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The POLYAMINE PATHWAY as a POTENTIAL TARGET for VASCULAR DISEASES: FOCUS on RESTENOSIS

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Abstract

Polyamines are organic polycations expressed by all living organisms, which are known to play an essential role in cell proliferation and differentiation. Recent studies revealed their involvement also in cell contractility and migration and in programmed cell death. These processes are known to contribute to restenosis, a pathophysiological process occurring in 10-20% of patients submitted to revascularization procedures. The advent of bare metal stents and of drug-eluting stents has significantly reduced but not eliminated the incidence of restenosis, which thus remains a clinically relevant problem.

Despite the potential role of the polyamine pathway as a therapeutic target due to its involvement in proliferation, apoptosis and migration of vascular cells, experimental inhibition of polyamine synthesis and/or uptake has been poorly investigated in animal models of vascular disease.

Here we review the current knowledge about molecular mechanisms related to polyamine functions, with particular reference to the role played by polyamines in vascular cell pathophysiology, together with experimental evidence obtained so far in animal models of (re)stenosis. We also evaluate the advantages of different routes of administration of polyamine synthesis/transport inhibitors and polyamine analogue molecules.

Increasing knowledge about the molecular mechanisms and functions of polyamines is expected to shed new light on their potential role as a therapeutic target for restenosis reduction.

Keywords: ornithine decarboxylase, arginase, DFMO, restenosis, neointima, negative remodelling.

Running Title: Polyamines and restenosis

Polyamine synthesis and functions:

The polyamines spermidine and spermine, and their precursor putrescine, are positively charged amines. They are essential for cell proliferation and play also a significant role in other processes, including cell migration and apoptosis, ion channel function and regulation of gene expression through a direct and/or indirect interaction with the nucleic acids DNA and RNA. Their major role is supported by the observation that these aliphatic polycationic molecules are produced through an ancient pathway by all living cells, with the exception of two orders of *Archaebacteria* [1]. Polyamines are produced by an intracellular highly regulated pathway or can be imported by the cell. The synthesis of polyamines starts by the conversion of ornithine into putrescine catalyzed by ornithine decaboxylase (ODC), the first rate-limiting enzyme in the biosynthetic pathway. Putrescine is then converted to the polyamines spermidine and spermine by spermidine and spermine synthases (Fig. 1) through the addition of one and two aminopropyl groups, respectively, with decarboxylated S-adenosylmethionine as aminopropyl donor [2]. Decarboxylated S-adenosylmethionine is synthetized from S-adenosylmethionine by S-adenosylmethionine decarboxylase (SAMDC or AdoMetDC), another rate-limiting enzyme in polyamine biosynthesis. The key role of ODC and SAMDC is demonstrated by the fact that their knockout in mice is lethal [3, 4]. The intracellular concentrations of polyamines are regulated also by polyamine degradation via the catabolic enzymes spermine oxidase (SMO), spermidine/spermine N^1-acetyltransferase (SSAT) and N^1-acetylpolyamine oxidase (APAO). Intracellular levels of polyamines are strictly regulated through a balance between their biosynthesis and the catabolic pathways, and alterations of this balance may contribute to the etiology of several pathological conditions, including cancer [5], due both to direct effects of polyamines on cell proliferation and to indirect effects of polyamine catabolites such as H_2O_2 and aldehydes formed by the catabolic oxidases SMO and APAO.

ODC activity is regulated by a family of antizymes, in which the most highly characterized member is called antizyme-1 (OAZ1), exerting an ODC inhibitory activity induced by polyamines [6]. The synthesis of ODC antizyme is stimulated by polyamines through a frameshifting mechanism, possibly through a direct interaction of polyamines with antizyme mRNA or via alternative mechanisms, including an interaction of polyamines with ODC antizyme at the translational level. The story has recently become even more complex with the finding that antizyme itself is regulated by an ODC-related protein termed antizyme inhibitor, lacking enzymatic activity [7].

The amount of polyamines in cells depends also on the activity of arginase I and II catalyzing the conversion of the cationic amino acid substrate L-arginine to L-ornithine (Fig. 1). L-arginine is the substrate for nitric oxide synthase (NOS) as well, and thus arginase I and II compete with NOS for the same substrate. L-ornithine is subsequently metabolized to putrescine by ODC, and to L-proline by the enzyme ornithine aminotransferase (OAT), respectively [8]. The presence of arginase I and II has been revealed in blood vessels from numerous animal species, as well as humans [9], and in particular, SMCs express exclusively arginase I, while ECs predominantly express arginase II [10, 11]. Due to its competition with NOS, arginase plays an important role in the regulation of vessel tone and of vascular homeostasis.

The major sources of exogenous polyamines are diet and intestinal luminal bacteria. Both endocytic and solute carrier-dependent mechanisms have been described for intestinal polyamine uptake. Polyamines are also exported from cells, mainly as N^1-acetyl derivates (N^1-acetylspermidine and N^1-acetylspermine), the intermediate products of polyamine catabolism [12]. These energy-dependent and selective polyamine transport systems have not yet been defined at molecular level in mammals, although different models have been proposed [13, 14].

A number of studies have highlighted the multiplicity of mechanisms and levels of action of polyamines in influencing cell functions in healthy and pathological settings. For example, polyamines can modulate the functions of DNA,

nucleotide triphosphates and proteins. In particular, polyamines stabilize double-stranded DNA by increasing the melting temperature and promote DNA bending, thus influencing the recognition of gene regulatory proteins by their response elements, and hence contribute to the regulation of gene transcription [15]. Polyamines can also modulate the functions of RNA because most polyamines exist in a polyamine-RNA complex in cells [2]. As a consequence, in addition to regulating gene transcription, polyamines also modulate gene expression post-transcriptionally. Post-transcriptional gene regulation, which includes processes such as mRNA transport, turnover, and translation, involves specific mRNA sequences (cis-element) that interact with transacting factors such as RNA-binding proteins and microRNAs [16]. For example, a recent interesting study revealed that polyamines are able to modulate the mRNA stability of JunD, a growth-inhibitory protein in intestinal epithelial cells, through the RNA-binding proteins HuR and AUF1, thus providing new insight into the molecular functions of cellular polyamines [17]. Moreover, polyamines are able to modulate ligand-receptor interactions and functions, including estrogen/ER [18], the nuclear receptors/vitamin D receptor-interacting proteins [19] and the glutamate/N-methyl-D-aspartate receptors [20].

Interestingly, polyamines seem to play a role also in epithelial-to-mesenchymal transition (EMT), a phenomenon linked to cell plasticity that has a pivotal function not only during embryogenesis, but also in adult tissue remodelling, wound healing, organ fibrosis and cancer progression [21]. Polyamine depletion alone through α-difluoromethylornithine (DFMO) enhances the EMT of Madin-Darby canine kidney (MDCK) cells, accompanied by the increased expression of genes coding for metalloproteinases, fibronectin and alpha-smooth muscle actin. This effect is potentiated by the simultaneous stimulation of cells with TGF-beta1 and involves the endoplasmatic reticulum-stress proteins.

In attempts to better understand the role of polyamines and of the enzymes involved in their metabolism, a number of transgenic or knock-out animal models have been produced. An extensive review of transgenic animals currently available for the analysis of polyamine metabolism has been published recently [22]. Among them, transgenic rats and mice overexpressing human ODC have been produced and used by the Alhonen group to study the role of ODC expression on the development of induced brain infarct. These transgenic rats had a 7-fold higher accumulation of putrescine in the brain in comparison to syngenic rats, but the levels of the higher polyamines spermidine and spermine remained constant and there were no significant effects on the development of the ischemic stroke lesion, showing that a several-fold increase in putrescine alone has no impact on this process [23].

Another transgenic rat line, overexpressing the mouse spermidine/spermine N^1-acetyltransferase (SSAT) gene under the control of the inducible mouse metallothionein I promoter, has been used to elucidate the role of putrescin and spermidine in the pancreas [24]. As a summary, in the large majority of cases, transgenic and KO animal models for the polyamine pathway unfortunately did not provide the expected results, probably due to compensatory mechanisms leading to homeostasis of cellular polyamine concentrations.

Arterial restenosis: molecular and cellular bases and current incidence:

Atherosclerotic stenosis and its ischaemic complications in the heart and brain require therapeutic strategies to restore blood flow. The treatment of atherosclerotic lesions can follow different revascularization strategies, including percutaneous transluminal angioplasty (PTA), stent deployment, cryoplasty [25], rotational or directional atherectomy, and interposition of venous, arterial or synthetic grafts. The interventional approach depends on the localization, number and length of vascular lesions, as well as on patient characteristics and concurrent risk factors.

Restenosis is arbitrarily defined as a greater than 50% narrowing of vessel diameter compared with a reference artery [26]. It can be considered as an excessive wound healing reaction following revascularization that leads to a new narrowing of the vascular lumen. Restenosis still represents the main limiting factor of the long-term success of

revascularization procedures. Restenosis rates are higher in patients presenting risk factors such as hypertension, diabetes [27] and hypercholesterolemia. The restenotic disease is supported by the contribution of different phenomena, whose prevalence depends on the kind of revascularization procedure applied. The different factors contributing to vascular restenosis include neointimal hyperplasia, constrictive remodelling and neoadventitia formation.

Neointimal hyperplasia after vascular injury is mediated by a cascade of inflammatory mediators and mitogenic and chemotactic factors and is supported by the contribution of heterogeneous cell types, including SMCs, fibroblasts, circulating progenitor cells, adventitial myofibroblasts and inflammatory cells. A detailed study revealed the existence of different subtypes of SMCs contributing to restenosis, and in particular of spindle-shaped SMCs in the tunica media, and of epitheloid SMCs in the neointima [28]. Neointimal formation is triggered by endothelial damage or denudation during angioplasty and leads to SMC switch from a contractile to a synthetic phenotype, followed by their proliferation and migration toward the source of stimulation.

Remodelling involves a spatial reorganization of elements of the vascular wall that can be compensative, leading to vessel lumen enlargement, or constrictive (also defined as negative), leading to vessel lumen narrowing. The constrictive process usually follows neointimal formation and can be initiated by the release of proteolytic enzymes during the inflammatory reaction to injury, leading to initial extracellular matrix degradation, and subsequently to new matrix deposition. Constrictive remodelling is also accompanied by the re-expression or up-regulation of contractile proteins by vascular cells, including those in the neointima layer [29].

Finally, the formation of neoadventitia has been highlighted as a cause of restenosis in distinct animal models of disease, including pig coronary angioplasty, demonstrating that it precedes late loss of lumen, constrictive remodeling and the formation of neointima [30].

In 1987, the PTA technique was significantly developed with the introduction of bare metal stents (BMS), with the aim of obtaining a long-term patency of the stenotic vascular lumen. After an initial optimism for the short-term results of clinical studies, long-term follow-up revealed a relevant percentage of patients incurring in-stent-restenosis, mainly related to an inflammatory reaction and neointima formation inside the stent. Moreover, constrictive remodelling can still occur also in the presence of a stent, in regions distal or proximal to a stented segment. Nonetheless, improvements in strut configuration, thickness, and materials have enhanced the capacity of delivery and reduced vessel damage. Subsequent development of drug eluting stents (DES), in which anti-proliferative drugs (e.g. paclitaxel, sirolimus, and the more recent zotarolimus and everolimus) [31] have been incorporated in the stent and gradually released, showed another long-term side-effect with respect to BMS, consisting in a delayed re-endothelialization after angioplasty, which leads to an excessive cell proliferation in the vascular wall and to thrombosis, thus requiring a more effective anti-platelet therapy [32].

Recent epidemiological studies report that the percentage of target vessel revascularization after coronary DES or BMS deployment is 5.9% and 11.8%, respectively [33].

The bioabsorbable stent concept has recently emerged in response to the complications with metal stents, but to date no investigational device has successfully overcome issues such as relatively low radial force and variable degradation rates [34].

Recent statistics released from the American Heart Association report that up to 20% of ischemic strokes results from atherosclerotic stenosis of the internal carotid artery. A meta-analysis of randomized controlled trials conducted to update the available evidence on the safety and efficacy of carotid endarterectomy (CEA) vs. carotid artery stenting (CAS) in the treatment of carotid artery stenosis supports the continued use of CEA as the standard care of carotid

artery stenosis, while CAS may represent a viable alternative in patients with high risk of cardiac complications [35]. Rates of restenosis of the revascularized carotid artery did not differ significantly between CEA and CAS interventions.
Despite the number of therapeutic strategies currently available to inhibit or reduce restenosis, the complex multifactorial nature of restenosis frequently leads to a failure of the attempts for its prevention in patients. Innovative strategies, e.g. based on local gene delivery, targeted inhibition of gene expression, or stem cell-mediated repair of injured vessels [36, 37] have been successfully tested in animal models of vascular stenosis. Additional studies will be necessary to verify the effectiveness of these strategies also in patients.

The potential role of polyamines in restenosis:

In vitro studies have revealed that polyamines are involved in a number of key phenomena occurring in restenosis progression, including SMC proliferation, migration, inflammation and apoptosis. On this basis, it can be argued that polyamine synthesis and uptake are potential targets for reduction of vascular restenosis.

Polyamines and cell proliferation: The role of polyamines in SMC proliferation has been dissected mainly through the use of inhibitors of their synthesis or through mutations of key enzymes. Thyberg et al. [38] demonstrated that the ODC inhibitor DFMO inhibits SMC proliferation and ODC expression and activity induced by platelet-derived growth factor (PDGF). Odenlund et al. [39] verified the mechanisms of action of DFMO and of N^G-hydroxy-L-arginine (NOHA), an inhibitor of arginase (Fig. 1), on proliferation in cultured SMCs and in endothelium-denuded rat arterial rings. Their data demonstrated that DFMO, but not NOHA, was effective in reducing the number of cells in culture without cytotoxic effects. This result was associated with decreased intracellular concentrations of putrescine and spermidine, but not spermine, thus suggesting that putrescine and spermidine are important for the regulation of cell cycle progression and proliferation of SMCs. The molecular mechanisms behind DFMO-mediated reduction of SMC proliferation was found to involve decreased expression of cyclin A, playing a role in the regulation of DNA replication in the S phase of the cell cycle. Polyamine synthesis in vascular SMCs is stimulated not only by PDGF, as described above, but also by thrombin, a serine protease known to be implicated not only in hemostasis and thrombosis, but also in the pathogenesis of restenosis and atherosclerosis [40]. This protease is able to induce SMC gene expression of ODC and cationic amino acid transporters (CAT) 1 and 2, responsible for cellular uptake of L-ornithine. Thrombin treatment thus increases both polyamine levels and cell proliferation in SMCs, which are both inhibited by DFMO, by CAT inhibitors and by the thrombin inhibitor hirudin [41].

Treatment with DFMO and NOHA, blocking polyamine biosynthesis, increases the amount of L-arginine available for NO formation and furthermore NO itself inhibits ODC [42]. Thus, treatment with polyamine synthesis blockers, such as DFMO and NOHA, inhibits polyamine formation and cell proliferation both via a direct mechanism and indirectly via elevated NO formation inhibiting ODC. Polyamine depletion (e.g. through ODC inhibition by nitric oxide or DFMO) has a cytostatic effect on vascular SMCs, which is mediated by the activation of the MAPK kinase (MEK)1/2 leading to the phosphorylation of their substrate p42/p44 MAPK, which in turn up-regulates $p21^{waf1/cip1}$, a cyclin-dependent kinase inhibitor known to regulate cell cycle progression [43].

Other studies highlighted in patients an association between OAZ1 polymorphisms and increased risk for in-stent restenosis [44]. Considering that OAZ1 negatively regulates the intracellular pool of polyamines by inhibiting their production and uptake, an alteration of OAZ1 expression or activity may have relevant consequences on cell proliferation.

Polyamines and cell migration: With respect to the link between polyamines and cell migration, it was been demonstrated that administration of the ODC inhibitor DFMO in combination with the AdoMetDC inhibitor CGP48664

to PDGF-AB- or foetal calf serum (FCS)-stimulated SMCs reduced their migration, but only if growth stimulators were administered at submaximal concentrations [45]. Cell migration is strictly related to contractility. Polyamines are known to inhibit the activities of voltage-dependent Ca^{2+} channels and phosphatases in intestinal and vascular SMCs, with implications for cellular growth and contractility [46, 47]. While both extracellular and intracellular polyamines reduce Ca^{2+} channel activity, polyamine uptake by SMCs induces increased Ca^{2+}-activated force through an inhibition of the myosin 20-kDa light chain phosphatase. Other studies showed that polyamines are able to inhibit agonist-induced cationic currents, contributing to their suppressing effect on the gastrointestinal smooth muscle excitability and contractility [48]. In gastrointestinal epithelial cells, lacking voltage-dependent Ca^{2+} channels, polyamines have been shown to regulate the expression of membrane K^+ channels [49]. Inhibition of polyamine synthesis in these non-excitable cells leads to membrane depolarization, decreased intracellular Ca^{2+} concentration, and inhibited cell migration. Polyamines thus affect intracellular Ca^{2+} and contractile function in multiple ways and are clearly able to stimulate cell migration, although the precise mechanisms may differ according to cell type.

Polyamines and inflammation: Vascular injury triggers acute and, in some cases, chronic inflammation with the release of mitogenic and chemotactic factors that play an important role in restenosis. In this context, some studies have highlighted a correlation between inflammation, polyamine biosynthesis and SMC proliferation. In particular, it has been demonstrated that the cytokines IL-4 and IL-13 induce arginase I expression, enhance polyamine formation and stimulate SMC proliferation [10]. The same group recently demonstrated that these cytokines are able also to up-regulate ODC expression and stimulate ODC activity, with a consequent increased proliferation of rat aortic SMCs [50], mediated by the ERK, PI3K and PKA pathways. The above mentioned arginase pathway is also active in human endothelial cells and is modulated by inflammatory molecules such as tumour necrosis factor-alpha and bacterial endotoxin [51]. On the other hand, polyamines are also endowed with an intrinsic anti-inflammatory activity, being able to down-regulate production of cytokines by macrophages [52], to mediate the effect of anti-inflammatory glucocorticoids and possibly to induce apoptosis of immune cells [53]. Mechanistic studies also indicated that the polyamine spermine is incorporated into macrophages and restrains the innate immune response [54]. Finally, polyamine catabolism through the amine oxidase-mediated degradation of spermine and spermidine may constitute a significant source of acrolein and of ROS, contributing to inflammation and to cell apoptosis. This apparent dual role of polyamines in the inflammatory reaction observed *in vitro* and *in vivo* requires a detailed analysis of the link and of the equilibrium between polyamines and inflammation in restenosis, not investigated so far.

Polyamines and apoptosis: The role of polyamines in cell apoptosis is quite controversial, as different studies have led to opposite conclusions. Some recent studies support a pro-apoptotic effect of polyamines. Among them, a study revealed that cell apoptosis induced by norepinephrine in rat cardiomyocytes is mediated by an early induction of ODC, and that these effects are prevented by DFMO [55]. These authors also revealed that the pro-apoptotic effect of polyamines is mediated by the involvement of AMP-activated protein kinase, AKT and p38 mitogen-activated protein kinases. A similar pro-apoptotic effect of polyamines has been highlighted in intestinal epithelial cells treated with camptothecin, a DNA topoisomerase I inhibitor [56]. The same authors further dissected the anti-apoptotic pathway mediated by DFMO treatment in a subsequent study [57], demonstrating that polyamine depletion through the DFMO-mediated ODC inhibition increased p53 but not its phosphorylation, thus highlighting the protective effect of polyamine depletion against DNA damaging agents. A pro-apoptotic role of polyamines has also been demonstrated in cardiomyoblasts in an *in vitro* model of simulated ischemia able to induce apoptosis [58]. Recent data show activation of JNK, iNOS induction and apoptosis in human cultured SMCs exposed to spermine [59].

In contrast to these studies, others revealed an anti-apoptotic effect of polyamines. One of the first demonstrations of this protective effect was obtained in thymocytes in which apoptosis was induced through Ca^{2+} ionophores and was blocked by spermine [60]. Other evidence of a putative protective role of polyamines in preventing apoptosis is provided by immunohistochemical observations conducted in breast tissue specimens in postmenopausal patients treated with DFMO for a period greater than 14 days, revealing an increased apoptosis in comparison to patients who received <14 days of treatment [61]. The anti-apoptotic protective effect of polyamines has been demonstrated also in a completely different experimental setting, represented by preconditioned rat hearts, in which polyamine synthesis and ODC up-regulation play a fundamental role in cardioprotection, including reduction of apoptosis in ischemic hearts [62].

Finally, it has also been reported that DFMO treatment of cultured rat vascular SMCs does not induce apoptosis in proliferating cells or increase the number of necrotic cells, as assessed by fluorescence microscopy [39], thus supporting the idea that DFMO acts via cytostatic rather then cytotoxic mechanisms. Nonetheless, it should be underlined that vascular cells in arteries submitted to surgical or balloon injury are stimulated by a number of biochemical and biomechanical factors, including pro-apoptotic stimuli, and consequently the results obtained *in vitro* on proliferating SMCs are not indicative of an *in vivo* pathophysiological setting.

As underlined elsewhere [63], DFMO could increase the resistance of tumor cells to apoptosis, thus representing a problem in cancer therapy. But this anti-apoptotic effect could be an advantage in restenosis prevention, as in this pathophysiological process, an excess of apoptosis could further stimulate cell proliferation in the injured vascular wall. Further studies are warranted to verify the effect of DFMO treatment on cell apoptosis in *in vivo* models of restenosis. Our preliminary data obtained in an arteriotomy model of carotid stenosis seem to exclude an effect of ODC inhibition on cell apoptosis (unpublished results).

Discrepancies among the above described data concerning the role of polyamines in apoptosis could be related to the different properties of the various cell lines used, to the environmental signals, to the experimental model and strategy, to the amount and duration of cell exposure to polyamines, and also to the distinct effect played by polyamines and by their upstream biosynthetic enzymes, such as ODC.

Some studies targeted the mediators responsible for the polyamine pro-apoptotic effects, and identified the production of reactive oxygen species (ROS) during polyamine catabolic reactions as one of the causes. For example, it has been found that polyamine-induced apoptotic cell death of microglia is triggered by an oxidative stress with acrolein, which is produced in polyamine degradation during a reaction catalyzed by amine oxidase [64]. Nonetheless, polyamines do not need to be oxidized for the induction of apoptosis, as their accumulation or depletion directly disrupt cellular functions [65].

The polyamine pathway as a potential therapeutic target in restenosis:

Considering the multiplicity of phenomena in which polyamines have been demonstrated to be involved, they are expected to play a key role in restenosis progression, as well as to show potential as therapeutic targets to prevent or reduce this recurrent pathophysiological phenomenon. A systematic screening of the OAZ1 gene, coding for the ODC antizyme-1, in a cohort of 205 patients yielded 21 variants of this gene. Among them, the variant characterized by the +2222A/G allele was associated in three independent studies with an increased risk of 6-month coronary in-stent restenosis, an increase in common carotid intima-media thickness and an increased risk of coronary heart disease, respectively [44]. The associations between genetic polymorphisms of OAZ1 and clinical outcomes involving the proliferation of SMCs support the hypothesis that the polyamine metabolism plays a role in vascular diseases.

Evidence for a role of polyamines in stenosis progression comes also from preclinical models of disease. Microarray-based studies of gene expression in a rat model of carotid arteriotomy revealed that vascular injury induced an acutely increased expression of genes involved in the polyamine metabolic pathway, including ODC, SSAT, AdoMetDC and arginase I [36].

Similar studies conducted in a rat model of carotid balloon angioplasty highlighted a significant increase of ODC at 4 and 7 days after injury [66].

A rapid and transient increase of ODC activity followed by an increase of polyamine content and by increased SMC proliferation has been observed also in rat aortas denuded with a balloon catheter [3].

These findings further support a role for growth- and migration-stimulatory polyamines in stenosis progression and suggest that their depletion could reduce this phenomenon.

There are currently only a few studies concerning the interference with polyamine biosynthesis in animal models of (re)stenosis. Endean ED et al. [67] inhibited the ODC activity in New Zealand rabbits submitted to carotid deendothelization and treated since 3 days before vascular injury with 2% DFMO in drinking water. Histological analysis revealed a significant reduction of intimal hyperplasia in DFMO-treated rabbits at 2 and 4 weeks after injury, while no difference was detected for media surface areas or thickness. The authors claimed that these results could be the result of inhibition of an early burst of ODC activity following vascular injury.

The key role of arginase I in the remodelling response after arterial injury and hence its interest as an attractive therapeutic target has been highlighted in a study conducted in a rat carotid balloon angioplasty experimental model [68]. The local application of arginase inhibitors S-(2-boronoethyl)-L-cysteine (BEC) or N^G-hydroxy-nor-L-arginine (L-OHNA) through pluronic gel F127 on injured carotids revealed for the first time that arginase is essential for vascular SMC proliferation and remodeling but not for apoptosis *in vivo*. Arginase inhibition reduced neointima formation after injury mainly through the increased expression of protein p21, a known mediator of G_1 arrest during cell cycle progression, and the blockade of polyamine biosynthesis. Of interest, arginase inhibitors were able to specifically reduce SMC but not EC growth, thus not repressing endothelial regrowth.

Interestingly, arginase activation and stimulation of cell proliferation and reduction of endothelial functionality have also been highlighted in a model of porcine carotid segments submitted to unidirectional high or oscillatory shear stress. All these phenomena, relevant in atherosclerosis development and in in-stent restenosis or vein bypass graft failure, were prevented by arginase inhibition [69].

The above illustrated indications obtained in animal models of (re)stenosis for a putative role of the polyamine pathway in neointima proliferation and in constrictive remodelling of the vascular wall does not necessarily imply the transferability of these results in patients, as the pathophysiological phenomena in restenosis preclinical models can differ in various animal species, and also depend on the severity of the injury. For example, in the rat carotid and canine models, a macroscopic thrombus is rarely found and the inflammatory reaction to angioplasty is remarkably limited. Conversely, in the rabbit iliac and porcine model, a macroscopic thrombus occurs, representing a scaffold for SMC colonization [70]. Moreover, it should be considered that in the large majority of experiments, arteries in animal models do not have a concomitant atherosclerosis, which most probably has an impact on restenosis progression induced by vascular injury.

It can be concluded that each animal model has advantages and disadvantages and that consequently an ideal animal model does not exist [70]. Nonetheless, preclinical models of vascular (re)stenosis represent an indispensable tool for research and testing of therapeutic protocols. The use of different animal models in a preclinical phase would probably provide more reliable data about the effectiveness of a potential therapeutic protocol before trials in patients.

The inhibitors of polyamine metabolism and the role of their route of administration *in vivo*:

The effectiveness of a therapeutic strategy for restenosis prevention or reduction based on administration of polyamine inhibitors depends on a number of factors, including the half-life of target molecules, the timing of drug administration, their mechanism of action, the route of administration and hence its local concentration and duration at the injury site.

Interference with the polyamine pathway can be obtained with the use of inhibitors of key enzymes (see [12] for an exhaustive review of the known inhibitors of polyamine metabolism). Alternatively, inhibitors of polyamine carriers and transporters can be used to limit their uptake, even if polyamine uptake proteins have not yet been identified in mammals. To overcome this limitation, recent studies identified a single chain variable fragment anti-heparan sulfate antibody as an inhibitor of cellular binding and uptake of polyamines and of polyamine-dependent cell proliferation [71].

Interference with the polyamine pathway can also be mediated by polyamine inhibitory analogues. These molecules basically exploit the self-regulatory nature of polyamine metabolism and are able to act trough different mechanisms [72], including the stimulation of antizyme production [73], finally leading to endogenous polyamine depletion.

DFMO competes with ornithine for binding to the active site of ODC, and is then decarboxylated by ODC to create a highly reactive intermediate that inactivates ODC by forming covalent linkages to identified enzyme amino acids [74]. *In vitro* experiments revealed that ODC inhibition produces a nearly complete depletion of putrescine and spermidine, while its effect on spermine concentrations is variable [72]. Clinical trials revealed that DFMO is well tolerated by patients (the maximum tolerated dose was 3 g per m^2), with only minor toxicities [72]. Previous studies demonstrated that the uptake of DFMO is by diffusion and therefore unpredictable and slow. DFMO is also rapidly excreted from the body [75]. Consequently, high doses of DFMO are necessary to obtain a prolonged and effective inhibition of ODC. Moreover, DFMO should be immediately available at high concentration for a sufficient period of time after vascular injury as its target, the ODC enzyme, has a very short half-life (about 10 min-1 h in mammalian systems) and is readily destroyed by 26S proteasome [72, 76]. On the basis of DFMO and ODC characteristics, locally applied DFMO could potentially prove successful in reducing restenosis induced by vascular injury. This route of administration could allow high concentration of this drug in the acute phase after injury, when vascular cells (including SMCs, fibroblasts, progenitor cells of various origin and myofibroblasts) are induced to proliferate to repair the injury. A limitation of the first wave of cell proliferation could effectively reduce the long-term restenosis, as suggested by results of other experiments in which a similar strategy has been successfully applied with locally-administered antisense oligonucleotides for intravascular gene therapy targeting genes or miRNA involved in cell proliferation, inflammation and apoptosis (e.g. c-myc, miR-21, MCP-1) [37, 77-79]. Ongoing experiments in our model of rat carotid arteriotomy are based on the local application of DFMO at the injury site through pluronic gel F127, an acquous polymer that is liquid at 4°C and that rapidly solidifies when in contact with rat tissues at 37°C (unpublished data). The local administration of DFMO leads to a high concentration of the drug at the injury site and minimizes unwanted collateral effects related to a systemic administration. Pluronic gel F127 has been previously demonstrated not to affect vascular remodelling [80]. This copolymer works as a rate-controlling barrier and serves as a vehicle for the sustained release of drugs. It has been demonstrated that over a period of 3 hours pluronic gel F127 releases 5.5% of the original dose of a drug at a gel concentration of 20-25%, or 3.8% of the original dose at a gel concentration of 30% [81]. Additional studies on pluronic gel characteristics revealed that the drug release from pluronic gel is not influenced by the properties of the drug (e.g. hydrophilic or hydrophobic) and is dependent solely on the gel concentration and on its dissolution rate, rather than on drug diffusion [82].

Also AdoMetDC, the enzyme involved in the second rate-limiting step in polyamine biosynthesis, has a short half-life [83], and consequently its inhibition should require a sustained local concentration of drugs at the vascular injury site. The AdoMetDC competitive agent SAM486A/CGP48664 has been demonstrated to have a low mitochondrial toxicity [84] but some haematological toxicities during clinical trials.

Conclusions:

After an initial enthusiasm, polyamine inhibitors have been largely abandoned as potential cancer chemotherapy agents after disappointing results obtained in clinical trials. Nonetheless, recent data demonstrate that polyamine inhibitors, alone or in combination with other molecules at low doses (e.g. non-steroidal anti-inflammatory drugs), can be effective as preventive agents for different kinds of cancer in preclinical animal models of disease and in clinical trials, including colorectal carcinogenesis [85, 86] and nonmelanoma skin cancer [87]. Interestingly, in a randomized placebo-controlled trial, DFMO induced a decrease of prostate putrescine levels and rate of prostate growth. The potential of this compound for prostate cancer or hyperplasia remains to be further studied [88].

On the basis of the recently obtained results in clinical trials for cancer chemoprevention and of the more clearly defined molecular mechanisms related to polyamine function, we foresee a renaissance in polyamine research also in the field of vascular hyperproliferative diseases. The inhibition of polyamine metabolic enzymes and transporters, in synergy with the use of polyamine analogues could prove effective in the reduction of restenosis induced by surgical injury or by stent deployment during angioplasty, especially if associated with a proper route of administration and a correct concentration, in order to reach a balance between decrease of hyperproliferation stimulated by vascular injury and the absolute requirement for polyamines in cell viability and functions.

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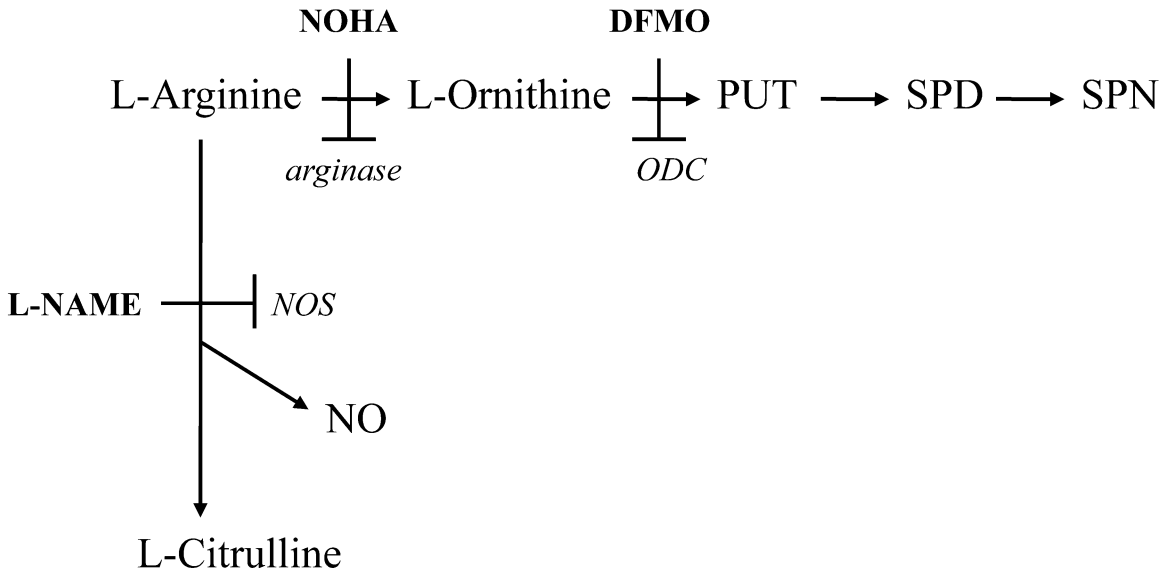
LEGENDS

Figure 1: The amino acid L-arginine serves as substrate for arginase catalyzing the conversion of L-arginine to L-ornithine. L-ornithine is decarboxylated by ornithine decarboxylase (ODC) to putrescine (PUT). Spermidine (SPD) and spermine (SPN) are then formed from putrescine. Arginase and ODC are inhibited by N^{G}-hydroxy-L-arginine (NOHA) and alfa-difluoromethylornithine (DFMO), respectively. Importantly, L-arginine serves as substrate for nitric oxide synthase (NOS) as well catalyzing the formation of NO and L-citrulline.

Figure 1


NOHA
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L-Arginine
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arginase
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