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Impact of nitric oxide in liver cancer microenvironment

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ABSTRACT

The pro- or antitumoral properties of nitric oxide (NO) are dependent on local concentration, redox state, cellular status, duration of exposure and compartmentalization of NO generation. The intricate network of the tumor microenvironment (TME) is constituted by tumor cells, stromal and immune cells surrounded by active components of extracellular matrix that influence the biological behavior and, consequently, the treatment and prognosis of cancer. The review describes critical events in the crosstalk of cellular and stromal components in the TME, with special emphasis in the impact of NO generation in the regulation of hepatocellular carcinoma (HCC). The increased expression of nitric oxide synthase (NOS) in tumors and NO-end products in plasma have been associated with poor prognosis of cancer. We have assessed the level of the different isoforms of NOS in tumors and its relation to cell proliferation and death markers, and cell death receptor expression in tumors, and apoptotic markers and ligands of TNF-α receptor family in blood from a cohort of patients with HCC from different etiologies submitted to orthotopic liver transplantation (OLT). The high levels of NOS2 in tumors were associated with low plasma concentration of apoptotic markers (M30 and M65), FasL and TNF-α in HCV patients. By contrast, the low levels of NOS2 in tumors from alcohol-derived patients was associated with increased Trail-R1 expression in tumors, and circulating Trail levels compared to observed in plasma from HCV- and alcohol + HCV-derived patients. This study reinforces the association between increased NOS2 expression and potential risk of low patients' survival in HCC. However, a differential functional relevance of NOS expression in HCC seems to be influenced by etiologies.

1. Cancer cells and stromal cells of the microenvironment

1.1. Cancer cells

Tumor microenvironment (TME) is constituted by tumor, stromal and immune cells surrounded by a complex meshwork of extracellular matrix (ECM) proteins and disrupted vasculature [1]. TME includes proliferative epithelial-like cancer cells, whose main function is tumor proliferation based on the process of transforming nutrients into tumor mass. Despite the relevance of tumor cell proliferation and survival, there are evidence reporting that the tumor is constituted by a heterogeneous population of cells with different levels of malignancy and functions. Among them, cancer stem cells (CSC) are characterized by self-renewing, multi-potent and tumor-initiating properties associated with chemoresistance, immunosuppression, and metastasis [2]. Similar to non-tumor stem cells, CSC can remain dormant or differentiate into several cell phenotypes, as mesenchymal stem cells (MSC) or embryonic ones. It has been proposed a potential influence of MSC on tumor initiation and progression, participating on generation of CSC, epithelial to mesenchymal transition, angiogenesis, drug resistance, and metastasis. Polarized MSC are classified depending on the cytokine profile, including tumor growth factor-β (TGF-β) or SMAD3-4, and

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toll-like receptors (TLR) expression. TLR4 primed-MSC or MSC1 have a proinflammatory phenotype, capable of inhibiting tumor growth and metastasis, while TLR3 primed-MSC, MSC2, have the classic immunosuppressive phenotype, with the role of improving the tumor growth and metastasis formation [3].

MSC can also up-regulate the metabolism of cancer cells through secretion of exosomes [4], as described in prostate cancer [5], or mediated by oxidative stress and the production of reactive oxygen species (ROS), NO and reactive nitrogen species (RNS), how it will be developed later.

1.2. Cancer-associated fibroblast

ECM proteins are secreted mainly by cancer-associated fibroblasts (CAF). These support cells can originate from tumor of host stromal and immune cells, being their main function to provide essential nutrients to the tumor and facilitate the matrix remodeling that can support tumor implantation [6]. Classically, a clear role in favor of oncogenesis has been attributed to CAF, despite in normal stroma there are fibroblasts that secrete ECM that provides a natural barrier against tumor progression [7,8]. Both, CAF and cancer-associated myofibroblasts, produce proteins such as collagen, fibronectin or α-smooth muscle actin, that modify ECM architecture. Consequently, the tumor cells change to invasive and metastatic morphologies [9], reported in breast [10] and pancreatic cancer investigations [11].

However, recent publications show that among CAF, there is a great heterogeneity of subpopulations with different roles in carcinogenesis. “Contractile” and “aggressive” CAF seem to have this well-known prooncogenic action, but others, such as “immune” and “desmoplastic” CAF, seem to develop an inhibitory action, reflecting the host response against the tumor [12].

1.3. Immune system

The immune system includes several populations of cells, including macrophages, lymphocytes, monocytes, and dendritic cells. They are relatively quiescent in normal conditions, but with a fast answer after infection or inflammation. This response typically involves changes in the expression of genes and results in both stimulatory and inhibitory roles on cancer growth, depending on the acquisition of new functions, the production of cytokines, lipid mediators and enzymes and the ability to migrate and undergo cellular division [13].

The presence of T and B-cells may represent a favorable prognostic factor, as it has been reported in cases of melanoma, colorectal, breast, and ovarian cancers [14,15]. Monocytes and macrophages can be recruited into tumor, accelerating tumor progression [16]. Tumor and stromal cells can influence macrophages to shift their phenotypes to classic M1 or alternative M2 macrophages [17]. While M1 macrophage is involved in the inflammatory response, pathogen clearance, and antitumor immunity, the M2 macrophage participates in an anti-inflammatory response, wound healing, and other pro-oncogenic actions. The tumor-associated macrophages (TAM) closely resemble the M2-polarized macrophages and participate in tumor development and progression regulating angiogenesis, invasion, metastasis, immunosuppression, and chemotherapeutic resistance [18]. MSC can promote the recruitment of TAM in tumor site producing chemokines as CCL2 [16] that results in tumor promotion and progression.

2. Crosstalk in the tumor microenvironment

The multidirectional interplay among epithelial cancer and stromal cells and immune compartment is fundamental for carcinogenesis, progression, and metastasis, and may lead to a different biological behavior of tumor cells during the cancer progression [19]. This complex microenvironment, called tumor niche, plays a key role in tumor progression and metastasis and is shown in Fig. 1.

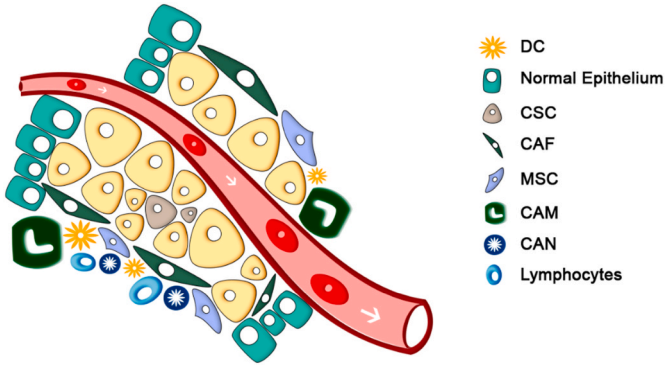

Fig. 1. DC, CSC, CAF, MSC, CAM and CAN constitute the tumor niche.

Cancer and stromal cells in TME interact each other influencing tumor development and metastasis [20]. MSC can migrate to inflammatory or damaged organs, or even incorporate into tumor tissues, interacting cells through paracrine signaling, driven by chemotactic gradients of cytokines/chemokines released from the own damaged tissues [21]. MSC have the capacity to differentiate to various cell types such as osteocytes, chondrocytes and adipocytes, providing structural support and secrete factors for tissue homeostasis [22]. However, crosstalk between tumor cells and MSC can also increase metastatic potential and promote epithelial-to-mesenchymal transition (EMT) in TME [22].

Metastasis and resistance to treatment depends on innate chemoresistance of cancer cells and, also, on the immune polarization, both related to CSC behavior and MSC modulation. The interrelationships between tumor and stromal cells in TME and their role in the oncogenesis of certain tumor subtypes have already been described. In a model of inflammation-induced gastric cancer, MSC was the responsible for CAF recruitment, mediated by TGF-β and stromal cell-derived factor-1 (SDF-1a) [23]. In an osteosarcoma model, cancer cells inhibited the osteogenic differentiation of MSC through TGF-β/Smad2/3 signaling and increased expression of β-catenin [24]. At the same time, an increased production of vascular endothelial growth factor (VEGF) and other pro-tumor cytokines as interleukin-6 (IL-6) was reported. Finally, in breast cancer, tumor cells were crucial in the attraction of marrow derived MSC, while breast cancer cells preferentially metastasize to the bone marrow, being key in both processes the presence of SDF-1a [25]. The same axis is probably important to guide the interaction between tumor and adipose-derived stem cells [26].

As previously exposed, cancer cells, through this crosstalk with TME non-neoplastic cells, induce the conversion of resident cells or chemoattracted cells into CAF, TAM, and cancer-associated neutrophils (CAN) [18]. CSC, probably stimulated by TME metabolic hypoxia and acidification, induces the expression and secretion of matrix metalloproteinases (MMP) from both cancer and stromal cells, and MMP, at the same time, promote EMT, apoptosis resistance, angiogenesis, lymphangiogenesis and tumor cell migration through the basement membrane and ECM [27]. MMP can also present protective effects, particularly at the disease’s early stages, such MMP-3, MMP-8, MMP-12, MMP-13, MMP-19 and MMP-26 [28], However, the expression of MMP is mostly related to tumor aggressiveness and metastatic potential, with differences in expression and functions among cancer types. For example, MMP-11 has been identified as pro-oncogenic in the early tumor stages, due to its anti-apoptotic properties and its effects on adipocytes. At the same time, it also has metastatic protective capacities during late stages [29,30]. These particularities could be a starting point for further research, so these enzymes could be used as biomarkers leading to an early disease diagnosis and recent studies report MMPs as therapeutic targets, with the focus of inhibiting or stimulating them [31,32].

Cellular components and ECM interact each other in the TME in a

dynamic fashion that results in changes of tissue remodeling, tumor metabolism, recruitment of additional stromal and immune cells. These dynamic characteristics impact on tumor progression, but also give yield potential therapeutic opportunities based on the disruption of TME component crosstalk.

3. TME in HCC

TME involves particularities in the specific setting of HCC widely studied in the context of liver cirrhosis. Accordingly, stromal and immune cells play a relevant role in the development of liver fibrosis that is the adequate environment to promote EMT and the rest of carcinogenic features in HCC [33]. Derangement of liver sinusoid during chronic liver diseases leading vascular obstruction and increased intrahepatic vascular resistance, contribute to development of portal hypertension and cirrhosis [34]. CAF, hepatic stellate cells (HSC), immune cells, and endothelial cells participate in the generation of ECM within TME, including proteins, proteolytic enzymes, growth factors and cytokines. On the other hand, non-cellular components modulate signaling pathways in tumor cells and stimulate invasion and metastasis. The crosstalk between these HCC cell lines is shown in Fig. 2.

3.1. CAF in HCC

Fibroblasts participate in wound healing, ECM generation, tissue maturation, and the inflammatory response [35]. CAF can arise from normal fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, bone marrow-derived progenitor cells, and pre-adipocytes [36]. In cirrhotic livers, there is a large number of activated fibroblasts, and these CAF contribute to tumor progression by producing growth factors such as epithelial growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and TGF-β, chemokines (SDF-1), cytokines (IL-6), and metalloproteinases (MMP-3 and MMP-9) [37]. Crosstalk between CAF and HCC could be mediated by miRNAs contained in exosomes. For example, low miR-150-3p levels secreted by CAF promotes HCC migration, invasiveness and poor clinical outcome [38]. Similarly, CAF-derived exosomal TUG1 has been related to migration, invasion, and glycolysis via the miR-524-5p/SIX1 axis [39], while the upregulation of mirR-335-5p by CAF has shown inhibition of HCC cells proliferation [40].

3.2. CAM in HCC

CAM can display the M1 (classic) or M2 (alternative) phenotype. In HCC, M2 can promote progression and metastasis through glypican-3, which is highly expressed in more than 70% of the cases [41]. CAM also lead to HCC progression by the secretion of TGF-β1 [42], IL-6 via STAT3 [43], MMP-9 [44], as well as angiogenesis [45]. CAM, in the early stage of carcinogenesis, display a suppressing phenotype, what has been shown in Hepa1-6 HCC. However, at advanced phases, CAM suffer an immune polarization that has been associated with tumor progression [46]. HCC cells also affect TME, for example releasing *Wnt* ligands that promote M2 polarization and tumor growth, invasion, and immunosuppression [47]. This polarization can be a sensible target to be treated in patients. In fact, Sorafenib has induced the repolarization of alternative macrophages to M1 phenotype through insulin-like growth factor-1 (IGF-1) signaling [48]. Finally, CAM infiltration was linked with programmed death-ligand 1 (PD-L1) overexpression in human HCC [49]. Although M1 have been considered to exert an anti-tumor role, they might promote PD-L1 expression in HCC cells, through the IL-1β pathway [50].

3.3. Mesenchymal stem cells in HCC

MSC are multipotent cells present in the TME, that not only can be recruited into the TME to become the HCC cell source but launch the inhibition or promotion effects on HCC progression through multiple molecular signaling pathways. MSC can lead to immune suppression by secreting varieties of cytokines, such as IL-10, TGF-β, nitric acid, indoleamine 2,3 dioxygenase and prostaglandin E2 (PGE2) [51]. IFN-γ and TNF-α inhibit the differentiation of dendritic cells (DC) and promote the polarization of M2 macrophages and tumor growth [52]. MSC interact with Natural Killer (NK) cells, changing their phenotype and inhibiting proliferation and cytokine secretion, and with T and B cells, inhibiting their proliferation and increasing their apoptosis [53,54]. MSCs can lead to CAF with increased cyclooxygenase-2 (COX-2) and PGE2 expression, what facilitates HCC progression in hypoxic TME [55,56]. MSC-derived CAF help the EMT process through integrin-related signaling pathways,

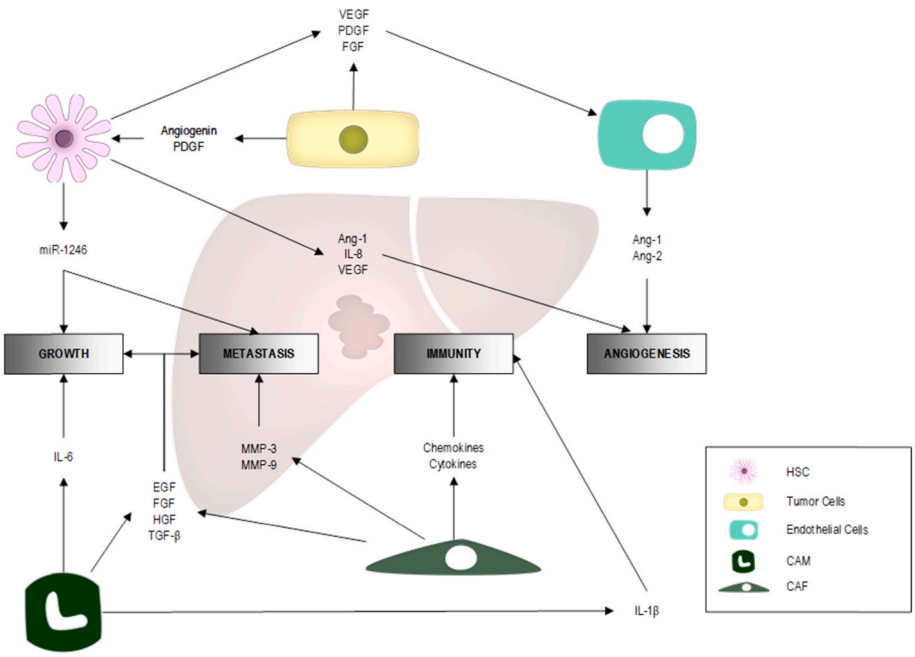

Fig. 2. Crosstalk between cellular components of TME in HCC.

and the high expression of osteopontin in the TME can mediate the MSC-CAF transition through the downstream of the MZF1-TGF-β1 pathway [57].

HCC cells located around the blood sinusoid and undergoing EMT commonly express the CAF biomarkers alpha-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP), indicating that both, TGF-β and hypoxia-inducible factor (HIF-1α), enriched under hypoxic condition, can induce CAF features in HCC cells [58]. However, the activation of CAF can be reversible as occurs during chronic hypoxia that may deactivate CAF, reduce contractile force and stiffness in the surrounding ECM, and alleviates cell invasion [59]. The reversible characteristics of CAF may provide therapeutic targets to be modulated in the HCC treatment.

3.4. Endothelial cells in HCC

Endothelial cells (EC) participate in controlling the size and elasticity of liver vessels, being important in tumor proliferation, stability and neoangiogenesis [60]. The process begins with structural abnormalities of tumor blood vessels, increasing permeability with the help of EC that carry angiogenic receptors such as VEGFR, EGFR, platelet-derived growth factor receptor (PDGFR), and α-chemokine receptors (CXCR) [61]. HIF led to the activation of VEGF, which also promotes angiogenesis [62]. Several studies have shown that VEGF and its receptor (VEGFR) are crucial for HCC development [63], and high VEGF blood levels have been found related to bad prognosis in HCC patients after surgical resection [64]. Angiopoietin-1 (Ang-1) and 2 (Ang-2) bind to their receptor, Tie2, to stimulate angiogenesis (Fig. 2) [65]. Ang-1 and Ang-2 expression was detected in hepatoma, HSC, EC, and smooth muscle cells, while Tie2 receptor was only identified in EC, HSC, smooth muscle cells, and monocytes [66]. Ang-2 serum levels have been found to be elevated in patients with liver cirrhosis and HCC [67], with a synergistic effect with VEGF in the development of angiogenesis in HCC in mice through the activation of MMP-2 and MMP-9 [68], and related to HCC invasion and metastasis [69].

3.5. HSC in HCC

HSC synthesize and release vitamin A, MMP, collagen, cytokines as IL-6 and IL-1β, defensin-1, chemokines as CCL5, CCL2 and growth factors (TGF-α/β, EGF, PDGF, bFGF) [70]. HSC are in a quiescent state until liver injury, increasing the expression of α-SMA, allowing to be in a transdifferentiated myofibroblast-like state, and with increased contractility that causes infiltration of the hepatic stroma and HSCs location around fibrous septa, sinusoids and capsules [71]. Several *in vitro* studies have shown tumorigenic effect of HSCs related to inflammation, chemotaxis, angiogenesis and metalloproteinase [72,73]. HSCs have increased tumor growth via nuclear factor-kappa β (NF-κB) and extracellular signal regulated kinase (ERK) pathways activation in xenograft mice model [74]. Angiogenin was shown to be *in vitro* and *in vivo* responsible for the crosstalk between HCC and HSC [75].

HSC also promotes angiogenesis through the secretion of Ang-1 and IL-8 [76], VEGF [77], HCC progression by IL-6 [78] and miR-1246 with the involvement of the Wnt/β-catenin pathway [79]. In addition, HSC can be related to Sorafenib resistance of HCC because of the laminin-332/α3 integrin axis and the ubiquitination of focal adhesion kinase [80]. FGF9 overexpression is associated with poor prognosis in HCC [81]. Moreover, tumor cells can induce the conversion of HSC into CAF through the secretion of miR-21, conducing to cancer progression via the secretion of the angiogenic factors VEGF, MMP2, MMP9, bFGF and TGF-β [82]. HSC have also been found to delay HCC progression by endosialin secretion in mouse models of HCC [83].

3.6. Immune cells in HCC

HCC arises almost exclusively in the setting of chronic necroinflammation, compensatory liver regeneration, induction of liver fibrosis and subsequent cirrhosis. The liver is a central immunomodulator that ensures protection, while deregulation of this controlled liver immunological network is a hallmark of chronic liver disease and HCC [84]. HCC that has high immune cell infiltration, activation of programmed death protein 1 (PD-1)/PD-L1, and activation of interferon-γ (IFN-γ) signaling pathway and granzyme B and perforin 1 expression could be grouped into an 'immune class' and constitute 30% of tumors. Two different subclasses can be found, an adaptive T cell response can identify the 'active immune' subtype, whereas the 'exhausted subclass' exhibits TGFβ-mediated immunosuppression and T cell exhaustion [85]. Innate immune mechanisms may support or neutralize tumor-related immune activation, being recognized drivers of disease progression in the liver, particularly during conditions such as fibrosis or cirrhosis. Tumor-infiltrating immune cells in the HCC play an important role in its clinical evolution. For example, myeloid cells as CAM and myeloid-derived suppressor cells (MDSC), abundantly present in the HCC TME, have been related to poor prognosis, promoting tumor initiation, development, angiogenesis, metastasis, and even therapeutic resistance [86]. In contrast, increasing numbers of infiltrating T-effector cells are habitually associated with a good prognosis [87]. The presence of infiltrating NK cells, high density of CD163+ macrophages and low density of $CD8^+$ T cells has been considered as a pro-inflammatory TME and associated with good clinical outcomes in HCC [88,89]. In this way, NK cells play a central role in hepatic immunity, accounting for 25–50% of the total number of liver lymphocytes. Both, circulating and tumor infiltrating NK cells, are correlated with better survival rates in HCC [90], contrary to other immune cells, such as MDSCs and regulatory T cells, which seem to disrupt the immune control of the HCC.

B cells can play a contradictory role in HCC development. They have humoral immune functions through antibodies, acting as antigen-presenting cells (APC) and helping T cells and enhancing the cellular immunity and an anti-tumor function. In addition, B cells can also secrete cytokines to act on other immune cells, modulating their function, as the case of DC cells, NK cells or neutrophils. The inflammation is a major condition for achieving differentiation within B regulatory cells [91]. Kupffer cells, resident macrophages in the liver, have been reported to mediate tumor growth in HCC by producing PD-L1 that interacts with PD-1 receptor in $CD8^+$ T cells, impairing $CD8^+$ T cell response [92]. In addition, Kupffer cells produce osteopontin, which is involved in inflammation, tumor progression, and metastasis [93]. MSC-derived extracellular vesicles can reduce the activation and proliferation of proinflammatory cells, such as Th1, Th17 and M1 macrophages, reducing the secretion of proinflammatory cytokines, while promoting the proliferation of anti-inflammatory cells, such as M2 macrophages and Tregs, and increasing the secretion of anti-inflammatory cytokines [94]. Therefore, this immune environment that forms part of the TME has a relevant influence on the prognosis of patients with HCC. The possibility of early identification and classification of patients based on immunological biomarkers opens the door to new research. Likewise, the possibility of modulating the clinical course of the disease by acting on the different cell populations of the TME would be interesting, which could have repercussions on the prognosis of these patients.

In this line, studies assessing the use of anti-PD-1/PDL-1 antibodies, which act as angiogenesis inhibitors, such as nivolumab and pembrolizumab, sequentially after the tyrosine kinase inhibitor Sorafenib has shown an increased median overall survival of about 22 months [95]. Similar to this, anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibodies could be used in patients with Sorafenib resistance, showing better clinical outcomes. The addition of the anti-angiogenic agent bevacizumab could also prolong overall survival of patients with advanced HCC after Sorafenib, but this should be studied more exhaustively [96].

3.7. Heterogeneity of HCC microenvironment

A greater heterogeneity of proteins and cells has been observed in patients with HCC compared to patients with healthy livers, related to different signaling pathways of this type of tumor [97]. Chemokines, a sub-family of cytokines, are one of the key molecular determinants of tumor heterogeneity in HCC and are involved in cell survival, growth, migration, and angiogenesis [98]. CAF also participate on this heterogeneity through COL1A1-ITGA2 and the YAP signaling pathway, which has been associated with morphological heterogeneity and decreased overall survival in HCC patients [99].

Additionally, recently published works have shown modulation of tumor heterogeneity by the local and systemic microbiota, which, in turn, can adapt to environmental changes. It has been shown that the intestinal microbiota and its metabolites can modify the expression of genes at the hepatic level, which could result in liver diseases, including HCC [100]. Cellular heterogeneity is a predominant feature in HCC, resulting from the TME and its inflammatory network, and plays a central role, limiting the availability of predictive biomarkers for diagnosis and clinical follow-up after the actual chemo and immunotherapy regimens, what complicates the criteria used to choose the most suitable therapeutic option.

4. Impact of dose- and cell compartment-dependent NO generation in the regulation of cancer

The role of NO in carcinogenesis is complex due its dual role, with pro- or anti-tumoral properties in a dose-, time- and compartment-dependent manner. NO is an ubiquitous, short-lived physiological messenger that plays important roles during tumor induction, growth progression and metastasis [101]. NO is synthesized by the enzymes NOS through a series of redox reactions involving L-arginine. The NOS expression has been detected in several cancers such as breast, bladder, stomach, prostate, lung, colon, pancreas and renal cancers [102–109]. NOS1 and NOS3 are constitutively expressed, and produce small quantity of NO, while NOS2 generates NO for extended periods of time, at high concentrations, being key as regulator and effector during inflammation and infection [110].

NO, as a radical species, primarily restricts its direct reactivity to metal complexes in iron-containing proteins and ROS [111]. In addition to oncogenic signaling mechanisms, NOS2-derived NO can also affect and tune anabolic and catabolic metabolism, including sugar, fatty acid, and amino acid metabolism that become functional mediators in TME. NO/RNS generation impacts the intracellular and intercellular signaling in TME where it might play pro- and antitumor mechanisms. There is an important variability in the rate of NO production and NO-susceptibility from different cell types [112]. Antitumor activity mediated by NO levels can be divided into three main categories: cGMP signaling (<100 nM NO), pro-oncogenic nitrosative signaling (100–500 nM NO), and nitrosative stress signaling (500–2000 nM NO) [113–115] (Fig. 3). Concentrations below 100 nM generally regulate cyclic-GMP activity and vascular tone via NO interaction with the heme protein soluble guanylyl cyclase (sGC) [116]. NO fluxes of 100–500 nM result from the chemical reactivity of nitrogen oxides derived from the reaction of NO with dioxygen (O_2), also a radical species [114], leading to the increase of numerous pro-tumorigenic signaling pathways, including activation of RAS/EGFR, TGF-β and Src signaling, as well as stabilization of HIF1 and Nuclear factor erythroid 2- related factor 2 (Nrf2) [117,118]. However, above 500 nM of NO, extensive nitrosative stress involve accumulation of DNA mutation or alter DNA repairing system associated with p53-dependent cell cycle arrest in tumors in which the presence of mutated p53 leads to poor prognosis in these patients [113,119].

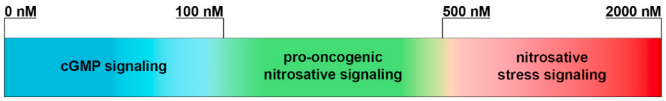

Fig. 3. Dose-dependent impacts of NO.

The complex network of the microenvironment constituents may also be differentially regulated by NO [120,121]. NO altered tumor cell metabolism and promotes chemotherapeutic resistance [122]. Tumor tissue can express different levels of NOS according to the disease stage. The tumor growth environment also contributes to establishment of NO steady state levels and activates important cell signaling pathways [123]. NO promotes angiogenesis, downregulates immune surveillance and encourages metastasis with gross pathological specimens demonstrating markedly elevated levels of NO or NOS expression in a wide variety of malignant tumors [124]. NO can also dysregulate different components of the epigenetic regulation machinery such as DNA methylation, histone post-translational modifications or non-coding RNAs involved in mRNA translation altering critical hallmarks of cancer [125]. Specifically, lncRNAs HEIH and UCA1 inhibit their target miRNAs and reverse the inhibition of NOS and promote tumor proliferation. miRNAs and NO jointly participate in the maintenance and renewal of CSC, M1 to M2 CAM transition, and CAF activation. NO exerts protumoral role on the immune polarization of CAM to a M1 (antitumoral profile) phenotype with the upregulation of NOS2 in melanoma [126]. NOS-derived NO also regulates CAM phenotype through metabolic reprogramming and cytokine release [127].

There is extensive literature reporting the influence of NO in the carcinogenesis participating in cancer induction and progression. NOS1 has been localized in the mitochondria attenuating ROS generation and apoptosis resistance in colorectal cancer [128]. The posttranslational modification by S-nitrosylation of phosphatase and tensin homolog (PTEN) increased AKT and the mammalian target of rapamycin (mTOR) inhibiting proapoptotic autophagy in HCC [129] or nasopharyngeal carcinoma [130]. Furthermore, NOS1 can be upregulated by CAF involving synergistic function of the Nrf2 and HIF1 in melanoma [131].

Increased NOS2 expression has been linked to poor patient outcomes for multiple types of malignancies and thus has become an important target for future therapeutic delivery [132]. NOS2 expression was associated with worse prognosis and low survival rates in patients with pancreatic ductal adenocarcinoma that correlates with reduced cell proliferation, migration and invasion rates in tumors developed in experimental models using NOS2 KO mice [116]. Sadozai et al. [133] highlighted the therapeutic benefits receiving agonistic T cell antibodies (e.g., OX40 or CD137 agonists) in T cell inflamed TME, as well as from macrophage targeted drugs (e.g., anti-CSF1R) and anti-CAF agents in TME including activated CAF and high infiltration of immunosuppressive CAM in pancreatic ductal adenocarcinoma. Poor clinical outcome related to high levels of NOS2 has also been shown in patients with metastatic breast cancer [134]. NO seems to contribute to chemoresistance through endoplasmic reticulum stress-related pathways in cultured triple-negative breast cancer cells, and in xenograft models in which NOS2 inhibition by L-NG-monomethyl Arginine acetate (L-NMMA) enhanced docetaxel-induced apoptosis [135]. Low production of NO by NOS2 also participates in tumor progression [136,137] in patients with metastatic HCC [138]. In fact, an association has also been found between the expression of NOS2 and HCC, specifically in relation to its aggressiveness, relapse and chemoresistance. NOS2 has been reported to activate Notch1 signaling in CSCs with CD24 and CD133 phenotype, thus allowing their self-renewal and growth [139].

NOS3 overexpression altered intracellular redox status involving thioredoxin (Trx) and glutaredoxin (Grx) disturbances in liver cancer cells [140]. In addition, the antitumoral properties of Sorafenib, tyrosine kinase inhibitor as recommended treatment in advanced stages of HCC, reduces Trx1 expression and S-nitrosylation of cell death receptors [141].

5. Expression of NO and disease progression in HCC

The concentration of NO-derived products in plasma from patients with HCC is increased [129]. However, the correlation of NOS expression in tumors, and relevant features of carcinogenesis has not assessed yet. One study investigated NOS2 expression in cancer tissue and pair-matched non-tumor liver tissue and found lower NOS2 expression in HCC [138]. NOS2 was positively associated with tumor proliferation, micro-vascularization and negatively with apoptosis, and significantly related to poor prognosis in HCC [142]. NO modulates different cancer hallmarks including apoptosis, cell cycle progression, invasion and metastasis [143]. As commented earlier, the pleiotropic effects of NO in biological systems are due to its reactivity with different cellular molecules, such as molecular oxygen, superoxide radical anion, DNA, lipids, proteins and transition metals, but also thought post-translational modifications as S-nitrosylation, nitration and phosphorylation on different proteins such as bile acid transports [144] and cell death receptors [145]. S-nitrosylation is a dynamic bidirectional process, and the relative balance of nitrosylated and denitrosylated proteins can serve as a biofeedback mechanism for physiological functions [146,147]. Deficiency in the denitrosylating enzyme S-nitrosoglutathione Reductase (GSNOR) has been associated with the development of cancer [148]. In particular, GSNOR deficiency causes S-nitrosylation and depletion of the DNA repair protein O6-alkylguanine-DNAalkyltransferase and increases rates of both spontaneous and diethylnitrosamine-induced HCC [149].

The exacerbation of NO production exerts antitumoral effects in liver cancer cells. NO has a significant effect on apoptosis in HCC cells by positive regulation of p38 and JNK pathway, but negative regulation of ERK signaling pathway through phosphorylation of substrates in p38, c-jun N-terminal kinase (JNK) and ERK [150]. The overexpression of NOS2 and NOS3 induces a switch between apoptosis and autophagy by disrupting the Beclin 1/Vps34 association and by increasing the Bcl-2/Beclin 1 interaction in liver cancer cells [129]. NO donor administration or NOS3 overexpression increased cell death receptors expression and reduced tumor cell growth after implantation of liver tumor cells in an *in vivo* model [151]. The infusion of NOS3-overexpressing vector in xenograft model of orthotopic liver cancer reduced tumor growth associated with the induction of nitrosative stress, DNA damage, p53 and expression of cell death [152].

HCC is closely associated with chronic inflammation, accumulation of genetic changes, alterations of the liver microenvironment, and generation of liver CSC [153]. The expression of stem cell markers CD24 and CD133 in the tumor of HCC patients was linked to higher NOS2 expression and poor prognosis of disease [139]. The expression of NOS2 in $CD24^{+}CD133^{+}$ liver CSC promoted NO/cGMP/PKG driving Notch signaling and stemness characteristics *in vitro* and *in vivo*, and accelerates HCC initiation and tumor formation in the murine HCC tumor model [139].

HCC frequently develops in patients with HBV or HCV. HBx gene has been implicated in HBV-linked HCC development through NOS2 induction [154]. NOS induction by HBx is related to NF-*k*B signaling [155]. NOS2 also upregulated HBx expression and activated HBx-mediated JNK signaling, and consequently promotes HCC development. On the other hand, HCV core-induced NOS2 and NO generation are implicated in the loss of DNA damage repair by the inhibition of DNA glycosylate activity [156] which impairment is likely to be mediated by posttranslational modification, as nitration or S-nitrosylation [157].

We have enrolled a cohort of 30 patients (2014–2017) (27 males and 2 females) with confirmed HCC by imaging associated with hepatitis C virus (HCV) infection (n = 12), alcohol abuse (n = 10) and alcohol + HCV (n = 7) etiologies that undergo OLT in the Hospital University "Virgen del Rocio" (Seville, Spain). The patients give the written consent for surgical intervention and for the use of remaining blood samples and peritumoral and tumoral sections from explants for measuring the parameters included in the present study. The privacy rights of human subjects were observed under written consent. The expression of NOS1, NOS2, NOS3, apoptosis (M30), cell proliferation (Ki67), tumor necrosis factor-receptor type 1 (TNF-R1), CD95 and TNF-related apoptosis-inducing ligand-receptor type 1 (TRAIL-R1) in tumor vs peritumor sections was assessed by immunohistochemistry (Table 1). The circulating levels of M30, M65, FasL (Fas ligand), TNF-α and TRAIL in plasma were assessed by enzyme-linked immunoassay-based assays (Table 2). The expression of tumor vs peritumor tissue markers showed that the expression of NOS2 was higher in HCV-derived tumors than in alcohol-derived tumors (Table 1). Interestingly, tumors from alcohol-related patients showed higher expression of Trail-R1 than in alcohol + HCV-derived patients (Table 1). In concordance, the concentration of apoptotic M30 marker and Trail in plasma was increased in alcohol-derived patients than that observed in HCV and alcohol + HCV-related patients (Table 2). Plasma levels of M65 and TNF-α were increased in alcohol + HCV that observed in HCV group (Table 2). Taking into consideration the low size of our cohort we could not exclude that the increased expression of NOS2 in tumors from HCV-derived patients impacted tissue markers related to cell death and proliferation assessed in the present study. However, the high levels of NOS2 in tumors were associated with low plasma concentration of apoptotic markers (M30 and M65), FasL and TNF-α in HCV patients. By contrast, the low levels of NOS2 in tumors from alcohol-derived patients was associated with increased Trail-R1 expression in tumors, and circulating Trail levels compared to observed in plasma from HCV- and alcohol + HCV-derived patients. Then, although the low number of patients does not allow any definitive conclusion, we might suggest that high expression of NOS2 appears to be associated to low apoptotic signaling in patients with HCC that might negatively impact the survival of patients submitted to OLT. In a previous study carried out in an extended cohort of patients submitted to OLT by HCC we showed that the expression of TNF-R1 and TRAIL-R1 was significantly reduced in differentiated HCC from HBV-infected patients, and reduced overall cell death receptor expression in tumors was related to tumor recurrence and low survival [158]. The extensive inflammatory response in patients with HCV-derived HCC might impact NOS2 and NOS3 upregulation [159,160].

Table 1
Differential expression of NOS enzymes, apoptosis- and cell proliferation markers, and cell death receptors in tumor vs peritumor sections from patients with HCC submitted to OLT. The expression of the different markers was assessed in tumor and peritumor sections by immunohistochemistry. Primary antibodies against NOS1 (sc-8309, Santa Cruz Biotechnology, Texas, USA), NOS2 (sc-7271), NOS3 (sc-654), M30 (M30 CytoDeath, Diapharma Group, Inc., Pennsylvania, USA), Ki67 (IR626, DAKO, Denmark), CD95 (sc-715), TNF-R1 (sc-7895) and TRAIL-R1 (sc-6823) were used. The corresponding commercial secondary antibodies, as well unspecific staining labelling retrieved using non-immunized fractions were selected accordingly to the primary antibodies used. Data represent differential values of fluorescence between tumor vs peritumor sections obtained in patients with HCC. All results are expressed as mean ± SEM. Dara were compared using analysis of variance (ANOVA) with the Least Significant Difference's test as post-hoc multiple comparison analysis (homogeneity of variances). The statistically significant differences ($p \leq 0.05$ level of significance) among etiologies in each studied variable were indicated using superscript ([a]when compared with HCV group; [b]when compared with alcohol; [c]when compared with Alcohol + HCV).

	HCV	ALCOHOL	ALCOHOL + HCV
NOS1	1.015 ± 0.1310	0.851 ± 0.1385	0.996 ± 0.1824
NOS2	1.346 ± 0.1845[b]	0.907 ± 0.1156[a]	1.321 ± 0.3697
NOS3	1.348 ± 0.3249	1.201 ± 0.2058	1.055 ± 0.1730
M30	0.929 ± 0.0914	1.153 ± 0.1778	0.984 ± 0.0750
Ki67	1.295 ± 0.1457	1.219 ± 0.1622	1.076 ± 0.1971
CD95	1.543 ± 0.2752	1.506 ± 0.3229	1.026 ± 0.2613
TNF-R1	1.102 ± 0.1398	1.106 ± 0.1112	1.324 ± 0.1830
TRAIL-R1	1.230 ± 0.1038	1.343 ± 0.0967[c]	1.015 ± 0.1015[b]

Table 2
Expression of M30, M65, FasL, TNF-α and TRAIL in plasma from patients with HCC at baseline. M30 Apoptosense® CK18 Kit and M65® ELISA CK18 Kit (Ref P10011 and P10020, Diapharma Group, Inc., Pennsylvania, USA), and circulating cytokines FasL, TNF-α and TRAIL (DFL00B, DTA00D and DTRL00, respectively, R&D System, Minneapolis, USA) were assessed by enzyme-linked immunoassay-based procedures. All results are expressed as mean ± SEM of different patients. Dara were compared using analysis of variance (ANOVA) with the Least Significant Difference's test as post-hoc multiple comparison analysis (homogeneity of variances). The statistically significant differences ($p \leq 0.05$ level of significance) among etiologies in each studied variable were indicated using superscript ([a]when compared with HCV group; [b]when compared with alcohol; [c]when compared with Alcohol + HCV).

	HCV	ALCOHOL	ALCOHOL + HCV
M30 (IU/L)	105.0 ± 33.84[c]	159.3 ± 36.33[c]	278.4 ± 76.92[a,b]
M65 (IU/L)	276.1 ± 62.54[c]	393.1 ± 99.91	411.6 ± 33.60[a]
FasL (pg/mL)	40.7 ± 9.03	44.5 ± 8.81	66.1 ± 16.03
TNF-α (pg/mL)	26.5 ± 5.69[c]	34.0 ± 7.83	40.5 ± 6.22[a]
TRAIL (pg/mL)	355.1 ± 42.80[b]	617.8 ± 113.65[a,c]	354.2 ± 110.70[b]

6. Clinical trials based on the impact of NO in the microenvironment

Different genetic studies have highlighted the role of NO polymorphism in cancer. NOS3 polymorphisms have been associated with susceptibility to colorectal cancer and treatment response. In this sense, the −786T > C and 894G > T polymorphisms are associated with the development of colorectal cancer in the Korean population [161]. In this particular setting, NOS3 polymorphisms appear to identify a subset of patients more responsiveness to bevacizumab-based chemotherapy in metastatic colorectal cancer [162]. In breast cancer, there were significant interactions between disease-free survival, adjuvant therapy, and NOS3 Glu298Asp and −786 polymorphisms. In particular, NOS3 genotypes leading to low NO generation have negative impacts in the progression of the disease when receiving chemotherapy, whereas there was reduced risk for those patients who did not receive adjuvant therapy [163].

The systemic administration of NO donor sodium nitrite and hypoxic cell radiosensitizer have not been associated with greater benefit in relation to radiological response in patients with brain metastases concurrent with radiotherapy [164]. According to the security profile of NO modulators, Arrieta et al. [165] developed a phase II clinical trial studying the effect of the addition of nitroglycerin, as a NO donor agent, combined with chemotherapy and concurrent chemoradiotherapy in patients with locally advanced non-microcytic lung cancer, getting an acceptable toxicity profile. Other studies showed, through phase I clinical trials, that the administration of nitroglycerin-type NO donors in transdermal patches, associated with chemotherapy, is safe in both lung cancer and rectal cancer patients, without significant adverse events [166]. However, the regulation of NOS2 signaling with or without chemotherapy appears to be more complicated than initially expected. In this sense, the inhibition of NOS2 by ASP9853 in combination with docetaxel was not tolerable and resulted in the possible potentiation of its neutropenia [167].

NO is also implicated in the adverse events of chemotherapeutic agents. Thus, in the case of anthracyclines and their widely known cardiotoxicity, a protective effect of the NOS3 TT894 genotype on ejection fraction was seen in high-risk patients, especially in those who did not receive dexrazoxane. This founding is remarkable, so that let stratify the probability of anthracycline associated with cardiotoxicity in patients with acute lymphoblastic leukemia using NO as biomarker [168].

7. Concluding remarks

NO plays a critical role in the crosstalk between tumor cells, MSC, CAM and CAF within TME which influence their biological behavior and, consequently, the progression of the malignancy and treatment response. In this sense, the identification of NO-related biomarkers might be useful to define signaling involved in HCC, as well as to identify tumor subsets associated with worse prognosis and treatment resistance. Our clinical data showed herein might suggest that NOS2 expression in tumor is related to altered apoptotic signaling that impact the survival of patients with HCC. Although NO donor, in monotherapy or in combined treatment, have been shown to be safe in some clinical trials, there are also evidence of their toxicity in certain circumstances. All these investigations are expected to generate relevant knowledge useful for the management of patients with HCC.

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.

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List of abbreviations

Alpha-smooth muscle actin α-SMA
Angiopoietin Ang
Antigen-presenting cells APC
Cancer-associated fibroblasts CAF
Cancer-associated neutrophils CAN
Cancer Stem Cells CSC
α-Chemokine receptors CXCR
Cyclooxygenase-2 COX-2
Cytotoxic T-Lymphocyte antigen 4 CTLA-4
Dendritic cells DC
Endothelial cells EC
Epithelial growth factor EGF
Epithelial-to-mesenchymal transition EMT
Extracellular matrix ECM
Extracellular signal regulated kinase ERK
Fas ligand, FasL
Fibroblast activation protein FAP
Fibroblast growth factor FGF
Glutaredoxin Grx
Hepatic stellate cells HSC
Hepatitis B virus HVB
Hepatitis C virus HCV
Hepatocarcinoma HCC
Hepatocyte growth factor HGF
Hypoxia-inducible factor HIF-1α
Insulin-like growth factor-1 IGF-1
Interferon-γ, IFN-γ
Interleukin IL
c-Jun N-terminal kinase JNK
L-NG-monomethyl Arginine acetate L-NMMA
Mammalian target of rapamycin mTOR
Matrix metalloproteinases MMP
Mesenchymal Stem Cells MSC
Myeloid-derived suppressor cells MDSC
Natural killer NK

Nitric oxide, NO
Nitric oxide synthase NOS
S-nitrosoglutathione reductase GSNOR
Nuclear factor erythroid 2- related factor 2 Nrf2
Nuclear factor-kappa β NF-κB
Orthotopic liver transplantation OLT
Phosphatase and tensin homolog PTEN
Platelet-derived growth factor receptor PDGFR
Programmed death-ligand 1 PD-L1
Programmed death protein 1 PD-1
Prostaglandin E2 PGE2
Reactive nitrogen species RNS
Reactive oxygen species ROS
Soluble guanylyl cyclase sGC
Stromal cell-derived factor-1 SDF-1a
Thioredoxin Trx
TNF-related apoptosis-inducing ligand-receptor type 1 TRAIL-R1
Toll like receptors TLR
Tumor-associated macrophages TAM
Tumor growth factor-β, TGF-β
Tumor microenvironment TME
Tumor necrosis factor-receptor type 1 TNF-R1
Vascular endothelial growth factor VEGF

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