



Inflammation and oxidative stress in atherosclerosis : role of S-nitrosothiols in the vascular responses

Eugenia Belcastro

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**Ecole Doctorale BioSE (Biologie Santé et son Environnement)
Doctorate in GenOMeC (Genetics, Oncology and Clinical Medicine)**

Thèse

Présentée et soutenue publiquement pour l'obtention du titre de

DOCTEUR DE L'UNIVERSITE DE LORRAINE

Mention : « Sciences de la Vie et de la Santé »

Eugenia BELCASTRO

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rôle dans les réponses vasculaires des S-nitrosothiols**

3 Novembre 2016

Membres du jury:

Rapporteurs:	Valérie Schini-Kerth	Pr, UMR 7213, CNRS, Université de Strasbourg
	Céline Demougeot	Pr, EA 4267, FDE, Université de Franche-Comté, Besançon
	Isabelle Lartaud	Pr, EA3452, Université de Lorraine, Nancy, Directeur de these
	Alfonso Pompella	Pr, Department of Translational Research NTMC, University of Pisa, Italy, co-directeur de thèse
	Yvan Devaux	Dr, Luxembourg Institute of Health (LIH), Luxembourg
	Marc Derive	Dr, INOTREM, Université de Lorraine, Nancy
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To strive, to seek, to find, and not to yield.

- Lottare, cercare, trovare, e non cedere -

Alfred Tennyson

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It's hard to write acknowledgment for many reasons. The first, because retracing my course of the research I can not think of those who have been my wing and how many people have helped me to form the person that I'm today and it is impossible to list them all in one page or so (but they know ...). The second because even with the enormous joy of cutting a milestone such as PhD graduation, you always fear of losing something, such as friends, "colleagues" of these three years, the professors in the fruitful relationship "*give and take*" (broadly defined), the environment itself of an experience that is fundamental part of the life.

The work of the doctoral thesis is one of the most challenging, not so much for its extension in time or for the continuing intellectual and physical effort that requires, rather because it is the result of a training program in which you choose to personally get involved. In such a way, the professional growth is just one of the challenges, inasmuch it is necessary also to know how to develop a set of transversal skills necessary to confront realities and situations, academic and non-academic, changing and uncertain.

Precisely, for me, this thesis is the expression of a human and scientific experience conceived through the encounter with many people and special places. I met in this "journey" many mentors, each of them have dedicated precious time to me, spent to discuss and find answers to my questions, to my beliefs and ideas.

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SCIENTIFIC WORKS

PUBLICATIONS

Published:

- Monocytes/macrophages activation contributes to b-gamma-glutamyltransferase accumulation inside atherosclerotic plaques. **Eugenia BELCASTRO**, Maria FRANZINI, Silvana CIANCHETTI, Evelina LORENZINI, Silvia MASOTTI, Vanna FIERABRACCI, Angela PUCCI, Alfonso POMPELLA and Alessandro CORTI. *Journal of Translational Medicine*, 2015, 13:325. (IF: 3.69, Article 1)

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- Oxidative stress enhances and modulates protein S-nitrosation in smooth muscle cells exposed to S-nitrosoglutathione. **Eugenia BELCASTRO**, Wen WU, Caroline PERRIN-SERRADO, Isabelle FRIES, Alessandro CORTI, Alfonso POMPELLA, Pierre LEROY, Isabelle LARTAUD, Caroline GAUCHER. Submitted to *Free Radical Biology and Medicine*, October 2016 (IF: 5.78, Article 2)

ORAL COMMUNICATIONS

- 5th International Conference on Translational Medicine - San Francisco, California (USA) 28-30, November 2016. "Oxidative stress enhances and modulates protein S-nitrosation in smooth muscle cells exposed to S-nitrosoglutathione".
- Workshop in NutriOx Network – University of Kaiserslautern (Germany), 21-23, September 2016. "Vascular oxidative stress toward S-nitrosothiols bioavailability".
- Riunione Scientifica "Scuola Dianzani" - University of Siena (Italy), 8-9, July 2016. "Oxidant stress conditions induce differential S-nitrosation of smooth muscle cell proteins by S-nitrosoglutathione (GSNO)".
- Italian Society of Pathology and Translational Medicine (SIPMet) - Alba (Italy), 11-12, September 2015. "Activated monocytes and M1-like macrophages release the b-GGT fraction of gamma-glutamyltransferase".
- Riunione Scientifica "Scuola Dianzani" - University of Cagliari (Italy), 20-21, March 2015. "Activated monocytes and M1-like macrophages release the b-GGT fraction of gamma-glutamyltransferase".
- Annual Progress Report Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC) – University of Siena (Italy), September 30, 2015. "Redox events during monocytes/macrophages activation and their potential contribution to progression of atherosclerosis".

- Annual Progress Report Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC) – University of Siena (Italy), September 16, 2014. “Inflammation and denitrosating enzymes: from the development of a cellular inflammatory model to pathological tissues”.

POSTER COMMUNICATIONS

- 20^é Congrès de la Société Française de Pharmacologie et de Thérapeutique (SFPT) – Centre Prouvé, Nancy (France), 19-21, April 2016. “S-nitrosoglutathione potentiates protein S-nitrosation under oxidative stress, a potential improvement of NO storage into smooth muscle cells”, **Eugenia BELCASTRO**, Wen Wu, Isabelle Fries, Alessandro CORTI, Alfonso POMPELLA, Pierre LEROY, Isabelle LARTAUD, Caroline GAUCHER.
- 15th International Conference on Oxidative Stress, Redox Homeostasis & Antioxidants - Institut Pasteur, Paris (France), 22-24, June 2015. “Opposite impact of inflammation and oxidative stress on vascular GGT activity”, **Eugenia BELCASTRO**, Caroline GAUCHER, Alessandro CORTI, Maria FRANZINI, Mario PONGAS, Caroline PERRIN-SARRADO, Alfonso POMPELLA, Isabelle LARTAUD.
- 19^{éme} Congrès de la Société Française de Pharmacologie et de Thérapeutique (SFPT) - Faculté de Médecine et de Pharmacie, Caen (France), 21-23, April 2015. “Opposite impact of inflammation and oxidative stress on vascular GGT activity”, **Eugenia BELCASTRO**, Caroline GAUCHER, Alessandro CORTI, Maria FRANZINI, Mario PONGAS, Caroline PERRIN-SARRADO, Alfonso POMPELLA, Isabelle LARTAUD.
- Journée Scientifique de l'école doctorale BioSE – Nancy (France), December 4, 2014. “Inflammation and denitrosating enzymes: from the development of a cellular inflammatory model to pathological tissues”, **Eugenia BELCASTRO**, Alessandro CORTI, Isabelle LARTAUD, Alfonso POMPELLA.
- 32^o National Congress - Italian Society of Pathology and Translational Medicine (SIPMet) – Palermo (Italy), 17-19, September 2014. “Human vulnerable carotid plaques show high levels of b-gamma-glutamyltransferase enzyme activity”, Maria FRANZINI, Sabrina CERAGIOLI, **Eugenia BELCASTRO**, Silvana CIANCHETTI, Mauro FERRARI, Fulvio BASOLO, Michele EMDIN, Aldo PAOLICCHI, Alfonso POMPELLA, Alessandro CORTI.

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL NAME
ABCA1	ATP-binding cassette transporter subfamily A member 1
ABGC1	ATP-binding cassette transporter G1
ADORA2A	Adenosine A2A receptor
agLDL	Aggregated low-density lipoprotein
APC	Antigen-presenting cell
Apo	Apolipoprotein
Arg-1	Arginase-1
ATF-1	Activating Transcription Factor-1
BH ₄	Tetrahydrobiopterin
CARKL	Carbohydrate kinase-like protein
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CHOP	CCAAT/Enhancer-Binding protein homologous protein
CMAF	Maf transcription factor
CR1	Carbonyl reductase 1
CRP	C-reactive protein
CVD	Cardiovascular diseases
Cys	Cysteine
Cys-Cys	Cystine
Cys-Gly	Cysteinyl-glycine
CysNO	S-nitrosocysteine
ECs	Endothelial cells
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelium nitric oxide synthase
ER	Endoplasmatic reticulum
FAD	Flavin adenine dinucleotide
FCMs	Foam cell macrophages
FMN	Flavin mononucleotide
GGT	γ-glutamyltransferase
GlyGly	Glycylglycine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Glutathione or L-γ-glutamyl-L-cysteinyl-glycine
GSNHOH	S-hydroxylaminoglutathione
GSNO	S-nitrosoglutathione
GSNOR	GSNO Reductase
GSO ₂ H	Glutathione sulfinic acid
GSSG	Oxidized glutathione

GTP-CH-I	Guanosine triphosphate-cyclohydrolase I
Hb	Hemoglobin
HDL	High density lipoprotein
HLA-DR	Human leukocyte antigen - antigen D related
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HNO	Nitroxyl
Hp	Haptoglobin
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFN- γ	Interferone gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KLF-2	Krüppel-like factor 2
LDL	Low-density lipoproteins
LOX-1	Low-density lipoprotein receptor-1
LPP	Lipoma preferred partner
LPS	Lipopolysaccharide
LRP	Low-density lipoprotein receptor-related protein
LXR α	Liver X receptor alpha
M1	Pro-inflammatory macrophage
M2	Anti-inflammatory macrophage
MAPK	Mitogen-activated protein kinases
MCP-1	Monocytes chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MerTK	Mer receptor kinase
MHCII	Major histocompatibility antigens of class II
mmLDL	Minimally modified low-density lipoproteins
MMP	Matrix metalloproteinase
Mox	Oxidized phospholipid-derived macrophages
N ₂ O ₃	Dinitrogen trioxide
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa B
NH ₂ OH	Hydroxylamine
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate-oxidases
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
oxLDL	Oxidized low-density lipoproteins
PDEs	Cyclic nucleotide phosphodiesterases
PDGF	Platelet derived growth factor
PDI	Protein disulfide isomerase
PKGs	Protein Kinase G

PLAG2G7	Phospholipase A2 Group VII
PMNs	Polymorphonuclear neutrophils
PPAR γ	Peroxisome proliferator-activated receptor gamma
PPP	Pentose phosphate pathway
Pr-SNO	S-nitrosated proteins
pSTAT1	Signal transducer and activator of transcription 1
RANTES	Regulated on activation, normal T cells expressed and secreted chemokine
RBP-J	Recombination signal-binding protein for immunoglobulin kappa J region
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S ⁻	Thiolate anion
S [·]	Radical thiyl
SAA	Seruma amyloid A
sGC	Soluble guanylyl cyclase
SIN-1	3-morpholiniosydnonimine
SMCs	Smooth muscle cells
SNAP	S-nitroso-N-acetylpenicillamine
SNO-Alb	S-nitrosoalbumin
SNO-Hb	S-nitrosohemoglobin
SR-A	Scavenger receptor
STAT6	Signal transducer and activator of transcription 6
TGF- β	Transforming growth factor beta
Th1	Type 1 helper lymphocytes
Th2	Type 2 helper lymphocytes
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
TrxR	Thioredoxin reductase
Trxs	Thioredoxin systems
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very Low Density Lipoprotein
XBP1	X-box binding protein 1

SPECIFIC FRENCH ABBREVIATION

FULL NAME

CML	cellules musculaires lisses
ERN	espèces réactives de l'azote
ERO	espèces réactives de l'oxygène

INTRODUCTION GÉNÉRALE

D'après l'Organisation Mondiale de la Santé, les maladies cardiovasculaires sont responsables de 30 % de la mortalité, constituant ainsi la première cause de décès au monde. L'athérosclérose est une pathologie caractérisée par la formation d'une plaque composée de lipides au niveau de la paroi des artères. Cette pathologie, associant inflammation et stress oxydant, représente le facteur de risque majeur de plusieurs maladies cardiovasculaires comme l'infarctus du myocarde (120 000 cas par an) et les accidents vasculaires cérébraux (130 000 victimes par an), et est responsable de 90 % des cas d'angor.

L'athérosclérose se développe en plusieurs stades évolutifs successifs : la strie lipidique, la lésion fibro-lipidique et la plaque compliquée. Les lipoprotéines et quatre types cellulaires: les monocytes-macrophages, les cellules endothéliales (ECs), les cellules musculaires lisses (CML) et les lymphocytes, sont les acteurs principaux de la genèse de la plaque. L'infiltration des lipoprotéines de basse densité (LDL), composées majoritairement d'apolipoprotéine B et de cholestérol (LDL-Cholestérol), dans l'intima constitue le point de départ de la formation de la strie lipidique. Leur oxydation (ox-LDL) joue un rôle clé dans l'activation pro-inflammatoire des macrophages, des ECs et des CML. En parallèle, la dysfonction de l'endothélium, avec diminution de la biodisponibilité du monoxyde d'azote (NO), est à l'origine de l'adhésion des monocytes à la surface de l'endothélium. La diminution de la biodisponibilité de NO est engendrée essentiellement par les espèces réactives de l'oxygène (ERO) qui oxydent directement NO en ions peroxynitrite ou découplent la NO synthase endothéliale empêchant ainsi la synthèse de NO et favorisant la production d'anions superoxyde.

Le développement de l'athérosclérose étant associé à une augmentation de la concentration plasmatique de cholestérol, le traitement actuel de cette pathologie fait appel aux statines hypolipémiantes. Cependant, les statines ne permettent pas d'enrayer la maladie mais tout au plus ralentissent son évolution. Ainsi, d'autres pistes comme les inhibiteurs/antagonistes du système rénine angiotensine aldostérone, système majeur de la régulation de l'homéostasie cardio-rénal, ont été envisagés car capables de diminuer aussi les marqueurs d'inflammation comme TNF- α , IL-6 et la protéine C-réactive [1] et de limiter le stress oxydant. Ces deux classes thérapeutiques (statines et inhibiteurs/antagonistes du SRAA) présentent des effets pléiotropes convergeant vers une augmentation de la biodisponibilité de NO [2] (**Fig. 1**).

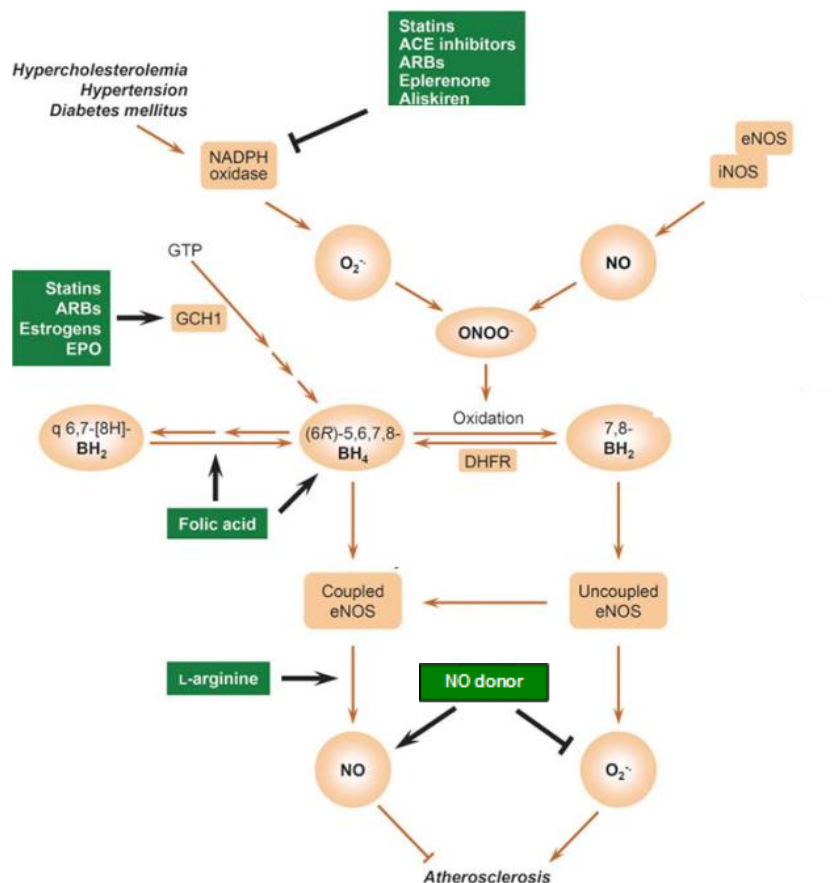


Figure 1. Potentiel des thérapeutiques cardiovasculaires pour l'athérosclérose via des actions "NO" dépendantes. La GTP cyclohydrolase I (GCH1) est l'enzyme limitante de la synthèse du (6R)-5,6,7,8-tetrahydro-L-bioptérin (BH₄), cofacteur des NO synthases inducible ou endothéliale (eNOS, iNOS). Le BH₄ est recyclé par réduction de la 7,8-dihydrobioptérine (BH₂) par la dihydrofolate réductase (DHFR), ou par la dihydroptéridine réductase à partir du quinonoïde 6,7-[8H]-BH₂. L'anion superoxyde ($O_2^{\cdot-}$) produit par la NADPH oxydase, oxyde le monoxyde d'azote (NO) en anion peroxynitrite ($ONOO^{\cdot-}$). Celui-ci oxyde à son tour le BH₄ en BH₂ provoquant le découplage de la eNOS qui produit $O_2^{\cdot-}$. La L-arginine, substrat de la eNOS et l'acide folique stabilisant le BH₄ améliorent la fonctionnalité de la eNOS. Les statines, les antagonistes du récepteur de l'angiotensine II de type 1 (ARBs), les œstrogènes et l'érythropoïétine (EPO) favorisent la synthèse de BH₄ en stimulant l'expression/activité de la GCH1. Les statines, les ARBs, les inhibiteurs de l'enzyme de conversion de l'angiotensine (ACE), l'éplérénone (antagoniste de l'aldostérone) et l'aliskiren (inhibiteur de la rénine) préviennent aussi l'oxydation du BH₄ en diminuant l'expression et/ou l'activité de la NADPH oxydase. NO est antiathérogénique donc les donneurs de NO limitant le stress oxydant pourraient prévenir l'athérosclérose. GTP : guanosine 5'-triphosphate. (D'après Li et al. 2009) [3]

La diminution de biodisponibilité de NO due à la dysfonction endothéliale ainsi que l'oxydation des LDL circulants seraient à l'origine de l'inflammation et donc de l'initiation de la formation de la plaque d'athérome. Ainsi, il apparaît comme essentiel d'étudier l'interaction inflammation/stress oxydant sur les types cellulaires (monocytes/macrophages et cellules musculaires lisses) composant la plaque d'athérome, en lien avec une restauration de la biodisponibilité de NO afin d'enrayer la formation de la plaque d'athérome. Différents donneurs de NO comme la molsidomine de la famille des sydnonimines, utilisée en clinique dans le traitement de l'angor, ont fait l'objet d'essais cliniques afin d'évaluer son intérêt thérapeutique pour l'amélioration de la fonction endothéliale de patients présentant un angor stable et éligibles à une angioplastie coronaire (étude MEDCOR) [4] [5]. Cette étude menée sur 165 patients recevant 16 mg de molsidomine par jour pendant un an après angioplastie coronaire n'a pas montré d'amélioration

significative des marqueurs de la dysfonction endothéliale (ICAM-1 soluble, protéine C réactive, myéloperoxydase, ox-LDL) hormis pour le rapport activité myéloperoxydase/antigène qui diminue significativement dans le groupe molsidomine. Cependant, la molsidomine ne semble pas avoir d'effets délétères sur la fonction endothéliale contrairement au dinitrate d'isosorbide [6] ou à la trinitrine [7]. D'autres donneurs de NO comme les *S*-nitrosothiols représentant la forme physiologique de stockage de NO sont actuellement en étude pré-clinique. En effet, les *S*-nitrosothiols ont montré des capacités à prévenir l'oxydation des LDL [8], à diminuer le stress oxydant en inactivant la NADPH oxydase et à induire une déplétion spécifique des macrophages dans la plaque d'athérome [9]. Finalement, ils pourraient prévenir la formation précoce de la plaque d'athérome et modifier modérément le profil des lipides circulants [10].

Parmi les *S*-nitrosothiols, nous nous sommes intéressés au *S*-nitrosoglutathion (GSNO), réserve de NO dans les tissus. GSNO est spécifiquement métabolisé par la gamma-glutamyl transférase (GGT) dont la concentration plasmatique est corrélée avec l'augmentation du risque de pathologie cardiovasculaire [11]. De plus la GGT a récemment été identifiée au sein des plaques d'athérome [12]. Ainsi ce travail de thèse est divisé en trois parties :

- Identification de la provenance (monocyte/macrophage ou cellule musculaire lisse) de la GGT au sein de la plaque d'athérome – rôle de l'inflammation
- Etude lien inflammation / stress oxydant sur monocytes/macrophages
- Evaluation de l'impact du stress oxydant sur la biodisponibilité de NO à partir de GSNO pour les cellules musculaires lisses, ainsi que l'évaluation de l'implication des deux enzymes redox, GGT et protéine disulfure isomérase (PDI), dans le métabolisme du GSNO et dans la *S*-nitrosation des protéines.

CHAPTER I

Introduction

1.1 Cardiovascular diseases and the atherogenesis process

Cardiovascular diseases (CVD) are the leading cause of mortality in developed countries and are likely to attain this status worldwide, accounting for 16.7 million deaths each year [13, 14]. Coronary artery disease and stroke, whose underlying pathological characteristic is atherosclerosis, are the most common forms of CVD. Atherosclerosis is a slowly progressing chronic disease of large and medium sized arteries which is characterised by the formation of atherosclerotic plaques consisting of accumulated modified lipids, leukocytes, foam cells, migrated smooth muscle cells (SMCs) and altered endothelial cells (ECs), leading to the formation of necrotic cores with calcified regions [15].

Since the term atherosclerosis was first introduced by Jean Lobstein in 1829 [16], it has long been believed that atherosclerosis involved the merely passive accumulation of cholesterol in arterial walls. Today, the picture of atherosclerosis is much more complex as it has been considered as a chronic inflammatory disease combined with oxidative stress, involving both the innate and adaptive immune systems, which modulate the initiation and progression of the lesions, and potentially followed by devastating thrombotic complications [17]. Understanding the principles of the inflammatory and oxidative processes is important for deciphering the complex processes involved in atherosclerosis progression. Atherosclerotic plaques are characterised by an accumulation of oxidized lipids in arterial walls combined with immunocytes infiltration. The degree of infiltration of inflammatory cells in atherosclerotic lesions is determined on the basis on monocyte recruitment, macrophage exit, and the balance between proliferation, survival, and apoptosis of several cells within the arterial walls [18, 19].

Moreover, oxidative stress, characterized by an increased production of free oxygen as well as nitrogen radicals, represents also basic pathogenetic processes of atherosclerosis and is closely related to endothelial dysfunction, promotes vascular inflammatory response and is involved in the initiation and progression of the disease. There is now consensus that atherosclerosis represents a state of heightened oxidative stress involving lipids and proteins in the vascular wall. Reactive oxygen species (ROS) are key mediators of signaling pathways that underlie vascular inflammation in atherogenesis, starting from the initiation of fatty streak development, through lesion progression, to ultimate plaque rupture [20]. Although there is considerable overlap and synergy between oxidative stress and pro-inflammatory conditions, it is not clear whether they can be controlled independently of each other or how they reciprocally affect each other.

1.1.1 Development of the atherosclerotic process

Atherosclerotic lesions have a slow evolution with an initial phase of endothelial damage leading to endothelial dysfunction, accumulation of oxidized lipids and cell infiltration into the intima. The formation of an atheromatous *core*, composed of oxidized lipids and phagocytic cells, and the formation of a fibrous

cap due to smooth muscle cells proliferation determine the so-called fibroatheroma, with or without calcification. Considering the importance of inflammatory processes in atherosclerosis development, the involvement of different infectious agents has been suggested [21]. Among the most studied pathogens, *Chlamidophila pneumoniae* [22], the herpes viruses, especially the human cytomegalovirus (CMV), [23] and the periodontal pathogens [24, 25] are mentioned. To date, however, no evidence is statistically valid to support the role of these agents in etiology of atherosclerotic disease.

Atherosclerotic lesions are localized in different vascular beds with different clinical manifestations, and in preferential sites such as the bifurcation/branching of vessels, where they create the favorable conditions to increase blood flow turbulence [26]. Over the decades, the progressive growth of the lesions in the vascular lumen will reduce the blood perfusion of the affected vessel, determining morphological (atrophy/remodeling) and functional (failure) modifications. This process occurs very slowly, and the majority of individuals, while being carriers of many atherosclerotic plaques, never develop clinical consequences of atherosclerosis in the arc of their life. The most dangerous and frequent consequences of atherosclerosis consist instead in acute events, such as myocardial infarction and cerebral stroke. These latter events lead to the sudden obstruction of blood flow due to thrombotic and bleeding events of the plaque which is independent of the size of the lesion. The occurrence of individual clinical consequences of atherosclerosis depends on the structural evolution of the plaque. In the case of chronic consequences proliferative/hyperplastic aspects prevail to reduce the lumen of the vessel. In the case of acute occurrences, plaques undergo structural alteration resulting in plaque breaks. The breaking or the ulceration (*i.e.* the loss of the endothelial lining) of the plaque allow the contact between the contents of the lesion, highly procoagulant, and the blood, triggering the hemostatic-coagulation and thrombosis processes of the vessel. At this stage the unstable plaque can announce with characteristics clinical syndromes (unstable angina, transient ischemic attacks) that precede the events of greater severity. Although in recent years great strides have been made to understand the mechanisms of atherosclerosis development and the related clinical syndromes, by developing animal and cell models, some aspects still remain to be clarified. The role of traditional risk factors (family history, high blood pressure, dyslipidemia, cigarette smoking, obesity, insulin resistance and diabetes mellitus) has now been established within epidemiological studies of large populations, but the absence of these risk factors does not fully protect from the development of the disease.

1.1.2 Morphology of the atherosclerotic plaque

Initial stages. The first signs of atherosclerosis occur with lipid accumulation, the so-called fatty streaks. They are formed from lipid-rich macrophages infiltrated below the endothelial layer. One of the most important underlying causes of this phenomenon is the oxidation of the lipid components of low-density

lipoproteins (LDL) forming oxidized LDL (oxLDL) (**Fig. 2A**). The oxidation can be minimal (minimally modified LDL, mmLDL) and do not inhibit the LDL recognition by LDL receptors expressed on macrophages. The oxidation can also be extensive, as it is the case for the apolipoprotein A (apoA), which is fragmented by oxidation and shows lysine residues covalently link to oxidized lipids. These oxLDL are no longer recognized by LDL receptors, but rather by neutralizing or "scavenger" receptors present on macrophages and smooth muscle cells of the media layer. The place and time of LDL oxidation are not clearly defined. Plasma circulating LDL are protected from oxidation, however they undergo oxidation during their infiltration in the vessel wall and their binding to the matrix proteins of endothelial cells and macrophages. The mmLDL and oxLDL can then exert a series of proinflammatory and prothrombotic activities leading first to the establishment and then to the expansion of the atherosclerotic plaque [27].

OxLDLs increase the expression of the intracellular adhesion molecules (ICAM-1) and the vascular adhesion molecule 1 (VCAM-1) on endothelial surface — resulting in monocytes recruitment, adhesion and transmigration — as well as the production of chemokines (**Fig. 2A**). This leads to monocytes diapedesis in lipid accumulating zones. Infiltrated monocytes subsequently differentiate into macrophages and overexpress a number of cell surface molecules, including scavenger receptors. These latter mediate the internalization of oxLDL and other modified LDLs resulting in the formation of lipid-laden foam cells.

The infiltration of monocytes is a defense mechanism to eliminate oxLDL, which are damaging for the vascular wall. However, the uncontrolled accumulation of macrophages (usually regulated by monocyte exit and apoptosis) may contribute to further progression of the damage. Infiltrated macrophages induce the recruitment of smooth muscle cells from the media through the production of growth factors, such as Platelet Derived Growth Factor (PDGF), Transforming Growth Factor β (TGF- β) and Fibroblast Growth Factor (FGF).

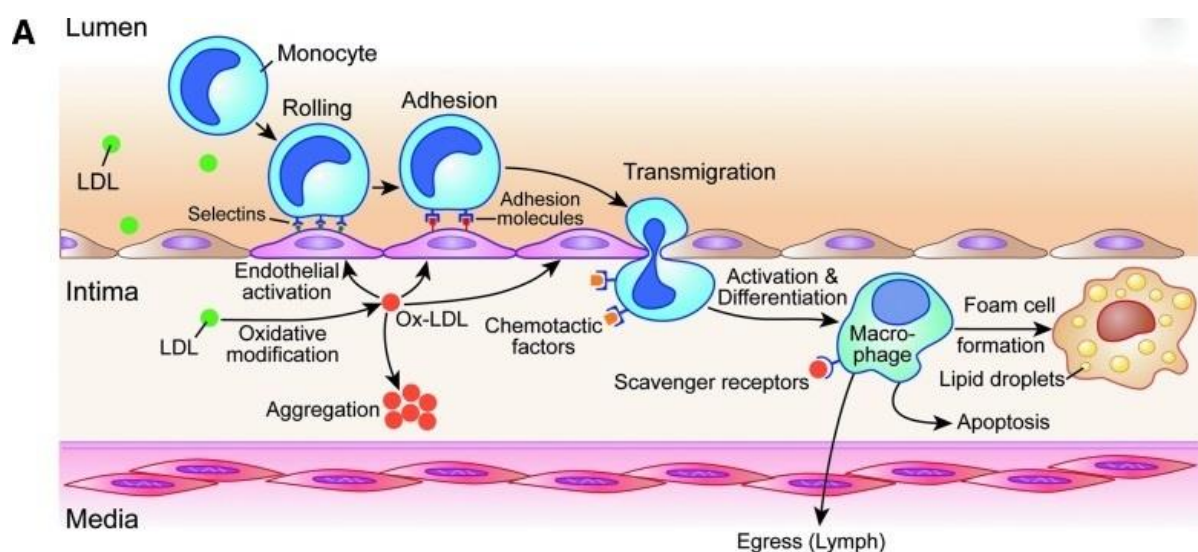


Figure 2. An overview of atherogenesis process. (A) The initial atherosclerotic stage [42].

More advanced stages (fibroatheroma).

During this stage, two major events are occurring inside the plaques:

- **Formation of a lipid core limited by a fibrous cap**
- **Phagocytosis of oxLDL by macrophages**

The lipid core is composed of foam cells undergoing apoptosis and oxidized lipids. This lipid core is delimited by a fibrous capsule combining matrix proteins such as fibronectin and collagen. Inside the lipid core, infiltrated macrophages engulf oxLDL and turn into foam cells. This process is mainly mediated by neutralizing or scavenger receptors and by the cluster of differentiation 36 (CD36), which binds many ligands including collagen, oxLDL and is involved in the phagocytosis process of macrophages. Phagocytosed cholesterol from oxLDL is esterified and stored in lipid-laden form, thus conferring the typical foamy appearance to macrophages [19]. Intracellular accumulation of free cholesterol triggers macrophage death [28].

As mentioned above, macrophages contribute to the migration of smooth muscle cells in the intima and in sub-endothelial layers. This phenomenon characterizes the later stages of the atherosclerotic plaque [29]. Smooth muscle cells can proliferate, but also bind and internalize oxLDL through their scavenger receptor and in this way, they also acquire the characteristics of foam cells [30, 31]. Moreover, these cells produce proteins of the matrix and contribute to the formation of fibrous tissue which characterizes the later stages of plaque. At this stage, frequently calcification areas are found and may be in the form of granules (microcalcifications or spotty calcifications) or, less often, of macrocalcifications (lamellar calcifications) with the appearance of hard and brittle flakes or splinters or sharp, and sometimes as real areas of ossification. The spotty calcifications prevail in the early stages of atherosclerosis, while macrocalcifications are typical of advanced forms. Responsible for the calcification (precipitation of calcium phosphate in the intima in the form of hydroxyapatite crystals) appear to be both the apoptotic vesicles derived from necrosis of foam cells and from smooth muscle cells, cells capable of morphological and functional pluripotency, so as to be able to acquire a fibroblast-like phenotype and also osteoblastic [32, 33, 34, 35, 36]. Moreover, it would appear to be due to a specific subpopulation of smooth muscle cells of the media indicated as "calcifying vascular cells." [35]. This active process acts in concert to initiate and propagate hydroxyapatite mineral deposition in the vessel media, leading to arterial stiffening and atherosclerotic plaque rupture [32, 37].

Moreover, activated T lymphocytes are present in the plaque and they may affect plaque progression. The type 1 helper (Th1) lymphocytes produce interferon gamma (IFN- γ) that has opposite effects on atherogenesis. These include the ability to reduce the expression of the scavenger receptor on monocytes and inhibit the proliferation of smooth muscle cells. However, IFN- γ induces the production of inflammatory cytokines by macrophages and induces the expression of major histocompatibility antigens of class II (MHCII). The ability of macrophages to present the antigen is related to the presence of antibodies

against oxLDL or proteins modified during the process of plaque formation. These antibodies can be measured in the circulation and constitute the possible markers of lesion progression [29].

Ulceration and plaque rupture. Contrary to the overall atherosclerotic process, the plaque rupture is a rapid and unpredictable event. Plaque rupture is the most dangerous event in the evolution of atherosclerosis because it is followed by coagulation cascade activation leading to the rapid formation of a thrombus, that may occlude the lumen of the vessel and cause ischemia and necrosis of perfused tissues. The persistent production of inflammatory mediators, including chemokines (Monocyte Chemoattractant Protein-1 [MCP-1] and Regulated on Activation, Normal T cell Expressed and Secreted chemokine [RANTES]), cytokines (interleukin 1 [IL-1], IL-6, tumor necrosis factor alpha [TNF- α] and IFN- γ), proteases (matrix metalloproteinases and cathepsins), and ROS formed by infiltrated immune cells, generates an inflammatory environment which promotes the recruitment, accumulation, and activation of additional inflammatory cells and smooth muscle cells, resulting in plaque expansion. As inflammation is a systemic phenomenon, the search for specific markers of inflammation of atherosclerotic plaque has led to identify a myriad of surrogate markers, variously located in the endless transcriptional program that triggers with the inflammatory response [38, 39]. Among those proposed, there are also serum amyloid A (SAA) [40] and C-reactive protein (CRP). This latter is one of the most studied among the inflammatory markers; indeed several studies have demonstrated a correlation between circulating CRP values and instability of coronary plaques [41].

Moreover, macrophage foam cells undergo cell death through apoptotic or nonapoptotic pathways (*e.g.* oncosis). Foamed cell lysis, impaired efferocytosis (clearance of apoptotic cells) and subsequent secondary necrosis promote the formation of necrotic cores and further inflammatory responses within advanced atherosclerotic plaques. In vulnerable plaques, these inflammatory mechanisms lead to the thinning of the protective fibrous cap and the expansion of the necrotic core, predisposing these lesions to mechanical destabilization and plaque rupture [42] (**Fig. 2B**). Whereas, the capsule thickness is critical in determining its fragility, the size of plaques is not prognostic of its breaking. Indeed, the size of the lipid core and the thickness of the capsule are determined by accumulation of foamed apoptotic cells, by the release of lipids and by the production of lytic enzymes by macrophages. Furthermore the progression of the plaque is favored by the formation of new blood vessels (angiogenesis). Neovascularization of the plaque seems to be very important in the establishment of micro-haemorrhages and in inducing fragility [27].

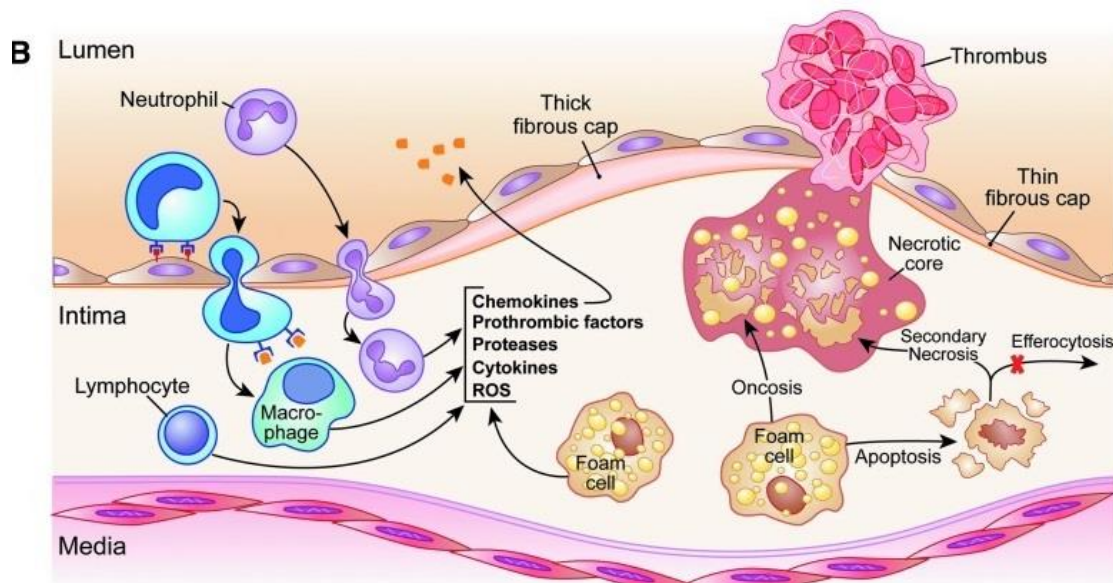


Figure 2. An overview of atherogenesis process. (B) The advanced atherosclerotic plaque [42].

Although substantial efforts have been made to dissect molecular mechanism of atherogenesis, a full understanding of the underlying mechanisms is still missing. However, activation of immune competent cells, leading to local and finally systemic inflammatory phenomena and the associated status of heightened oxidative stress are central events [43]. Oxidative stress is thought to have an important implication in the pathophysiology of injury in atherosclerosis through induction of various cellular/molecular reactions.

In atherosclerosis, during Th1-type response, IFN- γ is probably the most important trigger for high ROS production in macrophages [44] due to nicotinamide adenine dinucleotide phosphate (NADPH), oxidation by the granules of resting and phagocytizing cells [45]. Main reactive species are hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), but also reactive nitrogen species such as peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2) and trioxide (N_2O_3) [46]. IFN- γ signaling initiates a variety of cellular defense mechanisms such as pro-inflammatory cytokine production *via* nuclear factor kappa B (NF- κ B) signaling, enhancement of antigen presentation [47] and other important mechanisms, *e.g.*, neopterin formation *via* guanosine triphosphate (GTP)-cyclohydrolase I (GTP-CH- I) and indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan breakdown [48]. Under normal conditions, low levels of ROS are mainly byproducts from electron transport chain reactions in the mitochondria [49]. They are important regulators of several redox-sensitive pathways involved in the maintenance of cellular homeostasis [50], and act by modifying molecules, enzymes and transcription factors as well as by interfering with the endogenous antioxidant pool [46, 50, 51]. Depletion of endogenous redox buffer systems in conditions of overwhelming oxidative stress is critical, not only due to triggering of immune responses but also through induction of endothelial and smooth muscle dysfunction, and thus progression of atherosclerosis [52, 53].

Therefore, the oxidative stress plays not only an aetiopathogenetic role, but it is also strongly linked to the inflammatory process, since many reactive species of oxygen are mediators of inflammation or inflammatory cell functions. Oxidative stress also plays an important role in the genesis of plaque, *i.e.* the oxidation of LDL [54]. Although a complex inflammatory signaling regulates cellular activities inside the plaque, the oxidative-reductive reactions (redox) also contribute to the pathogenesis of plaque, modulating the proliferation of smooth muscle cells, apoptosis, and in general the remodeling of lesions through the regulation of proteases and antiproteases. Some aspects of endothelial dysfunction (dysregulation of nitric oxide synthesis and expression of adhesion molecules) are regulated by oxidative events, as well as other key steps of platelet function regulation and signal transduction [54]. For these reasons, even elements of the redox regulation have been considered as potential biomarkers of atherosclerosis and related risk.

1.2 Main cellular components in the atherosclerotic plaque

1.2.1 Endothelial cells in atherosclerosis

Blood vessels are made of three layers including the intima consisting of a single layer of endothelium, the media containing a mixture of SMCs and elastic fibers, and the adventitia composed of fibroblasts, collagen fibers, and perivascular nerves.

The atherosclerotic process begins from ECs, and so from the innermost layer of the arterial vessel. Consider the endothelial tissue as a simple lining of the vessels is very simplistic, indeed, because of the unique localization between circulating blood and the vessel wall, the endothelium has been suggested to play a crucial role in development and progression of atherosclerosis. It is known that the endothelium is in fact a metabolically active organ, playing a crucial role in the maintenance of vascular homeostasis by releasing a variety of vasoactive factors that can either dilate or constrict the blood vessels, depending on the type of the stimulus [55].

Vascular homeostasis entails keeping a tightly controlled balance between a vasodilatory state, which is often associated with anti-oxidant, anti-inflammatory and anti-thrombotic effects on one hand, and a vasoconstrictory state on the other, which is associated with pro-oxidant, pro-inflammatory and pro-thrombotic effects [56]. The vasodilatory state is mediated by factors such as nitric oxide (NO), endothelium-derived hyperpolarising factor (EDHF) and prostacyclins, while a vasoconstrictory state is mediated by factors such as endothelin-1 (ET-1), angiotensin II and thromboxane A₂ [55, 56]. Of these endothelial-derived factors, NO, which was originally identified as the endothelial-derived relaxing factor (EDRF), has since evoked much interest as it is considered to be the most potent endogenously synthesised vasodilator in the body, and a key marker of endothelial function and dysfunction.

Endothelial dysfunction, characterized by reduced NO bioavailability, is now recognised by many as an early, reversible precursor of atherosclerosis. Oxidative stress appears to be the common underlying

cellular mechanism in the ensuing loss of vaso-active, inflammatory, haemostatic and redox homeostasis in the body's vascular system. For these reasons, endothelial dysfunction has emerged as a potentially valuable prognostic tool in predicting the development of atherosclerosis. The progression from the early changes observed in compromised vascular endothelium (endothelial activation and dysfunction) to atherosclerosis is complex and multifactorial [57].

The healthy, intact endothelium is a highly selectively permeable barrier and does not promote leukocyte adhesion and invasion, or platelet aggregation and adhesion [58]. However, as the endothelium progresses to a dysfunctional state, vascular homeostasis becomes impaired, leading to reduced anti-oxidant, anti-inflammatory and anti-thrombotic properties (due to reduced NO bioavailability), enhanced endothelial permeability (barrier dysfunction), upregulated pro-inflammatory cytokine levels, and expression of adhesion molecules such as VCAM-1 and ICAM-1, which facilitate leukocyte adhesion to the endothelium [58]. Leukocyte adhesion represents one of the first steps in the initiation of atherosclerosis. After adhering to the endothelium, leukocytes (monocytes and lymphocytes) cross the endothelium and migrate into the intima [59, 60]. Migration to the intima is mediated by chemo-attractants such as MCP-1 [61]. Upon reaching the intima, monocytes transform into macrophages and express receptors that facilitate uptake of lipids. Uptake and accumulation of lipids lead to the transformation of macrophages into foam cells, which initiate an atherosclerotic lesion and further enhance release of inflammatory cytokines [59, 62]. Through these complex mechanisms, a cascade of events, which begins with the formation of an early atherosclerotic lesion, leading to an advanced lesion characterised by a plaque formation ensues [62].

Although endothelial cells represent the first cellular component involved in the atherosclerotic process, in this thesis work this latter aspect has not been widely investigated. Instead, the attention is mainly focused on the two cell types found in atherosclerotic lesion, macrophages and smooth muscle lineage cells, as amply described in sub-paragraphs below.

1.2.2 Monocyte/macrophages in atherosclerosis

Monocytes and macrophages play important roles in the initiation and progression of many chronic inflammatory diseases associated with oxidative stress, such as atherosclerosis.

A pivotal step in atherogenesis involves the subendothelial accumulation of monocyte-derived macrophages at predisposed sites of endothelial dysfunction and intimal lipoprotein retention, after transforming into foam cell macrophages (FCMs) [15]. In nascent lesions, these cells orchestrate the scavenging of lipids and cellular debris, as well as the local inflammatory equilibrium to ultimately define the likelihood of plaque complications [63, 64]. Therefore, monocyte-derived macrophages are instrumental to the atherogenic process and contribute to its initiation, progression and symptomatology. As plaque development may originate not only from persistent inflammation, but also from inadequate

anti-inflammatory responses, the macrophage polarization balance holds clear implications for lesion formation [65, 66]. Foam cells are not able to leave the initial plaque and contribute to the failure of inflammation resolution and further establishment of a complicated atherosclerotic plaque [67]. Macrophages can be commonly identified in the lesion shoulder and calcified plaque regions [68, 69]. Dying macrophages extensively contribute to the formation of the necrotic core and aggravation of the proatherosclerotic inflammatory response [70, 71].

Despite major advances in understanding the various functions of macrophages in atherosclerotic lesions [72, 73], it is generally accepted that the tissue microenvironment determines macrophage phenotypic polarization [74]. That is, on the one hand, macrophages specifically respond to extracellular cues ranging from bacterial components to oxidatively modified molecules [75], translating information by utilizing a range of cell surface receptors and their associated intracellular signaling [66]. On the other hand, macrophages also respond to changes in their intracellular environment such as cholesterol loading or endoplasmic reticulum-stress by inducing highly specified adaptive mechanisms and reactions [70, 76] using intracellular sensors such as redox-dependent transcription factors or nuclear hormone receptors.

Even though there are reports suggesting that macrophages can transdifferentiate into dendritic cells [77, 78, 79], it is commonly thought that plaque macrophages represent a population of terminally differentiated cells of monocytic origin; nevertheless, macrophages are influenced by multiple microenvironmental stimuli that could drive macrophage polarization towards more or less proinflammatory phenotype [80]. Several macrophage phenotypes could be observed in the plaque [81]. Indeed, macrophage phenotype is reversible and could be changed in response to different microenvironmental signals: macrophages are hallmarked by phenotypic heterogeneity and express a spectrum of activational programs that exist as a function of their immediate surroundings.

In the onset of atherosclerosis, LDL present in the circulation enter the activated or damaged vessel wall and become modified (*e.g.* oxidation resulting in oxLDL). These modified lipoproteins trigger an immune response and thereby attract blood monocytes. Circulating monocytes in the mouse exist as two equally abundant major subsets with differing cell surface marker and chemokine receptor expression patterns, that is, Ly6C^{high}CCR2+CX3CR1^{low} (“inflammatory”) vs. Ly6C^{low}CCR2–CX3CR1^{high} (“resident”). Ly6C^{high} monocytes are short-lived in the circulation and rapidly move into foci of acute inflammation, such as recent myocardial infarctions [82] and into early atherosclerotic plaques [83, 84]. By contrast, Ly6C^{low} monocytes persist longer in the circulation, where they engage in so-called patrolling behaviour, interacting with the endothelium without extravasation [85]. Ly6C^{low} monocytes show delayed incorporation into inflamed and damaged tissues, including infarcted myocardium [82]. In humans, blood monocytes are mainly divided into two populations based on cluster of differentiation 16 (CD16) and 14 (CD14) expression: CD14^{high}CD16^{low} macrophages (about 80-90%), that are the phenotypic equivalent of the Ly6C^{high} population in mice, whereas CD14^{low}CD16^{high} macrophages are the phenotypic equivalent of Ly6C^{low} [85, 86, 87].

However, functional equivalence with the mouse subpopulations is unclear and directly extrapolating any of the findings from mouse to humans is therefore premature. The majority of CD14^{high}CD16^{low} monocytes seem anti-inflammatory, as they produce the cytokine IL-10 in response to bacterial lipopolysaccharide (LPS). Conversely, the smaller CD14^{low}CD16^{high} population seems proinflammatory because it produces proinflammatory mediators in response to LPS and shows an increase in plasma levels during inflammatory conditions, including atherosclerosis [88]. Although different monocyte types may enter the atherosclerotic lesions, the majority of plaque infiltrating monocytes are from Ly6C^{high} origin [83, 84]. After migrating into the subendothelial space, monocytes differentiate into macrophages which engulf the modified lipids and become foam cells.

In vitro, the monocyte differentiation is driven by two growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF), leading to the formation of macrophages that are phenotypically similar to M1 and M2 subsets of macrophages [81]. The classic M1 phenotype of activated macrophages is characterized by a pro-inflammatory phenotype, by expressing a broad spectrum of proinflammatory cytokines (TNF- α , IL-1 β , IL-12, and IL-23) and chemokines (C-X-C motif chemokine (CXCL9, CXCL10, and CXCL11)) [89]. The anti-inflammatory M2 macrophages typically secrete high amounts of anti-inflammatory IL-10 and contribute to tissue remodeling, vasculogenesis and tumor development [90].

However, the M1/M2 system does not reflect the complexity of phenotypic subsets of macrophages. Depending on the activating stimulus, M2 macrophages can be divided into four subgroups: M2a, M2b, M2c and M2d are induced by M-CSF and IL-4 or IL-13, immune complexes together with LPS or IL-1 β , M-CSF and IL-10, and by TLR (Toll-like receptor) agonists through the adenosine A2A receptor (ADORA2A), respectively [91, 92].

Boyle et al. [93] described a new macrophage population (HA-mac) in hemorrhagic areas of human plaques that expressed high levels of CD163 but low levels of human leukocyte antigen (HLA)-DR. This population was shown to possess atheroprotective properties by sensing Hb-Hp (hemoglobin-haptoglobin) complexes *via* the receptor CD163 followed by further Hb clearance and reduction of oxidative stress. In principle, Hb has atheroprotective effects by preventing formation of foam cells [94] and activation of the nuclear transcription factor (erythroid-derived 2)-like 2 (Nrf2) that in turn induces expression of heme oxygenase 1 [95].

Another human atheroprotective macrophage subset called Mhem is closely related to HA-mac macrophages and also involved in Hb clearance through phagocytosis of erythrocytes. This subset is characterized by high expression of CD163 and heme-dependent activating transcription factor (ATF)-1 [96, 97]. The expression of these factors in turn leads to induction of other genes central to cholesterol efflux regulation. Indeed, the Mhem phenotype of macrophages is characterized by increased cholesterol efflux associated with increased production of IL-10 and apolipoprotein (Apo)E [98]. Furthermore, both Mhem

and HA-mac macrophages exhibit increased adaptation to intraplaque hemorrhage [96, 98]. This subset is also characterized by increased resistance against foam cell formation but enhanced ROS production. Collectively, Mhem, HA-mac and M(Hb) macrophages are able to reduce intraplaque hemorrhages that arise from plaque neovascularization and erythrocyte infiltration into the plaque. These macrophage subsets are involved in utilization and recycling of iron accumulated in the plaque [97].

Mice share with humans M1, M2 and M(Hb) macrophage phenotypes but have a specific proinflammatory subset called Mox macrophages. Mox macrophages were shown to be abundantly present in murine lesion, accounting for 30% of plaque macrophages of low density lipoprotein (LDL) receptor knock-out (LDLR^{-/-}) mice [99]. This macrophage population could be induced by oxidized phospholipids and produces high levels of heme oxidase 1 in Nrf2-dependent manner. Mox macrophages show proatherogenic properties through elevated production of IL-1 β and ROS.

Finally, recently discovered M4 macrophages could have a potential proatherogenic role in unstable plaques and may be involved in late atherosclerosis complications such as acute coronary syndrome and arterial thrombosis, since they are activated by platelet-derived CXCL4. They lose the Hb–Hp scavenger receptor CD163 that is essential for hemoglobin clearance after plaque hemorrhage. As a consequence, lack of CD163 leads to the inability to stimulate the atheroprotective enzyme heme oxygenase-1 in response to Hb–Hp complexes, and this could suggest a potential role of the M4 subset in atherosclerosis [100]. M4 macrophages possess proinflammatory properties since they express IL-6, TNF- α , and matrix metalloproteinase MMP-12 [101]. However, the actual role of M4 cells in atherogenesis is unknown and should be investigated.

An overview of macrophages subsets observed in human and mice is reported in **Table 1**.

Table 1. Overview of macrophage phenotypes observed in human and mice (*Chistiakov DA et al., International Journal of Cardiology, 2015*)

Phenotype	Induction	Cell markers	Cytokines, chemokines, and other secreted factors	Functions and properties	Presence in the plaque
M1	IFN- γ , TNF α , LPS	IL-1 β , IL-6, IL-12, IL-23, TNF α , CXCL9, CXCL10, CXCL11, Arg-2 (mouse)	IL-6, IL-10 ^{low} , IL-12 ^{high} , IL-23, TNF α , iNOS, ROS	Th1 response, tumor resistance	Human, mouse
M2a	IL-4, IL-13	Human: MR, IL1RN; Mouse: Arg-1, FIZZ1, Ym1/2	IL-10, TGF- β , CCL17, CCL22	Tissue remodeling	Human, mouse
M2b	IL-1 β , LPS	IL-10 ^{high} , IL-12 ^{low}	IL-6, IL-10 ^{high} , IL-12 ^{low} , TNF α	Immunoregulation	Human, mouse
M2c	IL-10, TGF- β , glucocorticoids	MR (human); Arg-1 (mouse)	IL-10, TGF- β , PTX3	Phagocytosis of apoptotic cells	Human, mouse
M2d	TLR + A ₂ R ligands	IL-12 ^{low} , TNF α ^{low}	IL-10, VEGF, iNOS	Angiogenesis, tumor progresion	Mouse
M4	CXCL4	MR, MMP7, S100A8	IL-6, TNF α , MMP12	Weak phagocytosis, minimal foam cell formation	Human
Mox	oxLDL	HMOX-1, Nrf2, Srxn1, Txnrd1	IL-1 β , IL-10	Weak phagocytosis, proatherogenic	Mouse
HA-mac	Hemoglobin/haptoglobin	CD163 ^{high} , HLA-DR ^{low}	HMOX-1	Hemoglobin clearance, atheroprotective	Human
M(Hb)	Hemoglobin/haptoglobin	MR, CD163	ABCA1, ABCG1, LXR α	Hemoglobin clearance, high cholesterol efflux, atheroprotective	Human
Mhem	Heme	ATF1, CD163	LXR β	Erythrophagocytosis, atheroprotective	Human, mouse

1.2.3 Smooth muscle cells in atherosclerosis

The primary functions of vascular smooth muscle cells are the contraction and the regulation of blood vessel tone, thus distributing blood flow and regulating blood pressure. Fully differentiated vascular SMCs display an elongated spindle-shaped morphology and express a unique repertoire of contractile proteins that serve as SMC markers, including α -smooth muscle actin (α -SMA), calponin, smooth muscle protein 22- α (SM22 α), SM myosin heavy chain (SMMHC) and smoothelin [102]. In contrast to skeletal and cardiac muscle cells that differentiate terminally, vascular SMCs retain a high degree of plasticity *in vivo* and *in vitro*.

During the so-called “phenotypic modulation”, SMCs can dedifferentiate from a contractile phenotype to a highly proliferative synthetic phenotype in response to local environmental cues including growth factors/inhibitors, mechanical influences, cell-cell/cell-matrix interactions, various inflammatory mediators, etc. [102]. Vascular SMCs differentiation is an important process during vascular development. The highly differentiated mature SMCs play critical roles in maintaining structural and functional integrity of blood vessels. Therefore, alterations in SMCs phenotype contribute to a number of major cardiovascular diseases such as atherosclerosis, hypertension and restenosis following angioplasty. Accumulation of SMCs is a hallmark of neointimal formation, and can be considered as the “soil” of atherosclerosis. Indeed, focusing the attention on atherosclerosis, the evolution of a fatty streak to an atherosclerotic plaque involves activation of macrophages by T lymphocytes through IFN- γ and cluster of differentiation 40 (CD40) [58, 103]. Once macrophages are activated, they produce cytokines, chemokines and growth factors, promoting SMCs migration and proliferation in the intimal layer [104]. SMCs then synthesize the collagen forming the fibrous cap that surrounds the lipid and necrotic core of the atherosclerotic plaque. The fibrous cap prevents exposition of the thrombogenic lipid core to arterial blood and provides mechanical strength and stability to the plaque [105]. Accumulation of activated inflammatory cells, such as macrophages and T lymphocytes, in the shoulder region of the plaque amplifies the inflammatory process by the release of pro-inflammatory cytokines. However, as the plaque matures, the SMCs and macrophages within the fibrous cap also release MMPs [106, 107] that degrade the extracellular matrix. All these events weaken the fibrous cap and render the plaque prone to rupture [15, 108].

SMC differentiation is orchestrated by a precisely coordinated molecular network that integrates a variety of factors including different environmental cues, signaling pathways, transcription factors, ROS, extracellular matrix (ECM), microRNAs, and chromosome structural modifiers [109, 110, 113]. In particular, hydrogen peroxide and superoxide anion are traditionally considered as harmful substances that cause cellular dysfunction in various systems, including the cardiovascular system [111].

The major source of ROS in the cardiovascular system is represented by nicotinamide adenine dinucleotide phosphate-oxidases (NADPH oxidases, NOX). To give an example, NOX1 and NOX4, two major NOX isoforms, have been identified in human and rodent aortic SMCs [112]. Interestingly, the literature

supports the important role of NOX4 in maintaining differentiated SMCs phenotype. In contrast, NOX1 is involved in signal transduction leading to SMCs hypertrophy and proliferation [112]. The role of ROS in SMC differentiation is further confirmed by a study showing that NOX4-produced H_2O_2 mediates the differentiation of mouse embryonic stem cells towards SMC lineage using collagen IV as a coating substrate [113]. In addition Nrf3, a nuclear factor erythroid 2-related factor, is found to promote NOX4-mediated ROS production and enhances SMC differentiation of embryonic stem cells by binding to promoter regions of *Pla2g7* (Phospholipase A2, group 7) gene [114]. These results demonstrate the importance of NOX4-ROS signals in SMC differentiation.

Moreover, the deposition of LDL in the vessel wall and their oxidative modification seem to initiate, or at least accelerate, the atherosclerotic process by several mechanisms, including SMC phenotypic modulation. OxLDL is detected in atherosclerotic plaques and in the plasma of atherosclerotic patients, where it contributes to disease evolution. Depending on the extent of its oxidation, oxLDL can induce proliferation, monocyte chemotaxis, apoptosis or necrosis of vascular endothelial cells and SMCs.

H_2O_2 is a major oxidative component of oxLDL-induced ROS and it can induce an increase in ROS formation, specific alterations in gene/protein expression, as well as apoptosis of vascular smooth muscle cells [115]. Indeed, several studies show that oxLDL and H_2O_2 can significantly decrease the expression of important proteins expressed in SMC and involved in their ECM adhesion and migration, such as lipoma preferred partner (LPP). At higher concentrations, OxLDL also upregulates the expression of oxidized low-density lipoprotein receptor-1 (LOX-1) and induces apoptosis of vascular SMCs [116], a process that may contribute to atherosclerotic plaque destabilization. Furthermore, in vascular SMCs, another LDL receptor, the low density lipoprotein receptor–related protein (LRP), mediating the binding and internalization of aggregated LDL (agLDLs) has been discovered [117]. LRP, contrary to the LOX-1, has multiple binding sites and is not regulated by intracellular cholesterol concentration. Therefore, LRP-mediated endocytosis can be considered as a low-specificity and high-capacity mechanism that allows the uptake of large amounts of ligand, *i.e.* agLDLs. LRP is highly expressed in atherosclerotic plaques and because of subendothelial LDL retention and aggregation, the uptake of agLDLs through LRP could have a crucial role in VSMC-lipid deposition in atherosclerotic plaques [30].

It is conceivable that a mix of mechanical cues, accompanied by oxidative stress and substrate composition, may dominate and account for the downregulation of several important mediators involved in SMC differentiation. An upregulation of these molecules may occur in the neointima following vascular injury, associated with increased cell migration and proliferation, presumably reflecting different changes in substrate composition and stiffness as well as different signaling pathways.

In summary, phenotypic switching of vascular SMCs from the contractile type towards proliferation and mobility is a physiological response to repair vessel damage. However, in atherosclerosis, the normal reaction of vascular SMCs to change the phenotype could be impaired by proinflammatory stimuli and

oxidative stress. In particular, it has been known for a decade that the loss of endothelial NO production impairs endothelium-dependent dilatation and promotes vasospasm in atherosclerotic arteries. More recent evidence indicates that dysfunction of the endothelial NO pathway may promote atherosclerosis in view of the protective effects of NO against leukocyte adhesion, oxidative processes, smooth muscle cell migration and proliferation. On the other hand, there is ample evidence to consider NO as a molecular aggressor in chronic inflammatory processes like atherosclerosis. This latter aspect will be widely discussed in the next section.

1.3 Atherosclerosis and decrease in nitric oxide bioavailability

1.3.1 Synthesis and role of nitric oxide

In the cardiovascular system, the deficiency of NO, which is the consequence of either endothelium dysfunction or NO oxidative consumption (increased oxidative or nitrosative stress, decreased antioxidant enzyme activity) is one of the key factors for the initiation and progress of many diseases, including atherosclerosis. As described previously, the endothelium plays an important role in this context, maintaining the balance between vasodilating substances with antiproliferative activity (such as NO) and vasoconstrictor substances with mitogenic properties (such as ET-1) and any disturbance to this balance may cause damage to the arterial wall, by promoting the appearance of endothelial dysfunction. The pathophysiology of endothelial dysfunction is a complex phenomenon, engaging different mechanisms: it leads to vasoconstriction, platelet aggregation, monocyte adhesion and smooth muscle cells proliferation, and has been related to a reduced NO bioavailability, an excess of ROS and an oxidative stress-dependent increase in ET-1 action.

NO, one of the most important substance produced by the endothelium, plays a key role in homeostasis maintenance. NO is a gaseous radical with a half-life of $\approx 6\text{--}30$ s, continuously synthesized from L-arginine by the nitric oxide synthase (NOS) [118].

There are three distinct isoforms of NOS which differ in structure and function [119]. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and are referred as Ca^{2+} -dependent enzymes [120] and generate small amounts of NO for signaling. They are present as dimers, containing a flavin adenine dinucleotide molecule (FAD), a flavin mononucleotide (FMN), a heme group and a tetrahydrobiopterin (BH_4), whose absence leads to the production of superoxide rather than NO. These cofactors, together with calmodulin and the NADPH, are needed of enzymatic activity [121]. The third type is the inducible isoform (iNOS), Ca^{2+} -independent and inducible by immunological stimuli [122]. This latter is activated and generates high amounts of NO in response to inflammation [123].

NO is a chemical messenger, particularly in vascular and immune systems playing a critical role in the regulation of a wide range of physiological processes. Its production within the cell is very finely

adjusted to ensure the correct action of NO. Indeed, in physiological conditions, low concentrations of NO (10 nM), which acts as a vasodilator and inhibitor of platelet aggregation, are produced by eNOS, while the activation of iNOS induced during physiopathological processes such as inflammation produces much higher concentrations of NO ($> 1 \mu\text{M}$). iNOS is expressed physiologically, but is induced by certain inflammatory cytokines (IL-1, INF γ , TNF- α), by LPS and oxidizing agents. This induction is inhibited by glucocorticoids and some cytokines (including TGF- β).

The effect of NO depends on the site of formation, its concentration and the type of targeted tissue. NO released from the endothelium stimulates soluble guanylyl cyclase, producing increased concentrations of cyclic guanosine monophosphate (cGMP). Cyclic GMP interacts with three types of intracellular proteins: cGMP-dependent protein kinases (PKGs), cGMP-regulated ion channels, and cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs). Thus, cGMP can alter cell function through mechanism dependent or independent of protein phosphorylation. Depending on the direction of NO release and the site of cGMP activation, differing biological effects can be observed. In vascular SMCs increased cyclic GMP concentrations activate cGMP-dependent kinases that decrease intracellular calcium, producing relaxation [124], whereas increased cGMP in platelets decreases platelet activation and adhesion to the surface of the endothelium [125].

NO can modulate protein activity through three main reactions:

- Nitrosylation, a reversible coordination of NO to transition metal ions in enzymes, such as ferrous (Fe^{2+}) heme prosthetic groups within the soluble guanylyl cyclase (sGC) enzyme, leading to enzyme activation and increased formation of cGMP.
- Protein nitrosation, where NO forms a covalent bond with cysteine (S-nitrosation) or tryptophan (N-nitrosation) residues. Modifications of free cysteine residues present at active sites of effector proteins and peptides subsequently change the activity or function of these proteins. This corresponds to a posttranslational modification of proteins as important as phosphorylation [126, 127]. Numerous studies have focused on the mechanistic aspect of S-nitrosation, however it has also been shown that denitrosation plays a major role in the control of S-nitrosated proteins (Pr-SNO) levels and NO release. S-nitrosothiols can undergo spontaneously or assisted transnitrosation from high molecular weight thiols to low molecular one and inversely.
- Nitration, introduction of a NO_2 group covalently bound to the aromatic ring of tyrosine or tryptophan residues. These changes are often related to loss of function, due to oxidation or nitration of functionally important residues, as well as the proteolytic degradation of damaged proteins. Nitration mainly consists in the formation of nitrotyrosine, occurring *via* peroxynitrite in the context of heavy tissular inflammation and oxidative stress. Nitrotyrosine is identified as an indicator or marker of cell damage, inflammation as well as high concentration of NO production and proteins modifications by NO-derived oxidants. Previous studies have demonstrated that

nitrotyrosine is enriched in human atherosclerotic lesions and low density lipoprotein (LDL) issued from human atheromas [128].

Endothelial dysfunction would seem to represent the earliest event of the atherosclerotic plaque formation, before a structural lesion of the vessel wall becomes visible [129]. However, the decrease of NO bioavailability can have different other origins, like (i) impairment of endothelial membrane receptors that interact with agonists or physiological stimuli to release NO, (ii) diminished levels or impaired utilization of L-arginine substrate, or BH₄ cofactor of the nitric oxide synthase, (iii) reduction in concentration or activity of nitric oxide synthase; (iv) enhanced degradation of NO by oxygen free radicals, (v) impaired diffusion from endothelium to SMCs, (vi) impaired interaction of NO with soluble guanylate cyclase and the subsequent limitation of the increase in intracellular cGMP level, (vii) generalized decrease in smooth muscle cell sensitivity to vasodilators [130]. In turn, diminished NO bioactivity may facilitate vascular inflammation that could lead to oxidation of lipoproteins and foam cell formation, smooth muscle proliferation, extracellular matrix deposition or lysis, accumulation of lipid-rich material, platelet activation, and thrombus formation. All of these consequences of endothelial dysfunction may contribute to the development and clinical expression of atherosclerosis [131].

Finally, several studies have highlighted the relationships between NO, inflammation and oxidative stress, *e.g.* (i) NO is released during inflammation processes (mainly from induction of the inducible NO-synthase), (ii) NO induces positive or negative effects on vascular homeostasis according to its environment and concentration (with deleterious impact during oxidative stress and inflammation), and (iii) the released NO itself can modulate inflammatory mediators. The reason for such dual effects relates to concentrations, duration of exposure, and production from NO-synthases but also release from the endogenous reservoir represented by S-nitrosothiols (RSNOs), an important storage form of NO. A summary of NO effects related to its concentration and physiopathological conditions are reported in figure 3 (**Fig. 3**).

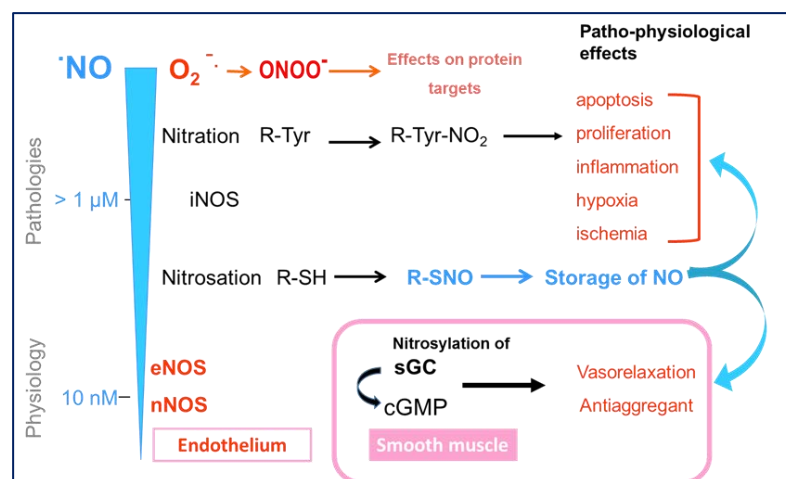


Figure 3. Vascular aspect: NO & physiopathological effects.

1.3.2 S-nitrosothiols: a class of nitric oxide donors

NO, despite being a very reactive molecule, appears to produce effects at distance from its synthesis site. These effects must be mediated by more stable molecules, capable of transporting and storing NO in its active form [132]. Stamler and colleagues postulated the existence of a “NO reserve” in the plasma, in which this radical is in equilibrium with S-nitrosothiols and/or peptides characterized by a covalent S-NO bond [133]. These adducts have been suggested to play an important role in NO transport, signal transduction pathways and regulation of gene expression [134]. Ignarro and co-workers showed that these adducts could stimulate the conversion of GTP to cGMP in guanylate cyclase and suggested that they are key intermediates in the action of various nitrovasodilating compounds such as sodium nitroprusside and nitroglycerin [135]. In fact, endothelium-derived relaxing factor may be a Pr-SNO adduct.

S-nitrosoalbumin (SNO-Alb) is the most abundant nitrosothiol in human plasma, with concentrations reported to be as high as 5 μM [134, 136]. Many of the high- and low-molecular-weight S-nitrosothiols can release NO either spontaneously or *via* metabolism, so they are able to mediate many of the biological functions of NO. In addition, Stamler and co-workers [137] proposed that the binding of oxygen to heme irons in Hb promotes also the binding of NO to specific cysteine residues located in the β-subunits of Hb, forming S-nitrosohemoglobin (SNOHb) [137]. Deoxygenation is accompanied by an allosteric transition in SNOHb that releases the NO group. Therefore, SNOHb has been proposed to participate in the regulation of blood flow [137] and platelet aggregability.

The formation of S-nitrosothiols may also play an important role in leukocyte adhesion to the microvascular endothelium. For example, S-nitrosothiols are known to inhibit leukocyte adhesion to microvascular endothelial cells *in vivo*, presumably *via* the release of NO. -SH groups are essential for normal leukocyte-endothelial cell adhesion [138]. S-nitrosation of these critical -SH groups on the surface of endothelial cells and/or polymorphonuclear neutrophils (PMNs) could decrease adhesion, thereby limiting leukocyte infiltration. Furthermore, the formation of endogenous antiadhesive S-nitrosothiols by NO-

derived nitrosating agents may be inhibited by $O_2^{\cdot-}$, suggesting that during inflammation enhanced $O_2^{\cdot-}$ production may promote PMN-endothelial cell adhesion [139]. Indeed it is well established that exogenous NO donors are very effective at inhibiting PMN adhesion *in vivo* [138, 140]. Formation of S-nitrosothiols may, on the other hand, promote or perpetuate chronic inflammation.

Lander and colleagues [141] have demonstrated that S-nitrosation of one specific cysteine residue on the p21 Ras protein (involved in cellular signal transduction and able to activate genes involved in cell growth, differentiation and survival) in lymphocytes is critical for guanine nucleotide exchange and downstream signaling resulting in the formation of proinflammatory cytokines such as TNF- α .

Among endogenous RSNOs, the attention was mainly focused on S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CysNO), the most represented low-molecular weight nitrosothiols *in vivo*.

GSNO — formed by the S-nitrosation of reduced glutathione (GSH) — is involved in the storage and transport of NO. It is mostly intracellular inside the vascular wall [142] and may release NO according to the environmental conditions. It exhibits higher stability than NO, mediates protein S-nitrosation processes and is thought to play an important role in vascular signaling and protection, especially in a context of inflammation [143]. The biological activity of GSNO and particularly its vasorelaxant effect have been reported in *ex vivo* isolated vessel models [144, 145], and is directly linked to its decomposition resulting in the release of NO.

Potential routes of decomposition of NO and potential biomarkers in human blood, their respective significance and their application fields are summarized in the respective figure (Fig. 4) and table (Table 2).

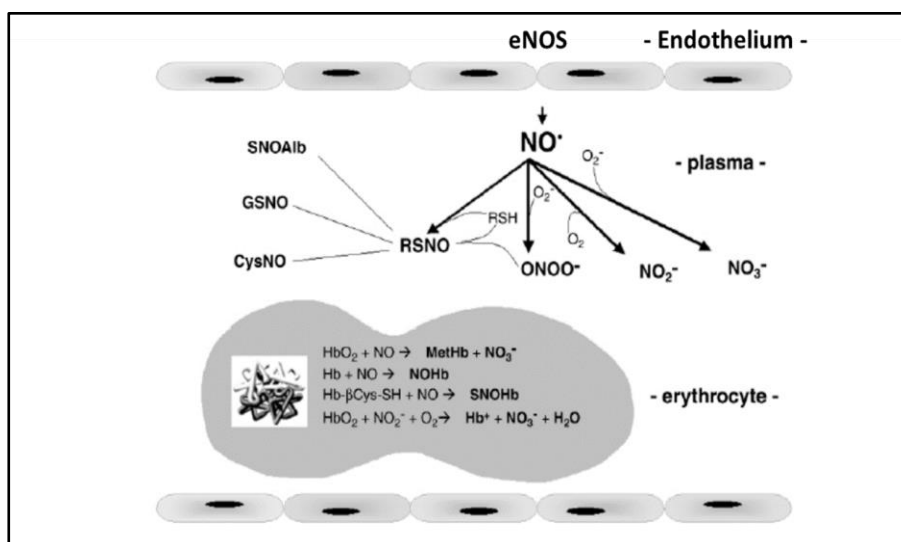


Figure 4. A model of intravascular metabolism of nitric oxide. NO produced by eNOS may diffuse into vascular lumen as well as the underlying smooth muscle. In plasma, NO may react with molecular oxygen to form nitrite (NO_2^-) or with superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$), which subsequently decomposes to yield nitrate (NO_3^-). Alternatively, the nitrosonium moiety of NO may react with thiols to form nitrosothiols (RSNO). Furthermore, NO may reach the erythrocytes to react with either oxyhemoglobin to form methemoglobin (metHb) and NO_2^- with deoxyhemoglobin to form nitrosylhemoglobin (NOHb), or with the Cys93 residue of the β -subunit to form S-nitrosohemoglobin (SNOHb). In addition, plasma NO_2^- could be taken up by erythrocyte, where it is oxidized in a Hb-dependent manner to NO_3^- (SNOAlb, S-nitrosoalbumin; GSNO, S-nitrosoglutathione; CysNO, S-nitrosocysteine, RSH, sulfhydryl group) [146].

Table 2. Potential markers of NO bioavailability in human blood

Potential Biomarker	Application Field	Reference(s)
NO ₂ ⁻	Sensitive and specific marker of acute change of eNOS activity in the human forearm vasculature.	[146]
NO ₃ ⁻	Rough estimate of total body nitrogen/NO turnover.	[534]
RSNO	Stabilize and transport NO; act as NO donor; mediate NO effects on proteins targets; inhibit leucocytes adhesion to microvascular endothelium through regulation of adhesion molecule expression; role in host defenses, affecting virus and bacteria. Under inflammatory conditions, they promote and perpetuate inflammation (<i>i.e.</i> S-nitrosation of Cys ¹¹⁸ residue on the p ²¹ Ras protein in lymphocytes yields the activation of reactive free radical-dependent signaling cascade)	[134, 138, 141]
SNO-Alb	Endogenous high molecular weight S-nitrosothiol, the most abundant protein in human plasma; physiological NO reservoir.	[134, 136]
SNO-Hb	Most abundant in the erythrocyte. Involved in the stabilization and delivery of NO in vascular bed. Participate in the regulation of vascular tone and blood flow and platelet aggregability.	[137]
CysNO	Endogenous low molecular weight S-nitrosothiol in the vascular space. It is an important means of NO storage in the organism.	[535]
GSNO	Endogenous low molecular weight S-nitrosothiol in the vascular space. Inhibits platelet aggregation, it has vasorelaxant effects. Physiological source of NO and it has a role in NO signaling (storage and transport) and protein S-nitrosation.	[133, 143, 192, 206]

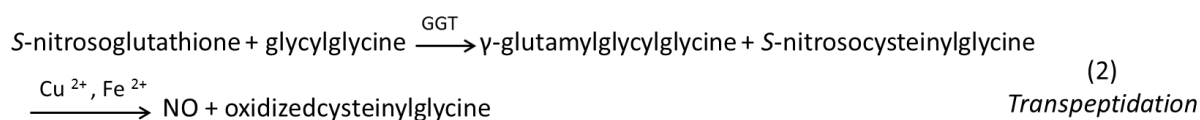
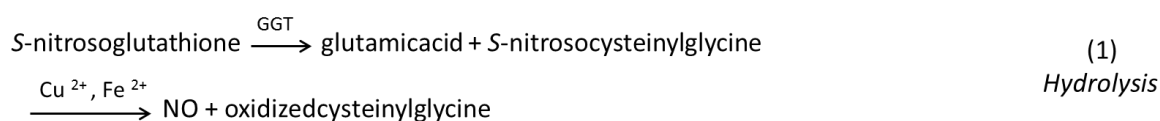
Abbreviations used: NO₂⁻ = nitrite; NO₃⁻ = nitrate; RSNO= S-nitrosothiol; SNO-Alb= S-nitrosoalbumin; SNO-Hb=S-nitrosohemoglobin; CysNO= S-nitrosocysteine; GSNO= S-nitrosoglutathione.

S-nitrosothiols are quite stable *in vitro* at 37°C and pH 7.4 [147] but they are degraded *in vivo*, especially by enzymatic activities. Cellular mechanisms of GSNO degradation are reported in the table below (**Table 3**). As from the literature, GSNO reductase (GSNOR), using NADH as a cofactor, produces an unstable intermediate, S-hydroxylaminoglutathione (GSNHOH), which can either react with GSH to produce oxidized glutathione (GSSG) and hydroxylamine (NH₂OH) or rearrange and then spontaneously hydrolyze to produce glutathione sulfinic acid (GSO₂H) and ammonia [148]. In either case, nitric oxide is not liberated during GSNO catabolism, and the nitroso moiety is reduced, effectively removing it from the “NO pool”. Carbonyl reductase 1 (CR1) metabolizes GSNO to an intermediate product, which can then react with GSH to produce NH₂OH and GSSG; thus, similar to GSNO reductase, CR1 does not liberate NO by its catalytic reaction [149]. Other important enzymes reported to be involved in GSNO decomposition are protein disulfide isomerase (PDI) and the thioredoxin system (Trxs).

An emerging literature has identified Trxs — composed by thioredoxin, thioredoxin reductase (TrxR) and NADPH — as major players in the reduction of low-molecular weight and protein S-nitrosothiols, participating in both denitrosation and transnitrosation reactions [150, 151, 152]. In its denitrosation capacity, reduced Trx reacts directly with either a low-molecular weight S-nitrosothiol (including GSNO) or protein SNO, thus liberating NO and thiols [153]. Through transnitrosation, the low-pK_a active-site thiol (Cys32) of Trx becomes S-nitrosated, leaving behind a low-molecular weight or protein thiol. The nitroso group now residing on Trx must then be turned over, through unknown mechanisms releasing nitroxyl (HNO) to form oxidized Trx. Oxidized Trx is then reduced by TrxR and NADPH. Unlike GSNOR and CR1, the thioredoxin system results in the release of NO and GSH coupled to the oxidation of NADPH.

PDI, another redoxin, has been shown to denitrosate GSNO [154]. NO released through this reaction combines with dioxygen in the hydrophobic environment of either the cell membrane or the PDI protein itself, to form dinitrogen trioxide (N₂O₃). In that way, PDI becomes a NO carrier *via* N₂O₃ mediating auto-S-nitrosation of its thiol active sites.

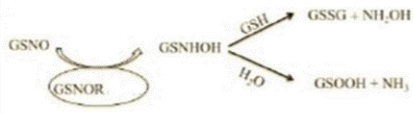
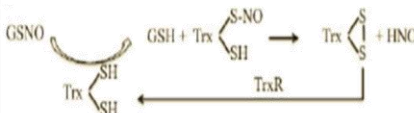
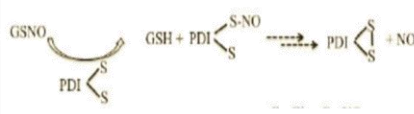
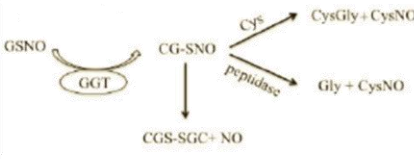
Finally, a cell membrane enzyme implicated in the metabolism of GSH, γ-glutamyltransferase (GGT), has also to be taken into account for specific GSNO catabolism [155, 156]. S-nitrosoglutathione is labile in the reducing environment of cytosol, on the other hand in the extracellular space GGT specifically catalyzes its breakdown releasing its γ-glutamyl residue through a hydrolytic pathway (1), and transferring this residue to an acceptor such as glycylglycine (GlyGly), in a transpeptidation reaction (2):



The presence of glycylglycine, added to serve as co-substrate for transpeptidation reaction [155], accelerates the decomposition rate of GSNO, thus producing S-nitrosocysteinyglycine more quickly. This latter being less stable than GSNO, it is rapidly decomposed in the presence of divalent metallic ions such as Fe^{2+} and Cu^{2+} leading to the release of NO and oxidized cysteinyglycine [155]. On the other hand, S-nitrosocysteinyglycine is itself provided with vasorelaxant activity, and the same is true for its metabolite S-nitroso-L-cysteine, produced by action of dipeptidases.

Indeed, in physiological conditions, the rapid vasorelaxant effects of GSNO are largely dependent on the presence of endothelium and are mediated through its GGT-dependent metabolism [157]. This is an important observation that should be taken into account in a pathophysiological perspective, because elevated GGT activity has been associated with various pathologies such as cystic fibrosis [158], hypertension [11] and atherosclerosis [159], and because therapeutics based on GSNO have already been proposed at the clinical level [160, 161].

Table 3. Cellular mechanisms of GSNO degradation

Enzymatic activities	Features	Reactions	Reference(s)
GSNO reductase (GSNOR)	Located in the cytosol NADH-dependent enzyme	 In either case, nitric oxide is not liberated during GSNO catabolism and the nitroso moiety is reduced, effectively removing it from the “NO pool”.	[148]
Carbonyl reductase 1 (CR1)	Located in the cytoplasm NADPH-dependent enzyme	 In this case, CR1 does not liberate NO in its catalytic reaction.	[149, 205]
Thioredoxin system (Trxs)	Located in the cytosol. Consists of Trx, NADPH and thioredoxin reductase (TrxR). Involved in denitrosation and transnitrosation reactions.		[136, 150, 151, 152]
Protein disulfide isomerase (PDI)	Located in the cytosol and on cell membrane surface (transferred to the membrane during oxidative stress). Involved in thiol-disulphide-exchange activity and in denitrosation reaction from RSNO, catalysed by the same active-site thiols involved in the thiol-disulphide-exchange activity of the enzyme.		[154]
γ-glutamyltransferase (GGT)	Located on cell membrane surface. Involved in hydrolysis and transpeptidation reactions. GSNO specific substrate		[155, 156, 214]

Abbreviations used: NADH= nicotinamide adenine dinucleotide; NADPH= nicotinamide adenine dinucleotide phosphate; GSNHOH= S-hydroxylaminoglutathione; GSH= glutathione; GSSG= oxidized glutathione; NH₂OH= hydroxylamine; NH₃= ammonia; Pr-SNO= S-nitrosated proteins; CysGlyNO= S-nitrosocysteinyglycine; oxidizedCysGly= oxidized cysteinyglycine; GlyGly= glycylglycine.

1.4 GSNO: a possible nitric oxide donor in atherosclerosis

Nitric oxide, as described previously, is highly reactive, has a short half life *in vivo* (estimated in the range of a few seconds) and mediates SMCs relaxation [162]. Therefore, in this context, several NO donor classes based on more complex chemical systems, such as nitrosamine, organic nitrates and metal-NO complexes, but also *S*-nitrosothiols, have emerged.

Such NO donating compounds are identified as “NO donors”. Three NO release mechanisms were elaborated depending on the chemical structure. The first type is “spontaneous” NO donation, which releases NO through thermal or photochemical self-decomposition (*e.g.* *S*-nitrosothiols, *N*-diazoniumdiolates, oximes) [163]. The second type is a “chemically catalized” NO release triggered by acid, alkali, metals and thiols [163]. This is the case *e.g.* of organic nitrates, nitrites, sydnonimines and *S*-nitrosothiols. The third type is an “enzymatically catalized” NO donation depending on enzymatic oxidation: *e.g.* *N*-hydroxyguanidines need metabolic activation by NO synthases or oxidases for NO release [163]. Moreover, some NO donors release NO in more than one way, for example some *S*-nitrosothiols can also generate NO by enzymatic catalysis [164], as previously described for GGT and GSNO.

The delivery of supplementary NO in the form of NO donor drugs represents an attractive therapeutic option for the treatment of cardiovascular diseases. Some NO-donor drugs are already in widespread clinical use, in particular the organic nitrates (*e.g.* nitroglycerin, isosorbide dinitrate or isosorbide-5-mononitrate), the organic nitrites (*e.g.* amyl nitrite), the ferrous nitro complexes (*e.g.* sodium nitroprusside) and the sydnonimines (*e.g.* molsidomine) [165]. Nitroglycerin and amyl nitrite are widely used in both prophylaxis and treatment of angina pectoris. Sodium nitroprusside is used in treating hypertensive emergencies as well as severe cardiac failure but never for a chronic treatment [166]. Other commercialized NO-donor drugs contain as active agent molsidomine, which is degraded in the liver in 3-morpholinosydnonimine (SIN-1) and liberates NO. Although these drugs are effective, they may however present some drawbacks. Indeed, continuous organic nitrate administrations can rapidly induce tolerance in patients, with reduced therapeutic effect of the drugs with time. Moreover, administration of sodium nitroprusside may be toxic since this drug is converted into cyanide and thiocyanate [167, 168]. Toxic accumulation of cyanide may lead to severe lactic acidosis, arrhythmia and excessive hypotension. SIN-1 decomposition can give concomitant large amounts of NO and superoxide leading to the formation of ONOO⁻, which has been associated to cytotoxic effects through oxidation and nitration reactions [169, 170, 171].

S-nitrosothiols are proposed as promising candidates as NO donors because they do not induce any tolerance or cyanide poisoning [166] and were shown to be effective in several disease models [172]. In contrast to the other NO donors described above, only initial small clinical studies have been reported for *S*-nitrosothiols, suggesting that they may be valuable therapeutic agents in a variety of cardiovascular disorders.

Recent studies have shown the implication of NO homeostasis in atherosclerosis and the potential therapeutic benefit of drugs which donate NO (*e.g.* organic nitrates, nicorandil and sydnonimines) or which increase the availability of endogenous NO (*e.g.* statins, angiotensin-converting enzyme inhibitors, L-arginine and tetrahydrobiopterin) [173, 9, 174]. The rationale for this implication lies on the potential effects of NO on macrophages, SMCs and ECs, main cellular components of atherosclerotic plaque.

On the other hand, it is known that NO by itself [175, 176] or in combination with superoxide anions [177] can stimulate the oxidation of LDL, which constitutes a critical triggering event in atherogenesis. Thus, *S*-nitrosothiols in spite of their advantages could also contribute to oxidative stress. Little was known about the effect of *S*-nitrosothiols on native LDL oxidation in normal ECs and SMCs. Therefore Jaworski et al. have compared the effects of *S*-nitroso-*N*-acetylpenicillamine (SNAP) and two other known NO donors, SIN-1 and sodium nitroprusside, on LDL oxidation either in an acellular system or in the presence of normal endothelial cells or smooth muscle cells [8]. They demonstrated that sodium nitroprusside strongly oxidized LDL in medium alone, as well as in endothelial or smooth muscle cell cultures, and it would have to be used carefully in therapeutic vascular treatments, especially under conditions of high concentration and prolonged administration. SIN-1 also oxidized LDL in the absence of cells and clearly enhanced the LDL oxidation in cultures, in agreement with other studies [177, 179]. SNAP was not able to oxidize LDL either in acellular system or in the presence of cells, showing that the amount of superoxide and other reactive oxygen species released by these cells did not suffice, contrary to those liberated by macrophages, to combine to NO providing oxidant activity.

It has been shown that other *S*-nitrosothiols, such as GSNO, could protect endothelial cells from the toxic effect of oxidized LDL [178]. In addition, SNAP was reported to be useful in the treatment of heart failure, reducing myocardial oxygen consumption [179], inducing vasodilation in rat femoral arteries [180] and inhibiting platelet activation and aggregation through cGMP accumulation [181, 182]. Taken together, these observations suggest that *S*-nitrosothiols could limit the progression of vascular disorders such as atherogenesis without increasing oxidative stress, and thus encourage their clinical applications in the therapeutic treatment of atherogenesis.

In this direction, Martinet and colleagues, on their previous work [183] demonstrated that the treatment of cholesterol-fed rabbits with the NO donor molsidomine preferentially eliminates macrophages exerting several beneficial effects on plaque structure and stability. In a more recent study, they investigated the underlying mechanisms and reported that such effect is mediated, at least in part, by the induction of endoplasmatic reticulum (ER) stress [9]. Macrophages and SMCs were treated *in vitro* with the NO donors spermine NONOate or SNAP, as well as with the well-known ER stress inducers thapsigargin, tunicamycin, dithiothreitol or brefeldin A. Several markers of macrophage death and induction of ER stress were measured. Macrophages and SMCs treated with spermine NONOate or SNAP showed several signs of ER stress, including upregulation of CHOP (CCAAT/Enhancer-binding protein homologous protein)

expression, hyperphosphorylation of eIF2 α , inhibition of *de novo* protein synthesis and splicing of XBP1 (x-box binding protein 1) mRNA. These effects were similar in macrophages and SMCs, yet only macrophages underwent apoptosis. Interestingly, selective induction of macrophage death could also be initiated with well-known ER stress inducers such as the Ca²⁺ homeostasis disruptor thapsigargin and the N-linked glycosylation inhibitor tunicamycin, reinforcing the finding that induction of ER stress during treatment with NO donors initiates selective macrophage death. In conclusion, Martinet et al., have provided evidence that NO-induced ER stress can induce macrophage cell death without affecting SMCs viability in atherosclerotic plaques, probably *via* inhibition of protein synthesis. From a clinical perspective, it is important to note that protein synthesis inhibitors, *e.g.* cycloheximide, cannot be administered systemically because they cause dramatic cell death in the liver [184]. Moreover, local drug delivery by means of coated stents avoids unwanted systemic effects, but does not guarantee permanent depletion of macrophages due to the fast release rates (hours to months) of the coated drug. In case of short-term drug delivery, it is only a matter of time before blood monocytes reinvade the “purified” plaque. NO donors are widely used by patients with coronary artery disease to relieve the symptoms of ischaemia evoked by atherosclerosis and can be administered safely for many years [173]. Therefore, it is conceivable that NO donors, if necessary in combination with a statin or local therapy (for example stent-based delivery of cycloheximide), would offer new opportunities for a long-term macrophage depletory effect in atherosclerotic plaques.

The table below reports a summary of the various NO donors and their application field described above (**Table 4**).

Table 4. Potential NO donors and their application field.

Potential NO donor	Feature	Application field	Species	Reference(s)
ORGANIC NITRATES Nitroglycerin (NTG)	Vasodilator	Angina pectoris Prophylaxis Symptomatic CAD Hypertension	Human (with large vessel coronary)	[166, 173]
Isosorbide mononitrate (ISMN)	Vasodilator	Decrease O_2^- production Prevent intimal lesion formation and ED	Hypercholesterolaemic rabbits	
Isosorbide dinitrate (ISDN)	Vasodilator	Combined with hydralazine → HF treatment (found efficacious and increased survival among patients with severe HF)	Human	
Nicorandil	Vasodilator	Angina pectoris Cardioprotective properties Improve fibrinolysis and reduce risk of thrombus formation	Human	
ORGANIC NITRITES Amyl nitrite	Vasodilator	Antihypertensive Angina pectoris Cyanide poisoning	Human	[166, 167]
IRON-NITROSYL COMPLEXES Sodium nitroprusside	Vasodilator	Hypertensive emergency and severe cardiac failure Source of nitrosium (NO^+), thus it behaves as nitrosating electrophilic species Oxidized LDL	Human ECs and SMCs (from porcine pulmonary arteries)	[8, 166]
SYDNONIMINES Molsidomine	Poor vasoactive	Decrease signs of oxidative stress and increase the features of stable atherosclerotic plaques in Cholesterol-fed rabbits ↑ Prevention of intimal hyperplasia ↓	Rabbits Human	[166, 169, 173]
3-morpholinosydnimine (SIN-1)	More vasoactive	Potent vasorelaxant and anti-platelet aggregating agent <i>In vitro</i> , SIN-1 simultaneously generates NO and O_2^- → ONOO $^-$ <i>In vivo</i> , endogenous oxidizing agents other than molecular oxygen (e.g. ferric haemproteins) can oxidize SIN-1, resulting in the release of NO without concomitant formation of superoxide Oxidized LDL +/- cells presence and enhanced the LDL oxidation in cultures	Human Human arteries and animal model	

Abbreviations used: CAD= coronary artery disease; O_2^- = superoxide anion; ED= endothelial dysfunction; HF= heart failure; LDL= low density lipoprotein; ONOO $^-$ = peroxynitrite.

Table 4. (continued)

Potential NO donor	Feature	Application field	Species	Reference(s)
SECONDARY AMINE/NO COMPLEX IONS Amine NONOate Spermine NONOate	Vasodilator	Stabilize NO drug storage in solid form Highly soluble in H ₂ O Release NO Controlling tumor cells proliferation NO donor induce signs of ER stress that can induce selective macrophages cell death, without affecting SMC viability, in atherosclerotic plaques	Human Human melanoma cells line (A375) Murine macrophage cell line (J774A.1) SMCs isolated from mouse or rabbit aorta	[9, 536, 537, 538]
S-nitroso-N-acetyl-D,L-penicillamine (SNAP)	Vasodilator Anti-platelet agent	Useful in the treatment of HF, reducing myocardial oxygen consumption Inducing vasodilation in rat femoral arteries and inhibiting platelet activation and aggregation through cGMP accumulation Not able to oxidize LDL either in acellular system or in the presence of cells NO donor induce signs of ER stress that can induce selective macrophages cell death, without affecting SMC viability, in atherosclerotic plaques	Human Rat arteries ECs and SMCs (from porcine pulmonary arteries) Murine macrophage cell line (J774A.1) SMCs isolated from mouse or rabbit aorta	[8, 9, 166]
Molsidomine <i>via</i> SIN-1 + Cholesterol lowering		Treatment of atherosclerotic plaques in cholesterol-fed rabbits → not affecting plaque size; increased the thickness of the subendothelial macrophage-free layer consisting of SMC, and normalized both superoxide production and ecSOD mRNA expression. Molsidomine + cholesterol lowering → decrease signs of oxidative stress and increases features of stable atherosclerotic plaques.	Rabbits	[183]
S-nitrosoglutathione (GSNO)	Vasodilator (not induction of tolerance or oxidative stress)	Direct and selective inhibition action in platelets Inhibition of ADP-induced platelet aggregation In women: decrease maternal mean arterial pressure, decrease platelet activation and uterine artery resistance Increase left ventricular function in healthy human Decrease embolism from symptomatic carotid plaques and after carotid endarterectomy, carotid angioplasty and vein graft, by limiting platelet activation Beneficial effects in the vasculature extend to systemic vasodilation and preeclampsia Prevent the angioplasty-induced increase in platelet expression of P-selectin and of glycoprotein lib/IIIa, without altering blood pressure Improve gas exchange in patients with cystic fibrosis and increase oxygene saturation acutely Topical administration increases clitoral blood flow (treatment for female sexual dysfunction) Act as NO carrier and it can be delivered transdermally, exerting a prolonged vasodilation effect on the skin microcirculation	Human Human (intravenous infusion) Human (intracoronary infusion) Human (aerosolized inhalant) Human (topical gel) Human healthy subjects and diabetic rats as poly-vinyl alcohol film	[160, 161, 193, 194, 195, 196, 198, 197, 199]

Abbreviations used: ER= endoplasmatic reticulum; SMC= smooth muscle cells; HF=heart failure; cGMP= cyclic guanosine monophosphate; ecSOD= extracellula superoxide dismutase; ADP= adenosine diphosphate;

Recently, indeed, a new class of compounds, the NO-donating statins, have been reported to associate the ability to donate bioactive NO to the capacity to inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), thus displaying a variety of biological effects in addition to those of statins, including anti-thrombotic and anti-inflammatory properties [185, 186, 187] (**Table 5**). Momi et al., [188] have compared the anti-inflammatory and anti-atherothrombotic properties of the NO-donating atorvastatin, NCX 6560, with those of plain atorvastatin in an animal model of severe endothelial dysfunction, oxidative stress and accelerated atherosclerosis (LDLR^{-/-} mice fed a cholesterol-rich diet and undergoing intravascular photochemically induced generation of oxygen free radicals). In this model, atorvastatin, despite its lipid-lowering and anti-inflammatory activity [189, 185] only partly prevented atherosclerosis and inflammation, while a NO-releasing atorvastatin showed superior activity. This suggests that in conditions of severe endothelial injury associated with the generation of oxygen radicals, the direct supply of NO allows the attainment of a significant anti-atherosclerotic effect by the enhancement of the vascular protective effects of atorvastatin unrelated to lipid lowering.

The conclusion, that NO donation confers superior anti-atherosclerotic activity to atorvastatin, is supported by several observations. The results showed a significant decrease in plasma IL-6 levels 1 week after the beginning of treatment with NCX 6560, while atorvastatin required at least 3 weeks. Then, the antiatherosclerotic activity of NCX 6560 was superior to that of atorvastatin, despite a similar reduction of serum cholesterol and similar drug plasma levels. The administration of NCX 6560, but not of atorvastatin, strongly enhanced the plasma levels of NO-degradation products and of cGMP, confirming the *in vivo* release of biologically relevant amounts of NO. Moreover, NCX 6560, but not atorvastatin, reduced the expression in the injured arterial wall of some inflammatory proteins, *e.g.* MMP-2, which participate in atherosclerotic vascular remodelling [190]. A direct effect of NCX 6560-released NO on the vascular wall is documented by the decrease of inflammatory markers (MMP-2, COX-2, and iNOS) in the vessel wall, by enhanced endothelium-dependent vasorelaxation, by the lowering of blood pressure in eNOS^{-/-} mice, and by the increase of phosphorylated eNOS in the vascular tissue [191].

In conclusion, this study showed that in a model of severe endothelial dysfunction, systemic peroxidation and inflammation and accelerated atherosclerosis, atorvastatin, even at high doses, displays a suboptimal anti-atherogenic and anti-inflammatory activity, while a NO-donating atorvastatin has strong and prompt anti-inflammatory and anti-atherosclerotic effects.

All these studies reinforce the idea of the importance of using "NO donors", focusing on their protective and beneficial effects, in order to lead novel strategies for the treatment of atherosclerosis or at least, the minimization of its development.

Table 5. Enhancers of NO availability and NO-donating statins and beneficial effects.

Enhancers of NO availability and NO-donating statins	Feature	Beneficial effects	Species	Reference(s)
STATINS	Decrease LDL cholesterol by inhibiting the enzyme 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA)	Improve vascular relaxation Reduce vascular inflammation and oxidative stress Decrease thrombosis and platelet aggregation Decrease adhesion of platelets and white cells to the vascular endothelium Stabilize vulnerable plaques and promote new vessel formation Block rac 1 isoprenylation (component of NAD(P)H oxidase complex) thus reducing the expression of NAD(P)H oxidase subunits → inhibition of endothelial production of O_2^- and a shift in the balance between NO and the free radical, by improving endothelial function	Human	[173]
Simvastatin	Decrease the concentration of LDL-cholesterol	Prevent the decreased bioavailability of endothelium-derived NO and downregulation of endothelial eNOS that result from elevated levels of native LDL → Elevated LDL reduces NO production, in part, by increasing the interaction between caveolin-1 and eNOS.	Human Bovine aortic ECs	
Atorvastatin	Decrease the concentration of LDL-cholesterol	Reduce caveolin-1 expression in ECs by inhibiting the interaction between caveolin-1 and eNOS, resulting in increased NO production.	In healthy normocholesterolaemic men	
NO-DONATING STATINS	Inhibit HMG-CoA Antithrombotic effect Antiinflammatory effect	Donate NO Enhanced anti-atherogenic and anti-inflammatory effects.	Rat smooth muscle cells LDLR ^{-/-} mice fed a cholesterol-rich diet	[185, 187, 188, 189]
NCX6560 (NO-donating statins) vs. Highest doses of atorvastatin		Decrease lipid plaques in the aorta Decrease macrophage infiltration Decrease plasma IL-6 levels (after 1 week) Decrease sICAM-1 and SAA Decrease of serum cholesterol and drug plasma levels Enhanced plasma concentrations of nitrites and nitrates, the NO degradation end-products Decrease platelet P-selectin expression in collagen- plus adrenaline-injected mice Decrease ROS formation in extracts of aorta Decrease injured arterial wall of some inflammatory proteins (e.g. MMP) Decrease inflammatory markers (e.g. MMP2, COX2, iNOS) in the vessel wall Decrease lipid plaques in the aorta Decrease macrophage infiltration lesser than NCX6560 Decrease plasma IL-6 levels (after 3 week) Decrease sICAM-1 and SSA lesser than NCX6560 Decrease of serum cholesterol Effect almost inactive to enhance plasma concentrations of nitrites and nitrates, the NO degradation end-products Decrease platelet P-selectin expression in collagen- plus adrenaline-injected mice lesser than NCX6560 Ineffective effect to decrease ROS formation in extracts of aorta	LDLR ^{-/-} mice fed a cholesterol-rich diet and undergoing intravascular photochemically induced generation of oxygen free radicals.	[188]

Abbreviations used: LDL= low density lipoprotein; RAC 1= ras related C3; NADPH= nicotinamide adenine dinucleotide phosphate; O_2^- = superoxide anion; eNOS= endothelial nitric oxide synthase; LDLR= low density lipoprotein receptor; IL-6= interleukin 6; sICAM= soluble intracellular adhesion molecule 1; SAA= serum amyloid A; ROS= reactive oxygen species; MMP= matrix metalloproteinase; COX-2= cyclooxygenase 2; iNOS= inducible nitric oxide synthase.

Finally, in the last few years, most of attention was focused on cellular responses to GSNO [192]. Indeed, GSNO does not induce any tolerance or oxidative stress. To date, there have been nearly 20 clinical trials investigating the therapeutic efficacy of GSNO in multiple pathological contexts, though most have focused on its effects in cardiovascular diseases. GSNO has been administered through intravenous infusion [160], as an aerosolized inhalant [161], and more recently, as a topical gel [193] and polyvinyl alcohol film [194]. The best-characterized effects of GSNO in humans are its direct and selective actions on platelets [195, 196]. GSNO has been shown to decrease embolism from symptomatic carotid plaques and after carotid endarterectomy [197], carotid angioplasty [195], and vein graft [198] by limiting platelet activation. Its beneficial effects in the vasculature extend to cardiac left ventricular function [160], systemic vasodilation [199], and preeclampsia [200] (**Table 4**).

Although with some discrepancies related to the analytical method used [201, 202], GSNO was found at nano- to low micromolar concentrations in extracellular fluids and tissues [201, 203, 204] and due to its nature of endogenous compound, it has been considered an “attractive candidate” for GSNO-based therapies [161, 205].

Despite GSNO is the main physiological source of NO in the tissue [206] and plays an important role in NO signaling and protein S-nitrosation, there are not enough experimental evidences in support of its employment for treatment of the atherosclerotic process. If one of the possible final aim for the “treatment” of atherosclerosis is to introduce new therapeutic interventions capable to reduce plaque macrophage content at different stages of atherosclerosis — either by reducing lipids in the circulation or from the plaque, or decreasing monocyte recruitment, or inhibiting macrophage activation, or inducing macrophage death in atherosclerotic plaques — the question arises spontaneously: *why do not employ GSNO?* Probably, it may venture the guess about a GSNO employment as a potential therapeutic agent in the prevention and treatment of atherosclerotic plaques. More in particular, it is conceivable that GSNO can be employed to counteract NO deficiency in cardiovascular disorders, and specifically in atherosclerosis, a condition characterized by a decrease of NO bioavailability and an increase in oxidative stress. In particular, given the multitude of factors that influence NO bioavailability in atherosclerosis, and its bioavailability is tightly regulated by multiple fine-tuned mechanisms, it is logical to target one or more of these processes to enhance NO levels. Indeed, the development and/or progression of atherosclerosis is associated with dysregulation of one or more of these mechanisms, resulting in the production of suboptimal levels of NO by the endothelium. Some of the mechanisms by which existing drugs or future therapeutics may increase NO bioavailability include supplementation of L-arginine or NO-releasing molecules, restoration of cofactors and cosubstrates for eNOS, stabilization of eNOS mRNA or protein, increasing eNOS expression or activity, reducing synthesis or increasing metabolism of methylarginines, reducing oxidative stress, and/or enhancing the clearance of harmful lipoproteins.

Precisely because GSNO ability to regulate NO bioavailability under oxidative stress conditions is poorly understood, to investigate and deepen this aspect can be regarded as a must. The elucidation of these mechanisms could open a way for the use of GSNO, as another useful NO donor in cardiovascular diseases associated with oxidative stress.

Finally, as previously described, GSNO is an endogenous low molecular weight S-nitrosothiol and is involved in storage and transport of NO. It exhibits higher stability than NO and mediates protein S-nitrosation processes and plays an important role in vascular signaling [207]. The biological activity of GSNO and particularly its vasorelaxant effect have been reported in *ex vivo* isolated vessel model [144, 145, 157] and are directly linked to its decomposition resulting in the release of NO. Indeed the potential therapeutic use of GSNO is relevant based on its unique capacity to deliver NO and beneficial effects, particularly in the cardiovascular diseases associated with oxidative stress, through its degradation by enzymatic activities. One of them, GGT (EC 2.3.2.2.) involved in the metabolism of GSH [208] has also been shown to metabolize specifically GSNO [155, 156], generating S-nitrosocysteinylglycine [156], reporting to have biological consequences in some cellular responses. The profile of GGT will be analyzed in depth in the following section.

1.5 Role of gamma-glutamyl transferase (GGT) in atherosclerosis

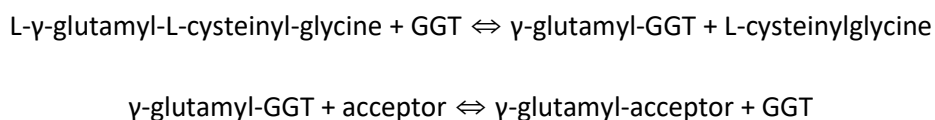
γ -Glutamyl transferase is an enzyme widely distributed and conserved in the living world: in bacteria [209], in plants [210] and throughout animal kingdom. In particular, in mammals, GGT is a membrane type II glycoprotein, including a heavy (380 amino acids, 55-62 kDa) and light (189 amino acids, 20-30 kDa) subunits, linked by non-covalent bonds. Through immunohistochemical studies, it has been shown that GGT is located on the membrane of nearly all cells and is preferentially expressed in epithelial tissues with secretory and absorption activity [211]. The highest GGT activity has been found in the kidney, on the luminal surface of the cells of the proximal convoluted tubule, while the cells of the distal tubule and glomeruli are practically free. In the liver, the GGT activity is found in the epithelial cell layer of the extrahepatic biliary tract and liver canaliculi, while, in the pancreas, the most of it is in acinar cells. In the brain the activity of GGT appears to contribute to the functionality of the blood-brain barrier by promoting the metabolism of leukotrienes and detoxification of xenobiotics [212]. GGT is expressed at the endothelial level, by exerting a role in the vascular relaxation, as shown by Dabhouli et al, in a rat aorta model, in which GGT activity mediates the release of NO from GSNO [157]. GGT activity is also present on the membrane and intracytoplasmic granules of platelets and granulocytes and lymphocytes, where its increased activity is considered as a marker of differentiation and malignant transformation [213].

The activity of serum GGT is probably due to its release from the cell membranes of various parenchymatous organs, and reflects the quantitative changes in production, release and removal of the circulating enzyme.

GGT is a highly glycosylated glycoprotein and, based on its primary sequence, six possible consensus sequences for *N*-glycosylation have been identified. *N*-glycosylation represents 25-30% of the total mass of GGT and it is tissue specific. In fact, purified GGT from different organs has a different molecular weight and, therefore, a different electrophoretic mobility. This heterogeneity of glycosylation suggest the occurrence of "isoforms" for the GGT enzyme, while there are no identified isoenzymes with respect to the amino acid sequence.

Being a cell surface enzyme from the Meister cycle, GGT catalyzes the cleavage of the γ -glutamyl amino acid of L- γ -glutamyl-L-cysteinyl-glycine (GSH) and its conjugates [214]. This is thought to occur through the formation of an intermediate γ -glutamyl enzyme, which then can form free γ -glutamate by hydrolysis, or transfer the γ -glutamyl to other acceptor molecules (*e.g.* amino acids) through transpeptidation activity.

Kinetic studies have suggested a mechanism of action of GGT, defined as "ping - pong", which could be represented by the following diagram:



GGT is specific to the type of bond and not to the substrate, since only the γ -glutamyl position is critical for the interaction with the GGT enzyme. Therefore all the γ -glutamyl-compounds are potential substrates for GGT. The main physiological GGT substrates are GSH, glutathione conjugates produced by GSH-transferase, and GSNO. As acceptor molecules, GGT only recognizes L-aminoacids (Cys, Met, Gln, Glu) or, preferably, dipeptides. The best acceptors are Cys, Met, Gln, and the aminoacylglycine dipeptides (CysGly, MetGly, GlnGly, CysGly, GlyGly) [215, 216].

GGT enzyme appears to be involved in various physiological functions and presents both anti-oxidant and pro-oxidant activities.

1.5.1 Anti-oxidant role of GGT

GGT is localized at the cell surface and only cleaves extracellular substrates. GSH and oxidized GSH (GSSG) are the most abundant physiological substrates, although GGT cleaves any gamma-glutamyl substrate including GSH S-conjugates [217].

The ratio of GSH to oxidized glutathione (GSSG) can be used as an estimate of the overall oxidation state of the cellular thiol redox environment [218, 219]. Indeed, the maintenance of the intracellular concentration of GSH is essential for cells survival [220]. The cytosol is considered to be a reducing environment with a cytosolic GSH/GSSG ratio equal to or greater than 100 [218]. Measuring the GSH/GSSG ratio or calculating the overall thiol redox potential based on these GSH/GSSG values, is a method often used to determine the level of (thiol) oxidative stress of cells or tissues. Shifting toward a more oxidized thiol redox status of cells and tissues (high GSSG, low GSH/GSSG ratio) correlates with disease processes like atherosclerosis and diabetes [42, 221, 222].

GSH has many important biological functions, including: conjugation of electrophilic compounds, maintenance of the intracellular redox state environment through thiol-disulfide exchange reactions, and scavenging of free radicals. The intracellular concentration of glutathione depends on the balance between its synthesis, adjusted by the contribution of constituent amino acids, and its consumption. Inasmuch GGT initiates the degradation of extracellular GSH, making possible the subsequent uptake of the composing amino acid by the cell (the γ -glutamyl cycle), it is considered as an ancillary enzyme of the antioxidant systems. From this point of view the enzyme could have a protective role against oxidative intracellular damage [223] (Fig. 5).

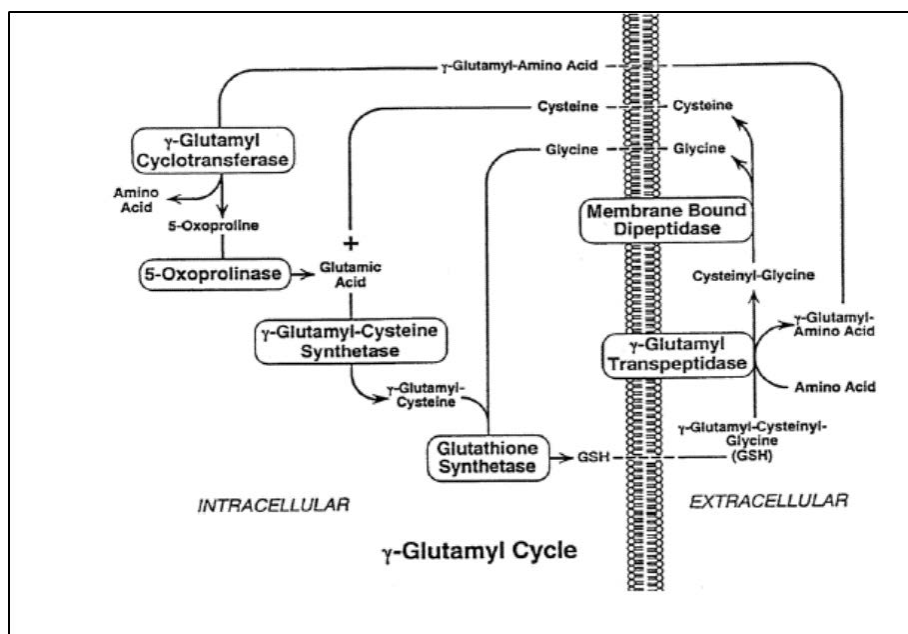


Figure 5. The γ -glutamyl cycle.

Moreover, GGT cleaves the γ -glutamate group from extracellular GSH and GSSG providing an additional source of cysteine for intracellular GSH synthesis. Indeed, another key function of GSH is represented by the transport of cysteine (Cys), which would be highly unstable in the extracellular environment as it self-oxidizes into cystine (Cys-Cys), favoring the formation of reactive oxygen species [224]. Following the degradation of glutathione into cysteinyl-glycine (Cys-Gly), GGT then allows the

recovery of cysteine. Cystine is the most effective acceptor of the transpeptidation reaction and also the tripeptide γ -glutamyl-cysteine (γ -GluCys) contributes to the recovery of cysteine. Cysteine is a limiting substrate for protein and glutathione synthesis since it is one of the essential amino acids [225]. GGT acts together with other membrane-bound enzymes (dipeptidases) capable of hydrolysing the peptide bond of CysGly for the recovery of the individual amino acids, to reconstitute glutathione or to effect the recovery of cysteine.

1.5.2 Pro-oxidant role of GGT

The substrate of GGT, GSH, is a tripeptide whose antioxidant properties are based on the reducing capacity of the thiol group (-SH) of the cysteine amino acid residue. The thiols, however, especially in their ionized form of thiolate anions ($R-S^-$), can give up electrons to reduce metal cations such as iron and copper. Indeed, the balance between antioxidant and pro-oxidant action depends on the availability of metal ions.

The pro-oxidant effects of GGT depend from high reactivity of the thiol group (-SH) of CysGly that is present in the form of a thiolate anion (S^-) at physiological pH. The latter reduces Fe^{3+} to Fe^{2+} , generating the radical thiyl ($S^\bullet$) of CysGly. Fe^{2+} , in turn, reduces molecular dioxygen to superoxide anion ($O_2^{\bullet-}$) which is converted into hydrogen peroxide (H_2O_2) either spontaneously or by superoxide dismutase (SOD). H_2O_2 , in the presence of free Fe^{2+} (Fenton reaction) can generate hydroxyl radicals, which together with the cysteinylglycine thiyl radical formed following the reduction of Fe^{3+} can initiate the chain reaction of lipid peroxidation, resulting in loss of structure and stability of the cell membrane. The same prooxidants also have important functions [226], *e.g.* regulate protease and antiprotease activities, modulate apoptosis or proliferation in relation to the cell line, and lead to transcriptional activation of NF-KB (Fig. 6) [227].

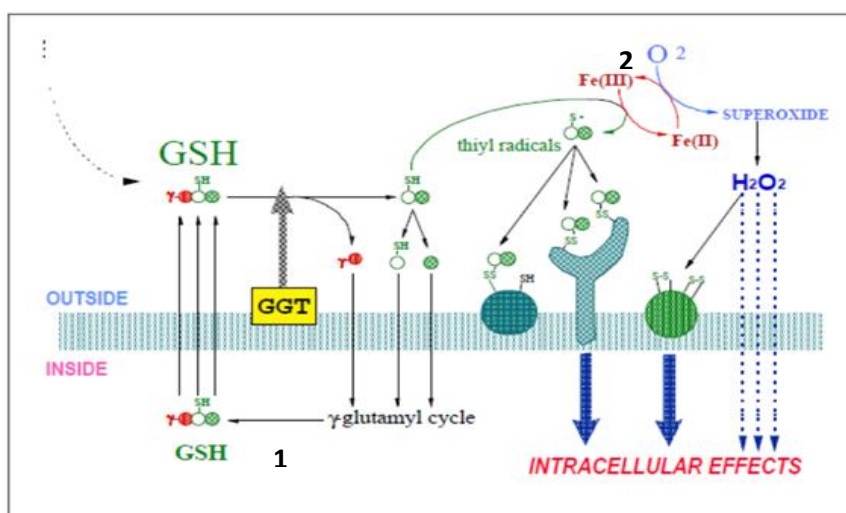


Figure 6. GGT implication in the recovery of extracellular GSH and associated prooxidant reactions [227].

- 1- recovery of precursors amino acids for intracellular GSH synthesis
- 2- pro-oxidant reactions in the extracellular environment

It is important to emphasize that these phenomena occur, at least initially, on the outer side of the plasma membrane of the cell, and therefore these phenomena are not subjected to anti-oxidant defense systems [228], rather their consequences are propagated throughout the cell.

While GSH, due to peculiar properties of the free carboxyl group of the γ -glutamic acid residue, is relatively ineffective in reduction of metal cations, its hydrolysis by GGT results in the release of a catabolite, CysGly, which is much more reactive and efficient in reducing metal cations, thus triggering the production of free radicals and reactive oxygen species. The pro-oxidant action of GGT is linked to the presence of redox active metals in the extracellular environment, which *in vivo* is strongly prevented thanks to the formation of complexes such as ferritin, transferrin and ceruloplasmin that do not allow metals to catalyze reactions with free radicals. In this regard, it should be underscored that GGT activity itself is able to reduce and to promote the release of metal ions from transferrin [229, 230] as well as from ceruloplasmin [231], *i.e.* two physiological sources of transition metal ions.

An excess production of ROS by GSH metabolism can induce DNA damage [232, 233] or trigger the process of lipid peroxidation, documented *in vitro* with linoleic acid [234] and isolated LDL lipoproteins [129] as substrates. Indeed, LDL oxidation is known to play a central role in atherogenesis and vascular damage and iron is a potential catalyst of LDL oxidation, provided that electron donors convert Fe^{3+} to Fe^{2+} . In fact thiol compounds such as cysteine and homocysteine are known to reduce Fe^{3+} and promote Fe^{2+} -dependent LDL oxidation [235]. A series of experiments showed that γ -glutamate residue of GSH affects interactions of the juxtaposed cysteine thiol with iron, precluding Fe^{3+} reduction and hence LDL oxidation [236, 237]. Both processes increase remarkably after addition of purified GGT which acts by removing γ -glutamate residue [230].

1.5.3 GGT in the atherosclerotic plaque

GGT has been successfully used as a predictor of cardiovascular risk, as studies conducted since the late '90s showed the association of relatively higher serum GGT levels with the progression of atherosclerosis and its complications [11]. Its accumulation in carotid [238] and coronary artery atherosclerotic plaques [12] was also documented. Histochemical studies had shown that intense GGT activity is detectable in the intimal layers of human atherosclerotic lesions, where it is apparently expressed by CD68+ macrophage-derived foam cells [236]. Moreover GGT-positive foam cells were found to co-localize with immunoreactive oxidized LDL, suggesting a possible role for GGT in the cellular processes of iron-mediated LDL damage. Interestingly, catalytically active GGT was also demonstrated in correspondence of microthrombi adhering to the surface of atheromas [236, 238, 239].

There are two possibilities for GGT origin in the plaque: an endogenous and an exogenous component. Concerning the first one, it could be dependent of the cellular elements of the plaque (inflammatory and smooth muscle cells). Recently, RT-PCR analysis revealed in plaque extracts the presence

of GGT mRNA transcribed from GGT-1 gene, one of the thirteen homologs of human GGT gene [159]. This could indicate that plaque GGT may at least in part also derive from local synthesis of the protein and a likely endogenous source of GGT could be represented by inflammatory cells [213, 240, 241].

On the other hand, as far as the exogenous component, this is a hypothesis supported from the finding that serum GGT, of patients suffering from CVD, seems to be transported by lipoproteins, including LDL [242, 243, 244]. A first characterization of GGT extracted from atherosclerotic plaques showed the presence of two distinct enzymatic forms based on molecular size, surface charge and precipitability. The form with high molecular weight (similar to complex between GGT and LDL found in the serum), with a high negative charge (high *N*-glycosylation) and precipitable with polyanions (in the conditions of serum LDL) may have serum origin [159]. Serum GGT can be divided into two fractions, either hydrophilic or hydrophobic with different characteristics of density, size and charge. The hydrophobic fraction is formed by a set of molecular complexes consisting of GGT transported by lipoproteins VLDL, LDL, HDL, and chylomicrons, thanks to the lipophilic domain located at the N-terminus domain of the GGT heavy chain, responsible for the normal enzyme insertion in the plasma membrane of cells. The hydrophilic form is constituted by GGT devoid of this lipophilic N-terminal domain, therefore not tied to any plasma transporter [242].

Using a method based on the separation of GGT complexes by size exclusion chromatography and identification of the enzyme activity, the distribution of GGT fractions in 200 blood donors (100 men and 100 women) was screened [245]. Four sub-fractions, corresponding to macro-molecular complexes with different molecular size, called big-GGT (b-GGT, > 2000kDa), medium-GGT (m-GGT, 940 kDa), small-GGT (s-GGT, 140 kDa) and free-GGT (f-GGT, 70 kDa) have been identified [245, 246] (**Fig. 7**).

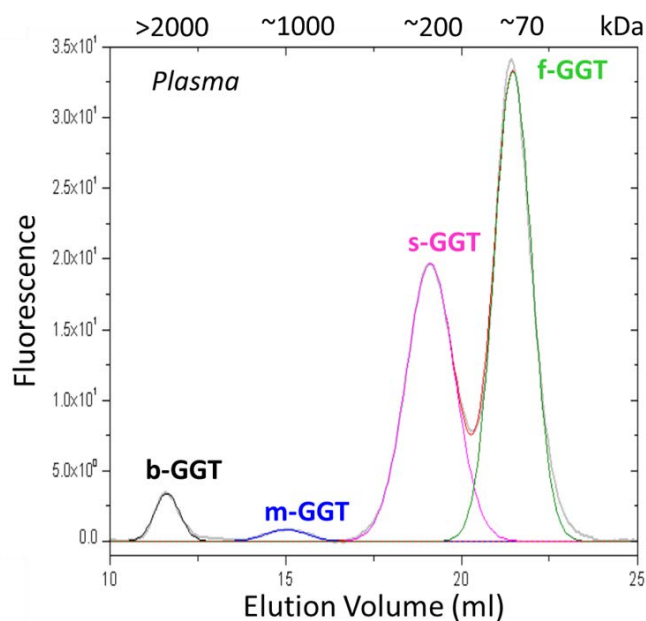


Figure 7. Plasma GGT elution profiles. High performance gel filtration chromatography method for GGT fraction analysis [245].

The molecular weights of b-, m-, and s-GGT fractions are respectively compatible with those of the complexes with lipoproteins VLDL, LDL, HDL, while that of f-GGT corresponds to the free enzyme form. Nevertheless, the gel filtration elution volumes of individual fractions (*i.e.*, their molecular weights) did not change, independently of the amount of associated GGT activity (**Fig. 7**). This suggests a specific interaction of GGT enzyme with each lipoprotein particle, rather than aspecific absorption of lipophilic GGT to circulating lipoproteins; the latter would produce a shift in elution volume peaks, as a consequence of the varying extents of GGT protein absorption. From the analysis of the activity of the four fractions as a function of the total GGT activity in both genders, the f-GGT fraction was the most represented at low values of total GGT, while an increase of GGT total concentration depended mainly on the s- and b-GGT fractions [245] (**Table 6**).

Table 6. Total and fractional GGT activities (mean $\pm$ SD, U/L) in both genders (Franzini M *et al.*, *Anal. Biochem.* 2008)

	Males (<i>n</i> = 20)	Females (<i>n</i> = 20)	<i>P</i>
GGT total	21.3 $\pm$ 11.1	13.1 $\pm$ 1.6	<0.01
b-GGT	2.6 $\pm$ 2.3	1.6 $\pm$ 1.1	NS
m-GGT	1.0 $\pm$ 0.7	0.7 $\pm$ 0.4	NS
s-GGT	8.1 $\pm$ 6.0	5.1 $\pm$ 4.4	NS
f-GGT	11.5 $\pm$ 4.1	8.2 $\pm$ 2.5	<0.01

Note. The parametric Student *t* test was applied. NS, not significant.

These observations are of particular interest, considering that serum levels of GGT have been repeatedly proposed to play an independent role in clinical evolution of CVD related to atherosclerosis, including stroke [11, 238]. A series of studies indicated that GGT is associated with overall mortality and cardiovascular events. The association occurs both in unselected populations [247, 248] and in patients with ascertained coronary artery disease, independently from potential confounders including liver disease and alcohol abuse [239, 249]. Interestingly, although conditions associated with increased atherosclerosis such as obesity, elevated serum cholesterol, high blood pressure and myocardial infarction are positive determinants of serum GGT activity, all studies mentioned above found that GGT predictive value for cardiovascular disease is independent from that of these determinants [239, 247, 250]. These findings were finally confirmed by a prospective study in a population of over 160,000 subjects [251]: serum GGT, with a dose–response relationship, was shown to predict the occurrence of incident fatal events during coronary heart disease, congestive heart failure and stroke [251] (**Fig. 8**).

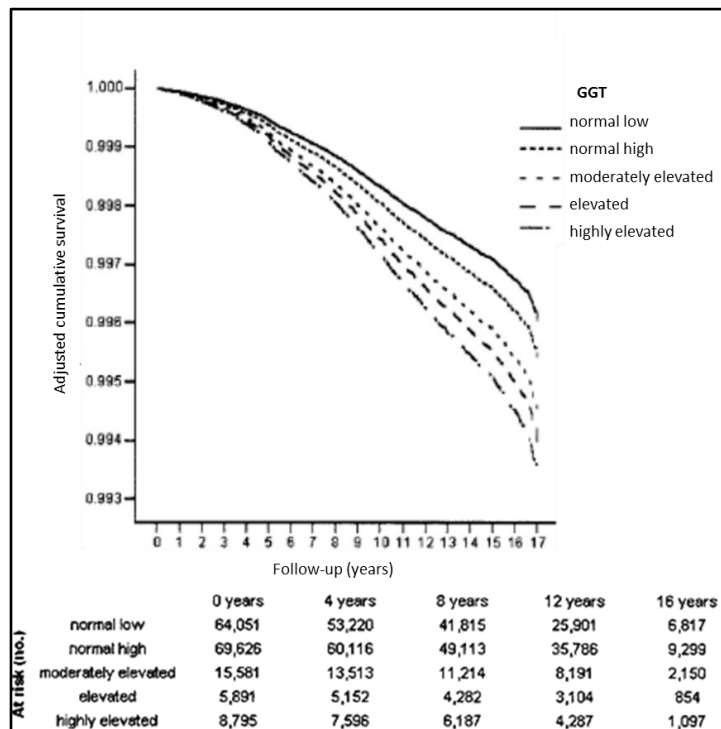


Figure 8. GGT and cardiovascular mortality. Adjusted cumulative survival from CVD mortality according to categories of GGT among 136,944 women and men (mean age 42 years) in the Vorarlberg Health Monitoring and Promotion Program (VHM&PP) estimated at the average values of covariates. Survival curves were calculated with a Cox proportional hazards model that was adjusted for sex, age, body mass index, systolic blood pressure, cholesterol, triglycerides, glucose, smoking, work status, and year of examination. Numbers at bottom of graph indicate participants available for analysis at given time points [251].

CHAPTER II

Macrophages and Inflammation

Macrophages execute central roles during the initiation, progression and vulnerability of the atherosclerotic plaque. Like other immune cells, macrophages are not static but show high plasticity and heterogeneity in response to a multitude of stimuli received from their micro-environment. This macrophage heterogeneity is reflected by functional polarization of differentiated macrophages and the dynamic switch between phenotypes. Under physiological condition, the M1/M2 population remains in mixture state while disoriented shift from M1 to M2 or *vice versa* results in disease progression.

This chapter will describe the fundamental differentiation and polarization process of macrophage lineage cells, but will also highlight the main differences in lipid and iron metabolism, as the critical role of glutathione and reactive oxygen species in M1 and M2.

Based on this broad background, the final aim will be to evaluate the link between macrophagic lineage cells as a potential source of the GGT found in atherosclerotic plaques, in order to understand whether macrophages may provide a source of b-GGT within the atherosclerotic plaque (*“Monocytes/macrophages activation contributes to b-gamma-glutamyltransferase accumulation inside atherosclerotic plaques”* [252] – The first article published is presented at the end of the chapter II).

2.1 Macrophages heterogeneity in atherosclerotic plaques

Macrophage functions can broadly be categorized by a series of dichotomies, for example, innate or acquired immunity, tissue destruction or repair, immigration or emigration, cholesterol accumulation or release, pro-inflammatory or anti-inflammatory phenotype. These functions could in principle be carried out by distinct subpopulations of macrophages or, in some cases, lineages related to different monocyte precursors as suggested in the previous chapter.

Actually, M1 and M2 macrophages are thought to represent the extreme polarization phenotypes of a continuum of pro- and anti-inflammatory macrophages all simultaneously present in atherosclerotic lesions. Pro-inflammatory macrophages were found in plaques several decades ago, while M2 macrophages were detected more recently [253]. Stöger et al. [254] studied the distribution of M1 and M2 macrophages in human plaques and showed that both types increase in numbers along plaque progression and are equally distributed in the fibrous cap region. M1-specific cell markers were preferentially detected in rupture-susceptible shoulder regions, whereas M2-specific markers were predominant in more stable plaque regions out of the lipid core in the adventitia [255]. M2-enriched lesion areas expressed high levels of IL-4, a cytokine that is essential for M2 polarization. The observed M2 macrophage population was relatively more resistant to the formation of foam cells and had increased ability to store engulfed cholesterol esters compared to M1 and resting macrophages. M1-specific markers were shown to be increased in carotid atherosclerotic lesions, while M2 markers were preferentially located in femoral

atherosclerotic plaques [256]. In addition, plaque M1 macrophages showed up-regulated expression of several MMPs [257]. Indeed, these observations suggest that M1 macrophages are preferentially accumulated in symptomatic and unstable plaques. Therefore, an important aspect is to link the presence of subset-specific surface markers to the actual function of the macrophages in the atherosclerotic plaque. Indeed, the contribution of subsets to the atherogenic process may be better understood, by studying the functions and signaling pathways of the plaque macrophage subsets in more depth, as described in detail in all following paragraphs.

2.1.1 GM-CSF and M-CSF: factors of macrophage differentiation in the plaque

The heterogeneity of macrophages population in atherosclerotic lesions has been a topic of great interest. As previously described, macrophage differentiation means the differentiation of monocytes to macrophages when monocytes infiltrate into the arterial wall and transform from round-shaped cells to irregularly shaped cells capable to intake antigens and migrate within the wall. In humans, blood monocytes are divided in two subpopulations based on CD14 and CD16 expression: CD14⁺ CD16⁻ are considered as the counterpart of the murine Ly6C^{high} monocytes (“inflammatory”) and CD14⁻ CD16⁺ are the phenotypic equivalent of the murine Ly6C^{low} monocytes (“resident”) [85, 86, 87] (**Fig. 9**).

In vitro, monocyte differentiation is driven by two growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) that leads to the formation of macrophages phenotypically similar to M1 (pro-inflammatory) and M2 (anti-inflammatory), respectively [81]. Both, pro and anti-inflammatory macrophages M1 and M2 have been detected in atherosclerotic plaques. M1 cells – widely known as “classically activated” – are characterized by a round morphology. They are involved in the management of Th1-dependent pro-inflammatory immune responses, because they produce a range of inflammatory cytokines – such as IL-1, IL-6, IL-8, IL-12, IL-23 and TNF- α – as well as ROS and NO. On the other hand, M2 cells, known as “alternatively activated”, are characterized by an anti-inflammatory phenotype, with a stretched, spindle-like morphology. They could be induced by Th2 cytokines, and produce IL-10 and various scavenger receptors such as CD36, macrophage scavenger receptor 1, macrophage receptor with collagenous structure and mannose receptor (MRC1 or CD206) [258]. In general, they promote angiogenesis, resolution and repair processes, and tumor progression [259, 90] (**Fig. 9**).

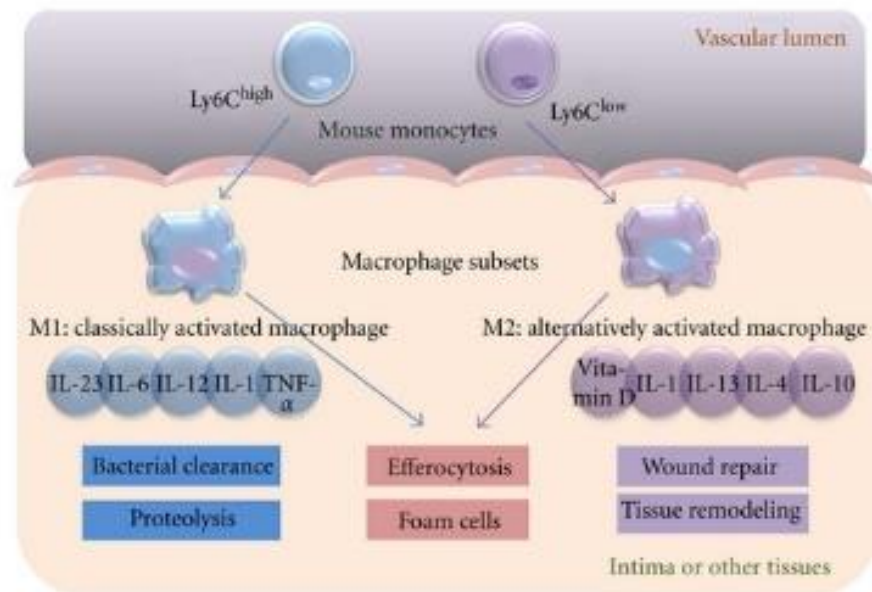


Figure 9. Signalling pathways in macrophages involved in atherosclerosis [19].

Implications for a M-CSF/GM-CSF balance in plaque macrophage differentiation *in vivo* are mainly based on *in vitro* studies showing this regulation in atherosclerosis-related cell types and detection in murine and human plaques. *In vitro* studies show that M-CSF is constitutively expressed under physiological conditions by EC, fibroblasts, macrophages and vascular SMC [260]. M-CSF expression is also shown to be increased after stimulation by pro-inflammatory stimuli (*e.g.* $TNF-\alpha$, $IFN\gamma$) [260] and oxLDL [261]. In murine and human lesions, M-CSF is detected both in healthy arteries and atherosclerotic lesions and in the latter correlates with macrophages and foam cells content as well as plaque progression [262]. In contrast, GM-CSF is poorly expressed by EC, vascular SMC and macrophages under basal conditions and requires inflammatory stimuli (*e.g.* $TNF-\alpha$ or IL-1) [260] or oxLDL [263] for induction. GM-CSF is co-localised only at very low levels with vascular SMCs and ECs in healthy human arteries, while it was increased in these cell types during macrophage accumulation and atherosclerosis development [264].

In line with these data, both factors may contribute to the macrophage heterogeneity observed in plaques. Since M-CSF is constitutively expressed, macrophages infiltrating early lesions are likely differentiated towards a M0 (intermediate state) and M2-like phenotype. As the plaque progresses, oxLDL and other inflammatory stimuli may increase the production of both M-CSF and GM-CSF, thereby producing an imbalance between these differentiation factors. Since GM-CSF expression seems to be specifically high in advanced lesions, this may favor a phenotypic switch towards pro-inflammatory M1-like macrophages upon plaque progression, similarly as observed in murine lesions [265]. Finally, monocyte-macrophage differentiation is also induced by chemokine (C-X-C motif) ligand 4 (CXCL4, or platelet factor 4) which primes monocyte differentiation to “M4 cells” another pro-inflammatory macrophage whose actual role in atherogenesis is unknown [266].

The current classification of macrophage differentiation reveals a far more complex picture. In fact, upon differentiation, macrophages are exposed to a wide variety of inflammatory cytokines in the inflammatory environment. Next, this aspect of the macrophage polarization based on published evidences will be discussed.

2.1.2 Macrophage polarization

Polarization of macrophages *e.g.* ability to switch phenotype and functional characteristics in response to external signals, designates their plasticity. M1 and M2 macrophages could be polarized from M0 macrophages by LPS and IFN- γ [267], or by IL-4 [268], respectively. Within the anti-inflammatory M2 set, several subsets are distinguished depending on the polarizing stimuli — such as M2a, M2b and M2c — inducible from M0 by IL-4/IL-13, immune complexes plus IL-1 β or LPS, and IL-10/ TGF- β / glucocorticoids, respectively [269]. Moreover, each M2 subset is characterized by a unique function. M2a macrophages express anti-inflammatory cytokines and contribute to tissue remodelling [258]; M2b macrophages produce high amounts of IL-10 and low amounts of pro-inflammatory cytokines IL-1b, IL-6, IL-12 and TNF- α , and are characterized by immunoregulatory properties [290]. The M2c subset expresses IL-10, TGF- β , pattern-recognition receptor pentraxin-3 and high levels of Mer receptor kinase (MerTK) essential for efferocytosis [270]. M2a, M2b and M2c macrophages were found in both humans and mice, whereas M2d subset was identified only in the mouse [271] (**Fig. 10**). This subset could be induced by treatment of M0 macrophages with toll-like receptor agonists that activate the adenosine A2 receptor [91].

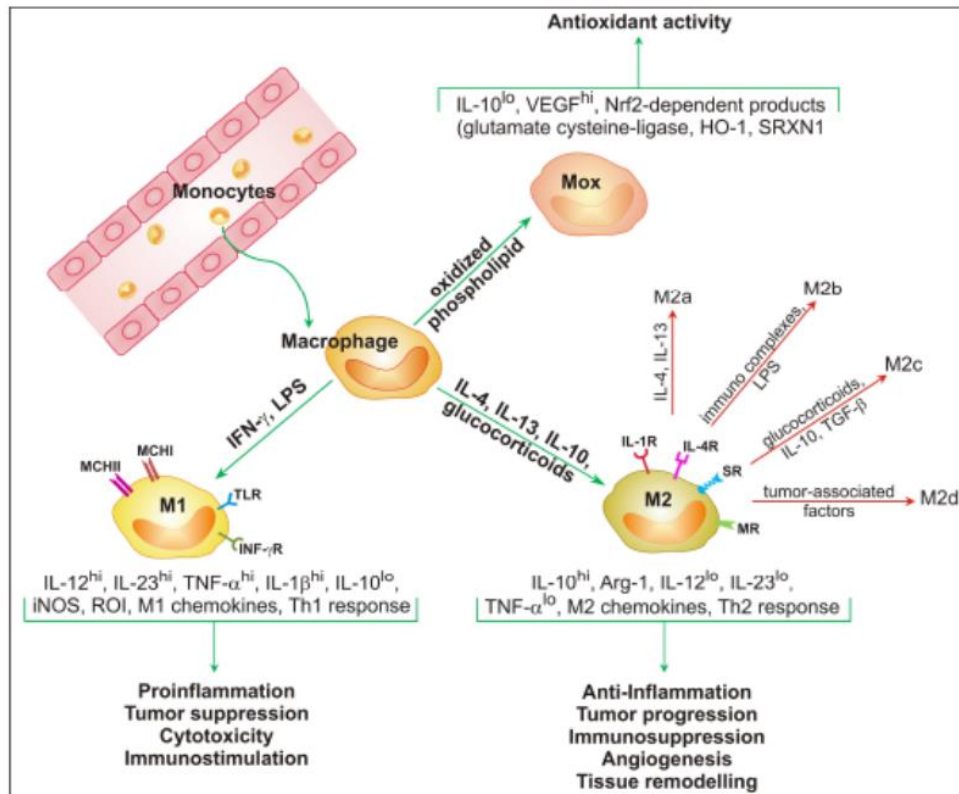


Figure 10. Polarization process of macrophages in the atherosclerotic lesions. M1: classical phenotype; M2: alternative phenotype; Mox: macrophages induced by oxidized low-density lipoproteins (oxLDL); ROI: reactive oxygen intermediates, iNOS: inducible nitric oxide synthase; Arg-1: arginase-1; HO-1: heme oxygenase-1; SRXN1: sulphiredoxin-1; VEGF: vascular endothelial growth factor [272].

Activation of the A2 receptor down-regulates the secretion of inflammatory cytokines like TNF- α , IL-1 β and IFN- γ , and induces as well a unique proangiogenic activity in M2d macrophages associated with production of vascular endothelial growth factor (VEGF), NO and IL-10 [273, 274]. Furthermore, the treatment of M0 macrophages with haeme degradation products could result in the polarization to the three phenotypically distinct macrophages HA-mac, M(Hb) and Mhem (Table 1), as described in the previous chapter (see chapter 1, section 1.2.2). Recently, additional plaque-specific macrophage phenotypes have been identified, termed as Mox, which it will be discussed in detail below.

2.1.3 Mox macrophages associated with atherosclerosis

The additional macrophage phenotype is represented by oxidized phospholipid derived macrophages (Mox) (Fig. 10). In particular, Mox subpopulation has been described to be produced by accumulation of oxidized phospholipids in atherosclerotic lesions.

Mox macrophages show different gene expression patterns and biological functions compared to M1 and M2 phenotypes. Expression of several genes in Mox macrophages is mediated by the redox-sensitive transcription factor Nrf2 [99]. It has been demonstrated that oxidative modification of

phospholipids is necessary for activation of Nrf2-dependent gene expression. Nrf2 goes to the nucleus and activates the genes involved in the synthesis of antioxidant enzymes including glutamate-cysteine ligase which is the first enzyme of the cellular GSH biosynthetic pathway [99]. In fact, an increased GSH/GSSG ratio in the Mox macrophages has been described compared to M1 and M2 phenotypes, suggesting that Mox macrophages have the ability to cope better with oxidative stress. Control of redox status in macrophages by Nrf2 may be important in the regulation of several cellular functions that influence tissue homeostasis and inflammation. Hence, an oxidizing environment such as in atherosclerotic lesions, induces the formation of a novel macrophage phenotype (Mox) that is characterized by Nrf-dependent gene expression and may significantly contribute to pathologic processes in atherosclerotic vessels. Defective redox regulation may lead to exacerbated cell death, as seen in chronically inflamed tissue [99].

2.1.4 Differential metabolism in GM-CSF and M-CSF differentiated macrophages

The clear metabolic differences existing between M1 (GM-CSF differentiation factor) and M2 (M-CSF differentiation factor) macrophages contribute to the shaping of their activation state [275].

Arginine catabolism is the most studied metabolism differentiating M1 and M2 macrophages is represented [276, 277]. L-arginine can be a substrate for either NOS which produce L-citrulline and NO, or arginase 1 (Arg-1) which produces polyamines, L-ornithine and urea. M1-derived NO is a major effector molecule in macrophage-mediated cytotoxicity, playing an important role in controlling bacterial and parasitic infections, whilst Arg-1 expression is linked to the wound healing actions of M2 macrophage population [276, 277].

M1 macrophages induce an anaerobic glycolytic pathway, which involves an increase in glucose uptake as well as the conversion of pyruvate to lactate [278]. In parallel, the pentose phosphate pathway (PPP) is also induced following IFN- γ /LPS activation. This pathway generates NADPH for the NADPH oxidase which is important for ROS production. In macrophages, increased lactate formation and activation of the PPP after phagocytosis had been already observed, suggesting potential importance of adapted metabolism on the activation cascade [279]. Additionally, down-regulation of the carbohydrate kinase-like protein (CARKL), which catalyzes the production of sedoheptulose-7-phosphate, is required for the development of a M1 phenotype [280]. Indeed, it has been observed that the drop of NADH levels in cells overexpressing CARKL during macrophage activation, results in a redox shift. PPP activity contributes to the reduction of redox couples *via* NADPH. Hence, increased GSH and NADH generation are observed during M1 activation while M2 activation resulted in an up-regulation of CARKL, which was not followed by increased GSH or NADH formation. These findings represent a functional distinction between the two polarization states that is CARKL dependent. Hence, CARKL can be considered as a kinase orchestrating pro- and anti-inflammatory immune responses through metabolic control. On the other hand, fatty acid oxidation and oxidative metabolism are the preferential pathways in IL-4-activated macrophages [281]. IL-4 activates the

transcription factor STAT6, which can trigger a pathway inducing mitochondrial respiration [280]. Upon activation, M2 macrophages can drive the pyruvate into the Krebs cycle and can induce expression of components of electron transport chain. Increased glycolysis in M1-polarized macrophages permits to quickly trigger microbicidal activity and cope with a hypoxic tissue microenvironment. In contrast, oxidative glucose metabolism in M2-polarized macrophages provides sustained energy for tissue remodeling and repair (**Table 7, see page 75**).

2.1.5. Lipid metabolism in GM-CSF and M-CSF differentiated macrophages

Both GM-CSF and M-CSF differentiated macrophages (M1 and M2, respectively) are able to take up lipids *in vitro* [282, 283] and *in vivo* [282]. This cholesterol load is accompanied by significant changes in the macrophage transcriptome. Currently, however, it is unclear whether there is a difference in lipid handling between these two types of macrophages. Only few studies focused on this subject and results so far are rather contradictory. Indeed, in primary murine macrophages, genetic profiles of M-CSF differentiated macrophages pointed favorably towards foam cells formation [264]. M-CSF upregulates enzymes involved in cholesterol biosynthesis and downregulates a transporter involved in cholesterol efflux, such as ATP-binding cassette transporter G1 (ABCG1). Moreover, in human monocyte-derived macrophages, oxLDL accumulation was shown to be higher in M-CSF differentiated macrophages than in GM-CSF differentiated cells and correlates with the upregulation of CD36 and SR-A, two membrane proteins involved in the uptake of modified lipids [284]. In particular, oxLDL loading of M-CSF differentiated macrophages causes a shift towards a M1-like phenotype *via* downregulation of the nuclear transcription Krüppel-like Factor 2 (KLF-2). In agreement with this, uptake of unmodified LDL was also found to be increased in M-CSF compared to GM-CSF differentiated human-derived macrophages [285]. In contrast, GM-CSF differentiated macrophages showed increased expression of several cholesterol efflux regulatory proteins, such as ABCG1, ATP-binding cassette transporter (ABCA1), peroxisome proliferator-activated receptor gamma (PPAR γ), liver X receptor alpha (LXR α), all involved in reducing foam cells formation. Moreover, based on CD68 and CD14 expression, Waldo et al. found lipid accumulation in both CD68⁺/CD14⁺ (M-CSF-like) and CD68⁺CD14⁻ (GM-CSF like) cells in advanced human atherosclerotic lesions [282]. In contrast to these studies, Kazawa et al. [283] found an increased uptake and storage of oxLDL in human GM-CSF differentiated macrophages, although they did find ABCA1 and LXR α to be upregulated. Therefore, future studies are necessary to further characterize the difference in lipid handling in these two macrophage subtypes and to determine the contribution of GM-CSF and M-CSF differentiated macrophage in *in vivo* foam cells formation in atherosclerosis progression. Anyway, macrophages recognize modified lipoproteins through toll-like receptor (TLR2 and TLR4). The scavenger receptor CD36, known to serve as TLR-co-receptor, binds oxLDL and triggers assembly of certain TLR heterodimers, leading to the induction of pro-

inflammatory mediators implicated in the deleterious effects of oxLDL [286]. The activation of TLR heterodimers, regulated by signals from CD36, leads to the activation of pro-inflammatory signaling pathways involving NFκB, MAP-kinase, ROS-dependent signaling, with an overall switching of macrophage phenotype towards M1. In addition, oxLDL-mediated stimulation of CD36 leads to activation of the inflammasome, which further aggravates vascular inflammation [287, 288] (**Table 7, see page 75**).

2.1.6 Iron metabolism in GM-CSF and M-CSF differentiated macrophages

Macrophages play an important role in iron homeostasis by recycling iron through phagocytosis of senescent red blood cells and their polarization is associated with differential regulation of iron metabolism [289]. On the other hand, iron can be used by bacteria for proliferation, virulence, and persistence [290].

M1 macrophages show a metabolic profile, which favors iron retention characterized by low heme uptake, high ferritin (iron storage) and low ferroportin (iron export). By this way, M1 macrophages reduce the labile iron pool, the metabolically active fraction of cytosolic iron that is available for metabolic purpose [291]. Therefore, sequestration of iron by M1 macrophages would have a bacteriostatic and tumoristatic effect. By contrast, M2 macrophages have a sustained heme uptake as well as a reduced iron storage and enhanced release of iron (low ferritin and high ferroportin) [289]. Iron release from M2 macrophages would favor tissue repair and cell proliferation (**Table 7, see page 75**).

2.1.7 Role of glutathione and reactive oxygen species in macrophages polarization

2.1.7.1 Glutathione and M1/M2 macrophages

In the last years GSH has been described to have an important role of in the immune response, by influencing the activity of both lymphocytes and macrophages [292, 293, 294]. Many *in vitro* and *in vivo* studies demonstrated that GSH depletion in antigen-presenting cells, such as macrophages, correlated with defective antigen processing and inhibited Th1-associated cytokine production. In murine studies, GSH depletion in antigen-presenting cells decreases the secretion of IL-12, known to regulate IFN-γ production, and leads to the switch from the typical Th1 cytokine profile towards Th2 response patterns [293, 295]. Hence, M1 cells have a higher ratio of reduced-to-oxidized glutathione (GSH/GSSG), with opposite effects of IFN-γ and IL-4 on the reductive status [296]. It is possible to regulate the amount of secreted IL-12 by modulating the intracellular GSH/GSSG balance and an IL-12^{low} M2-like phenotype can be changed into an IL-12^{high} M1-like phenotype. This has been demonstrated in a mouse asthma model where the release of Th2 cytokines orchestrate the recruitment and activation of the primary effector cells of the allergic response. [297]. However, infection by intracellular pathogens, such as *Mycobacterium tuberculosis*, provides an interesting example of an intra-macrophage pathogen able to modulate the T cell responses by

altering the intra-cellular redox state. In fact, Th1 immune response is down-regulated in patients with active tuberculosis infection [298], who have altered glutathione balance [299]. Alam et al. have demonstrated that IL-12 induction in native macrophages is controlled directly by the intracellular glutathione-redox and that manipulation of the macrophage redox state by N-acetylcysteine (NAC), a GSH precursor, can influence *in vitro* cellular immune response to pathogens of peripheral blood mononuclear cells obtained from patients with active tuberculosis [300]. These data support the critical importance of glutathione-redox status in regulating the induction of IL-12 which activates the Th1 T cell immune response, crucial for inducing protection against the intracellular pathogens (*e.g. Mycobacterium tuberculosis*). Therefore, it is possible that these pathogens, modulating the GSH/GSSG balance in macrophages, transform these cells in M2-like macrophages characterized by low IL-12 production, thus polarizing the immune environment to their favor. In fact, M1 macrophages display a microbicidal activity against a wide range of intracellular parasites. It can be observed that a low GSH/GSSG ratio characterizes a M2- like phenotype both in murine and human macrophages and that GSH replenishment can represent a strategy for repolarization of both macrophage species [293, 294] (**Table 7, see page 75**). Hence, it may be suggested that low macrophage GSH level can provide a consistent biomarker for human and murine M2 macrophages.

2.1.7.2 Reactive oxygen species and M1/M2 macrophages

The redox signalling has implication in macrophage polarization and the key roles of M1 and M2 macrophages in tissue environment provide the clue to explain the ROS abundance in certain phenotype. As previously described, M1 macrophages majorly clearing the pathogens and ROS may be crucial for the regulation of M1 phenotype, whereas M2 macrophages resolve inflammation, which favours oxidative metabolism. Therefore how ROS play its role in maintaining the homeostatic functions of macrophage and in particular macrophage polarization is not completely understood. Indeed, the regulation may be controversial, but the discrete role of ROS on macrophages may be impacted by the different sources of ROS as well as plasticity of macrophage itself.

As macrophages are endogenous scavengers for dying cells in various pathological conditions, interaction between macrophages with compartments determines the phagocytic function of macrophages. Dying cells produce high levels of ROS, which are released into extracellular area when cellular membrane is degraded during cell death. Attachment of dying cells to macrophages requires intercellular communication in which ROS may play a role. On the other hand, extracellular and intracellular ROS may differentially control the phagocytosis process of macrophage by regulating the ability and capacity of macrophages in the uptake and degradation of dying compartments. In this regard, ROS plays a critical regulatory role in determining the initiation and outcome of cellular phagocytosis.

As mentioned above, M1 macrophages possess a high bactericidal function as defense against invading pathogens is the primary function of M1. To clear the site of injury, M1 macrophages tend to trigger the bactericidal response, which involves the production of ROS and NO in contact with pathogen. The phagocytic function of M1 mainly depends on Nox2 gene. The production of ROS and NO derives from NADPH oxidase and nitric oxide synthase, both enzymes are generated from NADPH through pentose phosphate pathway [301], and Nox2 negatively regulates the phagosomal proteolysis [302]. The effective microbicidal function of M1 macrophages requires continuous production of ROS followed by delayed maturation of phagosomes. *In vitro* stimulation of M1 macrophages with LPS promotes recognition by TLRs, the primary LPS receptor, and, occasionally, LPS binds to phagocytic macrophage-1 antigen (MAC1) receptor independent of TLRs. The association of LPS with receptors drives the production of ROS and genes alterations. LPS-induced ROS generation is Nox-dependent and it further supports the ROS-induced TNF- α production, which is evidenced from the reduced TNF- α in PHOX^{-/-} mice model [303]. Besides, ROS may serve as secondary messenger in the LPS-induced signal transduction, facilitating the regulation of downstream pathways such as mitogen-activated protein kinase (MAPK) and NF- κ B. Activation of these pathways by H₂O₂ promotes expression of pro-inflammatory genes (**Table 7, see page 75**). Moreover, M1 macrophages activation is always correlated with upregulation of TNF- α mediated inflammatory response. Activation of TNF- α is deemed to depend on interaction of TNF with TNF receptors that triggers again the downstream signalling, MAPK and I κ B-kinases (IKK), that activates NF- κ B signalling [304]. In this case, H₂O₂ tends to accumulate in NF- κ B deficient cells when exposed to TNF, it further oxidizes the catalytic cysteine of MAPK phosphatases and triggers activation of MAPK cascades including JNK and p38 MAPK. Excessive H₂O₂ also promotes I κ B-kinase activation and drives tyrosine phosphorylation of I κ B α , leading to stimulation of NF- κ B signalling [305]. Given that ROS is closely related to the activation of MAPK and NF- κ B, ROS may partially regulate macrophage polarization towards M1.

In contrast to M1 macrophages, M2 activation stimulates increased arginase-1 activity and is accompanied by reduced ROS and NO generation. The functions of tissue remodeling and wound healing of M2 macrophages are explained to be attributed by the macrophages effect in expressing increased cathepsin S and cathepsin L and reduced Nox2 activity, which all improved the phagosomal proteolytic activity of M2 (IL-4) macrophages. Reduced Nox2 also improved the wound healing functions of M2 in degrading disulphide protein [306]. Furthermore, the interaction between M2 macrophages with apoptotic bodies triggers instability of NADPH oxidase Nox2 mRNAs through binding blockade of RNA-binding protein coding gene (*e.g.* SYNCRIP) to Nox2 3'-UTR. This further defects the ROS production and leads to M2 macrophage polarization [307] (**Table 7, see page 75**).

Moreover, although impact of redox signalling or ROS production in M1 macrophages activation seems to interfere with M2 macrophage priming, study by Zhang et al. postulated that ROS production is also important for M2 macrophage differentiation. Intervention of antioxidant butylated hydroxyanisole (BHA)

by inhibiting Nox-mediated $O_2^{\bullet}$ production before differentiation by M-CSF, blocked monocyte differentiation to M2, which suggests that ROS may implicate the early stage of M2 macrophage differentiation [308].

In summary, ROS has been shown to get involved in the functional and phenotypic regulation of macrophages. ROS is able to control the cell death, proliferation, motility, and phagocytic ability of macrophages. It is recently observed that ROS may play a complicated role in regulating macrophage polarization which can be summarized as follows:

- Involvement of ROS in regulating M1 is responsible for phagocytic activity and inflammatory response: multiple pathways are involved in generating NADPH, followed by Nox-dependant ROS production. The high ROS level used to mediate the phagocytic activity of M1 macrophages. Besides, ROS serves as a second messenger mediating the inflammatory response of M1 macrophages, primarily through MAPK and NF- κ B as well as inflammasome activation.
- Involvement of ROS in regulating M2 is responsible for inflammation resolving and wound healing activities: in contrast to M1, multiple pathways are involved in reducing NADPH and Nox activity followed by reduced ROS generation. The low ROS level was accompanied by reduced inflammatory mediators, increased M2-regulated genes responsible for inflammation resolution and increased disulphide protein degradation which enhanced wound healing effect of M2.

2.1.8 Specific phenotypic markers in M1 and M2 macrophages

The concept of polarization has been confirmed by clear and mentioned differences in cytokine production, NO metabolism, phagocytosis [90, 258, 259, 309, 310] and transcriptional profiles [311, 312, 313]. Macrophage polarization is also accompanied by specific changes in cell morphology and phenotype, as described above [90, 258, 259, 309, 310]. The emerging concept that macrophage functions are, at least in part, determined by the polarization status of the cells raised the question whether these distinct polarized macrophage subsets could be identified by specific phenotypic markers. Additionally, studies focusing on macrophage polarization are mostly performed *in vitro* and do not reflect the complexity of immune responses observed *in vivo* [314, 315]. For these reasons, the validation of specific phenotypic markers is highly relevant for further investigation of different aspects of human macrophage biology.

M1 and M2 macrophages are distinguished by the differential expression of several molecules, *e.g.*, iNOS, MMPs and arginase [316]. None of these antigens is suitable as a single-marker to identify polarized macrophages. According to the literature, CD80, CD14, CD68 (surface receptor for LDL) are phenotypical markers for M1-like macrophages [317]. The mannose receptor (CD206) and the scavenger receptor (CD163), mainly found in advanced human lesions near intraplaque hemorrhage areas [93], and whose expressions are enhanced by IL-4 [268, 318] and IL-10, respectively [319], are specific markers for

M2-like macrophages. However, cell markers alone do not fully define the overall subpopulations of macrophages [320]. Indeed, the same cell markers have also been identified as expressed by other cell types, for instance increased levels of CD163 are also found in the microglia of Alzheimer's disease patients', as well as in frontal and occipital cortices and brainstems of Parkinson's disease patients [321]. Moreover, several results in the literature, show that CD68 (clone PG-M1) may also identify dendritic cell subsets [322].

In summary, in order to identify macrophages subsets, a combination of markers is required [323]. CD68 or CD163 can be used to identify M1 polarized macrophages when in combination with signal transducer and activator of transcription 1 (pSTAT1) or recombination signal binding protein for immunoglobulin kappa J region (RBP-J), while the same in combination with CMAF (Maf transcription factor) — an essential transcription factor for interleukin IL-10 gene expression in macrophages [324] — identify M2 macrophages (**Table 7, see page 75**). This observation supports the notion that macrophage polarization is a dynamic process and the use of subset-specific phenotypic markers may open a new avenue for *in vitro* functional studies as well as a more accurate characterization of the macrophage infiltration in a variety of immune-mediated inflammatory diseases.

Finally, the findings considered above also suggest a functional plasticity of macrophages, being capable to change phenotype in response to microenvironmental stimuli in the plaque (*e.g.* cholesterol, oxLDL, oxidized phospholipids, chemokines, cytokines). Basically, two initial levels of regulation can be distinguished [325]:

- i) Factors that drive monocyte-to-macrophage differentiation such as M-CSF, GM-CSF and CXCL4;
- ii) Cell-derived cytokines that drive subsequent polarization of macrophages and highly influence their inflammatory phenotype.

However, it is unclear if different phenotypes come to coexist within the same location. Existing data suggests that plaque macrophages express features that are intermediate between the M1 and M2 phenotype extremes [80]. M0, the intermediate state, provides the dynamic plasticity to atherosclerotic macrophages to activate or downregulate an overlapping set of transcription factors in response to extrinsic signals and switch phenotype to adjust to environmental changing like lipids and ROS levels. Consequently, discriminating plaque macrophages solely as M1 or M2 is too simplistic. Studies identifying the presence of diverse macrophage subsets in plaques, followed by the examination of their phenotype and function *in vivo* and *in vitro*, can eventually be coupled back to the *in vivo* situation and in this way teach us more about the role of different macrophage subsets in the atherosclerotic process. Until now, detailed information about the role and function of the so far identified macrophage subsets in atherosclerotic processes is still minimal and mainly based on *in vitro* observations. Furthermore, as

detailed above, the field is still in need of good characterization of a panel of markers to identify subsets and correlate them to plaque phenotype characteristics. In any case, growing evidence indicates that understanding the mechanisms of macrophage plasticity and resolving functional characteristics of distinct macrophage phenotypes should help in the development of new strategies for treatment of chronic inflammation in cardiovascular disease.

Table 7. Characteristics of M1 and M2 macrophage subsets.

Phenotype	M1	M2
Functions and properties	Pro-inflammation (Inflammatory response)	Anti-inflammation (Tissue remodelling)
Inducers	GM-CSF	M-CSF
Polarization factors	IFN- γ , LPS, TNF- α	IL-4, IL-10, IL-13, glucocorticoids
Effectors and cytokines upon activation	IL-12 ^{high} , IL-23 ^{high} , TNF- α ^{high} , IL-1 β ^{high} , IL-10 ^{low} , iNOS, ROS, ROI, M1 chemokines, Th1 response	IL-10 ^{high} , Arg-1, IL-23 ^{low} , IL-12 ^{low} , TNF- α ^{low} , M2 chemokines, Th2 response
Surface molecules	CD80, CD14, CD68, GLUT, MHCII CD68 + pSTAT 1 or RBP-J CD163 + pSTAT 1 or RBP-J	CD206, CD163, CD36, SR CD68 or CD163 + CMAF
Arginine metabolism	Substrate for iNOS ↓ L-citrulline ↓ NO ↓ cytotoxicity KILLING RESPONSE	Substrate for arginase ↓ L-ornithine ↙ ↘ Polyamines L-proline ↓ ↓ cell proliferation collagene production PROMOTION OF TISSUE REPAIR
Sugar metabolism	↑Glycolysis→Pyruvate→Lactate	↑Free fatty acid→ β -oxidation, Krebs cycle, oxidative phosphorylation
Lipid handling	↑ ABCA1/G1 ↑ PPAR γ ↑ LxR α	↓ ABCG1 ↑ CD36 ↑ SR-A
Iron metabolism	Ferritin ^{high} (iron storage) Ferroportin ^{low} (iron export) → Iron Retention	Ferritin ^{low} (iron storage) Ferroportin ^{high} (iron export) → Iron Release
Redox state: GSH	> GSH/GSSG (IL-12 ^{high})	< GSH/GSSG (IL-12 ^{low})
ROS signaling	LPS ↔ TLRs PPP (CARKL↓) ↓ ↑NADPH ↓ ROS^{high} (↑ Nox2 activity) MAPK and NF-KB activation ↓ iNOS, TNF-α Inflammasomes (ATP-dependent) ↓ IL-1β, IL-18 ↑ INFLAMMATORY RESPONSE • ROS^{high}: ↑Phagocytic activity (<i>Nox2-dependent</i>) • ROS^{high} (<i>as second messengers</i>): mediate inflammatory response	↑ cathepsin S and L expression PPP^{low} (CARKL↑) Nox2 3'UTR \$ SYNCRIP ↓ ↓NADPH ↓ ROS^{low} (↓ Nox2 activity) • ROS^{low}: ↑Phagosomal proteolytic activity • ↓Nox2 activity: would healing improvement (↑disulphide protein degradation) • ↓Inflammatory mediators: TNF-α, IL-1β (↓ATP plasma membrane ion channel) • Cu, Zn-SOD action: ↑Arg-1, ↑FIZZ-1, ↑YM-1 (<i>regulated by redox-dependent STAT6 translocation</i>) ANTI-INFLAMMATORY & WOUND HEALING

For abbreviations, see list of abbreviations.

2.2 Macrophage lineage cells: a source of GGT in the atherosclerotic plaque?

Considering all the background above, one of the objective of my experimental work has been focused on the relation between monocyte/macrophage lineage cells and GGT, an independent risk factor for cardiovascular mortality related to atherosclerotic disease [251] (see chapter 1, section 1.5.3). Indeed, previous results indicate that neutrophils contain GGT in their granules and release it upon activation [158]. Importantly, human mononuclear cells have long been known to possess GGT activity [213, 326] and the enzyme is also present in GGT-positive CD68+ macrophage-derived foam cells in the intimal layers of human atherosclerotic plaques [12, 238]. In addition, GGT can be found as macro-molecular complexes with different size [246]. Only the big GGT (b-GGT, > 2000 kDa) fraction accumulates in atherosclerotic plaques, and correlates with other histological markers of plaque vulnerability—such as high macrophagic infiltration— as well as with levels of plasma b-GGT [15, 155, 213, 217, 228, 230, 236, 238, 326, 327, 328].

These pieces of evidence have prompted the need of characterizing the exact origin of GGT accumulating inside atherosclerotic plaques, in order to better understand its role in plaque instability and to assess whether monocytes/macrophages may contribute and represent a source of b-GGT within the atherosclerotic plaque.

2.3 Article 1

MONOCYTES/MACROPHAGES ACTIVATION CONTRIBUTES TO B-GAMMA-GLUTAMYLTRANSFERASE ACCUMULATION INSIDE ATHEROSCLEROTIC PLAQUES *(Journal of Translational Medicine, 2015, 13:325)*

Les lésions athérosclérotiques sont caractérisées par une abondance de monocytes et de macrophages. Ceux-ci peuvent acquérir des phénotypes et fonctions biologiques différentes en fonction du microenvironnement et de leur état métabolique. Le recrutement des leucocytes joue un rôle clé dans les processus complexes aboutissant à l'athérosclérose. Plus particulièrement, les monocytes envahissent les lésions athérosclérotiques et se différencient en macrophages. L'hétérogénéité des macrophages décelables dans les lésions athérosclérotiques a été, dernièrement, un sujet de grand intérêt. Une attention particulière a été portée aux deux grandes sous-populations de macrophages impliquées dans les processus pro-inflammatoires (phénotype "M1") et dans la résolution de l'inflammation et la réparation (phénotype "M2").

De plus, la gamma-glutamyl transférase (GGT) est un facteur de risque indépendant bien établi pour la mortalité cardiovasculaire liée à la maladie athéroscléreuse. Quatre complexes de GGT allant de la GGT libre (f-GGT, 70 kDa) à un complexe macromoléculaire de 2000 kDa (b-GGT) ont été identifiés dans le plasma. Cependant, seule la b-GGT s'accumule dans les plaques d'athérosclérose, et concorde avec l'apparition d'autres marqueurs histologiques de vulnérabilité. La présente étude a pour but d'évaluer si les monocytes et/ou les deux types de macrophages pro- ou anti-inflammatoires (M1 et M2) peuvent représenter une source de b-GGT au sein de la plaque athérosclérotique. L'expression et la libération de la GGT ont été étudiées dans des monocytes humains isolés à partir du sang périphérique de donneurs sains. Les facteurs de croissance GM-CSF et M-CSF ont été utilisés pour induire la différenciation des monocytes en macrophages M1 et M2, respectivement, tandis que la GGT dans la plaque a été étudiée dans des échantillons de tissu obtenus de patients subissant une endoarterectomie carotidienne. Les résultats révèlent que les macrophages de type M1 expriment des niveaux plus élevés de GGT par rapport aux macrophages de type M2 et aux monocytes. De plus, les macrophages de type M1, mais pas les M2, sont capables de libérer la fraction b-GGT après activation avec des stimuli pro-inflammatoires. L'analyse par western blot de la b-GGT extraite des plaques a confirmé la présence de GGT en parallèle de la présence des macrophages.

Ces résultats indiquent que les macrophages caractérisés par un phénotype pro-inflammatoire peuvent contribuer à l'accumulation intra-plaque de la b-GGT, qui à son tour peut jouer un rôle dans la progression de l'athérosclérose en modulant les processus inflammatoires et favorisant l'instabilité de la plaque.



RESEARCH

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Monocytes/macrophages activation contributes to b-gamma-glutamyltransferase accumulation inside atherosclerotic plaques

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Abstract

Background: Gamma-glutamyltransferase (GGT) is a well-established independent risk factor for cardiovascular mortality related to atherosclerotic disease. Four GGT fractions have been identified in plasma, but only b-GGT fraction accumulates in atherosclerotic plaques, and correlates with other histological markers of vulnerability. The present study was aimed to evaluate whether macrophagic lineage cells may provide a source of b-GGT within the atherosclerotic plaque.

Methods: GGT expression and release were studied in human monocytes isolated from peripheral blood of healthy donors. The growth factors GM-CSF and M-CSF were used to induce differentiation into M1-like and M2-like macrophages, respectively. Plaque GGT was investigated in tissue samples obtained from patients undergoing carotid endarterectomy.

Results: We found that M1-like macrophages express higher levels of GGT as compared to M2-like, and that both monocytes and M1-like macrophages—but not M2-like—are able to release the b-GGT fraction upon activation with pro-inflammatory stimuli. Western blot analysis of b-GGT extracted from plaques confirmed the presence of a GGT immunoreactive peptide coincident with that of macrophages.

Conclusions: Our data indicate that macrophages characterized by a pro-inflammatory phenotype may contribute to intra-plaque accumulation of b-GGT, which in turn may play a role in the progression of atherosclerosis by modulating inflammatory processes and favouring plaque instability.

Keywords: Gamma-glutamyltransferase, b-GGT fraction, Monocytes, Macrophages, Atherosclerosis

Background

Monocyte/macrophage lineage cells represent one of the main cellular components of atherosclerotic lesions. Atherosclerosis is a progressive and chronic inflammatory disease characterized by cellular and molecular alterations within the arterial wall which lead to lumen narrowing and/or to plaque rupture with superimposed acute thrombosis [1, 2]. Such condition is by far the most frequent underlying cause of coronary, carotid and

peripheral arterial disease [3]. The inflammatory reactions taking place in plaques implicate highly complex processes that are still not completely understood. However, it is known that the recruitment of leukocytes play a key role in these events, and monocytes in particular invade atherosclerotic lesions and differentiate into macrophages. The heterogeneity of macrophages detectable in atherosclerotic lesions has been a topic of great interest lately, with particular reference to the two major macrophage subpopulations involved in pro-inflammatory processes ("M1" phenotype) and in resolution and repair ("M2" phenotype), respectively. Actually, M1 and M2 macrophages are thought to represent the extreme polarization phenotypes of a continuum of pro- and

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anti-inflammatory macrophages all simultaneously present in atherosclerotic lesions [4–6]. M1 macrophages were demonstrated to be the predominant phenotype in rupture prone shoulder regions of human plaques, while M2 macrophages-associated markers were predominant in the adventitia [7]. On the other hand M2 macrophages were identified in more stable cell-rich areas, away from the lipid core of human atherosclerotic plaques [8, 9]. It was suggested that—in response to the changing environment in developing atherosclerotic plaques—macrophages may shift transiently between different phenotypes. Nevertheless a number of questions remain to be answered, e.g. little is known about the propensity of individual macrophage phenotypes to become foam cells, even if some reports suggest that both M1 and/or M2 macrophages may be foam cell precursors [5, 10].

Epidemiological studies showed that total serum gamma-glutamyltransferase (GGT) activity is an independent risk factor for cardiovascular mortality related to atherosclerotic disease [11] and a correlation between plasma GGT levels and the presence of multivessel disease has been proposed [12]. We recently identified four different GGT fractions in human blood [13] and an association between the fraction with highest molecular weight (b-GGT) with known cardiovascular risk factors—such as LDL-cholesterol, triglycerides, glucose and C-reactive protein—was demonstrated [14]. More recently, a first characterization revealed that b-GGT consists of membrane microvesicles with physical properties resembling those of exosomes [15].

Importantly, b-GGT has been detected also inside atherosclerotic plaques [16] and it has been suggested that this may be a factor in pathogenesis of atherosclerosis. Indeed, GGT enzyme activity is involved in the modulation of inflammatory mediators such as leukotrienes [17] and S-nitrosoglutathione (GSNO) [18] and it has been repeatedly documented that in the presence of glutathione and transition metal cations (in particular Fe^{3+} and Cu^{2+}), GGT activity is able to trigger the production of reactive oxygen species (superoxide, hydrogen peroxide), thus promoting LDL oxidation and other pro-oxidant reactions potentially involved in the progression of atherosclerotic lesions [19–21]. The necrotic core of the plaque, where both GGT [19, 20] and iron [22] are present at adequate concentrations, represents an environment favourable to the evolution of such reactions.

Human mononuclear cells have long been known to express GGT activity [23, 24] and GGT-positive CD68+ macrophage-derived foam cells are present in the intimal layers of human atherosclerotic plaques [25, 26]. The levels of intra-plaque b-GGT activity were demonstrated to correlate with both plasma b-GGT and levels of macrophagic infiltration, a histological marker of plaque

vulnerability [27]. Against this background, the aim of the present study was to assess whether monocytes/macrophages may represent a source of b-GGT within the atherosclerotic plaque.

Methods

Chemicals

Unless otherwise indicated, all reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human cytokines and growth factors were purchased from PeproTech (London, UK).

Ethics, consent and permissions

The study was approved by the Institutional Ethics Committee of the University Hospital of Pisa, and conformed to the Declaration of Helsinki. Patients gave their verbal informed consent to participate and the data were analysed anonymously.

Isolation and activation of monocytes

Human peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque®-1077 density centrifugation from the blood of healthy donors. Monocytes were enriched by plating (10×10^6 cells/wells) PBMCs in 6-well plates (Sarstedt) with RPMI 1640 medium containing 2 mmol/L L-glutamine and 10 % v/v fetal calf serum, the latter ultracentrifuged ($100,000 \times g$, 120 min, 4 °C) before addition to remove serum-associated b-GGT (data not shown). After 2 h, non-adherent cells were removed and adherent cells were washed five times with RPMI 1640. Cytospin preparations of trypsinized cells were prepared to evaluate the purity of adherent monocytes (74.5 ± 3.4 monocytes, 25.0 ± 3.6 lymphocytes, 1.1 ± 0.4 neutrophils; $n = 8$). Monocytes-enriched cell preparations were then incubated with ionomycin (1 μM , 15 min), LPS (5 $\mu\text{g}/\text{ml}$, 24 h) or a combination of TNF α /IL-1 β (both 10 ng/ml, 24 h), as described by others [28–30]. All cells were kept in a humidified incubator with 5 % $\text{CO}_2/95$ % air and cell viability was assessed by Trypan blue exclusion. Finally media were collected and centrifuged at $300 \times g$ (5 min, 4 °C) and $10,000 \times g$ (10 min, 4 °C) before GGT determinations. Further details are reported in the figure legends.

Polarization of monocytes into M1-like or M2-like phenotype

In vitro differentiation of monocytes into macrophages with properties similar to M1 and M2 cells (“M1-like” and “M2-like”, respectively) was obtained as repeatedly described [10, 31–34]. Briefly, PBMCs isolated by Histopaque®-1077 density centrifugation were left to adhere for 24 h in RPMI 1640 medium integrated with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$

streptomycin and 10 % v/v fetal calf serum. After 24 h, non-adherent cells were removed, while adherent cells were cultured for additional 6 days in complete medium integrated with 50 ng/ml recombinant human granulocyte/macrophage colony stimulating factor (GM-CSF) or with 50 ng/ml macrophage colony stimulating factor (M-CSF), respectively. Where indicated, cells were incubated for additional 24 h with a combination of TNF α /IL-1 β (both 10 ng/ml) [28–30] and media were collected as described above.

In another set of experiments, adherent monocytes were incubated with M-CSF for 6 days in the presence of a mouse anti-IL10 antibody (1 μ g/ml; Abcam) or the corresponding isotype control (1 μ g/ml; Abcam). Other experiments were finally performed by incubating adherent monocytes with TNF α (10 ng/ml) and/or IL-10 (20 ng/ml) in complete medium for 48 h. Where indicated, cells were pre-treated (20 min) with IL-10, then TNF α was added to incubation media. Cell viability was assessed by Trypan blue exclusion. Further details are reported in the figure legends.

Plaques and monocytes staining

Samples of carotid plaques were from patients undergoing carotid endarterectomy at the General and Vascular Surgery (University Hospital, Pisa, Italy) and were already fully characterized [27]. The patients were asymptomatic, since endarterectomy is recommended in case of severe carotid artery stenosis (>70 %) even in absence of neurological symptoms [35]. Surgically excised carotid plaques were collected on ice and dissected into 5-mm segments, then a segment of the plaque was frozen at -20°C in Optimal Cutting Temperature (OCT) medium for in situ evaluation of enzymatic activity. Plaque sections were stained as described [27]. Briefly, serial 3 μ m paraffin sections were stained with hematoxylin-eosin and Masson's trichrome method. Immunohistochemistry was performed on adjacent paraffin-embedded sections. Macrophages were identified by immunoperoxidase staining by using a specific monoclonal antibody raised against CD68 antigen (Dakopatts, Glostrup, Denmark) and the immunoreaction was visualized by 3-diaminobenzidine

substrate. GGT immunostaining was performed by using a previously characterized polyclonal antibody directed against the heavy chain of human GGT antibody at the appropriate dilution (1:1600) [36]. Basing on plaque histology [27], two (#1, #2) representative thin-cap fibroatheromas with large necrotic core and medium-high macrophage infiltration score, and one (#3) stable, thick-cap fibroatheroma with small necrotic scores and low macrophage infiltration score, were selected (Table 1).

Cytochemical staining for GGT activity was performed on air-dried buffy coat films fixed in a phosphate-buffered acetone formaldehyde mixture (PBAF) and incubated with GGT substrate gamma-glutamyl-4-methoxy-2-naphtylamide and Fast Garnet GBC, as previously described [24]. Nuclei were counterstained with methyl green.

Microvesicles isolation

Portions of plaque tissue were snap-frozen in liquid nitrogen, mechanically disrupted and homogenized in 5 % volume of cold hypotonic buffer (PBS diluted 10 folds) using a Potter homogenizer. Isolation of microparticles and exosomes from monocytes/macrophages supernatants as well as from homogenates of atherosclerotic lesions was performed by a differential centrifugation procedure [15]. Samples were first centrifuged at $2000\times g$ for 10 min at 4°C , then supernatants were centrifuged at $10,000\times g$ (45 min, 4°C) to remove large debris. Again, supernatants were collected, transferred into ultracentrifuge tubes and centrifuged at $100,000\times g$ (120 min, 4°C). Pellets were then washed ($100,000\times g$, 120 min, 4°C) and finally resuspended in physiological saline.

Western blot analysis

For western blot determinations of GGT, isolated monocytes, lymphocytes, platelets and cultured endothelial HMEC-1 cells—harvested in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.8)—or plaque derived exosomes were used. Non-adherent lymphocytes were isolated from plated PBMCs, whereas platelets were obtained from buffy-coat derived platelet concentrates. All samples were thoroughly washed in order to remove

Table 1 Histological features of the selected plaques used

N.	Cap thickness	Necrotic core	CD68+ macrophages	Fibrous tissue	Calcification	Intra-plaque activities (mU/g tissue)	
						b-GGT	Total GGT
#1	Thin and ulcerated	High	Medium	Low	Low	28.89	100.73
#2	Thin and ulcerated	High	Medium	Low	Low	14.61	47.02
#3	Thick	Medium	Low	Medium	High	9.43	35.58

contaminating plasma GGT. Endothelial HMEC-1 cells were grown in MCDB 131 medium (Life Technologies) supplemented with 1 µg/ml hydrocortisone, 10 ng/ml EGF (Life Technologies) and 10 % v/v fetal calf serum, and cultured at 37 °C in a 5 %/95 % CO₂/air atmosphere.

All samples were separated by 8 % SDS-PAGE [37] and incubated with rabbit anti-GGT IgG directed against the C-terminal 20 amino acids of human GGT heavy chain prepared as described [36]. Visualization of protein bands was obtained using a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the ECL detection system (Roche, Basel, Switzerland). Bands were analyzed with a Bio-Rad ChemiDoc apparatus equipped with the QuantityOne software.

Fractional GGT analysis by high-performance gel filtration chromatography

Determination of GGT fractions was performed as previously described [13, 38] by a FPLC system (AKTA-purified-10, GE-Healthcare). Separation and quantification of GGT fractions was performed by gel-filtration chromatography (Superose 6 10/300, GE Healthcare) followed by post-column injection of the fluorescent substrate gamma-glutamyl-7-amido-4-methylcoumarin. Intensity of the fluorescence signal was expressed in arbitrary fluorescence units (f.u.) and the area under chromatographic peaks was proportional to GGT activity. The elution volume for b-GGT fraction is 12.9 ml, corresponding to a molecular weight (MW) of 2000 kDa [13].

Other determinations

GGT activity was determined according to Huseby and Strömme [39]. Cytokines were measured using specific enzyme-linked immunosorbent assay kits (TNF-α, R&D Systems; IL-6 and IL-10, Affymetrix eBioscience) according to the manufacturer's instructions. Protein content was determined by the Bradford's method using the Bio-Rad protein assay reagent. Statistical analysis of data was performed by Student's *t* test for paired observations and one-way ANOVA with Newman-Keuls test for multiple comparisons.

Results

Monocytes activation

In agreement with earlier evidence [24], different levels of GGT activity were detected when monocytes isolated from the blood of healthy donors were stained for the enzyme (Fig. 1a), and a mean value of 10.1 ± 2.0 mU/mg of protein in the whole homogenate was calculated. In order to understand whether such cells were able to release GGT, isolated monocytes were exposed to activating substances and GGT activity was measured in the

incubation media. As reported in Fig. 1, both the combination of pro-inflammatory cytokines TNFα/IL-1β (Fig. 1b) and bacterial lipopolysaccharide (LPS; Fig. 1c) significantly increased ($p < 0.05$) GGT activity in the incubation media as compared to controls. In a separate set of experiments also the calcium ionophore ionomycin induced a significant ($p < 0.05$) release of GGT activity in the incubation media (data not shown). GGT activity was also detected in the media of the corresponding control samples (Fig. 1b, c), possibly ensuing from a weak activation of monocytes during isolation/incubation procedures. As regard cell-associated GGT activity, TNFα/IL-1β treatment did not produce any appreciable effect (Fig. 1d), whereas a significant decrease was induced by LPS (Fig. 1e; $p < 0.05$).

Macrophages activation

M1- and M2-like macrophages were obtained by incubating aliquots of monocytes (isolated from the same buffy coat) with GM-CSF or M-CSF, respectively. As previously described [32], after 6 days of culture the majority of M1-like macrophages presented with a classical adherent, "fried egg" morphology, whereas M2-like macrophages primarily presented with a stretched, spindle-like morphology (data not shown). The analysis of cytokines concentration in culture media revealed significantly ($p < 0.01$) higher levels of TNFα and IL-6 (Fig. 2a, b) and lower ($p < 0.05$) levels of IL-10 (Fig. 2c) for M1-like as compared to M2-like macrophages. M1-like macrophages showed also significantly ($p < 0.005$) higher levels of cell associated GGT activity (Fig. 3a) and protein (Fig. 3b) as compared to M2-like macrophages. SDS-PAGE analysis revealed that the GGT heavy subunit presented with the same apparent MW in both differentiated macrophages and monocytes (Fig. 3b).

The exposure to pro-inflammatory cytokines TNFα/IL-1β induced a significant increase ($p < 0.05$) of both released (Fig. 4a) and cell-associated GGT (Fig. 4c) only in M1-like macrophages, whereas no appreciable effect was detectable in M2-like cells (Fig. 4b, d).

Regulation of GGT expression in M1-like and M2-like macrophages

In order to characterize the possible mechanisms underlying the lower expression of GGT in M2-like macrophages, we evaluated the involvement of IL-10. As expected, 48 h incubation of monocytes with TNFα resulted in increased levels of cellular GGT activity (Fig. 5a) and protein (data not shown), but this effect was completely abolished when also IL-10 was added to incubation media (Fig. 5a). In a separate set of experiments, M2-like macrophages were incubated during their

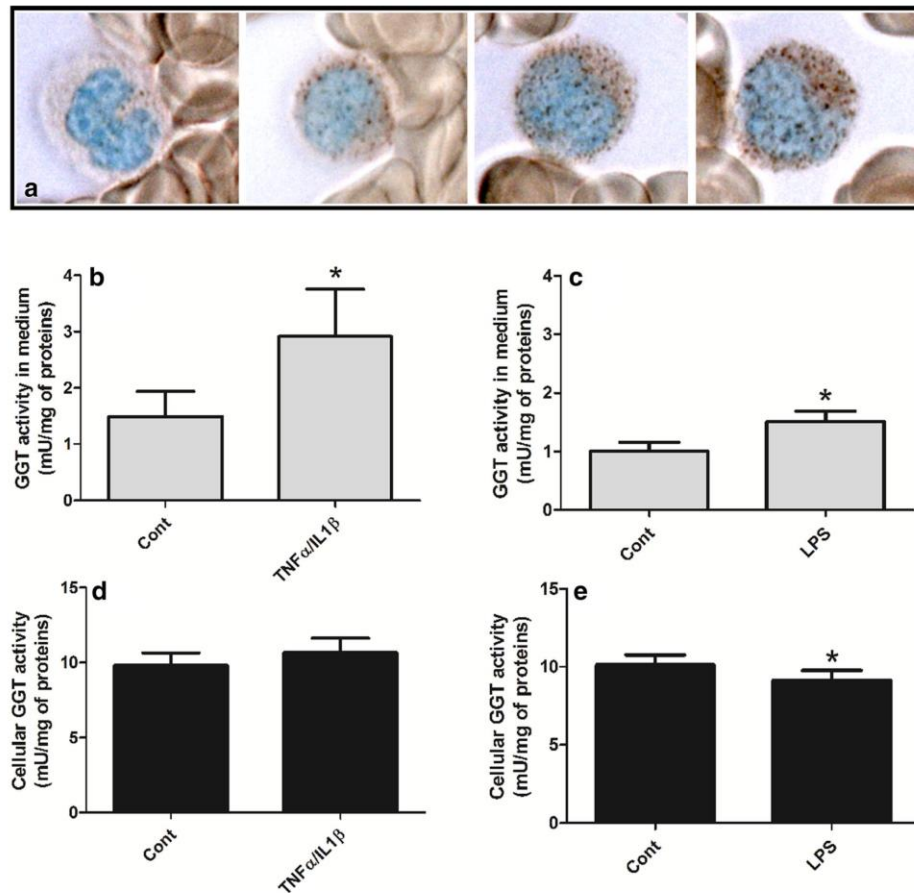


Fig. 1 Cytochemical staining for GGT enzyme activity expressed in activated monocytes. Monocytes from the blood of healthy donors expressing different levels of GGT activity. The azo-dye reaction product (reddish-brown) varies in intensity from slight to very strong, resulting in diffuse staining or granular positivity. Nuclei were counterstained with methyl green. Original magnification: $\times 100$ (a). Monocytes isolated from fresh buffy coats were incubated in the presence of TNF α /IL-1 β (both 10 ng/ml; 24 h) or LPS (5 μ g/ml; 24 h). GGT activity was measured in the 10,000 $\times$ g centrifuged supernatants (b, c) and in cellular homogenates (d, e). Results—expressed as mU/mg of cellular proteins—are mean $\pm$ SD of five to nine separate determinations. Data were analyzed by Student's t test for paired data; * $p < 0.05$ as compared with the corresponding control

differentiation with an anti-IL-10 antibody. As shown in Fig. 5b the addition of the blocking antibody was associated with induction of significantly higher levels of both GGT activity ($p < 0.05$) and protein (data not shown).

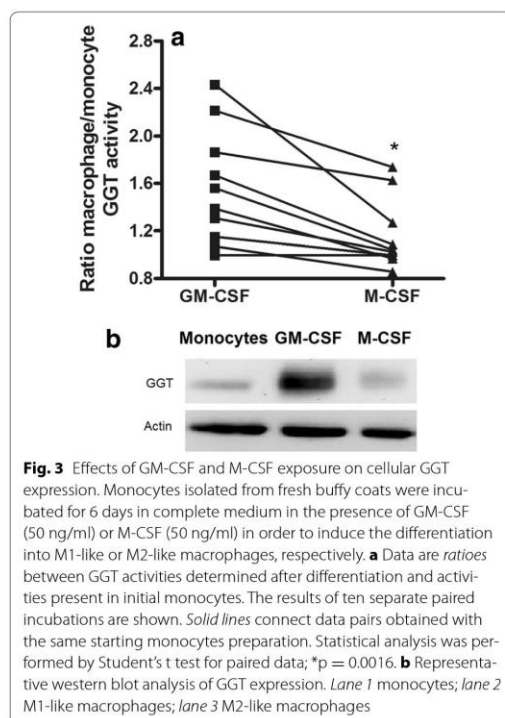
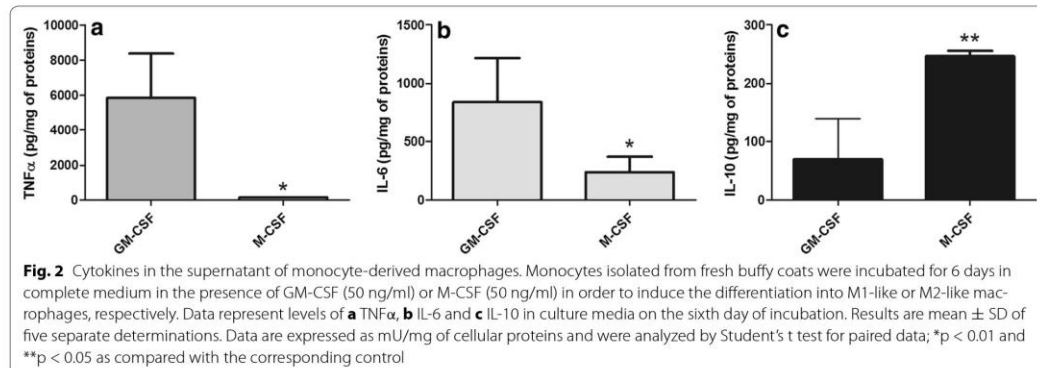
Release of b-GGT fraction in monocytes/macrophages supernatants

Incubation media from activated monocytes and M1-like macrophages were ultracentrifuged at 100,000 $\times$ g and incubation media were analyzed by gel-filtration

chromatography. As shown in Fig. 6 a peak corresponding to the b-GGT fraction was detected in the incubation media of both monocytes (Fig. 6a) and M1-like macrophages (Fig. 6b). In both cases, the exposure to the pro-inflammatory cytokines TNF α /IL-1 β increased the release of b-GGT.

GGT expression in atherosclerotic plaque

In agreement with previous evidence [26, 27], the immunohistochemical staining of plaques sections revealed the



presence of variable amounts of immunoreactive GGT, mainly localized to areas corresponding to necrotic core and macrophage-rich sites (Fig. 7).

The fractional GGT analysis of plaque homogenates (Table 1) confirmed the presence of a major peak

corresponding to the b-GGT fraction (Fig. 8a–c). In order to further characterize the properties of such fraction, plaque b-GGT was isolated by ultracentrifugation from all the selected samples, and the GGT protein was compared by SDS-PAGE with that expressed by monocytes, lymphocytes, platelets and endothelial cell line HMEC-1 (Fig. 8d). Using a specific antibody against the heavy subunit of GGT, a single GGT band with the same apparent molecular weight was observed in monocytes, lymphocytes and platelets, while two bands were present in HMEC-1 cells, a heavier one and one slightly lighter than those observed in the other cell types. Plaques homogenates showed three bands: one comparable with the heavier band of HMEC-1, one with the same apparent MW of monocytes, lymphocytes and platelets, and one lighter than all the other observed bands. Interestingly, the second band was clearly detectable only in samples with a higher macrophage score (#1 and #2).

Discussion

GGT activity is a well-established independent risk factor for coronary and cerebrovascular events both in unselected populations [11] and in patients with diagnosed coronary artery disease [12]. GGT activity has been detected inside human atherosclerotic plaques associated both to CD68+ macrophage-derived foam cells [25, 26] and to a microvesicles-like fraction called b-GGT [16, 27]. The b-GGT fraction is the heaviest of the four GGT fractions identified in plasma [38] and it is the sole identified within plaque homogenates [27]. In a recent study b-GGT has been demonstrated to consist of membrane microvesicles [15], sharing the physical properties of exosomes. Interestingly, intra-plaque b-GGT activity correlates with the histological markers

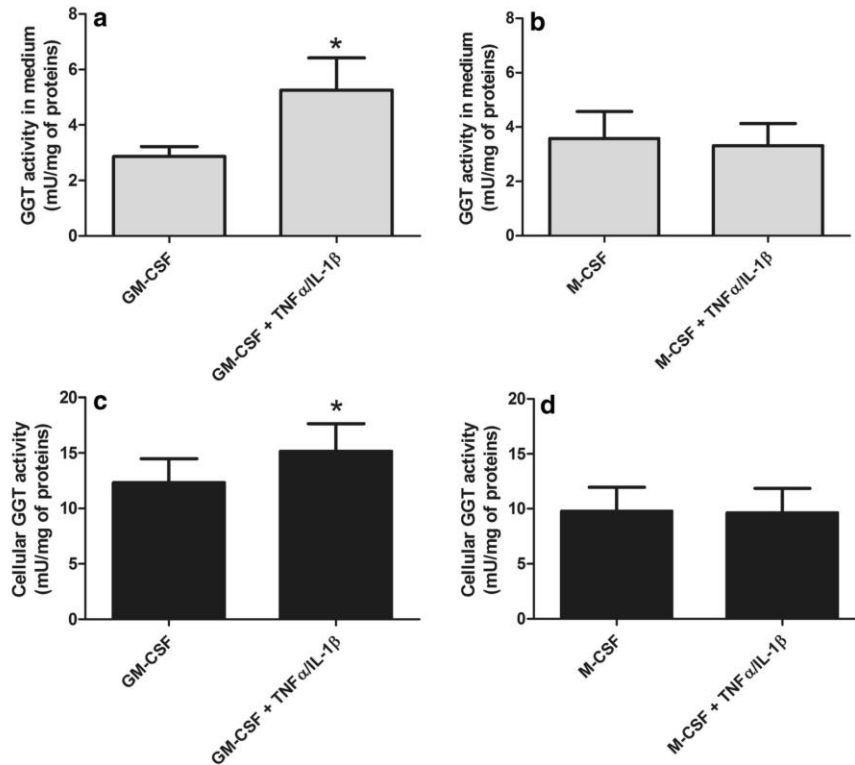


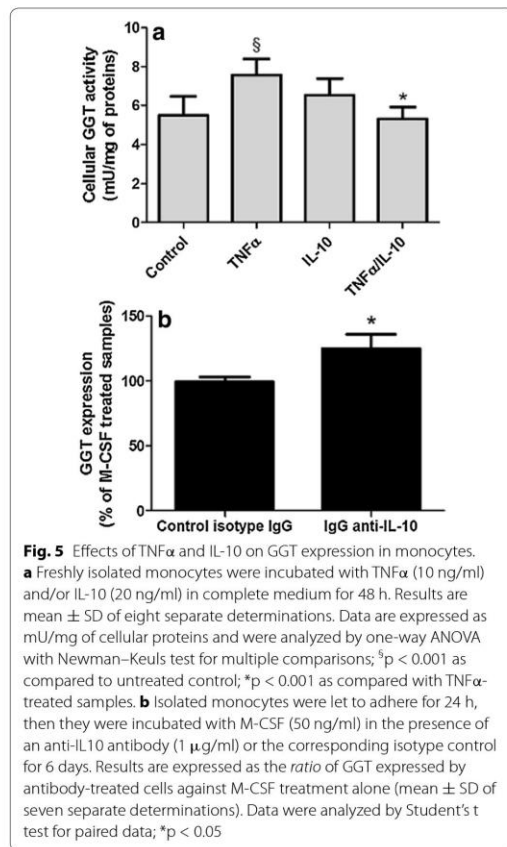
Fig. 4 GGT release by activated macrophages. Monocytes isolated from fresh buffy coats were incubated for 6 days in complete medium in the presence of GM-CSF (50 ng/ml) or M-CSF (50 ng/ml) in order to induce the differentiation into M1-like or M2-like macrophages, then they were incubated in the presence of TNFα/IL-1β (both 10 ng/ml) for additional 24 h. GGT activity was measured in the 10,000×g centrifuged supernatants (a, b) and in cellular homogenates (c, d). Results are mean ± SD of eight separate determinations. Data are expressed as mU/mg of cellular proteins and were analyzed by Student's t test for paired data; *p < 0.05 as compared with the corresponding untreated control

of plaque vulnerability—such as high macrophagic infiltration—as well as with levels of plasma b-GGT [17–27]. These pieces of evidence have prompted the need of identifying the origin of GGT accumulating inside atherosclerotic plaques, in order to better understand its role in plaque instability.

It was proposed that part of b-GGT present in atherosclerotic plaques might derive from the bloodstream through damaged endothelium [16, 27]. On the other hand an endogenous release of b-GGT by cellular elements of plaque—such as macrophages—is also conceivable. Indeed our results demonstrate that both monocytes (Figs. 1, 6) and pro-inflammatory M1-like macrophages (Figs. 4, 6) are able to release b-GGT upon activation. These results are in agreement with what

already observed for neutrophils—whose contribute to inflammatory exudate b-GGT in cystic fibrosis airways has been assessed [37]—and adds evidence in support of the connection between GGT and inflammation. GGT is expressed by inflammatory cells [24] and its expression is induced by inflammatory cytokines (e.g. TNFα, IFNs) and oxidative stress. Two major inflammatory mediators, cysteinyl-leukotriene LTC4 and S-nitrosoglutathione, are GGT substrates, and it was even proposed that soluble GGT may effect a cytokine-like function [40].

The potential contribution of macrophages to plaque b-GGT accumulation was further investigated by SDS-PAGE analysis of samples belonging to a previously characterized series of carotid plaques [27]. A single



peak corresponding to b-GGT was detected in all the plaques studied (Fig. 8a–c), but SDS-PAGE analysis revealed a heterogeneous composition of such fraction (Fig. 8d). In this respect it should be considered that GGT protein is transcribed from a single gene (*ggt1*), nevertheless several distinct glycosylation forms have been described, resulting in different molecular weights [41]. It was also demonstrated that inflammatory cells present in the vessel wall might induce modifications in protein sialylation status [42]. The presence of different bands corresponding to distinct MWs (Fig. 8d) may thus reflect both a heterogeneous origin for intra-plaque b-GGT as well as an intra-plaque modification of GGT protein (e.g. proteolysis, sialylation). SDS-PAGE analysis nevertheless confirmed that inflammatory as well as endothelial cells might contribute to b-GGT accumulation within the atherosclerotic plaque.

SDS-PAGE does not allow discriminating among different “inflammatory” sources for b-GGT, and it is conceivable that macrophages, lymphocytes and platelets all can contribute to intra-plaque b-GGT. In particular, the demonstrated correlation between intra-plaque b-GGT activity and the levels of macrophages infiltration [27] and the fact that both monocytes (Figs. 1, 6) and M1-like macrophages (Figs. 4, 6) are able to release b-GGT upon activation, suggest that macrophages play a major role in b-GGT accumulation inside the atherosclerotic plaque.

As previously observed for neutrophilic infiltrates in cystic fibrosis [37], b-GGT accumulation in the atherosclerotic plaque should not be interpreted as the mere epiphenomenon of an exogenous contribute (i.e., from the bloodstream), or as the effect of inflammation-related oxidative stress. Rather, the expression and the release of b-GGT by macrophages may be considered as one of the effects associated with the immune response, possibly involved in the modulation of selected mediators (e.g. GSNO, leukotrienes). Moreover, in selected conditions GGT activity has been shown to promote the production of reactive oxygen species and other prooxidants, resulting in redox reactions potentially involved in the progression of atherosclerosis [19–21]. M1 macrophage content of atherosclerotic plaques is positively associated with increased inflammation and clinical incidence of ischemic stroke [9]. High numbers of M1 macrophages were detected in symptomatic plaques, whereas smaller numbers, predominantly staining for M2 macrophage markers, were detected in asymptomatic plaques [5, 7–9]. The higher levels of GGT expression and b-GGT release observed from M1-like macrophages may thus play a role in plaque instability.

Altogether, our data indicate that inflammatory conditions are able to increase the expression and release of GGT enzyme, which in turn could play a role in modulating inflammation in the plaque environment. The higher cellular GGT expression in GM-CSF-induced M1-like macrophages, as compared to M-CSF-induced M2-like ones (Figs. 1a, b), appears to further support such hypothesis. Our data are in agreement with earlier evidence obtained with the human leukemic cell line KG-1, where incubation with GM-CSF resulted in a significant increase in GGT enzyme activity, while M-CSF did not produce any appreciable effect [43]. As far as treatment of monocytes with GM-CSF and M-CSF it has been reported that both GM-CSF and M-CSF receptors activate Ras-dependent signal transduction pathways and are both capable of activating AP-1 [44]. GM-CSF [45] and to some extent M-CSF

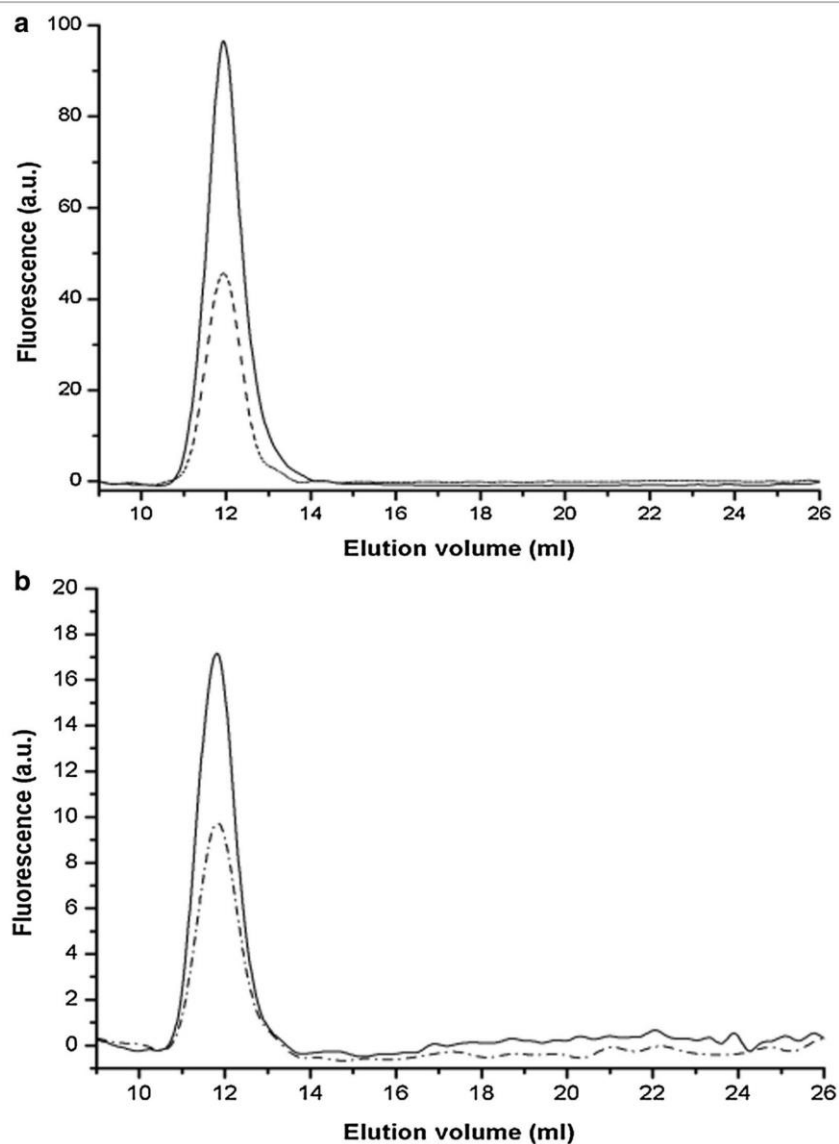
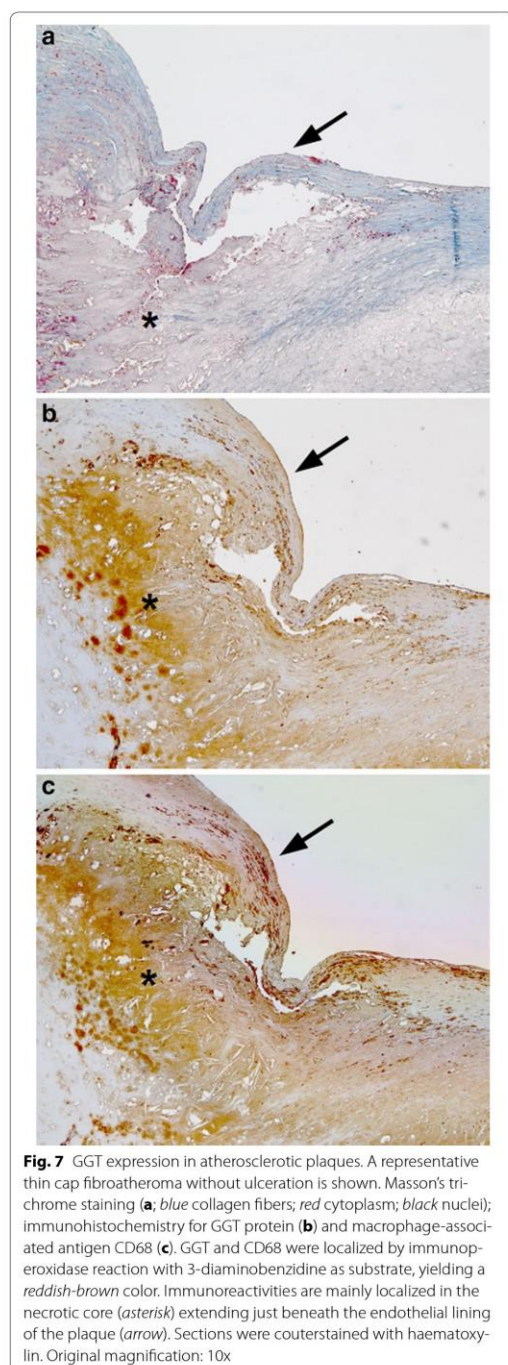


Fig. 6 Gel filtration chromatography of b-GGT released by TNF α /IL-1-activated monocytes (**a**) and M1-like macrophages (**b**). Monocytes and GM-CSF-differentiated M1-like macrophages were incubated with TNF α /IL-1 β (both 10 ng/ml, 24 h), then supernatants were pooled and ultracentrifuged (100,000 $\times g$, 120 min, 4 $^{\circ}C$). The resuspended final pellets were analyzed by gel filtration chromatography. Representative elution profiles of controls (dashed lines) and TNF α /IL-1 β -activated cells (continuous line) are shown



[46] are also known to modulate NF- κ B activation, but with some important differences in terms of timing, duration and factors involved [47]. In agreement with previous evidence [33, 47], our M1-like macrophages produced higher levels of pro-inflammatory TNF α and IL-6 and lower levels of anti-inflammatory IL-10, whereas the opposite was observed with M2-like macrophages (Fig. 2). Indeed, it has been proposed that Ras signalling is involved in GGT expression [48], and synthesis of GGT mRNA is induced by cytokines—including TNF α —through a NF- κ B-dependent pathway [49, 50]. The differential basal expression of cytokines in M1-like and M2-like macrophages could thus account for the differential GGT expression observed. Indeed TNF α was able to significantly increase GGT expression in monocytes, but this effect was inhibited in the presence of IL-10 (Fig. 5a), i.e. a cytokine known to inhibit NF- κ B pathway [51, 52]. Accordingly, when monocytes were differentiated into M2-like cells, the addition of an anti-IL-10 antibody was associated to higher levels of GGT expression (Fig. 5b). The lack of GGT induction in M2-like cells following TNF α /IL-1 β might be therefore explained by such inhibitory effect of endogenous IL-10 (Fig. 4c, d), whose expression is markedly higher in M2- as compared to M1-like cells [47]. These observations support the hypothesis that the balance of pro-inflammatory/anti-inflammatory cytokines such as TNF α and IL-10 may differentially modulate GGT expression.

Besides GM-CSF or M-CSF treatments, macrophage M1 or M2 phenotypes have also been obtained by other in vitro procedures. M-CSF was shown to induce M1 differentiation in the presence of IFN- γ and/or bacterial products such as LPS [53], whereas three distinct M2 subtypes were induced by treatments with IL-4 or IL-13 ("M2a" phenotype), or immune complexes in combination with IL-1 β or LPS ("M2b"), or IL-10, TGF β and glucocorticoids ("M2c") [54]. Interestingly, the *ggt5* gene (formerly designated as GGTLA1/GGT-rel or GGL) was also recently shown to be highly expressed in GM-CSF differentiated macrophages [33]. GGT5 is a member of GGT family supposed to be mainly responsible for the conversion of LTC4 to LTD4 [55].

Conclusions

The results of the present study indicate that macrophages characterized by a pro-inflammatory phenotype can release b-GGT and contribute thus to its accumulation inside atherosclerotic plaques. Our observations may explain the existing association between b-GGT activity in thin-cap fibroatheromas and greater macrophage infiltration [27]. Further studies are however

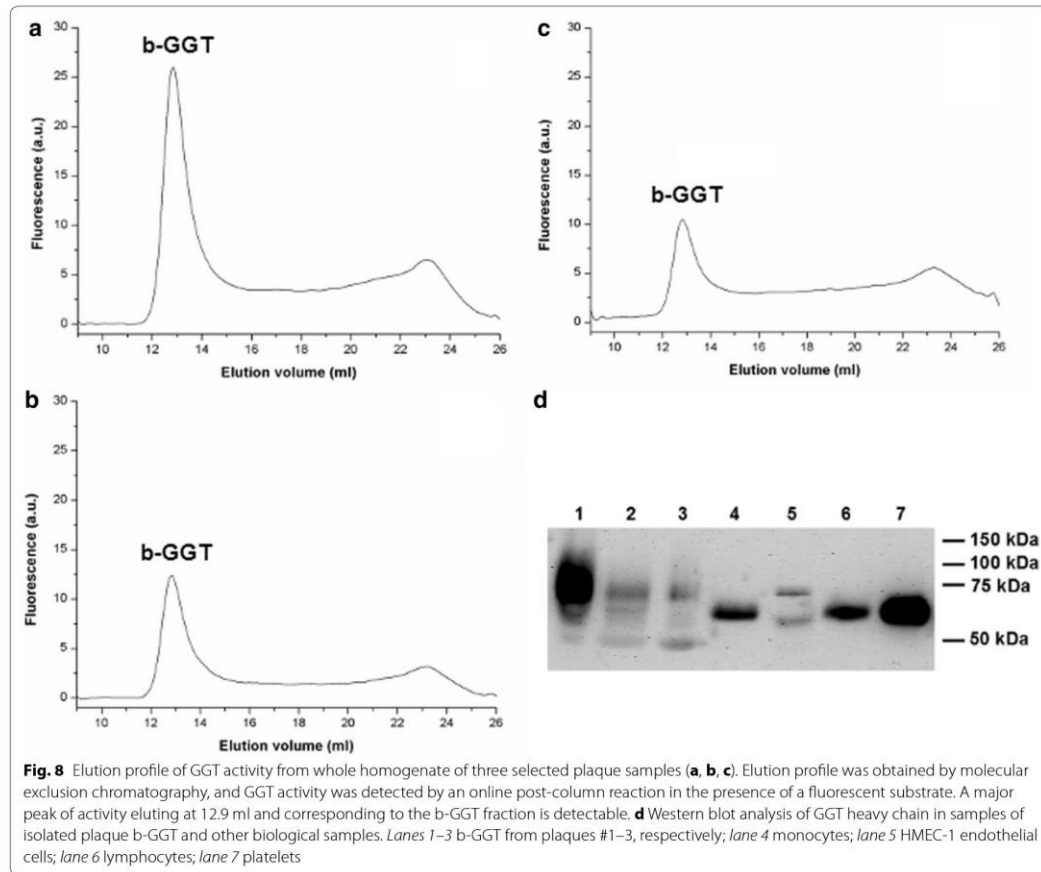


Fig. 8 Elution profile of GGT activity from whole homogenate of three selected plaque samples (**a**, **b**, **c**). Elution profile was obtained by molecular exclusion chromatography, and GGT activity was detected by an online post-column reaction in the presence of a fluorescent substrate. A major peak of activity eluting at 12.9 ml and corresponding to the b-GGT fraction is detectable. **d** Western blot analysis of GGT heavy chain in samples of isolated plaque b-GGT and other biological samples. Lanes 1–3 b-GGT from plaques #1–3, respectively; lane 4 monocytes; lane 5 HMEC-1 endothelial cells; lane 6 lymphocytes; lane 7 platelets

needed to fully identify the different sources of intra-plaque b-GGT and their actual role in the pathogenesis/progression of atherosclerosis.

Abbreviations

AP-1: activator protein 1; b-GGT: big-gamma-glutamyltransferase; FPLC: fast protein liquid chromatography; GGT: gamma-glutamyltransferase; GM-CSF: granulocyte/macrophage colony stimulating factor; HMEC-1: human dermal microvascular endothelial cells; IL: interleukin; LPS: bacterial lipopolysaccharide; M-CSF: macrophage colony stimulating factor; NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells; PBMCs: peripheral blood mononuclear cells; TNF α : tumor necrosis factor alpha.

Authors' contributions

EB designed and performed the experiments, wrote and reviewed the manuscript; MF analyzed the data, wrote and reviewed the manuscript; SC conceived and designed the experiments; EL performed the experiments and analyzed data; SM performed chromatographic determinations and data analysis; VF analyzed the data; APu collected plaque samples and performed histopathological characterization; APo contributed to analysis and interpretation of data, drafted and reviewed the manuscript; AC conceived and

designed the experiments, analyzed the data and drafted and reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare no conflict of interest.

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2.4 Supplementary study: Monocytes/macrophages differentiation and oxidative stress markers.

As described in the previous chapter, M1 and M2 showed an opposite inflammatory profile and are together present in atherosclerotic plaques. However, even if the pathogenesis of atherosclerosis is driven by inflammation and oxidative stress, little is known on the redox status of M1 and M2. Redox status concerns mainly the production of ROS, reactive nitrogen species (RNS), antioxidant enzymes activity and glutathione concentration. Evidences in the literature have shown that pro-inflammatory M1 macrophages produce high bactericide concentrations of NO, a RNS, resulting from an increased expression of inducible nitric oxide synthase (iNOS). They also secrete large quantities of a major pro-inflammatory cytokines, the IL-1 β [259] and ROS [329]. In contrast, M2 macrophages are characterized by anti-inflammatory phenotype and demonstrate elevated activity of Arg-1, which is essential in the quenching of inflammatory state [330]. If pro-inflammatory M1 are able to produce a large amount of ROS compared to M2, they also should bear higher antioxidant defenses.

To our knowledge, there are not many studies linking macrophages differentiation with its redox status (production of prooxidant and antioxidant molecules). Thus, the aim of this section is to evaluate the redox profile of macrophage populations, with particular regard to M1 and M2 in comparison to monocytes. In order to better understand the complex macrophage differentiation and activation processes, some oxidative and nitrosative stress markers and selected antioxidant enzyme activities of macrophages populations have been considered.

2.4.1 Main results

2.4.1.1. *Markers of differentiation*

Human monocytes from peripheral blood of healthy donors were stimulated during 6 days with two different growth factors GM-CSF (50 ng/ml) and M-CSF (50 ng/ml), to induce differentiation into M1 or M2 macrophages, respectively [252, 331]. Macrophages differentiation characterization was assessed with immunofluorescence studies to evaluate specific cell membrane markers, such as CD32 for M1 and CD163 for M2 [332, 323, 333, 334]. As shown in Figure 1, M1 are positive for their specific marker CD32 and M2 show specificity for the CD163 antigen.

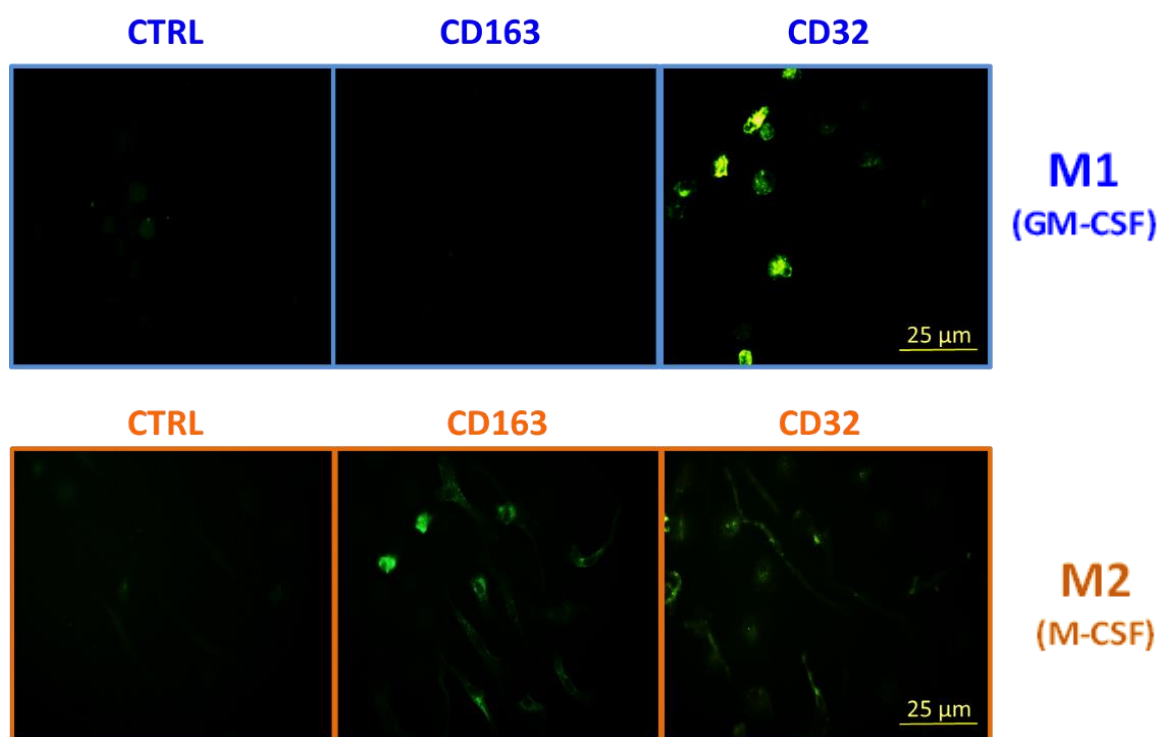


Figure 1. Immunofluorescent detection of specific markers of M1-like and M2-like macrophages. Cells, seeded in 8-well plates and differentiated with GM-CSF or M-CSF, were fixed with paraformaldehyde 4% (m/v) and labeled with 1:50 diluted goat CD32 and mouse CD163 antibodies (Santa Cruz Biotechnology) for 60 min in blocking solution (PBS, 8% (v/v) milk, 1% (v/v) TRITON X-100). Then a 1:100 dilution of a secondary anti-mouse FITC and anti-goat PE antibodies (Santa Cruz Biotechnology) were incubated with cells during 30 min. n=1 (Magnification: 40x, scale bar: 25µm).

2.4.1.2. Production of free radicals

Nitric oxide, a generic macrophage marker, has been evaluated in the intracellular and extracellular compartments of monocytes, M1 and M2 macrophages through nitrite ions quantification using the Griess reaction. The extracellular NO concentration was under the limit of detection of the Griess reaction for monocytes, M1 and M2 macrophages. Moreover, in the intracellular compartment M1 and M2 macrophages showed a NO quantity between 4 and 5 mmol/mg of proteins, respectively, whereas intracellular NO from monocytes was under the limit of detection (Fig. 2). This result is another proof of monocytes differentiation into macrophages. However, even if M1 are pro-inflammatory macrophages and M2 anti-inflammatory macrophages, no difference in intracellular nitrite ions amount was seen between M1 and M2 macrophages.

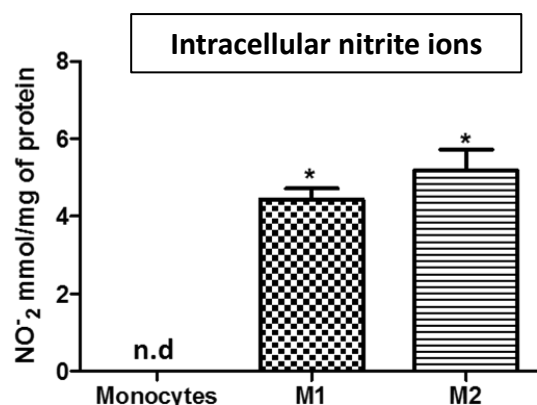


Figure 2. Intracellular nitrite ions production. Monocytes were differentiated in M1 and M2 by GM-CSF and M-CSF, respectively during 6 days. After differentiation, cells were lysed in Tris-HCl buffer (10mM, pH=7.8) and nitrite ions were quantified with the Griess reaction. The amount of intracellular nitrite ions was expressed as a ratio of total protein mass. Data are shown as mean $\pm$ sem, n=6-8 (M1-like and M2-like). * $p < 0.05$ vs monocytes, one-way ANOVA test + Bonferroni post-test.

Basing on several evidences in the literature that support the critical role of ROS in the functioning monocytes and macrophages, respectively, [308, 335], intracellular and extracellular ROS have been measured by the oxidation of 2',7'-dichlorodihydrofluorescein (DCF). As shown in Figure 3, monocytes, M1 and M2 macrophages produced ROS in the intracellular and extracellular compartments. The level of ROS produced is weaker in the extracellular compartment compared to the intracellular compartment. However, no statistically differences were seen between monocytes, M1 or M2. These results indicate that the basal level of ROS is the same for the three types of immune cells, but do not predict ROS production when cells are stimulated.

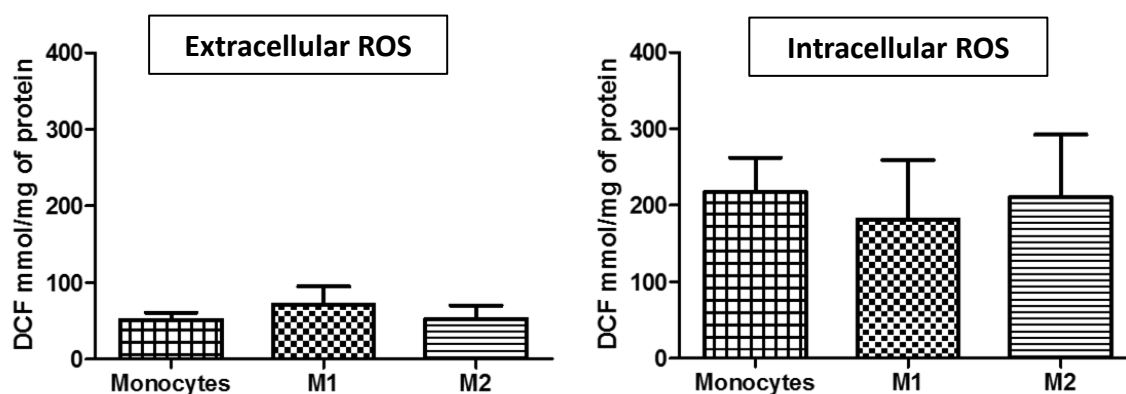


Figure 3. Quantification of ROS in intracellular and extracellular compartments. Monocytes were differentiated in M1 and M2 by GM-CSF and M-CSF, respectively during 6 days. After differentiation, in the cell culture supernatants and in the cells lysed in lysis buffer (Tris-HCl 0,5 M, NaCl 1,5 M, Triton X-100, SDS 10 % and ultra-pure H₂O₂), extracellular and intracellular ROS were quantified through the oxidation of 2',7'-dichlorodihydrofluorescein (DCF).

The amount of ROS was expressed as a ratio of total protein mass. Data are shown as mean $\pm$ sem, n=5 (monocytes) and n=10 (M1 and M2). One-way ANOVA test.

2.4.1.3. Antioxidant defenses

Glutathione is the major intracellular antioxidant and partly reflects the redox status of the cells. Intracellular GSH has been quantified by the 2,3-naphthalene dicarboxaldehyde probe. M1 and M2 showed a significant increase of intracellular GSH amount compared to monocytes (Fig. 4). However, the intracellular quantity of GSH is the same in M1 and M2 macrophages. The increased GSH concentration could indicate that macrophages have larger antioxidant defenses compared to monocytes.

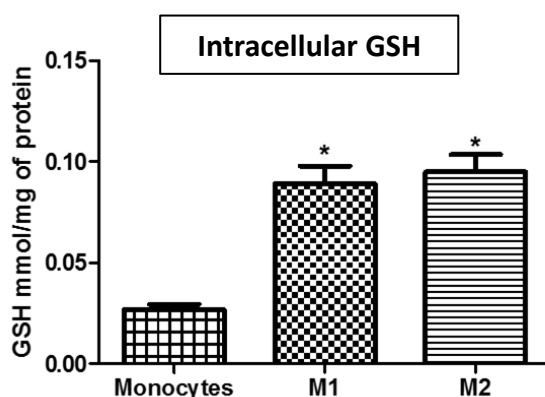


Figure 4. Quantification of intracellular GSH. Monocytes were differentiated in M1 and M2 by GM-CSF and M-CSF, respectively during 6 days. After differentiation, cells were lysed in perchloric acid buffer 3.3% and GSH concentration was quantified with 2,3-naphthalene dicarboxaldehyde probe (NDA). The amount of intracellular GSH was expressed as a ratio of total protein mass. Data are shown as mean $\pm$ sem, n=6 (monocytes, M1 and M2). * $p < 0.05$ vs monocytes, one-way ANOVA test + Bonferroni post-test.

GSH homeostasis in the cell is regulated by redox enzymes, such as glutamate-cysteine ligase and glutathione synthase for its synthesis and GGT for its catabolism. GGT activity has already been evaluated in Article 1. Considering that our focus is on the analysis of the redox status of the cells, the *de novo* synthesis of GSH was not investigated. Therefore, we focused on the glutathione peroxidase (GPx), an antioxidant enzyme used to detoxify from ROS, mainly H_2O_2 , through the oxidation of two GSH into GSSG. Then, we get interest in the glutathione reductase (GR), which is the critical enzyme for the reduction of one GSSG back to two GSH molecules. Moreover, GSH is also able to react with critical Cys residues in proteins, by forming mixed disulfides (GSSR) and this process can be reverted by glutathione S-transferase (GST). More details on cell antioxidant defenses will be given in the chapter III.

Therefore, in order to understand the changes in thiol status content, the GSH-dependent enzymes activities were evaluated in monocytes, M1 and M2 lysed in Tris-HCl buffer (10mM, pH=7.8) (Fig. 5), as follows:

- GPx activity was evaluated using the Paglia and Valentine method, with minor modifications [336]. Briefly, cells lysate (100 μ L) was mixed in 0.1 M of potassium phosphate buffer, pH=7, in presence

of 1 mM of EDTA, 1mM of sodium azide, 1 mM of reduced glutathione, 2 U/mL of glutathione reductase and 0.2 mM of NADPH. The enzymatic reaction was initiated by addition of 0.1 mL of 1.5 M cumene hydroperoxide. The oxidation of the NADPH was followed spectrophotometrically at 340 nm between 3 and 5 min after initiation of the reaction.

- GR activity was performed using 100 μ L of cell lysate mixed in 0.06 M of sodium phosphate buffer, pH=7.6, containing 3 mM of EDTA and 2 mg/mL of bovine serum albumin and adding with 3.25 mM of GSSG and 0.1 mM of NADPH. The oxidation of the NADPH was followed spectrophotometrically at 340 nm between 3 and 5 min after initiation of the reaction [337].
- GST activity was measured using 10 μ L of cell lysate mixed in 0.1 M of potassium phosphate buffer, pH=6.5, in presence of 1 mM of reduced glutathione and 1 mM of chloro-2,4 dinitrobenzene (CDNB), which forms GS-dinitrobenzene (GS-DNB). The absorption of GS-DNB adduct was followed spectrophotometrically at 340nm between 3 and 5 min after initiation of the reaction [338].

As shown in Figure 5, activities for all the investigated enzymes were detected in monocytes and macrophages. The GPx activity was significantly higher in the anti-inflammatory M2 compared to M1 and monocytes (Fig. 5A). Whereas the GR activity was the highest in pro-inflammatory M1 compared to M2 (Fig. 5B). Finally, GST activity was much lower in both macrophage types compared to monocytes (Fig. 5C).

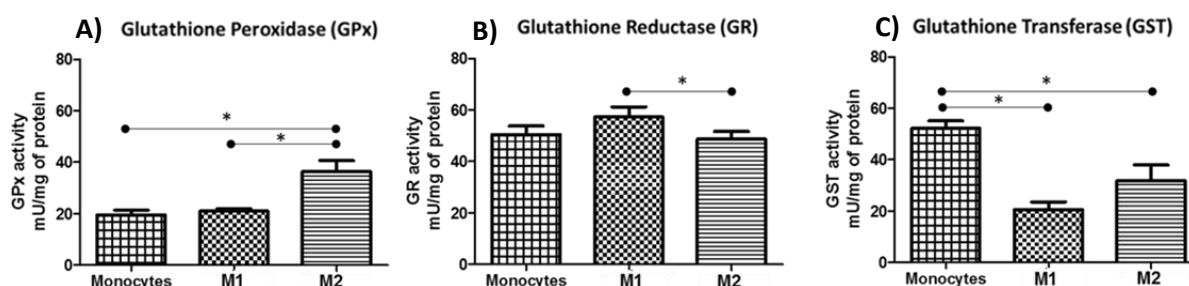


Figure 5. Evaluation of GSH-dependent enzyme activities. Monocytes were differentiated in M1 and M2 by GM-CSF and M-CSF, respectively during 6 days. After differentiation, cells were lysed in Tris-HCl buffer (10mM, pH=7.8) and undergone to 5 cycles of freezing/thawing in a mixture of ice and salt. After a further homogenization with Dounce (20 strokes) in the ice, a centrifuge at room temperature (2500 rpm, 5 minutes) and a resuspension of the sample in lysis buffer, the enzyme activities were evaluated. Data are shown as mean $\pm$ sem, n=6 (monocytes, M1 and M2). *p < 0.05, one way ANOVA test + Bonferroni post-test.

2.4.2 Discussion

To complete the phenotypic macrophage characterization started in the first published article, we assessed the redox status of differentiated macrophages in comparison with each other and monocytes.

The characterization of monocytes differentiation into M1-like and M2-like macrophages was attested by immunofluorescence labeling of CD32 and CD163, specific markers for each macrophage

subtype, respectively. Then, NO quantification confirmed macrophage differentiation, as NO was only quantified in M1 and M2. These results showed that macrophages present an active NO synthesis. However, to prove that inducible NOS is present and active, mainly in pro-inflammatory (M1) macrophages, a combination of inflammatory stimuli (TNF- α , IL-1 β) were used to stimulate our cells. No increase in NO production was observed (data not shown). However, all the three types of immune cells are able to produce ROS in the extracellular and intracellular compartments, even if no significant variations was observed. Antioxidant molecule like GSH and antioxidant enzymes (GPx, GR and GST) showed a different profile in monocytes, M1 and M2 (Table 8). In our results the concentration of intracellular GSH increased within the differentiation process, whereas GST activity disappeared.

Table 8. GSH and antioxidant enzymes in monocytes, M1 and M2 macrophages.

	Monocytes	M1	M2
GSH	+	++	+++
GPx	+	+	++
GR	++	+++	++
GST	+++	+	++

Furthermore, the increase GPx activity could be specific for M2 differentiation, whereas GR is specific for M1 differentiation process. The redox status characterization can be associated to other phenotypic markers within the differentiation of monocytes into macrophages. Therefore GPx, GR and GST can be used to discriminate between M1 and M2 phenotype. Furthermore, the significant increase of intracellular GSH concentration could indicate that macrophages are ready and able to protect themselves from oxidative stress. The higher intracellular GSH concentration in M1 could be due to a higher activity of GR. In contrast, M2 macrophages are characterized by increased activity of GPx, which is also confirmed by the literature [339]. This increase of GPx activity will help the anti-inflammatory macrophage to detoxify ROS under oxidative stress. In fact, the glutathione system is the major antioxidant in cells where glutathione peroxidase together with GSH act and neutralize lipid peroxides and hydrogen peroxide [340, 341, 342, 343, 344, 345].

Overall, it is conceivable to think that the GSH status may be connected to macrophagic development. Indeed, it is known that the redox status in the cells plays an important role in the cellular development, such as proliferation, differentiation and apoptosis [218, 346, 347]. Many studies have been reported that most of the cells represent redox shift to more oxidizing environments with a low reducing state during development [218, 348, 349]. In general, the redox state decreased according to the following rank order: proliferation > differentiation > apoptosis. Under the cellular development, the balance between the reducing and oxidizing equivalents determines the intracellular redox state. Therefore, the

redox state in the cells could modulate the ratio of the reversible oxidized and reduced biological redox couples, such as [NADH/NAD⁺], [NADPH/NADP⁺], [GSH/GSSG], [thioredoxin-SH₂/thioredoxin-SS], and [protein-SH/protein-SS] [218]. Among these redox couples, GSH is a major reducing agent that can catabolize H₂O₂ and other peroxides by enzymatic coupling reactions and protect the protein thiol groups from oxidation. Precisely thiol groups are involved in LDL oxidation [350, 351, 352], and this latter by arterial cells, including macrophages, is known to play a major role in atherogenesis [353, 354]. In fact with particular regard to GSH, several studies showed that macrophage GSH content affect cellular oxidative status and thus their capacity to oxidize LDL and to accumulate cholesterol, leading foam cells formation. As shown by Rosenblat et al. [355], the macrophage GSH content and cellular GPx activities (selenium-dependent enzyme) are inversely related to cell-mediated oxidation of LDL [356]: upon selenium supplementation to a murine macrophage-like cell line (J-774 A.1), the cellular GPx activity and the GSH content increased, and cell mediated oxidation of LDL was reduced. Both, GSH and GPx, can protect macrophages from the toxic effects of oxidized LDL and thus may affect the development of the atherosclerotic lesion. These results suggest that the major effect of cellular glutathione on macrophage-mediated oxidation of LDL is attributed to the cellular GSH content. Furthermore, Rosenblat et al. [357] demonstrated that these phenomena are also operative *in vivo*. In fact, using the apo e^{-/-} mouse model, as these atherosclerotic mice are under oxidative stress and develop atherosclerosis within 3–4 months [358, 359], they showed whether manipulation of the macrophage GSH content could affect their oxidative status, their capability to oxidize LDL and, finally, the development of atherosclerotic lesions in these mice.

In conclusion, to complete and confirm these preliminary data, further experiments, with particular regard to the thiol status are needed, in particular the evaluation of GSSG concentration and the GSH-dependent enzyme activities analysis in monocytes, M1 and M2 under inflammatory conditions. Moreover, these results will be also supplemented with an experimental planning under oxidative stress conditions, in order to obtain a complete overview. Finally, experiments are running to evaluate iNOS expression, another important macrophage marker, under basal and inflammatory conditions (stimulation with TNF- α /IL-1 β , 10 ng/ml 24 h).

CHAPTER III

Vascular smooth muscle cells and Oxidative stress

During development and progression of atherosclerosis, there is an important cross-talk between inflammation, generation of reactive oxygen and nitrogen species, and lipid metabolism leading to vascular remodeling following injury [360, 361, 362]. At physiological levels, NO plays an important role in the maintenance of vascular homeostasis, and inhibits the proliferation and migration of vascular SMCs. By contrast, during atherosclerosis, the decrease in NO bioavailability caused mainly by an increase of oxidative stress and endothelial dysfunction main lead SMCs proliferation and migration to vascular remodelling.

As previously described in the first chapter (section 1.4), several NO-related therapeutics have been developed to counteract the decrease in NO bioavailability. Among them, only S-nitrosothiols, which are physiological stores of NO, avoid any tolerance phenomena and oxidative stress. However, even if RSNO do not induce any oxidative stress, NO donors are still considered as potential oxidative stress enhancers leading to the formation of peroxynitrite producing deleterious proteins nitration, especially under oxidative stress conditions. The ability of GSNO to regulate NO bioavailability under oxidative stress condition is poorly studied. The following chapter will focus on the possible GSNO employment to counteract NO deficiency occurring within oxidative stress. First the selection of the most adapted oxidative stress model will be discussed in face of the literature, as well as the “phenotypic switching” of SMCs occurring during atherosclerosis and induced by ROS signaling. Besides, the relationship between S-nitrosothiol metabolism by SMCs under oxidative stress will be experimentally investigated. Precisely, the Pr-SNO, as a biomarker of the NO pool [133] will be used to evaluate the capacity of GSNO to deliver NO to SMCs cultured in an oxidative stress environment. As nitrosation can also modulate signaling pathway, the different classes and function of S-nitrosated proteins will be identified and analyzed. (Article 2 submitted to Free Radical Biology and Medicine, *“Oxidative stress enhances and modulates protein S-nitrosation in smooth muscle cells exposed to S-nitrosoglutathione”* and presented at the end of the chapter III)

As little is known on the possible interferences between free radicals and the metabolism of GSNO, a deeper appreciation of these mechanisms will open a way for the use of GSNO in cardiovascular diseases associated with oxidative stress.

3.1 Phenotypic switching of smooth muscle cell in atherosclerosis

SMCs exhibit phenotypic and functional plasticity in order to respond to vascular injury. In the case of vessel damage, SMCs are able to switch from “contractile” phenotype to “proliferative” (or “proinflammatory”) phenotype. This change is accompanied by a decrease in the expression of

smooth muscle (SM)-specific markers responsible for contraction and the production of proinflammatory mediators, which induce proliferation and chemotaxis. However, during chronic inflammatory diseases like atherosclerosis, arterial SMCs become aberrantly switched to proliferative phenotype, which leads to SMC dedifferentiation, proliferation and extracellular matrix formation for migration within plaque areas. This proatherosclerotic switch is a complex and multistep mechanism that may be induced by a variety of proinflammatory stimuli, oxidative stress and hemodynamic alterations. Disturbances in hemodynamic forces could initiate the proinflammatory switch in SMC phenotype even before the onset of symptoms of atherosclerosis. Proinflammatory signals play a crucial role in further dedifferentiation of SMCs in affected vessels and propagation of pathological vascular remodelling. The majority of SMCs within lesions appear to be derived from phenotypic modulation of preexisting SMCs in response to environmental changes like lipids internalisation, lipid peroxidation products, ROS, inflammatory cytokines, altered cell-cell and cell matrix contacts, exposure to circulating blood products, platelet-derived products, growth factors, and perhaps specific negative regulators of SMC differentiation.

The first chapter (section 1.2.3) focused on the mechanisms that regulate the differentiated state of the SMC under normal circumstances and how these regulatory processes are altered during the formation of intimal lesions. The evolved model shows that regulation of SMC differentiation is extremely complex and involves constant interplay between environmental cues and the genetic program. Therefore, this next section will focus on the molecular mechanisms that control phenotypic switching of SMC in atherosclerosis.

Molecular mechanism of the phenotypic switching: some key players

The phenotypic switching of SMC is characterized by a decrease in expression of SM contractile proteins such as SM α -actin and SM-myosin heavy chain (MHC). The mechanism of transcriptional repression of the SM22 α gene (an important contractile protein) for example was found to be involved in the changes of regulation of SMC marker genes [363, 364]. Of major significance, Owens et al. previously demonstrated the mutation of a highly conserved G/C repressor element 5' to the proximal CARG element (a DNA consensus sequences present within the promoters of SMC genes that plays a pivotal role in controlling their transcription) in the SM22 α promoter. Moreover, they also found in the promoters of many other SMC marker genes [102], nearly abolished down-regulation of this gene *in vivo* in response to vascular injury [365] or in atherosclerotic lesions of ApoE^{-/-} mice [366]. Further studies showed that the Krüppel-Like Factor-4 (Klf4), a transcriptional regulator activated by oxidized phospholipids, led to the inhibition of expression of SM22 α and other SM marker genes [367, 368, 369]. During atherosclerosis Klf4 is

upregulated and activated in SMCs [368] and responsible for SMC dedifferentiation inducing atherosclerotic plaque progression and vascular remodelling [370]. Finally, Klf4 is also involved in the upregulation of vascular SMC-mediated extracellular matrix (ECM) gene expression such as type VIII collagen [368].

In quiescent SMCs, NF- κ B, a pro-inflammatory transcription factor, exists as an inactive p50-p50 homodimer that is constitutively bound to DNA, thereby repressing proinflammatory genes [371]. However, inflammatory stimuli cause formation of the p65-p50 heterodimer that activate the transcription of proinflammatory genes [372]. Activation of NF- κ B in SMCs plays a crucial role in their phenotypic switching. Indeed, NF- κ B drives the expression of IL-8 [163], MCP-1 [373], the chemokine (C-XC motif) ligand 1 (CXCL1) [374], VCAM-1 [374], ICAM-1 [375] and metalloproteases (MMP-1, -2, -3 and -9) [376, 377, 378]. CXCL1 could attract neutrophils to inflamed sites [379], while MMPs are required for ECM degradation and increasing migration of SMCs [380].

Finally, many changes occur in ECM composition during SMC phenotypic switching. Normally, SMCs are surrounded by ECM (basal lamina) composed of laminin, collagen IV and perlecan. In steady state, basal lamina provides signals that support SMC “contractile” phenotype and prevent dedifferentiation, growth and proliferation [381]. In contrast, in the case of vessel damage and vascular proliferative diseases, the composition of basal lamina changed, with appearance of osteopontin, fibronectin and syndecan-4, that in turn are able to mediate proatherogenic dedifferentiation of SMCs.

Finally, the modification of SMCs environment by inflammation and oxidative stress will affect the vascular wall. Thus, in the next section, the impact of ROS signaling on SMCs phenotypic modulation will be investigate.

3.2 ROS signaling and smooth muscle cells

Vascular oxidative stress is a mechanistic link between macrophage infiltration, MMP activation and SMC apoptosis in plaque instability. The cellular redox environment is a balance between the production of ROS, RNS and reactive sulphur species (RSS), and their removal by antioxidant enzymes and small molecular-weight antioxidants. ROS include free radicals such as superoxide anion ($O_2^{\cdot-}$), perhydroxyl radical ($HO_2^{\cdot}$), hydroxyl radical ($\cdot OH$) and other species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid ($HOCl$) [382]. RNS are derived from nitric oxide through the reaction with $O_2^{\cdot-}$ to form $ONOO^-$. RSS are easily formed from thiols by reaction with ROS [383]. ROS generation has been further localized to the tunica media, suggesting a prominent role for SMC cells in their production [384]. Directly exposing SMC cells to ROS-generating

systems stimulates migration, proliferation, and growth, implicating ROS in these processes [385, 386, 387, 388]. Under physiological conditions ROS are produced in a controlled manner at low concentrations and function as inter- and intracellular signaling molecules [389, 390]. By contrast, in pathological conditions, increased activity/expression of ROS generating enzymes or decreased defenses by antioxidants in the vasculature result in increased bioavailability of ROS, reduced bioavailability of NO (which is reoriented to ONOO⁻ formation), increasing oxidative stress and vascular damage [391, 392].

3.2.1 Enzymatic sources of ROS

There are multiple potential enzymatic sources of ROS in vascular cells, including xanthine oxidase, uncoupled nitric oxide synthases, cyclooxygenase, and cytochrome P-450, which can potentially generate and/or release ROS in amounts that have a major influence on the control of vascular function [393, 394] (**Fig. 11**). In order to reduce ROS-induced oxidative damage, the cells normally possess intracellular molecules and enzymes to keep the homeostasis of ROS at a low signalling level. The antioxidant defence system includes enzymatic (*i.e* superoxide dismutase (SOD), catalase (CAT), GPx, GR, GST, TrxR), metal chelating and free radical scavenging activities to neutralize these radicals after they have been formed.

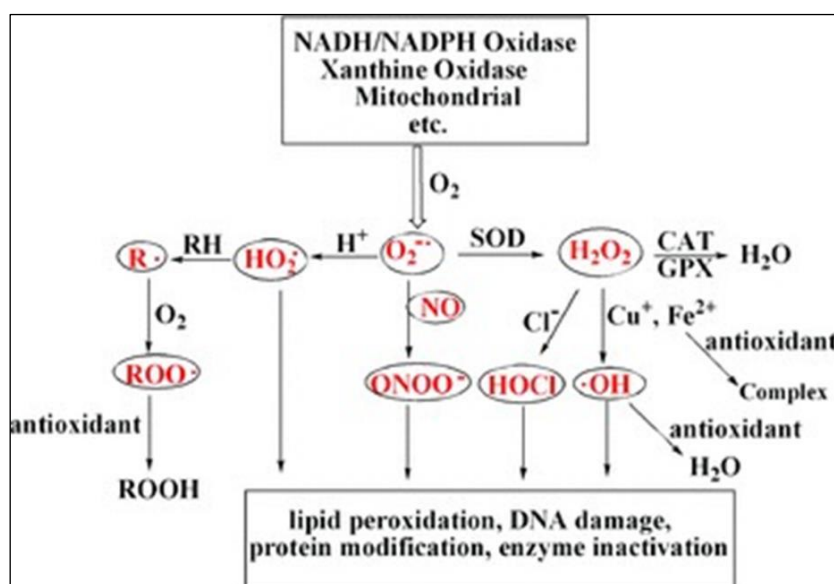


Figure 11. Summary of ROS types and sources, and action point of antioxidants (R•, lipid alkyl radical; RH, lipid; ROO•, lipid peroxy radical; ROOH, lipid hydroperoxide) [395].

However, currently, there is substantial evidence that NADPH oxidases (NOX) and mitochondria are significant sources of ROS generation in SMC [396, 397, 398] (**Fig.11**). Indeed, as previously cited in the first chapter, NOX 1 and NOX 4 have been identified in human and aortic

smooth muscle cells, by participating in SMC proliferation and differentiation, respectively [112]. NADPH oxidases are the primary source of intracellular ROS in atherosclerosis [399], as confirmed by several studies, *i.e.* using mice deficient in catalytic subunits NOX 1 or NOX 2 or the cytosolic subunit p47^{phox} [400, 401, 402, 403]. However, NOX homolog-specific changes in gene expression occur at different stage of atherosclerosis development. In atherosclerotic human coronary arteries, NOX 4 expression progressively increases from stage I to stage IV [404]. NOX 1 expression is increased early after wire injury, whereas NOX 4 is increased later [405]. In a recent study, Xu et al., examining the contribution of NADPH oxidase in plaque-derived SMCs, identified a relationship between NOX 4 expression and lesion progression, ROS levels, apoptosis, and cell cycle arrest. These results implicated NOX 4 in the senescence and apoptosis of plaque-derived SMCs, suggesting an integral role for NOX 4 in plaque stability [406].

In recent years, several reports supported the idea that cellular ROS levels can function as a “second messengers” regulating numerous cellular processes, including proliferation [407, 408, 409, 410]. The second-messenger properties of ROS are believed to activate signaling pathways by activating tyrosine kinases, tyrosine phosphatases, MAP kinases, or ion channels [411]. This dual function of ROS, as signaling molecules or oxidative stress inducers, could result from the differences in their concentrations, pulse duration, and subcellular localization. Indeed, although higher levels of ROS can be toxic, low levels of ROS may serve as signaling molecules regulating many cellular processes, including proliferation.

3.2.2 Non enzymatic sources of ROS

In biological systems, ROS can also arise from non-enzymatic sources. In fact as peroxides reactivity with thiols show slow rates, alternative processes may be involved. One possibility is that protein-binding interactions might put the catalytic site of oxidases together with thiols, in a manner that exposes them to the high concentrations of peroxide needed to drive direct reactions of peroxide with thiols. Alternatively, catalysts, such as transition metals, might promote peroxide reactions with reactive protein thiol groups. Oxidized forms of NO, including ONOO⁻ and NO₂⁻, have chemical properties that are potentially involved in this regulation through their reactions with protein sites such as thiols and tyrosine, unsaturated fatty acids, and other molecules (GSH, tetrahydrobiopterin, etc.) [412].

ROS generated during SMC proliferation could originate also from growth factors, such as PDGF [413]. Increased expression of PDGF and its receptors has been found in lesions of atherosclerosis [413]. Furthermore, PDGF is known to activate redox factor 1 (Ref-1) by altering its redox status, enhance AP-1 activity, and increase cell-cycle-regulatory protein expression, facilitating progression from G0/G1 to S phase in SMCs [414]. Besides, TGF-β, epidermal growth factor, insulin-

like growth factor, basic fibroblast growth factor and angiotensin-II (Ang-II) could also initiate SMCs proliferation [415, 416, 417, 418, 419].

3.2.3 Renin angiotensin system as source of ROS

With particular regard to Ang-II, both clinical and experimental evidence support a potential role of the renin-angiotensin-aldosterone system (RAAS) in contributing to phenotypic switching in SMCs. Cross-talk between the main mediators of the RAAS has been shown to participate in the development of the vascular dysfunction, with SMCs being key participants in this cross-talk [420]. SMCs were shown to express angiotensin I receptor type 1 (AT1R) that binds Ang-II, a vasoconstrictor [421]. Previous studies have supported a role for Ang-II in the generation of oxidative stress in the vasculature *via* the induction of superoxide *via* NADPH oxidase [422]. In addition, Ang-II has been shown to stimulate the increase of a variety of proinflammatory mediators such as MCP-1 and VCAM-1 mRNA expression in rat SMCs aortas [423]. In these experiments, the increased expression of both VCAM-1 and MCP-1 by Ang-II could be blocked by NADPH oxidase inhibitors and catalase, suggesting that NADPH oxidase may be contributing to oxidative stress and regulation of vascular inflammatory genes *via* the generation of H₂O₂. Thus, Ang-II stimulates adhesion of monocytes to SMCs and promotes changes from the contractile quiescent phenotype towards SMC proliferation and migration [424]. Ang-II, through the formation of oxidative stress and increased levels of proinflammatory genes in the vessel wall, may serve also as a molecular link between hypertension and the pathogenesis of atherosclerosis [425].

3.2.4 ROS and lipoprotein oxidative modifications

The last aspect to analyze is the involvement of free radicals in lipoprotein oxidative modifications, which is supported by several lines of evidence [426]. Indeed, macrophages accumulate oxLDL *via* scavenger receptors, resulting in cellular cholesterol accumulation and the subsequent foam cell formation. For SMC, in addition to the expression of several LDL receptors already described in the first chapter (section 1.2.3), the cellular cholesterol accumulation results, on one hand, in an increase in cholesterol biosynthesis and esterification, and on the other hand in a decrease in cholesteryl esters (CE) hydrolysis and cholesterol efflux, fully regulated by specific enzymatic activities (HMG-CoA reductase), acyl coenzyme A: cholesterol acyltransferase (ACAT) and neutral cholesteryl ester hydrolase (NCEH)). Therefore, the disturbances in cholesterol metabolism favor accumulation of cholesterol and cholesteryl esters in vascular cells, and thus may contribute to the formation of SMC foam cells. Indeed, the idea that free radicals may modulate the activities of some enzymes involved in cellular cholesterol metabolism is supported by several studies. Gesquiere

et al. observed that *in vitro* treatment of SMC by free radicals led to an increase in the activities of both HMG-CoA reductase and ACAT activities, in contrast NCEH activity was decreased. They propose that the changes in the activities of enzymes involved in cholesterol homeostasis could be the result of a free radical-mediated decrease in cyclic AMP (cAMP) concentration [427]. In fact, cAMP was also shown to modulate the LDL receptor activity [428, 429], HMG-CoA reductase [428, 430], ACEH [431] and NCEH [432] and also cholesterol efflux have also been demonstrated to be regulated by a cAMP-dependent pathway [433, 434].

In summary, the oxidative stress-mediated cholesterol accumulation in SMC may be related to a decrease in cAMP concentration. In light of these results, all of these metabolic modifications may contribute to cholesterol accumulation in SMC and, extrapolating to the *in vivo* situation, they may lead to the resulting foam cells found in atherosclerotic plaque.

3.2.5 Antioxidative systems: enzymatic defenses against ROS

Superoxide, in addition to spontaneous conversion, is converted to hydrogen peroxide by superoxide dismutase enzymes (MnSOD, CuZnSOD, and Ec-SOD). CAT and GPx neutralize H_2O_2 to water. Hydroperoxides are also neutralized by thioredoxin/thioredoxin reductase, glutaredoxin/glutaredoxin reductase, and the six-member family of peroxiredoxins (Prxs) [435]. More specifically, the generation of ROS usually induce thiols oxidation forming disulfide bound in proteins or GSSG. Among ROS, H_2O_2 could influence the redox state protein thiols, two-electron reactions. The reduced form of cysteine in proteins can undergo oxidation reactions to form sulfenic (RSOH), sulfinic (RS_2OH), and sulfonic acids (RS_3OH). Sulfinic and sulfonic forms are believed to be irreversible, whereas the sulfenic form can conjugate with other reduced thiols (RSH) to form a disulfide bridge (RSSR). Then, cellular antioxidant systems can reduce the disulfide bond and generate the reduced form of the cysteine in proteins. In addition, superoxide can initiate one-electron reactions that can alter the redox state of metal cofactors (*e.g.* Fe and Zn) present in many kinases and phosphatases, thereby affecting their activities. The generation of oxidized thiols can be reversed by the thioredoxin/thioredoxin reductase systems or the protein disulfide isomerases family (PDI).

PDI has emerged as a key redox-sensitive player in protein folding and ER stress. For instance, it is known that ER stress generally increases cellular ROS generation [436] in part by increasing calcium release, which increases ROS production by the mitochondria [437]. In particular, ER lumen is an oxidative environment compared with the cytosol, with a high ratio of GSSG:GSH [438]. A recent study by Wu et al. [439] however, did not find increased free H_2O_2 in the ER compared with the cytosol using the redox probe HyPer (hydrogen peroxide), suggesting that much of the observed redox potential is protein bound. The oxidative ER environment is conducive to the formation of disulfide bonds and a key role is played by PDI, whose expression increases at the plasma membrane

under oxidative stress conditions [Belcastro et al. to be submitted, section 3.5, Article 2]. PDI catalyzes the formation and breakage of disulfide bonds between cysteine residues of proteins in a process known as oxidative folding. It is composed of 4 thioredoxin domains and contains redox-sensitive cysteines whose oxidation state can influence the protein binding and activity of PDI. PDI has two active sites, both of which are characterized by the presence of a CGHC motif, which either forms a disulfide for the enzyme to become active as an oxidase, or a dithiol for the enzyme to act as an isomerase. In its reduced form, PDI acts as an isomerase, whereas oxidation of PDI enables it to form disulfide bridges [440]. Reduced PDI can bind another ER oxidoreductase, ER oxidoreductin 1 (Ero1), which is capable of oxidizing PDI and produces H_2O_2 in the ER as a consequence [441]. Ero1 is thought to be essential for PDI activity by generating internal disulfide bonds and transferring them *via* PDI to target proteins [442]. The oxidation of PDI by Ero1 is thought to occur through disulfide exchange that results in the formation of reduced Ero1 [442]. Ero1 can be oxidized rapidly in the presence of FAD and oxygen, indicating that oxygen is the ultimate electron acceptor [443]. The reactivation of Ero1 by molecular oxygen generates ROS in the ER. How the cell is protected from the damage caused by ER-generated ROS is poorly understood; however, recent data suggest that GSH might have an important role in this process. Recent evidence suggests that the inhibition of PDI in cells can contribute to ER stress and apoptosis. Toldo et al. [444] found that overexpression of PDI protects against myocardial damage in an acute myocardial infarction (MI) mouse model. The authors suggest that PDI activity is antiapoptotic, in part, by increasing the activity of SOD1.

3.2.6 Antioxidative systems: non enzymatic defenses against ROS

The neutralization of ROS by additional intracellular small-molecular-weight antioxidants includes cysteine, vitamin C (ascorbic acid), and vitamin E (atocopherol) [445]. However, changes in the antioxidant enzyme activities or small-molecular-weight antioxidant levels or both could perturb the cellular redox environment, which in turn could affect the redox regulation of the cell-cycle progression. In fact, another critical role played by ROS is on the redox regulation of cell-cycle progression. It is known, that the mammalian cell cycle has five distinct phases; G0 (quiescent state), whereas the proliferative state encloses the G1, S, G2, and M phases. In response to mitogenic stimuli, quiescent cells enter the proliferative cycle and may transit back to the quiescent state. Reentry into quiescence is essential to prevent aberrant proliferation as well as to protect the cellular life span. Precisely, the thiol-disulfide exchange reaction can regulate many of the cell-cycle-regulatory protein functions during the redox regulation of the cell-cycle. Indeed, the progression through the cell-cycle phases is regulated by sequential and periodic activation of positive regulators, cyclins, and cyclin-dependent kinases (CDKs), *i.e.* the progression from G0/G1 to S is largely regulated by the D-type cyclins (cyclin D1 and D2) in association with CDK4-6.

In addition, ROS signaling is known to regulate many of the transcription factors that influence the development. For instance, Xu et al., using SMCs derived from a murine model of atherogenesis (ApoE ^{-/-}/ LDLR ^{-/-} mice), examining the contribution of NADPH oxidase in plaque-derived SMCs, observed a decreased in cellular growth, an hypophosphorylation retinoblastoma protein (Rb), an increased expression of cyclin-dependent kinase inhibitors, and decreased expression of cyclins D1 (G0 / G1 arrest) [406, 447]. In conclusion, ROS, altering the redox state of several proteins, can affect the redox regulation of cell-cycle proteins during progression from one cell-cycle phase to the next.

3.2.7 Antioxidant glutathione effects and glutathione related post-translational modifications

Glutathione homeostasis

The most abundant molecule among endogenous antioxidant is the tripeptide glutathione, which is critical for the maintenance of the cellular redox balance [447, 448]. GSH is synthesized *in vivo*, by the consecutive action of two ATP-dependent enzymes, from the precursor amino acids cysteine, γ -glutamate and glycine (**Fig. 12**). The first enzyme, glutamate–cysteine ligase (GCL) formerly called γ -glutamylcysteinesynthase (GCS) is an heterodimeric rate-limiting enzyme. GCL is sensitive to oxidative stress, whose expression is regulated by the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), a transcription factor that regulates a wide array of antioxidant responsive element driven genes in various cell types [449]. The glutathione synthase (GS) is the second enzyme required for GSH synthesis catalysing the addition of glycine to γ -glutamyl-cysteine and consuming ATP. Finally, the chemical structure of GSH with γ -Glu and Cys provides peculiar characteristics ranging from insusceptibility to proteolysis to redox thiols catalysis, respectively. The overall rate of GSH synthesis is controlled by several factors including: (i) availability of L-cysteine [340]; (ii) relative ratio between the two subunits of GCL [450]; (iii) feedback inhibition of GCL by GSH [451]; (iv) ATP provision. All cell types synthesize GSH, however, the main source of the tripeptide in the body is the liver. In contrast to GSH synthesis, which occurs intracellularly, GSH degradation occurs exclusively in the extracellular space, on the surface of cells that express GGT (chapter I, section 1.5), the only enzyme that catabolizes GSH and GSH adducts (*e.g.* oxidized glutathione, glutathione S-conjugates and glutathione complexes).

Therefore, the intra- and extracellular GSH levels are determined by the balance between its production, consumption, and cellular export. This redox cycle, known as the GSH cycle (**Fig. 11**), incorporates other important antioxidant, redox-related enzymes. Due to important physiological functions of GSH, these processes are tightly controlled at transcriptional, translational, and post-translational levels.

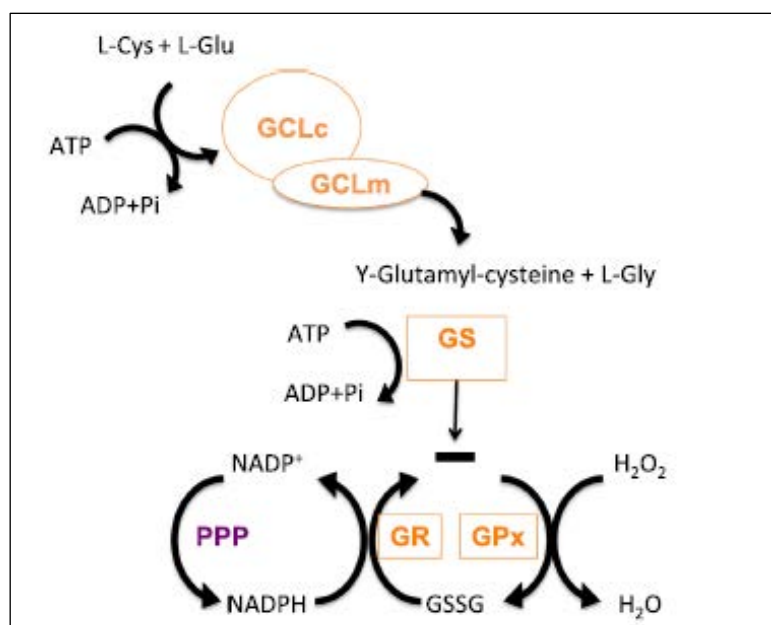


Figure 12. GSH biosynthetic route and GSH cycle. GSH biosynthesis occurs in two ATP-dependent steps. The first limiting step is the formation of a covalent bound between γ -Glu and L-Cys catalysed by glutamate–cysteine ligase (GCL), formed by two subunits GCLc and GCLm. The addition L-Gly in the second step is catalysed by glutathione synthase (GS) to form γ - glutamyl-cysteinyl-glycine (GSH). Then GSH is used by GPx to detoxify H_2O_2 , generating GSSG that could be recycle by glutathione reductase (GR) consuming NADPH, which is reduced from pentose phosphate pathway (PPP) [452]

GSH physiological functions include: (i) maintaining the reduced status of cysteine residues on proteins; (ii) the storage of cysteine reserves; (iii) involvement in the metabolism of oestrogens, leukotrienes, and prostaglandins; (iv) participation in the production of deoxyribonucleotides; (v) participation in the maturation of iron–sulfur cluster in proteins; (vi) signal transduction from the environment to cellular transcription machinery, (vii) maintaining the cellular redox potential [453]. Indeed, GSH is required for several cell processes interconnected with alterations in the maintenance and regulation of the thiol-redox status, due to its capability to exist in different redox species [454]. Under physiological conditions, the reduced GSH concentration is 10 to 100 folds higher than the oxidized species (GSSG and GSSR).

Glutathione and oxidative stress

GSH scavenges free radicals, ROS and RNS (hydroxyl radical, lipid peroxy radical, superoxide anion, and hydrogen peroxide) directly and indirectly through enzymatic reactions. The one-electron reduction with radicals is not chemically favourable, because it generates the unstable thiyl radical $\text{GS}^\bullet$. However, the reaction is kinetically driven in the forward direction by the removal of $\text{GS}^\bullet$ through a reaction with thiolate anion (GS^-) and O_2 . The first reaction leads to the generation of GSSG^- , which in the presence of O_2 , generates GSSG and $\text{O}_2^{\cdot-}$. Ultimately, the $\text{O}_2^{\cdot-}$ production will be dismuted in H_2O_2 by the SOD enzyme. H_2O_2 will then be kept in charge by catalase or GPx [455]. GSH

does not react directly with hydroperoxides, however, it is a cosubstrate of the selenium-dependent GPx, which has been recognized as the most important mechanism for the reduction of H_2O_2 and lipid hydroperoxides such as malonyl dialdehyde and 4-hydroxy-2-nonenal [456]. GPx catabolized hydroperoxides converting two GSH into GSSG, its oxidized form. GSH is then recycled by the GR through FAD and NADPH consumption.

Therefore, the GSH/GSSG redox couple is of great importance in maintaining cellular redox status. The estimated *in vivo* redox potential for the GSH/GSSG couple ranges from -260 mV to -150 mV depending on the conditions [457]. Thus, changes in the GSH/GSSG ratio are fundamental in the fine tuning of signal transduction such as cell cycle regulation [218]. Under oxidative stress, the concentration of GSH decreases leading to irreversible cell degeneration and death. In fact, shifting the GSH/GSSG redox toward the oxidizing state activates several signalling pathways including protein kinase B, calcineurin, NF- κ B, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinase, thereby reducing cell proliferation and increasing apoptosis [458].

In recent years, additional roles for the antioxidant function of GSH related to signal transduction have emerged. The most common covalent protein post-translational modifications of cysteine residues are the modification of the cysteine by NO, the S-nitrosation (described in the section 3.4) or thiol by incorporation of a glutathione moiety, S-glutathionylation (**Fig. 13**). Protein S-glutathionylation provides protection of protein cysteines from irreversible oxidation and serves to transduce a redox signal by changing structure/function of the target protein [459, 460, 461]. This process is observed either under physiological redox signalling or oxidative stress. Protein S-glutathionylation involves the reaction of protein cysteine residue or oxidized derivative such as S-nitrosyl (S-NO), sulfenic acid (S-OH), thiyl radical ($\text{S}\cdot$) with GSH. The reversal of S-glutathionylation (*i.e.*, de-glutathionylation) is catalysed by glutaredoxin (Grx) at the expense of GSH as a cosubstrate [462].

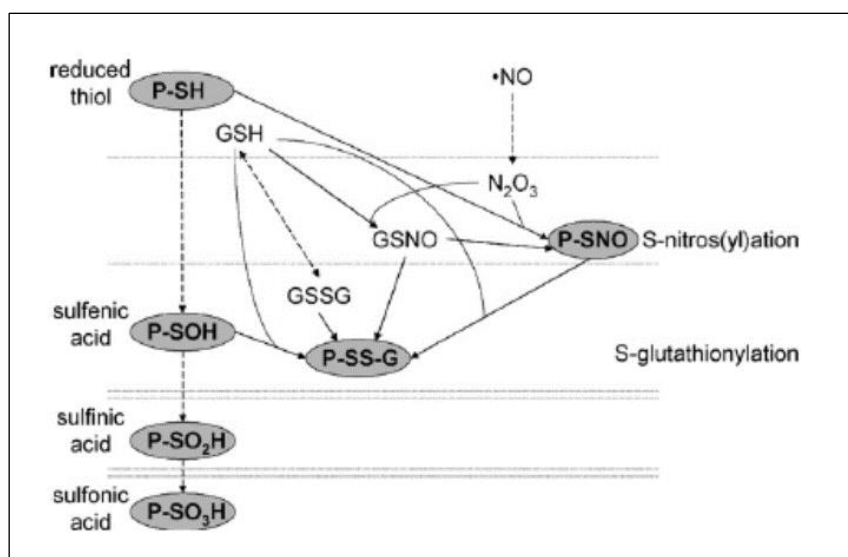


Figure 13. Chemical pathways leading to the formation of protein S-nitros(yl)ation and S-glutathionylation. Horizontal dotted lines separate one-electron oxidative states. Dashed lines represent oxidations by oxidative species not depicted, including molecular oxygen (O_2). For the sake of simplicity, lines representing reaction of P-SH with GSSG or GSNO to yield mixed disulfide protein (P-SS-G) have been omitted. GSH: reduced glutathione; GSNO; S-nitrosoglutathione; GSSG: oxidized glutathione [463].

In conclusion, the scenario depicted, clearly indicates that GSH is a *weighty protagonist* of the huge network governing the decision between life and death, through the modulation of cellular redox state. A simplified version of several roles of GSH described and not, are reported in table 8.

Table 9. Role of glutathione

ANTIOXIDANT DEFENSE	METABOLISM	REGULATION
Scavenging free radicals and other reactive species	Synthesis of leukotrienes and prostaglandins	Intracellular redox status
Removing hydrogen and lipid peroxides	Conversion of formaldehyde to formate	Signal transduction and gene expression
Preventing oxidation of biomolecules	Production of D-lactate from methylglyoxal	DNA and protein synthesis, and proteolysis
	Formation of mercapturates from electrophiles	Cell proliferation and apoptosis
	Formation of glutathione-NO adduct	Cytokine production and immune response
	Storage and transport of cysteine	Protein glutathionylation
		Mitochondrial function and integrity

3.3 Different models to induce oxidative stress

In the past decades, cell cultures and animals models have been established in order to better understand the effects and mechanisms of ROS and antioxidants. To induce oxidative stress, two alternative approaches may be followed to disturb the prooxidant-antioxidant balance: either increasing the radical load or inhibiting the antioxidant defenses. The radical load can be increased by exposing the cells to γ -irradiation, elevated oxygen tension (hyperoxia), extracellular $O_2^{\bullet-}$ and/or H_2O_2 , or by using free radical-generating azo compound, such as 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) or by using free radical-generating drugs, such as paraquat and menadione. These drugs contain a quinone structure and can take part in an intracellular oxidation reduction cycle by which $O_2^{\bullet-}$ is generated. However, in addition to $O_2^{\bullet-}$, reactive semiquinone radicals are also formed that may contribute to the stress induced by these compounds [464, 465].

In the following subparagraphs several oxidative stress models will be described, with a particular attention for the AAPH model employed within article 2.

3.3.1 Exposure to extracellular H_2O_2

H_2O_2 added directly to the culture medium results in short-term exposure to a quickly decreasing concentration of H_2O_2 . H_2O_2 is rather stable in most culture media, but in the presence of cells, its concentration diminishes quickly, depending on cell population density and cellular catalase contents. H_2O_2 readily penetrates the cellular membrane and inside the cell, where it is detoxified by catalase. Sensitivity towards the cytotoxic effects of H_2O_2 is inversely correlated with catalase activity when relatively high bolus doses of H_2O_2 are given [466]. Glutathione peroxidase may act as the predominant defense enzyme against lower H_2O_2 concentrations [467]. H_2O_2 itself is a relatively inert species and most of its deleterious effects, such as induction of lipid peroxidation and DNA damage, are due to its ability to stimulate $\bullet OH$ formation by the transition metal-catalyzed Haber-Weiss reaction. The availability of transition metals in the cells is supposed to be limiting for $\bullet OH$ formation. Therefore, the formation of $\bullet OH$ can only occur at those intracellular sites where these transition metals are available in the reduced form. For continuous $\bullet OH$ formation, it is necessary to keep the transition metals in its reduced form. This can be achieved by $O_2^{\bullet-}$ (the classical Haber-Weiss reaction) but also by other reducing agents — such as ascorbate and GSH.

3.3.2 Exposure to extracellular $O_2^{\bullet-}$

$O_2^{\bullet-}$ can be added directly to the culture medium in the form of potassium superoxide. In this way a high but quickly diminishing concentration of $O_2^{\bullet-}$ can be obtained. Due to spontaneous as well

as enzyme catalyzed dismutation, $O_2^{\bullet -}$ is converted into H_2O_2 , which in turn may give rise to $^{\bullet}OH$, if transition metal ions are present. The effects of $O_2^{\bullet -}$ will be almost similar to those of H_2O_2 . However, extracellular $O_2^{\bullet -}$ will not readily penetrate the cellular membrane, in contrast to the hydroperoxyl radical $HO_2^{\bullet}$, the uncharged form of $O_2^{\bullet -}$, which is supposed to induce lipid peroxidation [468]. Theoretically, $^{\bullet}OH$ is also able to penetrate the cellular membrane; however, this radical is so reactive that it is most likely to react with components of the plasma membrane. Moreover, when the effect of 'pure' $O_2^{\bullet -}$ is to be studied, catalase should be added to the culture medium to remove H_2O_2 and to prevent $^{\bullet}OH$ formation. The purity of the catalase preparation is extremely important, indeed many catalase preparations appear to be contaminated with SOD activity. To obtain a level of $O_2^{\bullet -}$ for a longer period of time, it is possible to generate $O_2^{\bullet -}$ enzymatically by adding: xanthine (X) and xanthine oxidase (XO) to the culture medium. In this way it is possible to generate continuous fluxes of $O_2^{\bullet -}$ for a longer period of time and the progress of the X/XO reaction can be followed by monitoring the formation of urate, the primary product of the X/XO reaction. In such experiments, it was observed that the formation of urate slows down after 30-40 minutes, due to inactivation of XO [469]. Using this system, a mixture of $O_2^{\bullet -}$ and H_2O_2 will be generated, the ratio of which will depend on SOD and catalase activities present in the culture system.

There are several factors that modulate the effect of extracellular H_2O_2 and $O_2^{\bullet -}$. When cells are exposed to H_2O_2 and $O_2^{\bullet -}$ in complex medium, the composition of the medium may modulate the ultimate effect. For example, pyruvate, present in almost all cell culture media, is known to scavenge H_2O_2 [470] resulting in a reduced effective dose. On the other hand, ascorbic acid, which is present in high concentrations in some standard culture media, may act as an antioxidant or prooxidant molecule. Ascorbic acid can stimulate $^{\bullet}OH$ formation probably due to its ability to reduce transition metals. Another important factor influencing H_2O_2 toxicity is the cell population density. Cells at high densities appear to be much less susceptible to H_2O_2 than cells at low densities [471]. This effect is caused by the ability of the cells to catabolize H_2O_2 .

Besides the above-mentioned effects, the addition or generation of $O_2^{\bullet -}$ or H_2O_2 in complex medium might result in the formation of many other secondary reactive species. Therefore, using these types of models, it is recommended to use the simplest possible medium. For short-term exposures, when $O_2^{\bullet -}$ or H_2O_2 are added directly to the cultures, the exposure time may be as short as 30 min. It makes no sense to 'expose' cells for many hours or even overnight, since the effective exposure is much shorter. Short exposure times have the advantage that complicating factors, such as fetal calf serum, can be omitted from the medium. It is also important, however, during relatively short exposure times, to add glucose and glutamine to the exposure media, in order to provide the

cells with their major energy sources. In contrast, for longer exposure times, when a more chronic stress is desirable, it may be necessary to include serum proteins in the exposure medium.

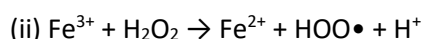
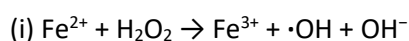
Therefore, application of peroxides leads only a transient oxidative stress depending on the ability of the cultured cells to detoxify the peroxide. Using $O_2^{\bullet-}$ or H_2O_2 models to generate ROS *in vitro* render difficult the quantification of generated reactive oxygen species or to clearly define the identity of ROS inducing the oxidative stress. Precisely for these reasons and for the purpose of this experimental thesis work, developing an oxidative stress model using SMCs (A-10 cell line) reproducible and under controlled condition, we do not favour these kind of models.

3.3.3 Oxidative stress and metal ions

Detailed studies have shown that redox active metals like iron (Fe), copper (Cu), chromium (Cr), cobalt (Co) and other metals undergo redox cycling reactions and possess the ability to produce reactive radicals such as superoxide anion radical and nitric oxide in biological systems. Disruption of metal ion homeostasis may lead to oxidative stress, causing interference with signal transduction pathways that play important roles in cell growth and development [472]. The mechanism of metal-induced formation of free radicals is tightly influenced by the action of cellular antioxidants. Many low molecular weight antioxidants (ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), GSH, carotenoids, flavonoids, and other antioxidants) are capable of chelating metal ions reducing thus their catalytic activity to form ROS. In particular, the oxidative stress models induced by Fe and Cu will be described below.

Iron induces oxidative stress

Iron occurs in two oxidation states Fe^{2+} (ferrous ion) and Fe^{3+} (ferric ion). The ferrous ions are soluble in biological fluids and generated in the presence of hydroxyl radicals. They are also unstable in aqueous media and tend to react with molecular oxygen to form ferric ions and $O_2^{\bullet-}$. This oxidized form of iron is insoluble in water at neutral pH and precipitates in the form of ferric hydroxide [473]. The toxic effects of free ferrous ion is substantiated by its ability to catalyze *via* the Fenton reaction, the generation of damaging reactive free radicals [474]. Indeed, ferrous ion is oxidized by H_2O_2 to ferric ion, forming a hydroxyl radical and a hydroxide ion (i). Ferric ion is then reduced back to ferrous ion by another molecule of hydrogen peroxide, forming a hydroperoxyl radical and a proton (ii):

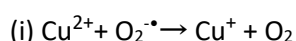


The hydroxyl radical is highly reactive with a half-life in aqueous solution of less than 1 ns [475] and it is able to abstract a hydrogen atom from polyunsaturated fatty acids to initiate lipid peroxidation. When the metal is iron and copper, the production of hydroxyl radical according to the Fenton reaction is the most abundant species *in vivo* and it reacts close to its site of formation. Although Fenton chemistry is known to occur *in vitro*, its significance under physiological conditions is not fully understood.

Copper induces oxidative stress

The most oxidation numbers of copper in living organisms are Cu^{2+} (cupric ion) and Cu^+ (cuprous ion). Copper is a cofactor of many enzymes involved in redox reactions, such as cytochrome c oxidase, ascorbate oxidase, or superoxide dismutase. In addition to its enzymatic roles, copper is used in biological systems for electron transport [476]. Copper can catalyze ROS formation *via* Fenton and Haber-Weiss chemistry. Under physiological conditions, copper free exists very rarely inside cells. Copper can induce oxidative stress by two mechanisms. First, it can directly catalyze the formation of ROS *via* a Fenton-like reaction [476, 478]. Second, exposure to elevated levels of copper significantly decreases glutathione levels [479].

The cupric ion, in the presence of superoxide anion radical or biological reductants such as ascorbic acid or GSH, can be reduced to cuprous ion (i), which is capable of catalyzing the formation of reactive hydroxyl radicals through the decomposition of hydrogen peroxide *via* the Fenton reaction (ii) [480, 481, 482]:



Again, copper-induced formation of ROS can cause peroxidation of lipids, as clearly demonstrated *in vitro* studies. GSH can suppress copper toxicity by directly chelating the metal [483], maintaining it a reduced state, unavailable for redox cycling. Disruption of copper homeostasis resulting in elevated pools of copper may contribute to a shift in redox balance towards more oxidizing environment by depleting glutathione levels [484]. The depletion of glutathione may enhance the cytotoxic effect of ROS and allow the metal to be more catalytically active, thus producing higher levels of ROS. The large increase in copper toxicity following GSH depletion clearly demonstrates that GSH is an important cellular antioxidant acting against copper toxicity [485].

Iron and copper are two divalent metallic ions very important in the organism: iron and copper are cofactors of many enzymes involved in redox reactions and metabolic processes. The

metal-induced oxidative stress could be a good oxidant stress model. However, they are both implicated in the non-enzymatic decomposition of S-nitrosothiols and proteins causing chemical reduction of the S-NO bond [486]. Therefore, considering the object of this second part of the thesis, the aforesaid model cannot be applied.

3.3.4 Oxidative stress and generator of free radicals: 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH)

The 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) is a water soluble azo small molecule that is often employed in the study of lipid peroxidation and for the characterization of antioxidants *in vitro*. Spontaneous decomposition of AAPH, at physiological temperature (37°C), produces one mole of nitrogen and two moles of carbon radicals (R•). The carbon radicals could either combine to produce stable products or react with molecular oxygen to generate peroxy radicals (ROO•) or with polyunsaturated lipids of cell membranes for their peroxidation [487]. At 37°C and pH 7, the half-life of AAPH is about 175 h; consequently, the ROO• generation rate is virtually constant for the first few hours [488], and is directly proportional to free base concentration. Because AAPH is water soluble, the rate of free-radical generation from AAPH can be easily controlled and measured. AAPH has been used *in vitro* to determine both antioxidant properties of compounds [489, 490] and the total defense against free radicals [491, 492]. It has been extensively used as a free-radical initiator for biological studies and the haemolysis induced by AAPH provides a good approach for studying membrane damage induced by free radicals [493]. This small molecule has been used, as a source of thermal free radical, in the study of oxidations of red blood cells, plasma, whole blood, HeLa cells, various tissues, and even the whole body [494], reporting that AAPH is able to cause various types of pathological changes through cellular oxidative damage.

The use of this azo compound, on different cell types cultures, is supported by several lines of evidence. Indeed, Hyuck et al. evaluated intracellular oxidants in human premonocytic U937 cells after exposure to AAPH, in order to analyze the role of cytosolic NADP⁺-dependent isocitrate dehydrogenase (IDCH) in cellular defense against the lipid peroxidation-mediated oxidative damage [495]. In the same year, Aldini et al. investigated the molecular mechanisms of procyanidins (polyphenols) as cardioprotective agents on endothelial stress model based on AAPH stimulation. They studied the efficacy of procyanidins to protect ECs against ONOO⁻ and to modulate the endothelium-dependent NO release in human internal mammary artery [496]. More recently, Scarpato et al. investigated the cytotoxic and genotoxic effects of ROO• and ONOO⁻ generated from AAPH and SIN-1 in a human microvascular endothelial cell line and human peripheral lymphocytes, respectively [497]. Furthermore, AAPH was also used to study the effects of oxidative stress on the

cardiovascular system during chick embryo development [498]. In particular, when nine-day-old (stage HH 35) chick embryos were treated with different concentrations of AAPH inside the air chamber, it was established that the LD₅₀ value for AAPH was 10 mmol/egg. At this concentration, AAPH was found to significantly reduce the density of blood vessel plexus that was developed in the chorioallantoic membrane of HH 35 chick embryos. Impacts of AAPH on younger embryos were also examined and discovered that it inhibited the development of vascular plexus on yolk sac in HH 18 embryos. AAPH also dramatically repressed the development of blood islands in HH 3+ embryos. These results implied that AAPH-induced oxidative stress could impair the whole developmental processes associated with vasculogenesis and angiogenesis. Furthermore, He et al. observed heart enlargement in the HH 40 embryo following AAPH treatment, where the left ventricle and interventricular septum were found to be thickened in a dose-dependent manner due to myocardiac cell hypertrophy. Therefore, oxidative stress, induced by AAPH, could lead to damage of the cardiovascular system in the developing chick embryo. Finally, AAPH was used in vascular muscle cell line to study the direct effects of free radicals on SMCs cholesterol metabolism [499], cAMP concentration and in cholesterol homeostasis related cAMP-dependent enzymes activity [427].

Based on these informations and excluding the oxidative stress models based on ROS and metal ions for the reasons mentioned above (instability, interference with *S*-nitrosothiols), AAPH can be considered as a model applicable to our study for stable and reproducible induction of oxidative stress in SMC without any interference with a *S*-nitrosothiol treatment.

3.4 Smooth muscle cells and protein *S*-nitrosation

Apart from ROS and the interaction of NO with ROS (causing the production of several RNS, which occurs only under conditions of excessive ROS production), and besides its direct role in vascular function, NO also participates in redox signaling by modifying lipids (*via* nitration of fatty acid) and proteins (*via* *S*-nitrosation of cysteine residue) [500, 501]. In respect to the latter, NO can directly modify sulfhydryl residues of proteins through *S*-nitrosation, resulting in the formation of *S*-nitrosothiols. As already described (chapter 1, section 1.3), *S*-nitrosation is a redox dependent, thiol-based, reversible post-translational protein modification that involves attachment of an NO moiety to a nucleophilic protein sulfhydryl group and it is involved in redox based cellular signalling [502, 503, 504, 505] (**Fig.14**). *S*-nitrosation is analogous to phosphorylation, glutathionylation, palmitoylation, acetylation and other physiological modifications of proteins.

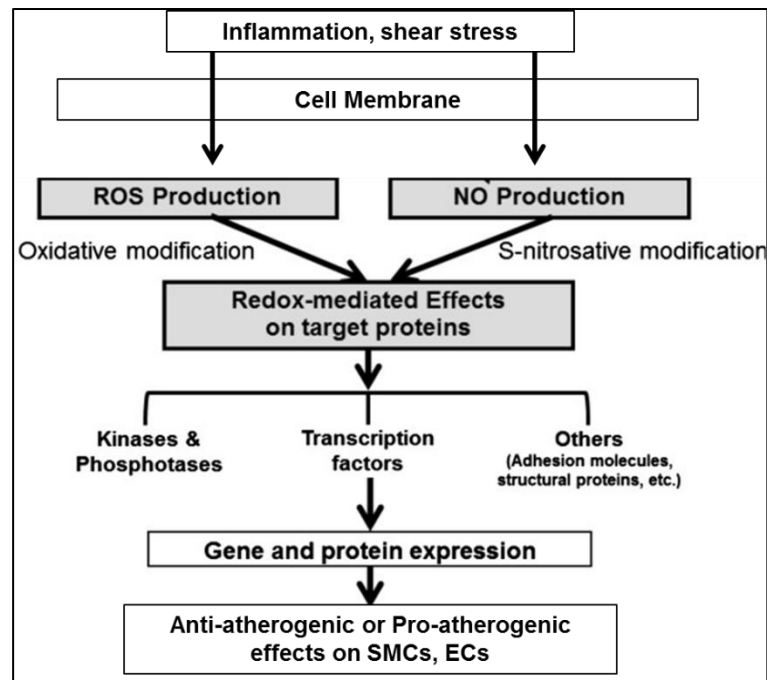


Figure 14. Mechanisms for oxidant stress-induced modifications on target proteins in cardiovascular diseases. In this process, ROS triggers oxidative modification and NO triggers S-nitrosation of many target molecules, together with activation of pro-oxidant and antioxidant enzymes to regulate the redox status of SMCs and ECs (Adapted from [506]).

There are emerging data suggesting that S-nitrosation of proteins plays an important role both in normal physiology and in a broad spectrum of human diseases. For instance relates to the regulation of vascular tone, the balance between Ang-II and NO appears crucial for maintaining the homeostasis of the cardiovascular and renal systems. In fact, when this homeostatic balance becomes perturbed, the actions of Ang-II predominate over those nitric oxide [507]. In this regards, in the laboratory, an important point under investigation is precisely the S-nitrosation effect on Ang-II receptors by GSNO (the S-nitrosothiol thoroughly studied in our laboratory), in the cerebral circulation, to understand the real impact of this important post-translational mechanism [Bouressman et al. to be submitted].

There is cross-talk between S-nitrosation, phosphorylation and other post-translational signaling mechanisms that affect protein interactions. In many cases, pathophysiology correlates with hypo- or hyper S-nitrosation of specific protein targets, rather than a general cellular insult due to loss of or enhanced nitric oxide synthase activity. In addition, the dysregulated S-nitrosation results from a modification of NO availability (quantity and/or localization). NO availability results not only from alterations of the expression, compartmentalization and/or activity of nitric oxide synthases, but also reflect the contribution of denitrosylases, including GSNO-metabolizing enzymes, like GSNO reductase releasing GSNHOH, a non NO active molecule, or GGT releasing cys-gly-NO, an

active NO molecule [157]. If on one hand, the irreversible oxidation of thiols can block the physiologic modification by S-nitrosation or S-glutathionylation and thereby interfere with normal physiologic signaling [508], on the other hand, it has been suggested that NO can protect cells from oxidative stress, whereas loss or inhibition of NOS enhances oxidative stress. By the way, the emerging picture shows that protein S-nitrosation not only leads to changes in protein structure and function, but also prevents these thiol(s) from further irreversible oxidative/nitrosative modification.

3.4.1 S-nitrosation influences cell signalling and redox regulation

The protein S-nitrosation is a redox reversible process with high spatial and temporal specificity. A determinant that governs the specificity of post-translational protein modification by NO is provided by the colocalization of NO sources and targets proteins, which is based at least in part on specific protein–protein interactions with NO synthases. Conversely, the S-nitrosation is also a temporal signaling event, which depends on the formation of NO by NOS and other nitrosylating equivalents.

Many Cys-containing proteins such as signaling molecules and transcriptional factors are potential targets that undergo a range of ROS-dependent or RNS-dependent oxidative and nitrosative modifications of this Cys-containing proteins. Physiologically, NO through S-nitrosation of proteins regulates numerous cellular responses. NO exerts as an antioxidant by inhibiting NADPH oxidase activity *via* S-nitrosation [509]. NO was shown to promote the ROS scavenging activity of thioredoxin-1 *via* S-nitrosation on Cys69 residue [510, 511]. Indeed, ECs under physiological shear stress increased protein S-nitrosation [512, 513] independently of cGMP-dependent signaling. In contrast, ECs after TNF- α and mild oxidized LDL treatment reduced S-nitrosation [514]. Early researches demonstrated that AP-1 (an important transcription factor that regulates gene expression in response to a variety of stimuli and it controls a number of cellular processes including differentiation, proliferation, and apoptosis [515]) activity was altered by S-nitrosation [516] and by oxidation of Cys residues [517]. Furthermore, H₂O₂ treatment inhibited AP-1 activity and decreased eNOS promoter activity [121]. NF- κ B, AP-1, and p53 all contain reactive thiols in their DNA binding regions, the modification of which alters their binding to DNA.

Regulation of protein interactions with chromatin can also involve S-nitrosation. For example, in neuronal development, brain-derived neurotrophic factor activates nNOS, which nitrosates histone deacetylase 2 (HDAC2; cysteines 262 and 274), causing HDAC2 to dissociate from chromatin [518]. This increases histone acetylation, permitting transcription of beneficial target genes regulating dendritic growth [518]; but it can also have potentially adverse effects, including increased expression of metastatic tumor antigen 1 [519]. Additionally, cell cycle regulation appears to involve S-nitrosation and denitrosylation of critical proteins involved in the formation of the mitosis process

[207]. As with other cellular effects, however, the role of S-nitrosation signaling in epigenetics and cell-cycle regulation is only beginning to be understood.

Furthermore, a number of nuclear regulatory protein interactions are modified by S-nitrosation. For example, the Hypoxia-inducible factor 1 α (Hif1- α) is stabilized by physiological S-nitrosothiol levels through S-nitrosation of protein von Hippel Lindau (C162), preventing its degradation [520]. Then HIF1- α can interact with HIF1- β to bind to hypoxia-responsive elements in gene promotor regions resulting in transcription of genes such as VEGF. S-nitrosation has also a variety of effects to alter NF- κ B activity, including S-nitrosation of both NF- κ B subunits p50 (C 62) and p65 (C 38); as well as I κ B kinase [521, 522]. The net effect of these reactions is generally to increase cytosolic NF κ B-I κ B interaction, and/or preventing nuclear translocation of NF- κ B; these effects prevent interaction of NF κ B with inflammatory gene promoters, inhibiting inflammation.

Therefore, it should be noted that on one hand S-nitrosation could have a strong impact on protein signaling in a targeted way to cause specific changes, and eventual alterations in this process can promote the occurrence of disorders in many diseases. On the other hand, it is also true that S-nitrosation in normal and disturbed cell function presents, in principle, novel therapeutic opportunities in a wide range of human diseases, such as cardiovascular diseases associated with oxidative stress. Indeed, in a context characterized by a significant decrease of NO bioavailability and an increase of oxidative stress, which is the basis of most pathologies, S-nitrosothiols represent promising candidate as NO donors to maintain an appropriate level of NO and treat NO deficiency, compared to other NO-related therapeutics developed, in fact many of them produce tolerance phenomena and oxidant stress (chapter I, section 1.4).

3.4.2 GSNO: a key player in the S-nitrosation

Among S-nitrosothiols, most of our attention is focused on GSNO, the endogenous/physiological storage and transport form of NO. Precisely for these reasons, the regulation of the cardiovascular system by this intracellular low molecular weight appears to be of particular physiologic interest and is under active investigation in our laboratory. Our common purpose is to evaluate the ability of GSNO to regulate NO bioavailability, in different cell-free or cell-based systems and in different conditions, in order to develop GSNO pharmaceutical forms, that actually are still lacking, for the treatment of vascular diseases. Indeed, despite its powerful antiplatelet activity, vasodilator effects, antimicrobial and antithrombotic effects (chapter I, section 1.4), this molecule is not yet present in any pharmaceutical composition. This may be related to the fast and unpredictable rate of decomposition of GSNO. *In vitro*, GSNO decomposition is promoted by factors such as pH, temperature and metal ions. *In vivo*, it is effected by enzyme activities such as GSNO reductase, CR1, PDI, Trx systems and GGT.

Therefore, considering its stability, limited by enzymatic and non-enzymatic degradations, too low for clinical application to provide a long-lasting effect and to deliver appropriate NO concentrations to target tissues, GSNO, and RSNO in general, have to be protected. GSNO encapsulation is an interesting response to overcome degradation and provide protection, but it raises difficulties for encapsulation due to its hydrophilic nature and the instability of the S-NO bound during the formulation process. In a previous study in our laboratory [523], the direct encapsulation of GSNO within polymeric nanoparticles has been described and demonstrated the conservation of the activity of this fragile molecule after the formulation process. Even if the potential of the S-nitrosothiol GSNO for NO supplementation is limited by its poor stability and high hydrophilicity (the obtained release may not be sustained enough for a chronic *in vivo* therapeutic effect), these studies represented a first starting point for chronic oral delivery of GSNO, providing opportunities for vascular diseases treatment.

3.4.3 GSNO: NO storage and transportation

It is documented in the literature that NO stores implicated in physiological responses has been proposed to explain endothelium-dependent relaxing effects that persist after blockade of NO synthase by inhibitors [524, 525]. NO store is also involved in long lasting hyporesponsiveness to vasoconstrictors elicited by endotoxin [526] and by NO donors [527, 528]. Alencar et al, using rat aorta exposed to GSNO displayed, even after washout of the drug, a persistent increase in cysteine-NO residues and in NO content, a persistent attenuation of the effect of vasoconstrictors, and a relaxant response upon addition of low-molecular-weight thiols [145]. Rat mesenteric and porcine coronary arteries exposed *in vitro* to GSNO, as well as aorta and mesenteric arteries removed from rats treated *in vivo* with GSNO *via* infusion, displayed similar modifications of contraction [145]. Together, these studies support the idea that S-nitrosation of cysteine residues is involved in long-lasting effects of NO on arterial tone. They suggest that S-nitrosation of tissue thiols is a mechanism of formation of local NO stores from which biologically active NO can be subsequently released [145, 529]. Basing on these considerations, in which NO stores are involved in the vascular response to vasoconstrictors and in the regulation of blood tone, the identification of the modification of NO stores can be used as a model to evaluate the therapeutic efficiency of free NO donors or NO donors related delivery systems. Precisely, in another work [530], we evaluated the efficiency of oral delivery of GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCP) through the formation of NO stores in rat aorta. After validation of NO absorption through a cell model of intestinal barrier, the selected GSNO-acNCP were orally administrated to Wistar rats. The efficient loading, protection and sustained release of GSNO provided by these formulations allows GSNO to reach the blood stream and contributes to form a reservoir of NO by transnitrosation inside the vascular wall.

Therefore, 17 h after oral administration of this formulation to Wistar rats, we observed vascular hyporeactivity to the vasoconstrictor phenylephrine. This is probably in relation with the formation of a releasable NO store, as it was possible to mobilize this NO store using N-acetylcysteine (NAC), a thiol which can displace NO from cysteine-NO residue to induce relaxation. These new drugs delivery systems of NO donors may be particularly adapted for oral treatment of cardiovascular disease treatment.

At this stage, it is conceivable to think that GSNO could be one of the best NO donor to use in cardiovascular field, precisely because it is a physiologic source of NO, exhibits higher stability than NO, does not induce any tolerance or oxidative stress, is well suited to the chemical formulation, and mediates protein S-nitrosation processes, playing an important role in vascular signaling [207]. Even if the role of ROS in oxidative modifications and that of NO in the S-nitrosation of many target molecules emerge from the literature, little is known on the possible interferences of free radicals with the metabolism of GSNO and subsequent NO-mediated molecular events.

For this reason, the last part of this chapter will focus on the possible GSNO employment to counteract NO deficiency occurring with oxidative stress and on the S-nitrosation effects on specific target molecules involved in smooth muscle cells phenotype. Considering, that SMCs are involved in the contraction and the regulation of blood vessel tone, thus distributing blood flow and regulating blood pressure and in signaling communication, the identification of the nitrosated proteins under oxidant conditions could help us to understand better eventual changes in their phenotype, function and biological process in the cardiovascular disorders associated with oxidative stress.

A rat aorta embryonic smooth muscle cell (A10 cell lines) exposed *in vitro* to the already discussed free radical generator, AAPH, will be used as oxidative stress model. The effects will be evaluated on levels of expression/activity of selected GSNO-metabolizing enzymes and levels of NO release. Moreover, considering that the protein S-nitrosation can be modified itself and in turn modulates signaling pathway under oxidative stress, the GSNO impact on S-nitrosation will be assessed. The extent and distribution of GSNO-induced S-nitrosation of cellular proteins will be also analyzed through biotin switch method [531] in combination with proteomic approaches [532, 533]. Analysis reveals that many additional proteins identified are involved in SMCs cytoskeleton and contractile machinery.

These studies underlie the significant role of S-nitrosation by GSNO, suggesting novel mechanisms for the protein S-nitrosation in diseases and adding to an expanding list of potential therapeutic targets.

3.5 Article 2

OXIDATIVE STRESS ENHANCES AND MODULATES PROTEIN S-NITROSATION IN SMOOTH MUSCLE CELLS EXPOSED TO S-NITROSOGLUTATHIONE

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Les S-nitrosothiols, comme le GSNO, principale forme physiologique de stockage du NO dans les tissus présentent une liaison réversible de NO à un groupement thiol réduit (-SH) et sont de potentiels agents thérapeutiques dans le cadre du traitement des maladies cardiovasculaires présentant une réduction de la biodisponibilité de NO. Le stress oxydant est concomitant au développement des maladies cardiovasculaires, cependant peu d'études documentent la capacité de GSNO à restaurer la biodisponibilité du NO dans les tissus vasculaires sous stress oxydant. La présente étude évalue l'impact du stress oxydant sur la biodisponibilité pour les CML de NO à partir de GSNO, en tenant compte de l'implication d'enzymes redox, la GGT et la PDI, impliquées dans le métabolisme de GSNO et dans la S-nitrosation des protéines.

Le stress oxydant a été induit *in vitro* sur des CML à l'aide d'un générateur spontané de radicaux libres, le 2,2'-azobis (2-amidinopropane) (AAPH). Les effets du stress oxydant sur la libération de NO ainsi que sur l'expression / l'activité de la (GGT) et de la PDI ont été évalués. L'état redox des thiols cellulaires a été quantifié au travers des concentrations intra et extracellulaires de glutathion et des groupements thiols réduits (-SH) des protéines. La capacité de GSNO à induire la S-nitrosation des protéines a été globalement quantifiée puis les protéines S-nitrosées ont été identifiées par spectrométrie de masse.

Comme attendu, le stress oxydant diminue la concentration intracellulaire de glutathion et des groupement thiols réduits au sein des protéines. Cependant, l'activité de la GGT est diminuée 3.5 fois, tandis que l'expression membranaire de la PDI est augmentée de 1.7 fois sans que ces modifications n'aient d'effet sur le catabolisme extracellulaire de GSNO. L'addition de GSNO sur les CML en condition de stress oxydant restaure les groupements thiols réduits des protéines et produit une S-nitrosation des protéines plus grande qu'en condition basale.

En outre, l'analyse par spectrométrie de masse des protéines S-nitrosées a révélé un nombre plus élevé de protéines S-nitrosées par GSNO en condition de stress oxydant (51 protéines, vs 32 en condition basale) comprenant un nombre plus élevé de protéines du cytosquelette (17, vs 8 en condition basale) impliquées dans la contraction vasculaire, la morphogenèse et le mouvement cellulaire. En outre, des classes supplémentaires de protéines impliquées dans l'adhésion cellulaire, le transfert / support et des protéines de transport) sont S-nitrosées uniquement en condition de stress oxydant.

Dans l'ensemble, même si le stress oxydant a modulé différemment la GGT et la PDI, des niveaux plus élevés de protéines S-nitrosées par GSNO ont été identifiées. En conclusion les résultats peuvent inciter à l'identification de biomarqueurs appropriés pour l'évaluation de la bioactivité de GSNO dans le traitement des maladies cardiovasculaires.

Oxidative stress enhances and modulates protein S-nitrosation in smooth muscle cells exposed to S-nitrosoglutathione.

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Abstract

Among S-nitrosothiols showing reversible binding between NO and -SH group, S-nitrosoglutathione (GSNO) represents potential therapeutics to treat cardiovascular diseases (CVD) associated with reduced nitric oxide (NO) availability. It also induces S-nitrosation of proteins, responsible for the main endogenous storage form of NO. Although oxidative stress parallels CVD development, little is known on the ability of GSNO to restore NO supply and storage in vascular tissues under oxidative stress conditions.

Aortic rat smooth muscle cells (SMC) were stressed *in vitro* with a free radical generator (2,2'-azobis(2-amidinopropane) dihydrochloride, AAPH). The cellular thiol redox status was reflected through levels of reduced glutathione and protein sulfhydryl (SH) groups. The ability of GSNO to deliver NO to SMC and to induce protein S-nitrosation (investigated *via* mass spectrometry, MS), as well as the implication of two redox enzymes involved in GSNO metabolism (activity of gamma-glutamyltransferase, GGT, and expression of protein disulfide isomerase, PDI) were evaluated.

Oxidative stress decreased both intracellular glutathione and protein -SH groups (53% and 32% respectively) and caused a 3.5 fold decrease in GGT activity, while PDI expression at the plasma membrane was 1.7-fold increased without any effect on extracellular GSNO catabolism. Addition of GSNO (50 μ M) increased protein -SH groups and protein S-nitrosation (50%). Mass spectrometry analysis revealed a higher number of proteins S-nitrosated under oxidative stress (51 proteins, vs 32 in basal conditions) including a higher number of cytoskeletal proteins (17, vs 8 in basal conditions) related with cell contraction, morphogenesis and movement. Furthermore, proteins belonging to additional protein classes (cell adhesion, transfer/carrier, and transporter proteins) were S-nitrosated under oxidative stress.

In conclusion, higher levels of GSNO-dependent S-nitrosation of proteins from the cytoskeleton and the contractile machinery were identified under oxidative stress conditions. The findings may prompt the identification of suitable biomarkers for the appraisal of GSNO bioactivity in treatment of CVD.

Keywords: Oxidative stress, S-nitrosoglutathione, Protein S-nitrosation, Gamma-glutamyl transferase, Protein disulfide isomerase, Mass spectrometry.

1. Introduction

Cardiovascular diseases like atherosclerosis, pulmonary hypertension, thrombosis, ischemia and cardiac arrhythmia are usually associated with oxidative stress and a reduced bioavailability of nitric oxide (NO) [1]. To overcome this aspect, several NO-related therapeutics have emerged over the past few decades, such as nitrosamines [2], organic nitrates [3], and N-diazeniumdiolates [4]. However, these compounds induce undesirable effects, such as tolerance and hypotension, and are often considered as oxidative stress enhancers in an environment rich in oxygen and/or radical species where they may favour the formation of peroxynitrite ions (ONOO^-), a reactive nitrogen species (RNS) producing deleterious proteins nitration [5, 6, 7, 8]. Other NO compounds, such as S-nitrosothiols may represent safer alternatives [9, 10]. Several investigations on the therapeutic potential of S-nitrosothiols have focused on S-nitrosoglutathione (GSNO), the physiological storage form of NO in tissues, due to the absence of recorded side effects in preclinical studies [11, 12, 13]. However, even though S-nitrosothiols are not prooxidant *per se*, the ability of GSNO to regulate NO bioavailability under oxidative stress conditions has not yet received sufficient attention.

Oxidative stress in the vessel wall has been shown to involve the *tunica media*, where smooth muscle cells (SMC) can produce reactive oxygen species (ROS) – *e.g.* superoxide anion, $\text{O}_2^{\bullet-}$ – following the activation of their own NADPH oxidase. SMC probably represent a privileged target of ROS [14]. Exposure of SMC cells to ROS-generating systems actually stimulates migration, proliferation, and growth [15, 16, 17, 18]. SMC also are a main target of (endothelium-derived or exogenous) NO, which exerts in this way its vasorelaxing effects.

NO, besides its direct role in vascular function, also participates in redox signaling by modifying proteins *via* S-nitrosation. The S-nitrosation, which is the formation of a covalent bond between NO and the sulfhydryl group of a cysteine residue, is a redox dependent, thiol-based, reversible posttranslational modification of proteins [19, 20, 21]. There are emerging data suggesting that S-nitrosation of proteins plays an important role both in physiology and in a broad spectrum of pathologies [22]. Pathophysiology correlates with hypo or hyper S-nitrosation level of specific protein targets. This dysregulation of protein S-nitrosation results from a modification of NO availability (quantity and/or localization). NO availability results not only from alterations of the expression, compartmentalization and/or activity of NO synthases, but also reflects the contribution of denitrosylases, including GSNO-metabolizing enzymes, like GSNO reductase releasing GSNOH, a non active NO-related molecule, and the gamma-glutamyl transferase (GGT) releasing cys-gly-NO, an active NO-related molecule [23]. Redoxines like protein disulfide isomerase (PDI) known to reverse thiol oxidation can also catabolise GSNO to release NO [24].

In the present study, we aimed to assess the suitability and potency of GSNO as a NO donor in an oxidative stress environment. Its metabolism by two specific redox enzymes (GGT and PDI), the cellular thiol redox status and protein S-nitrosation were thus analyzed in SMC exposed to oxidative stress induced by a free radical generator, *i.e.* 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). We more specifically evaluated whether oxidative stress modulates the bioactivity of GSNO, by favouring release of NO and S-nitrosation of potentially critical protein targets related with cell contraction, morphogenesis and movement.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All reagents were of analytical grade and all solutions prepared with ultrapure deionized water (>18.2 mΩ.cm). BCA Protein Assay Kit was purchased from Pierce and protease inhibitor cocktail from Roche. The Ez-Link Biotin-HPDP and the high capacity neutravidin agarose resin were obtained from Fisher Scientific. All other reagents came from Sigma, France country not precised for other companies.

2.1.2. Synthesis of S-nitrosoglutathione

GSNO was synthesized as previously described [25]. Briefly, reduced glutathione (GSH) was incubated with an equivalent amount of sodium nitrite under acidic conditions (0.626 M HCl). The concentration of GSNO was calculated using the specific molar absorbance of S-NO bond at 334 nm ($\epsilon = 922 \text{ M}^{-1}\text{cm}^{-1}$) and the Beer Lambert Law.

2.2. Cell culture and oxidative stress model

Vascular smooth muscle cells derived from embryonic rat aorta (A-10 line, ATCC, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 4.5 g/L glucose, 2% (v/v) glutamine (200 mM), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, as well as phenol red. They were cultured at 37°C under 10% (v/v) CO₂ in a humidified incubator and used between passages 25 and 30. For all experiments, cells were seeded in a 6-wells plate at 6,400 cells/cm², 48 h before stimulation. Oxidative stress was induced on cells during 2 h at 37°C by addition of 50 mM of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) in an incubation medium containing 5% (v/v) fetal bovine serum, 4.5 g/L glucose, 2% (v/v) glutamine (200 mM), 100 U/mL penicillin and 100 µg/mL streptomycin. After oxidative stress induction, 50 µM of GSNO (or the same volume of PBS) was added for an additional incubation period of 1 h at 37°C.

In another set of experiments, 15 min before the end of oxidative stress induction, 20 mM of serine borate complex (SBC), or 100 μ M of bacitracin were added to inhibit GGT or PDI, respectively.

2.3. *S-nitrosoglutathione metabolism*

2.3.1. *Quantification of extracellular S-nitrosothiols*

After cell incubation (see section 2.2.), media were collected for quantification of nitrite ions and total *S*-nitrosothiols (including residual GSNO) using the Griess and Griess-Saville methods. Briefly, 100 μ L of sample were diluted with 100 μ L acetoacetic solution (pH= 2.5). Then, for nitrite ions quantification, 40 μ L of sulfanilamide solution in 0.4 M HCl were added (Griess assay). For *S*-nitrosothiols quantification, the sulfanilamide solution was supplemented with 0.2% (w/v) HgCl_2 (to cleave the S-NO bond: Griess-Saville assay). Finally, the diazonium salt formed was reacted with 10 μ L of a 0.6% (w/v) N-(1-naphthyl) ethylenediamine solution to form a chromophoric azo product that absorbs at 540 nm. To calculate the concentration of *S*-nitrosothiols, free nitrite ions quantified by Griess assay were subtracted from those obtained with the Griess-Saville assay.

2.3.2. *Quantification of intracellular S-nitrosothiols*

Intracellular *S*-nitrosothiols were quantified using the DAN fluorescent probe (lower limit of quantification than the Griess method). After incubation (see section 2.2.), cells were washed with PBS and lysed with 500 μ L of 0.4% (m/v) Triton X-100 in 0.1 M HCl. The intracellular *S*-nitrosated proteins were quantified by the DAN (nitrite ions) or DAN- Hg^{2+} (*S*-nitrosothiols) using standard curves (0.1 - 1 μ M) of sodium nitrite and GSNO, respectively [26]. The concentration of nitrite ions (DAN) was subtracted from the DAN- Hg^{2+} quantification to obtain the intracellular *S*-nitrosothiols concentration, which was normalized upon the intracellular proteins concentration (see section 2.7).

2.4. *Thiol redox status*

The redox potential of the culture medium was measured by using a redox electrode (Hanna Instruments) combined with a reference Ag/AgCl electrode, ($E = 0.207$ V).

2.4.1. *Quantification of reduced membrane thiols*

After incubation, media were withdrawn and cells washed twice (PBS). Cells were then incubated for 10 min in the dark with 750 μ L of 1 mM 5-5'-dithio-bis(2-nitrobenzoic) acid (DTNB) prepared in PBS. Then, 200 μ L from each well were transferred in triplicate in a 96-wells plate and absorbance read at 405 nm (EL800, Universal Microplate Reader, Biotek Instruments). Membrane thiols concentration was calculated using a GSH standard curve ranging from 3.25 μ M to 32.5 μ M and expressed relatively to protein quantity (see section 2.7).

2.4.2. Quantification of intracellular reduced glutathione

Intracellular GSH was measured as previously described [27, 28], with some adaptations. Cells were lysed in a cold 3.3 % (v/v) perchloric acid solution and centrifuged for 15 min at $10,000 \times g$. Acidic supernatants were neutralized with 10 M NaOH and diluted 10 times in 0.1 M HCl containing 2 mM EDTA. Sixty μL of diluted samples or standard GSH solutions (0.65–3.25 μM) were transferred to a 96-wells plate; 120 μL of 0.4 M borate buffer (pH= 9.2) and 20 μL of 5.4 mM 2,3-naphthalene dicarboxaldehyde (NDA) solution prepared in ethanol were then added into each well. Microplate was incubated 25 min on ice in the dark. The fluorescence intensity of GSH-NDA adducts was measured using a microplate reader (Synergy 2 model, Biotek Instruments, Colmar, France) with excitation set at 485 ± 20 nm and emission at 528 ± 20 nm and expressed relatively to protein quantity (see section 2.7).

2.4.3. Quantification of intracellular protein reduced thiols

Intracellular protein reduced thiols were quantified using the DTNB method. After treatments, cells were lysed in a 3.3% (v/v) cold perchloric acid solution and centrifuged for 15 min at $10,000 \times g$. The pellets were resuspended in PBS containing 0.5% (V/V) sodium dodecylsulfate (SDS). Cells were then incubated 10 min in the dark with 700 μL of 1 mM DTNB. After incubation, 200 μL were transferred in triplicate in a 96-wells plate and the absorbance was read at 405 nm. Intracellular thiols concentration was calculated using a GSH standard curve ranging from 3.25 μM to 32.5 μM and expressed relatively to protein quantity (see section 2.7).

2.5. Determination of gamma-glutamyltransferase activity

Gamma-glutamyl transferase activity was kinetically determined using the synthetic GGT substrate L- γ -glutamyl-3-carboxy-4-nitroanilide (GCNA). After treatments, incubation media were replaced with 750 μL of 1 mM of GCNA in 100 mM Tris buffer (pH= 7.4) containing 20 mM glycyl-glycine and 10 mM MgCl_2 , with/without 50 mM AAPH or 20 mM of the GGT inhibitor serine-borate complex (SBC). Cells were then incubated at 37°C , and 50 μL of incubation medium were transferred in a 96-wells plate each 30 min and absorbance read at 405 nm. At the end of the kinetic assay, cells were lysed in 500 μL 0.1 M HCl containing 0.4% (m/v) Triton X-100 for protein quantification (see section 2.7).

2.6. Cell membrane PDI expression

Cells were scrapped in 50 mM Tris buffer (pH= 8) added with 50 mM 2-mercaptoethanol and protease inhibitor cocktail. After 30-min incubation on ice, cell lysates were centrifuged at $17,600 \times g$ during 20 min. The pellet containing membrane proteins was resuspended in 50 mM Tris buffer

(pH= 8) added with 1% (v/v) SDS and agitated for 30 min on ice. Membrane proteins were then centrifuged for 20 min at $21,000 \times g$. Finally, proteins were precipitated with 100% cold acetone for 1 h at -20°C . After centrifugation ($3,000 \times g$, 10 min), the pellet was resuspended in 50 mM Tris-HCl buffer (pH= 6.8) containing 0.15 M NaCl, 1% (w/v) SDS and 1% (w/v) Triton X-100. Proteins were quantified (see section 2.7) and 10 μg were deposited on a SDS PAGE with 10% separative gel and 4% concentrating gel. After migration, proteins were transferred on a polyvinylidene membrane and labelled with anti-PDI (sc-20132 Santa Cruz biotechnology) or anti-actin antibody diluted 1/1000 or 1/2000, respectively. Secondary antibody conjugated with HRP (sc-2004, Santa Cruz biotechnology) diluted 1/5000 was used to quantify PDI/Actin ratio using Image J 1.47V software (NIH, USA).

2.7. Total protein quantification

Protein determination was performed using the Pierce BCA Protein Assay Kit, following instructions of the manufacturer. A standard curve ranging from 0.025 to 1 $\text{mg}\cdot\text{mL}^{-1}$ was built with bovine serum albumin to calculate protein concentration.

2.8. Purification and identification of S-nitrosated proteins

Cells incubated in a 75 cm^2 flask were lysed in 500 μL of 50 mM Tris (pH= 6.8) containing 0.15 M NaCl, 1% (v/v) NP-40, 0.1% (v/v) SDS, 1 mM EDTA, 0.1 mM neocuproine and protease inhibitor cocktail. S-nitrosated proteins were purified by the biotin switch technique as previously described [29, 30], with some adaptations. Briefly, free thiols in cell lysates were blocked with 50mM of *N*-ethylmaleimide (NEM). Then, S-nitrosated proteins were labeled with pyridyl disulfide-biotin (*N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide biotin, biotin-HPDP) after cutting the S-NO bond with sodium ascorbate. Biotin-HPDP-labeled proteins were purified with NeutrAvidin beads (High Capacity NeutrAvidin Agarose Resin) and eluted in a buffer containing 1.5% (v/v) 2-mercaptoethanol.

After purification, S-nitrosated proteins were identified by mass spectrometry, as follows. Samples diluted 4-fold in 6 M urea, 50 mM Tris (pH= 8.0) were processed for cysteine reduction and alkylation, followed by overnight digestion in 10 vol of 50 mM Tris (pH= 8.0), 1 mM CaCl_2 containing 100 ng sequencing-grade trypsin (Promega). Protein digests were purified through C18 mini spin columns (Pierce, Thermofisher scientific, France), resuspended in 8 μL of 2% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and analyzed through label-free LC-MALDI as previously described [31]. Proteins and peptides were identified based on fragmentation spectra by interrogation of the whole Swissprot database through the public Mascot server (taking in account protein scores above 80.0 and peptide scores above 20.0 at first rank, allowing one trypsin miscleavage and considering

cysteine carbamidomethylation and methionine oxidation as optional). Finally, identified proteins were classified using the Panther database [32].

2.9. Statistical analysis of data

Results are expressed as means $\pm$ standard error of the mean (sem). Statistical analyses were performed using either the Student t-test (for enzyme activity/expression or inhibition) or two-way ANOVA ($p_{\text{condition}}$ basal *versus* AAPH, $p_{\text{treatment}}$ with or without GSNO and $p_{\text{interaction}}$ between condition and treatment) followed by a Bonferroni's multiple comparisons test. The GraphPad Prism software (GraphPad Software version 5.0, San Diego, USA) was used.

3. Results

3.1. Oxidative stress biomarkers

A 3-h incubation of SMC in presence of 50 mM AAPH significantly increased the redox potential of the culture medium from 256 ± 19 (basal) to 484 ± 8 mV (AAPH) ($n = 3$, Student t test, $p < 0.05$ vs basal). Addition of GSNO (50 μ M) during the final 1-h incubation had no impact on this redox potential, neither in control nor under AAPH exposure. No variation of the pH (7.4) was observed all along the experiment.

Under oxidative stress, the GGT activity decreased 3.5 fold, from 1.35 ± 0.20 to 0.39 ± 0.14 nmol/min/mg of proteins ($n = 3$, Student t test, $p < 0.05$). At variance, PDI localization at the plasma membrane increased 1.7 fold, from 0.72 ± 0.02 to 1.22 ± 0.31 (PDI/actin ratio, $n = 4$, Student t test, $p < 0.05$).

The intracellular GSH content significantly decreased with oxidative stress (**Fig. 1-A**), while extracellular GSH increased (**Fig. 1-B**) ($p_{\text{condition}} < 0.0001$ for both). The addition of GSNO produced a very slight (1.9 %) but significant increase in extracellular GSH ($p_{\text{treatment}} = 0.0395$), both in basal and oxidative conditions ($p_{\text{interaction}}$ ns), while this did not permit and recover intracellular levels of GSH, which remained at a low value under AAPH exposure ($p_{\text{treatment}}$ and $p_{\text{interaction}}$ ns, **Fig. 1-A**).

Reduced thiols at the plasma membrane (0.015 ± 0.002 nmol/ μ g of proteins) did not change with oxidative stress. Intracellular protein thiols evolved differently according to conditions and treatment ($p_{\text{interaction}} < 0.0001$, **Fig. 1-C**). They decreased with oxidative stress in the absence of GSNO. The addition of GSNO under oxidative stress almost doubled the intracellular protein thiol.

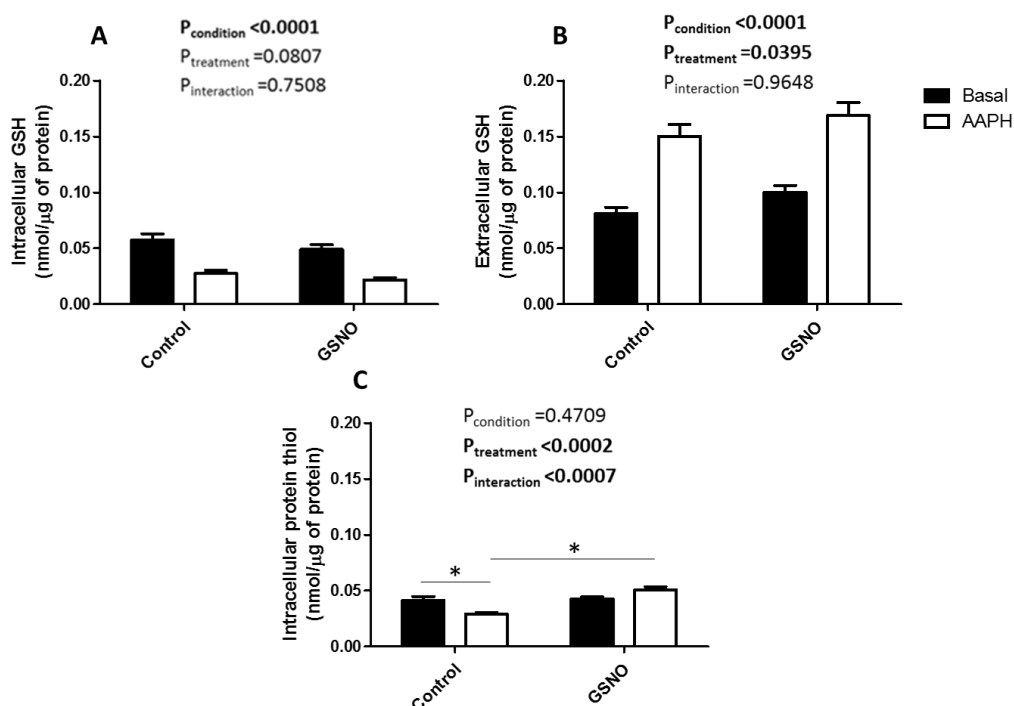


Fig. 1. Intracellular and extracellular thiol status in basal and oxidative stress conditions. Smooth muscle cells were incubated for a total of 3 h without (basal) or with 50 mM AAPH. In each condition, 50 μM GSNO or GSNO+AAPH were added during the 3rd h of incubation. Intracellular reduced thiols (**C**) were quantified by reacting precipitated proteins with DTNB. Intracellular (**A**) and extracellular GSH (**B**) were quantified with the NDA probe in the supernatant after protein precipitation. Results are presented as means ± sem of three independent experiments and compared using a two way ANOVA ($p_{\text{condition}}$ (Basal vs AAPH), $p_{\text{treatment}}$ (Control, GSNO) and $p_{\text{interaction}}$); * $p < 0.05$ (Bonferroni's multiple comparisons test).

3.2. Extracellular GSNO metabolism and intracellular formation of S-nitrosothiols.

After one hour in contact with cells, only approx. 20 μM of GSNO (**Fig. 2-A**) – out of the 50 μM initially added, and approx. 12 μM of nitrite ions (**Fig. 2-B**) were found in the extracellular space, indicating that GSNO is partly metabolized by SMC to release NO (detected as nitrite ions).

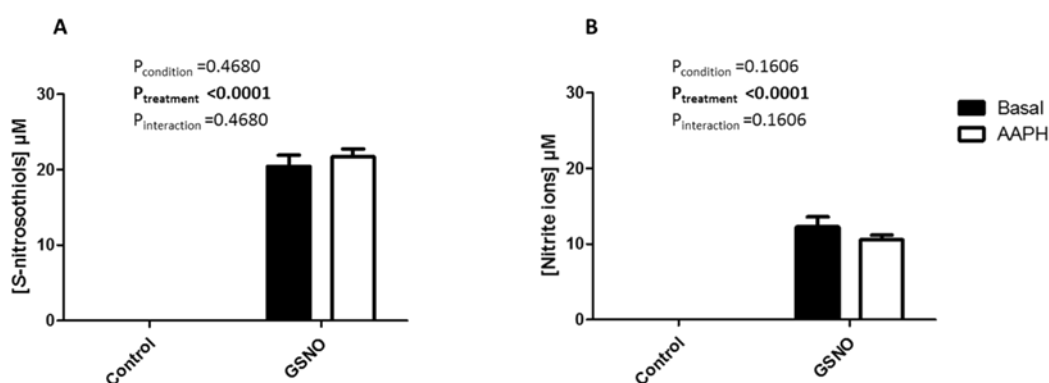


Fig. 2. Extracellular metabolism of S-nitrosoglutathione. Smooth muscle cells were incubated for 3 h without (Basal) or with 50 mM AAPH. In each condition, 50 μM GSNO or GSNO+AAPH were added during the 3rd hour of incubation. S-nitrosothiols **(A)** and nitrite ions **(B)** were quantified by the Griess-Saville and Griess methods, respectively. Results are presented as means $\pm$ sem of three independent experiments and compared using a two way ANOVA ($p_{\text{condition}}$ (Basal vs AAPH), $p_{\text{treatment}}$ (Control, GSNO) and $p_{\text{interaction}}$).

GGT and PDI inhibition under basal conditions led to an increased extracellular content of GSNO to $27 \pm 0.5 \mu\text{M}$ for SBC and $28 \pm 0.5 \mu\text{M}$ for bacitracin ($p < 0.05$ versus GSNO in the absence of inhibition, t-test) and a decrease in extracellular nitrite ions concentrations ($10 \pm 0.4 \mu\text{M}$ for SBC ($p > 0.05$) and $7 \pm 0.9 \mu\text{M}$ for bacitracin ($p < 0.05$ versus GSNO in the absence of inhibition, t-test), attesting a decrease in GSNO catabolism. Similar profiles were obtained with GGT and PDI inhibition under oxidative stress.

At the intracellular level, addition of GSNO for 1 hour induced formation of S-nitrosothiols, which nearly doubled under oxidative stress compared to basal condition **(Fig. 3)**. Both enzymes were implicated in the formation of intracellular S-nitrosothiols in both conditions. Inhibition of GGT by SBC led to a half time decrease in the content of S-nitrosothiols, both in basal condition ($2.1 \cdot 10^{-4} \pm 0.2 \cdot 10^{-4} \text{ nmol/} \mu\text{g}$ of proteins, $p < 0.05$ versus GSNO in the absence of inhibition, t-test) and under oxidative stress ($6.6 \cdot 10^{-4} \pm 0.4 \cdot 10^{-4} \text{ nmol/} \mu\text{g}$ of proteins, $p < 0.05$ versus GSNO in the absence of inhibition, t-test). PDI inhibition by bacitracin showed the same profile with a S-nitrosothiols formation decreasing to $2.90 \cdot 10^{-4} \pm 0.05 \cdot 10^{-4} \text{ nmol/} \mu\text{g}$ of proteins in basal condition ($p < 0.05$ versus GSNO in the absence of inhibition, t-test) and to $4.50 \cdot 10^{-4} \pm 0.5 \cdot 10^{-4} \text{ nmol/} \mu\text{g}$ of proteins under oxidative stress ($p < 0.05$ versus GSNO in the absence of inhibition, t-test).

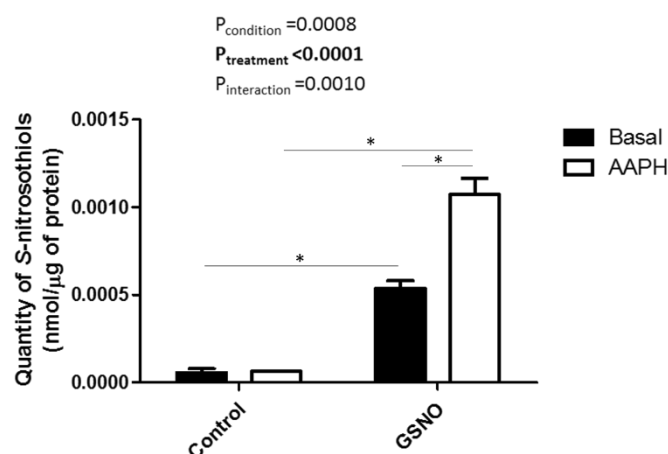


Fig. 3. Intracellular formation of S-nitrosothiols. Intracellular S-nitrosothiols were quantified by the DAN/Hg²⁺ method after incubation of smooth muscle cells for 3 h without (Basal) or with 50 mM of AAPH. In each condition, 50 μM GSNO or GSNO+AAPH were added during the 3rd hour of incubation. Results are presented as means ± sem of three independent experiments and compared using a two way ANOVA ($p_{\text{condition}}$ (Basal vs AAPH), $p_{\text{treatment}}$ (Control, GSNO) and $p_{\text{interaction}}$); * $p < 0.05$ (Bonferroni's multiple comparisons test).

3.3. Identification of S-nitrosated proteins

Purification and identification of proteins undergoing S-nitrosation revealed that 32 proteins were nitrosated under basal conditions, whereas 51 were S-nitrosated under oxidative stress. GSNO-nitrosated proteins were mainly present in macromolecular complexes and organelles under basal conditions, while the membrane and extracellular region pools were prevailing under oxidative stress (Table 1).

Table 1. Distribution of S-nitrosated proteins among distinct cell compartments upon treatment with GSNO (50 μ M) of smooth muscle cells cultured under basal or oxidative stress (50 mM AAPH) conditions.

Cell compartment	Percentage of total identified proteins	
	Basal + GSNO	AAPH + GSNO
Cell junction		2.20
Membrane		43.50
Macromolecular complex	48	2.20
Extracellular matrix		2.20
Cytosol	8	6.50
Organelle	44	2.20
Extracellular region		41.30

The identified proteins belonged to 20 different classes for basal conditions, and to 23 for oxidative stress. Three additional classes were S-nitrosated by GSNO under oxidative stress, designed as cell adhesion, transfer/carrier and transporter proteins (**Fig. 4**). Among these classes, importin subunit beta-1 and procollagen c-endopeptidase enhancer 1, involved in the cellular cycle and proliferation of vascular smooth muscle cells, respectively [33, 34], have been identified.

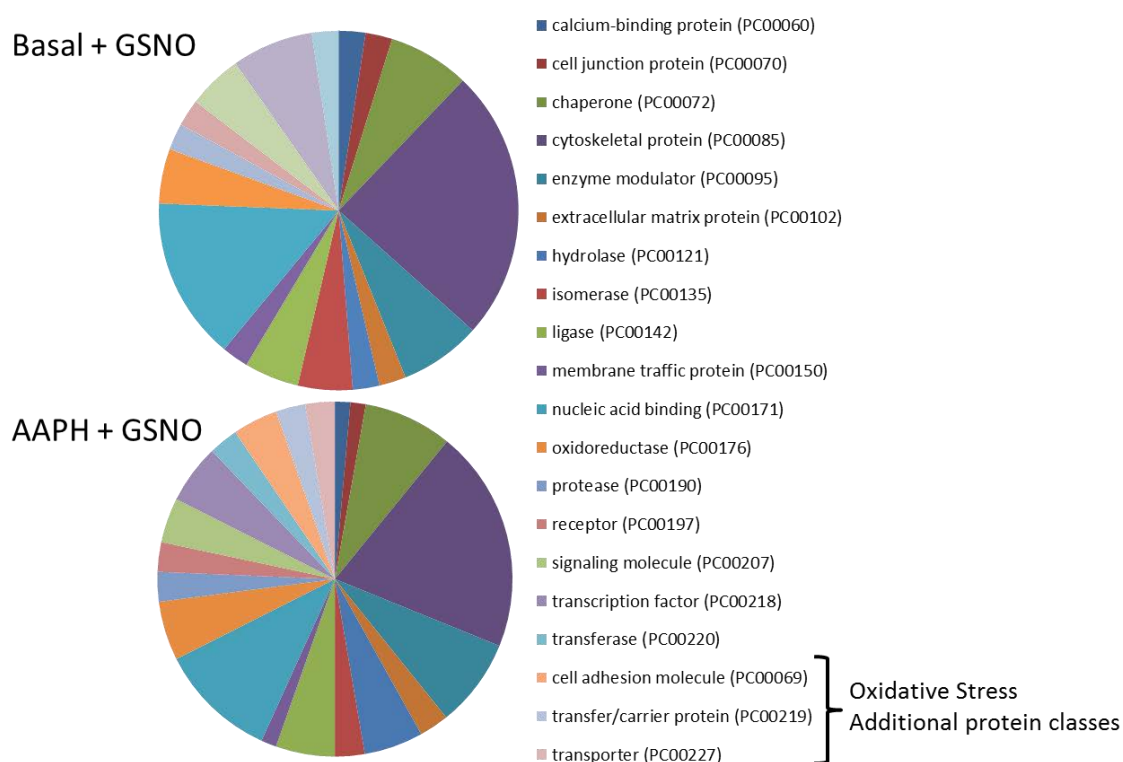


Fig. 4. Identification and classification of smooth muscle cells proteins S-nitrosated in basal or oxidative stress conditions. Proteins were S-nitrosated by 50 μ M GSNO in cells exposed or not to oxidative stress (50 mM AAPH). After purification (biotin switch technique), proteins identified by mass spectrometry were classified using the Panther database.

In both culture conditions, the two major classes of S-nitrosated proteins were nucleic acid binding transcription factors and cytoskeletal proteins. Nucleic acid binding transcription factors accounted for 15% of identified proteins in basal conditions – with 6 different proteins – and 11% of identified proteins under oxidative stress, with 8 different proteins including elongation factor 2 or cellular nucleic acid-binding protein. S-nitrosoglutathione induced S-nitrosation of a high proportion of SMC contractile proteins: 8 different cytoskeletal proteins were identified in basal conditions, and additional 9 (total: 8 + 9 = 17) under oxidative stress. Such proteins are structural constituents of cytoskeleton variably implicated in muscle contraction, as well as in cell morphogenesis and movement (**Table 2**).

Table 2. Molecular function and biological implications of cytoskeletal proteins S-nitrosated by 50 μ M GSNO under basal or oxidative stress conditions.

Basal + GSNO	GSNO + AAPH		
Panther family/subfamily		Molecular function	Biological process
	Elongation Factor 1-Gamma	Structural constituent of cytoskeleton	Cell communication
Actin, Aortic Smooth Muscle			Movement and morphogenesis and organization Mitosis
Alpha-Actinin-1		Structural constituent of cytoskeleton Actin binding	Movement
Calponin-2			Movement and morphogenesis and organization
Filamin-A			Muscle contraction
	Filamin-B		Movement and morphogenesis and organization
Filamin-C			Muscle contraction Movement
	Inverted Formin-2	Structural constituent of cytoskeleton	Morphogenesis and organization
	Lipoma-Preferred Partner		
Myosin Light Polypeptide 6			
	Pdz And Lim Domain Protein 5	Structural constituent of cytoskeleton Actin binding	Movement and morphogenesis Muscle contraction
	Tropomyosin	Motor activity Structural constituent of cytoskeleton	Morphogenesis and organization
	Talin-1	Structural constituent of cytoskeleton	Movement, organization and morphogenesis Mitosis
	Transgelin	Structural constituent of cytoskeleton Actin binding	Movement, organization and morphogenesis Muscle contraction
	Tubulin Beta-2a Chain	Structural constituent of cytoskeleton	Movement, Morphogenesis, Organization
Lim Domain And Actin-Binding Protein 1			Movement, organization and morphogenesis Muscle contraction
Pdz And Lim Domain Protein 5			

4. Discussion

The present study was designed to evaluate the bioactivity of GSNO in vascular SMC exposed to oxidative stress. Experiments were thus planned in order to assess the efficiency of GSNO-dependent NO release, and to verify possible quantitative/qualitative changes induced by oxidative conditions in cellular protein S-nitrosation.

From an experimental point of view, two main approaches can be used to induce oxidative stress, *i.e.* either by inhibiting cellular antioxidant defenses or by increasing the free radical load. The latter can be obtained by exposing cells to extracellular ROS like $O_2^{\bullet-}$ or H_2O_2 . Prolonged enzymatic generation of $O_2^{\bullet-}$ can be sustained *e.g.* by the xanthine/xanthine oxidase system, which however can introduce a major bias in the results, as itself can denitrosate S-nitrosothiols [35]. H_2O_2 prooxidant effects are mediated by the formation of hydroxyl radical, $^{\bullet}OH$, through the transition metal-catalyzed Fenton reaction. However, metal cations are also known to catalyze direct degradation of S-nitrosothiols, preventing its use in our study. On the other hand, the water soluble azo compound 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) can be considered as a 'clean' and reproducible free radical generator, as it spontaneously decomposes at 37°C into one mole of nitrogen and two moles of carbon-centered radicals. AAPH-derived radicals can either combine with each other to produce a stable product, or react with molecular oxygen to generate peroxy radicals ($ROO^{\bullet}$), or with polyunsaturated lipids of cell membranes thus starting lipid peroxidation [36]. A number of studies have employed AAPH to investigate antioxidant defenses in cellular systems [37, 38]. More recently, cytotoxic and genotoxic effects of $ROO^{\bullet}$ originating downstream of AAPH have been studied in a human microvascular endothelial cell line [39]. The effects of oxidative stress on the development of the cardiovascular system were also investigated after administration of AAPH in the air chamber of chicken embryos [40]. As far as SMC are concerned, AAPH was used to study the direct effects of free radicals on cyclic AMP-related cholesterol homeostasis [41]. On this background, the exposure of vascular SMC to AAPH was chosen as a simple and reproducible model of oxidative stress.

AAPH-induced oxidative stress caused no significant change in the extracellular GSNO metabolism, as the consumption of added GSNO and the corresponding NO release were largely the same in both conditions. In principle, the GSNO degradation might ensue from a direct oxidation by AAPH radicals. However, when checked in the absence of cells, the direct AAPH-mediated oxidation of GSNO (50 μM) actually released $4 \pm 0.1 \mu M$ nitrite ions, *i.e.* much less than the concentrations detected in the presence of SMC ($18.3 \pm 0.6 \mu M$), suggesting that most of the observed GSNO metabolism in oxidative stress conditions in SMC occurs through the activity of diverse cellular enzymes.

Oxidative stress induced a remarkable increase in the formation of intracellular S-nitrosothiols under GSNO addition, a finding whose interpretation requires additional considerations. GGT is a critical enzyme in GSNO metabolism [42], essential for the release of NO and its subsequent utilization in S-nitrosothiols formation. Indeed, intracellular concentrations of S-nitrosothiols detected within GGT inhibition were markedly decreased. However, even if the GGT activity was decreased by oxidative stress, it was still implicated in the same extent in GSNO extracellular catabolism and intracellular S-nitrosothiols formation. Actually, GGT inhibition did not entirely suppress GSNO metabolism, as it could not restore the initial extracellular concentration of GSNO: approx. 26 μ M GSNO were detected at the end of the incubation with GGT inhibitor, vs 50 μ M initially added. Taken together, these findings indicate that GGT activity is certainly involved in the extracellular metabolism of GSNO, but other enzymes must also be implicated in the process. As regards PDI, its inhibition produced similar results: enzyme inhibition did not entirely suppress GSNO extracellular metabolism and approx. 28 μ M GSNO (of 50 μ M initially added) were still detectable in the extracellular compartment at the end of incubation. Cellular GGT and PDI activities appear therefore to stay implicated in GSNO catabolism, even if they were inversely (GGT activity decreases, while PDI expression at the membrane level increases) impacted by oxidative stress.

The observed increase in S-nitrosation of SMC proteins under oxidative stress conditions was rather unexpected, as prooxidants should oxidize reduced thiols to disulfides and/or other sulfur species, which are then unavailable for nitrosation. The oxidation of thiols during oxidative stress might prevent S-nitrosation thus interfering with NO-based physiologic signaling [43]. However, it has also been showed that S-nitrosation is a protection of thiols from oxidation by NO [44].

In our experiments, oxidative stress (in the absence of added GSNO) caused both a decrease in intracellular GSH and a decrease in SH groups in cellular proteins, accompanied by an increase in extracellular GSH. The latter was likely the result of the AAPH-induced decrease in GGT activity at the SMC plasma membrane level, resulting in a lower consumption of extracellular GSH. In fact, the AAPH-induced decrease of GGT activity can be explained as the effect of either direct inactivation of the enzyme protein by AAPH radicals or the large increase in the extracellular redox potential. Direct oxidation of plasma membrane proteins by AAPH is a known phenomenon [45]. Loss of cellular GGT activity was also reported following exposure of lung epithelial cells to hyperoxia-induced lipid peroxidation [46], and indeed, AAPH is itself known to induce lipid peroxidation [47].

The decrease of protein SH groups induced by AAPH was reversed by GSNO (**Fig. 1-C**). In principle, the addition of GSNO in an oxidative stress environment would rather be expected to enhance oxidative stress by production of peroxynitrite anions. In our systems, no peroxynitrite ion was detected in smooth muscle cells in basal conditions upon GSNO addition, using DHR probe.

AAPH-induced oxidative stress increased the intracellular peroxynitrite ion concentration up to $6.7 \pm 0.8 \mu\text{M}$; however, the concomitant addition of GSNO did not modify peroxynitrite ion concentration ($5.9 \pm 0.4 \mu\text{M}$). The protection offered by GSNO to intracellular protein -SH groups could be explained by the release of GSH concomitantly to the release of NO. Released GSH can be incorporated in the intracellular GSH pool to support GSH-dependent antioxidant defenses. However, our data did not show any recovery of intracellular GSH levels after GSNO addition. This phenomenon was probably due to direct oxidation of GSH by AAPH challenge. Furthermore, our data showed that GSNO is able to recover the oxidation of protein thiols caused by oxidative stress (**Fig. 1-C**). In this perspective, the ability of GSNO to protect reduced protein thiols from oxidative stress, making them again available to react with NO, may represent the mechanism explaining the increased formation of intracellular S-nitrosothiols (**Fig. 3**) observed under oxidative conditions.

Protein S-nitrosation is considered as an important mechanism for post-translational modulation of protein function, and several studies have described such modulatory effects on a series of cys-containing proteins, being potential targets of RNS-dependent nitrosative modifications [48, 49, 50]. In addition to direct modulation of protein function, protein S-nitrosation can also represent a mean for constitution of 'NO stores' in tissues. Indeed, different studies support the idea that S-nitrosation of tissue thiols is a mechanism for the constitution of local reservoirs from which biologically active NO can be subsequently released [51, 52, 53]. Identification and assessment of such NO stores could provide a valuable biomarker for evaluation of the therapeutic efficiency of NO donors.

The increase in S-nitrosation mostly concerned proteins belonging to plasma membrane and extracellular region, which is not surprising considering that the AAPH-dependent oxidative challenge was originated in the extracellular compartment. In particular, the detailed pattern of S-nitrosated proteins indicates that most of the proteins selectively S-nitrosated under oxidative stress conditions are of primary relevance for the performance of SMC functions, which are often altered in vascular diseases, such as cell communication, cytoskeletal organization, contraction, morphogenesis and movement. Interestingly, the pathway involves actin cytoskeleton dynamics of several key regulatory proteins including Calponin-2 (CNN2), Myosin Light Polypeptide 6 (MYL6), Transgelin (TAGLN) and Lipoma Preferred Partner (LPP). The role of S-nitrosation in regulating these proteins is not completely understood particularly during oxidative stress. Each of these proteins has been shown to play a role in regulating and modulating smooth muscle contraction or nitric oxide signaling. *In vitro* S-nitrosation of skeletal muscle myosin, for example, increases the force of the actomyosin interaction while decreasing its velocity indicating a relaxed state [54]. The calcium binding protein CNN2 has been shown to participate in regulating smooth muscle contraction by binding to actin,

calmodulin, troponin C and tropomyosin. The interaction of calponin with actin inhibits the actomyosin Mg-ATPase activity [55, 56]. This tonic inhibition of the ATPase activity of myosin in smooth muscle is blocked by Ca^{2+} -calmodulin, which inhibits CNN2 actin binding [57]. MYL6 regulates light chain of myosin and it does not bind calcium, but it is always involved in muscle contraction and skeletal muscle tissue development. Transgelin (also designated SM22 α and p27) is a smooth muscle protein that physically associates with cytoskeletal actin filament bundles in contractile smooth muscle cells. Studies in transgelin knockout mice have demonstrated a pivotal role for transgelin in the regulation of Ca^{2+} independent contractility [58] and it is proposed to be necessary for actin polymerization and bundling [59]. Moreover, LPP, a nucleocytoplasmic shuttling protein, is located in focal adhesions and associates with the actin cytoskeleton [60]. LPP can function as an adaptor protein that constitutes a platform that orchestrates protein-protein interactions and contributes to the migratory phenotype of SMC [61]. As ROS have also been shown to enhance cell migration [62, 63] and GSNO has been shown to decrease smooth muscle cells migration capacity [64], we can speculate that LPP S-nitrosation could protect against oxidative stress induced cell migration.

Therefore, considered together, all these proteins, almost quite implicated in Ca^{2+} -dependent contractility and in NO signalling, constitute a potential interactome and discovering their behavior as S-nitrosated proteins may further help our understanding of several processes, such as contraction-relaxation signaling of SMC or their phenotype switching, in the vascular system.

In conclusion, our study documented that oxidative stress can significantly modify smooth muscle cells metabolism of GSNO, an endogenous NO-donor presently under active investigation as a potential therapeutic agent. In particular, oxidative stress was shown to increase the extent, and profoundly modify the pattern of GSNO-dependent protein S-nitrosation, with the additional involvement in the process of several proteins critical for SMC homeostasis and function. These data can represent a valuable basis for the identification of biomarkers of GSNO bioactivity in the vascular system, as well as for the appraisal of possible beneficial effects of this NO donor in the treatment of cardiovascular diseases.

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CHAPTER IV

Discussion Générale, Conclusions et Perspectives

4.1 Discussion Générale

L'athérosclérose est une maladie chronique à évolution lente caractérisée par la formation de plaques d'athérome. Elle est considérée comme une maladie complexe et multifactorielle et des biomarqueurs circulants de l'inflammation (*e.g* la protéine C-réactive) sont considérés comme des indicateurs possibles de l'état de la maladie athéroscléreuse [1]. Plusieurs facteurs, à la fois génétiques (tels que l'hypercholestérolémie familiale, l'homocystinurie, les maladies de la coagulation, l'hypertension, les maladies métaboliques) et environnementaux (alimentation, le tabagisme, le stress, mode de vie) ont un rôle étiologique important. Dans l'étiopathogénie de l'athérosclérose, un rôle important est joué par l'interaction entre les lipoprotéines modifiées et oxydées, les cellules inflammatoires telles que les monocytes, les macrophages et les lymphocytes T, et les constituants normaux de la paroi artérielle. Cette pathologie associe stress oxydant (production excessives d'espèces radicalaires de l'oxygène et/ou de l'azote, ERN) et inflammation, tous deux responsables de l'initiation et du développement de la strie lipidique, jusqu'à la rupture de la plaque [20]. La synergie entre inflammation et stress oxydant joue un rôle important dans (i) la dysfonction endothéliale induisant une diminution de la biodisponibilité de NO, (ii) l'oxydation des LDL, (iii) le remodelage de la lésion (régulation de protéases et d'antiprotéases) et (iv) la prolifération des CML.

En outre, la diminution de la biodisponibilité de NO au cours du développement de l'athérosclérose peut être due à plusieurs facteurs en lien avec la dysfonction endothéliale et le stress oxydant. Il est documenté que le déficit de la biodisponibilité de NO a été impliqué dans les maladies cardiovasculaires, précisément dans l'hypertension et l'athérosclérose [3, 58], et que sa surproduction est liée à d'autres maladies comme le cancer et l'inflammation [59]. Donc le maintien des concentrations stables de NO est vital pour la santé.

Afin de contrer la carence en NO, des donneurs de NO comme les *S*-nitrosothiols peuvent être envisagés. Les *S*-nitrosothiols sont également connus pour protéger contre le stress oxydant grâce essentiellement à la *S*-nitrosation [8], modification post-traductionnelle des protéines consistant en la formation d'une liaison covalente entre la fonction thiol du résidu de la cystéine et NO.

Parmi les *S*-nitrosothiols, le GSNO, forme physiologique de stockage de NO dans les tissus [206], est spécifiquement métabolisé par la GGT [156], semble être un bon candidat.

La corrélation entre l'augmentation des concentrations sériques de GGT et les facteurs de risque cardiovasculaire a récemment été démontrée [251]. Seule la b-GGT (complexe macromoléculaire) s'accumule au sein des plaques athérome humaines et concorde avec l'apparition d'autres marqueurs histologiques de vulnérabilité de la plaque [12], suggérant qu'elle pourrait représenter un biomarqueur pour l'athérosclérose.

Sur la base de ce qui précède, l'attention de ce travail de thèse a été concentrée sur l'analyse du lien entre l'inflammation et le stress oxydant dans l'athérosclérose, étudiant le rôle des *S*-nitrosothiols dans

les réponses vasculaires. En particulier, en mettant l'accent sur les macrophages et le CML, qui sont les principaux types cellulaires retrouvés dans les lésions athérosclérotiques et semblent être colocalisés avec la GGT, le plan expérimental de thèse a été divisé en deux parties :

- Le premier objectif visait à identifier l'origine de la GGT accumulée dans la plaque d'athérome, et à élucider entre les stimuli inflammatoires et oxydants, qui est responsable de l'accumulation de GGT dans les plaques d'athérosclérose, afin de comprendre si les macrophages peuvent fournir une source potentielle de b-GGT dans la plaque athéroscléreuse (premier article) [252].
- Le deuxième objectif est étroitement lié au précédent et a été consacré à la restauration de la biodisponibilité de NO à partir de GSNO dans les cellules musculaires lisses sous stress oxydant, qu'a l'évaluation de l'implication des deux enzymes spécifiques, la GGT, déjà mentionné, et la PDI, impliquées dans le métabolisme du GSNO et dans la S-nitrosation des protéines, cette dernière utilisée comme biomarqueur du pool de NO [133].

Des études récentes ont montré que les cellules inflammatoires de patients présentant une pathologie cardiovasculaire contiennent des granules de GGT qui ont libérées lors d'une stimulation [158]. En effet, le travail effectué dans notre laboratoire a montré que la GGT libérée est associée à d'autres protéines pour former de grands agrégats, avec des caractéristiques semblables à celles des exosomes ou microparticules [158]. Il existe donc un lien entre les concentrations sériques élevées de GGT, probablement d'origine inflammatoire et l'identification de la GGT intra-plaque. Le relargage de la GGT peut donc interférer avec le métabolisme physiologique de GSNO dans le sang et / ou dans les tissus.

Cependant, il est également bien documenté que les cellules inflammatoires stimulent la production de molécules d'adhésion endothéliales et de facteurs chimiotactiques qui augmentent le recrutement des monocytes et des macrophages ainsi que leur accumulation dans les lésions athérosclérotiques. Les données actuelles identifient plusieurs populations de macrophages dans la plaque d'athérome présentant différents phénotypes en lien avec l'inflammation (pro-inflammatoire : M1, anti-inflammatoire : M2) [80] ou avec les modifications redox de l'environnement (Mox) [65].

Par conséquent, sur la base du premier objectif du travail, un grand intérêt a été porté sur l'hétérogénéité des macrophages au sein des lésions athérosclérotiques. Une première approche expérimentale a été d'isoler les monocytes humains à partir du sang périphérique de donneurs sains. La différenciation des monocytes en macrophages M1 et M2 a été induite par les facteurs de croissance GM-CSF et M-CSF, respectivement. Suite à la différenciation, nous avons étudié l'expression et la libération de la GGT au sein de ces cellules et l'analyse de la GGT dans la plaque a été réalisée dans des échantillons de tissu obtenus de patients ayant subi une endartériectomie carotidienne. Les résultats ont révélé que les macrophages de type M1 expriment des niveaux plus élevés de GGT par rapport aux macrophages de type M2 et aux monocytes. De plus, les macrophages de type M1, mais pas les M2, sont capables de libérer la

fraction b-GGT après activation par des stimuli pro-inflammatoires (combinaison de cytokines pro-inflammatoires, $\text{TNF-}\alpha/\text{IL-1}\beta$). L'analyse par western blot de la b-GGT extraite des plaques a confirmé la présence de GGT en parallèle de la présence des macrophages.

Ces résultats indiquent que les macrophages caractérisés par un phénotype pro-inflammatoire (M1) peuvent contribuer à l'accumulation intra-plaque de la b-GGT, qui à son tour peut jouer un rôle dans la progression de l'athérosclérose en modulant les processus inflammatoires et favorisant l'instabilité de la plaque. Ces données sont en accord avec celles observées pour les neutrophiles, qui contribuent tous à la libération de b-GGT dans les exsudats inflammatoires de la mucoviscidose [158]. Cela ajoute un autre support évident pour la connexion entre GGT et inflammation.

Afin de compléter la caractérisation phénotypique des macrophages, nous avons commencé à évaluer le statut redox des macrophages différenciés, M1 vs M2, et par rapport aux monocytes. En effet, on en sait peu sur l'état d'oxydo-réduction de M1 et M2. Mais comme confirmé par la littérature et les données préliminaires obtenues (chapitre II, section 2.4), ERO, ERN et GSH sont connectés et influent sur le développement des macrophages. En effet, on sait que l'état d'oxydo-réduction dans les cellules joue un rôle important dans le développement cellulaire, tels que la prolifération, la différenciation et l'apoptose [218, 346, 347]. Cependant, les résultats sont préliminaires et doivent être complétés par des expériences complémentaires, en particulier en condition de stress oxydant.

Effectivement, comme déjà mentionné, une augmentation du stress oxydant et de la dysfonction endothéliale au cours de l'athérosclérose, conduisent à la prolifération et la migration des CML. Le remodelage vasculaire consécutif à l'hyperprolifération de ces cellules, est la conséquence d'une dédifférenciation cellulaire d'un phénotype contractile à sécrétoire, augmentant leur capacité proliférative et migratoire [21]. De plus, le stress oxydant provoque une diminution de la biodisponibilité de NO et comme décrit précédemment (section 1.4), plusieurs molécules donneuses de NO ont été développées pour contrer cette diminution de biodisponibilité. Cependant, même si certains donneurs de NO comme GSNO n'induisent pas le stress oxydant, ils sont encore considérés comme de potentiels activateurs de stress oxydant conduisant à la formation d'ions peroxynitrite susceptibles de produire des effets délétères sur les protéines en particulier dans des conditions de stress oxydant [169, 170, 171]. Bien que le stress oxydant soit concomitant avec le développement de maladies cardiovasculaires, on en sait peu sur la capacité de GSNO à restaurer la biodisponibilité de NO dans les tissus vasculaires sous stress oxydant. Tout comme on en sait peu sur les interférences possibles entre les radicaux libres et le métabolisme de GSNO. Une appréciation plus profonde de ces mécanismes pourrait donc ouvrir une voie à l'utilisation de GSNO dans les maladies cardiovasculaires associées au stress oxydant. En outre, la littérature a également montré que la S-nitrosation de protéines impliquées dans le phénotype des cellules musculaires lisses est très importante [506], surtout si on prend en considération que les CML sont impliquées dans la contraction et la régulation du tonus des vaisseaux sanguins, répartissant ainsi le flux sanguin et la régulation de la

pression artérielle. En conséquence, l'identification des protéines nitrosées en condition de stress oxydant pourrait révéler de nouvelles cibles thérapeutiques des donneurs de NO et aider à mieux comprendre les processus pathologiques impliqués dans le développement des maladies cardiovasculaires associées au stress oxydant.

Ainsi, la deuxième approche expérimentale présentée dans ce manuscrit était de développer un modèle de stress oxydant, sur des cellules musculaires lisses. Il est connu que pour induire un stress oxydant, deux approches alternatives peuvent être suivies pour perturber l'équilibre pro-oxydant-antioxydant: soit augmenter la charge radicalaire ou inhiber les défenses antioxydantes. La charge radicalaire peut être augmentée en exposant les cellules à une irradiation gamma, une élévation de la tension d'oxygène (hyperoxie), extracellulaire $O_2^{\bullet}$ et / ou H_2O_2 , ou en utilisant des composés azoïques générant des radicaux libres, tels que AAPH ou en utilisant des médicaments générateurs de radicaux libres (chapitre III, section 3.3). Dans ce cas, afin de développer un modèle de stress oxydant sur CML (A-10 lignée cellulaire) dans des conditions reproductibles et contrôlées, nous avons utilisé l'AAPH, une molécule soluble dans l'eau, utilisée dans l'étude de la peroxydation lipidique, dans la caractérisation des antioxydants *in vitro* et capable de provoquer différents types de changements pathologiques à travers des dommages oxydatifs. En outre, l'utilisation de ce composé, sur différents types de cultures cellulaires, est soutenue par plusieurs sources de données [495-499]. Comme décrit dans le deuxième article, les cellules ont été exposées à l'AAPH et les effets du stress oxydant sur la libération de NO ainsi que sur l'expression / l'activité de la PDI et de la GGT respectivement, ont été évalués. En outre, l'état redox des thiols cellulaires a été quantifié au travers des concentrations intra et extracellulaires de glutathion et des groupements thiols réduits (-SH) des protéines. Enfin, la capacité de GSNO à induire la S-nitrosation des protéines a été globalement quantifiée puis les protéines S-nitrosées ont été identifiées par spectrométrie de masse.

Comme attendu, le stress oxydant diminue la concentration intracellulaire de glutathion et des groupements thiols réduits au sein des protéines. Les résultats montrent que le stress oxydant a provoqué une modulation différente de la GGT (diminution de l'activité) et de la PDI (expression accrue à la membrane plasmique) sans aucun effet sur le catabolisme extracellulaire du GSNO. Cependant, l'addition de GSNO sur les CML en condition de stress oxydant restaure les groupements thiols réduits des protéines et produit une S-nitrosation des protéines plus grandes qu'en condition basale. En outre, l'analyse par spectrométrie de masse des protéines S-nitrosées a révélé un nombre plus élevé de protéines S-nitrosées par GSNO en condition de stress oxydant comprenant un nombre plus élevé de protéines du cytosquelette impliquées dans la contraction vasculaire, la morphogenèse et le mouvement cellulaire. Encore plus intéressant, des classes supplémentaires de protéines impliquées dans l'adhésion cellulaire, le transfert / support et des protéines de transport sont S-nitrosées uniquement en condition de stress oxydant.

Dans l'ensemble, même si le stress oxydant a modulé différemment la GGT et la PDI, des niveaux plus élevés de protéines S-nitrosées par GSNO ont été identifiés. Ces études soulignent le rôle important de

la S-nitrosation par GSNO, suggérant de nouveaux mécanismes pour la S-nitrosation des protéines dans les maladies cardiovasculaires. Ils peuvent inciter à l'identification de biomarqueurs appropriés pour l'évaluation de la bioactivité de GSNO dans le traitement des maladies cardiovasculaires associées au stress oxydant. Ces études peuvent être le point de départ pour soutenir l'idée d'utiliser GSNO pour le traitement / prévention des maladies cardiovasculaires, notamment l'athérosclérose, en réduisant l'accumulation des macrophages et en restaurant les niveaux de NO. Ce vaste travail révèle plusieurs questions qui demeurent en suspens et nécessiteront d'être posées à l'avenir.

4.2 Conclusions et perspectives

Ce travail visait à étudier l'impact de l'inflammation et du stress oxydant sur les cellules majoritaires au sein de la plaque d'athérome : les macrophages et les cellules musculaires lisses. L'inflammation a dans un premier temps été considérée sur les monocytes/macrophages avec la mise en place d'un protocole de différenciation des monocytes en macrophages de type M1 (proinflammatoire) et M2 (antiinflammatoire). L'impact d'un stimulus inflammatoire sur l'expression/activité de la GGT a été évalué. Du point de vue du stress oxydant, évalué uniquement sur les cellules musculaires lisses le but était de montrer que le S-nitrosoglutathion pouvait être utilisé comme donneur de NO dans un environnement défavorable.

Nous avons démontré que :

- L'inflammation, induite par IL1- β et TNF- α , provoquait la dégranulation des macrophages de type M1 (pro-inflammatoires) et ainsi la libération de la GGT intracellulaire sous la forme d'un macrocomplexe nommé b-GGT. Cette première publication a permis d'émettre une hypothèse sur la provenance de la GGT identifiée au sein de la plaque d'athérome.
- Le stress oxydant amplifiait les phénomènes de nitrosation des protéines induites par le S-nitrosoglutathion au sein des cellules musculaires lisses. L'identification de ces protéines a révélé que l'appareil contractile était la cible privilégiée du S-nitrosoglutathion et que le stress oxydant impliquait aussi la nitrosation de protéines de transport et de trafic intracellulaire. Cette deuxième publication a permis d'envisager GSNO comme donneur de NO potentiel restaurant le pool de NO vasculaire faisant défaut dans le cadre de l'athérosclérose.

Au regard de ces deux études, différents paramètres méritent approfondissement et d'autres n'ayant pu être étudiés mériteraient notre attention. En effet, l'athérosclérose est une pathologie mêlant inflammation et stress oxydant, ainsi, les macrophages et les cellules musculaires lisses subissent ces deux conditions. Le stress oxydant induit la différenciation des monocytes en Mox présentant des défenses antioxydantes suractivées. Cependant, à l'heure actuelle, nous ne disposons d'aucune donnée sur l'impact du stress oxydant sur les macrophages de type M1 et M2. Notre étude préliminaire ayant montré que les macrophages M1 et M2 présentent un profil redox différent (concentration intracellulaire en GSH, activité des enzymes antioxydantes), il serait intéressant d'étudier leur réponse face à un environnement oxydant induit par exemple avec de l'AAPH. Cette étude permettrait aussi de faire le lien avec celle menée sur les cellules musculaires lisses en condition de stress oxydant. L'impact de S-nitrosoglutathion dans cet environnement oxydant, au

regard de l'activité de la GGT, en combinaison avec l'étude des protéines nitrosées au sein des macrophages pourrait être un point de confirmation quant à la capacité du *S*-nitrosoglutathion à restaurer la biodisponibilité de NO au sein du compartiment vasculaire. L'étude de l'évolution du phénotype des macrophages placés en condition de stress oxydant en présence de *S*-nitrosoglutathion ainsi que l'identification des protéines *S*-nitrosées pourraient mettre en évidence de nouvelles protéines cibles de NO et ouvrir de nouvelles portes pour l'utilisation des donneurs de NO en thérapeutique (Fig. 15).

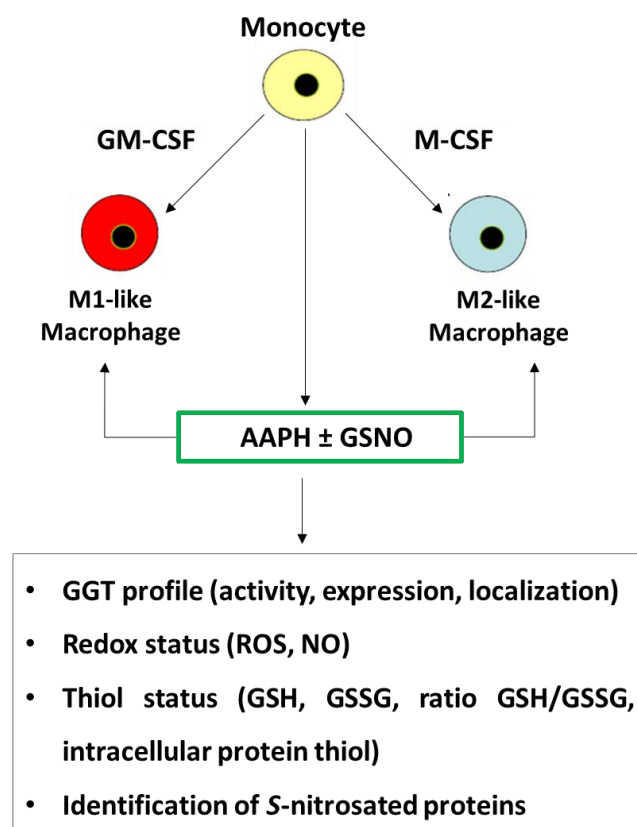


Figure 15. Présentation des perspectives expérimentales concernant le développement d'un modèle de stress oxydant sur les macrophages.

L'autre composante de l'athérosclérose, l'inflammation a un impact sur la prolifération et la différenciation de cellules musculaires lisses. Pour le moment, aucune donnée n'est disponible quant à l'impact de l'inflammation sur l'activité/expression/localisation de la GGT au sein des cellules musculaires lisses. Lors de nos études préliminaires, nous avons montré (données non présentées dans ce manuscrit), que l'activité de la GGT était augmentée, au sein des cellules musculaires lisses, par l'inflammation. Cependant, aucune libération de la GGT dans le milieu extracellulaire n'a pu être mise en évidence. Ces premiers résultats, combinés à ceux menés sur macrophages attestant la relargage de la GGT sous stimulus inflammatoire, augurent que l'utilisation du *S*-nitrosoglutathion en

tant que donneur de NO dans le cadre du traitement de l'athérosclérose permettrait la libération préférentielle de NO au niveau de la plaque d'athérome. L'inflammation induisant la prolifération et la dédifférenciation des cellules musculaires lisses, il serait opportun d'évaluer l'impact du S-nitrosoglutathion sur ces deux phénomènes en condition inflammatoire (Fig. 16).

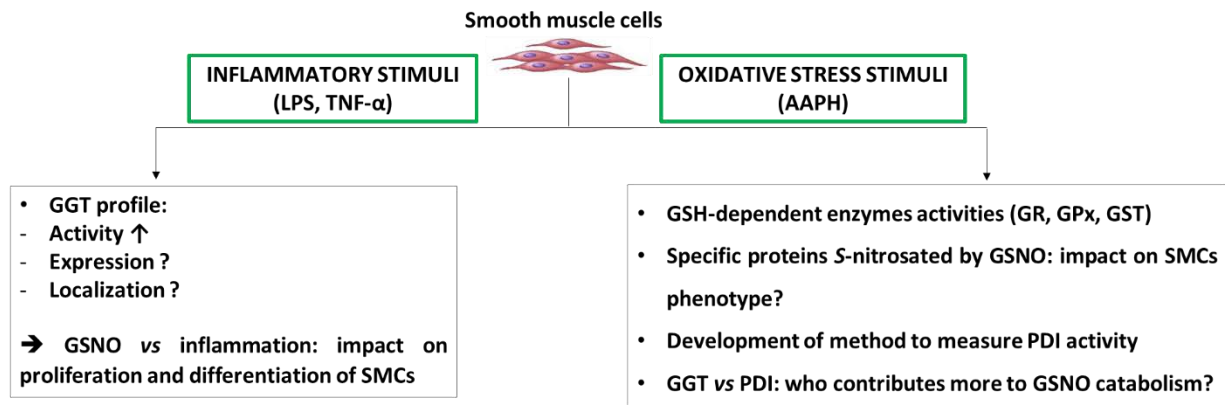


Figure 16. Présentation des perspectives expérimentales sur les cellules musculaires lisses.

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Inflammation et stress oxydant dans l'athérosclérose: rôle dans les réponses vasculaires des S-nitrosothiols.

L'athérosclérose est une maladie chronique à évolution lente caractérisée par la formation de plaques d'athérome, consistant en l'accumulation de lipoprotéines de basse densité (LDL), de leucocytes, de cellules spumeuses, la migration des cellules musculaires lisses (CML) et l'altération des cellules endothéliales (ECs). Ces phénomènes conduisent à la formation d'un noyau nécrotique incluant des régions calcifiées. La genèse de l'athérosclérose et de l'instabilité de la plaque d'athérome sont le résultat d'une synergie entre inflammation et stress oxydant. Les données actuelles identifient plusieurs populations de macrophages dans la plaque d'athérome présentant différents phénotypes en lien avec l'inflammation (pro-inflammatoire: M1, anti-inflammatoire: M2) ou avec des modifications redox de l'environnement (Mox). Stress oxydant et inflammation sont liés et jouent un rôle important dans (i) la dysfonction endothéliale induisant une diminution de la biodisponibilité du monoxyde d'azote (NO), (ii) l'oxydation des LDL, (iii) le remodelage de la lésion (régulation de protéases et d'antiprotéases) et (iv) la prolifération des CML. Les CML sont le deuxième type cellulaire le plus abondant dans la lésion athérosclérotique après les macrophages, leur hyperprolifération est la conséquence d'une dédifférenciation cellulaire d'un phénotype contractile à sécrétoire, augmentant leur capacité proliférative et migratoire. Les donneurs de NO, comme les S-nitrosothiols, connus également pour protéger contre le stress oxydant grâce essentiellement à la S-nitrosation, peuvent contrer la carence en NO. Parmi eux, le S-nitrosoglutathion (GSNO), forme physiologique de stockage de NO dans les tissus, spécifiquement métabolisé par la gamma-glutamyl transférase (GGT) peut être envisagé. La corrélation entre l'augmentation des concentrations sériques de GGT et les facteurs de risque cardiovasculaire a récemment été démontrée. En particulier, seule la b-GGT s'accumule dans les plaques d'athérome, et concorde avec l'apparition d'autres marqueurs histologiques de vulnérabilité de la plaque. Étant donné que, les macrophages et les CML sont les principaux types cellulaires retrouvés dans les lésions athérosclérotiques et semblaient être colocalisés avec la GGT, l'attention de ce travail de thèse a été centrée sur la compréhension de la provenance de la GGT et son rôle dans le métabolisme du GSNO au sein de la plaque d'athérome. Une première partie de ce manuscrit vise à identifier l'origine de la GGT accumulée dans la plaque d'athérome, et à élucider entre le stimulus inflammatoire et oxydant, qui est responsable de l'accumulation de GGT dans la plaque d'athérome. La deuxième partie a été consacrée à la restauration de la biodisponibilité de NO dans les CML en condition de stress oxydant avec un intérêt particulier porté sur l'identification des protéines S-nitrosées.

Mots clés: Inflammation, stress oxydant, athérosclérose, GGT, S-nitrosoglutathion, S-nitrosation

Inflammation and oxidative stress in atherosclerosis: role of S-nitrosothiols in the vascular responses.

Atherosclerosis is a slowly progressing chronic disease characterized by the formation of atherosclerotic plaques consisting of accumulated low density lipoprotein (LDL), leukocytes, foam cells, migrated smooth muscle cells (SMCs) and altered endothelial cells (ECs), leading to the formation of necrotic cores with calcified regions. Atherosclerosis genesis and subsequent instability of atherosclerotic plaques result from a synergy between inflammation and oxidative stress. Current data identified several macrophage populations within the atherosclerotic plaque showing different inflammatory phenotypes (pro-inflammatory: M1, anti-inflammatory: M2) or functions in response to redox changes in the environment (Mox). The oxidative stress linked to inflammation plays an important role in (i) endothelial dysfunction, with reduced nitric oxide (NO) bioavailability, (ii) LDL oxidation, (iii) lesion remodeling (regulation of proteases and antiproteases) and (iv) SMCs proliferation. Indeed, SMCs are the second more abundant cell type, after macrophages, in the atherosclerotic lesion because their dedifferentiation from contractile to secreting phenotype increased their proliferation and migration capacity. NO donors, like S-nitrosothiols, also known to protect from oxidative stress by S-nitrosation, could counteract this NO deficiency. Among them, the S-nitrosoglutathione (GSNO), a physiological storage form of NO in tissues, specifically catabolized by the gamma-glutamyltransferase (GGT) is considered. Recently, it has been shown that the increased serum level of GGT is an independent risk factor for cardiovascular mortality related to atherosclerotic disease. In particular, only the big fraction (b-GGT) has been detected inside human atherosclerotic plaques associated to CD68⁺ macrophage-derived foam cells. As macrophages and SMCs are the main cell types found in atherosclerotic lesion and seemed to be colocalized with GGT, the attention of this thesis work was focused on the understanding of GGT provenance and its role in the GSNO metabolism within the atherosclerotic plaque. A first part of the thesis was to identify the origin of GGT accumulating inside atherosclerotic plaques, and to decipher between inflammation and oxidative stress stimuli, which one is responsible of GGT accumulation in atherosclerotic plaques. The second part was dedicated to the restoration of NO bioavailability within SMCs under oxidative stress with a focus on the identification of S-nitrosated proteins.

Keywords: Inflammation, oxidative stress, atherosclerosis, GGT, S-nitrosoglutathione, S-nitrosation