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**Oxidative/nitrative Stress in the Pathogenesis of Systemic Sclerosis: are Antioxidants Beneficial?**

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**Abstract**

Systemic sclerosis (SSc) is a multisystem autoimmune disease: characterised from the clinical side by progressive vasculopathy and fibrosis of the skin and different organs and from the biochemical side by fibroblast deregulation with excessive production of collagen and increased expression of nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4). The latter contributes to an overproduction of reactive oxygen species that via an autocrine loop maintains NOX4 in a state of activation. Reactive oxygen and nitrogen species are implicated in the origin and perpetuation of several clinical manifestations of SSc having vascular damage in common; attempts to dampen oxidative and nitrative stress via different agents with antioxidant properties have not translated into sustained clinical benefit. Objective of this narrative review is to describe the origin and clinical implications of oxidative and nitrative stress in SSc, with particular focus on the central role of NOX4 and its interactions, to re-evaluate the antioxidant approaches so far employed to limit disease progression, to appraise the complexity of antioxidant treatment and to touch on novel pathways elements of which may represent specific treatment targets in the not so distant future.

**Key words: systemic sclerosis, NOX, Nfr2, oxidative stress, antioxidant**

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**List of abbreviations**

LA: alpha-lipoic acid
ABI: ankle brachial index
Ang II: angiotensin II

ARE: antioxidant responsive elements
ADMA: asymmetric dimethyl arginine:
CFR: coronary flow reserve
CAT: catalase
DAMP: damage associated molecular pattern:
D*cSSc:* diffuse cutaneous systemic sclerosis:
DHLA: Dihydrolipoic acid
PEN: penicillamine
Endothelial cells: EC
eNOS:endothelial nitric oxide synthase
ET-1: endothelin-1
FAD: flavin adenine dinucleotide
FPP: farnesyl-pyrophosphate
FMD: flow mediated dilation
GGPP: geranyl-geranyl-pyrophosphate
GSH: Glutathione:
HR: Hazard ratio
HMG-CoA: reductase Hydroxy-3-methylglutaryl-coenzyme A reductase
HNN: hydroxynonenal
HO-1: haemo- oxygenase-1
iNOS*:* inducible nitric oxide synthase
IL: interleukin
LcSSc: Limited cutaneous systemic sclerosis
L-NAME: N-Nitroarginine methyl ester
MDA: Malondialdehyde
NAC: N-acetylcysteine
NOX: nicotinamide adenine dinucleotide phosphate oxidase
NMD: nitroglycerine mediated dilatation
NPC: nitrosoperoxocarbonate
NT: nitrotyrosine
Nrf2: nuclear erythroid-derived factor-2
NFkB:nuclear factor kappa B
OR: odds ratio
PDGF: platelet-derived growth factor
PDGFR:platelet-derived growth factor receptor:
PI3K: phosphoinositide-3 kinase
PKC: protein kinase C
RNS: reactive nitrogen species

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ROS: reactive oxygen species
RP: Raynaud's phenomenon
SOD: superoxide dismutase
SSc: systemic sclerosis
BH4: tetrahydrobiopterin:
TLR: Toll-like receptor
TGF-β:transforming growth factor beta
VEGF:vascular endothelial growth factor

**Introduction**

Systemic sclerosis (SSc) is a chronic autoimmune disorder characterized by a progressive non inflammatory vasculopathy with intimal hyperplasia affecting small and large arteries [1] and by excessive production of collagen, fibronectin and other matrix proteins which accumulate in the skin and internal organs, resulting in fibrosis[2]. Free radical production is enhanced in SSc in relation to some clinical manifestations as indicated in the following paragraphs. [3] While the review focuses on the interplay between reactive oxygen species (ROS) generated by NOX and TGF-beta, inflammasome and Nrf2 in the pathogenesis of fibrosis, we must touch on how the immune system is affected by ROS in SSc.

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**Notes on Oxidative/Nitrative Stress and Immunity in Systemic Sclerosis**

Oxidative stress is a measure of the prevailing levels of reactive oxygen species (ROS) in biological systems determined by the relative rates of their formation and their removal by plasma and cellular repair mechanisms [3]. In given micro-environments excess ROS produced by neutrophils, endothelial cells and monocytes contributes to free radical

attack on membrane and/or lipoprotein lipids in a process called lipid peroxidation[4].

Nitric oxide (NO•) generated from arginine by the action of nitric oxide synthases (NOS) regulates several biological processes including vasomotor tone. Upon reaction with superoxide anion ($O^2$•-), NO• forms peroxynitrite (ONOO-), which interacts with $CO^2$ to form nitrosoperoxocarbonate ($ONOOCO^2$-). Aromatic amino acyl (tryptophanyl, tyrosyl, phenylalanyl), cysteinyl and methionyl residues of proteins are sensitive to modification by different forms of reactive nitrogen species (RNS), depending upon the availability of $CO^2$ and pH [3]. ONOO- is not only responsible for the nitration of tyrosyl residues in proteins [5] but also for the formation of nitro-fatty acids that may modulate metabolic- and anti-inflammatory pathways [6,7]

Oxidative stress may lead to cell/tissue damage by several mechanisms: by the oxidative modification of cell macromolecules (DNA, proteins, lipids) that may occur by direct metal-catalyzed oxidation of amino acyl side chains and by the formation of covalent adducts of the products of carbohydrate oxidation, advanced glycoxidation end products (AGE), with DNA and proteins of crucial importance for cell viability. Auto-fluorescence of SSc skin reveals the presence of AGE in relation to carotid radial pulse wave velocity and capillary flow percentage change during occlusion[8].

Moreover, the peroxidation of polyunsaturated fatty acids in phosphatidylcholine and other phospholipids in cell membranes and in low density lipoprotein yield 1) γ-hydroxy-alkenals generically called ALE that include acrolein, malonyldialdehyde and 2) an α,β-unsaturated hydroxyalkenal called and 4-hydroxy-2-nonenal (4-HNE) that may form adducts with cysteine,lysine and histidine residues inducing post-translational modifications in those proteins bearing such residues (5) and 2)a family of cyclic molecules called isoprostanes, generated independently of cyclooxygenase, that are sensitive and specific markers of oxidative stress and that have powerful vasoactive properties [3].

The engagement of oxidation-specific epitopes (OSE), including AGE/ALEs and relevant adducts with DNA and proteins to scavenger receptors including RAGE induce the activation of NF-#B [10] in endothelial cells, macrophages and dendritic cells thereby promoting inflammatory and immune responses. The relevance of RAGE

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stimulation in SSc is highlighted by the report concerning the secretion of alarmins (S100A8 and S100A9) from monocytes and neutrophils that bind to TLR4 and RAGE, thereby recruiting and activating leukocytes into inflamed tissues[9].

The oxidative modification of self-epitopes with the consequent sensitization to mixed self/non-self neoepitopes and the breaking of immunological tolerance to their native counterparts, leads to immuno-mediated cell and tissue damage and the appearance of autoantibodies. Various pro-oxidative agents (bleomicin, superoxide anions, hydroxyl radicals, hypochlorous acid and peroxynitrite) injected in immune deficient mice induced the development of serum anti-centromeric protein-B and anti-DNA topoisomerase 1 autoantibodies. On the other hand, sera of oxidatively treated mice (hypochlorite or hydroxyl radical) and of patients with diffuse SSc contained high levels of ALE that triggered endothelial production of $H^{2}O^{2}$ and fibroblast hyperproliferation[9].

**Nicotinamide Adenine Dinucleotide Phosphate Oxidase in Systemic Sclerosis**

The main source of ROS in the organism is the NOX family, composed of five different NOX isoforms. NOX enzymes are characterized by six transmembrane domains and two cytosolic domains respectively for NADPH and for flavin adenine dinucleotide[10]. NOX reduces molecular oxygen, forming superoxide anion $O^{2}$•- and hydrogen peroxide (H202), using NADPH as an electron donor that is transferred first to the FAD and then to a heme group. Of the different NOX isoforms, NOX4 is particularly relevant to SSc: 1) though present on the surface of fibroblasts, vascular smooth muscles cells (VSMC) and endothelial cells (EC)[11]NOX4 is constitutively expressed on dermal fibroblast [12] as it lacks cytosolic regulatory subunits, and therefore depends almost exclusively on the amount of p22phox expressed on the cellular membrane[13]; 2) NOX4 does not generate $O^{2}$•- but almost exclusively H2O2; the latter does not react with NO• to yield $ONOO^{-}$[14]. Other cell types produce enhanced of ROS in SSc such as circulating neutrophils, monocytes [15] and T lymphocytes [16].

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## Nicotinamide Adenine Dinucleotide Phosphate Oxidase and Toll Like Receptors

Toll-like receptors (TLR) are a family of pattern recognition receptors (PRR) that sense invading pathogens or endogenous damage signals and once stimulated initiate the innate and adaptive immune response. Damage associated molecular patterns (DAMP) appearing during the course of oxidation and nitration including post-translational modification of certain connective tissue proteins and heat shock proteins also stimulate TLR[17,18]. The intracellular signalling triggered by TLR activation is mediated by the cytoplasmic adaptor molecule, MyD88 and by the serine/threonine kinases of the IL-1R–associated kinase family, resulting in translocation of NF-κB to the nucleus and synthesis of type I interferons and inflammatory cytokines[19]. TLR4 is a better sensor for oxidative stress than other TLRs[20]. Interestingly, TLR4 and its co-receptors, MD2 and CD14, are over-expressed in affected skin from patients with diffuse cutaneous SSc; the same study demonstrated in a mouse model that chronic TLR4 stimulation is associated with increased expression of the TGF-β gene[21]. Others have also demonstrated overexpression of TLR9 (an endosomal TLR) in the skin of SSc patients of SSc mice in parallel with overexpression of TGF-β[22]. Conversely in vitro studies show that TGF-β stimulated the expression of NOX4 both in normal dermal and in SSc dermal fibroblasts via the PKC-δ and the Smad2/3 pathways[23] and that TGF-β increases the expression of NOX4 in normal human lung fibroblasts[24].

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## Nicotinamide Adenine Dinucleotide Phosphate Oxidase and Inflammasomes

The inflammasome is a multiprotein oligomer that promotes the maturation and secretion of pro-inflammatory cytokines involved in the inflammatory response; the phagocyte inflammasome NLRP3 requires a two step signal for its activation: a priming transcriptional step that involves NF-κB pre-activation by TLR2, TLR3, TLR4, and TLR7 and a post-translational step that allows the oligomerization of the inflammasome components followed by the maturation and secretion of IL-1β[25]. ROS participates indirectly through the activation of TRL involved in the priming step and directly at the

post-translational step[26]. ROS of NOX origin are critical for NLRP3 inflammasome activation though mitochondrial ROS are also relevant[27].

The inflammasome is involved in the pathogenesis of SSc: a clinical study reported over-expression of NLRP3 in affected skin of SSc patients compared to healthy individuals: NLRP3, caspase-1, IL-1β, IL-18 and (endothelin-1) ET-1 were over-expressed in affected skin of limited and diffuse SSc whereas limited SSc patients showed a significant increase in eNOS, iNOS and TGF-β. The expression of NLRP3, IL-1β, IL-18 and ET-1 correlated with dermal fibrosis in limited SSc[28]. In vitro studies on SSc fibroblasts show that inflammasome activation favoured the expression of 40 genes involved in the synthesis of signalling. Moreover inhibition of caspase-1 in dermal and lung SSc fibroblasts abrogated the secretion of collagens, IL-1β, and IL-18[29]. A definitive proof for inflammasome involvement in fibrosis comes from the mouse model of bleomycin-induced skin fibrosis where NLRP (-/-) mice failed to develop fibrosis after bleomicin exposure[30]

**Nicotinamide Adenine Dinucleotide Phosphate Oxidase & Antioxidant Responsive Elements**

The activity of NOX4 is also closely related to that of nuclear erythroid-derived factor-2 (Nrf2), the most important nuclear transcriptional factor involved in the expression of genes coding for the synthesis of antioxidant enzymes[31]. In fact, Nrf2, interacting with the Maf protein, binds the antioxidant responsive elements (AREs) in proximity of the promoters of the genes coding for glutathione (GSH) biosynthesis and regeneration (glutamate-cysteine ligase), for the thioredoxin system (eg thioredoxin reductase), for the detoxification of ROS (eg NADPH quinone oxidoreductase-1) and for heme and iron metabolism (eg heme oxygenase-1)[32]. This cross-talk is fundamental to restore the oxidative-redox balance upset by excessive NOX4 generated ROS: the protective role of NOX4 on the cardiovascular system[33] is due to the ability to promote growth, proliferation and migration of endothelial cells[34]. In this scenario, NOX4 producing H2O2 induces p38MAPK; this in turn activates eNOS with consequent production of

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NO• that restores endothelial and vascular function[35]. Moreover, thanks to its ability to react with cysteine residues forming disulfide bridges, $H_2O_2$ is an important mediator for many transcription factors and enzymes involved in the regulation of oxidative stress[14].

**Nicotinamide Adenine Dinucleotide Phosphate Oxidase and Fibrogenesis**

Several fibrogenic cytokines such as TGF-β, platelet-derived growth factor (PDGF) and angiotensin II (Ang II) induce the expression of NOX4 in various cell lines enhancing thus the production of ROS.

The TGF-β induced NOX4 expression through the Smad 2/3 and the phosphoinositide3-kinase (PI3K) pathways[36] is particularly relevant at the pulmonary level: hypoxia stimulates the release of TGF β from epithelial cells that, fibrosis aside, stimulates threefold the expression of NOX4 on EC and VSMC favoring thus the transformation of VSMC into myofibroblasts[37]; this causes a severe structural remodeling the intima of the pulmonary vessels[38] that leads to pulmonary hypertension, one of the lethal complications of SSc.

Platelet, macrophage and fibroblast derived PDGF[39] synergizes with TGF-β: in fact PDGF increases the production and mediates some of the effects of TGF-β, whereas TGF-β stimulates PGDF in a positive feedback loop[40]. The intimal hyperplasia characteristic of the early stages of SSc is associated with an over expression of PDGF receptor (PDGFR) in EC and VSMC[41] that behaves as an autoantigen against which activating autoantibodies can be formed[42]. Upon activation, PDGFRs trigger a Ras, ERK1/2 and PI3K dependent intracellular pathway that stimulates NOX4 to produce $H_2O_2$ which in turn activates fibroblasts resulting in further extracellular matrix deposition[12,43].

AngII is a peptide that regulates vasomotor tone: its precursor angiotensinogen is produced by the liver and is subsequently transformed into AngII through cleavage first by the renin and subsequently by the angiotensin converting enzyme. Increased expression of Ang II is detected within the skin lesions of SSc[44]. By binding to its type

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1 receptor Ang II activates NOX4 which produces $H^2O^2$ and induces the decoupling of the eNOS, stimulates the production of TGF-β and activates PKC which by means of ERK1/2 induces the deposition of proteins of the extracellular matrix with consequent fibrosis[45].

**Oxidative and Nitrative Stress markers in Systemic Sclerosis**

A recent meta-analysis including 47 articles and 12 oxidative stress markers confirmed an oxidant/antioxidant imbalance in SSc:oxidative markers such as NO•, asymmetric dimethyl arginine (ADMA), malondialdehyde (MDA), carbonyls and ROOH were higher in SSc than in control groups whereas the antioxidant vitamins E and C, and thiols were lower alongside a decreased enzymatic activity of superoxide dismutase (SOD) and catalase (CAT)[46]. Another meta-analysis confirmed overproduction of isoprostanes in SSc[47].

More recently an imbalance of the vanin/pantetheinase system emerged as a contributor to oxidative stress in SSc; the hydrolysis of vasculoprotective pantethine generates pantothenic acid that has profibrotic effects and cysteamine (then cystamine) that has pro-oxidant effects[48]. In turn cystamine directly[49] and indirectly via the Nfr2 pathway[50] inhibits directly γ-glutamyl synthetase, the rate-limiting enzyme of glutathione (GSH) synthesis that has strong free radical scavenging effects. Diffuse cutaneous (dcSSc) patients overexpress vanin-1 in the skin and the circulatory system, and display elevated levels of serum pantothenic acid in relation to disease severity [51].

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**Micro/macrovascular Disease in Systemic Sclerosis: Functional Studies**

The initial functional and structural hallmarks of microvasculature changes in SSc are Raynaud’s phenomenon (RP) and the morphological abnormalities seen at capillaroscopy [52]. Endothelial dysfunctionmay be present at this early stage, whereby the loss of NO● bioavailability determines an impairment of endothelium-dependent

arterial vasodilation alongside a pro-inflammatory and pro-thrombotic state[53]. A systematic review and meta-analysis on 7 studies and 283 SSc patients dealing with functional vascular measurements revealed impaired flow mediated dilation (FMD) with wide heterogeneity[54] though one study did not favour the impairment. A later meta-analysis including 35 studies and 1292 patients came to similar conclusions though 4 studies did not favour the association. On the other hand nitroglycerine mediated vasodilatation was not affected[55]. Microvascular involvement underlies subclinical cardiac disease: a study on 19 asymptomatic SSc patients revealed decreased coronary flow reserve (CFR) compared to controls ($p<0.001$); CRF was lower in dcSSc than in the limited cutaneous SSc patients (lcSSc) ($p=0.05$); interestingly CRF was inversely correlated with the time of onset of RP[56]. Functional microvascular impairment may be simultaneously present with functional macrovascular impairment[57] but the recent meta-analysis onfive studies concluded that ankle brachial index did not discriminate SSc from healthy controls [55].

**Micro/macrovascular Disease in Systemic Sclerosis: Morphological Studies**

According to different methodologies the prevalence of macrovascular involvement can be as high as 58% [58]. A systematic review and meta-analysis on atherosclerosis measured as intima media thickness of carotid arteries on 14 studies including 666 SSc patients revealed greater intima media thickness with wide heterogeneity [54]. The same systematic review concluded that sub-clinical atherosclerosis was present in specific districts such as the renal, radial and ulnar arteries [54] with the latter two leading to ischaemia, ulceration or amputation of fingers [59-61].

A population based study calculated the hazard ratio (HR) for developing acute myocardial infarction at 3.49, the HR being higher at 8.95 within the first year of SSc diagnosis [62] whereas a later study identified SSc as an independent risk factor for acute myocardial infarction (HR 2.45) [63]. An early case control autopsy study revealed a

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predilection for small coronary arteries or arterioles present in 17% of SSc against 2% of controls ($P$<0.01)[64]; this matches the data of a very recent study in which SSc patients had lower myocardial perfusion during adenosine stress compared to controls (p=0.008) implying microvascular myocardial disease[65].

**Oxidative Stress and Vascular Manifestations in Systemic Sclerosis**

As mentioned previously ROS and more specifically isoprostanes are overproduced in SSc and bear major clinical relevance [66]: 1) isoprostanes inversely correlated with post-occlusion hyperemia, expressed either as raw data (p = 0.007) or as an increase compared to baseline (p = 0.04) whereas endothelium-independent response did not change [67]; 2) urinary isoprostane strongly correlated with nail fold morphological capillaroscopic pattern (p=0.002) and lung involvement (p=0.003), showing increasing levels with the progression of pulmonary severity [68]; 3) similarly serum isoprostanes correlated negatively with pulmonary function (percentage vital capacity and diffusion capacity for carbon monoxide) and positively with renal vascular damage assessed by colour flow Doppler ultrasonography [69]. Among other less specific oxidative stress markers,plasma hydroperoxides correlated with the semi-quantitative capillaroscopy rating score (p < 0.05) [70].

**Nitrative Stress and Vascular Manifestations in Systemic Sclerosis**

SSc patients also show overproduction of nitrate [71,72] in relation to markers of endothelial damage and disease activity [72]; dcSSc patients display higher plasma concentration of crude plasma NT than patients with lcSSc, in relation to the severity and the duration of the disease; moreover NT staining is increased in skin biopsy sections from dcSSc compared to lcSSc [73]. At variance another study did not detect NT differences between dcSSc and lcSSc disease, though in the whole SSc population NT levels correlated inversely with the carbon monoxide diffusion capacity (p < 0.02) [74]. To gain further insight in on the nitrative stress pathway, histologically graded skin biopsies from 33 patients with SSc (ten grade 0, ten grade 1, eight grade 2, and five grade

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3) and eight healthy controls were reacted with antibodies against eNOS and inducible NOS (iNOS) and NT. The degree of staining was assessed using a semi-quantitative system and a staining score was developed for the endothelial cells (EC) of different vessel types in different areas of dermis at all grades. In biopsies from patients with SSc, superficial microvessel ECs showed a peak of eNOS expression in grade 1 skin which fell as the grade increased. By contrast, iNOS staining increased with the grade of skin lesion, a pattern paralleled by endothelial NT detection. These findings suggest that at some point during the progression of the skin lesions, dermal ECs undergo a metabolic switch from constitutional expression of eNOS to cytokine activation of iNOS that, in the presence of adequate substrate availability (arginine) releases up to 1000 fold more NO• than eNOS; this excess NO• reacts with $O^{2}$•- forming more ONOO- that my nitrate skin proteins as well as contributing to EC malfunction [75]. This has to be reconciled with the notion whereby several stimuli failed to activate iNOS in human EC[76] whereas iNOS is truly inducible in murine ECs [77]

**Human interventional Studies with Agents bearing Antioxidant Properties**

With the knowledge that SSc patients overproduce ROS in relation to specific clinical features, several authors undertook interventional trials with different agents bearing antioxidant properties as indicated below.

**Vitamin E and A: mechanisms of action**

Vitamin E (α-tocopherol) is a lipid soluble membrane antioxidant that protects against lipid peroxidation by scavenging free radicals and superoxide[78] though it may also increase intracellular superoxide dismutase and catalase via NF-κB modulation[79]. Its efficacy in SSc has been tested in isolation or in combination with various agents.

Vitamin A (retinol) derives from β-carotene, a polyunsaturated hydrocarbon present in the hydrophobic domains of LDL, member of the large carotenoid family that includes also abscisic acid and crocetin. As an antioxidant Vitamin A quenches NO2• and the

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peroxyl radicals: this effect strongly depends on the $O^2$ partial pressure and it is possibly overcome by pro-oxidant toxic effects at the high $O^2$ partial pressure in the lungs. As a transcriptional modulator, Vitamin A (as all-*trans*-RA) regulates in a dose-dependent manner collagen gene expression in fibroblasts from normal subjects and from SSc patients[80].

**Vitamin E and A in systemic sclerosis**

A randomized, double-blind, placebo-controlled trial compared the efficacy of 500 or 1000 mg of vitamin E once daily versus placebo in SSc: after three weeks of treatment neither dose of vitamin E had any effect on the clinical (blood flow variation in response to cold) or the biochemical outcomes [81]. Vitamin E has been used in combination with vitamin C (a water soluble and chain breaking antioxidant that promotes the regeneration of vitamin E), beta-carotene (two molecules of dietary vitamin A joined together), selenium (cofactor to several intracellular enzymes with antioxidant activity: glutathione peroxidase, thioredoxin reductase and iodothyronine deiodinases), and methionine in a placebo-controlled double-blind crossover study. The efficacy of the above mentioned concoction, “BioAntox”, was evaluated on the frequency and the duration of RP attacks, on the thermographic response to a standard cold challenge and on free radical markers in lcSSc; after the 20-week study period there was no effect on clinical outcomes despite an increase of the levels of antioxidant molecules on active treatment[82]. In a further study vitamin E (800 UI/day) was paired with pentoxyphylline (800 mg/day) in a 24 week open-label trial to examine their effect on a possible decrement of the modified Rodnan skin score (from a baseline of at least 15 points), an improvement of ischemic ulcers and laboratory parameters; after 16 weeks the average skin score had decreased from 25.7 to 18.7 and remained such at 24 weeks; however the intervention with two agents limits our understanding of the effect of Vitamin E [83]. Finally, a randomized trial compared intravenous cyclophosphamide (500mg/$m^2$ of body surface area monthly) combined with vitamin E (400IU/day) and vitamin C (1g/day) versus antioxidant

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vitamins without cyclophosphamide: after six months the skin thickening of SSc patients receiving antioxidants with cyclophosphamide was significantly inferior than that of SSc receiving cyclophosphamide alone but it is not clear how much of the benefit can be ascribed to either vitamin [84]. The topical administration of vitamin E twice weekly, in addition to a standard medication protocol, induced faster pain resolution and healing of digital ulcers in 15 treated SSc patients compared to 12 untreated controls (n = 12)[85].

With regards to carotenoids, protracted topical tretinoin resulted in considerable improvement in mouth and facial skin tightening in few patients[86]; etretinate, a lipophilic, aromatic retinoid induced a 75% skin score decrement in SSc patients at 0.5 mg/kg per day, in isolation (7 cases) or in combination with other drugs (systemic corticosteroids, immunosuppressants, d-penicillamine, methotrexate, bucillamine and/ or UVA irradiation) (5 cases) compared to 19 SSc patients not receiving etretinate, but only ointments and vasodilators (6 cases) or other drugs (corticosteroids, immunosuppressant) (13 cases)[87].

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**N-Acetylcysteine: mechanism of action**

Acetylation of the aminoacid L-cysteine yields N-Acetylcysteine (NAC), the direct antioxidant activity of which resides within the thiol group; however, the scavenging effect in vivo depends on the reaction rate of NAC towards the oxidants formed at any given intracellular or extracellular site and on the relative concentrations of antioxidant and oxidant present at any given moment. With this in mind NAC has reasonable direct antioxidant activity against $NO^{2}$, hypohalous acids deriving from peroxidases and HOX but negligible activity against $H^{2}O^{2}$, $O^{2}•-$, and $ONOO^{-}$,[88]. On the other hand NAC behaves as an indirect antioxidant by boosting the intracellular content and consequently the natural antioxidant defence of GSH that has been depleted for any reason. GSH is a tripeptide (c-L-glutamyl-L-cysteinylglycine, GSH) synthesised and maintained at

elevated (mM) intracellular concentrations[89]. NAC achieves this as a precursor of cystein, the rate-limiting factor in cellular GSH[90]. The latter is as a substrate or cofactor for a large number of cellular antioxidant enzymes: glutathione reductase, glutaredoxin, glutathione peroxidase, peroxiredoxin, glyoxalases 1 and 2, glutathione transferase[91]. Given in acute or chronic regimens NAC significantly replenished the GSH pool in certain organs such as liver, skin, lung, and brain, preventing the damaging effects of GSH impoverishment on these organs. NAC has the ability to restore the intracellular thiol pool that in turn regulates the redox state[92].

**N-Acetylcysteine in systemic sclerosis**

A parallel, double-blind, placebo-controlled, prospective study carried out on 22 SSc failed to show any clinical response after 1-year of oral NAC (up to 10g/day) [93]; the inefficacy of NAC via the oral route (1.8 g daily) on digital blood flow and RP was confirmed in a short tem double-blind, placebo-controlled trial on 42 SSc patients [94]. Converselyone group has consistently shown the benefits of intravenous NAC (15 mg/ Kg/h for 5 consecutive hours every 14 days) in the medium term management of SSc: in one open label study carried over 3 years they documented a reduction of attacks and severity of RP and of digital ulcers [95], a reduction of the resistance index of the renal arteries alongside an improvement of the diffusion capacity for carbon monoxide [96,97].

**Penicillamine: mechanism of action**

Of the effects that PEN has on collagen metabolism [98] few were exploited in the management of SSc: PEN interferes with collagen biosynthesis, with the formation of intra and inter molecular cross links and accelerates collagen turnover [99-101]. Moreover, PEN seems to have also antioxidant proprieties: it is structurally similar to the α-amino acid cysteine but with two methyl groups attached to the same α carbon as the thiol group that can act as an electron donor scavenge free radicals in a way similar to GSH[102]. Elevated ROS beyond that required for the normal biochemical processes

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would deplete the reduced form of PEN as the first line of antioxidant defence against ROS. In turn PEN would lessen the reduced form of glutathione (GSH) because of its lower redox potential and be converted to a GSSG adduct. Under normal circumstances, the GSSG adduct is reduced back to GSH by the action of cellular anti-oxidative enzymes including glutathione reductase and glutathione peroxidase[103].

**Penicillaminein systemic sclerosis**

Between 1966 and 1988 several uncontrolled studies, using different dosages and duration of treatment, concluded that PEN decreased skin thickness in patients with systemic sclerosis[104-107] These evidences led other authors to design studies with with more representative samples and more reliable endpoints. The ambiguous results obtained aroused a fervent debate at the beginning of the 2000s, causing various controversies on this topic [108,109]. In fact the only one double blind, randomized, prospective study, investigating the effect of low dose (125 mg every other day) and high dose (822 mg daily) of PEN, failed to show any clinical improvement in systemic sclerosis[110] whereas many other observational and retrospective studies [111-113] demonstrated that PEN reduces skin sclerosis, slows new visceral organ involvement and improves 5 years survival. In fact, at a median dose of 750 mg per day, PEN is associated with a statistically significant reduction in skin, renal, cardiac and pulmonary involvement and overall mortality [111-113]. Unfortunately 20% of patients on PEN developed membranous glomerulopathy with proteinuria with a 40% mortality [114] and many others developed several autoimmune disorders[115].

**Probucol: mechanism of action**

Probucol is a bis-phenol lipophilic lipid-lowering agent that inhibits oxidation of low-density lipoproteins from which its antioxidant activity that was initially attributed to its phenol moieties[116]; the latter however do not protect against 2-electron

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oxidation that is instead achieved by the two sulphur moieties of probucol[117]. Probucol increases the expression of HO1 gene and activity in balloon-injured rabbit aorta and rabbit aortic smooth muscle cells[118]: the promoter region of the HO1 gene contains multiple copies of ARE controlled by the redox-sensitive transcription factor Nfr2[119]: the phenol moieties of probucol are involved in this effect[120]. Moreover probucol inhibited lipopolysaccharide-induced nuclear NF-κB activation in paw tissue as well as NF-κB activity in cultured macrophages indicating that probucol may block the NF-κB transcriptional pathway[121]. Furthermore, probucol inhibits protein argininemethyltransferase I expression, increase dimethylarginine dimethylaminohydrolase activity, reduce the asymmetric dymethil arginine concentration and restore the activity of eNOS in cultured EC[122]whereas in animal models it enhances catalase and glutathione peroxidase activities[123].

**Probucol in systemic sclerosis**

One trial compared the efficacy of probucol (500 mg/day) or nifedipine (20 mg/day) on the frequency or severity of RP attacks in primary or SSc related RP over a 12 week period: patients on probucol experienced less frequent and less severe attacks of RP than those on nifedipine [124].

**Statins: mechanism of action**

Statins are cholesterol lowering drugs that inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, fundamental step in the synthesis of endogenous cholesterol. Statins are pleiotropic drugs in that apart from lowering cholesterol they have also anti-inflammatory, antioxidant, anti-fibrotic and anti-platelet properties. These pleiotropic effects are due to their ability to suppress pro-oxidant enzymes such as NOX, to induce the nitric endothelial oxide synthase (eNOS) and enhance levels and activity of endogenous antioxidant systems.

Endothelial NOS generates NO• by converting L-arginine into L-citrulline in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and other cofactors such as calmodulin, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin (BH4)[125]; it maintains arterial vasodilation and exerts

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anti-inflammatory, anti-proliferative and antithrombotic activities. Statins regulate eNOS expression through several mechanisms. One is the inhibition of mavelonate formation, the precursor of isoprenoids such as geranyl-geranyl-pyrophosphate (GGPP) and Farnesyl-pyrophosphate (FPP). The GGPP activates the Rho A pathway that destabilizes the eNOS mRNA[126], and inhibits the PI3K/Akt pathway, preventing thus the phosphorylation of eNOS[127]. FPP, through the sterol regulatory building protein reduces the transcription of caveolin-1 and activates eNOS[128].

Statins have a dual antioxidants capacity: they can suppress pro-oxidant enzymes and activate antioxidant ones. With regards to the former capacity, atorvastatin down regulated NOX1 expression and Rac1 membrane translocation in VSMCs resulting in reduced ROS generation[125,129]. In a reverse fashion, withdrawal of cerivastatin from VSMCs in culture induced the translocation of Rac1 to the membrane, with a subsequent activation of NOX complexes and increase in ROS production[130]. Moreover, porcine coronary arteries exposed to high glucose levels generated increased ROS that may was suppressed by statins through reduction of $p22^{phox}$ mRNA levels[131]. In the cellular membrane, NOX oxidase co-localizes with ceramide and acid sphingomyelinase to form membrane rafts; statin treatment prevents the oxidised low density lipoprotein induced formation of membrane rafts consequently reducing ROS generation from human coronary artery endothelial cells[132]. In a rat model, statin suppressed NOX activity through a mevalonate-dependent prevention of Rac activation, favouring increased NO bio-availability and improved endothelial function[133]. In humans, short-term treatment with atorvastatin rapidly inhibits NOX activity hence ROS generation in saphenous venous grafts from patients undergoing coronary artery bypass independently of the lipid lowering effect of the drug[134].

With regards to stimulating anti-oxidant enzymes, simvastatin partially restored renal levels of the three major cellular antioxidant defence systems (SOD, GSH-Px, and catalase) in diabetic animals[135,136] whereas in rats chronically treated with the eNOS inhibitor L-NAME statins improve endothelial function by increasing SOD levels[137]. Rosuvastatin antagonizes the deleterious effects of Ang II on the vascular system by

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down regulating the expression of NOX4 and increasing the expression of SOD[138]. Atorvastatin increase the gene expression and activity of catalase in aortic VSMCs from rats and in senescent HUVECs[125].

Furthermore, by inhibiting pro-oxidant enzymes and restoring eNOS coupling, statins act also at transcriptional level: in fact, reduction of ROS generation prevents ROS induction of NFkB; in addition statins increase the levels of the inhibitor IkBα and decrease those of IKK, reducing the binding capacity of NFkB that does not engage in the transcription of genes coding for pro-inflammatory cytokines[139].

**Statins in systemic sclerosis**

Stemming from this evidence, several studies investigated the protective role of statins in SSc and a recent meta-analysis determined that statins treatment is associated with significant biochemical and clinical improvements[140]. All studies included in the final analysis showed a significant reduction of Interleukin-6 (IL-6), E-selectin, vascular endothelial growth factor (VEGF), endothelin 1, and basic fibroblast growth factor[140-147] Only one study measured NO that significantly increased following treatment with simvastatin at a dose of 20 mg daily for 12 weeks[140]. Four of the studies in the meta-analysis evaluated flow mediated vasodilation. Of these, three cohort studies showed a significant improvement in endothelium dependent dilatation though the only randomized control trial present in the meta-analysis not revealed any improvement in endothelium dependent and independent vasodilatation after 8 weeks of treatment with 20 mg of Simvastatin. In all the studies considered in the meta-analysis, no significant improvements was shown in regard of other vascular parameters, such as endothelium independent vasodilatation, arterial stiffness, ankle/brachial index or carotid intimal medial thickness.

**Animal and in vitro Studies with Agents bearing Antioxidant Properties**

**Vitamin E**

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Conditioned media from SSc patients and isoprostanes inhibited endothelial cell tube formation *in vitro*, the equivalent of angiogenesis in vivo, via activation of the thromboxane A2 receptor and the Rho-associated kinase pathways that suppresses vascular endothelial growth factor (VEGF) activity. The addition of vitamin E increased cell tube formation [148].

**Edaravone**

Edaravone is a phenolic compound used in neurology to limit hydroxyl radical-dependent and radical-independent peroxidation of brain lipids [149]; it quenches ROS generated from neutrophil NOX and by mitochondria[150]rather than by inhibiting neutrophil function[151]. In animal models of ischemia-reperfusion edaravone suppresses the oxidative/inflammatory response secondary to ROS [152]. In the tight skin mouse and in the bleomycin-induced SSc mouse edavarone reduced skin and lung fibrosis alongside the reduction of fibrogenic cytokines and ONOO [153].

**Lipoic acid**

Alpha-lipoic acid (LA) is a naturally occurring dithiol compound enzymatically synthesized in mitochondria from octanoic acid[154]. From the free radical perspective, in cells containing mitochondria, LA is reduced to dihydrolipoic acid (DHLA) via an NADPH-dependent reaction with lipoamide dehydrogenase whereas in cells lacking mitochondria LA is reduced to DHLA via thioredoxin reductase [155]. Two relevant aspects of LA are that both its oxidized and reduced forms are powerful antioxidants and being amphiphilic ad that LA exerts its antioxidant effects in the cytosol as well as in the plasma membrane[156]. In particular LA scavenges hydroxyl radicals and hypochlorous acid and prevents protein carbonyl formation but most importantly it neutralizes free radicals without becoming one itself [157,158].

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Another relevant aspect of LA is its ability to reduce the oxidized forms of other antioxidants such as GSH, vitamin C and E. As an intracellular antioxidant, GSH buffers the thiol redox state: this requires a steady intracellular level of GSH either by substrate availability or by the transcriptional regulation of the GSH gene[159]. Accordingly LA increases cysteine uptake[160] reducing thus the ratio of cystine to cysteine (as cysteine is the rate-limiting substrate for this reaction) [161] and activates Nrf-2 that mediates the gene expression and synthesis of GSH[159].

Moreover LA regenerates vitamin E either directly by reacting with tocopheroxyl radical or indirectly by reducing dehydroascorbate, which in turn reduces alpha tocopherol. LA also reduces ubiquinone to ubiquinol, an essential component of the mitochondrial electron transport chain[158]. A stable availability of intracellular GSH prevents the age related oxidation of the sulphur amino acids cysteine and methionine, maintaining them in a reduced form[162] LA has regulatory effects on gene transcription: apart its effect of Nrf-2, LA inhibits IκB degradation and NF-κB-dependent gene expression independently of its antioxidant function[157,163]. Finally because of its two thiol groups, LA chelates several divalent metal ions in vitro and can form stable complexes with $Fe^{2++}$ [164] minimising thus iron induced oxidative stress[165]. We know thattotal plasma and dermal fibroblast thiols are reduced in SSc [148].

In vitro experiments have shown that LA and its metabolite dihydrolipoic acid (DHLA) behave as antioxidants in dermal fibroblasts in that they quench the production of ONOO- that in turn is accompanied by a reduction of PDGFR phosophorilation and consequently lower expression of Col I; the authors suggest that LA and DHLA may act on redox-sensitive transcription factors that control the expression of phosphatases though their data cannot be extrapolated to the in vivo scenario [166].

**Pantethine**

As mentioned earlier pantethine is a vasculoprotective compound made up of two pantetheines joined by a disulphide bridge produced from vitamin B5 (pantothenic acid)

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by the addition of cysteamine; the vanin/pantetheinase pathway [48] may hydrolyse pantethine into pantothenic acid that is profibrotic and cysteamine that is pro-oxidant. Over activation of the vanin-1/pantetheinase pathway occurs in wild-type BALB/c mice with hypochlorous acid (HOCl)-induced SSc [167]. Pantethine administration restored the production of gluthatione and decreased the generation of ONOO- in fibroblasts and EC alongside a decrease in skin and lung fibrosis [167].

**Asiatic acid**

Asiatic acid is a pentacyclic triterpenoid extracted from Centella asiatica, a herbaceous perennial plant from the family of Apiaceae: it inhibits TGF-β1-induced collagen expression in human keloid fibroblasts, via PPAR-gamma activation [168]. In the hypochlorous acid-induced murine model of systemic sclerosis, asiatic acid alleviated pulmonary fibrosis and slowed disease progression compared to untreated mice. Moreover, trans-differentiation of fibroblasts into myofibroblasts was significantly reduced in the lungs of SSc mice treated with asiatic acid[169].

**Tanshinone IIA**

Tanshinone IIA is a natural diterpene quinone with antioxidant and anti-inflammatory properties, isolated from the root of *Salvia miltiorrhiza*. It exerted inhibitory effects on IL-17-induced ERK phosphorylation and functional activation (proliferation, collagen type I and III synthesis, and migration) of dermal vascular smooth muscle cells isolated from SSc patients[170].

**Crocetin**

Crocetin is an apocarotenoid dicarboxylic acid extracted from *Crocus* flowers and *Gardenia jasminoides* fruits. Crocetin inhibited the proliferation of normal and SSc fibroblasts and the trans-differentiation of SSc fibroblasts into myofibroblasts; in the bleomicin mouse model of SSc repeated intraperitoneal injection of crocetin alleviated

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skin and lung fibrosis in association with decreased levels of mRNAs for type 1 collagen and endothelin-1 in skin and lung[171].

**Epigallocatechin-3-gallate**

Epigallocatechin-3-gallate (EGCG) is the ester of epigallocatechin and gallic acid (trihydroxybenzoic acid), a type of phenolic acid. EGCG is a polyphenol found in high content in the leaves of green tea and white tea. Orally administered EGCG is poorly absorbed. EGCG down-regulates, in a dose-dependent manner, basal levels of type I collagen and TGF-β-stimulated production of type I collagen, fibronectin and connective tissue growth factor (CTGF) in fibroblasts isolated from normal subjects and patients affected by SSc. More interestingly EGCG suppressed TGF-β-induced ROS production in all fibroblasts and inhibited NF-κB activation in response to TGF-β or PDGF-BB[172,173].

**Curcumin**

Curcumin is a phenolic diarylheptanoid deriving the spice turmeric (*Curcuma longa*). Curcumin protected rats against lung fibrosis induced by bleomycin[174] and induced apoptosis in scleroderma lung fibroblasts, but not in normal lung fibroblasts[175]. Curcumin acted via induction of the Nrf2/ARE pathway, increasing the expression of intracellular detoxifying enzymes in fibroblasts[176]. Furthermore, curcumin exerted a marked inhibitory activity on TGF-β signaling in SSc fibroblasts, by counteracting TGF-β-induced phosphorylation of Smad2, but not Smad3[177]. Unfortunately the potential utility of curcumin as a therapeutic agent is limited by its chemical instability, insolubility in water,[178] and poor bioavailability after oral administration[179].

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## Appraising the Complexity of Antioxidant Treatment

Before considering treatment with antioxidant agents it must be appreciated that ROS and RNS develop in different intracellular compartments within different cell types and in different organs before occurring systemically [180]. From the previous paragraphs it appears that intracellular NOX and extracellular TGF-β have reciprocal effects: NOX4 mediates TGF-β induced pro-fibrotic responses [181] whereas TGF-β specifically increases NOX4 gene expression [182]. In keeping with the first pathway treatment with small interfering RNA against NOX4 prevented the expression of TGF-β target genes such as fibronectin, collagen I and connective tissue growth factor [142]; with regards to the second pathway TGF-β upregulates NOX4 expression hence ROS via the classical Smad2/3 and the PI3K intracellular pathways[183]. Moreover, intracellular ROS may increase JNK and p38 activation aiding further the effect of TGF-β-induced signalling [184] whereas extracellular ROS may directly convert inactive TGF-β to its active form, a crucial step in TGF-β signalling [185]: in fact HNN, the major reactive aldehyde formed during lipid peroxidation, when added to cultured macrophages up-regulates the expression and release of TGF-β[186]. Finally TLR4 may be sense ROS[20] and mediate NFkB activation via non-receptor tyrosine kinases[19] and via inflammasomes [29]; ROS/RNS generated in the course of oxidation and inflammation may also induce post-translational modifications in connective tissue proteins that in turn may act as danger signals for TLR4 and further perpetuate the production of TGFβ hence of fibrosis[18]. It remains to be ascertained whether these in vitro pathways are also active in vivo. Apart from this complex interplay, antioxidant agents, including vitamins, react with $O^{2\bullet-}$ almost one billion times slower than $NO^{\bullet}$; this means that the reaction of $O^{2\bullet-}$ with $NO^{\bullet}$ is energetically favoured leading to a constant loss of bioavailable $NO^{\bullet}$.

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**Targeting Nfr2e**

To restore or to increase the intracellular antioxidant environment seems an attractive goal: unfortunately only a limited number of agents able to induce the transcription of Nfr2 and enhance the synthesis of detoxifying enzymes. Amongst these curcumin [176] probucol [118] and LA[159]seem suitable candidates. Curcumin however has limited pharmaco dynamic and pharmaco kinetic properties [178,179], probucol has the added benefit of suppressing NF-κB activation [121], whereas LA not only activates Nrf-2 [159], but increases cysteine uptake [160], maintains in reduced form other intracellular antioxidants including vitamin E [159] and inhibits NF-κB-dependent gene expression [144,150] making it an all-round and desirable antioxidant agent.

**Targeting NAPDH oxidase**

Silencing NOX4 with small inhibitory RNA improved bleomycin induced lung fibrosis in the specific mouse model[187]; GKT-137831, a specific NOX1 and NOX4 inhibitor under clinical development[188] decreases CCN2 and α-SMA expression and collagen gel interaction in SSc fibroblasts[189]. Azithromycin (AZM), apart from its antibiotic activity, exerts antioxidant and antifibrotic properties in the bleomycin mouse model of lung fibrosis[190]; in particular AZM degrades the proteasome activating NOX4, preventing the TGFβ-induced myofibroblast differentiation and limiting lung fibrosis[191]. Diphenyleneiodonium, a pan-Nox inhibitor, inhibited gene expression of collagen type and fibronectin in human dermal fibroblasts and limited skin fibrosis and myofibroblast activation in the bleomycin mouse model [192].

Outside SSc, atorvastatin and rosuvastatin have the capacity to inhibit the NOX system [193,194]; in a mouse model of diabetes probucol downregulated NOX expression[195] and in human renal proximal tubular epithelial cells it prevented epithelial-mesenchymal transition [196] though the latter is debated[197]; it is envisaged that probucol might have similar effect of SSc fibroblasts. LA may dampen NOX activity either when being reduced [198] or via inhibition of the signalling pathways leading to NOXactivation [199].

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**Concluding Remarks**

According to their lipophilic/hydrophilic nature, their dose and their route of administration, antioxidants or agents with antioxidant properties may achieve in vivo different plasma, tissue or intracellular level, and for each compartment the therapeutic level may vary. On the other hand, one issue is to quench extracellular or intracellular ROS/RNS, another is to target and switch off the genes and/or the enzymatic systems producing them. In this respect statins, probucol and LA may have something new to offer in the management of SSc. Knowledge of the cellular/sub-cellular localisation of potential target enzymes, of the equilibrium constant of the biochemical reactions involved and of the pharmacokinetics and pharmacodynamics of different antioxidants are necessary pre-requisites for the planning of randomised clinical trials. These might explore different doses and/or combinations of new and old antioxidants with adequate follow-up, taking into account the timing of the intervention with regards to disease duration, disease activity and organ involvement. The heterogeneity of the SSc populations to be enrolled ought to be overcome by joining forces in multicenter trials, possibly free from the influence of the pharmaceutical industry. It is envisaged that an expert in free radical chemistry and biology should be included amongst the team members to advice less savvy clinicians on the intricacies of redox biology in vivo. The new information accrued over the last decade on ROS/RNS and their possible manipulation at gene level makes this an interesting time for antioxidant intervention in a disease such as SSc that still defies conventional treatments.

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Table. 1. Interventional study with antioxidant

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| Study (year) | type | Patients (df/lc) | | drugs | | | | Duration (weeks) | results |
|---|---|---|---|---|---|---|---|---|---|
| | | control | intervention | type | dosage | administration | | | |
| Cracowski JL (2005) | Randomized double-blind, placebo-controlled trial | | 20 (6/14) | Vitamin E | 500 mg<br>1000 mg | daily | os | 3 | X: body cooling Test;<br>urinary F2-isoprostane |
| Herrick AL (2000) | Placebo-controlled double-blind crossovertrial | | 33 (33/0) | methionine<br>selenium<br>vitamin A<br>vitamin C<br>vitamin E<br>allopurinol<br>vs<br>placebo | 100 mg<br>600 ug<br>9000 UI<br>540 mg<br>220 mg<br>300 mg | daily | os | 20 | X: body cooling Test;<br>frequency and duration of RP attack;<br>vWF levels |
| de Souza RB (2009) | | 9 (0/9) Cyp 500 mg/ $m^2$ e.v. monthly | 12 (9/3) | vitamin E<br>pentoxyphylline | 800 UI<br>800 mg | daily | os | 24 | ↓: MRSS<br>X: ischemic ulcers; ERS |
| Ostojic P (2010) | | | 13 (0/13) | Vitamin E<br>Vitamin C<br>+<br>CyP | 400 UI<br>1000 mg<br>500 mg/$m^2$ | daily<br>monthly | os<br>e.v. | 24 | ↓: STPR<br>X: MRSS; DLCO; FVC |
| Furst (1979) | Randomized double-blind, placebo-controlled trial | | 11 (8/3) | NAC | 10 gr | daily | os | 48 | X:MSDSS |
| Correa MJ (2014) | Randomized double-blind, placebo-controlled trial | 21 (12/9) placebo | 21 (9/12) | NAC | 1800 mg | daily | os | 4 | X: frequency and duration of RP;<br>digital blood flow; |
| Rosato E (2009) | Open-label trial | - | 50 (35/15) | NAC | 15 mg/ kg/h for 5 hours | biweekly | e.v. | 144 | ↓: ischemic ulcers; frequency and duration of RP attack; |
| Rosato E (2011) | Retrospective | - | 41 (23/18) | NAC | 15 mg/ kg/h for 5 hours | biweekly | e.v. | 96 | ↑: DLCO; VC; TLC<br>X: FEV1; HRTC score |
| Rosato E (2009) | Open-label trial | - | 40 (21/19) | NAC | 15 mg/ kg/h for 5 hours | once | e.v. | - | ↓: renal artery RI * |
| Denton CP (1999) | Randomized open-label crossovertrial | - | 20 (5/15)* | probucol<br>vs<br>nifedipine | 500 mg<br>10 mg | daily | os | 12 | ↓: frequency and duration of RP attack;<br>LDL oxidation |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sadik HY (2010) | Randomized double-blind, placebo-controlled trial | 18 (3/15) placebo | 36 (19/17) | atorvastatin | 20 mg | daily | os | 8 | X: frequency and duration of RP attack; digital blood flow; NVC alteration; vWf; hsPCR |
| Jimenez SA (1991) | Prospective observational | - | 69 (69/0) | PEN | 750 mg | daily | os | 180 | ↓: MRSS |
| Clements PJ (1999) | Randomized double-blind-controlled trial | 68 (68/0) D-PEN 125 mg alternate days^ | 66 (66/0) | PEN | 822 mg | daily | os | 24 | X:MRSS; renal crisis; overall mortality |
| Steen VD (2001) | Prospective observational | - | 278 (278/0) | PEN | 750 mg | daily | os | 24 | ↓:MRSS: overall mortality |
| Derk CT (2008) | Retrospective | - | 84 (84/0) | PEN | 750 mg | daily | os | 96 | ↓: cardiac, renal and pulmonary involvement (MSDSS); MRSS,TBS |

Abbreviations: df: diffuse; lc: limited; X: No effect; ↓: reduced; ↑increased; vWF: von Willebrand factor; RP: Raynaud phenomena; MRSS: Modified Rodnan Skin Score; Cyp :cyclophosphamide; ESR: erythrocyte sedimentation rate; STPR: skin thickening progression rate; MSDSS: Medsger Scleroderma Disease Severity Scale; DLCO: diffusing coeffcient for carbon monoxide ;FVC: forced vital capacity; VC: vital capacity; TLC: total lung capacity; NAC:N-Acetylcysteine; HRTC: high resolution computed tomography; RI: resistance index; NVC: Nailfoldvideocapillaroscopy; hsPCR: high sensitive protein C reactive, TBS:total body surface. PEN: penicillamine. *This results was significant only for patients with early/active NVC pattern or MRSS<14. And not for patients with more advanced disease. ^This dosage was considered ineffective and the authors used this group as a “placebo group”

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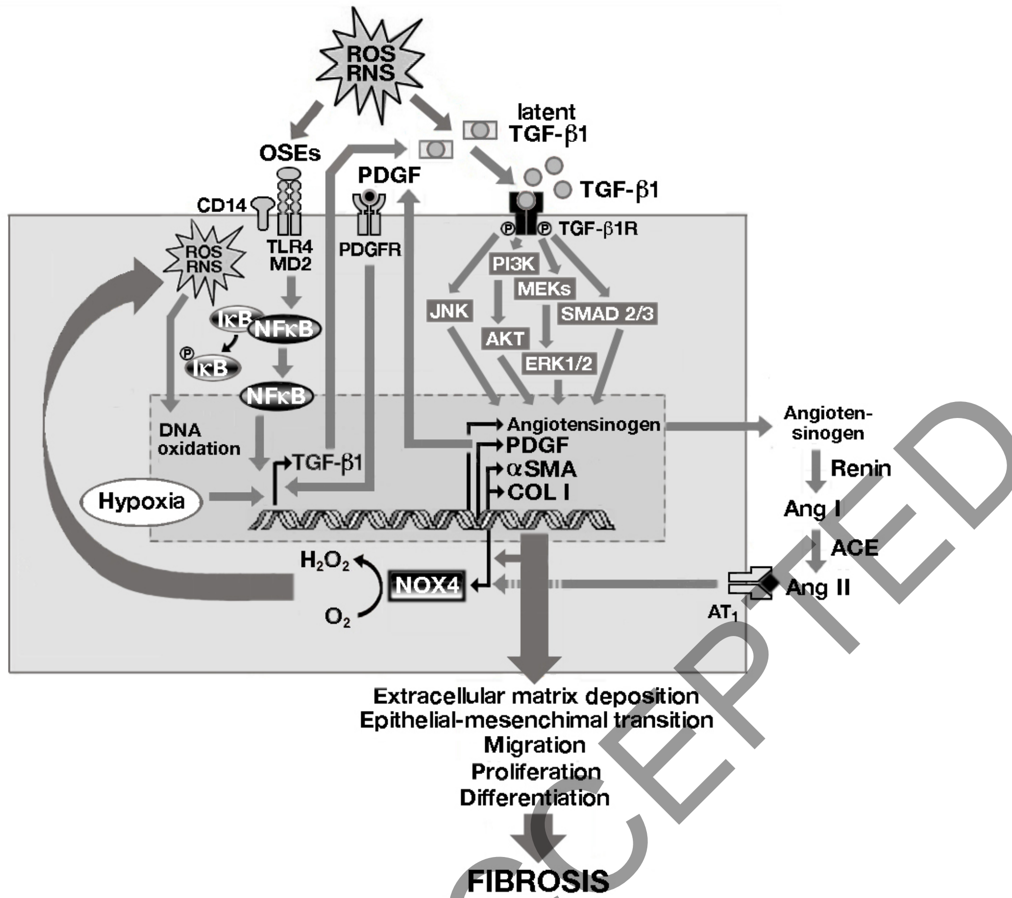


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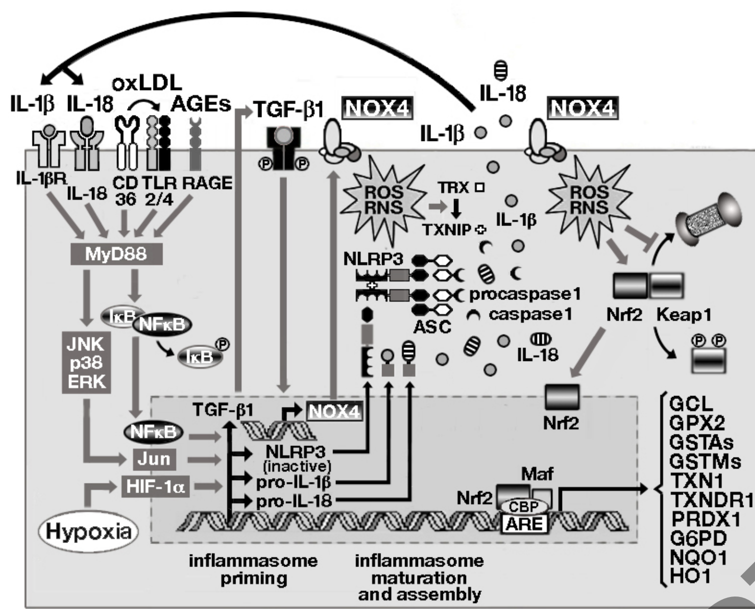


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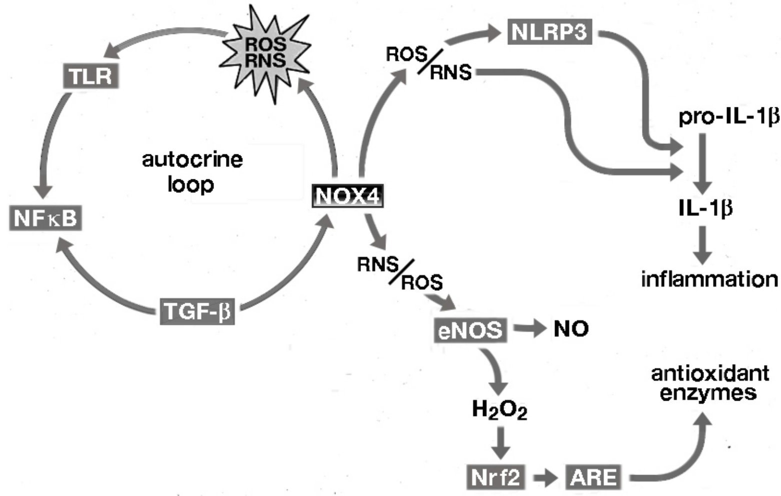


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