Title Page

Nitric Oxide prevents aortic neointimal hyperplasia by controlling macrophage polarization.

Running Title: Neointimal formation prevention by eNOS

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Abbreviations:
NOS3: Endothelial Nitric Oxide Synthase 3.
NOS2: Inducible Nitric Oxide Synthase 2.
MAEC: Murine Aortic Endothelial Cells.
VSMC: Vascular Smooth muscle Cells.
MMP-13: Matrix Metalloproteinase-13.

Abstract

Aims: NOS3 prevents neointima hyperplasia by still unknown mechanisms. To demonstrate the significance of endothelial Nitric Oxide in the polarization of infiltrated macrophages through the expression of MMP-13 in neoíntima formation.

Methods and Results: After aortic endothelial denudation, NOS3-null mice show elevated neointima formation, detecting increased mobilization of LSK progenitor cells, and high ratios of M1 (proinflammatory) to M2 (resolving) macrophages, accompanied by high expression of, IL5, IL6, MCP1, VEGF, GM-CSF, IL-1 β, and interferon-γ. In conditional c-Myc knockout mice, in which M2 polarization is defective, denuded aortas showed extensive wall thickening as well. Conditioned medium from NOS3-deficient endothelium induced extensive repolarization of M2 macrophages to an M1 phenotype, and vascular smooth muscle cells proliferated and migrated faster in conditioned medium from M1 macrophages. Among the different proteins participating in cell migration, MMP-13 was preferentially expressed by M1 macrophages. M1-mediated VSMC migration was inhibited when macrophages were isolated from MMP-13 deficient mice, whereas exogenous administration of MMP-13 to VSMC, fully restored migration. Excess vessel wall thickening in mice lacking NOS3 was partially reversed by simultaneous deletion of MMP13, indicating that NOS3 prevents neointimal hyperplasia by preventing MMP13 activity. An excess of M1 polarized macrophages that coexpress MMP13 was also detected in human carotid samples from endarterectomized patients. **Conclusions:** These findings indicate that at least M1 macrophage-mediated expression of MMP-13 in NOS3 null mice induces neointima formation following vascular injury, suggesting that MMP-13 may represent a new promising target in vascular disease.

Abbreviations:

NOS3: Endothelial Nitric Oxide Synthase 3.
NOS2: Inducible Nitric Oxide Synthase 2.
MAEC: Murine Aortic Endothelial Cells.
VSMC: Vascular Smooth muscle Cells.
MMP-13: Matrix Metalloproteinase-13.

Vascular stents and percutaneous balloon angioplasty, despite their undoubted benefits, often have adverse effects, including restenosis of the vessel wall [1, 2]. Arterial wall restenosis is caused by mechanical disruption of the vascular endothelium, which, by unknown mechanisms, induces extensive smooth muscle and endothelial cell migration and proliferation, resulting in increased arterial wall thickness and a narrowing of the lumen [3-6].

The increased cell migration, cell proliferation and platelet aggregation that lead to neointima formation are triggered by NO-dependent endothelial dysfunction [7]. The ability of endothelial NOS (eNOS, NOS3) to limit these events was demonstrated by viral and non-viral transfection in rabbits with NOS3, which significantly reduced neointima formation and endothelial regeneration, and by pharmacological inhibition in rats [8-11].

The early response to arterial injury involves the recruitment, infiltration and activation of M1 macrophages, which produce proinflammatory cytokines, chemokines, and basement-membrane-degrading extracellular-matrix metalloproteinases (MMPs), resulting in increased cell migration and proliferation. Polarization to M2 (resolving) macrophages induces the expression of several genes involved in wound healing and re-endothelization. Differentiated macrophages are thought to be able to switch between the M1 and M2 phenotypes, a phenomenon known as macrophage plasticity. This would imply that cells that initially promote an inflammatory response can later have anti-inflammatory properties, and suggests that controlling the signals that trigger macrophage polarization presents potential for the therapeutic regulation of neointima formation in injured vessels [12-15].

Macrophage inducible NOS (iNOS, NOS2), drives the expression of several proinflammatory genes in M1 macrophages [16]. Nitric Oxide induces the expression and activity of collagenase MMP13 [17-2122, 23], which regulates proliferation and migration of several cell types. The effect of NO-mediated matrix metalloproteinase expression on neointima formation is largely unknown. Here we describe the contribution of macrophage polarization through MMP-13-mediated expression on neointimal formation in the absence of NOS3.

Results

Lack of NOS3 promotes neointima formation

In order to explore the role of intimal derived endothelial NOS3 on neointimal formation, we used a non wire-surgical denudation procedure, to prevent destruction of smooth muscle cells from tunica media, and a wire denudation injury for validation purposes, in aortas from WT and NOS3 null mice. Over a 30-day period after endothelial denudation, aortas lacking NOS3 showed significant neointimal formation when compared with WT aortas in both procedures (Fig.1A).

We calculated the intima, intima/media, lumen, total vessel areas and the amount of smooth muscle cells at the time points indicated finding significant differences between wild type and NOS3 null aortas at 15 and 30 days after denudation, revealing an impaired remodeling of NOS3 knockout mice. Endothelial regeneration was also defective in these mice (Fig.1A right panels). The protective effect of NOS3 was further verified in this model by intraperitoneal injection of the NO donor sodium nitroprusside (SNP) into NOS3 null mice, showing significant regression of the neointimal layer (Fig. 1B), while in WT mice injection of the NOS inhibitor L-NAME increased intimal-media thickness, mimicking the effect of NOS3 gene deletion (Fig. 1C). SNP and L-NAME had no effect in endothelial denuded WT and NOS3 null mice respectively (Figs 1B, and 1C).

Transplantation of NOS3 bone marrow into irradiated WT animals had no effect on neointima formation (Supplemental Fig. 1B), whereas transplantation of WT bone marrow into NOS3 deficient mice significantly reduced wall thickness, compared with NOS3 null mice transplanted with NOS3 null bone marrow, and with non irradiated/bone marrow transplanted NOS3 null mice (Supplemental Fig. 1C), indicating that bone marrow from NOS3 expressing mice may participate in the prevention of neointimal formation in response to endothelial injury.

To explain why bone marrow from WT mice had such impact on NOS3 denuded aortas, we analyzed bone marrow LSK (multipotent hematopoietic progenitor cells) and myeloid progenitor cells from WT and NOS3 null animals, finding no differences in cell content between strains in basal conditions (Supplemental Fig. 1D). However, in response to injury, at 7 days after endothelial denudation, NOS3 knockout mice contain twice LSK progenitor cells respect to their WT counterparts in circulating blood (Supplemental Fig. 1E, left), indicating that in the absence of NOS3 more progenitor cells are mobilized, and thereby NOS3 may prevent neointimal formation by controlling the migration of bone marrow derived cells in response to endothelial denudation (Figs 1 and Supplemental 1C). Nevertheless, no differences were detected in myeloid progenitor cell mobilization between mice (Supplemental Fig. 1E, right), suggesting a key role of lymphoid cell mobilization in NOS3 null mice.

Lack of NOS3 induces accumulation of M1 pro inflammatory macrophages after denudation.

Aortic denudation provoked an inflammatory response characterized by leukocyte infiltration into the vessels. No differences in CD68+ cell content was observed in both strains, although in the absence of NOS3 there is an imbalance M1>M2 of pro inflammatory macrophages per lesional area (CD68+/iNOS+), whereas in WT aortas extensive M2 infiltrated macrophages at 14 days after injury (CD68+/MRC1+) were detected, thus contributing to the shortening of inflammation (Fig. 2A, and Supplemental Figure 2A).

This was also evidenced in mice conditionally deficient for macrophage specific c-Myc expression (Fig. 2B), which blocks their polarization to the M2 phenotype [24], and in

which extensive aortic wall thickening and M1 macrophage accumulation, was also detected when compared to NOS3 WT mice (Fig. 2C, and Supplemental Figure 2B).

Lack of NOS3 increases the levels of cytokines and chemokines that promote polarization and accumulation of proinflammatory M1 macrophages

The data shown below explains why NOS3 prevents abnormal remodeling of the vessel wall, by dampening accumulation of proinflammatory vs resolving macrophages at the injured area. To investigate the role of NOS3-deficiency in the accumulation pro-inflammatory macrophages, we first measured 20 cytokines in plasma collected from NOS3-WT and -null mice after aortic denudation at the times indicated (Fig. 3A). Increased levels of IL5, IL6 and MCP1 were detected in the first 24 h after denudation. By contrast, the levels of monocyte chemotractant MCSF and CCL5 proteins remained unchanged (Supplemental Figure 3). To address whether vascular endothelial cells are responsible of such profile, we analyzed cell culture supernatants collected from wounded NOS3-WT and -null MAEC monolayers. In these conditions, we also observed significant levels of proinflammatory cytokines and chemokines including, IL6, GM-CSF, VEGF, IFNγ and IL-1β in NOS3 null MAEC (Fig. 3B).

To asses whether NOS3 deficiency may also induce M2 repolarization to an M1 phenotype, we cultured WT M1- and M2-polarized bone marrow derived macrophages in conditioned medium from endothelial cells isolated from WT or NOS3-null mouse aortas. Conditioned medium from NOS3-null MAEC induced WT M2 polarized macrophages to express high levels of iNOS (Fig. 3C, upper panel), a classical marker of M1 proinflammatory activated macrophages (Fig. 3C bottom), together with marked levels of proinflammatory cytokine IL-6, when compared to the levels found in M2 macrophages incubated with conditioned medium from WT MAEC, as detected by immunoblot (Fig. 3D).

Taken together, lack of NOS3 induces pro-inflammatory macrophage accumulation by inducing M1 polarization, and repolarization of M2 macrophages to the M1 phenotype.

M2 polarized macrophages slow VSMC proliferation and migration in the absence of NOS3

VSMC proliferation and migration are key steps during neointimal formation. To test whether proinflammatory or resolving macrophages may have an effect on these processes, we incubated VSMC with conditioned medium from M1 and M2 polarized macrophages, finding that cells proliferate more and migrate faster with conditioned medium from M1 respect to M2 macrophages (Figs. 4A and B), and confirmed by exogenous administration the NO donor Sodium Nitroprusside to M2 polarized macrophages (Fig. 4C). Interestingly M1 and exogenous M2 macrophages incubated with SNP, express high levels of MMP-13 mRNA, and secreted MMP-13, a protein implicated the migration of different cell types, when compared with polarized M2 macrophages (Fig. 5A). To test whether macrophage mediated MMP-13 may regulate VSMC migration, we found no significant differences when macrophages were isolated from MMP-13 null mice (Fig. 5B), whereas exogenous administration of MMP-13 restored the migratory phenotype (Fig. 5C). Taken together, M1 macrophage-mediated MMP-13 expression and activity induce VSMC migration in the absence of NOS3, shedding light about the mechanism by which NOS3 prevents neointimal formation.

MMP13 expression regulates neointima hyperplasia in NOS3 injured vessels

To test whether MMP-13 plays a role in the absence of NOS3, we generated a double NOS3/MMP-13 null mouse strain (Fig. 6A). While the absence of MMP-13 in NOS3 expressing vessels (MMP-13 null mice), has no effect on neointima formation, lacking of MMP13 in the NOS3-null background (double NOS3/MMP-13 null mice), prevented

the aortic wall thickening seen in mice singly deficient for NOS3 30 days after endothelial aortic denudation (Fig. 6B).
Transplantation of MMP-13 deficient bone marrow (either from MMP-13 null mice or double MMP-13/NOS3 knockout mice) into irradiated NOS3 null animals significantly reduced wall thickness, when compared with NOS3 null denuded aortas from non irradiated mice (Fig. 6C), indicating that MMP-13 expressing bone marrow derived cells may regulate neointimal formation in NOS3 null mice.
Consistent with these findings, we detected high levels of MMP13 in cross sections of human aortic endarterectomies, together with high numbers of M1 (CD68+ iNOS+) polarized macrophages, as detected by confocal microscopy, and extensive neointimal hyperplasia, whereas in healthy human arteries, MMP-13 was barely detected (Fig. 6D). A proposed mechanism is shown in Supplemental Figure 4.

Discussion

We show for the first time a matrix metalloproteinase as a target of NOS3 in the prevention of arterial neointimal hyperplasia. In the absence of NOS3, aortic endothelial disruption increases neointimal formation by promoting inflammation through an imbalance of M1 pro-inflammatory vs M2 resolving macrophage infiltrates, as result of significant accumulation of several pro-inflammatory cytokines in plasma and in endothelial cells from NOS3 deficient mice. M1 macrophages expressing MMP-13 induce VSMC migration and proliferation, which are inhibited by M1 macrophages from MMP-13 null mice, and reversed by exogenous replacement of the missing protease. Furthermore, in double NOS3/MMP-13 deficient animals, lack of MMP-13 was sufficient to prevent NOS3 null dependent neointimal formation. These data, together with the detection of extensive MMP-13 and M1 macrophage co-localization in human carotid endarcterectomies suggest MMP-13 as a new target to prevent human neointima hyperplasia.

NOS3 prevents neointimal hyperplasia by limiting smooth muscle cell proliferation through as-yet undefined mechanisms [25]. Arterial wall thickness is reduced in animal models of vascular injury by experimental approaches, including NO delivery by exogenous systemic or local administration of NO donors, or by viral-mediated transfection with NOS3 [26, 27]. However, no preclinical findings have been successfully implemented so far. Recent contributions elegantly describe the effect of NO in the ubiquitin-proteasome system [28-30], and the inhibition of the PDGF-surviving pathway during flow dependent vascular remodeling [31]. Our work is the first to describe the role of NOS3 in the resolution of the immune response elicited in the vessel as result of endothelial denudation, identifying MMP-13 as a new target.

After endothelial denudation, monocytes infiltrate in response to chemokines produced by a variety of cells, including immune and endothelial cells. M-CSF induces recruitment and differentiation of circulating monocytes into macrophages [13]. The relevance of NOS3 to macrophage polarization has not been described so far. We found high levels of MCP-1 but not MCSF or CCL5 in plasma from NOS3 null mice, which may explain why monocyte recruitment is not affected by the absence of NOS3 (Fig 2A, Total WT vs Total KO). However, the extensive expression of GM-CSF and IFN-gamma in the absence of NOS3, may explain the increased M1 proinflammatory macrophage polarization in these mice.

The levels of interleukin-6 (IL-6) detected in vascular endothelium and plasma from NOS3 deficient denuded aortas are of significant interest. Over the past decade a large number of studies have demonstrated the importance of IL-6 in the progression of several inflammatory cardiovascular complications including unstable angina [32], coronary artery disease [33], and myocardial infarction [34]. Coronary artery atherosclerosis is characterized by infiltration of activated macrophages, which generate high levels of IL-6. IL-6 is associated with increased mortality and also it serves to identify patients who may benefit most from early invasive strategies [35, 36]. Furthermore, heart transplanted patients treated with simvastatin have low levels of IL-6 and improved levels of endothelial function [37]. In this regard, inhibition of NO-mediated endothelial-cell-driven relaxation is also strongly associated with increased levels of IL-6 [38, 39]. The levels of IL-6 found in denuded aortas from NOS3 null mice, further support the importance of IL-6 in the progression of neointimal formation.

Matrix Metalloproteinases play an important role in the proliferation and migration of several cell types [18, 21], but the impact of NOS3-mediated MMP regulation of neointimal hyperplasia is yet unknown. Here we found that MMP-13 expression is increased in M1 and NO-treated M2 macrophages, and induces migration of VSMC. MMP-13 null M1 macrophages by contrast, had no effect on cell migration, unless exogenous MMP-13 is administered, pointing MMP-13 as a target of M1 macrophage derived VSMC migration.

Macrophage expression of MMP-13 can be stimulated by pro-inflammatory cytokines, including endothelial IL6, which is strongly upregulated in the absence of NOS3, thereby promoting pro-inflammatory macrophage accumulation and increased macrophage-meditated MMP-13 expression, as detected, and as described in osteoblasts [40], fibroblasts [41], and cancer cells. High levels of MMP-13 were detected in carotid M1 infiltrated macrophages from patients undergoing carotid endarcterectomy, with extensive neointimal hyperplasia. To this regard, in patients undergoing percutaneous transluminal coronary angioplasty, neointima hyperplasia might be reduced by promoting M1 proinflammatory macrophage polarization to M2 resolving macrophages, in which expression of MMP-13 may play a pivotal role.

Lack of MMP-13 neither induce neointimal hyperplasia in mice expressing NOS3, nor promotes M1-macrophage mediated VSMC migration, whereas increased aortic thickness shown in the absence of NOS3 is significantly reduced in double NOS3/MMP-13-deficient mice. Taking all these findings together, we propose that NO may prevent neointimal formation in mice by at least targeting MMP-13.

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Disclosures: none

References

1. Young JJ. Neointimal formation following drug-eluting stents: physiology, timeline, and the influence of drug delivery systems. *Reviews in cardiovascular medicine*. 2007;8 Suppl 1:S3-10.
2. Popowich DA, Varu V and Kibbe MR. Nitric oxide: what a vascular surgeon needs to know. *Vascular*. 2007;15:324-35.
3. Bendeck MP, Zempo N, Clowes AW, Galardy RE and Reidy MA. Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circulation research*. 1994;75:539-45.
4. Iaconetti C, Polimeni A, Sorrentino S, Sabatino J, Pironti G, Esposito G, Curcio A and Indolfi C. Inhibition of miR-92a increases endothelial proliferation and migration in vitro as well as reduces neointimal proliferation in vivo after vascular injury. *Basic research in cardiology*. 2012;107:296.
5. Kim J, Zhang L, Peppel K, Wu JH, Zidar DA, Brian L, DeWire SM, Exum ST, Lefkowitz RJ and Freedman NJ. Beta-arrestins regulate atherosclerosis and neointimal hyperplasia by controlling smooth muscle cell proliferation and migration. *Circulation research*. 2008;103:70-9.
6. Zaidi SH, You XM, Ciura S, O'Blenes S, Husain M and Rabinovitch M. Suppressed smooth muscle proliferation and inflammatory cell invasion after arterial injury in elafin-overexpressing mice. *The Journal of clinical investigation*. 2000;105:1687-95.
7. Cui B, Huang L, Fang Y, Guo R, Yin Y and Zhao X. Transplantation of endothelial progenitor cells overexpressing endothelial nitric oxide synthase enhances inhibition of neointimal hyperplasia and restores endothelium-dependent vasodilatation. *Microvascular research*. 2011;81:143-50.
8. Cooney R, Hynes SO, Sharif F, Howard L and O'Brien T. Effect of gene delivery of NOS isoforms on intimal hyperplasia and endothelial regeneration after balloon injury. *Gene therapy*. 2007;14:396-404.
9. Sharif F, Hynes SO, Cooney R, Howard L, McMahon J, Daly K, Crowley J, Barry F and O'Brien T. Gene-eluting stents: adenovirus-mediated delivery of eNOS to the blood vessel wall accelerates re-endothelialization and inhibits restenosis. *Molecular therapy*. 2008;16:1674-80.
10. Marks DS, Vita JA, Folts JD, Keaney JF, Jr., Welch GN and Loscalzo J. Inhibition of neointimal proliferation in rabbits after vascular injury by a single treatment with a protein adduct of nitric oxide. *The Journal of clinical investigation*. 1995;96:2630-8.
11. Le Tourneau T, Van Belle E, Corseaux D, Vallet B, Lebuffe G, Dupuis B, Lablanche JM, McFadden E, Bauters C and Bertrand ME. Role of nitric oxide in restenosis after experimental balloon angioplasty in the hypercholesterolemic rabbit: effects on neointimal hyperplasia and vascular remodeling. *Journal of the American College of Cardiology*. 1999;33:876-82.
12. Stoger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, Biessen EA, Daemen MJ, Lutgens E and de Winther MP. Distribution of macrophage polarization markers in human atherosclerosis. *Atherosclerosis*. 2012;225:461-8.
13. Hoeksema MA, Stoger JL and de Winther MP. Molecular pathways regulating macrophage polarization: implications for atherosclerosis. *Current atherosclerosis reports*. 2012;14:254-63.
14. Baitsch D, Bock HH, Engel T, Telgmann R, Muller-Tidow C, Varga G, Bot M, Herz J, Robenek H, von Eckardstein A and Nofer JR. Apolipoprotein E induces antiinflammatory phenotype in macrophages. *Arteriosclerosis, thrombosis, and vascular biology*. 2011;31:1160-8.

15. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schutz G, Lumeng CN and Mortensen RM. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. *The Journal of clinical investigation*. 2010;120:3350-64.
16. Kobayashi Y. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. *Journal of leukocyte biology*. 2010; 88:1157-62.
17. Tarin C, Gomez M, Calvo E, Lopez JA and Zaragoza C. Endothelial nitric oxide deficiency reduces MMP-13-mediated cleavage of ICAM-1 in vascular endothelium: a role in atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:27-32.
18. Lizarbe TR, Garcia-Rama C, Tarin C, Saura M, Calvo E, Lopez JA, Lopez-Otin C, Folgueras AR, Lamas S and Zaragoza C. Nitric oxide elicits functional MMP-13 protein-tyrosine nitration during wound repair. *FASEB journal*. 2008;22:3207-15.
19. Lopez-Rivera E, Lizarbe TR, Martinez-Moreno M, Lopez-Novoa JM, Rodriguez-Barbero A, Rodrigo J, Fernandez AP, Alvarez-Barrientos A, Lamas S and Zaragoza C. Matrix metalloproteinase 13 mediates nitric oxide activation of endothelial cell migration. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:3685-90.
20. Zaragoza C, Soria E, Lopez E, Browning D, Balbin M, Lopez-Otin C and Lamas S. Activation of the mitogen activated protein kinase extracellular signal-regulated kinase 1 and 2 by the nitric oxide-cGMP-cGMP-dependent protein kinase axis regulates the expression of matrix metalloproteinase 13 in vascular endothelial cells. *Molecular pharmacology*. 2002;62:927-35.
21. Zaragoza C, Balbin M, Lopez-Otin C and Lamas S. Nitric oxide regulates matrix metalloprotease-13 expression and activity in endothelium. *Kidney international*. 2002;61:804-8.
22. Wang H and Keiser JA. Expression of membrane-type matrix metalloproteinase in rabbit neointimal tissue and its correlation with matrix-metalloproteinase-2 activation. *Journal of vascular research*. 1998;35:45-54.
23. Wang Z, Kong L, Kang J, Vaughn DM, Bush GD, Walding AL, Grigorian AA, Robinson JS, Jr. and Nakayama DK. Interleukin-lbeta induces migration of rat arterial smooth muscle cells through a mechanism involving increased matrix metalloproteinase-2 activity. *The Journal of surgical research*. 2011;169:328-36.
24. Pello OM, De Pizzol M, Mirolo M, Soucek L, Zammataro L, Amabile A, Doni A, Nebuloni M, Swigart LB, Evan GI, Mantovani A and Locati M. Role of c-MYC in alternative activation of human macrophages and tumor-associated macrophage biology. *Blood*. 2012;119:411-21.
25. Ekshyyan VP, Hebert VY, Khandelwal A and Dugas TR. Resveratrol inhibits rat aortic vascular smooth muscle cell proliferation via estrogen receptor dependent nitric oxide production. *Journal of cardiovascular pharmacology*. 2007;50:83-93.
26. Havelka GE, Moreira ES, Rodriguez MP, Tsihlis ND, Wang Z, Martinez J, Hrabie JA, Kiefer LK and Kibbe MR. Nitric oxide delivery via a permeable balloon catheter inhibits neointimal growth after arterial injury. *The Journal of surgical research*. 2012.
27. Kapadia MR, Chow LW, Tsihlis ND, Ahanchi SS, Eng JW, Murar J, Martinez J, Popowich DA, Jiang Q, Hrabie JA, Saavedra JE, Keefer LK, Hulvat JF, Stupp SI and Kibbe MR. Nitric oxide and nanotechnology: a novel approach to inhibit neointimal hyperplasia. *Journal of vascular surgery*. 2008;47:173-82.

28. Tsihlis ND, Kapadia MR, Vavra AK, Flannery WD, Oustwani CS, Jiang Q and Kibbe MR. Nitric oxide may inhibit neointimal hyperplasia by decreasing isopeptidase T levels and activity in the vasculature. *Journal of vascular surgery*. 2013;58:179-86.
29. Tsihlis ND, Kapadia MR, Vavra AK, Jiang Q, Fu B, Martinez J and Kibbe MR. Nitric oxide decreases activity and levels of the 11S proteasome activator PA28 in the vasculature. *Nitric oxide*. 2012;27:50-8.
30. Tsihlis ND, Oustwani CS, Vavra AK, Jiang Q, Keefer LK and Kibbe MR. Nitric oxide inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia by increasing the ubiquitination and degradation of UbcH10. *Cell biochemistry and biophysics*. 2011;60:89-97.
31. Yu J, Zhang Y, Zhang X, Rudic RD, Bauer PM, Altieri DC and Sessa WC. Endothelium derived nitric oxide synthase negatively regulates the PDGF-survivin pathway during flow-dependent vascular remodeling. *PloS one*. 7:e31495.
32. Kang WC, Il Moon C, Lee K, Han SH, Suh SY, Moon J, Shin MS, Ahn T and Shin EK. Comparison of inflammatory markers for the prediction of neointimal hyperplasia after drug-eluting stent implantation. *Coronary artery disease*. 2011;22:526-32.
33. Paulsson J, Dadfar E, Held C, Jacobson SH and Lundahl J. Activation of peripheral and in vivo transmigrated neutrophils in patients with stable coronary artery disease. *Atherosclerosis*. 2007;192:328-34.
34. Suzuki H, Kusuyama T, Sato R, Yokota Y, Tsunoda F, Sato T, Shoji M, Iso Y, Koba S and Katagiri T. Elevation of matrix metalloproteinases and interleukin-6 in the culprit coronary artery of myocardial infarction. *European journal of clinical investigation*. 2008;38:166-73.
35. Lindmark E, Diderholm E, Wallentin L and Siegbahn A. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. *Jama*. 2001;286:2107-13.
36. Tedgui A. The role of inflammation in atherothrombosis: implications for clinical practice. *Vascular medicine (London, England)*. 2005;10:45-53.
37. Weis M, Pehlivanli S, Meiser BM and von Scheidt W. Simvastatin treatment is associated with improvement in coronary endothelial function and decreased cytokine activation in patients after heart transplantation. *Journal of the American College of Cardiology*. 2001;38:814-8.
38. Myung SC, Han JH, Song KK, Kang GH, Lee SY, Kim TH, Lee MY, Kim HW and Kim SC. The effects of interleukin-6 on the contraction and relaxation responses of the cavernous smooth muscle from rats. *European journal of pharmacology*. 2008;589:228-32.
39. Orshal JM and Khalil RA. Reduced endothelial NO-cGMP-mediated vascular relaxation and hypertension in IL-6-infused pregnant rats. *Hypertension*. 2004;43:434-44.
40. Kusano K, Miyaura C, Inada M, Tamura T, Ito A, Nagase H, Kamoi K and Suda T. Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. *Endocrinology*. 1998;139:1338-45.
41. Solis-Herruzo JA, Rippe RA, Schrum LW, de La Torre P, Garcia I, Jeffrey JJ, Munoz-Yague T and Brenner DA. Interleukin-6 increases rat metalloproteinase-13 gene expression through stimulation of activator protein 1 transcription factor in cultured fibroblasts. *The Journal of biological chemistry*. 1999;274:30919-26.

Significance

Arterial wall restenosis may be caused by mechanical disruption of the vascular endothelium, inducing extensive smooth muscle and endothelial cell migration and proliferation, resulting in increased arterial wall thickness and a narrowing of the lumen. We show the impact that arterial endothelium expressing Nitric Oxide has in the modulation of the inflammatory response, elicited as result of endothelial disruption, in which endothelial nitric oxide synthase (NOS3) plays a critical role. **NOS3 regulates macrophage polarization by preventing accumulation of pro-inflammatory macrophages**, thereby contributing to the resolution of inflammation, and limiting restenosis. The significance of this finding is based in the effect on proinflammatory macrophage **derived MMP-13 stimulation of smooth muscle cell proliferation and migration in the absence of NOS3, proposing** MMP-13 as a novel target in the prevention of restenosis.

Legends

Figure 1. Lack of NOS3 promotes neointimal formation and impairs endothelial regeneration in denuded abdominal aortas A. Hematoxilin-Eosin (H&E) staining of aortic rings form WT and NOS3 knockout mice after endothelial denudation (0, 15, and 30 days. Lower panels: WT and NOS3 aortic rings 30 days after wire injury (30W)). Right. Intima/media, lumen, intima, total vessele areas, endothelial cell and vascular smooth muscle cell counts of the same aortas (n=6 mice/group/day by triplicate. Mean ± SD; *p<0.05 NOS3 KO 15 vs WT 15; **p<0.05 NOS3 KO 30 vs WT 30; ***p<0.05 NOS3 KO 30W vs WT 30). Scale bars 50 μm".**B.** Intima/media area of denuded aortas from WT, NOS3 KO, and NOS3 KO mice weekly injected with 0.25 mg/kg/day of the NO donor sodium nitroprusside (SNP), or 50 mg/kg/day L-NAME as indicated (n=6 mice/group/day by triplicate, mean ± SD; *p<0.05 NOS3 KO 30 vs NOS3 KO 30+SNP). **C.** Intima/media thickness of denuded aortas from WT mice injected with 50 mg/kg/day L-NAME, or 0.25 mg/Kg/day SNP as indicated (n=6 mice/group/day by triplicate, mean ± SD; * p<0.05 WT 30 vs NOS3 KO 30; **p<0.05 WT 30 vs WT 30+L-NAME).

Figure 2. Mice lacking NOS3 show increased levels of pro-inflammatory M1 macrophages after aortic endothelial denudation. A. WT and NOS3 KO aortic CD68+/MRC1- (M1) and CD68+/MRC1+ (M2) infiltrated macrophages at 7 and 15 days after endothelial denudation. Representative images show "en face" confocal microscopy detection of CD68+ (FITC, green) MRC1+(Cy3, red), and nuclei (Hoecht, blue). Data was graphically represented as percentage of lesional area, (n=6 mice/group/day by triplicate; mean ± SD, * p<0.05 M1 WT 14 vs M1 NOS3 14, ** p<0.05 M2 WT 14 vs M2 NOS3 14. **B.** Bone marrow macrophages from WT and c-Myc conditional null mice were polarized to M1 and M2 macrophages, and the levels of c-myc mRNA were evaluated by quantitative RT-PCR (n=6 mice/group by triplicate, mean ± SD * p<0.05 M2 WT vs M2 c-Myc KO). **C.** Intima/media area of aortas collected from WT and c-Myc conditional null mice at 0, 15 and 30 days after endothelial denudation (n=6 mice/group by triplicate. mean ± SD; * p<0.05 WT 30 vs Myc 30).

Figure 3. Vascular endothelium regulates macrophage polarization. A. Plasma levels of pro-inflammatory cytokines isolated from WT and NOS3 null mice after aortic endothelial denudation at the times indicated (n=3 mice/group/day by triplicate, mean ± SD *** p<0.05 WT vs NOS3 KO). **B**. Multiplex analysis of secreted cytokines from injured WT and NOS3-null endothelial cell monolayers, and collected at the times indicated (n=3 by triplicate, mean ± SD *** p<0.05 WT vs NOS3 KO). **C**. **Upper**. Expression of iNOS by qRT-PCR in polarized M1 and M2 macrophages, and incubated for 24h and 48h with conditioned medium from MAEC WT and NOS3 KO (n=3 by triplicate, mean ± SD * p<0.05 M1 24h vs M2 24h; ** p<0.05 M1 48h vs M2 48h). **Bottom**. qRT-PCR expression of iNOS from bone marrow macrophages 24 hours after polarization into M1, M2, and M2 treated macrophages with the NO donor SNP (n=3 by triplicate, mean ± SD * p<0.05 M1 vs M2; ** p<0.05 M1 vs M2+SNP). **D.** Expression of IL6 from M1 and M2 polarized macrophages and incubated for 24 or 48h with conditional medium from WT or NOS3 KO MAEC as indicated. Expression of GAPDH was assayed as control (n=3 by triplicate).

Figure 4. M1 macrophages regulate VSMC proliferation and. A. Proliferation of VSMC after 24 hours of incubation with conditioned medium from M1 and M2 polarized macrophages (n=3, by triplicate mean ± SD * p<0.05 Control vs M1; ** p<0.05 M1 vs M2). **B.** VSMC monolayers were injured, and migration of cells was evaluated at the times indicated (n=3, by triplicate mean ± SD * p<0.05 M1 vs M2) Representative micrographs of migrating wounded cells taken at the times indicated are depicted. **C.** VSMC migration as in B, in which the contribution of exogenous NO administration

(Sodium Nitropruside, SNP) to M2 polarized macrophages was evaluated (n=3, mean ± SD * p<0.05 M2 vs M2+SNP). Scale bars 100 μm.

Figure 5. M1-mediated VSMC migration depends on macrophage-dependent MMP-13 expression. A. Expression of MMP-13 in polarized macrophages. Immunoblot detection of MMP-13 (upper), gelatin zymography (middle) from macrophage cell culture supernatants (upper), and macrophage MMP-13 mRNA detection (lower graph), 24 hours after polarization into M1, M2, or into M2 macrophages and incubated with the NO donor SNP (n=3, by triplicate, mean ± SD * p<0.05 M2 vs M2+SNP; ** p<0.05 M1 vs M2). **B.** VSMC migration in the presence of polarized M1, M2 and M2 +SNP macrophage supernatants isolated from MMP-13 null mice (n=3, by triplicate mean ± SD). **C.** VSMC migration as in B, in which cell monolayers were also incubated with exogenous recombinant active MMP-13 ((n=3, by triplicate, mean ± SD * p<0.05 MMP-13 KO M2 4h vs MMP-13 KO M2 + MMP-13 4h; ** p<0.05 MMP-13 KO M2 6h vs MMP-13 KO M2 + MMP-13 6h).

Figure 6. Lack of MMP-13 prevents neointimal thickening of NOS3 null mice. A. DNA electrophoresis from WT and double NOS3- and MMP-13-deficient mice, showing the different allelic combinations, as indicated. **B.** Aortic intima/media thickness from WT, MMP-13-null mice, NOS3 null mice, and NOS3/MMP-13 null mice, 15 and 30 days after endothelial denudation (n=10 mice/group/day, by triplicate mean ± SD; * p<0.05 NOS3 null 30 vs NOS3/MMP-13 null 30). **C.** Aortic intima/media thickness from a 30 day injured and transplanted NOS3-deficient mice with bone marrow from MMP-13 null or double MMP-13/NOS3 null mice (n=6 mice/group/day by triplicate. Mean ± SD * p<0.05 NOS3 healthy control (open bar) vs NOS3 KO denuded 30 days; ** p<0.05 NOS3 KO denuded 30 days vs NOS3 KO irradiated/BM MMP-13 KO; *** p<0.05 NOS3 KO denuded 30 days vs NOS3 KO irradiated/BM MMP-13/NOS3 KO). **D.** Immunohistochemical detection of MMP-13 in cross sections of carotid samples from endarcterectomized patients, and healthy mammary arteries. The white box corresponds to the carotid magnified region as also shown. Scale bars: 50 μm. Bottom. Confocal microscopy detection of CD68 (green, FITC), and NOS2 (red Cy3) in the same carotid section as above. Co-localization of both signals is represented in yellow (Merged). Arrows indicate co-localization of macrophage infiltrates and MMP13.