invaginations of the nuclear membrane filled of α-SMA [52]. The role of the nuclear invaginations is currently quite completely unknown, but it has been hypothesized that these structures could be involved in cellular and nuclear mechanotransduction, nuclear transport, calcium signaling, cell differentiation [52–54]. Moreover, in agreement with our results, it has been found that TNF-α suppresses α-SMA expression and stress fiber formation in dermal fibroblasts and that persistent inflammation, mediated by TNF-α, might prevent normal matrix deposition and myofibroblast-dependent wound contraction mediated by TGF-β1 in physiological wound healing [55]. The inhibition of stress fiber formation would turn the cells into a phenotype more migratory and less able to generate tractional forces [51], with possible consequences and delay in the healing process.

Additionally, in the case of α-SMA, NIR laser treatment after IL-1β and TNF-α partially prevented the cytokine effect and some stress fibers reappeared inside the cells. α-SMA expression following red- or NIR-laser treatment has been widely studied being connected with the effectiveness of laser therapy in promoting wound healing and avoiding scarring. The results have been controversial, showing both down- and up-regulation of α-SMA expression [56–58]. This variability in results is possibly due to the many different models (from cell cultures to animal models both normal and representing serious diseases, such as diabetes), laser sources, treatment protocols and parameters, and times at which analysis of α-SMA expression was performed. Interestingly, some studies in which the analysis of α-SMA expression was performed at different healing times after laser treatment showed

that α-SMA expression changed in the different healing phases and resulted significantly different from controls only at specific time points [59]. The only unambiguous result is that laser irradiation is able to modulate α-SMA, but the modulation depends on many factors, among which the healing phase and corresponding cell phenotype (e.g., the phenotype of fibroblasts in the inflammatory phase is different from what they assume in the remodeling phase). This means that further studies are needed to develop treatment protocols suitable for the different patient's conditions and, in case of wounds, healing phase. However, the data of the present study indisputably demonstrate that, even at the cytoskeletal level, the source and the treatment parameters used are effective in counteracting the changes induced by cytokine stimulation, thus returning the cells to the basal state.

Compared to controls, the IL-1β and TNF-α stimulated fibroblasts showed increased fibronectin (FN) expression and assembly observed in the same samples. The increase in FN, a major ECM component, could be expected since the pro-inflammatory cytokines IL-1β and TNF-α, together with TGF-β, are considered potent fibrogenic initiators [60]. The increase in expression of α5β1 observed in the same samples is consistent with that of FN, considering that α5β1 is a membrane integrin able of binding FN [61].

A number of studies investigated the expression and role of α5β1 and its ligand FN in fibroblasts during inflammation and wound healing. In the healing process, α5β1-mediated fibroblast-FN interaction is crucial: α5β1 is involved in myofibroblast differentiation and granulation tissue formation by promoting FN assembly in a fibrillar structure. In the granulation tissue, a reduced ability to bind FN via integrin α5β1 might allow fibroblasts to migrate in the early FN-rich matrix and invade the wound [62]. On the other hand, some studies demonstrated that α5β1 integrin is able to confer strong cohesivity to 3D cellular aggregates linking adjacent cells together via FN, and that the FN with its dimeric structure is essential for this process [63]. Moreover, it has been suggested that α5β1-FN interaction contributes to clot retraction [63]. Therefore, α5β1 and FN play a crucial role in wound healing, and alterations in their expression can lead to healing impairment and fibrosis. These conditions can affect ECM remodeling by stimulating collagenase production and stimulating/inhibiting collagen/glycosaminoglycan biosynthesis depending on the target cells and experimental conditions.

The effects of the pro-inflammatory cytokines IL-1β and TNF-α on ECM remodeling and their role in fibrosis have been studied for many years with controversial results. In the present study, the cytokine-stimulated dermal fibroblasts showed increased expression of MMP-1 and collagen I, which have key roles in ECM degradation and building, respectively, thus modulating ECM turnover. In agreement with literature, the intracellular distribution of MMP-1 was associated with mitochondria [64] and, probably, the cytoskeleton. In fact, a relation between actin system dynamics and MMPs has been speculated because it has been observed that cytoskeleton changes often precede MMPs modulation and actin microfilament dynamics might be linked to the expression of MMP genes [65]. Regarding collagen I, contrary to what observed for FN, in stimulated fibroblasts it showed an intracellular localization and no extracellular fibrils were observed. If an increase in MMP-1 expression following IL-1β and/or TNF-α stimulation has been unanimously reported [39,66–68], the effects the two cytokines have on collagen synthesis remain uncertain. Many studies reported that both IL-1β and TNF-α inhibit collagen I synthesis [67,69–71], but other studies demonstrated that IL-1β and TNF-α increased collagen I synthesis in human renal fibroblasts [72] and in murine intestinal myofibroblasts [38], respectively. A proposed scenario is that, in some conditions, the antifibrotic effect of TNF-α is overwhelmed by its central role in driving inflammation [66].

Fibroblasts stimulated with IL-1β and TNF-α and then exposed to NIR laser radiation recovered features more similar to unstimulated controls as regards the expression and distribution of α5β1, FN, collagen I, and MMP-1. Therefore, laser treatment was also able to counteract the cytokine effects on α5β1 integrin and the proteins involved in ECM turnover and remodeling after injury. It is noteworthy that, in the case of FN, not only the expression returned to levels comparable with unstimulated controls, but in samples treated with

laser radiation the fibrils showed a more ordered and parallel distribution. This effect of laser radiation on FN and collagen fibril organization has already been described [73] and could be connected with the laser radiation's ability to prevent fibrotic scars. The influence of red-NIR laser radiation on the expression of α5β1 integrin, FN, collagen, and MMP-1 has already been investigated in studies concerning laser application in the management of inflammatory response and wound healing. The results of these studies are controversial. A recent study on a model of diabetic wounded fibroblast cells showed that PBMT (660 nm wavelength) downregulated the expression of the genes *FN1*, *ITGA5*, and *ITGB1*, encoding for FN, α5, and β1 integrin subunits, respectively [74]. In a study on an immunosuppressed rat wounded model, PBMT by an 810 nm pulsed laser induced an increase in FN expression [75]. Enhanced FN expression was found also in human fibroblasts irradiated with a 940 nm diode laser [58]. A research on the effects of different protocols of PBMT in the healing of open wounds in rats showed that all the protocols used induced an increase in collagen deposition, but at different extent, depending on wavelength and fluence applied [43]. Using similar fluence but a different wavelength, a decrease in collagen production was found in wounded human skin fibroblasts [76]. In a rat model of wound healing, collagen deposition did not increase 3 days after laser treatment, but it increased significantly at day 7 after treatment [77]. Sakata et al. [28] found that, in chondrocytes stimulated with IL-1β, MMP-1 increased and then decreased after NIR irradiation applied post-stimulation, in complete agreement with what has been observed in the present study on fibroblasts activated by IL-1β and TNF-α.

From the outcomes of the studies mentioned above, it is evident that expression and function of α5β1 integrin, FN, collagen, and MMP-1 can be modulated through application of PBMT. However, results so uneven as those reported in the literature about PBMT effects demonstrate once again that it is very difficult to compare studies carried out using different experimental models, laser sources, and treatment parameters. Laser source and treatment protocol should be characterized for their biological effects before application for the management of specific pathological conditions.

In this paper, an in vitro model of fibroblast activation via stimulation with the pro-inflammatory cytokines IL-1β and TNF-α has been proposed and used to test the anti-inflammatory effect of a dual wavelength NIR laser source widely used in clinics to promote healing and reduce inflammation and pain. Like all in vitro models, a limit of the proposed model is to provide a very partial representation of what happens in vivo during inflammation and healing (a single cell population and two pro-inflammatory cytokines vs. many cell populations and a plethora of pro- and anti-inflammatory molecules). However, it can be considered representative of the early stage of the inflammation phase after an injury, when M1 macrophages produce great amounts of IL-1β and TNF-α. In the normal evolution of inflammation, macrophage phenotype is expected to shift from M1 to M2, with increased TGF-β production and a decrease in IL-1β and TNF-α levels [60]. Therefore, the proposed model can be considered also representative of altered evolution of inflammation with persistence of high levels of TNF-α, compared to TGF-β levels, due to the failure to switch from M1 to M2.

Using this model, the effectiveness of PBMT by a dual wavelength NIR laser source (MLS-MiS) in reducing inflammation has been tested, and the results obtained show that PBMT, administered through the laser source and protocol here described, is significantly effective in preventing the effects of IL-1β and TNF-α, thus modulating the cell inflammatory response and favoring cell return to the basal physiological state.

The anti-inflammatory effect of red-NIR laser radiation has been already reported in a number of studies but, to the best of our knowledge, it is the first time that it is evaluated and confirmed in an "in vitro" model of IL-1β and TNF-α activated dermal fibroblasts. Moreover, the significant anti-inflammatory activity of the laser emission tested in the present research is consistent with our previous studies carried out with the same laser source. In an in vitro model of myoblasts, it was found to increase the expression of NLRP10, a potent inhibitor of inflammasome activation and IL-1β and IL-18 produc-

tion, as well as that of PP1, which regulates many important cell functions and favors cell recovery from stress to basal state [25]. In vivo, the same laser emission was able to reduce inflammatory infiltrate and accelerate the healing of ulcers in feline stomatitis [78] while, in a rat model of neuropathic pain induced by trauma, it significantly lowered inflammation and pain and preserved the myelin sheath [79]. It is well known that, when released by cells under pro-inflammatory stimuli, IL-1β and IL-18 induce the production of other pro-inflammatory cytokines, such as interferon-γ (INF γ), TNFα, IL-6, etc., thus triggering a cascade of events which further increase and perpetuate inflammation. Therefore, the ability of the proposed laser treatment to inhibit IL-1β and IL-18 release, through increased NLRP10 production, could explain its effectiveness in controlling fibroblast activation induced by IL-1β and TNF-α stimulation, thus damping excessive inflammatory response. Further studies could help to define treatment protocols specific for each different healing phase.

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4. Topic 3: Safety assessment of potential chemotherapeutic agents on endothelium

4.1. Safety of xenobiotics and drugs on endothelium

In the introduction we mentioned the various causes that can lead to endothelial and vascular dysfunction. Among these it is relevant to mention endothelial damage induced by drugs and xenobiotics, which can promote or accelerate the onset of some CVDs, including atherothrombosis, systemic and pulmonary hypertension or diabetes (Wojcik et al., 2015). Since the bloodstream is the main vehicle of therapeutic agents, endothelium results to be particularly exposed and sensitive to local or systemic damages, compared to other tissues or organs. Many are the clinical trials suspended and drugs withdrawn from the market (Fitzgerald, 2004; Wojcik et al., 2015) due to adverse effects on the cardiovascular system, such as increased risk for thromboembolic events. The class of drugs that are most clinically debated for their undesirable effects on the endothelium are chemotherapeutic agents (Soultati et al., 2012); despite their implementation which allows today for the treatment of a high percentage of malignancies, patients show higher risk of CVD (Shenoy et al., 2011) and vascular complications, exacerbating in some cases the clinical profile. Furthermore, cancer patients with concomitantly impaired systemic endothelial function may be particularly susceptible to the detrimental effects of anticancer medications (Morbidelli et al., 2016). Cancer therapy-induced endothelial cytotoxicity is due to the fact that these molecules may target specific signaling pathways that promote cancer cell proliferation, but also protect ECs. An example of chemotherapeutic dugs that display negative effects on ECs we find doxorubicin, cisplatin, 5-florouracil (Morbidelli et al., 2016). Endothelial toxicity is a common feature of various novel biological chemotherapeutics, for example, anti-Her2 (trastazumab) or anti-VEGF (bevacizumab, sunitinib, sorafenib) agents (Wojcik et al., 2015; Ewer et al., 1999; Sandoo et al., 2014). In clinical trials, 25% of patients receiving trastazumab (herceptin) developed systolic dysfunction and Her2 inhibition was also shown to cause impairment of vascular function through a reduction in NO bioavailability and an increase in ROS production (Wojcik et al., 2015; Sandoo et al., 2014).

In conclusion, the development of molecules capable of inducing robust anti-tumor responses concurrent with minimal systemic side effects should constitute an important criterion in preclinical drug development and approval process, through the introduction of endothelial toxicity profiling methods. Without such a strategy, commercially available therapeutic agents may impair the vasoprotective mechanisms of the endothelium, possibly promoting the development of cardiovascular diseases (Wojcik et al., 2015).

4.2. Aim of the study

The focus of the present study was to assess and compare the safety profile of two carbonic anhydrase IX (CA-IX) inhibitors, SLC-0111 and AA-06-05 on cultured human endothelial cells. CA-IX plays a pivotal role in regulation of pH in solid hypoxic tumor milieu catalyzing carbonic acid formation by hydrating CO_2 (Figure 4.1). An acidification of tumor microenvironment contributes to tumor progression via multiple processes, including reduced cell-cell adhesion, increased migration and matrix invasion (Pastorekova and Gillies, 2019).
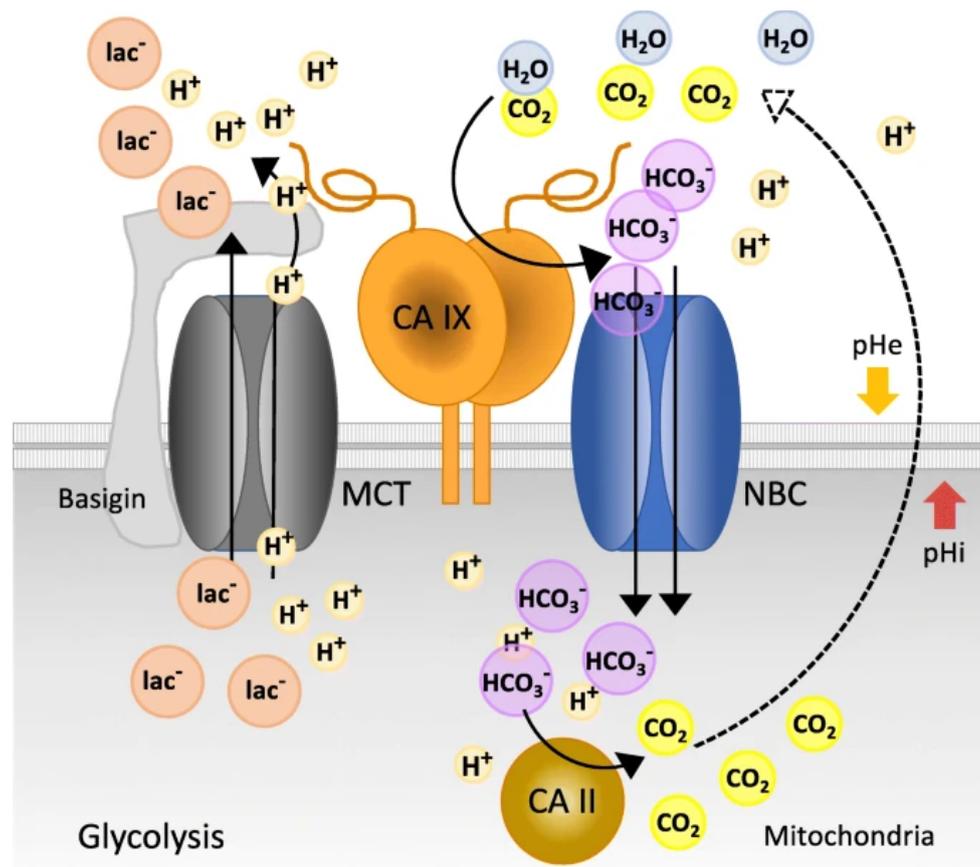
Figure 4.1. Schematic model of the CA-IX role in pH regulation in hypoxic cancer cells (image taken from Pastorekova and Gillies, 2019). In hypoxic conditions cancer cells activate alternative metabolic pathways, from general mitochondrial oxidative phosphorylation to anaerobic glycolysis, leading to the production of acidic metabolites. Since these organic acids may result toxic for the cell, they are extruded through specific transporters, acidifying the surrounding matrix, sometimes reaching pH values below 6.5. CA-IX contributes to the adaptation of cancer cells to acidosis, creating a differential pH across the plasma membrane (external acidic pH and intracellular alkaline pH). The cell surface domain of the enzyme catalyzes the conversion of extracellular CO_2 into H^+ and $HCO3^-$. Protons remain outside the cell, contributing to the acidification of the matrix, while bicarbonate ions are translocated through the membrane into the cytoplasm by specific transporters. Here, they interact with protons deriving from different metabolic pathways forming H_2O and CO_2 which leaves the cell by diffusion. Subtraction of intracellular protons by the imported bicarbonate ion helps to increase the intracellular pH to the permissive values for metabolic activity and cell proliferation.

Keeping in mind the overexpression of CA-IX in several hypoxic tumors and its limited expression in normal cells of gut epithelium, in the last years, CA-IX has been validated as a promising new anticancer target and many efforts have been made to develop selective inhibitors for biomedical applications. Benzene-sulfonamides derivates are the most common molecules employed in designing CA-IX inhibitors. Specifically, SLC-0111 is a ureido benzene-sulphonamide, currently in phase 2 clinical trial for the treatment of solid tumors associated to hypoxic microenvironment (Pacchiano et al., 2011; Carta et al., 2017). Inhibitor AA-06-05 has been developed from the lead compound SLC-0111 by a divalent isosteric replacement approach, by means of introduction within the ureido moiety of a selenium element (Angeli et al., 2017). A recent study (Ciccone et al., 2020) reported an evident anti-proliferative and anti-invasive effect of both SLC-0111 and AA-06-05 in A549 and MDA-MB-231 cancer cells. However, a deeper characterization of the effect of these two compounds on endothelium was required.

The data obtained in this study document the likelihood for CA-IX inhibitor AA-06-05 to be developed as new anticancer drug, but a particular attention should be paid to its potential side effects on endothelial cells due to its targeting on other CA isoforms as CA-I, with ubiquitous localization and physiological significance.

4.3. Paper: "Effect of Carbonic Anhydrase IX inhibitors on human endothelial cell survival"

Follows in the next page

Effect of Carbonic Anhydrase IX inhibitors on human endothelial cell survival

Shirley Genah[a], Andrea Angeli[b], Claudiu T. Supuran[b], Lucia Morbidelli[a,*]

[a] Department of Life Sciences, University of Siena, Siena, Italy
[b] Dipartimento NEUROFARBA, Sezione di Scienze Farmaceutiche, Università di Firenze, Sesto Fiorentino, Firenze, Italy

ABSTRACT

The vascular endothelium is one of the first barriers encountered by drugs and xenobiotics, which, once administered, enter the blood stream and diffuse to all organs through blood vessels. The continuous exposure of endothelial cells to drugs and chemical compounds turns out to be a huge risk for the cardiovascular system, as these substances could compromise endothelial vitality and function and create irreparable, localized or systemic damages. For this reason, a special attention should be paid to the safety of developing drugs on the cardiovascular system.

In this study we focused our attention on carbonic anhydrase (CA)-IX inhibitors. CA-IX is an enzyme overexpressed in tumor cells in response to hypoxia, which is involved in pH control of the neoplastic mass microenvironment and in tumor progression. Specifically, we evaluated the safety on human umbilical vein endothelial cells (HUVEC) of CA-IX inhibitor AA-06-05, compared to its lead compound SLC-0111, for which the efficacy on tumor cells has already been proven. In this analysis we detected an impairment in viability and mitochondrial metabolism of HUVECs treated with AA-06-05 (but not with SLC-0111) in the concentration range 1–10 μM. These data were accompanied by an increase in the expression of the cell cycle negative regulator, p21, and a down-regulation of the pro-survival proteins ERK1/2 and AKT, both in their phosphorylated and total forms.

The data obtained document the likelihood for CA-IX inhibitor AA-06-05 to be developed as new anticancer drug, but a particular attention should be paid to its potential side effects on endothelial cells due to its targeting on other CA isoforms as CA-I, with ubiquitous localization and physiological significance.

1. Introduction

In solid and metastatic tumors, the continuous division and accumulation of cells create a very tight environment that hinders access to blood vessels for oxygen provision [1]. In hypoxic conditions cancer cells activate alternative metabolic pathways, from general mitochondrial oxidative phosphorylation to anaerobic glycolysis, leading to the production of acidic metabolites. Since these organic acids may result toxic for the cell, they are extruded through specific transporters, acidifying the surrounding matrix, sometimes reaching pH values below 6.5 [1,2].

The acidification of the extracellular milieu brings a clear selective advantage for the development of tumor mass [3]. First, the acidic and hypoxic environment counteracts healthy cells, compromising their viability in the areas surrounding the tumor tissue. Secondly, acidosis leads to the activation of metalloproteases involved in remodeling stromal and tumor cell surface proteins, promoting cancer cell motility and metastasis, and in extracellular matrix degradation for new blood vessel formation [4]. Extracellular and intracellular acidosis threatens cell viability as low pH levels can disrupt various biological activities, but cancer cells can adapt to these changes by up-regulating key pH regulatory factors, such as carbonic anhydrase IX (CA-IX) [5].

CA-IX is a tumor-associated, cell-surface glycoprotein induced by hypoxia [5,6], belonging to the α-carbonic anhydrase family of zinc metalloenzymes that catalyzes the reversible hydration of carbon dioxide (CO_2) to bicarbonate ions (HCO_3^-) and protons (H^+) [6.7]. In mammals, 16 different α-CA isozymes were described so far: eight cytosolic forms (CA-I-III, CA-VII, CA-XIII as well as the non-catalytic CA-VIII, -X and -XI proteins); five membrane-bound/transmembrane isozymes with extracellular active site (CA-IV, CA-IX, CA-XII, CA-XIV and CA-XV); two mitochondrial forms (CA-VA and CA-VB); one secreted isozyme, CA-VI (in saliva and milk) [6,7].

* Corresponding author at: Department of Life Sciences, University of Siena, Via A. Moro 2, 53100, Siena, Italy.
E-mail address: lucia.morbidelli@unisi.it (L. Morbidelli).

CA-IX contributes to the adaptation of cancer cells to acidosis, creating a differential pH across the plasma membrane (external acidic pH and intracellular alkaline pH). The cell surface domain of the enzyme catalyzes the conversion of extracellular CO_2 into H^+ and HCO_3^- [7,8]. Protons remain outside the cell, contributing to the acidification of the matrix, while bicarbonate ions are translocated through the membrane into the cytoplasm by specific transporters. Here, they interact with protons deriving from different metabolic pathways forming H_2O and CO_2 which leaves the cell by diffusion. Subtraction of intracellular protons by the imported bicarbonate ion helps to increase the intracellular pH to the permissive values for metabolic activity and cell proliferation [8].

Keeping in mind the overexpression of CA-IX in several hypoxic tumors and its limited expression in normal cells of gut epithelium, in the last years, CA-IX has been validated as a promising new anticancer target and many efforts have been made to develop selective inhibitors for biomedical applications [9,10]. Although benzene-sulfonamides derivates are the most common molecules employed in designing CA-IX inhibitors, different compounds have been studied in the past years, such as glucosyl-based sulfamates [11] or inhibitors based on the uricosuric agent Probenecid [12]. This drug is used to treat gout and hyperuricemia by increasing uric acid excretion in urines. Considering Probenecid ability to interfere with CA catalytic activity, many derivates have been synthesized with good affinity for CA-IX and CA-XII [12].

Among such new anti-tumor candidates reported as CA-IX inhibitors, SLC-0111 and AA-06-05 are being examined here. SLC-0111 is a ureido benzene-sulphonamide, currently in phase 2 clinical trial for the treatment of solid tumors associated to hypoxic microenvironment [13,14]. SLC-0111 has a very high affinity with this enzyme, equal to a K_I of 45.0 nM, calculated by stopped-flow carbon dioxide hydration assay [14,15]. Inhibitor AA-06-05 has been developed from the lead compound SLC-0111 by its bioisosteric modification, i.e., the replacement of the oxygen atom from the ureido functionality by a selenium one [16,17]. Specifically, AA-06-05 was obtained by the replacement in SLC-0111 of the oxygen, within the ureido moiety, with a selenium atom. Besides having certain affinity with CA-IX (K_I = 63.0 nM), AA-06-05 also inhibits the two human isoforms of this enzyme, the ubiquitous CA-I (K_I = 152.3 nM) and the cytosolic CA-II (K_I = 66.3 nM) [17].

The effectiveness of anticancer drugs in treating a solid tumor is dependent on delivery of the drug to virtually all cancer cells within the tumor. Orally given drugs, after absorption, pass in the blood stream and diffuse in all the organs through blood vessels. Hence, drugs and their metabolites are continuously facing endothelial cells [18]. The cardiovascular system has proven to be particularly sensitive to a large variety of drugs and chemical compounds, as they can produce several damages such as direct myocardial lesions, pro-arrhythmic alterations and impairment of vascular integrity and tone [19]. For this reason, cardiovascular safety remains the major concern in drug development and it is fundamental for any medication to be screened for toxic effects on endothelial cells [19].

Since vascular endothelium is not a stable barrier but can be influenced by inflammatory mediators, oxidative stress or growth factors [18,19], it is also critical to have information on the reactivity of endothelial cells to xenobiotics under the stimulation with permeabilizing factors as vascular endothelial growth factor (VEGF), especially close or within a tumor mass. Indeed, VEGF is produced and released by growing tumors in hypoxic environment to induce the formation of new blood vessel (namely angiogenesis) with aberrant and hyper-permeable features [20]. When developing new anti-tumor agents, we need to test the safety of the novel molecule on both quiescent endothelium and VEGF-stimulated endothelial cells.

The focus of the present study was to assess and compare the safety of two CA-IX inhibitors, SLC-0111 and AA-06-05 on cultured human endothelial cells under quiescent condition and following stimulation with VEGF to mimic tumor microenvironment with activated endothelial cells.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and grown in endothelial growth medium (EGM-2), containing VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000 (Lonza, Basel, Switzerland), 10 % Fetal Bovine Serum (FBS; Hyclone, Euroclone, Milan, Italy), 2 mM glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin (Merck KGaA, Darmstadt, Germany). Cells were cultured at 37 °C with 5 % CO_2 in Petri dishes coated with gelatin derived from bovine skin (Sigma-Aldrich, St. Louis, Missouri, US).

Cell stimulation with test substances was performed with endothelial cell growth basal medium-2 (EBM-2, Lonza) supplemented with 1 % FBS, 2 mM glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin (Merck KGaA). For all the experiments, HUVEC cells were used until passage 6–7.

2.2. Pharmacological inhibitors

The chemical properties and synthesis of the ureido benzene-sulphonamide, SLC-0111 (PubChem CID: 310360), have been described previously [14]. As already discussed, AA-06-05 is a SLC-0111 congener obtained by a divalent isosteric replacement approach, by means of introduction within the ureido moiety of a selenium element [17] (Fig. 1).

For in vitro studies, SLC-0111 and AA-06-05 were dissolved in a solution of 10 % dimethylsulfoxide (DMSO) to a final concentration of 0.01 M. Aliquots were stored at −20 °C and properly diluted into culture medium prior HUVEC stimulation.

2.3. MTT survival test

HUVEC metabolic activity was quantified through MTT assay following the protocol described by Monti et al. [21]. 4×10^3 cells/100 μl were seeded in 96-multi well plates, pre-coated with gelatin, in EGM-2

SLC-0111

Chemical Formula: $C_{13}H_{12}FN_3O_3S$

Molecular Weight: 309,32

AA-06-05

Chemical Formula: $C_{13}H_{12}FN_3O_2SSe$
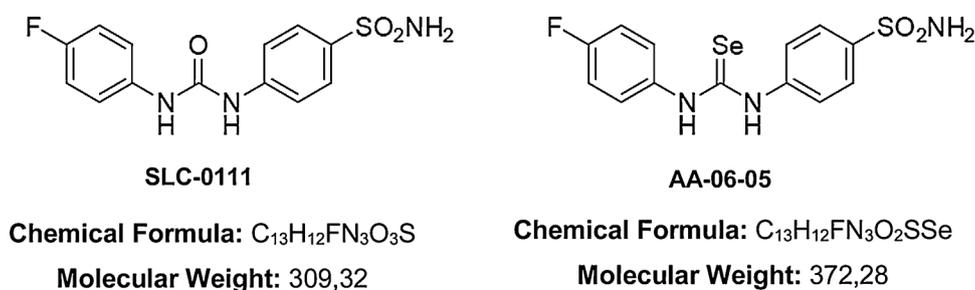
Molecular Weight: 372,28

Fig. 1. Structure, chemical formula and molecular weight of the two CA-IX inhibitors, SLC-0111 and AA-06-05.

medium supplemented with 10 % FBS. After 24 h of incubation to allow cell adherence, cells were stimulated for 48 h with the CA inhibitors diluted in EBM-2 with 0.1 % FBS. Specifically, for each compound, cells were challenged with 10-fold serial dilutions from 10 μM to 0.01 μM, in the absence/presence of VEGF (R&D Systems, Minneapolis, MN, USA) used at 25 ng/mL.

After two days, medium was removed and cells were incubated for 4 h with fresh medium in the presence of 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After solubilization of the resulting formazan derivative in DMSO, absorbance was measured with a micro-plate absorbance reader (Infinite 200 Pro, Tecan Life Sciences, Switzerland) at 540 nm. Data are reported as 540 nm relative absorbance/well.

2.4. Cell proliferation assay

HUVEC viability was evaluated by cellular proliferation assay [21]. 1.5×10^3 cells/100 μl were seeded on the bottom of 96 multiwell plates, pre-coated with gelatin, in EGM-2 medium supplemented with 10 % FBS. After 24 h of incubation, for cell adherence, cells were treated for 48 h with test substances SLC-0111 and AA-06-05, properly diluted in EBM-2 medium with 0.1 % serum. Specifically, for each of the two compounds, cells were challenged with 10-fold serial dilutions from 10 μM to 0.01 μM, with or without 25 ng/mL VEGF. At the end of incubation, cells were fixed, stained and counted at $20 \times$ magnification in five random fields. Results are expressed as the number of cells counted per well.

2.5. Western blot

Western blot was performed on culture lysates of sub-confluent HUVECs seeded in 60 mm Petri dishes (3×10^5 cells/dish) [21]. After 24 h of incubation at 37 °C, cells were treated for 8 h with the appropriate stimuli diluted in EBM-2 with 0.1 % FBS. Cells were stimulated with the two CA inhibitors, specifically with 10 μM of SLC-0111 and $1-10$ μM of AA-06-05. In order to simulate tumor hypoxia microenvironment, HUVECs were stimulated with 100 μM Cobalt Chloride (CoCl$_2$, Sigma Aldrich) diluted in EBM-2 with 5% FBS for 18 h [22].

After stimulation, cells were washed twice with cold Dulbecco's Phosphate Buffered Saline (Sigma Aldrich) and scraped on ice with CelLytic™ MT Cell Lysis Reagent supplemented with 2 mM Na$_3$VO$_4$ and 1x Protease inhibitor cocktail for mammalian cells (Sigma Aldrich). Cell lysates were centrifuged at $16,000 \times g$ for 20 min at 4 °C and the supernatants were then collected. Protein concentration was determined using the Bradford protein assay (Sigma-Aldrich). Electrophoresis with equal amounts of proteins (40 μg) was carried out in NuPAGE™ 4–12 % Bis-Tris precast Gels (Thermo Fisher Scientific, Waltham, Massachusetts, US).

Proteins were then blotted onto nitrocellulose membranes, blocked (1 h) in a solution of 5% (wt/vol) milk and then incubated overnight at 4 °C with the primary antibodies diluted 1:1000 in 1% (wt/vol) milk: anti-CA-IX (MW: 50 kDa; #MAB713, Merck Millipore, Merck KGaA, Darmstadt, Germany), anti-CA-I (MW: 28 kDa; #ab108367, Abcam, Cambridge, UK), anti-p21 (MW: 21 kDa; #05345, Merck Millipore), anti-p53 (MW: 53 kDa; #SC-126, Santa Cruz, Dallas, Texas, US), anti-total and phosphorylated AKT (MW: 60 kDa; Cell Signaling, Danvers, Massachusetts, US; #9272 and #9271, respectively), anti-total and phosphorylated ERK1/2 (MW: 42/44 kDa; Cell Signaling, #9102 and #9101, respectively). After 1 h of incubation with the secondary antibody anti IgG HRP (diluted 1:2500, Promega, Madison, Wisconsin, US), the immunoreaction was revealed by ImageQuant LAS 4000 chemiluminescence system (GE Healthcare, Chicago, Illinois, US). Results were normalized to those obtained by using an antibody against β-Actin (MW: 42 kDa; #A5441, Sigma Aldrich) diluted 1:10000. Immunoblots were analyzed by densitometry using NIH Image J 1.48v software.

2.6. Statistical analysis

Each experimental condition was run in triplicate and data are reported are the mean ± SD of at least three separate independent experiments. Statistical significance between means was determined using Student's t test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. HUVEC express basal levels of CA-IX and CA-I which are upregulated by VEGF

Firstly, we evaluated the presence of CA-IX in HUVEC under normoxic conditions. The expression of this isozyme in endothelial cells has been compared with that in human breast adenocarcinoma cells MDA-MB-231, for which an inhibitory effect has already been demonstrated for the lead compound SLC-0111 and its derivative AA-06-05 [13,17].

We found that HUVECs constitutively expressed CA-IX, while CA-IX expression was 68–70 % higher in MDA-MB-231 cells compared to HUVECs (Fig. 2A and B), a result compatible with the involvement of CA-IX in oncogenesis and tumor progression.

Following this initial approach, we evaluated CA-IX and CA-I expression in two conditions commonly associated with tumor microenvironment: high levels of the endothelium permeabilizing factor VEGF and hypoxia. For this purpose, HUVECs were stimulated with 25 ng/mL VEGF or treated with 100 μM CoCl$_2$ for 18 h to mimic hypoxia. As we can see in Fig. 2C and D, CA-IX enzyme, constitutively expressed in HUVEC, was not modulated by VEGF. Interestingly, following VEGF stimulation, we observed a significant increase in CA-I levels compared to the basal condition. CoCl$_2$ treatment produced a significant increase in CA-IX and, more significantly, of CA-I. In the same experimental conditions, no expression of CA-II was detected (data not shown).

3.2. The two CA-IX inhibitors differently affect HUVEC proliferation and their metabolic activity

HUVEC viability and proliferation capacity have been tested in the presence of the two compounds SLC-0111 and AA-06-05 for two days. This assay revealed a significant impairment in cell proliferation exposed to compound AA-06-05 with a pattern proportional to the concentration of the inhibitor. By contrast, for compound SLC-0111 a not significant reduction in cell number was observed (Fig. 3A and B). However, at longer stimulation times (72 h), the lead compound SLC-0111 also produced a toxic effect on HUVEC, reducing their proliferation and viability (data not shown). Results on cell proliferation were then confirmed by the evaluation of HUVEC metabolic activity by MTT assay. Again, AA-06-05 seems to be the compound with the most negative activity on HUVEC mitochondrial metabolism (Fig. 3B).

The results described above could be further corroborated by the images in Fig. 3C which show HUVEC status after 48 h of stimulation with the two CA-IX inhibitors at different concentrations. Compound AA-06-05 exerted the most impairing effects at concentrations of 5 and 10 μM when a drastic decrease in cell number and a morphological change can be noticed. Indeed, HUVECs acquired an elongated morphology and many refractive elements could be found, indicating a progressive detachment of cells from the surface and a possible phenomenon of cell death.

Finally, the activity of the two compounds was assessed on endothelial cells activated by VEGF to mimic the tumor microenvironment characterized by endothelial hyper-permeability and ignition of angiogenesis [20]. HUVEC were stimulated with VEGF (25 ng/mL) in the absence and presence of 10 μM of the test compounds. In the presence of VEGF, the two inhibitors continued to show survival impairment (Fig. 4). In particular, AA-06-05 inhibitory effect was not restored
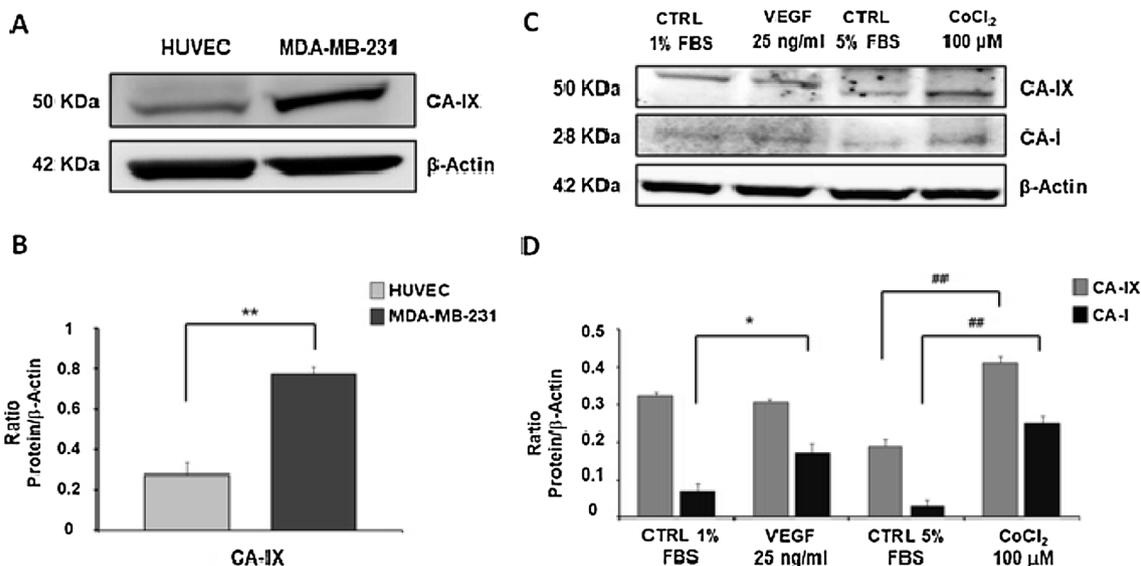
Fig. 2. Basal expression of CA-IX in HUVEC and MDA-MB-231 mammary tumor cells (A, B). Expression of CA-IX and CA-I in HUVECs activated by VEGF or treated with CoCl$_2$ for 18 h to simulate hypoxia (B, C). Whole cell lysates were collected and Western blots (A, C) were performed to assess protein abundance. Immunoblots were analyzed by densitometry and the results were normalized to β-Actin (B, D). Data represent means +/- SD (n = 2) **p < 0.01 HUVEC vs MDA-MB-231 (B); *p < 0.05 VEGF treatment vs Control 1% FBS; ## p < 0.01 CoCl$_2$ treatment vs Control 5% FBS (D).

by VEGF, which, on the other hand, produced an additive inhibitory effect, as VEGF may sensitize HUVEC to the cytotoxic effect of the CA inhibitor.

3.3. AA-06-05 inhibitor induces cell cycle arrest and cell death

Following the above results, we evaluated the molecular mechanisms underneath the cytotoxic effect of compound AA-06-05 on HUVECs. To this purpose, cells were stimulated for 8 and 48 h with 1 − 10 μM of AA-06-05 and 10 μM of reference inhibitor SLC-0111. Typical markers of cell cycle arrest and death, p53 and p21, were analyzed by Western Blot.

From the blots in Fig. 5A and C we can observe, compared to basal condition, an increase of p21 protein expression in HUVEC cells treated with the two inhibitors at all test concentrations. Specifically, AA-06-05 increased p21 expression in a concentration dependent manner with a similar pattern both at 8 and 48 h of stimulation. However, HUVECs administered with 10 μM of SLC-0111 expressed a significantly reduced quantity of p21, about 50 % compared to the amount obtained with AA-06-05 at the same concentration (Fig. 5B and D). Regarding p53, a mild baseline expression could be noticed in all samples, which however was not modulated following treatment with inhibitory compounds.

Ultimately, we evaluated the modulation of pro-survival pathways in response to 48 h of HUVEC treatment with the two CA-IX inhibitors.
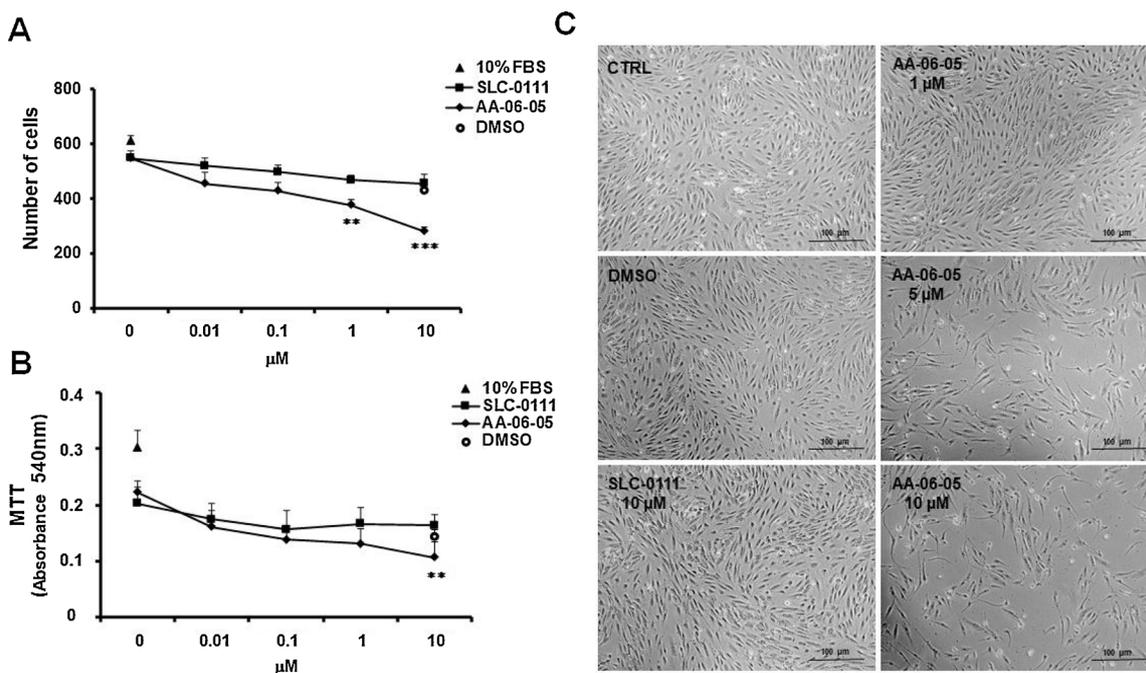
Fig. 3. AA-06-05 negatively affects HUVECs survival. Cells were exposed for 48 h with test substances diluted in EBM-2 1% FBS. (A) data on cell proliferation (B) results of the MTT assay. Data represent means +/- SD (n = 3) **p < 0.01 and ***p < 0.001 vs basal control condition. In panel (C), representative images of alive HUVECs taken after 48 h of incubation with test compounds.
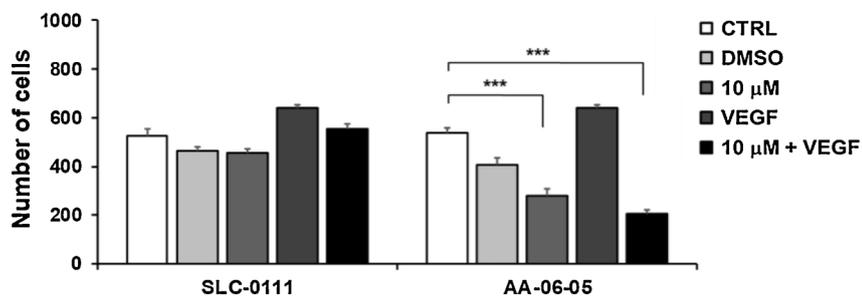
Fig. 4. Effect of the two CA-IX inhibitors on HUVECs activated by VEGF. Cells were stimulated for 48 h with test substances SLC-0111 (10 μM) and AA-06-05 (10 μM) in the absence and presence of VEGF (25 ng/mL). HUVECs were fixed, stained and counted. Data represent means +/- SD. (n = 2). ***p < 0.001 as specified in the graph.
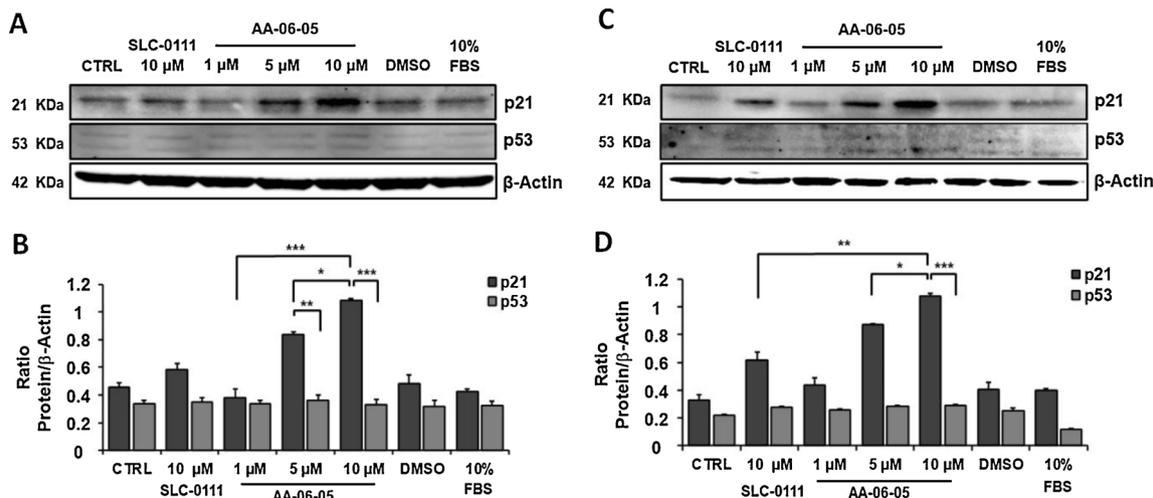
Fig. 5. Effect of CA-IX inhibitors on cell cycle regulators in HUVECs. Cell whole cell lysates were collected after 8 h (A) and 48 h (C) of exposure to the test compounds and Western blot was performed to assess protein abundance of p21 and p53. (B, D) Immunoblots were analyzed by densitometry and the results were normalized to β-Actin. Data represent means +/- SD. (n = 2) *p < 0.05, **p < 0.01 and ***p < 0.001 as specified in the graph.
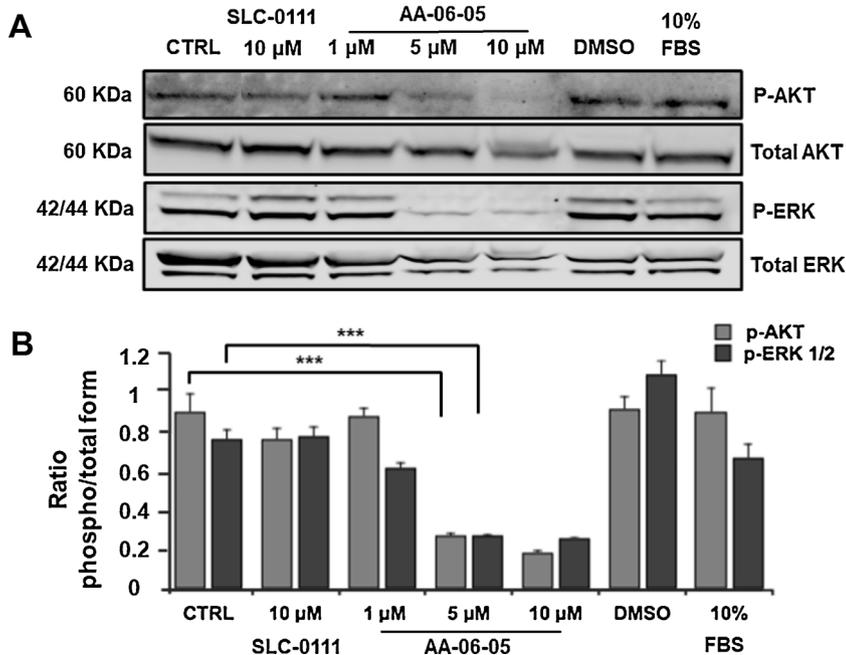
Fig. 6. Effect of CA-IX inhibitors on pro-survival pathways in HUVECs. Cell whole cell lysates were collected after 48 h of exposure to the test compounds and Western blot (A) was performed to assess protein abundance of phosphorylated AKT and ERK1/2. (B) Immunoblots were analyzed by densitometry and results of the phosphorylated proteins were normalized to the corresponding total form. Data represent means +/- SD. (n = 2), ***p < 0.001 as specified in the graph.

To this purpose, typical markers of these pathways, such as ERK1/2 and AKT, were evaluated by Western Blot. In line with data obtained so far, in response to treatment with compound AA-06-05, we can observe a dose-dependent downregulation of both ERK1/2 and AKT total expression and their activation by phosphorylation (Fig. 6A and B). Specifically, a nearly complete downregulation of pro-survival proteins

was obtained at the concentrations of 5 and 10 μM.

4. Discussion

Summing together the results obtained so far, it is confirmed the safety of the lead compound SLC-0111 on cultured human endothelial

cells, while compound AA-06-05 causes a deleterious effect on cells and therefore its potential therapeutic use should be considered with cautions. The impairing behavior of AA-06-05 can be explained by its scarce selectivity. As previously discussed, while compound SLC-0111 is very selective for CA-IX, AA-06-05 interacts with high potency with two additional cytosolic isoforms of carbonic anhydrase (CA-I and CA-II) which are present in many tissues where they are involved in cell respiration and regulation of the acid/base homeostasis [7] accomplishing vital functions for the cell. In humans, CA-II is usually expressed by epithelial cells of the gastrointestinal tract and lungs, osteoclasts and kidney cells [7]. However, there is no proof of CA-II expression in human umbilical vein endothelial cells under basal conditions and our results corroborate the lack of CA-II expression. In this study we demonstrated that HUVECs express CA-I following VEGF activation and in CoCl$_2$ induced hypoxia. This observation can explain the additive cytotoxic effect of compound AA-06-05 on endothelial cells stimulated with VEGF. Therefore, it can be concluded that AA-06-05 inhibition, compared to that observed for its lead compound SLC-0111, operates by interfering with CA-I and CA-IX functioning, reflecting negatively on HUVEC viability and metabolic capacity.

The inhibition of proliferation and mitochondrial metabolism, due to treatment with AA-06-05, correlates with an increased expression of the p21 protein, marker of cell cycle arrest [23]. Indeed, this protein is known to be a potent cyclin-dependent kinase inhibitor that regulates cell cycle progression at the checkpoint level between G1 and S phases [24]. Although p21 is a well-known p53-downstream gene, in our study cell cycle arrest appears to be independent of p53 activation.

Under resting conditions, the phosphorylated, and therefore the active form, of ERK1/2 is implicated in cell proliferation and survival. Specifically, it performs these tasks by inhibiting the apoptosis process through degradation or sequestration of its effectors [25]. Similarly, AKT kinase, once activated by phosphorylation, is important in HUVECs for many cellular functions including proliferation, migration, cell growth and metabolism [26]. In this work we observe, in response to AA-06-05, a down-regulation of these two proteins, both in their total expression and in their activation. These data confirm that endothelial cells, following treatment with the CA-IX inhibitor AA-06-05, undergo a reduction in viability, proliferation and metabolism, as demonstrated by the MTT and proliferation data.

In conclusion it can be inferred that CA-IX has no essential function in endothelial cells grown in normoxia conditions. Indeed, its inhibition by lead compound SLC-0111 does not produce negative consequences on cell viability and proliferation, whereas the deleterious effect observed for the compound AA-06-05 could be attributed to its combined action on CA-IX and CA-I enzymes, further exacerbated by VEGF stimulation.

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.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.104964.

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5. Conclusions

The arguments of this thesis concern the stromal components of tissues in particular vascular endothelium and fibroblasts and their reciprocal interaction, in order to define new therapeutic strategies for diseases accompanied by exacerbated inflammation and endothelial dysfunction.

In the first topic of this thesis we have demonstrated that erucin, as a smart H_2S-donor molecule, is able to promote angiogenesis which represents a fundamental process in numerous physiological functions, including tissue healing. Specifically, concentrations of erucin in the nanomolar range are able to promote ECs migration and organization in capillary-like networks *in vitro*, through the early activation of eNOS - ERK1/2 - Akt pathways. Subsequently, at longer stimulation times erucin leads ECs to produce and release soluble factors such as VEGF and FGF-2 which, on the one hand, feed the angiogenesis process creating a positive feedback, and, on the other, affects fibroblast responses.

As discussed in the introductory chapters, endothelial dysfunction induced by chronic exposure of ECs to hyperglycemic conditions is associated with a reduction in the production and release of the two gaseous transmitters, NO and H_2S (Rajendran et al., 2013). Specifically, insufficient production of H_2S plays a crucial role in impaired microvascular endothelial function and angiogenesis *in vivo* (Cheng et al., 2018), and recent studies demonstrated that up-regulation of H_2S biosynthesis is able to rescue endothelial dysfunction in diabetic animal models (Cheng et al., 2017; Durante, 2019). Therefore, pathways involved in the up-regulation and release of hydrogen sulphide may represent a valuable target for the development of therapeutical countermeasures aimed at restoring EC function in hyperglycemic or diabetic conditions. In this scenario we demonstrated erucin ability, alone or in combination with VEGF, to protect endothelial cells from high glucose-induced damage and recover impaired functional responses to physiological levels, such as *in vitro* migration and angiogenesis on Matrigel. It should be noted that in our experimental conditions, glucose levels of 25-30 mM, corresponding to 450 - 540 mg/dL were used in cell cultures to accelerate the occurrence of diabetic complications. These values are, however, far above those observed in diabetic patients (typically 7.8 to 10 mM; 140–180 mg/dL), this represent a limit when extrapolating results from these experimental systems to the human disease. Preliminary data obtained in this study could, however, represent a solid basis for possible *in vivo* observations. Indeed, among the future objectives of this project, animal models of diabetes could be employed to further and better characterize erucin profile in the context of endothelial dysfunction induced by hyperglycemia.

Secondly, we contextualized erucin effect in the process of wound healing by analyzing its effect on two cell types involved in tissue remodeling and epithelialization: fibroblasts and keratinocytes. While keratinocytes seemed to respond well to the effective concentration of

the compound in terms of migration and survival, fibroblasts have not been affected directly by erucin. However, an indirect consequence on NHDF proliferation has been observed, albeit in a preliminary way, presumably mediated by an increase in the release of FGF-2 by ECs stimulated with the isothiocyanate. Although the project is still in progress and further experimental investigations are required, preliminary data suggests that erucin could be exploited, in a context of defective tissue healing, to favor the progression towards the proliferative phase through the induction of the granulation tissue, also considering its direct pro-angiogenic properties.

In addition to endothelial dysfunction and insufficient angiogenesis, among the various factors that disrupt tissue repair process we also find excessive inflammatory state which cause pathologic conditions ranging from healing delay (e.g., chronic wounds) to fibrosis (Qing, 2017). In the second part of this thesis we focused our attention on fibroblasts, activated by macrophage release of IL-1β and TNF-α, which modulate the recruitment and behavior of immune cells through a release of cytokines, vasoactive and pro-angiogenic factors, and regulate tissue remodeling by synthesizing ECM components and assuming contractile properties (Wynn and Vannella, 2016; Zhao et al., 2016). Inflammation dysregulation and persistence of fibroblast activation state, with excessive production of pro-inflammatory agents, ECM components and vasoactive factors, leads to altered repair process which, at the tissue level, is characterized by redness, heat, edema, pain, and can lead to fibrosis (Hinz, 2016; Qing, 2017; Zhao et al., 2016). Thus, eliminating or controlling the excessive inflammation can represent a turning point in the transition to the following stages of healing and can effectively resolve many chronic wounds. Due to side effects raised by long-term administration of anti-inflammatory drugs, the research is continuously active to define alternative therapies. Among them, physical countermeasures such as photobiomodulation therapy (PBMT) are considered effective and safe (Hamblin, 2017). Therefore, the aim of the second topic was to study the cellular and molecular events associated with the anti-inflammatory activity of PBMT by a dual-wavelength NIR laser source, on human dermal fibroblasts exposed to a mix of inflammatory cytokines (IL-1β and TNF-α) followed by laser treatment. Results demonstrated laser ability to revert fibroblast inflammatory phenotype by reducing to basal levels pro-angiogenic factors, as VEGF, and inducible inflammatory key enzymatic pathways, as iNOS and COX-2/mPGES-1/ PGE2, by retaining NF-kB transcription factor in a cytoplasmic localization. These molecular changes are accompanied by a shift in cell morphology attributed to a re-distribution of fundamental cytoskeletal proteins (Tubulin, F-actin, and α-SMA) to basal localization following laser treatments. Like all *in vitro* models, a limit of the proposed approach is to provide a very partial representation of what happens in vivo during inflammation and healing. However, it can be considered representative of the early stage of the inflammation phase after an injury, when M1 macrophages produce great amounts of IL-1β and TNF-α. In the normal evolution of inflammation, macrophage phenotype is expected to shift from M1

to M2, with increased TGF-β production and a decrease in IL-1β and TNF-α levels (Wynn and Vannella, 2016; Zhao et al., 2016). Therefore, the proposed model can be considered also representative of altered evolution of inflammation with persistence of high levels of TNF-α, compared to TGF-β levels, due to the failure to switch from M1 to M2.

In the third and final topic of this thesis we outlined the importance of assuring endothelial safety during drug development. Orally given medications pass in the blood stream and diffuse in all the organs through blood vessels, therefore drugs and their metabolites are continuously facing endothelial cells. The cardiovascular system has proven to be particularly sensitive to a large variety of drugs and xenobiotics, which can promote or accelerate the onset of some CVDs, including atherothrombosis, systemic and pulmonary hypertension or diabetes (Wojcik et al., 2015) and impair of vascular integrity and tone. For this reason, cardiovascular safety remains the major concern and it is fundamental for any medication to be screened for toxic effects on endothelial cells. The class of drugs that are most clinically debated for their citotoxicity on the endothelium are chemotherapeutic agents (Soultati et al., 2012). Recently, carbonic anhydrase IX (CA-IX) emerged as an ideal target for solid hypoxic tumor treatment and in the last paper presented, the safety profile of two CA-IX inhibitors, SLC-0111 and AA-06-05 on human endothelial cells was assessed. Of the two molecules, SLC-0111 is currently in phase 2 clinical trial for the treatment of solid tumors associated to hypoxic microenvironment, while AA-06-05 has been developed from the lead compound SLC-0111 by a divalent isosteric replacement approach (Pacchiano et al., 2011; Carta et al., 2017; Angeli et al., 2017). Despite AA-06-05 displays remarkable anti-tumor activity *in vitro* on tumor epithelial cells from breast (MDA-MB-231) and lung (A549) cancer at concentrations of 50 µM (Ciccone et al., 2020), its potential therapeutic use should be considered with cautions due to the serous cytotoxic effects on endothelium. Indeed, ECs treatment with AA-06-05 in the concentration range 1-10 µM leads to a significant impairment in cell viability and proliferation, further exacerbated by VEGF stimulation. These data are accompanied by an increase in the expression of the cell cycle negative regulator, p21, and a down-regulation of the pro-survival proteins ERK1/2 and Akt, both in their phosphorylated and total forms.

In conclusion vascular endothelium plays a pivotal role in the maintenance of many biological functions, among these, angiogenesis which is the formation of new capillaries from pre-existing vasculature, is a finely tuned process aimed at supplying nutrients and oxygenation to growing or healing tissues following injury. In the context of wound healing, ECs are continuously engaged in a crosstalk with other cell types, immunity cells or fibroblasts, to assure a correct progression in the process of regeneration. Disruption of endothelial functions, which can occur for several reasons, including systemic administration of certain dugs, leads to a variety of pathological cardiovascular consequences which are commonly featured by a dysregulation in gaseous transmitters

production such as NO and H_2S. Experimental data proved exogenous H_2S-donors ability to recover several pathological conditions associated with EC dysfunction, such as hypertension or diabetes (Ciccone et al., 2021). Endothelial dysfunction and associated inflammation continue, however, to be the main causes of morbidity and mortality all over the world (Rajendran et al., 2013). Therefore, new therapeutical approaches are needed to prevent endothelial dysfunction or revert cardiovascular disorders, and sulfur compounds with natural origin, such as erucin described in this thesis, represent helpful pharmaceutical/nutraceutical tools to be used in therapy or as a template for the ideation of advanced H_2S-donor molecules with improved pharmacodynamic and/or pharmacokinetic properties.

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7. Supplementary Material

Reviews in the order:

1. Ciccone V., **Genah S.**, Morbidelli L. Endothelium as a Source and Target of H2S to Improve Its Trophism and Function. Antioxidants (Basel). 2021 Mar 19;10(3):486. doi: 10.3390/antiox10030486. PMID: 33808872; PMCID: PMC8003673.

2. Morbidelli L., **Genah S.**, Cialdai F. Effect of Microgravity on Endothelial Cell Function, Angiogenesis, and Vessel Remodeling During Wound Healing. Front Bioeng Biotechnol. 2021 Sep 22;9:720091. doi: 10.3389/fbioe.2021.720091. PMID: 34631676; PMCID: PMC8493071.

3. **Genah S.**, Monici M., Morbidelli L. The Effect of Space Travel on Bone Metabolism: Considerations on Today's Major Challenges and Advances in Pharmacology. Int J Mol Sci. 2021 Apr 27;22(9):4585. doi: 10.3390/ijms22094585. PMID: 33925533; PMCID: PMC8123809.

Review

Endothelium as a Source and Target of H$_2$S to Improve Its Trophism and Function

Valerio Ciccone [ID]**, Shirley Genah and Lucia Morbidelli *** [ID]

Department of Life Sciences, University of Siena, Via A. Moro 2, 53100 Siena, Italy;
ciccone3@student.unisi.it (V.C.); shirley.genah@student.unisi.it (S.G.)
* Correspondence: lucia.morbidelli@unisi.it; Tel.: +39-0577-235381

Abstract: The vascular endothelium consists of a single layer of squamous endothelial cells (ECs) lining the inner surface of blood vessels. Nowadays, it is no longer considered as a simple barrier between the blood and vessel wall, but a central hub to control blood flow homeostasis and fulfill tissue metabolic demands by furnishing oxygen and nutrients. The endothelium regulates the proper functioning of vessels and microcirculation, in terms of tone control, blood fluidity, and fine tuning of inflammatory and redox reactions within the vessel wall and in surrounding tissues. This multiplicity of effects is due to the ability of ECs to produce, process, and release key modulators. Among these, gasotransmitters such as nitric oxide (NO) and hydrogen sulfide (H$_2$S) are very active molecules constitutively produced by endotheliocytes for the maintenance and control of vascular physiological functions, while their impairment is responsible for endothelial dysfunction and cardiovascular disorders such as hypertension, atherosclerosis, and impaired wound healing and vascularization due to diabetes, infections, and ischemia. Upregulation of H$_2$S producing enzymes and administration of H$_2$S donors can be considered as innovative therapeutic approaches to improve EC biology and function, to revert endothelial dysfunction or to prevent cardiovascular disease progression. This review will focus on the beneficial autocrine/paracrine properties of H$_2$S on ECs and the state of the art on H$_2$S potentiating drugs and tools.

Keywords: vascular endothelium; hydrogen sulfide; endothelial dysfunction; hypertension; atherosclerosis; diabetes; angiogenesis; wound healing; H$_2$S donors

1. Vascular Endothelium

The vascular endothelium is the tissue that lines the inside of the circulatory system (blood vessels, lymphatic vessels and heart). The cells, arranged in a single layer oriented on the longitudinal axis of the vessel, assume a flattened shape and lay side by side with each other to form a complete monolayer. Structurally, the endothelial cell (EC) apical domain is in direct contact with blood or lymph, while the basolateral domain anchors to the basal lamina, which connects EC to the underlying tissues, such as the medial or muscular layer and the adventitia, rich in fibrous tissue.

The vascular endothelium acts as a selectively permeable barrier between extravascular and intravascular compartments and provides a non-thrombogenic surface for the cardiovascular system [1]. Nowadays, the endothelium can no longer be considered a passive barrier. Indeed, its anatomical position allows it to integrate the physical and neurohumoral signals from the blood and surrounding tissues for regulating vascular tone and permeability, cell adhesion, inflammation, smooth muscle phenotype and proliferation, as well as thromboresistance and blood fluidity [2,3].

The endothelial lining represents a wide area for the exchanges between blood and tissues (about 350 m^2 in humans) [3]. Electron microscopy observations reveal the continuous nature of arterial endothelium, characterized by tight junctions among adjacent cells in order to limit macromolecule exchange, and by a complex micro-vesicular system

involved in macromolecular transport. In spite of its apparent morphological lack of complexity, the endothelium is characterized by heterogeneity, with differences in permeability, reactivity, and biosynthesis in relation to the type of vascular district and organ considered [1–3].

2. Role of Endothelium in Physiology

The endothelium's role as a semipermeable barrier is one of its fundamental and basic functions: it regulates macromolecule transport between the lumen and vascular smooth muscle tissue [4]. Several mechanisms control the passage of macromolecules across the endothelial barrier: (i) through ECs themselves (transcellular flux); (ii) through the cell–cell junctions (paracellular flux); and (iii) via vesicular transport.

Most biological transmitters consist of large molecules with anionic and hydrophilic features, unable to diffuse across the membrane bilayer. The majority of those transmitters are believed to move through intracellular junctions between cells, or via vesicular transport, thanks to the formation of transient channels resulting from vesicle fusion. The reorganization of the intercellular junctions, which involves actin and myosin reconfiguration or direct collapse of junctional connections, appears to be the main process by which ECs increase their permeability to solutes and water [1].

The function of the endothelium is not limited to the internal surface lining of vessels or to constitute the vascular wall in the microcirculation, but it produces and releases vasoactive factors such as nitric oxide (NO), prostacyclin (PGI2), hydrogen sulfide (H_2S) and endothelin (ET) which, in the appropriate concentration and balance, maintain adequate vascular tone and blood fluidity, giving the endothelium itself an antithrombotic phenotype [5,6].

The synthesis of NO by ECs is constitutive, but it can be augmented by a wide variety of compounds, including acetylcholine, angiotensin II (AngII), bradykinin, histamine, arachidonic acid. NO has a very short half-life and is synthesized from L-arginine and oxygen by the NO synthase enzyme (NOS). The endothelial isoform of this enzyme, eNOS, constitutively expressed, appears to be Ca^{2+}/calmodulin-dependent. Once synthesized, NO rapidly spreads to vascular smooth muscle cells where it stimulates soluble guanylate cyclase (sGC), with an increase in cGMP formation and consequent vascular relaxation, while its autocrine function is related to the control of EC trophism and angiogenesis [7].

NO is not the only endothelial-dependent vasodilator. The endothelium also constitutively generates PGI2, which relaxes the underlying smooth muscle cells by activating adenylate cyclase and increasing cAMP. PGI2 is released in high quantities following the binding of transmitters, such as thrombin, histamine, serotonin, on cell surface receptors. The endothelium also produces and releases a hyperpolarizing factor (EDHF) whose chemical nature is still debated. EDHF's function is to hyperpolarize vascular smooth muscle cells, causing these cells to relax and allowing dilation of blood vessels [6].

In addition to NO and carbon monoxide (CO), H_2S is an endogenous gasotransmitter involved in the regulation of the cardiovascular, nervous, gastrointestinal, and renal systems, with a great impact on inflammatory and immune responses [8]. Recently, it has been proposed as one of the EDHFs. H_2S exerts a multitude of physiological effects on the wall of the vessels, acting in an autocrine/paracrine manner. It is produced by vascular cells and exhibits antioxidant, anti-apoptotic, anti-inflammatory and vasoactive properties. Indeed, it reduces arterial blood pressure, limits the formation of atheromatous plaques, and promotes the vascularization of ischemic/injured tissues [8].

In some pathophysiological circumstances including hypoxia, tissue hypoperfusion or arterial hypertension, some vasoconstrictor factors may be released from the endothelium as endothelins (ETs), thromboxane A2, and prostaglandin H2. Moreover, vascular endothelium participates at the regulation of vessel tone and trophism and blood flow through the processing of angiotensin, via the expression of angiotensin converting enzyme isoforms ACE-1 and ACE-2, responsible for the balance between AngII/AT-1 receptor and Ang(1–7)/Mas receptor [9].

ECs respond to the increase in blood flow through the release of NO and PGI2 by the same cells. Indeed, shear stress causes rapid activation of eNOS and increases its gene transcription; it also endorses ECs to release factors that inhibit coagulation, leukocyte migration, and smooth muscle cell proliferation, simultaneously promoting EC survival. Conversely, low shear stress and turbulent blood flow promote a pathological feature in the endothelium responsible for atherosclerosis ignition, documenting the pivotal role of ECs in finely controlling vascular functions [2,6].

A healthy functioning endothelium also provides protection against radical species of oxygen and nitrogen (ROS/RNS). It is now clear that increased levels of ROS and RNS are harmful to cells and tissues and are involved in a wide range of cardiovascular diseases having endothelial dysfunction as an underlying phenomenon. This boosted the concept of oxidation as synonymous with cell damage and senescence. The post-translational modifications involving RNS share a common ancestor—high NO concentrations mainly synthetized by inducible NOS (iNOS), upregulated in response to various endotoxin or cytokine signals. Several pathological states are linked to the deregulation of NO levels, indicating that aberrant production of NO and its products can have deleterious consequences on cells [10]. Again, one of the functions related to a healthy endothelium is the scavenging activity of reactive species through the production of antioxidant products as H_2S or protective enzymatic pathways.

The intimate surface of a healthy endothelium is both anticoagulant and antithrombotic: ECs secrete a wide range of molecules relevant to the regulation of blood clotting and platelet functions, as PGI2 and NO. Damage to the vessels or exposure to certain cytokines and proinflammatory stimuli overturns the equilibrium towards a procoagulant and prothrombotic EC phenotype, through the exposure of basal membrane components and/or tissue factor, and reduced presence of glycosaminoglycans or tissue factor inhibitor [5].

Endothelial trophism is guaranteed by the response to vasoactive and growth factors produced by surrounding tissues or autocrinally by the same ECs. Among the various examples, we and others have contributed to characterizing the beneficial effects on vascular endothelium by NO derived from eNOS, bradykinin, substance P, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), prostaglandin E2, H_2S [11–15]. The molecular mechanisms responsible for cell survival, proliferation, migration and functioning include eNOS/NO/cGMP/protein kinase G (PKG), PI-3K/Akt, MAPK/ERK1/2 and gene transcription of autocrine factors as FGF-2 [7].

Epigenetics is an emergent mechanism involved in the regulation of vascular biology and endothelial trophism. Through chromatin structure modification, epigenetics can modify endothelial functions with an impact on cardiovascular disease, being the regulatory functions of epigenetics also active on endothelial precursor cells and circulating angiogenic cells [16,17]. DNA methylation, variants, histone post-translational modifications, and recently discovered RNA-based mechanisms represent the major pathways involved in the molecular basis of epigenetics. VEGF-A and NOS are the key players in regulating and maintaining cardiovascular functions. Their expression can be controlled by epigenetic mechanisms. In particular, VEGF-A epigenetic control can occur mainly through changes in histone code by RNAs. VEGF-A acts through VEGFR2, which in turn is regulated by promoter DNA methylation [18]. Furthermore, accumulating evidence indicates that epigenetic pathways play an important role in eNOS gene regulation [19].

These findings suggest the importance to deeply understand the epigenetic mechanisms involved in the regulation of vascular functions both in physiology and in pathological conditions.

3. Endothelial Dysfunction

Endothelial dysfunction refers to a systemic condition in which the endothelium loses its physiological properties, including the control of vasodilation, fibrinolysis and platelet aggregation. Key features of the endothelial dysfunction are: (1) the reduced local production of NO due to impaired activity (uncoupling) of eNOS, and of other vasodilating

mediators as H$_2$S; (2) the decrease in anticoagulant factors such as heparin; (3) the increase in the secretion of reactive species, von Willebrand factor, and tissue factor; (4) the overexpression of adhesion molecules for leukocytes and platelets [20,21]. All these factors concur to compromise the physiological vascular homeostasis. Due to the impairment of the main protective transmitters NO and H$_2$S, the resulting endothelial dysfunction is associated with increased ROS and RNS levels and vascular oxidative stress [22,23]. The perpetuation of this condition then leads to retraction and death of the endothelium with increased permeability and exposure of the components of the basement membrane, that further amplifies the picture of vascular inflammation [4,20,22].

Endothelial dysfunction risk factors are represented by pathological states such as hypertension, diabetes, and hyperlipidemia, and improper lifestyles such as high-fat diets, tobacco and alcohol consumption, and physical inactivity [24]. Most cardiovascular diseases share endothelial dysfunction as a hallmark: atherosclerosis, diabetes complications, thrombosis, and hypercoagulation [20]. Moreover, physiological ageing through the phenomenon of mild chronic inflammation ("inflammaging") is accompanied by endothelial dysfunction [25]. Inflammatory factors such as tumor necrosis factor-α (TNF-α), inteleukin-6 (IL-6), intercellular adhesion molecule 1 (ICAM-1), and loss of the antioxidant mechanism are among the most influential promoters of vascular impairment [21].

Mounting evidence suggests that epigenetic mechanisms may contribute to vascular complications in many pathological conditions, such as diabetes or atherosclerosis, linked to altered endothelial trophism and functions [17,26]. The inflammatory phenotype in ECs induces the transcription of many cytokines and adhesion molecules, in a nuclear factor-kappa B (NF-κB)-dependent manner. Epigenetic modifications in the NF-κB promoter region produce an increased expression of p65 subunit of NF-κB, and a hyper-activation of the NF-κB pathway [27]. Another mechanism could involve histone deacetylase 2 (HDAC2), which interacts and deacetylates Nrf2. Oxidized low-density lipoproteins (ox-LDLs) are able to downregulate HDAC2 expression, resulting in increased production of eNOS-dependent reactive species [16]. Furthermore, shear stress represented by blood flow alteration modifies EC gene expression and function. Dunn et al. demonstrated that disturbed blood flow stimulates DNA methyltransferase-1 (DNMT-1) expression in endothelial cells with aberrant DNA methylation at the promoter of flow-inducible genes, contributing to atherosclerosis [28]. Finally, recent data showed the role of noncoding RNA in regulating the expression of endothelial adhesion molecule [29].

Acute or chronic infections both by bacteria and viruses have cardiovascular consequences for their direct or indirect effects on vascular endothelium, through bacterial products or cytokines released by tissue and immune system cells [30,31], and through epigenetic regulation [32]. The recent pandemic due to SARS-CoV-2 supports this concept [33,34].

Furthermore, our studies and those of others have revealed that endothelial dysfunction is associated to impaired EC survival and physiological angiogenic outcomes with subsequent rearrangement of the microcirculation that contributes to the emergence of various pathological conditions and healing disorders [7,35]. ECs play a key role in the adaptation of tissues to damage, revealing their plasticity. A change in endothelial functions following ischemia can induce the transition to a mesenchymal phenotype characterized by functional, metabolic and gene expression signatures. Indeed, the mesenchymal phenotype, with increased cell migration and clonal expansion, participates in regenerating a functioning vascular network [36].

Considering endothelial function as a "barometer for cardiovascular risk", it is crucial to identify the molecular determinants underlying endothelial integrity and functionality. Seeing endothelium as an exchange regulator between the vascular wall and surrounding tissues, it is expected that dysfunctional ECs can determine damage to other tissues [37]. Indeed, a detailed assessment of the cellular and molecular mechanisms at the base of vascular function, and, particularly, of endothelial dysfunction, will help the diagnosis

and treatment choice for a broad array of human disorders, including cardiovascular and neurodegenerative diseases [21,24,38–40].

4. Biochemistry of H_2S Production

H_2S is a gas physiologically produced by tissue and vascular cells. The enzymes responsible for the synthesis of H_2S are cystathionine β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST). The first two enzymes use L-cysteine as a substrate and are dependent on pyridoxal-5′-phosphate. 3-MST, on the other hand, works in association with cysteine aminotransferase which, starting from L-cysteine and α-ketoglutarate, produces 3-mercaptopyruvate. These enzymes are differentially expressed in the various tissues. In particular, the expression of CSE and 3-MST predominates in the cardiovascular system [8,41]. CSE is only present in the cytoplasm, while CBS and 3-MST both have a cytosolic and mitochondrial form, with the latter predominating. CSE is the principal enzyme responsible for H_2S biosynthesis, located in vascular smooth muscle cells and found mainly in ECs [42,43]. This can explain why the concentration of H_2S in the vascular tissues is around 100 times greater than in other tissues [44], suggesting a crucial role in vascular homeostasis, endothelial function, and trophism [45]. In addition, H_2S can be also generated via a reduction in thiols and thiol-containing molecules, in a non-enzymatic manner [8]. Compared to CBS, knockout mice for CSE have no severe phenotype and normal lifespan. Their phenotype is mainly cardiovascular, with hypertension and endothelial dysfunction [46].

Information has become available about the regulation of the expression and activity of these enzymes (Figure 1). It has been reported that NADPHox4 derived ROS (via heme-regulated inhibitor kinase/eIF2/activating transcription factor 4 (ATF4) signaling) enhance the expression of CSE [47]. Blood flow has been reported to exert divergent effect of H_2S producing enzymes, depending on the type of endothelium and flow stress. While laminar flow was initially demonstrated to enhance the expression of CSE and 3-MST in ECs [47,48], recent data are more complex. In particular, laminar flow (high shear stress) has been demonstrated to inhibit CSE expression via KLF2 regulated miRNA-27b [49], while turbulent flow (low shear stress) seems to upregulate CSE [50,51]. In rat aortic ECs, calcium-sensing receptors increase CSE expression in a phospho-calmodulin kinases II-dependent manner to inhibit platelet activation [52]. Transcription factors specifically controlling CSE expression are among the others NF-κB in lipopolysaccharide (LPS)-stimulated macrophages [53], specificity protein 1 (Sp1) in smooth muscle cells [54] and Elk1 in beta pancreatic cells [55]. Nuclear factor of activated T cell (NFAT) binding sites have been identified in the CSE promoter. Intermittent hypoxia exposure reduces Ca^{2+}-dependent activation of calcineurin/NFAT to lower CSE expression and impair vasodilation, while NFAT activation lowers CSE expression at the cell and microvascular levels [56].

OxLDL in one of the major stimuli to cause the endothelial damage that leads to atherosclerosis. One of the epigenetic mechanisms underlying CSE reduced expression at both mRNA and protein levels by oxLDL is increased histone deacetylase 6 (HDAC6) [57]. Recently this finding has been extended to blood pressure control, by assessing the role of tubastain A in AngII induce hypertension, and documenting that upregulation of CSE and H_2S through HDAC6 inhibition can be a valid therapeutic strategy [58].

Additional information on CSE gene and enzyme control is provided in the chapters below and is summarized in Figure 1.

H_2S plasma levels are kept at appropriate concentrations by three elimination systems. The first pathway involves mitochondrial oxidative metabolism which converts H_2S into thiosulfate, followed by further conversion into sulfate and then sulfite. The second metabolic pathway is cytosolic methylation to dimethyl sulfide via thiol S-methyltransferase. Finally, the binding of H_2S to hemoglobin leads to the formation of sulfhemoglobin [45].
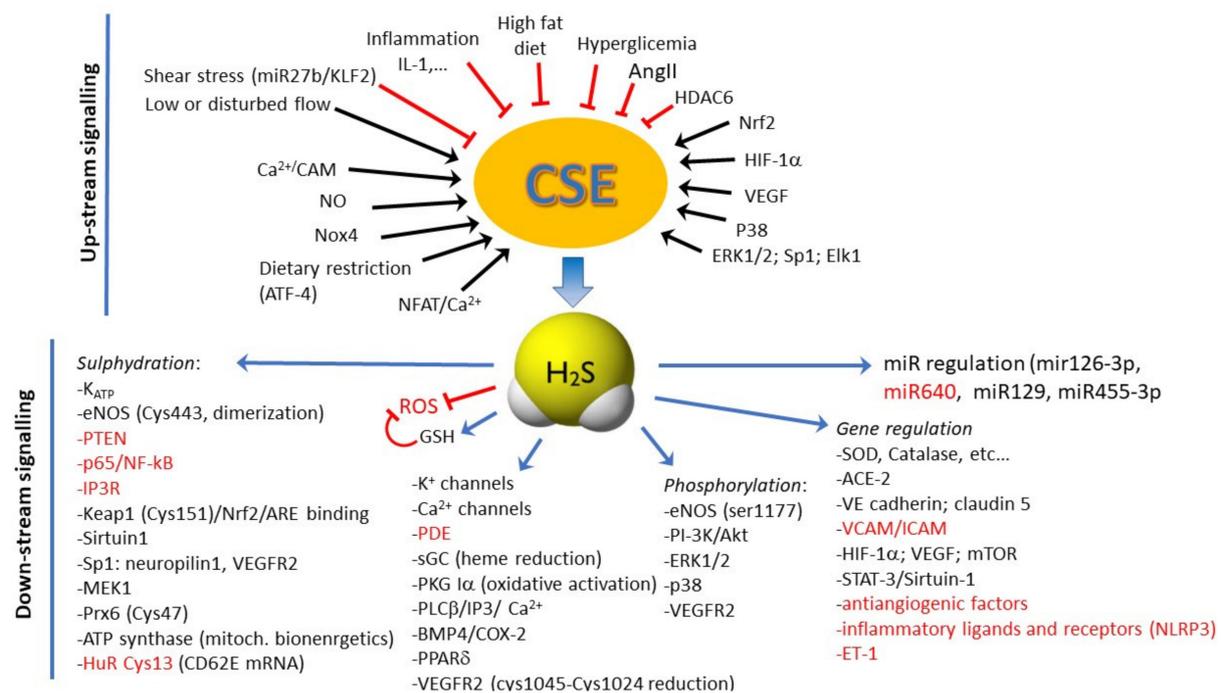
Figure 1. Summary of the molecular mechanisms controlling CSE expression and function in endothelial cells (up-stream signaling) and of the multiple downstream signaling activated or inhibited by H₂S in ECs. Red target or lines means inhibition. Note that some signals are both up- and downstream, strengthening the central role of CSE/H₂S in controlling vascular trophism and functions. ACE-2, angiotensin converting enzyme 2; Akt, protein kinase B; AngII, angiotensin II; ARE, antioxidant responsive elements; ATF4, activating transcription factor 4; BMP4, bone morphogenetic protein 4; cGMP, cyclic guanosine monophosphate; COX-2, cyclooxygenase-2; CSE, cystathionine γ-lyase; Elk1, ETS Like-1 protein; eNOS, endothelial NO synthase; ERK1/2, extracellular signaling regulated kinase $\frac{1}{2}$; ET-1, endothelin-1; HIF-1a, hypoxia inducible factor-1a; HuR, human antigen R; ICAM, intercellular adhesion molecule; IL-1, interleukin 1; IP3, inositol-3-phosphate; IP3R, inositol-3-phosphate receptor; K$_{ATP}$, ATP-sensitive K+ channels; Keap1, Kelch-like ECH associated protein 1, KLF2, Krüppel-like Factor 2; MAPK, mitogen-activated protein kinase; MEK1, MAP kinase kinase 1; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor-kappa B; NLRP3, nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain containing protein 3; NO, nitric oxide; Nox4, NADPH oxidase 4; Nrf2, nuclear factor erythroid 2-related factor 2; p38, p38 mitogen-activated protein kinases; PDE, phosphodiesterase; PI-3K, phosphoinositide 3-kinase; PKG, protein kinase G; PLCβ, phospholipase Cβ; PPARδ, peroxisome proliferators-activated receptor δ; Prx6, thioredoxin-dependent peroxiredoxin; PTEN, phosphatase and tensin homolog; sGC, soluble guanylate cyclase; SOD, superoxide dismutase; Sp1, specificity protein 1 transcription factor; STAT3, signal transducer and activator of transcription 3; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

5. Molecular Signaling Activated by H₂S into ECs

H₂S in ECs performs a protective action on vessels in an autocrine/paracrine manner. It plays a role in the regulation of vasodilation, angiogenesis, inflammation, oxidative stress and apoptosis [59]. Three are the main mechanisms through which H₂S exerts it biological effect: (i) reactive oxygen species/nitrogen species scavenging; (ii) interaction with metal centers; (iii) persulfidation (called also S-sulfhydration).

H₂S acts through a post-translational modification—the S-sulfhydration of cysteine residues, which modifies the structure and activity of the target proteins [60]. The mechanism is persulfidation on reactive cysteine residues (-SH) of target proteins to form a persulfide group (-SSH). An example is the persulfidation of the ATP-dependent K channel (K$_{ATP}$) in ECs and smooth muscle cells, responsible for fast hyperpolarization and vasorelaxation [61]. Indeed, evidence has been provided in support of H₂S function as an EDHF, exerting more remarkable vasorelaxation in the peripheral resistance arteries [62].

H$_2$S also reduces ROS levels through their direct inactivation and by enhancing antioxidant defense mechanisms. One of the mechanisms underlying oxidative protection is given by the H$_2$S regulation of the Keap1/Nrf2 pathway. Normally, the transcription factor Nrf2 is inhibited by its binding to Keap1 in the cytoplasm. In conditions of oxidative stress, H$_2$S promotes the translocation of Nrf2 into the nucleus by means of the S-sulfhydration of the Keap1 inhibitor, causing the dissociation of the Keap1/Nrf2 complex. Nrf2 in the nucleus activates the antioxidant responsive element. Consequently, the transcription of many antioxidant genes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase is induced, requiring hours or days to produce a biological effect [63].

Among other targets, H$_2$S decreases inflammation by inhibiting transcription factors such as NF-κB through persulfidation [64], thus decreasing the expression of pro-inflammatory mediators. Most persulfidation reactions lead to target inhibition as phosphatase and tensin homolog (PTEN) [65], except for MEK1 activity, which in HUVEC leads to increased activity with DNA damage repair and senescence impairment [66]. Recently, Prx6 has been identified as a further target of sulfhydration on Cys47, which controls its decamerization and peroxidase activity [49], while the "S-sulphydrome" was identified among the many target proteins, and β3 integrin was identified as the key element of endothelial mechanotransduction [67].

The interaction of H$_2$S with the NO/NOS pathway involves different modalities: as inhibition of PDE in smooth muscle cells, PI-3K/Akt-dependent phosphorylation of eNOS in Ser1177 [68] and stabilization of eNOS in the dimeric state through enzyme persulfidation [69]. Additionally, heme reduction in sGC enzyme with facilitated response to NO [70] and activation of protein kinase G Iα (PKG Iα) through disulfide bond formation [71] have been reported to potentiate the NO/cGMP pathway.

H$_2$S also stimulates endothelial proliferation and migration, aiding the process of angiogenesis and wound repair. H$_2$S acts at several levels on the mechanisms responsible for angiogenesis, including the control of VEGF expression, through upregulation of the transcription factor hypoxia inducible factor-1α (HIF-1α) or direct modulation of the PI-3K and Akt pathways in ECs (signaling pathways also activated by VEGF) [41,72,73]. In angiogenic ECs, H$_2$S has also been reported to activate signal transducer and activator of transcription 3 (STAT3) [74], mammalian target of rapamycin (mTOR) and the VEGFR2 pathway [75]. It has been reported that CBS silencing in ECs reduces VEGF signaling through VEGFR2 and neuropilin-1 downregulation [76].

A schematic summary of the molecular mechanisms activated or inhibited by H$_2$S in ECs is reported in Figure 1. As a note, the effects of H$_2$S are context and tissue-dependent, sometimes producing divergent functional effects. It is plausible that this depends on endothelial heterogeneity, tissue microenvironment, and physio-pathological conditions where there is the influence of epigenetic mechanisms.

6. Cardiovascular Diseases Associated with Altered Levels of H$_2$S

6.1. Hypertension

Altered levels of H$_2$S have been reported in both experimental models and clinical studies on patients with severe hypertension, where lower plasma H$_2$S levels are described along with reduced content of CBS and CSE (see [77,78] for recent reviews). A human cohort study demonstrated a reduced H$_2$S plasma level in hypertensive patients, suggesting H$_2$S as a potential therapeutic target and diagnostic marker [79].

The intimate relationship between H$_2$S-associated endothelial dysfunction and hypertension comes from the observation that CSE KO mice develop hypertension particularly with impaired endothelium-dependent relaxation in resistance mesenteric arteries [46].

The levels of H$_2$S-producing enzymes are reduced in the vessel wall of spontaneous or drug induced hypertensive animals [80–82]. Exposure of cultured endothelial cells to AngII and, similarly, to H$_2$O$_2$, downregulated the expression and activity of CSE with induction of endoplasmic reticulum stress [83]. In a mouse model of Ang II-induced hypertension,

H_2S reversed the aortic endothelial dysfunction and reduced NO bioavailability, while blockade of endogenous H_2S exacerbated these alterations [84]. Other studies demonstrated that administration of H_2S donors decreases blood pressure and reverses vascular remodeling through the suppression of smooth muscle cell proliferation and collagen deposition in the vessel wall [61,85–89]. H_2S treatment noticeably reestablishes eNOS function and NO bioavailability in N^ω-nitro-l-arginine methyl ester (L-NAME)-induced hypertensive rats [90]. From a mechanistic point of view, H_2S improves endothelial function through the inhibition of oxidative stress, suppression of renin angiotensin system, downregulation of bone morphogenic protein 4/cyclooxygenase-2 (BMP4/COX-2) pathway, or activation of the PPARδ/PI-3K/Akt/AMPK/eNOS cascade, thus contributing to the antihypertensive mechanism of H_2S in renovascular hypertensive rats [91–93]. In SHR, administration of H_2S significantly decreases blood pressure and abrogates endothelial dysfunction through inactivation of NLRP3 inflammasome and oxidative stress [94]. In another disease model, lead-induced hypertension in rats, H_2S treatment normalizes blood pressure and ameliorates endothelial dysfunction with an inhibition of oxidative stress [95].

Recently, it has been proposed that H_2S can regulate EC pathological behavior through epigenetic mechanisms. H_2S induces miR-129, which inhibits DNA methyltransferase-3 (DNMT3) and IL-17, found to be overexpressed in hypertension [96].

Summarizing all results, it can be concluded that the CSE/H_2S signaling pathway may represent a potential therapeutic target for hypertension.

6.2. Diabetes

The relation between diabetes-induced endothelial dysfunction and H_2S impairment is now well established (for review see [77,97]). H_2S levels have been observed to be reduced in rats with diabetes induced by streptozotocin (STZ) and in subjects with type 2 diabetes mellitus [98–102]. In line with these findings, a high fat diet downregulates and dietary restriction induces (via ATF4) CSE expression [103,104]. Hyperglycemia lowers H_2S levels due to the high H_2S catabolism favored by the extremely oxidizing environment or the reduced gasotransmitter production due to a lower expression of the generating enzymes or their inhibition such as oxidative inactivation of the 3-MST at endothelial level [105]. The lack of H_2S bioavailability supports the accumulation of intracellular ROS, which are not completely scavenged by H_2S due to its consumption in high-glucose-treated ECs [106]. The consequent oxidative status favors mitochondrial dysfunction and mitophagy, cell damage, and apoptosis [107–110].

Endothelial dysfunction in diabetes correlates with angiogenesis impairment. CSE expression and H_2S levels are strongly diminished in wound granulation tissues of obese diabetic mice [111], thus explaining the angiogenesis impairment described in wounds and critical limb ischemia in diabetes [112–114]. The availability of H_2S donors or CSE upregulators could be an innovative therapeutic strategy to promote endothelial function and proper neovascularization of wounds. However, despite the protective effect of H_2S on endothelial function and wellness, the stimulation of angiogenesis in atherosclerosis plaques by high CSE expression could have a negative outcome, resulting in plaque vulnerability and rupture [115]. The choice of the proper strategy and best control of H_2S at tissue level is still a critical point to be resolved at the experimental and clinical level.

7. Molecular Mechanisms Regulated by H_2S in Support of EC Function and Trophism
7.1. Antioxidant and Anti-Inflammatory Properties

Several studies document that H_2S limits vascular permeability, directly or indirectly through antioxidant and anti-inflammatory actions. The multiplicity of the mechanism downstream H_2S production is schematically reported in Figure 1. Vascular hyperpermeability was inhibited in mice undergoing cardiac arrest and blood–brain barrier disruption following H_2S inhalation [116]. This protective effect was linked to reduced expression of VEGF and metalloproteinase-9 and increased angiopoietin-1. Another study documented

scavenging of ROS and activation of Akt [117]. Data, however, are not all in the same direction, documenting that the final effect is context dependent.

The protective effect of H_2S in conditions such as hypertension, atherosclerosis, and vascular diabetic complications may be related to multiple actions by the gasotransmitter: H_2S inhibition of ROS production, blunting of ROS by direct scavenging, upregulation of glutathione, and antioxidant enzymes [101,118]. H_2S reduces ROS levels in ECs exposed to high glucose, preventing their apoptosis and damage [108,119]. Gene transfer of CSE or administration of exogenous H_2S in diabetes models reduced ROS levels and improved endothelial dependent vasorelaxation, while CSE KO was responsible for a greater impairment of endothelial function [106]. Many studies support the inhibitory effect of H_2S on endothelial inflammation [120]. The autocrine/paracrine action of endothelial-derived H_2S has been documented by endothelial specific deletion of CSE, which predisposes to vascular inflammation and atherosclerosis [50].

In ECs exposed to high glucose, a suppression of NF-κB activity and reduction in ICAM-1 levels were found upon NaHS pretreatment [121]. Moreover, stimulation of ECs with high glucose significantly promotes ET-1 secretion, which was reduced by administration of H_2S [122].

Recently, inhibition of necroptosis together with ROS downregulation have been described in ECs exposed to hyperglycemia [123]. Inhibition of adhesion molecules such as ICAM-1 in ECs has been described in response to NaHS through NF-κB inhibition [124], while CSE inhibition increased leukocyte adherence to the endothelium [125]. The anti-inflammatory activity of H_2S is not only related to the impairment of adhesion molecules as vascular cell adhesion molecule (VCAM) and ICAM, but also to the inhibition of inflammatory mediator production, such as IL1-β, TNF-α, IL-6 and monocyte chemoattractant protein-1 by ECs and monocytes/macrophages [126,127]. IL-1β, in turn, was found to be increased in atherosclerotic plaques, and induces the phosphorylation of Ser377 and inactivation of CSE [127].

In cultured ECs, the stability of eNOS regulated by miR-455-3p and NO production is induced by H_2S. Moreover, H_2S levels and miR-455-3p are incremented in human atherosclerosis plaques, implying that H_2S could be involved in the miR-455-3p/eNOS/NO pathway controlling atherosclerosis development [128].

Sirtuin-1 has been reported to prevent premature senescence of ECs, protecting from dysfunction [129]. Exogenous H_2S directly induces sirtuin-1 sulfhydration and stability, reducing aortic inflammation and formation of atherosclerosis plaques [130].

Further studies demonstrated that H_2S reduces the severity of atherosclerosis in a mouse model of disturbed blood-flow, through the upregulation of ACE-2 and increase in Ang(1–7) levels [131]. At cellular level, in LPS-activated ECs, H_2S promotes the upregulation of the beneficial side of the renin-angiotensin system [131], documenting a multitargeting effect of H_2S.

An original mechanism of action of H_2S in controlling endothelial dysfunction in atherosclerosis was recently proposed [50]. In both cultured ECs and in mice, endogenous CSE-derived H_2S leads to sulfhydration and dimerization of the RNA-binding protein human antigen R (HuR), described to be inhibited in atherosclerosis [50]. The administration of SG1002, a slow polysulfide donor, in ECs isolated from CSE knockout mice, re-established HuR sulfhydration with subsequent inflammatory marker (CD62E) downregulation. Moreover, SG1002, administered to ApoE$^{-/-}$ CSE knockout mice exposed to partial carotid ligation, limited plaque formation, demonstrating an H_2S-induced antiatherogenic effect [50].

Based on the above results, it appears that H_2S donors may be a potential promise for the treatment of endothelial inflammation related disorders [132].

7.2. Proangiogenic Effect

Several studies report the effects of H_2S, derived from endogenous biosynthesis or released by exogenous donors, on the process of angiogenesis and in the wound heal-

ing context, mainly at low micromolar concentration range, mimicking the physiological concentration of the gasotransmitter [15,68,105]. Additionally, CSE overexpression promotes in vitro angiogenesis [68,133], while CSE silencing, KO or pharmacological inhibition blocks in vitro and in vivo neovascularization responses [133,134]. In addition to the activation of the autocrine eNOS pathway [7], the exposure of ECs to VEGF produces an increase in CSE-dependent H_2S [134].

Recently, a role of 3-MTS participation in angiogenesis occurrence has been demonstrated in vitro [135]. A connection between 3-MTS-derived H_2S and EC metabolism has been demonstrated: 3-MTS downregulation decreased mitochondrial respiration and ATP production, increased glucose uptake, and perturbed the whole EC metabolome [135].

Pro-angiogenic effects of H_2S are evident as increased EC proliferation, migration, and tube formation in vitro. Exogenous H_2S has been shown also to promote in vivo angiogenesis in models of chicken chorioallantoic membrane and to induce neovascularization in mouse subcutaneous Matrigel plugs [15,134]. In a model of cutaneous burn injury and wound healing, topical administration of a H_2S-saturated physiological solution has been demonstrated even to significantly increase the wound closure [134].

Therefore, various studies have investigated the cellular signaling pathways involved in the pro-angiogenic effect of H_2S to discover its molecular targets (Figure 1). Hydrogen sulfide has been shown to activate multiple signaling pathways with a key role in the contribution of EC migration during angiogenesis. Exposure of ECs to H_2S donors induced increased phosphorylation of Akt, ERK1/2, and p38 MAPK, resulting in their activation [15,134].

There is also evidence about the effect of H_2S on the activity of eNOS, promoting its phosphorylation on Ser1177 and consequent NO production inside ECs [133,136,137]. Ultimately, H_2S and NO emerged as being mutually dependent in inducing angiogenesis of ECs and vasorelaxation [68].

In addition, a reverse mechanism appeared to also be effective in controlling EC viability: NO, produced by eNOS, is able to induce CSE activation, resulting in further production of H_2S in ECs [138].

However, the direct molecular target of H_2S on angiogenesis remains to be elucidated. From studies of mass spectrometry and additional investigations, it emerged that a disulfide bond between Cys1045 and Cys1024 in the intracellular kinase core of VEGFR-2 serves as a molecular switch for H_2S to regulate the function of VEGFR-2 [139]. In particular, data revealed that HS^- (in aqueous solution, H_2S is a mixture of H_2S and HS^-) breaks an inhibitory disulfide bond, bringing VEGFR-2 in an active conformation, probably promoting the activation of downstream signaling [139,140].

An alternative theory about H_2S interaction with its target molecules is associated with S-sulfhydration, a post-translational modification of cysteine residues, induced by H_2S on target proteins, involved in signaling pathways [141]. An example of this mechanism is S-sulfhydration of eNOS on Cys443 by NaHS, resulting in increased activity and stability of eNOS and promotion of its phosphorylation, with higher NO bioavailability in ECs to promote their survival and trophism [69].

7.3. Wound Healing Promotion

H_2S has been reported to accelerate the healing of gastric ulcers and skin burn wounds [15,134,142]. Topical application of H_2S improved recovery from burns in wild-type rats, while genetic ablation of CSE delayed healing in mice [134]. H_2S improves angiogenesis and wound healing in db/db mice by promoting transcription of VEGF, epidermal growth factor (EGF), HIF-1α and eNOS, by upregulating VEGF and platelet-derived growth factor (PDGF) proteins and receptor phosphorylation [143,144]. H_2S accelerates wound healing in STZ-induced diabetic mice with the formation of granulation tissue and increased levels of anti-inflammatory factors and VEGF [59]. Additionally, attenuation of inflammation has been attributed to H_2S, thus improving diabetic wound healing in ob/ob mice [111]. Accordingly, H_2S facilitates wound closure through the inhibition of neutrophil

extracellular traps (NET) release-coupled neutrophil death (NETosis) in db/db mice [114]. Interestingly, H$_2$S improved wound healing via restoration of endothelial progenitor cell functions and activation of angiopoietin-1 in db/db mice [113].

Recent epigenetic data document that treatment of ECs with H$_2$S or upregulation of CSE rescued migration impairment due to high glucose, through a pathway involving miR126-3p upregulation and DNA methyl trasferase-1 downregulation [145].

8. Therapeutic Strategies to Improve H$_2$S Concentration at Endothelial Level

The use of H$_2$S donor compounds or gene therapy to increase the expression of enzymes responsible for the endogenous synthesis of H$_2$S has the aim of restoring endothelial function and preventing the onset of pathologies associated with endothelial damage. Several efforts have been made to synthetize effective H$_2$S donors showing different H$_2$S releasing kinetics and site of action. The main objective has been to control blood pressure and correct endothelial dysfunction, vascular inflammation and redox state and to improve neovascularization and healing of wounds (Figure 2). Furthermore, H$_2$S donor drugs have been evaluated as hypoglycemic agents in type 2 diabetes. H$_2$S has been shown not only to protect cells from damage induced by hyperglycemia, but to prevent the onset of type 2 diabetes, preserving the functionality of β-pancreatic cells and regulating the sensitivity of target organs to insulin [146].
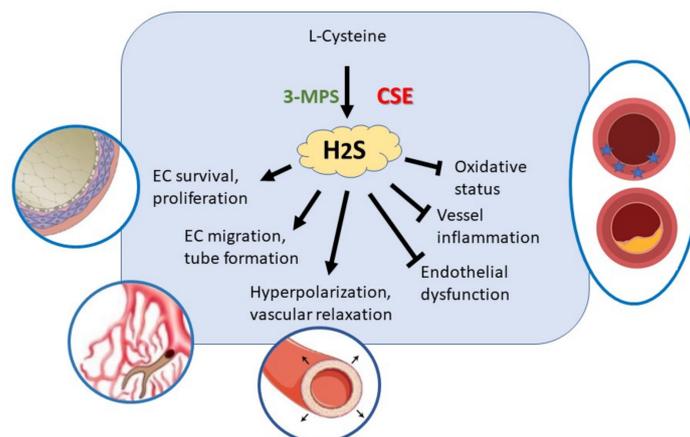
Figure 2. H$_2$S exerts autocrine/paracrine actions in vascular endothelial cells in order to maintain their trophism and physiological functions.

Due to the divergent responses induced by different concentrations of H$_2$S, it is important for H$_2$S donor drugs to maintain the plasma concentration of H$_2$S at physiological levels, in the nanomolar order. Therefore, an ideal H$_2$S donor should possess two qualities: slow and gradual production and intracellular release of H$_2$S.

Indeed, NaHS, widely used for experimental purposes as H$_2$S donor, is unsuitable for clinical use, due to its fast kinetics of H$_2$S release, difficulties to titer the dosage, and its toxic effects [147,148].

A slow-releasing H$_2$S donor was developed, GYY4137, demonstrating its vasodilating property in aortic, renal, and cardiac arteries in an L-NAME-induced hypertension model [85]. The antiatherogenic and endothelium-dependent vasodilating effects of GYY4137 were reported in ApoE$^{-/-}$ mice, through decreasing vascular inflammation (lower ICAM-1, IL-6 and TNF-α expression) and oxidative stress [149]. An additional antithrombotic action was demonstrated in mice [150]. Among the others, GYY4137 was proposed in post-ischemia remodeling. The beneficial effects on cardiac functions were correlated to greater vessel density in the infarcted area [151].

The mitochondria-targeted H$_2$S donors AP123 and AP39 have been demonstrated to prevent hyperglycemia induced oxidative stress and metabolic alteration in microvascular ECs, suggesting their use in vascular complications of diabetes [119]. The slow releasing

H$_2$S donor AP39 remarkably reduced systemic blood pressure, heart rate and arterial stiffness in L-NAME treated rats [88].

The positive interaction and synergistic action between NO and H$_2$S [152] lead to the development of H$_2$S-NO hybrid donor as ZYZ-803, recently reported to promote angiogenesis with a crosstalk between STAT-3 and CAMKII [153]. The authors reported an increased blood flow and vascular density in the hind limbs of mice exposed to femoral artery ligation.

The orally available prodrug SG1002 is an inorganic mixture (sodium polysulthionate) which in vivo was demonstrated to increase both H$_2$S and NO levels [154]. Its protective effects have been demonstrated in animal models of atherosclerosis and acute limb ischemia, and patients with heart failure [50,132,155,156].

Among the established drugs, ACE inhibitors bearing a SH group, such as captopril, can promote blood pressure reduction though the sulfhydryl moiety beside the primary pharmacological target. Zofenopril demonstrates vasorelaxant and proangiogenic properties in addition to its ACE inhibitory activity. Indeed, we have contributed to demonstrating that its active moiety, zofenoprilat, can be considered an H$_2$S donor and an upregulator of CSE expression at the EC level [80,157–159].

A further therapeutic option is represented by H$_2$S-releasing derivatives of a number of drugs, such as non-steroidal anti-inflammatory drugs [160]. Alongside its antithrombotic properties, H$_2$S releasing-aspirin was recently demonstrated to exert pro-proliferative and anti-apoptotic actions on cultured ECs together with anti-inflammatory and anti-oxidative features [161].

The use of orally active compounds able to endogenously produce H$_2$S, such as *N*-acetylcysteine (NAC) and taurine, has been proposed, but clinical trials unfortunately were not followed up with published data (Table 1). NAC, a well-tolerated compound, clinically employed to enhance cellular levels of glutathione, is rapidly cleaved in vivo to yield cysteine. On the other hand, in vivo and ex vivo studies demonstrate that the sulfur amino acid taurine markedly and dose-dependently increased the expression of both CSE and CBS, with a higher effect on CSE upregulation [162]. A reduction in blood pressure in patients with prehypertension has been described [163].

Table 1. Clinical trials on endothelial dysfunction-related diseases with H$_2$S donors/enhancers.

Identifier Year (Location)	Condition or Disease	Drug	Phase	Status Results
NCT01232257 2011 (The Netharlands)	HYPERTENSION (Chronic Kidney Disease, Chronic Kidney Failure, End Stage Kidney Disease, End Stage Renal Disease)	*N*-acetylcysteine (NAC)	Phase 3	Completed No results posted
NCT03179163 2020 (USA)	HYPERTENSION	Captopril	Phase 1/2	Recruiting
NCT03410537 2018 (China)	DIABETES TYPE 2 (Lower Extremity Artery Disease)	Taurine vs. Placebo	ND	Recruiting No results posted
None	ATHEROSCLEROSIS/ THROMBOSIS	-	-	-
None	ANGIOGENESIS/ WOUND HEALING	-	-	-

The search of clinical trials listed in ClinicalTrials.gov was performed combining the keywords: H$_2$S, hydrogen sulfide, endothelial dysfunction and the disease listed in the second column. Other not recent studies performed on patients are reported in the text (see [154]).

In the complex, only very few clinical studies on H$_2$S donors or enhancers administered in endothelial dysfunction-related disorders are currently registered in NIH ClinicalTrials.gov as listed in Table 1, but no results have been posted or are available on PubMed.

In a manner similar to NAC, cysteine/cysteine-rich undenatured whey protein supplement improved pressure ulcer recovery in a small group of diabetic patients [164].

The development of natural compounds, present in the diet, as a H_2S source is interesting, such as polysulfides. Diallyl trisulfide (DATS), diallyl disulfide (DADS) and diallyl sulfide (DAS) are the active principles of the Alliaceae family, such as garlic, which is recognized worldwide as a popular remedy of hypertension. These polysulfides have been demonstrated to exert vasodilating properties in relation to H_2S release [165], behaving as anti-hypertensives in L-NAME-treated rats [166]. In animal experiments, DATS improved cardiac function in aortic constricted mice, via an upregulation of VEGF, reduced angiostatin and increased myocardial vascular density [167]. Systemic administration of DATS or local transplantation of DATS-treated or CSE-overexpressing bone marrow cells improved capillary density, cell survival and blood perfusion in ischemic hindlimb of db/db mice [168]. Administration of DATS improved neovascularization in STZ-induced diabetic mice through increased NO availability [169].

Erucin [4-(methylthio) butyl isothiocyanate] is a natural isothiocyanate particularly abundant in Eruca sativa Mill. (rocket salad), an edible cruciferous plant belonging to the family of Brassicaceae. Isothiocyanates (ITCs) in general represent a source of different beneficial biological effects on human health, and most are investigated in relation to their chemo-preventive and anti-cancer properties [170–172]. Numerous studies demonstrated a general anti-inflammatory and antioxidant activity [173], together with protective properties for the cardiovascular system, where ITCs exhibit vasorelaxing and antihypertensive activity and a protective effect against endothelial dysfunction [174–176]. Several biological effects of ITCs may be associated with their ability to release H_2S inside cells in a slow and long-lasting manner, leading to the definition of "smart H_2S-donors" [175,177]. H_2S release from ITCs occurs in a specific manner, depending on the presence of thiols, and it is particularly facilitated in the cell cytosol, where high concentrations of organic thiols, glutathione (in 1–10 mM range), and cysteine (in 30–200 µM range) are present [175,176]. Natural isothiocyanates, including erucin, may therefore represent a possible exogenous source of H_2S, which, if gradually released, could mimic the physiological concentrations of the endogenous gasotransmitters. On the other hand, they can be the base for the design of synthetic H_2S donor hybrids with antioxidant property and interesting pharmacological development [178].

Considering the requirement of dressings able to protect ulcers with high exudate levels and to promote wound healing (i.e., in diabetic patients), medicated dressings have been designed and developed. In particular, a functional sodium alginate dressing with H_2S-releasing properties (SA/JK-1) was fabricated incorporating a pH-dependent donor, JK-1 molecule, into a sodium alginate sponge [179]. The resulting construct provided a moist healing protection able to continuously release H_2S under acidic pH and absorbing exudate at the wound interface. In vitro, the construct was demonstrated to be biocompatible and effective in improving fibroblast migration and proliferation. When tested in animal model of full thickness dermal defect, SA/JK-1 promoted granulation tissue formation, angiogenesis, collagen deposition, and re-epithelization [179]. Overlapping results were demonstrated by the same group with hyaluronic acid hydrogels doped with H_2S which was shown to induce M2 macrophage polarization [180]. Another example is represented by silk fibroin porous scaffold loaded with GYY4137, reported to facilitate in vitro bone cell trophism and angiogenesis [181]. These data demonstrate that H_2S-medicated wound dressing/biomaterial may represent promising strategies for non-healing wounds or bone healing and regeneration. Extensive animal and clinical studies are, however, necessary for assessing their safety and validation.

9. Concluding Remarks

H_2S is nowadays considered an important transmitter able to maintain vascular homeostasis. Most of its activities are due to autocrine/paracrine actions by ECs, with a fine control of its plasma concentrations. The availability of H_2S depends on the activity

of endothelial and other cells that express the key enzymes involved in the gasotransmitter release, the reactivity of H_2S and its inactivation by redox systems and the efficacy of elimination reactions. On top of these, the production of H_2S by the gut microbiota and intestinal epithelium is important to consider, due to the increasing recognition of circulating molecules coming from this source and finely controlling the cardiovascular system performance [96,182].

There is still demand for the availability of safe and effective synthetic H_2S donors or enhancers, and natural products or nutraceuticals are helping to fulfil this demand. Indeed, few clinical trials based on H_2S exogenous sources have been interrupted or have not published their results for unknown reasons.

Since endothelial dysfunction and inflammation continue to be the main causes of morbidity and mortality all over the world, knowledge of the molecular and biochemical mechanisms underlying cardiovascular pathologies and their complications is still required, as well as the definition of new treatment options to prevent endothelial dysfunction or revert cardiovascular disorders [183,184]. The recent pandemic evidenced this unresolved medical need [185].

What it is expected from novel molecules in order to be druggable is the exhibition of H_2S levels near the physiological ones, and many compounds are actually druggable H_2S-donors, but it seems that no clinical trials are currently running against endothelial dysfunction (Table 1). Sulfur compounds with natural origin represent helpful pharmaceutical/nutraceutical tools to be used in therapy or as a template for the ideation of advanced H_2S-donor molecules with improved pharmacodynamic and/or pharmacokinetic properties [132,178].

Although experimental data clearly document a protective effect of H_2S donors against endothelial dysfunction, further clinical studies are needed. To the best of our knowledge, there are no clearly active clinical trials on patients affected by pathologies due to endothelial dysfunction and treated with H_2S donors. Even a modest improvement in endothelial function and viability would be a therapeutic success due to the lack of drugs against this diffuse condition predisposing to cardiovascular pathologies.

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Effect of Microgravity on Endothelial Cell Function, Angiogenesis, and Vessel Remodeling During Wound Healing

Lucia Morbidelli[1]*, Shirley Genah[1] and Francesca Cialdai[2]

[1]Department of Life Sciences, University of Siena, Siena, Italy, [2]ASA Campus Joint Laboratory, ASA Research Division & Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy

Wound healing is a complex phenomenon that involves different cell types with various functions, i.e., keratinocytes, fibroblasts, and endothelial cells, all influenced by the action of soluble mediators and rearrangement of the extracellular matrix (ECM). Physiological angiogenesis occurs in the granulation tissue during wound healing to allow oxygen and nutrient supply and waste product removal. Angiogenesis output comes from a balance between pro- and antiangiogenic factors, which is finely regulated in a spatial and time-dependent manner, in order to avoid insufficient or excessive nonreparative neovascularization. The understanding of the factors and mechanisms that control angiogenesis and their change following unloading conditions (in a real or simulated space environment) will allow to optimize the tissue response in case of traumatic injury or medical intervention. The potential countermeasures under development to optimize the reparative angiogenesis that contributes to tissue healing on Earth will be discussed in relation to their exploitability in space.

Keywords: wound healing, angiogenesis, angiogenic factors, endothelial cells, microgravity, drugs, cell therapy, physical therapy

INTRODUCTION

The skin is the largest organ by surface area in the human body. Its structure is highly organized with different cell types (epithelial, stromal, and endothelial cells-ECs), which finely cooperate in order to guarantee a constant functioning and structural homeostasis of the organ. Indeed, the skin is the first defensive barrier of our body that protects internal tissues from microbial infections, mechanical damages, UV radiations, dangerous substances, and high temperatures. Therefore, the maintenance of its integrity is fundamental for our survival and skin repair, following a mechanical or physical injury, implying very complex and delicate processes to recover its integrity and barrier function (Sorg et al., 2017; Rodrigues et al., 2019). Here we report the state-of-the art of tissue wound healing phases and factors, the role of angiogenesis and the potential pharmacological, cellular, and physical countermeasures acting on ECs. The aim of the article is to understand how to apply all these findings in the space environment as the one that astronauts face during long duration missions. Indeed during space travels the possibility for astronauts to hurt themselves during their routine activities is not excluded. The availability of countermeasures with verified effectiveness and safety in unloading conditions will guarantee the optimal tissue healing and health recovery in extreme environments without the urgency to rapidly return to Earth. The review article is based on the recent/most cited

FIGURE 1 | Phases of physiological wound healing. Following tissue injury, hemostasis occurs. Coagulation is responsible for fibrin clotting and cessation of blood flowing. Platelets activate and secrete PDGF, EGF, TGF-β, CXCL4, and PF4. The following phase is represented by inflammation, also induced by tissue debris and bacteria. Vessels dilate in a NO-dependent manner. Neutrophils (0–3 days) exert antimicrobial activity, secreting MMPs and cytokines as TNF-α and IL-1/4/6. Monocytes are attracted (1–7 days) and transform in M1 macrophages involved in host defense with phagocytic activity. ROS are generated to eliminate bacteria. Then, M2 phenotype occurs with the production of growth factors involved in tissue repair. The proliferation phase is characterized by angiogenesis occurrence due to release of VEGF and FGF, EC proliferation and migration and fibroblast proliferation. Fibroblasts participate in angiogenesis through secretion of proteinases and deposition of ECM proteins. Reepithelialization starts and actively continues during the remodeling phase, characterized by collagen conversion and vascular regression. The release of TGF-β induces wound contraction and closure, and tissue remodeling, till the recovery of tissue integrity. When inflammation persists, wounds do not heal and chronic ulcers occur.

papers of PubMed and Scopus databases on the topic of reparative angiogenesis, wound healing, and related countermeasures.

WOUND HEALING PHASES

Wound healing involves a coordinated interaction of cells, proteins, growth factors, small molecules, proteases, and extracellular matrix (ECM) components aiming at restoring tissue morphology and functioning. The network of communications between stromal, endothelial, and immune cells is the key for determining the course of healing and recovery of tissue function and features (Rodrigues et al., 2019). Skin repair process can be divided into sequential phases: hemostasis, inflammation, proliferation, and remodeling (**Figure 1**). Although different growth factors, cytokines, and predominant cell types characterize each phase at different times, a considerable amount of overlap can occur (Sorg et al., 2017; Rodrigues et al., 2019). The four phases require different kinetics: 1) coagulation and hemostasis, starting immediately after injury; 2) inflammation, shortly after, lasting few days; 3) proliferation, occurring in several days; 4) wound remodeling, lasting days or even many months (**Figure 1**) (Reinke and Sorg, 2012; Velnar and Gradisnik, 2018).

The first response to a wound is constriction of the injured blood vessels, accompanied by activation of platelets and

coagulation to form a fibrin clot to stop blood flow and provide a scaffold for incoming inflammatory cells. Indeed, in response to a mechanical injury, coagulation and hemostasis activate to prevent exsanguination and form a supportive matrix (including fibrin, fibronectin, vitronectin, and thrombospondins) that represents a substrate for invading cells, required later during the process. Upon injury, the microvasculature is disrupted leading to fluid accumulation, inflammation, and the development of hypoxia (Veith et al., 2019). At this stage, platelet-derived chemotactic factors released by platelet α-granules recruit leukocytes and monocytes to the area of injury to start the inflammatory phase finalized to tissue debris removal and bacteria killing (Etulain, 2018; Ridiandries et al., 2018). Leukocytes produce and release chemokines and cytokines (interleukin IL-1α, -1β, -6 and tumor necrosis factor-alfa, TNF-α), which, together with reactive oxygen species (ROS) release, amplify the inflammatory response (Reinke and Sorg, 2012; Veith et al., 2019). This second phase, often associated with edema, erythema, heat, and pain, aims to prepare the wound bed for the growth of new tissue. However, inflammation can become problematic if it is prolonged or excessive.

Once the lesion is cleaned out, the wound enters the third proliferative phase, where the aim is to fill and contract the wound edges and cover the gap. The proliferative phase is characterized by granulation tissue formation, collagen deposition, angiogenesis, and reepithelialization. Granulation tissue consists of layers of fibroblastic cells separated by a

collagenous extracellular matrix containing capillary buds and inflammatory cells. The recruited neutrophils and macrophages release growth factors, such as transforming growth factor-beta (TGF-β), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), responsible for activating resident ECs toward an angiogenic phenotype. Angiogenesis consists of EC proliferation, migration, and branching to form new blood vessels. Neovascularization, regulated predominantly by hypoxia-induced VEGF released by macrophages and fibroblasts, is needed to deliver nutrients and maintain the granulation tissue bed (Tonnesen et al., 2000, Li et al., 2003; Reinke and Sorg, 2012). Additionally, soluble factors induce endothelial precursor cells (EPC) recruitment in the granulation tissue and promote fibroblast proliferation and migration with changes in the ECM architecture. In this intermediate phase, macrophages continue to supply growth factors, promoting angiogenesis and stimulating resident fibroblasts to invade the wound and proliferate and remodel ECM through the synthesis of collagen, fibronectin, laminin, and metalloproteases, providing strength and elements to the injured tissue (Gurtner et al., 2008; Darby et al., 2014; Etulain, 2018; Ridiandries et al., 2018; Rodrigues et al., 2019). In the complex, the granulation tissue is composed by a high density of fibroblasts, granulocytes, macrophages, and capillaries (for the 60% of the mass) and loosely organized collagen bundles. It is well documented that angiogenic factors are present in wound fluid and promote repair, while antiangiogenic factors inhibit repair (Li et al., 2003; Schultz and Wysocki, 2009). During healing of the tissue defect, the edges of the wound are progressively brought together by the retraction of granulation tissue. This is due to the effect of TGF-β1 on fibroblasts which are induced to differentiate in myofibroblasts, contractile cells with stress fibers containing α-smooth muscle actin. This phenomenon, called wound contraction, is of great clinical importance in reducing the size of the wound. Reepithelialization simultaneously occurs and involves the proliferation of both unipotent epidermal stem cells from the basal layer and dedifferentiation of terminally differentiated epidermal cells (Gurtner et al., 2008; Rodrigues et al., 2019).

The final stage of the repair process, the remodeling or maturation phase, is characterized by the transition from granulation tissue to scar formation and maturation. Wound remodeling takes place when components of ECM undergo changes (e.g., replacement of collagen III by stronger collagen I) and myofibroblasts reduce the scar surface (Tomasek et al., 2002; Darby et al., 2014).

The process concludes with a decrease in cell density and a gradual arrest of angiogenesis, involving cell apoptosis and release of antiangiogenic factors (Reinke and Sorg, 2012; Sorg et al., 2018). When all these steps proceed in a regular and coordinated manner, a morphological and functional recovery of the injured tissue is obtained. However, if one or more of these mechanisms is impaired or delayed, healing is not guaranteed, and ulcer can occur with the risk of infections and chronic damage.

ANGIOGENESIS DURING WOUND HEALING

Among the different events that occur in the proliferative phase, angiogenesis is particularly important, because it forms the basis for tissue survival and recovery in the wound. Angiogenesis is defined as the formation of new vessels from preexisting ones and the microcirculation is the main site of vascular remodeling and angiogenesis. Angiogenesis appears to occur by two mechanisms, namely nonsprouting (intussusceptive) and sprouting angiogenesis (Ribatti and Crivellato, 2012). During intussusception, endothelial protrusions of opposing capillary walls extend towards each other and fuse creating an interendothelial contact (Burri et al., 2004). Sprouting angiogenesis is however the major form of neovascular growth, which requires migration, proliferation, and differentiation of ECs under the stimulation of specific glycoproteins called angiogenic factors, mainly VEGF and FGF (Hughes, 2008; Vandekeere et al., 2015). The development of blood vessels, which is a requirement for growth and regeneration, depends on a highly structured communication of ECs with their surrounding tissue. *In vivo* vascularization is based on complex cell-matrix and cell-cell interactions, where the ECM seems to play a pivotal role (Neve et al., 2014; Mongiat et al., 2016; Tracy et al., 2016).

The principal stimulus for angiogenesis occurrence is a lack of nutrients and oxygen, which characterize the hypoxic condition arising during tissue growth or following tissue injury and impaired blood flow (Ahluwalia and Tarnawski, 2012). Hypoxia is indeed able to promote hypoxia inducible factor1-α (HIF1-α) upregulation at nuclear level, which is responsible for angiogenic factor overexpression and vasoactive molecules upregulation. Under the action of the angiogenic factors VEGF, FGF, and PDGF, ECs undergo receptor activation and modification of intracellular pathways and cytoskeleton structure, leading to cell proliferation and chemotaxis (Ahluwalia and Tarnawski, 2012).

The first step is the binding of proangiogenic factors including VEGF, FGF-1 and 2, PDGF, and angiopoietins and stromal-derived growth factor (SDF-1), to their receptors on ECs of existing vessels, triggering complex and intricate intracellular signaling cascades. Heparin sulfate proteoglycans and syndecans also play a key role in regulating the angiogenic activity of VEGF and FGF-2 (Corti et al., 2019). Activated ECs secrete matrix metalloproteinases (MMPs) to degrade the capillary basement membrane (BM) and allow their migration and proliferation outside of the original blood vessel. Therefore, new vessels are formed as capillary sprouts and are then extended and remodeled. Finally, ECs interconnect to form a loop or a tube and the recruitment of pericytes stabilizes the newly formed vessels in a mature conformation (Carmeliet, 2003; Sorg et al., 2018).

In this framework, EC migration plays an important role for vascular remodeling and is a necessary condition for angiogenesis to occur. EC migration is a coordinated process that involves changes in cell adhesion, signal transduction, and cytoskeleton

dynamic reorganization (Li et al., 2003; Velnar and Gradisnik, 2018).

The regulation of this process is achieved by three types of mechanisms: chemotaxis or migration towards a concentration gradient of soluble chemoattractants; haptotaxis or rather migration in response to a gradient of immobilized ligands; and mechanotaxis which is the migration induced by mechanical forces. Specifically, chemotaxis of ECs is driven by growth factors (VEGF and FGF-2, among the most important), haptotaxis is related to increased EC migration activated in response to integrins (e.g., αvβ3 and αvβ5), bound to ECM components, and mechanotaxis is associated to a polarization of cytoskeleton and cell-ECM interactions in the blood flow direction (Lamalice et al., 2007).

Due to the combined action of cell migration and proliferation, the nascent vessels grow in the hypoxic/ischemic wounded tissue particularly rich of angiogenic stimuli. Circulating EPCs concur to the nascent vessels, being recruited by the factors released by the hypoxic environment, primarily VEGF and SDF-1 (Zhu et al., 2016). The process arrives at its end when the bud cavitates and blood starts to flow, bringing oxygen and nutrients to the tissue and taking away CO_2 and waste catabolites.

The Complexity of Wound Angiogenesis

Sprouting angiogenesis has recently been a subject of intense research since it is a requirement for growth and regeneration. The process has many sequential hierarchical steps that require the close interaction of EC with both cellular and acellular components of the surrounding tissue (Hughes, 2008). *In vivo*, capillaries are embedded in a microenvironment that consists of the ECM and cellular components including fibroblasts as well as immune cells. The ECM is a complex, noncellular network constituted by distinct components that is found in two different locations, i.e., the interstitium as interstitial ECM and, in association with epithelial and endothelial tissues, as the BM (Neve et al., 2014).

During early phases of wound healing, capillary sprouts invade the fibrin/fibronectin-rich wound clot and within a few days organize into a microvascular network throughout the granulation tissue. Growth factors that are released from the ECM trigger sprouting angiogenesis. These include a range of angiogenic factors, the most important being VEGF-A (Henning, 2016). Angiogenic stimuli activate the ECs to migrate into the avascular tissue (Eming and Hubbell, 2011). ECs express VEGF receptor-2 (VEGFR-2) that responds to the VEGF-A gradient. Once an angiogenic stimulus occurs, MMPs break down the BM of the blood vessel, mainly near the trigger sites (Davis and Senger, 2008). During sprouting, ECs are triggered by the VEGFR-2/VEGF-A binding to temporarily transform into migrating tip cells. These cells are polarized and have well-developed filopodia that enable them to interact with the ECM via integrins. These proteins (primarily αvβ3) of the ECs filopodia surface have an adhesive function during the endothelial migration (Davis and Senger, 2008). ECM proteins are important for adhesion and migratory processes of the endothelial tip cells and therefore promote angiogenesis (Neve

et al., 2014; Mongiat et al., 2016). The endothelial tip cells move into the surrounding avascular extracellular matrix towards the angiogenic stimulus (Stratman et al., 2009). To enable this process, the MMPs form tunnels in the ECM to facilitate endothelial migration (Sacharidou et al., 2012).

Membrane-type 1-matrix metalloproteinases (MT1-MMP), synthesized by the ECs themselves, are responsible for most of the proteolysis of the ECM (Stratman et al., 2009). Endothelial stalk cells follow the tip cells into the ECM where they proliferate and elongate the developing capillary sprout as well as establish its internal lumen. The tubular lumen is formed by intraendothelial vacuoles that fuse. For the development of the vacuoles, MT1-MMP and the integrins αvβ3 and α5β1 play important roles (Welch-Reardon et al., 2014). Tight and adherens cell junctions are established between the stalk cells of the newly built tube and, consequently, a new vessel arises.

Due to their roles in cell-matrix interactions and especially matrix remodeling, fibroblasts are crucial in vascular development through transmitting biochemical signals and mechanical forces that affect cell survival, shape, and orientation (Kamei et al., 2006; Costa-Almeida et al., 2015). Stalk ECs synthesize, in cooperation with surrounding fibroblasts, basement membrane proteins, namely laminin, collagen IV, perlecan, nidogen, collagen XVIII, and fibronectin. The BM envelops and stabilizes the newly developing capillary sprout, serving as an acellular barrier against the capillary microenvironment and ensuring the correct polarity of ECs (Davis and Senger, 2008). Maturation and stabilization as well as remodeling of the dynamic capillary structures follow initial angiogenesis. As tubules mature, their ECs transform into quiescent phalanx cells (Senger and Davis, 2011). When collagen accumulates in the granulation tissue to produce scar, the density of blood vessels diminishes.

In dermal wounds with robust healing, the angiogenic activity during the proliferative phase initially creates a disorganized vascular network with highly tortuous vessels pathways, often reaching higher vessel numbers than normal (DiPietro, 2016). Following this peak in neovascularization, there is increased expression of antiangiogenic factors, such as Sprouty2 and pigment epithelium-derived factor (PEDF), leading to vascular regression and pruning (Wietecha et al., 2011; Wietecha et al., 2015). Maturation and stabilization of the new vascular network require the involvement of pericytes and vascular smooth muscle cells (Bergers and Song, 2005). A key growth factor is PDGF-BB, which acts on pericyte differentiation (Hellberg et al., 2010). Additionally, there is the contribution of angiopoietin-2/Tie2 receptor in pericytes which regulate angiogenesis and maturity of vascular networks (Teichert et al., 2017).

Signaling Pathways in Endothelial Cells During Angiogenesis

ECs receive multiple stimuli from the environment that eventually induce them to progress along all the stages of angiogenesis, until new vessels are formed in the wound bed. Angiogenic signals that promote EC survival, proliferation, migration, and finally differentiation are the result of a

complex framework, involving cell-ECM and cell-cell interactions, and action of soluble mediators (Muñoz-Chápuli et al., 2004; Lamalice et al., 2007). ECs express a large number of receptors that make them responsive to several growth factors and cytokines involved in the promotion of angiogenesis, but the most important and specific for their action is VEGFR (Hofer and Schweighofer, 2007). In particular, the binding of VEGF-A, secreted by cells in the hypoxic environment, to VEGFR-2 is the major way by which EC migration is promoted (Olsson et al., 2006). Specifically, like other tyrosine kinase receptors, VEGFR-2 undergoes ligand (VEGF-A) induced dimerization and oligomerization, which activates its intrinsic tyrosine kinase activity, resulting in auto- and transphosphorylation on specific tyrosine residues in the cytoplasmic domain, ultimately being responsible for cell proliferation and migration. These pathways involve activation of the small GTPases of the Rho family, PI-3K/Akt, p38 MAPK, FAK, and ERK1/2 signaling cascades (Muñoz-Chápuli et al., 2004; Webb et al., 2004; Lamalice et al., 2007; Yang et al., 2017).

Intermediate messengers are upregulated during angiogenic cell activation as gasotransmitters nitric oxide (NO) and hydrogen sulfide (H_2S). PI-3K/Akt-activated eNOS produces NO, which is a major regulator of EC migration and angiogenesis, by inducing expansion of EC surface, after vasodilatation, associated with a more proper response of endothelium to angiogenic and promigratory stimuli (Dimmeler et al., 2000; Morbidelli, 2016b). In addition, a particular attention has been put on hydrogen sulfide (H_2S). Endothelial-associated H_2S producing enzymes are activated or upregulated by hypoxia and VEGFR-2 signaling cascades, as recently reviewed (Ciccone et al., 2021).

Endothelial Dysfunction and Impaired Healing

All the above mechanisms however are working in healthy endothelial cells. Physiological conditions such as ageing and pathologic conditions as metabolic syndrome or diabetes are characterized by endothelial impaired functions, called endothelial dysfunction, with reduced ability of ECs to release vasoactive and protective factors like NO and to undergo beneficial and sufficient angiogenesis. These disorders are indeed accompanied by altered healing, chronic ulcers, and infections that in extremis may need amputation (Brem and Tomic-Canic, 2007). Beside the inability of ECs to promote a reparative angiogenesis, we have to consider the microenvironment composition. Recent analysis of the exudate of venous nonhealing leg ulcers has found increased antiangiogenic factors, increased VEGF proteolytic products, and increased levels of soluble VEGFR-1, known to neutralize VEGF-A activity (Lauer et al., 2000; Drinkwater et al., 2002; Eming et al., 2004; Eming et al., 2010).

All these findings push scientists to identify novel biochemical targets for the development of therapies to promote endothelial proper functions during reparative angiogenesis. The research is nowadays active in characterizing novel drugs or therapies and their delivery systems to be used in pathological conditions

characterized by endothelial dysfunction and impaired angiogenesis and wound healing.

PHYSICAL-CHEMICAL FACTORS CONTROLLING WOUND HEALING

The different steps of tissue repair are strictly regulated by a multitude of biochemical and physical factors, including gravitational/mechanical forces acting at cellular and tissue level. Interruption, failure, or alteration in one or more phases of the repair process can lead to the formation of nonhealing chronic wounds or fibrotic scars, accompanied by pain and inflammation (Guo and DiPietro, 2010).

In general terms, the factors that influence repair can be categorized into local and systemic. Local factors are those directly influencing the characteristics of the wound itself like oxygenation/hypoxia, infection occurrence, vascular insufficiency, or presence of foreign bodies. On the other hand, systemic factors are the overall health or disease state of the individual affecting his or her ability to heal, such as age and gender, stress, nutrition, alcoholism and smoking, immunocompromised conditions, and diseases (as diabetes) (Guo and DiPietro, 2010). Although many factors related to a patient's conditions cannot be changed, local factors can be controlled and improved in order to obtain the best therapeutic result.
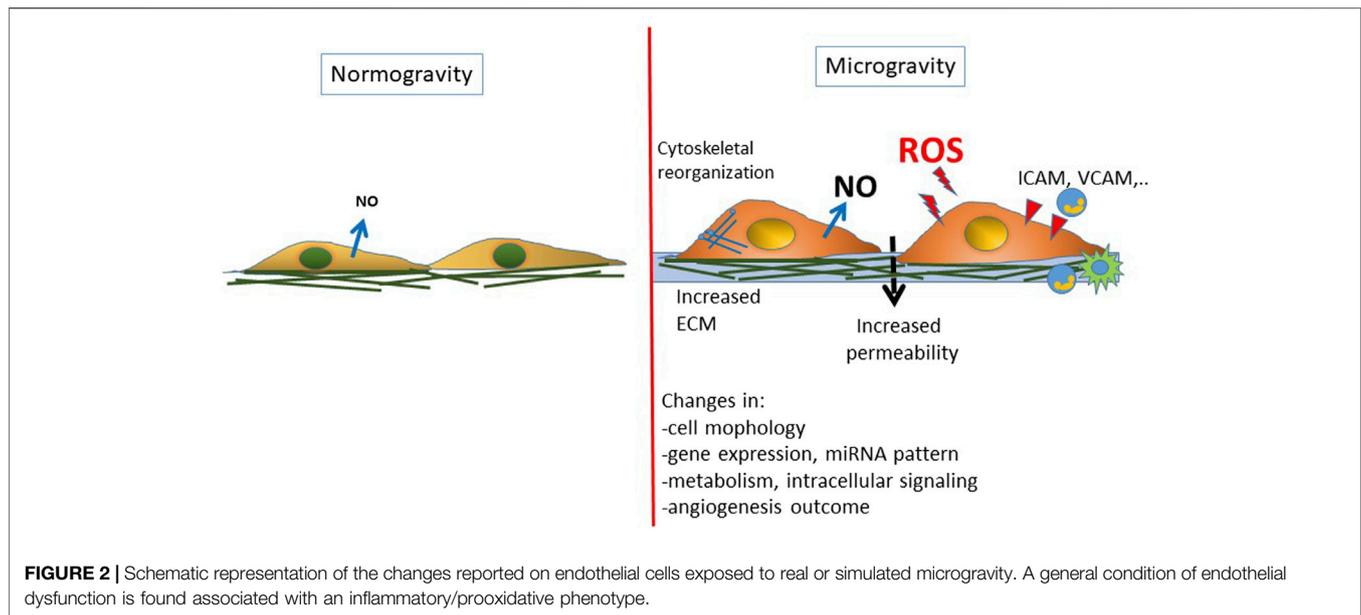
Among the exogenous factors, there are mechanical stressors as pressure, vibrations, and loading. ECs are particularly sensitive to changes in gravitational forces and the mechanisms of mechanotransduction are now known (Maier et al., 2015; Li et al., 2018).

In particular, studies on unloading condition can be performed at different levels as single cell, cell cocultures or organoids, and living animals, as rodents or invertebrates (leeches), exposed to real microgravity in vehicles operating in terrestrial orbits or to terrestrial models of unloading (Cialdai et al., 2020). Animal hindlimb unloading (HLU) or bed rest experimental studies in humans are established models designed to mimic unloading conditions in space.

In Vivo Experiments of Unloading Effect on Wound Healing

Very few are the reports on the effects of mechanical unloading and physical deconditioning on wound healing *in vivo*. Several studies have indicated that spaceflight can adversely affect tissue repair in muscle and bone (Pospishilova et al., 1989; Ilyina-Kakueva and Burkovskaya, 1991; Kaplansky et al., 1991; Bolton et al., 1997; see Genah et al., 2021a for review). In relation to skin repair, Davidson et al. (1998) conducted a study to determine the effects of microgravity on wound healing in rats, in term of granulation tissue and collagen formation. They found a reduced capacity of wounds to heal, being the cells less responsive to added growth factors.

Radek et al. demonstrated that in rats exposed for 2 weeks to HLU before excisional wounding, the healing process was delayed

FIGURE 2 | Schematic representation of the changes reported on endothelial cells exposed to real or simulated microgravity. A general condition of endothelial dysfunction is found associated with an inflammatory/prooxidative phenotype.

on day 2 with respect to ambulatory controls. Although the levels of proangiogenic growth factors FGF-2 and VEGF were similar between the two groups, wound vascularization in HLU animals was significantly reduced at day 7. To further examine this disparity, total collagen content was assessed and found to be similar between the two groups (Radek et al., 2008). Recently, inhibition of cell proliferation and angiogenesis was verified in skeletal muscles of rats exposed to hindlimb unloading through RNA sequences analysis (Cui et al., 2020).

Taken together, these results suggest that keratinocyte and EC functions may be impaired during the wound healing process under periods of prolonged inactivity or bed rest.

Effect of Weightlessness on Endothelial Cell Transcriptome, Morphology, and Function

ECs are mechanosensitive cells undergoing morphological and functional changes in response to fluid shear stress, cyclic tensile strain, and substrate stiffness. In space their alterations contribute to cardiovascular deconditioning and immune dysfunction commonly faced by astronauts during spaceflight. Ground-based and space experimentation has provided a body of evidence about how ECs can respond to the effect of simulated and real microgravity (Byfield et al., 2009; Chancellor et al., 2010; Maier et al., 2015; Li et al., 2018). Exposure of ECs to microgravity in space can change morphology and gene expression, displaying heterogeneous cell size and shape (Kapitonova et al., 2012), 3D growth (Pietsch et al., 2017), energy and protein metabolism deficiency (Chakraborty et al., 2018), significant suppression of genes associated with host defense (Chakraborty et al., 2014), and alterations in genes involved in cell adhesion, oxidative phosphorylation, and stress responses (Versari et al., 2013) (**Figure 2**). To date, only a limited number of studies in space have been performed and the impact of real microgravity on EC functions still remains unclear.

Most of the data derive from ground-based microgravity simulators, as rotating wall vessel (RWV) (a 2D clinostat) (Goodwin et al., 1993) and random positioning machine (RPM) (a 3D clinostat) (Morbidelli et al., 2005; Wuest et al., 2017).

Specifically, markers for leukocyte adhesion and recruitment, adhesive counterreceptors and inflammatory cytokine expression pattern are altered in RWV (Wang et al., 2015), documenting a proinflammatory phenotype. Nevertheless, the outcomes are controversial in literature. For instance, a decreased expression of intercellular adhesion molecule-1 (ICAM-1) has been found in ECs cultured in RPM for 24 h (Grenon et al., 2013). However, upregulation of ICAM-1 transcription was found after 30 min clinorotation and the clustering of ICAM-1s on cell membrane was observed when ECs have been activated by TNF-α and cultured in RWV (Zhang et al., 2010).

Mechanistically, the cytoskeletal remodeling has been considered the transducer of cellular responses to microgravity (van Loon, 2009; Long et al., 2015). The reorganization of cytoskeleton proteins following microgravity exposure includes microtubule and actin filament (F-actin) upregulation (Zhang et al., 2017; Buravkova et al., 2018). Significantly downregulated amount of actin (Carlsson et al., 2003; Corydon et al., 2016), depolymerization of F-actin (Kang et al., 2011), and clustering of the stress fibers at the cellular membrane and around the nucleus (Infanger et al., 2007; Grenon et al., 2013) have been reported, and the actin rearrangement is typically RhoA dependent (Shi et al., 2017). The controversial results probably depend on the EC type used in the experiments, stimulation facility, and time of exposure.

Cytoskeletal rearrangement was accompanied by the overexpression of ECM proteins, including collagen I, fibronectin, osteopontin, and laminin (Infanger et al., 2006; Grimm et al., 2009; Buravkova et al., 2018), but again also downregulation has been described (Corydon et al., 2016).

Furthermore, intracellular signaling and cell–cell communication are crucial for EC functional alterations in microgravity. For example, NO has been reported to be upregulated by RPM and is deemed to be responsible for angiogenesis and cardiovascular deconditioning experienced by astronauts during spaceflight (Siamwala et al., 2010; Grenon et al., 2013). The increased eNOS activation found in 2D or 3D clinostat cultures in ECs seems to be due to PI-3K pathway (Shi et al., 2012), actin remodeling (Siamwala et al., 2010), and caveolin-1- (Cav-1-) mediated mechanotransduction (Shi et al., 2016).

Concerning angiogenesis outcome, in agreement with the results reported by other authors (Kang et al., 2011; Xu et al., 2018), our studies demonstrated that microgravity induces significant changes in EC behavior with reduced cell survival, induction of apoptosis, and angiogenesis impairment (Morbidelli et al., 2005; Maier et al., 2015).

Controversial results were however reported both in differentiated ECs depending on the district (micro- or macrocirculation) and in mesenchymal stem cells (MSC). Simulated microgravity promotes angiogenic output in HUVEC via RhoA-dependent rearrangement of actin and cytoskeleton (Shi et al., 2017). It has been demonstrated that microgravity could stimulate mature ECs and MSC to produce IL-8 and VEGF as well as other paracrine factors involved in angiogenesis. This is responsible for the regulation of EC functions by creating a specific microenvironment in support of EC proliferation and migration (Dittrich et al., 2018; Ratushnyy et al., 2018).

Recently, in human ECs cultured under simulated microgravity achieved with a clinostat, a total of 1,870 miRNAs were found to be differentially expressed with respect to normal gravity. The functional association of identified miRNAs targeting specific mRNAs revealed that a series of miRNAs (hsa-mir-496, hsa-mir-151a, hsa-miR-296-3p, hsa-mir-148a, hsa-miR-365b-5p, hsa-miR-3687, hsa-mir-454, hsa-miR-155-5p, and hsa-miR-145-5p) differentially regulated the genes involved in cell adhesion, angiogenesis, cell cycle, JAK-STAT signaling, MAPK signaling, NO signaling, and VEGF signaling, finally favoring angiogenesis (Kasiviswanathan et al., 2020). In a study on endothelial progenitor cells, cultured in simulated microgravity, a facilitation of functional angiogenic properties has been reported, with increased HIF-1α and eNOS/NO induced FAK/ERK1/2 pathway upregulation in HUVEC (Kong et al., 2021).

In real gravity study, cultured ECs were kept on board of the SJ-10 Recoverable Scientific Satellite for 3 and 10 days (Li et al., 2018). Space microgravity suppressed the glucose metabolism; modulated the expression of cellular adhesive molecules such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and CD44; and depressed the secretion of proangiogenic factors and proinflammatory cytokines. Moreover, space microgravity induced the depolymerization of actin filaments and microtubules, promoted vimentin accumulation, restrained collagen I and fibronectin deposition, regulated the mechanotransduction through focal adhesion kinase and Rho GTPases, and enhanced the exosome-mediated mRNA transfer. As previously seen in simulated microgravity, neither three-dimensional growth nor enhanced NO production has been observed in real microgravity (Li et al., 2018).

Moreover, some preliminary results from *in vitro* studies in modeled microgravity indicate that the cross-talk between fibroblasts and ECs, a building block in the healing evolution, is impaired. The production of angiogenic growth factors is altered as well (Cialdai et al., 2017), with a consequent inability of ECs to form tube-like structures. The complex cross-talk between fibroblasts and ECs and its role in tissue healing are beyond the focus of the present paper.

Despite these controversial results in cultured cells, probably due to different cell source and microgravity protocols, astronauts spending a long time in International Space Station (ISS) are more vulnerable to vasculopathies, associated to endothelial dysfunction (Zhang and Hargens, 2018; Garrett-Bakelman et al., 2019; Navasiolava et al., 2020), thus strengthening the finding of defective angiogenesis and tissue repair in space environment.

PHARMACOLOGICAL, CELLULAR, AND PHYSICAL COUNTERMEASURES

In current clinical practices, a series of drugs can be employed to control symptoms related to wound healing like inflammation, oedema, pain, and steroidal or nonsteroidal anti-inflammatory drugs. Furthermore, dressings and topic products are used to create and keep a humid environment, providing the ideal conditions for a correct wound healing process (Dreifke et al., 2015). However, side effects, or even opposite effects on wound healing, such as hypertrophic scarring, contraction, and necrosis, can limit their employment, especially considering their long-term use, raising the necessity for alternative countermeasures (Dreifke et al., 2015).

Emerging skin regeneration techniques involving scaffolds activated with growth factors, bioactive molecules, and genetically modified cells are being exploited to overcome wound healing technology limitations and to implement personalized therapy design. Results are however partial and under consolidation. The following sections report the state of the art or the ultimate findings on various types of countermeasures for recovery of tissue integrity (**Table 1**). However, not all these countermeasures can be adopted in a space environment, due to their shelf life and stability in space, the necessity to be produced/manipulated in real time by highly prepared operators or in dedicated facilities, and the consideration of the specific personal need.
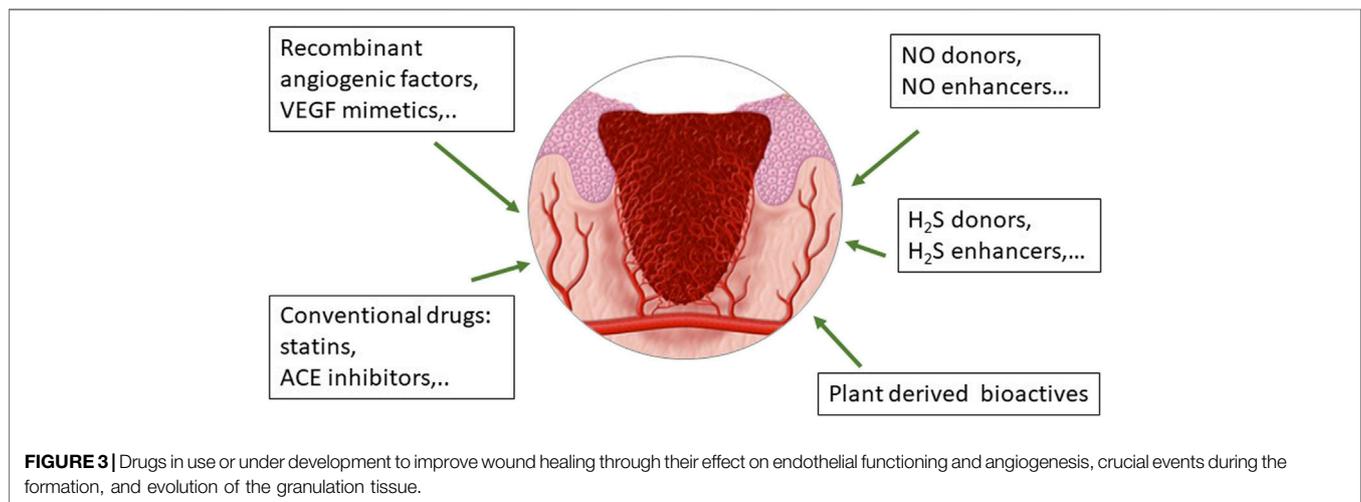
Pharmacological Countermeasures

As reported above, different growth factors as FGF, VEGF, EGF, and PDGF are needed to orchestrate neovascularization and the whole wound healing process and many attempts have been made to use recombinant growth factors in dermal wound healing with different approaches and formulations (see Veith et al., 2019 for a comprehensive review). To the best of our knowledge, there are only a few drugs of protein nature

TABLE 1 | Countermeasures to improve or accelerate wound healing through enhanced angiogenesis.

Type of therapies	Specific clues
Protein therapeutics	Growth factors: PDGF, EGF, FGF, and VEGF
	Nongrowth factor proteins/glycoproteins: insulin, erythropoietin, SDF-1, syndecans
	Peptides: antimicrobial peptides, vasointestinal peptide
	Blood-derived factors: PRP, hemoglobin
Gene and nucleic acid-based therapies	Gene therapy for growth factors microRNA
Drugs and bioactives	Statins
	NO donors
	ACE inhibitors
	Natural compounds (astragaloside, centelloids, and asiaticoside)
	Nutraceuticals
Polymers for dressing or scaffold preparations	Dextran hydrogels
	Hyaluronan oligosaccharides
Stem cell–based therapies (in the form of naïve or genetically modified cells, cell secretome, exosomes, and EV)	Adipose-derived stem cells
	Bone marrow–derived mesenchymal stem cells
	Induced pluripotent stem cells
	Endothelial precursor cells
Physical therapies	Ultrasound/low pressure shock-waves
	Laser therapy/photobiomodulation
	Electrical stimulation
	Hyperbaric oxygen
	Vacuum-assisted closure
	Hypergravity

For details on the state of the art of the single therapies, see Dreifke et al. (2015) *and* Veith et al. (2019). *For hypergravity use in unloading conditions, see* Physical Countermeasures *section.*

FIGURE 3 | Drugs in use or under development to improve wound healing through their effect on endothelial functioning and angiogenesis, crucial events during the formation, and evolution of the granulation tissue.

approved to promote wound healing with potential proangiogenic properties. The first is represented by recombinant PDGF prepared as a skin preparation (Regranex Gel, 0.01% becaplermin). Regranex Gel is the first and only FDA-approved recombinant PDGF therapy for use on diabetic neuropathic ulcers. Regranex contains becaplermin, a human PDGF that is indicated for the treatment of lower extremity ulcers that extend into the subcutaneous tissue or beyond and have an inadequate blood supply. PDGF initiates healing by attracting repair cells to revitalize wounds (Pierce et al., 1991). Indeed,

PDGF works by stimulating fibroblast proliferation, increasing granulation tissue and the rate of reepithelialization and revascularization, and promoting collagen production (Heldin and Westermark, 1999).

Other attempts have included studies with gene therapy or recombinant growth factors administered directly in wounds or delivered with scaffolds, nanomaterials or cells (Veith et al., 2019), but the results still need to be confirmed both in clinical studies on an appropriate number of patients/ pathological conditions and, on top of this, in a space environment.

Conventional drugs are small molecules that could be an optimal alternative to recombinant growth factors to be used in space, since many of them are part of the on-board pharmacy and their pharmacological and toxicological characterization is robust (**Figure 3**). Among the small molecules, particular attention has been posed to statins, widely used to lower cholesterol level due to their inhibitory activity on the liver enzyme 3-hydroy-3-methyl-glutaryl-coenzyme A reductase. Statins are molecules with pleiotropic and multitarget activity, demonstrating a high protective profile on ECs and anti-inflammatory and antioxidative properties, among others (Khaidakov et al., 2009) (**Figure 3**). Statins have been established to possess proangiogenic features, protecting the cardiovascular system against ischemic injury (Kureishi et al., 2000). Simvastatin has been characterized for its healing activity in experimental diabetic ulcers, demonstrating a proangiogenic action with NO pathway upregulation (Bitto et al., 2008). The efficacy of statins has been demonstrated through daily oral administration and by topical treatment, being able not only to produce neovascularization but also lymphangiogenesis (Asai et al., 2012). Recently, novel drug delivery systems for statin local release are under evaluation, demonstrating their efficacy and potential clinical application in chronic ulcers (Rezvanian et al., 2016; Yasasvini et al., 2017). The research on the best formulation and treatment protocols is still active and no report has explored their employment in space environment.

As stated above, NO has been identified to exert a pivotal role in wound healing. NO levels increase significantly following skin injury and then gradually decrease as healing progresses (Childress and Stechmiller, 2002). At the same time pathological conditions characterized by H_2S deficiency as diabetes are known to be associated with impairment of skin healing and progressive ulcerations (Ciccone et al., 2021). These considerations encouraged us to develop strategies with the ability to release both gasotransmitters in a tunable manner for the control of endothelial function and angiogenesis.

Considering the central role of NO in angiogenesis, we have contributed to demonstrate the beneficial effect on endothelial cell functions and angiogenesis by a series of synthetic molecules: 1) a peptidomimetic of VEGF, as QK; 2) the NO donors metal NONOates; and 3) the angiotensin converting enzyme (ACE) inhibitor zofenoprilat, which shares the common feature of releasing NO and H_2S in a controlled manner (Finetti et al., 2012; Monti et al., 2016; Monti et al., 2018) (**Figure 3**).

The peptidomimetic analogue of VEGF, QK, reproduced all the angiogenic functions of the whole molecule, including its ability to promote the eNOS pathway (Finetti et al., 2012). Being a peptidomimetic, QK could be a promising tool to be used in space, for its easy manipulation and stability.

The metal-nonoate Zn(PipNONO)Cl shows an interesting kinetic of NO release (both fast and prolonged) due to the ability to upregulate eNOS and H_2S producing enzymes in ECs, thus contributing to improve endothelial function (Monti et al., 2018). Its role in human skin tissue repair is under evaluation within space agencies programs (LM personal communication). The H_2S facilitating properties on tissue repair have been recently strengthened by the findings that H_2S improves wound healing by induction of angiogenesis (Ciccone et al., 2021) and restoration of EPC functions in type 2 diabetic mice (Liu et al., 2014).

Concerning conventional drugs used in clinics for other purposes, we have demonstrated endothelium protective, proangiogenic, and anti-inflammatory properties by the active moiety of the ACE inhibitor zofenopril, namely zofenoprilat, which through its SH group behaves as a H_2S donor and promoter (Monti et al., 2013; Monti et al., 2016) (**Figure 3**). Its indication in wound healing however has never been verified.

Beside the recombinant growth factors and conventional drugs, an interesting therapeutic approach for wound healing management is represented by ethnopharmacology and traditional remedies, which are present in the various cultures and provide preparations and active principles with anti-inflammatory, antibacterial and proproliferative/proangiogenic properties (Morbidelli, 2016a; Morbidelli et al., 2018) (**Figure 1**). Plant-derived active principles have been isolated and demonstrated to reduce scar formation as astragaloside IV from *Astragalus membranaceus* and centelloids and asiaticoside from *Centella asiatica* (Chen et al., 2012; Bylka et al., 2014). Concerning the beneficial effects of Mediterranean diet, olive oil–based diet has been demonstrated to improve cutaneous wound healing of pressure injury in mice through the reduction of inflammation and stimulation of redox equilibrium (Schanuel et al., 2019). In this scenario, we have contributed to evaluate the protective effect of extra-virgin olive oil polyphenol hydroxy-tyrosol and its metabolic derivative which positively controls angiogenesis and the endothelial-to-mesenchymal transition (Terzuoli et al., 2020). Another example of nutraceuticals which exerts antioxidant and protective effects on EC function affected by high glucose is erucin, derived from *Eruca sativa* Mill. seeds. Its endothelial positive outcomes are correlated to increased H_2S intracellular levels (Martelli et al., 2021). Studies are needed to further validate these findings in experimental models and in the clinic and to define other nutraceuticals with protective effects on ECs and promoting reparative angiogenesis.

Cell Therapies

The use of stem cells, alone or by the help of a scaffold, provides better and faster healing of burn wounds, with decreased inflammation, slow scar progression, and reduced fibrosis. Stem cell homing at the wound site results in granulation tissue formation, immunomodulation, neovascularization, apoptosis inhibition, and induction of epithelial cell proliferation with skin regeneration. While these findings are clear in animal models, the validation of their clinical use is at the beginning (Veith et al., 2019; Phua et al., 2021). Sources of MSCs in adults are bone marrow, adipose tissue, and umbilical cord blood. Additionally, induced-pluripotent-stem-cell- (iPSC-) derived MSC have been reported to be suitable for cell therapy. However, their effectiveness in human patients remains to be established (Jo et al., 2021; Mazini et al., 2021; Ullah et al., 2021). The proposal of proangiogenic paracrine secretion by stem and precursor cells cultured in microgravity has been provided (Ratushnyy et al., 2018; Kong et al., 2021).

Dermal fibroblasts are the major cell type in skin dermal layers. As said above, they actively participate in skin regeneration and these cells are becoming attractive candidates for cell-based therapies in wound healing. Due to their heterogeneity linked to variable activation by inflammatory stimuli, tissue niche of origin and different scar forming properties, their potential clinical exploitation is far (Xue et al., 2021).

Due to the plethora of secretory products involved in angiogenesis (as VEGF and PDGF), tissue remodeling, and wound healing (Pierce et al., 1991; Frechette et al., 2005; Lacci and Dardik, 2010), platelet therapy has been applied in regenerative medicine and wound healing from decades (Martinez-Zapata et al., 2016). Recently, in cultured fibroblasts and in an experimental model of dermal injury in leech exposed to clinostat induced microgravity, we have demonstrated the efficacy of platelet rich plasma (PRP) in accelerating healing by acting on fibroblast migration (Cialdai et al., 2017; Cialdai et al., 2020).

While the use of cells in a space environment seems not feasible due to the many risky procedures (self-harvesting, *in vitro* culture and checking of safety, inoculation in the wound or systemically), the use of autologous PRP appears a very promising approach in the astronauts not only for dermal repair but also for regenerative bone, tendon, and endodontic treatment, where improvement of angiogenesis is a necessary step for proper repair.

Microvesicles and Exosomes

Recently, a therapeutic role for extracellular vesicles (EV) derived from stem cells has been described in animal wound models (Dalirfardouei et al., 2021). Extracellular vesicles and in particular exosomes contain various bioactive molecules as proteins, enzymes, and nucleic acids, thus providing the wound with all the necessary stimuli to promote angiogenesis and cell proliferation and regulate inflammation and collagen remodeling, ultimately leading to tissue healing (**Table 1**). The use of stem-cell-derived exosomes seems more feasible with respect to cell therapy, without potential problems related to the use of living cells, which would make their use in space quite impossible. Additionally, they are not rejected by the immune system and have a homing effect and their dosage can be easily controlled. *In vivo* studies on animal models demonstrate the beneficial effects of EVs on accelerating wound closure and reepithelization in a dose-dependent manner. Various studies demonstrate induction of angiogenesis through the conventional mechanisms of PI-3K/Akt, MAPK/ERK1/2, and JAK/STAT pathways. Interestingly, the upregulation of TGF-β2/SMAD2 pathway involved in scar inhibition has been reported (Dalirfardouei et al., 2021). They can also act as carriers for other interventions and be combined with scaffolds. Also this innovative approach should be, however, thoroughly verified before clinical utilization in particular with respect to EV manufacturing, treatment protocols, and long-term follow-up (An et al., 2021).

Physical Countermeasures

In recent years, many therapies aiming at stimulating the healing process are in clinical use, such as ultrasound, laser therapy, and other forms of photobiomodulation, electrical stimulation,

hyperbaric oxygen, and vacuum-assisted closure (Dreifke et al., 2015; Nesi-Reis et al., 2018; Priyadarshini et al., 2018; Micheli et al., 2019; Xu et al., 2021). The efficacy of physical approaches is due to the fact that all the cell types involved in wound healing (fibroblasts, keratinocytes, and ECs) are sensitive to mechanical forces at cellular and molecular level acting as mechanotransducers.

ECs in particular respond to mechanical stimuli such as shear, strain, and stretch. This property can be exploited to induce reparative angiogenesis in pathological conditions associated with insufficient angiogenesis. In cultured ECs and in a mouse model of wound healing, it has been demonstrated that low-pressure shock waves induced angiogenesis. Increased EC migration and proliferation were associated with enhanced Ca^{++} influx and PI-3K which is usually observed when ECs are exposed to stretch. Shock wave treated mice showed enhanced wound-induced angiogenesis documented by increased vascular area and vessel length. Accelerated wound closure was observed compared to control mice (Sundaram et al., 2018).

Photobiomodulation, i.e., lasers emitting red/IR radiation, promotes angiogenesis in wound healing (de Madeiros et al., 2017). In a previous experimental paper, ECs were exposed to simulated microgravity or pulsed Nd:YAG laser radiation to assess their behaviour and morphology. Increased fibronectin and laminin could be the cause for impaired ECM rebuilding and altered cell adhesion/migration in microgravity (Monici et al., 2011) in accordance with previous data on impairment of angiogenesis (Morbidelli et al., 2005). On the contrary, the exposure to Nd:YAG laser pulses induced the formation of a highly ordered array of fibronectin fibrils on EC surface and cell spreading to form a monolayer (Monici et al., 2011). Recently, we have additionally demonstrated the anti-inflammatory effect of NIR laser on dermal fibroblasts stimulated with inflammatory cytokines, through an inhibition of NF-kB transcription pathway (Genah et al., 2021b). All these results suggest a beneficial effect of photobiomodulation as an effective healing option with proangiogenic and anti-inflammatory properties. Its inflight applicability should however be verified.

Among different countermeasures implemented to minimize the effects of microgravity, a promising one could be artificial gravity. We have demonstrated that discontinuous hypergravitational stress did not significantly affect cell survival in macrovascular and microvascular ECs. In both cell populations, we found similar changes in cytoskeleton and αvβ3 integrin distribution that in microvascular ECs were combined with an increased anaerobic metabolism and cell detachment from the substratum (Monici et al., 2006; Morbidelli et al., 2009).

Exposure to artificial gravity provides protection against microgravity induced apoptosis and oxidative stress in retinal endothelial cells of rodents flown on ISS (Mao et al., 2018).

A profound rearrangement of the cytoskeleton network, dose-dependent increase of FAK phosphorylation, and Yes-associated protein 1 (YAP1) expression was found in dermal microvascular ECs exposed to hypergravity, suggesting improved motility and proangiogenic response. Transcriptome analysis showed changes in the expression of genes associated with cardiovascular homeostasis, NO production, angiogenesis, and inflammation

(De Cesari et al., 2020). These results show that adaptation to hypergravity has opposite effects compared to microgravity on the same cell type, suggesting it as a potential physical countermeasure. Its real application is however far away.

In summary, the efficacy of physical countermeasures, alone or combined with other therapies, remains to be defined in cultured cells and in integrated and innovative tissue models in order to be effective and safe in a spaceflight arrangement.

CONCLUSION AND PERSPECTIVES

While the events and mechanisms controlling wound healing are well known and characterized, the pharmacological interventions to prevent or treat healing dysfunction are few and nowadays still under evaluation and validation on Earth. The data available in relation to unloading conditions document that the impaired wound healing results from the following mechanisms: 1) persistent inflammation with neutrophil infiltration (Dovi et al., 2003; Radek et al., 2008) and overall alteration of the inflammatory phase; 2) altered blood flow with more permeable vessels (McDonald et al., 1992), presumably linked to increased NO and VEGF levels (Shi et al., 2012; Dittrich et al., 2018); 3) an altered neovascularization that may result from impaired EC proliferation and migration in response to angiogenic factors, increased EC apoptosis, and altered gene expression and signalling pathways (Morbidelli et al., 2005; Radek et al., 2008; Li et al., 2018; Kasiviswanathan et al., 2020); 4) exposure to simulated microgravity also resulting in enhanced ROS production that may contribute to unloading-induced oxidative stress. The systemic oxidative status can be derived from radiation-induced immune system alterations especially relevant in long-duration space flight (Rizzo et al., 2012).

Considering the space environment and the critical issues characterizing long duration space travels (unloading, confinement, scarce hygiene, and radiations) is mandatory to further study angiogenesis and wound healing in space, to precisely define the target for therapeutic interventions and to validate efficient and safe countermeasures and treatment protocols. The combination of various stressors needs to be characterized. Recently, a paper by Mao et al. (2019) revealed the synergistic worsening effect of combined unloading and radiation exposure on oxidative stress and dysfunction of retinal ECs, events responsible for sight loss associated to space permanence.

Additionally, for a series of countermeasures, it is difficult to imagine their exploitation in a space environment since they require freshly isolated living cells or facilities and competences to extract/cultivate cells from the injured subject. In long duration missions, to deliver in time, ad hoc cells/tissues will be not possible and proangiogenic/regenerative countermeasures should be available on shelf/on board or rapidly achieved. Therefore, the effort of national and international space agencies goes in this direction and this review reflects the state of the art on the specific phenomenon of angiogenesis contribution on wound healing and the potentiality of developing effective and safe countermeasures. Up-to-date techniques are needed both for the study of the mechanisms of angiogenesis alterations in space environment and for the validation of countermeasures to improve wound healing. Examples are tissue engineering, cocultures, 3D multicellular structures, lab-on-chip approaches (Grimm et al., 2014; Ma et al., 2014; Huang et al., 2020), which are starting to substitute experimental animals due to ethical issues. Thorough comprehension of the molecular and biochemical mechanisms underlying cellular responses is coming from omics techniques and RNA sequence analysis of samples from simulated and real microgravity experiments (Ma et al., 2014; Mao et al., 2018; Cui et al., 2020; Kasiviswanathan et al., 2020). The expectations from these types of experiments are high.

Nevertheless, it is important to stress the concept that all the information obtained for space research can be exploited on the Earth for fragile (aged, diabetic) or bed-ridden patients, whose clinical characteristics are very similar to astronauts. A common feature that accompanies space travelers and aged/fragile patients is indeed endothelial dysfunction, which, among the others, is responsible for angiogenesis impairment and not efficient healing of wounds and ulcers.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, investigation, and writing (original draft preparation) were carried out by LM; writing review and editing were done by LM, SG, and FC; supervision and funding acquisition were provided by LM. All authors have read and agreed to the published version of the manuscript.

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Review

The Effect of Space Travel on Bone Metabolism: Considerations on Today's Major Challenges and Advances in Pharmacology

Shirley Genah [1], Monica Monici [2] and Lucia Morbidelli [1,*]

1   Department of Life Sciences, University of Siena, I-53100 Siena, Italy; shirley.genah@student.unisi.it
2   ASA Campus Joint Laboratory, ASA Research Division, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, I-50139 Florence, Italy; monica.monici@unifi.it
*   Correspondence: lucia.morbidelli@unisi.it; Tel.: +39-0577-235-381

Abstract: Microgravity-induced bone loss is currently a significant and unresolved health risk for space travelers, as it raises the likelihood for irreversible changes that weaken skeletal integrity and the incremental onset of fracture injuries and renal stone formation. Another issue related to bone tissue homeostasis in microgravity is its capacity to regenerate following fractures due to weakening of the tissue and accidental events during the accomplishment of particularly dangerous tasks. Today, several pharmacological and non-pharmacological countermeasures to this problem have been proposed, including physical exercise, diet supplements and administration of antiresorptive or anabolic drugs. However, each class of pharmacological agents presents several limitations as their prolonged and repeated employment is not exempt from the onset of serious side effects, which limit their use within a well-defined range of time. In this review, we will focus on the various countermeasures currently in place or proposed to address bone loss in conditions of microgravity, analyzing in detail the advantages and disadvantages of each option from a pharmacological point of view. Finally, we take stock of the situation in the currently available literature concerning bone loss and fracture healing processes. We try to understand which are the critical points and challenges that need to be addressed to reach innovative and targeted therapies to be used both in space missions and on Earth.

Keywords: space pharmacology; bones; osteopenia; microgravity; space medicine

1. Introduction

Today, it is well established that the space "exposome"—the set of environmental factors related to space to which an astronaut is constantly exposed during a mission—can significantly influence human physiology [1]. Among the factors that place humans most at risk in space are microgravity and ionizing cosmic radiations (galactic cosmic radiation or GCR), deriving from the sun and other celestial bodies or events [2]. The human body results from a very long evolution shaped by gravitational forces; bones are needed to support body weight and muscles to carry out movements that counteract gravity [3,4]. The cardiovascular system has also adapted; our body is mainly composed of fluids, and a complex and delicate balance of hydrostatic forces is required to flow blood against gravity and maintain a constant supply of oxygen to the brain [4,5]. The transition to a condition where the gravitational force lacks causes a long series of physiological changes and related disorders.

Moreover, gravity plays a pivotal role in cell biology, acting on cytoskeletal organization and cell structure. Cells can convert mechanical inputs into biochemical signals, initiating downstream signaling cascades in a process known as mechanotransduction. Therefore, any changes in mechanical loading, for example, those associated with microgravity, can consequently influence cell functionality and tissue homeostasis [6], leading to altered physiological conditions.

Physiological changes in microgravity can either be immediate or manifest in the long-term with some permanent effects that never resolve completely. Within the first 72 h upon exposure to weightlessness, certain physiological systems exhibit altered function. These include baroreceptor reflex, neurovestibular system, and gastrointestinal tract, leading to problems like nausea and vomiting, space motion sickness (SMS), sleep disturbances and headaches [7]. The majority of these systems will adapt to microgravity by resetting to a new equilibrium state within a relatively short time. Upon return to a gravitational field from space, the same rapid effects may be seen in reverse [8].

Many of these immediate indispositions can interfere with crewmember critical operational procedures associated with entering orbit or returning to Earth. The lack of readiness in managing certain situations due to adaptation disturbances can represent a risk for mission success, and it is necessary to find effective and rapid countermeasures, both pharmacological and otherwise.

Other physiological systems manifest weightlessness effects on a longer term (weeks to months). For short-duration missions, these changes may be minor or even undetectable. On longer flights, the effects can become more pronounced. The recovery of astronauts from these symptoms depends on the time of exposure to stress factors related to the space environment; the longer they stay, the longer the recovering time [8,9]. Among long-term effects on human physiology, we find muscle atrophy, bone demineralization and, therefore, alteration in calcium balance, immune function dysregulation, endocrine disorders, such as insulin resistance [10,11], and cardiovascular deconditioning that leads to orthostatic intolerance [12]. Currently, these physiological changes present major obstacles to long-term space missions. Therefore, it is fundamental to develop countermeasures aimed at preventing or slowing down the progression of these complications.

This review discusses alteration of human physiological parameters related to bone metabolism due to long-term weightlessness exposure and examines the effectiveness of both pharmacological and non-pharmacological countermeasures.

2. Bone Loss and Osteoporosis

Reduction in bone density related to microgravity is due to an imbalance of bone remodeling induced by changes in bone cells. Studies of ground-based simulated microgravity showed that morphology and functions of osteoblasts, osteoclasts, osteocytes and mesenchymal stem cells were all changed, suggesting that bone cells respond and adapt to the altered gravity condition by changing their morphology and functions.

Much research demonstrated that modeled microgravity increased osteoclast activity, receptor activator of nuclear factor-kappa-B ligand (RANKL)-mediated osteoclastogenesis and bone resorption capacity [13–15]. Nabavi and colleagues showed that unloading causes many negative effects in osteoblasts that are likely due to signaling cascades from the cell surface to the nucleus [16]. According to their results, osteoblasts show an altered microtubule organization, reduction in the size and number of focal adhesions (FA) and FA proteins (paxillin, vinculin and zyxin). Unloading has been shown to disrupt F-actin stress fiber formation, and a significant expansion in cell area was observed in osteoblasts exposed to microgravity, related to the altered F-actin tensional forces and decreased mechanical stiffness. Additionally, a significant change in nuclear morphology also has been observed [16]. Therefore, the absence of gravitational force affects the normal distribution of major cytoskeletal networks and the overall geometry of osteoblasts, influencing parallel cell function.

Osteocytes are remarkably versatile cells and are involved in all aspects of skeletal biology, including the response to loading, regulation of bone turnover and the control of mineral metabolism [17,18]. These cells are housed individually in cavities (lacunae) located inside the mineralized bone and communicate with each other, and with bone surface cells, by gap junctions connecting dendritic-like cellular extensions housed in connecting canaliculi [19]. Osteocytes regulate bone remodeling by sensing, responding and translating mechanical stimuli into biochemical signals that affect other bone cell functions

(e.g., osteoblast, osteoclast) [17,18]. In the presence of loading stimuli, osteocytes produce a typical signaling glycoprotein, sclerostin, which provides for negative regulation of bone formation by inhibiting the Wnt-β-catenin pathway in osteoblasts [20,21]. Usually, Wnt binding to its frizzled receptor complexed with low-density lipoproteins 5 or 6 (Lrp5/Lrp6) initiates a signaling cascade resulting in B-catenin translocating to the nucleus, where it activates transcription factors regulating target genes involved in osteoblast proliferation and differentiation [19–21]. The Wnt-signaling pathway is not only involved in osteoblastic cell differentiation and bone formation but also inhibits bone resorption by blocking the receptor activator of nuclear factor-κB-ligand (RANKL)/RANK interaction [1,22]. Many studies in the literature have reported that when reduced mechanical loading and microgravity occur, the production of sclerostin by osteocytes increases notably, blocking Wnt binding and thus resulting ultimately in reduced osteoblast and increased osteoclast activity [23–25].

Alongside this finding, recent data have demonstrated that osteocytes can remodel their perilacunar and canalicular matrix and participate in the release of skeletal calcium stores, a process called "osteocytic osteolysis", which occurs in response to diverse stimuli [7]. Blaber et al. found that bone loss induced by microgravity in mice was not only due to osteoclastic hyperactivation but also caused by osteocytic osteolysis and osteoblastic cell cycle inhibition [26]. Taken all this evidence together, it can be hypothesized that osteocytes function in two ways to respond to microgravity. On one hand, they sense the gravity changes and begin osteocytic osteolysis themselves. On the other hand, they send signals to other effector cells (e.g., osteoclast, osteoblast) to regulate their functions.

Blaber and colleagues also proposed the hypothesis that at the basis of bone formation decrease, there is also an alteration in the differentiation of bone marrow mesenchymal and hematopoietic stem cell lineages [27,28]. The group conducted in vivo studies using 16-week-old female mice flown for 15-days on the International Space Station (ISS). It analyzed femoral bone marrows, which displayed large cavities, clearly indicating unloading-associated bone resorption, and demonstrated a general downregulation pattern of gene expression required to differentiate bone marrow mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) progenitor lineages. This phenomenon is revealed in multiple cell types, including osteoclasts and osteoblasts required for bone remodeling and mineral homeostasis, erythrocytes required for the transport of oxygen and iron throughout the body, and megakaryocytes required for the formation of platelets [27,28].

Microgravity-induced bone loss has been suggested to be more severe than osteopenia on Earth, and prolonged exposure to unloading conditions can raise the risk of osteoporosis and bone fractures [29]. Bone loss onset is usually very fast and can be detected within the first months in microgravity, whereas bone density recovery and re-mineralization are slower at 1 g on Earth and, in some cases, are not even restored to previous levels [30]. Bone demineralization during space flights also increases urinary calcium excretion and raises the risk of kidney stone formation. In physiological conditions, blood calcium and phosphate kinetics are regulated by the parathyroid gland through the secretion of parathyroid hormone (PTH), whose effects concern bone turnover, kidneys homeostasis and intestine Ca^{2+} absorption by stimulating the production of active vitamin D [31]. As a consequence of bone demineralization, due to unloading, Ca^{2+} released from bones reaches high blood levels causing suppression of PTH secretion. This inhibition leads to a reduction in vitamin D production at the intestine level, a decrease in kidney Ca^{2+} reabsorption and hypercalciuria, raising the risk of kidney stone formation [32].

Despite interindividual heterogeneity in bone mass, not all sites of the skeleton are similarly affected by unloading. Weight-bearing bones at 1xg are majorly affected by the space environment compared to non-weight-bearing bones. Indeed, the tibial cancellous bone showed more striking and earlier bone loss than the tibial cortex, probably because of more local remodeling [30]. According to National Aeronautics and Space Administration (NASA), the proximal femoral bone loses 1% to 1.5% of its mass per month or

roughly 6% to 10% over a 6-month stay in space. Recovery after returning to Earth takes at least 3 or 4 years [33].

Microgravity-induced osteopenia is a significant and unresolved health risk for space travelers. For this reason, many countermeasures are employed today to reduce bone loss during long-duration space flights and include diet supplementations, physical activity and pharmacological interventions.

3. Countermeasures

3.1. Diet Supplements

Although the most important cause for bone loss in space is unloading of the body weight, when considering the long duration of spaceflight, there are several other factors able to influence calcium metabolism, including alterations in diet intake and low sunlight exposure [34]. Calcium and vitamin D have long been recognized as essential nutrients for bone health and maintenance.

The principal function of vitamin D in calcium homeostasis is to increase calcium absorption from the bowel. A deficiency of this element, due to poor exposure of astronauts to sunlight and ultraviolet radiations, may lead to insufficient Ca^{2+} intake, diminished intestinal absorption, decreased serum Ca^{2+} levels, which cause poor mineralization of bones [34]. Ca^{2+} intake is generally associated with the ingestion of specific foods, such as dairy products, nuts and seeds, vegetables like kale, broccoli and watercress and salmon. Unfortunately, despite many attempts to implement astronauts' regimes, diet components are not sufficiently absorbed. Furthermore, the presence of intolerances to dairy products may play a negative role in assuming adequate calcium levels. Lactose intolerance has been associated not only with poor Ca^{2+} intake but also with low bone mass and increased risk of fracture [35]. In these cases, diet supplements are needed to reach optimal nutritional needs of calcium. Astronauts necessitate sufficient oral intake of vitamin D and Ca^{2+} during spaceflights, and the amount necessary to minimize a negative balance is approximately 1000 mg/d for calcium and 800–1000 IU/d for vitamin D, which is currently recommended for space flights up to one year [34,36].

3.2. Physical Exercise

Until 2004 early countermeasures on the ISS included an interim resistance exercise device (iRED), treadmills and exercise bicycle. In 2004, NASA installed the advanced resistance exercise device (aRED) with higher loads to increase forces on bone, particularly the spine and proximal femur [37]. Smith et al. showed in 2012 that resistance exercise coupled with adequate dietary supplements could maintain bone mass in most regions during short space missions. In this study, involving 13 crew members on ISS missions from 2006 to 2009, eight members had access to iRED, and five had access to the advanced resistive exercise device aRED. All crew members were provided with a specific program prescribing 2.5 h of exercise per day (6 days/week), including aerobic training and resistance exercise. Diet was designed to provide adequate intake of seven nutrients of interest (proteins, water, energy, sodium, calcium, iron, and potassium). The authors concluded that improved nutrition and resistance exercise during spaceflight could attenuate the expected bone resorption observed on prolonged missions [1,38].

3.3. Pharmacological Countermeasures

Physical activity and nutrient integration are usually coupled and associated with pharmacological therapies to prevent bone loss. Today, treatments for space osteoporosis include antiresorptive drugs aiming at actively reducing osteoclast numbers by inhibiting osteoclastogenesis or inducing apoptosis, thereby reducing resorption levels. The list of pharmacological countermeasures and related dosages is summarized in Table 1.

Table 1. List of pharmacological countermeasures, related dosages and administration routes proposed for the treatment of microgravity-induced osteopenia.

Activity	Class	Drug	Dosage	Administration
Anti-resorptive agents	Bisphosphonates	Alendronate	70 mg/week	Oral
		Risedronate	35–75 mg/week	Oral
		Ibandronate	150 mg/month	Oral
		Pamidronate	60–90 mg/month	Intravenous
		Zoledronic acid	4–5 mg/year	Intravenous
	Monoclonal antibodies	Denosumab	60 mg/6 months	Subcutaneous
Anabolic drugs		Teriparatide	20 µg/day	Subcutaneous
			2.5–5 mg/ day	Oral

3.3.1. Antiresorptive Agents

Among antiresorptive drugs, bisphosphonates are primary agents in the current pharmacological arsenal against osteoclast-mediated bone loss. Structurally, bisphosphonates are chemically stable derivatives of inorganic pyrophosphate. Second- and third-generation bisphosphonates (alendronate 70 mg, risedronate 35–75 mg, ibandronate 150 mg, pamidronate 60–90 mg, zoledronic acid 4 mg) have nitrogen-containing R2 side chains [39]. Bisphosphonates are preferentially incorporated into sites of active bone remodeling, and the primary pharmacological action is the suppression of bone resorption by inducing selective osteoclast apoptosis. Specifically, bisphosphonates attach to hydroxyapatite binding sites on bone surfaces undergoing active resorption [39,40]. When osteoclasts begin to resorb bone that is impregnated with a bisphosphonate, the released drug during resorption impairs the ability of the osteoclasts to form the ruffled border, to adhere to the bony surface, and to produce the protons necessary for continued bone resorption [39,40].

LeBlanc and colleagues studied the administration of alendronate as a supplement to exercise to protect bone during long-duration spaceflight. According to their results, the combination of the aRED exercises (2.5 h, 6 days/week) and oral administration of alendronate (Fosamax® 70 mg/week) attenuated the expected decline in essentially all indices of altered bone physiology during spaceflight [41]. Today, the combination of aRED exercises, vitamin D and calcium supplementation together with bisphosphonates administration seems to be the best countermeasure to employ to prevent astronaut bone loss during space flights.

An interesting molecular target that could slow down bone resorption is RANKL (receptor activator of NF-kB ligand), a protein expressed by osteoblasts, bone marrow, stromal cells and chondrocytes. The interaction of RANKL with its receptor on osteoclasts RANK is involved in cell differentiation and activation [42]. The monoclonal antibody denosumab shows antiresorptive activity by selectively blocking the binding of RANKL to RANK on osteoclasts [43]. In the FREEDOM clinical trial, denosumab subcutaneous administration at the dose of 60 mg every six months was effective in reducing fracture risk in women with postmenopausal osteoporosis. Over 3 years, denosumab significantly reduced the risk of new vertebral fracture by 68%, nonvertebral fracture by 20% and hip fracture by 40% relative to placebo. Furthermore, denosumab also significantly improved bone mineral density at various skeletal sites, including the total hip, lumbar spine, femoral neck and trochanter bone [43,44]. For its proven efficacy and safety, denosumab is being considered by NASA as a potential countermeasure for bone loss due to microgravity and results from experiments regarding its employment in space are still pending.

Antiresorptive drugs are potent agents in preventing bone loss and reducing fracture risk. However, long-term use of those medications, such as what might be done in space travel, can produce rare but potentially serious adverse effects, such as osteonecrosis of the

jaw and atypical femoral fractures [44,45]. When thinking about drug therapy, especially in conditions that could alter per se pharmacokinetic parameters, such as space environment [46], several variables must be considered. These include the safety profile, route of administration and frequency: in fact, a drug with an easy route of administration, such as oral or intranasal, could ensure better adherence to the drug regimen, while a reduced frequency in assumption could decrease the onset of undesirable effects due to a continuous and repeated exposure to the medication [47]. To meet this last requirement, a medication must demonstrate a prolonged pharmacological action and, to date, some drugs that fall into the bisphosphonate class possess this property; indeed, some of them, in addition to an oral route of administration, also has a weekly (alendronate or risedronate) or even monthly (pamidronate) dosage. In particular, zoledronic acid is a bisphosphonate generally indicated for the treatment and prevention of osteoporosis and Paget's disease [48]. The frequency in its administration changes according to the type of pathology considered. For the prevention and treatment of osteoporosis, it corresponds to an infusion of 4–5 mg of zoledronic acid, respectively, every one or two years [49]. Although the route of administration is complicated and requires the intervention of specialized healthcare personnel, its unusual frequency in dosage may suggest its use as a single infusion pre-launch. The same reasoning could be applied to the monoclonal antibody denosumab, whose dosage schedule is a subcutaneous administration of 60 mg every 6 months [43,44].

3.3.2. Anabolic Agents

Considering adverse effects produced by antiresorptive agents, the focus and medical interest are shifting to developing pharmacological countermeasures acting through different mechanisms of action. Among these, anabolic drugs exert their function by increasing bone formation rather than inhibiting resorption.

Currently available anabolic agents improve bone mass and reduce fractures through stimulation of the parathyroid hormone receptor-1 on osteoblasts and their precursors. In a healthy organism, PTH functions as an essential endocrine regulator of calcium and phosphate concentrations in the extracellular space, which is crucial for maintaining serum and urinary calcium levels within the physiological range [50]. The first anabolic agent approved by Food and Drug Administration (FDA) to treat osteoporosis in postmenopausal women and in men, who are at high risk for fracture, is recombinant teriparatide (rh-PTH (Forteo®)) an analog of human PTH. Subcutaneous daily administration of 20 µg of teriparatide for 19 months to women with low bone mass and a history of prior fracture resulted in an almost 10% increase in vertebral bone mineral density (BMD). Usually, teriparatide is also administered in combination with vitamin D (400–1200 IU) and calcium (1000 mg) supplementation [51]. Many other studies confirm the efficacy of teriparatide in preventing bone fractures and increasing BMD in osteoporosis patients and safety, considering no severe adverse reactions have been reported after long-term administration [52].

Nowadays, the administration of teriparatide through daily subcutaneous injections seems to be the most employed method. However, in environments such as the one aboard the ISS, it perhaps may not be indicated due to the difficulty in administration, which could hamper the compliance of the therapeutic regime. For this reason, it would be appropriate to evaluate different routes of administration. Several studies in the literature have demonstrated that oral formulations at higher doses (2.5 and 5 mg) of teriparatide show an efficacy, pharmacokinetic and safety profile comparable to the subcutaneous formulation [53,54]. To enhance absorption and bioavailability of therapeutic peptides, a new delivery system has been developed by Altaani and colleagues [55] in a preliminary in vivo study, which relies on teriparatide encapsulation in oleic acid-based nanoemulsions to be administered orally.

As discussed in previous paragraphs, microgravity-induced bone loss and demineralization raises the risk for kidney stone formation, as elevated blood Ca^{2+} levels suppress PTH secretion manifesting with hypercalciuria [56,57]. Therefore, studies with teriparatide,

as an additional countermeasure for circulating PTH fall in microgravity and hypercalciuria, would be interesting in future space missions.

3.3.3. Combination and Sequential Therapies

The effect of teriparatide, as an anabolic agent aimed at increasing BMD, is confined to a well-defined therapeutic window. Among the factors that mitigate the bone-forming activity, it must be considered that PTH, in addition to the bone-forming effect exerted on osteoblasts and osteocytes, also displays an indirect resorptive action by stimulating osteoclasts through a well-known molecular mechanism, already described elsewhere [50,58]. Furthermore, PTH's ability to stimulate new bone formation waned with time and repeated dosing [59]. Indeed, several studies have shown that long-term administration of teriparatide beyond 24 months leads to increased resorptive markers and to a reduction in pro-forming bone markers, shifting the balance in the opposite direction. For this reason, using hrPTH is limited to a maximum period of 2 years, beyond which the anabolic effect on bones declines in favor of a catabolic action [60,61].

Teriparatide therapeutic regime, described in the previous paragraph, may be compatible with the duration of the current missions since astronauts rarely stay on the ISS for more than a year. To date, only the cosmonaut Valery Polyakov currently holds the overall record for the longest space mission, having completed a stay of 438 days aboard the ISS [62]. The problem arises when evaluating the possibility of long-term space flights, such as future expeditions to Mars, where extensive use of teriparatide alone will not only be able to counteract microgravity resorptive effects but instead could cause a synergic catabolic activity alongside the one induced by microgravity, leading to a dangerous risk for human health. For long-term treatment of osteoporosis, researchers have focused their attention on developing combined and sequential drug therapies with both anabolic and antiresorptive mechanisms of action.

In 2014, a clinical trial (DATA study) on postmenopausal osteoporotic women showed how the combination of teriparatide, 20 µg daily, and denosumab, 60 mg every 6 months, for 24 months could increase BMD at the femoral neck, total hip and spine significantly more than either teriparatide or denosumab alone [63]. Soon after, an extension study followed, aimed at evaluating the effect of sequential therapies on osteoporosis: women originally assigned to 24-months of teriparatide received 24-months of denosumab; subjects originally randomized to 24-months of denosumab shifted to 24-months of teriparatide; and subjects, who originally received both drugs, received an additional 24-months of denosumab alone [64]. BMD continued to increase transitioning from teriparatide to denosumab, whereas switching from denosumab to teriparatide resulted in progressive or transient bone loss. According to the authors, 2 years of combined therapy followed by 2 years of denosumab alone is associated with the largest cumulative BMD increases at the hip and radius, an important clinical outcome since increases obtained were greater than any currently available therapy taken for a similar duration. The additive effect of these two drugs appears to be linked to the ability of denosumab to fully inhibit teriparatide-induced bone resorption but only partially inhibit anabolic bone formation [64]. This combined and sequential approach could be evaluated for its effectiveness in long-term space travels. Ground-based simulated studies are needed and should be carried out to validate the potential of this 4-year therapeutic regimen. However, observations in real space conditions could be difficult due to the excessively long time astronauts should spend on the ISS. To date, no information is available regarding the effects of space exposome on human physiology over such prolonged times. Furthermore, it is unclear what effect stopping this therapy may have once the astronaut returns to normal gravity conditions.

3.4. Melatonin

All pharmacological countermeasures described above are effective in reducing bone loss associated with unloading conditions. However, they show some undesirable effects, even serious ones, which may arise after long-term use. For this reason, efforts have been

made to find more riskless drugs for the prevention of bone loss during space flight. In this regard, researchers have focused their attention on melatonin, a hormone produced by the pineal gland, synthesized almost exclusively in the dark. Melatonin is known for its wide variety of physiologic functions, including hypothalamic control of circadian rhythms, body temperature, bone homeostasis and displays effects on both cardiovascular and immune systems [65–67]. The implication of melatonin in the maintenance of skeletal apparatus physiology has been raised by several studies associating a decrease in the nocturnal production of melatonin, due to aging or to light exposure at night, with increased risk of osteoporosis [68,69]. The effects of melatonin on bone marker turnover were further supported in the MelaOst trial demonstrating a significant improvement in BMD in postmenopausal women with osteopenia following nightly melatonin administration at the dose of 3 mg/day, for one year [69,70].

Today, molecular mechanisms responsible for melatonin anabolic effects on bone density have been extensively investigated and can be summarized in Figure 1. Briefly, melatonin can act on osteoblasts, favoring proliferation and differentiation, and simultaneously manifest inhibitory effects on osteoclast differentiation and bone-resorbing activity [65,69]. Melatonin binding to its G-protein coupled receptor (melatonin receptor 2 or MT2R), located on the cell membrane of hMSCs and pre-osteoblasts, induces a signaling cascade that leads to the activation, by phosphorylation, of MEK and ERK1/2, favoring cell proliferation and the upregulation of factors involved in cell differentiation via Wnt/β-catenin signaling pathway [65,66]. These factors, such as bone morphogenetic proteins 2 and 4 (BMPs), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN), display anabolic action as they are positively involved in controlling the bone formation and osteoblasts and osteocyte differentiation from precursor cell lines [65,66,69].
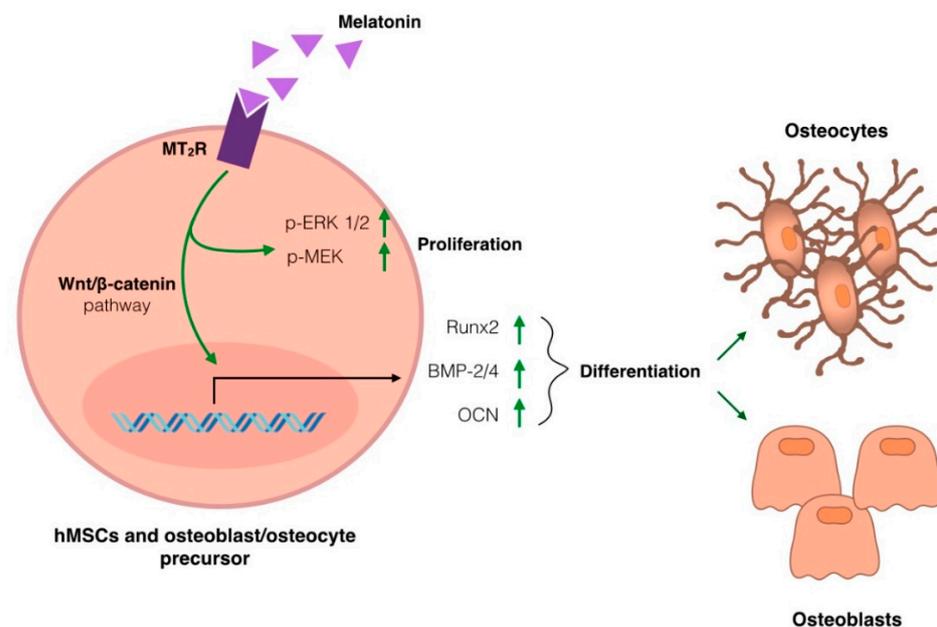

Figure 1. Molecular mechanism underlying melatonin's anabolic effect on bone density. Melatonin binding to its G-protein coupled receptor (MT$_2$R), located on the cell membrane of hMSCs and pre-osteoblasts, induces a signaling cascade that leads to the phosphorylation of MEK and ERK1/2 and, favoring (green arrows) cell proliferation and the upregulation of factors involved in cell differentiation via Wnt/β-catenin signaling pathway. The factors involved in controlling the bone formation and osteoblasts and osteocyte differentiation from precursor cell lines are bone morphogenetic proteins 2 and 4 (BMPs), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN).

Recent literature also unraveled the mechanism underlying melatonin's antiresorptive effects (Figure 2). In bone tissue, differentiation and activation of osteoclasts are generally influenced by interactions with osteoblastic lineage cells. Osteoclasts express the receptor activator for NFkB (RANK), which is a receptor for RANKL found on osteoblasts and bone marrow cells and is required for osteoclastogenesis and osteoclast activation [71,72]. RANKL signaling is usually inhibited by osteoprotegerin (OPG), a decoy receptor produced by stromal cells, as negative feedback to control osteoclastogenesis. Therefore, the ratio between RANKL:OPG expression is critical in this process [71,72]. Melatonin intracellular signaling leads to the modulation of genes that influence osteoclast differentiation and activity; specifically, a downregulation of RANKL and upregulation of OPG have been observed in cell culture studies after stimulation with melatonin, hence shifting the RANKL:OPG ratio towards an anti-osteoclastogenic activity [69]. This finding is also supported by clinical trials showing that in women treated with melatonin supplements, ratios of type-I collagen crosslinked N-telopeptide NTX (a bone resorption marker) to OCN trended downward compared to placebo. This is an important finding because as women transition through menopause, the NTX:OCN ratio increases such that osteoclast activity outpaces osteoblast activity leading to bone loss [73]. Recent studies on fishbone scales and chick calvariae models demonstrated that melatonin is also able to upregulate, in osteoblasts and their precursors, the transcription and production of another hormone, calcitonin, involved in bone remodeling [74,75]. However, more in-depth studies must be performed to verify this pattern also in human bone cells.
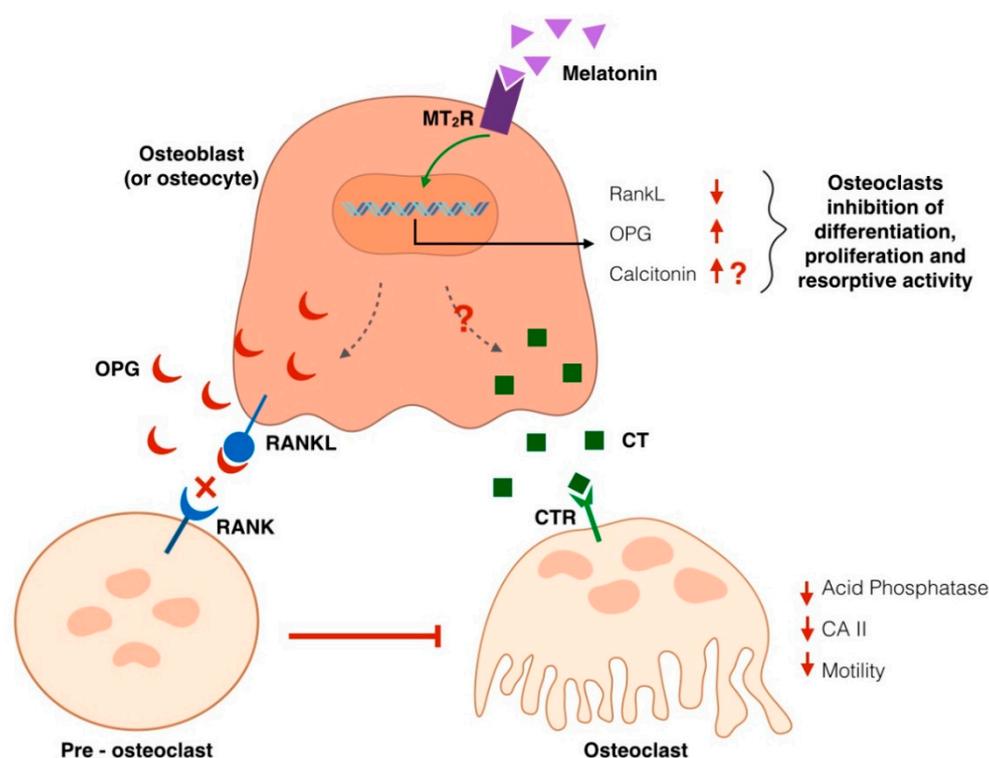

Figure 2. Molecular mechanism proposed for melatonin's antiresorptive effect on bone density. Melatonin binding to its G-protein coupled receptor (MT2R), located on the cell membrane of osteoblasts/osteocytes and mesenchymal cells, induces a signaling cascade culminating in the modulation of genes that influence osteoclast differentiation and activity; specifically, a downregulation of RANKL and upregulation of osteoprotegerin (OPG), a decoy receptor produced by stromal cells as negative feedback to control osteoclastogenesis. Melatonin has also been proposed to produce an upregulation of calcitonin, whose binding to its receptor (calcitonin binding receptor or CTR) on osteoclasts induces a rapid cell contraction, causes inhibition of osteoclast motility. Calcitonin also inhibits (red arrows) other pathways associated with osteoclast activity, such as the release of acid phosphatase and the expression of carbonic anhydrase II (CA II), a cytosolic enzyme involved in the maintenance of an acidic environment, necessary for osteoclast resorption.

Calcitonin is a 32-amino acid linear polypeptide usually produced in humans primarily by the parafollicular cells of the thyroid gland. It acts as an osteoclast-inhibiting hormone, and today, salmon calcitonin is a currently FDA-approved medication as a second-line drug for postmenopausal osteoporosis and as a first-line treatment for hypercalcemia and Paget's illness [76,77]. Focusing on calcitonin antiresorptive activity, the binding of this hormone to its receptor (calcitonin binding receptor or CTR) induces a rapid cell contraction and, therefore, causes inhibition of osteoclast motility which negatively affects cell capacity to resorb bone surfaces. However, this effect is temporary, and osteoclasts have been shown to gradually escape this inhibition after several hours [78–80]. Besides its action on cell motility, calcitonin also inhibits other pathways associated with osteoclast activity, such as the release of acid phosphatase and the expression of carbonic anhydrase II, a cytosolic enzyme involved in the maintenance of an acidic environment, necessary for osteoclast resorption [79–81]. Calcitonin also has been demonstrated to interfere with osteoclast differentiation from precursor cells and the fusion of mononucleated precursors to form multinucleated osteoclasts in bone marrow cultures [79,80].

Considering evidence indicating melatonin as a new therapeutic approach for the treatment of osteoporosis on Earth, through its dual anabolic and antiresorptive action, its use in space medicine could be advantageous for the treatment not only of osteopenia related to microgravity but also for the reestablishment of all those alterations linked to circadian rhythms. It is known. Indeed, changes in lighting and work schedules during spaceflight missions can impact circadian clocks and disrupt sleep, especially in the early stages of adaptation to living conditions on the ISS, hence compromising the mood, cognition and performance of orbiting astronauts [82,83]. To date, using melatonin in space has been mainly considered as a non-pharmacological remedy for the treatment of circadian misalignment and sleep deficiency. However, its clinical use for the treatment of microgravity-induced osteoporosis was first hypothesized by Ikegame and colleagues in 2019, thanks to their studies on interactions between osteoclasts and stromal cells in real microgravity conditions, using a new experimental bone model based on goldfish scales flown on the ISS [74]. Their interesting studies provide evidence that melatonin suppresses osteoclast bone-resorbing activity in bone tissues under microgravity conditions via the upregulation of calcitonin and the downregulation of RANKLin osteoblasts. Taking together all the aspects described in the previous paragraph, a more in-depth study of the dual, anabolic and antiresorptive effect of melatonin on bone metabolism is, therefore, highly encouraged in both simulated and real microgravity conditions.

4. Fractures and Bone Healing

In the previous paragraphs, the effect that the loss of bone mass can cause on the resistance and strength of the entire skeletal system has been brought to light several times. Indeed, one of the main consequences of severe bone mass loss and demineralization due to unloading conditions is the raised risk of both vertebral and nonvertebral fractures. Astronauts spending six months in spaceflight have on average a 10% loss in BMD, which is 10-fold greater than the BMD loss observed in postmenopausal women on Earth [1,24]. Beyond fractures due to progressive osteopenia induced by microgravity, it must also be considered that traumatic injuries, such as bone fractures and wounds, can naturally occur during routine operational procedures or extravehicular activities (EVA) [84,85]. Despite the evident medical need, however, the systemic response to fracture injury and the mechanism of human bone repair in unloading conditions is poorly investigated.

In normal conditions, tissue regeneration involves a coordinated interaction of cells, proteins, proteases, growth factors, small molecules and extracellular matrix (ECM) components to restore tissue morphology and functioning. Just like skin repair, also the bone healing process can be divided into multiple steps, and a communication network between stromal, endothelial, bone and immune cells is very important in determining the course of healing and recovery of tissue function [86–88]. Briefly, the first response to a bone fracture is constriction of the injured blood vessels and activation of platelets that form a fibrin

clot (or hematoma) to cease blood flow and provide a scaffold for incoming cells by releasing signaling and growth factors, which, in turn, activate the migration of inflammatory cells and repair cells, such as fibroblasts, osteoblasts, stem cells and vascular endothelial cells [88]. The recruited fibroblasts begin to lay down the stroma that helps support vascular ingrowths while the responding macrophages remove tissue debris. Inflammatory cells also release growth factors and cytokines signals to recruit mesenchymal stem cells (MSCs), which proliferate and differentiate into osteoprogenitor cells and then into osteoblasts and osteoclasts to form and remodel newly formed bone tissue [86,87]. Much evidence in the literature highlights the pivotal role of blood vessels in the process of bone repair and osteogenesis, which indicates intimate molecular crosstalk between endothelial cells and osteoblasts [88,89]. Angiogenesis is required at different steps and, among the key roles played in the process, it furnishes oxygen and nutrient supply for the regenerating tissue, while endothelial cells (ECs) secrete osteogenic growth factors [90] to promote osteogenesis and osteoblast differentiation from their precursors. Newly formed blood vessels ensure steady transport of circulating osteoclast and osteoblast precursors to remodeling sites, regulating, therefore, osteoprogenitor cell invasion [90,91].

Many studies in the literature have reported that cell lines responsible for bone repair (MSC, fibroblasts, macrophages, osteoblasts, osteoclasts, and ECs), taken individually, are highly sensitive to microgravity and undergo morphological, functional, and biochemical changes under these conditions [16,27,92,93]. Besides osteoblast, osteoclast, and MSC alterations discussed previously, simulated microgravity studies have shown that ECs undergo important cytoskeletal remodeling and show impairment in proliferation and survival. However, there are still controversial data about ECs capacity to migrate and organize in 3D structures, and therefore, to sustain full angiogenic responses in unloading conditions [94], especially in the processes of wound or fracture healing.

Given the above, it could be deduced that if individual cell types manifest alterations in their morphology and function, the entire regeneration process may somehow be compromised by the lack of canonical gravitational forces. To date, very limited data in this regard is available. However, all studies performed on murine or rat models, both in real and simulated microgravity conditions, provide strong evidence and convey the same conclusion that the fracture healing is compromised in microgravity conditions and manifests in both histological and morphometric alterations and differences between animals healed in space or on Earth [95–97]. While these data demonstrate that microgravity has a deleterious effect on bone healing, the direct translation of these results to human bone healing is difficult due to the numerous differences between rodent and human bone microstructure and healing process. Furthermore, the molecular crosstalk among these cell lines in bone regeneration following fracture injuries is not fully known in conditions of real or simulated microgravity, and it is not yet clear how these physiological and key interactions can change. In vitro experiments using simulated μG devices or in vivo studies are, therefore, highly encouraged as elucidating molecular mechanisms at the basis of altered interaction between bone cells, endothelial and immune cells, in microgravity, could lead to the identification and development of specific countermeasures, both pharmacological or otherwise like bandages or specific dressings biomaterials, to improve and accelerate the regeneration process in space [98].

Besides studying the phenomenon from a molecular point of view, it would also be interesting to evaluate whether nonsurgical options currently in use on Earth to promote and facilitate bone regeneration in patients with healing deficiencies could be applied to space medicine for the treatment of mild fractures, which, in long-term spaceflights, could compromise astronaut performance. Among nonsurgical treatments, we find physical stimulation therapies, such as extracorporeal shockwave treatment (ESWT), low-intensity pulsed ultrasound (LIPUS) or low-level laser therapies (LLLT) [99–102]. The advantage of using nonsurgical countermeasures would lie in their non-invasive nature and easy application, which could allow the astronaut, in the event of minor fractures, rapid self-medication.

Advances in regenerative medicine are focused on the employment of mesenchymal stem cells (MSCs) to improve bone regeneration with interesting results due to their self-renewal and differentiation capacity and their ability to secrete bioactive molecules and regulate the behavior of other cells in different host tissues [103]. Instead, other research lines are evaluating pharmacological approaches based on the regulation of molecular mechanisms, such as antagonists of the WNT pathway [104,105], or using bone tissue morphogenic factors [106,107]. However, these lines of research have yet to find a consolidated use for the treatment of bone healing deficiencies on Earth, and their employment in space medicine will be subsequently validated.

5. Conclusions

Bone plays an important role as a structure that supports the body and represents a mineral reservoir for calcium and phosphate. The skeletal apparatus is in continuous re-shaping, and its homeostasis is finely tuned by a balance in bone resorption and formation operated by osteoblasts, osteoclasts and osteocytes [3,4]. In a microgravity environment, because of reduced loading stimuli, this equilibrium is lost, and bone resorption prevails on bone formation, leading to bone mass loss at a rate of about ten times that of Earth osteoporosis [1,24]. Microgravity-induced osteopenia is, therefore, a significant and un-resolved health risk for space travelers, which leads to a raised likelihood for irreversible changes that weaken skeletal integrity and to an increment in the onset of fracture injuries and renal stones formation [84,85].

Today several pharmacological and non-pharmacological countermeasures to this problem have been proposed, including physical exercise, diet supplements and adminis-tration of antiresorptive (Bisphosphonates or denosumab) or anabolic drugs (teriparatide). However, each class of pharmacological agents presents several limitations as prolonged and repeated employment of both antiresorptive and anabolic agents singularly is not exempt from the onset of serious side effects, which limit their use within a well-defined therapeutic window. Antiresorptive and anabolic drug therapeutic schedules, described in previous paragraphs, may be compatible with permanence in space comparable to that of the current missions since astronauts rarely stay on the ISS for more than a year. The problem arises when evaluating the possibility of long-term space flights (>2 years), such as future expeditions to Mars, where extensive and repeated use of antiresorptive agents may produce potentially serious adverse effects, such as osteonecrosis of the jaw and atypical femoral fractures [44,45], and anabolic drugs (teriparatide) could cause a synergic bone catabolic activity alongside to the one induced by microgravity, leading to a dangerous risk for human health [60,61].

For long-term treatment of osteoporosis on Earth, researchers have focused their attention on developing sequential drug therapies with both anabolic and antiresorptive mechanisms of action, which could prove to be very effective and useful in future explo-ration missions. In particular, recent clinical trials [63,64] demonstrated that two years of combined therapy (teriparatide + denosumab) followed by two years of denosumab alone is associated with the largest cumulative BMD increases at the hip and radius. However, the potential use of this pharmacological approach in space flights has yet to be validated through studies in real or simulated microgravity conditions. Furthermore, the 4-year therapeutic regimen applied in clinical studies on Earth to treat menopause osteoporosis is hardly applicable to observations in real space conditions due to the excessively long time astronauts should spend on the ISS. For this reason, the search for both safe and effective drugs for the long-term treatment of microgravity-related osteopenia, given longer space expeditions, remains an open challenge that requires major efforts to be resolved.

In the last decade, several studies have brought to light the role of melatonin in regu-lating and maintaining skeletal apparatus physiology. Today, the molecular mechanism at the basis of melatonin anabolic effects on bone density have been extensively investigated and can be summarized in a dual opposed action of both anabolic and antiresorptive activity: on one side, melatonin favors cell proliferation and osteoblasts and osteocyte

differentiation from precursor cell lines; on the other hand, it hampers osteoclast differentiation [65,69]. Considering evidence indicating melatonin as a new therapeutic approach for the non-pharmacological treatment of osteoporosis on Earth, its use in space medicine, under the form of dietary supplement alongside vitamin D and calcium intake, could be advantageous for the treatment not only of osteopenia related to microgravity but also for the reestablishment of all those alterations linked to circadian rhythms due to changes in lighting and work schedules during spaceflight missions, that could compromise mood, cognition and performance of orbiting astronauts [82,83]. Preliminary data that emerged in the last years, and reported in this review, are definitely encouraging, but stronger clinical data in this regard are still lacking and are highly needed.

Another problem related to bone tissue homeostasis in microgravity is its capacity to regenerate following bone fractures due to both weakening of the tissue, caused by unloading related BMD loss, and accidental events, for instance, during the accomplishment of particularly complex operations. Following the above discussion, it has emerged that today there are still considerable gaps in this regard, and it is not known how human bone tissue responds during wound regeneration both at the molecular/cellular level, analyzing the crosstalk among these cell lines involved the process, and at the anatomical–morphological level. Gathering more detailed information on these processes in microgravity conditions could result advantageous for the identification and development of specific countermeasures, both pharmacological and otherwise. Therefore, we strongly believe that further studies must be performed in this direction, especially in view of long-term space operations, where reentry on Earth for bone trauma handling and care is highly unlikely.

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