## Review

# Unraveling the association between oxidative stress, nitric oxide signaling, and hypoxia inducible factor in chronic limb-threatening ischemia

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**Abstract**

**Objective:** Chronic limb-threatening ischemia (CLTI) is a serious medical condition characterized by decreased blood supply to the lower limbs, which causes tissue loss and amputation. The pathophysiology of CLTI has been associated with oxidative stress, nitric oxide (NO) imbalance, and the hypoxia-inducible factor (HIF) signaling pathway. This review focuses on how oxidative stress causes endothelial dysfunction, impairment of NO bioavailability, and HIF stability, subsequently aggravating tissue hypoxia and ischemia. Furthermore, this review explores the treatment implications of focusing on these pathways to enhance CLTI outcomes. The aim of the review was to explore the role of oxidative stress, NO imbalance, and HIF pathway dysregulation in CLTI pathophysiology and implications for treatment.

**Methods:** PubMed, Google Scholar, E-library, and the websites of medical journals (Medicine, Biology, and Biochemistry) were used as electronic databases for the literature search. Overall, 109 articles out of the 214 that were initially discovered satisfied the inclusion requirements.

**Results:** Oxidative stress contributes to CLTI progression via endothelial dysfunction, impaired nitric oxide bioavailability, and HIF stability, worsening tissue hypoxia and ischemia.

**Conclusion:** Targeting oxidative stress, nitric oxide, and HIF pathways may enhance CLTI outcomes.

**Key words:** Oxidative stress, chronic limb-threatening ischemia, endothelial dysfunction, nitric oxide, hypoxia inducible factor

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**List of abbreviations**

| | |
|---|---|
| ABPI | Ankle-Brachial Pressure Index |
| AGEs | Advanced Glycation End Products |
| BH4 | Tetrahydrobiopterin |
| cGMP | Cyclic Guanosine Monophosphate |
| CLTI | Chronic Limb-Threatening Ischemia |
| EC | Endothelial Cell |
| GSH | Glutathione |
| HDLs | High-Density Lipoprotein |
| HIF | Hypoxia Inducible Factor |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| NOXs | NADPH Oxidases |
| oxLDL | Oxidized Low-Density Lipoprotein |
| PAD | Peripheral Arterial Disease |
| PHD | Prolyl Hydroxylases |
| PKC | Protein Kinase C |
| ROS | Reactive Oxygen Species |
| SNP | Single-Nucleotide Polymorphisms |
| TBI | Toe-Brachial Index |
| VEGF | Vascular Endothelial Growth Factor |
| VNTR | Variable Numbers of Tandem Repeats |

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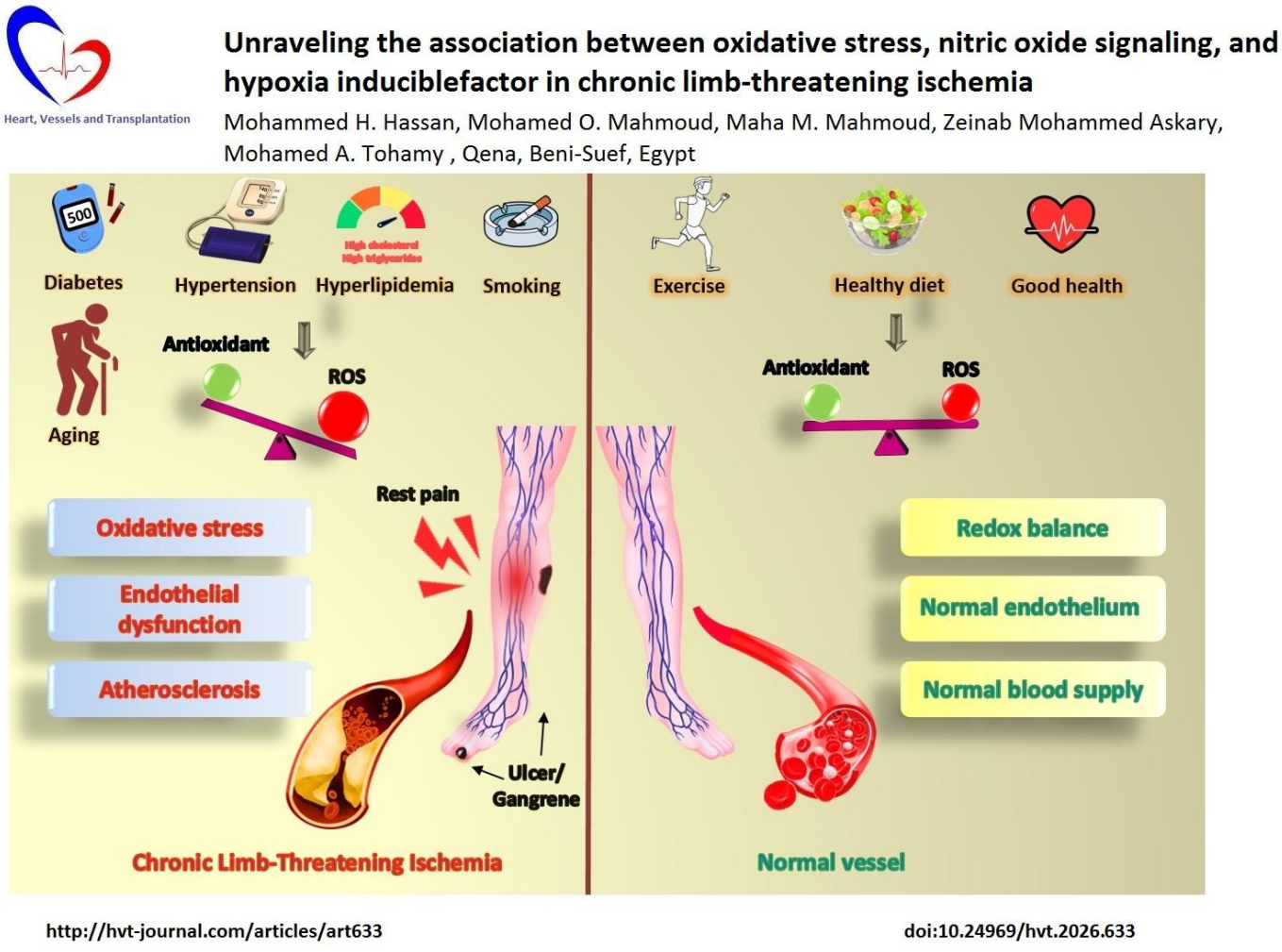
## Highlights

- CLTI pathophysiology involves a complex interplay of oxidative stress, impaired nitric oxide signalling, and HIF pathway activation.

- Oxidative stress and nitric oxide imbalance contribute to endothelial dysfunction and HIF-mediated responses, exacerbating CLTI progression.

- Understanding the crosstalk between these pathways may reveal therapeutic targets to improve limb outcomes in CLTI patients.

## Graphical abstract




**Graphical abstract: Significance of reactive oxygen species in vascular function (This figure was created by Canva.com)**

### Introduction

Chronic limb-threatening ischemia (CLTI), a severe clinical subgroup of peripheral arterial disease (PAD) is characterized by ischemic rest discomfort, non-healing ulcers, or gangrene (1). Using CLTI-specific ICD-10-CM coding, an updated study on the incidence and prevalence of CLTI in the Medicare population found that the estimated yearly incidence was 0.33%, while the estimated 2-year prevalence was 0.74% (2).

Patients who have undergone surgery for CLTI show that in comparison to males, women are underrepresented and have fewer risk factors and comorbidities. However, CLTI targets women above the age of 75 (3). In order to diagnose CLTI, ischemic rest discomfort or tissue loss must be present together with an established PAD. The presence of pain should last longer than two weeks and be linked to at least one abnormal hemodynamic parameter, such as flat or minimal pulsatile volume recording (PVR) waveforms, ankle brachial index < 0.4, absolute AP < 50 mmHg, absolute toe pressure < 30 mmHg, and transcutaneous pressure of oxygen < 30 mmHg (4). Patients with small vascular disease caused by diabetes mellitus, hypertension, or chronic renal disease have lower ankle-brachial pressure index sensitivity, and vascular stiffness is less likely to affect the toe vessels, so in this case, the toe-brachial index (TBI) can be employed, where TBI < 0.7 is considered an abnormal result (5). For CLTI classification, there are many classification guidelines; however, the Fontaine and Rutherford categories are most frequently used by vascular experts to classify the severity of CLTI, whereas the WIfI category evaluates the risk of lower extremity amputation based on the size of the wound, the degree of ischemia, and the existence of a foot infection (6). Although CLTI occurs in just 11 to 20% of all documented PAD cases, it is very serious, with a reported 15-30% 1-year limb amputation rate and 10-25% mortality rate (7). Therefore, further research into illness triggers and the development of novel treatment options is crucial.

Free radicals, including the reactive oxygen species (ROS), reactive nitrogen species (RNS), and sulfur reactive species, do not necessarily represent harmful substances; they play vital physiological roles as mediators in cellular communication and immune responses, on condition that the equilibrium between the amount of free radicals and the effectiveness of antioxidant defenses is maintained. Once this equilibrium is disrupted, a state of oxidative stress occurs, leading to the destruction of deoxyribonucleic acid (DNA), proteins, and lipids (8, 9). Endothelial dysfunction, the first stage in the pathophysiology of atherosclerosis, which can be driven by oxidative stress, is associated with predisposing risk factors such as diabetes mellitus, hypertension, hypercholesterolemia, and smoking, resulting in many cardiovascular issues (10). Oxidative stress can lead to endothelial dysfunction through a number of mechanisms, including inducing endothelial cell (EC) apoptosis, increasing EC adhesion to monocytes, altering EC angiogenesis potential, ROS-triggered inflammation, ROS-triggered mitochondrial dysfunction, and compromising nitric oxide (NO) endothelium-dependent vasorelaxation (11, 12). The latter process is the key pathway that we will focus on.

Endothelial nitric oxide synthase (eNOS), the most common NOS isoform in the vasculature and responsible for the majority of NO generated within tissue, causes dilation of all types of blood vessels by activating soluble guanylyl cyclase and raising cyclic guanosine monophosphate (cGMP). NO is an effective suppressor of platelet aggregation and adhesion, as well as leukocyte adhesion; therefore, it can defend against atherogenesis and fibrous plaque development in the vasculature (13, 14). An imbalance between NO production and ROS generation triggers impairment of vessel dilation, inflammatory responses, platelet aggregation, and smooth muscle cell proliferation, which impairs blood and oxygen delivery (15).

The majority of multicellular creatures have developed molecular processes that allow them to adapt to hypoxic circumstances, which occur when oxygen demand exceeds supply. These mechanisms involve the activation of genes that generate proteins that improve oxygen supply and modify the metabolic process in a hypoxic tissue (16). The hypoxia-inducible factor (HIF), a transcriptional regulatory protein, is one of the main cellular adaptive mechanisms (17). A family of HIF-dependent adaptive genes, including those involved in the regulation of angiogenesis, metabolic processes, erythropoiesis, and vascular tone, is coordinated by HIF accumulation driven by hypoxia. Increased blood and oxygen flow to the ischemic tissue is the outcome of these genes' expression (18).

This review evaluating the relationship between oxidative stress, NO, and hypoxia-HIF in CLTI aims to summarize how these interrelated pathways contribute to disease pathogenesis and prospective therapeutics.

**Methods**

The electronic databases PubMed, Google Scholar, E-library, and the websites of medical journals (medicine, biology, and biochemistry) represented the literature search. The following search terms were used: oxidative stress, chronic limb-threatening ischemia, chronic limb ischemia, critical limb ischemia, endothelial dysfunction, nitric oxide, hypoxia inducible factor.

Articles published between 2010 and 2025 met the requirements for inclusion in the narrative review. Articles about the connection between oxidative stress, NO, and hypoxia-HIF in CLTI were among the literary references. Articles without full text, significant content, or about acute lower limb ischemia were excluded from consideration. Overall, 214 items were found during the data search. After preliminary screening to ensure they aligned with the research's objectives, 109 were chosen for additional examination as a result.

**Oxidative stress: the major cause of CLTI**

In the majority of cells, mitochondria have been the main source of intracellular oxidant generation, followed by the nicotinamide adenine dinucleotide phosphate (NADP) oxidases (NOXs), xanthine oxidase, and heme oxygenase 1 sources. Overproduction or buildup of ROS, such as superoxide, hydroxyl, and peroxyl radicals, as well as hydrogen peroxide and hypochlorous acid, triggers a state of oxidative stress (19). ROS are crucial for the oxidation of low-density lipoprotein (LDL), inflammatory responses, and changes in vascular tone. They also raise the expression of cell adhesion molecules on endothelial cells, such as P and E-selectins and vascular cell adhesion molecules-1 (VCAM-1), which attract leukocytes into the inner endothelial space (20). The loosening of the endothelial membrane facilitates the invasion of monocytes and oxidized LDL (oxLDL) from the vessel lumen to its wall. After leaving the vessel's lumen, monocytes transform into macrophages and take up oxLDL, becoming foam cells. Over time, foam cells disintegrate and form the plaque's lipid core (21). The fibrous section, including the stabilizing cap, is formed by vascular smooth muscle cells (VSMCs) that are triggered by chemokines and adhesion factors. As these cells migrate to the site of inflammation, they generate collagen and elastin, which help to solidify the formed atherosclerotic plaque (22) (Fig. 1). When the endothelium wears down or a plaque layer cracks, a chain reaction develops, leading to platelet-rich thrombus development and further narrowing of the blood vessel lumen (23). Restricted flow of blood in peripheral tissues results in diminished oxygenation and supply to peripheral tissues, leading to claudication or rest discomfort, which develops into chronic limb ischemia (24).

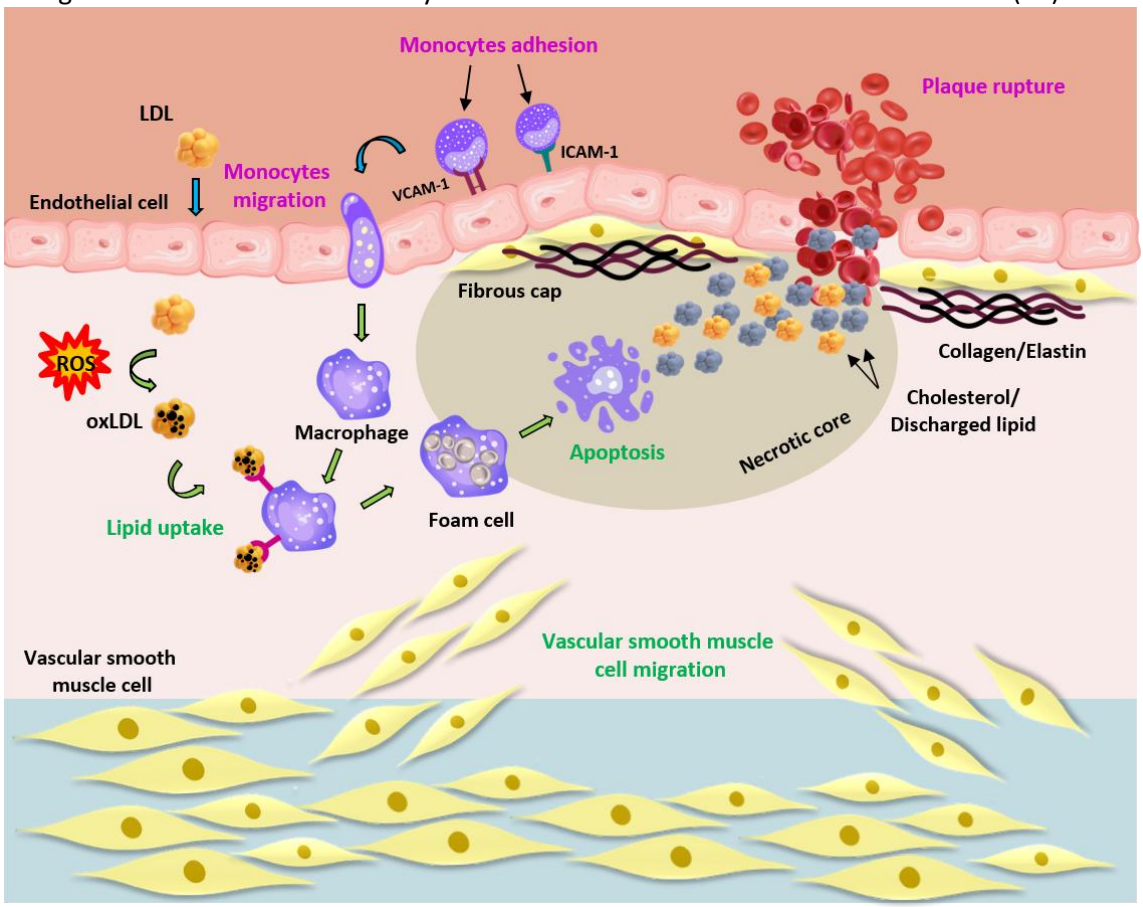



**Figure 1. Oxidative stress triggers the formation of atherosclerotic plaque (This figure was created by Canva.com)**
ICAM-1 – intercellular cell adhesion molecule 1, oxLDL – oxidized low-density lipoprotein, ROS-reactive oxygen species, VCAM1- vascular cell adhesion molecule-1

Therefore, many variables, including endothelial dysfunction, leukocyte activation and inflammation, and altered microvascular flow due to oxidative stress, all affect the final clinical picture of CLTI (25).

**Oxidative stress and NO bioavailability**

NO generation is the primary mechanism by which the endothelium sustains its defense function against vascular disorders (26). As a biologically vasoactive substance, NO is involved in blood pressure control, vascular permeability regulation, vasodilation, and blood flow-dependent dilatation (27). Various cofactors are necessary for NOS to produce NO from its substrate L-arginine, including tetrahydrobiopterin ($BH_4$). NOS uncoupling is the process by which the oxygenase domain of NOS monomers produces superoxide anions rather than NO in the lack of $BH_4$ or its oxidation to $BH_3$ or $BH_2$ and L-arginine deficiency (28). Furthermore, excessive ROS levels may readily inactivate NO to produce peroxynitrite, which lowers the quantity of functional NO. Importantly, peroxynitrite is a potent oxidant that promotes atherogenesis and has the ability to uncouple eNOS, increasing superoxide and decreasing NO bioavailability (29) (Fig. 2). The excessive level of generated ROS can restrict vascular development and remodeling by generating endothelial dysfunction, which includes decreased vasomotor activity, monocyte infiltration, poor endothelial barrier integrity, and increased risk of thrombosis (30, 31). Increasing stenosis may compromise the adaptive ability of arteriogenesis, leading to degradation of newly formed arterial frameworks and exacerbating muscle cell destruction. Physical activity has been limited owing to ischemic discomfort, and further diminishing exercise capacity occurs (32).

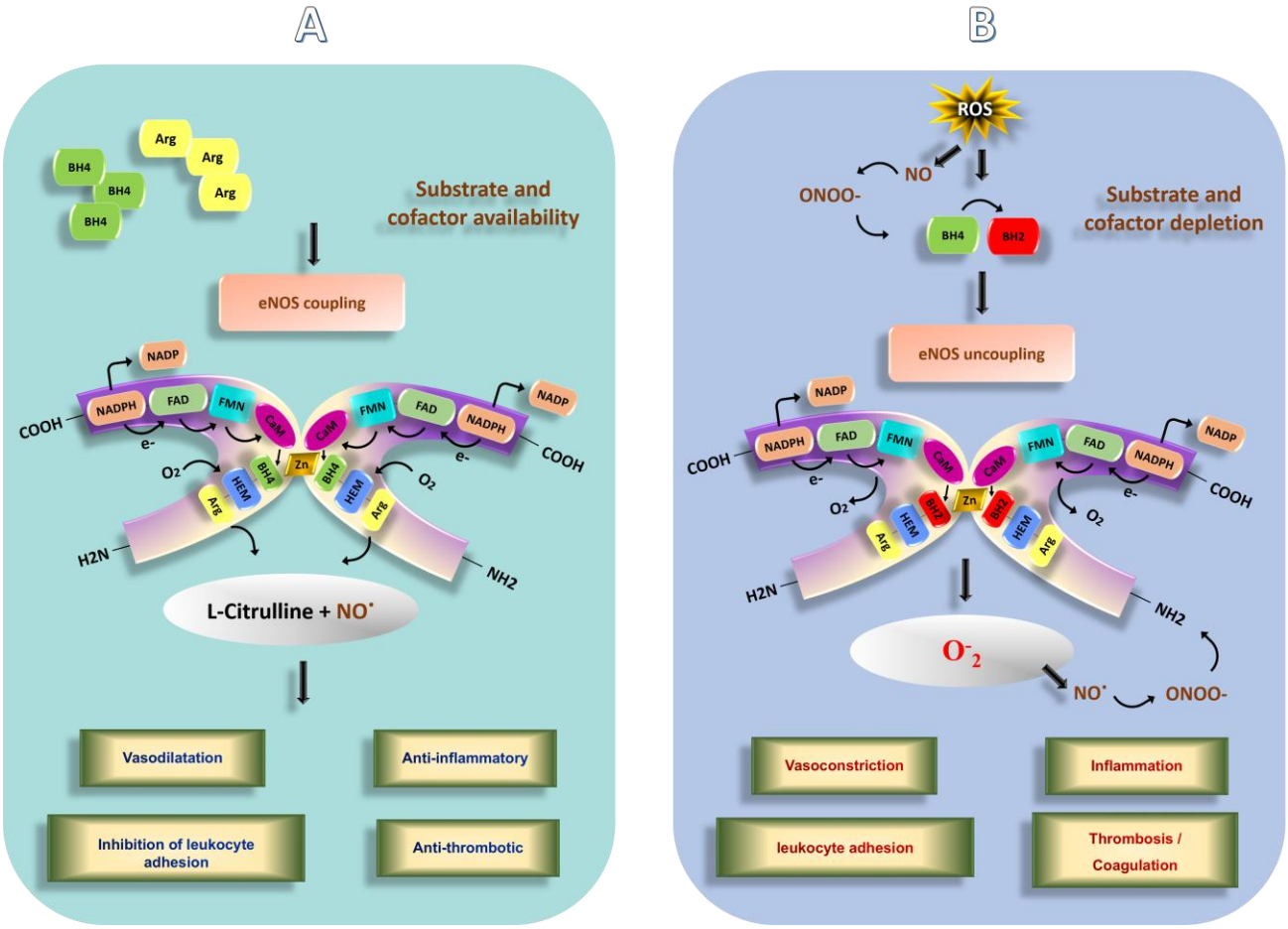



**Figure 2. The impact of oxidative stress on nitric oxide availability. A: The eNOS can synthesize NO from its precursor in the presence of its cofactor. B: In oxidative stress condition eNOS undergoes uncoupling and forms superoxide instead of NO. (This figure was created by Canva.com)**

Arg – arginine, BH – tetrahydrobiopterin, CaM -calmodulin, eNOS - endothelial nitric oxide synthase, FAD – flavine adenine dinucleotide, FMN- flavine mononucleotide, NADP –oxidized nicotinamide adenine dinucleotide phosphate, NADPH –reduced nicotinamide adenine dinucleotide phosphate, NO – nitric oxide, ROS-reactive oxygen species

NO production can be impacted by NOS polymorphisms that alter NOS expression or activity. Although NO is generated by neuronal (NOS1), inducible (NOS2), and endothelial (NOS3) nitric oxide synthases, NOS3 is the main isoform for NO production in the circulatory system. The NOS3 gene has several variation regions, such as insertions or deletions of nucleotides, single-nucleotide polymorphisms (SNPs), and variable numbers of tandem repeats (VNTRs) (33). The 7q35–7q36 locus of human chromosome 7 contains the NOS3 gene (34). The SNP database contains over 1700 genetic variations, some of which are recognized as effective because they impact NOS3 transcription or function, such as the SNPs rs2070744, rs1799983, rs3918226, and a VNTR in intron 4, which have been extensively researched (35). The SNP rs1799983 is found in exon 7 and is referred to as Glu298Asp because a guanine is altered to thymine at position 894 of the NOS3 gene, which results in a substitution of glutamine (Glu) to aspartate (Asp) at position 298 of the protein (36). According to Joshi et al. (37), endothelial cells with the variation of Asp allele for the Glu298Asp polymorphism showed reduced NOS3 activity. The SNP rs2070744, often known as the -786T>C SNP, has cytosine for the substitution of thymine at location 786 of the NOS3 promoter (38). Miyamoto et al. (39) found that people with the –786T→C mutation had considerably lower serum nitrite/nitrate levels than people without the mutation (i.e., carriers of the T allele), indicating that this SNP decreases NOS3 transcriptional activity. The SNP rs3918226 (665C>T) involves the replacement of C with T in the promoter region (40). This alteration reduces promoter function by 20–40%, which was proved by Salvi et al. (41) using HeLa and HEK293T cells transfected with the NOS3 promoter incorporating either the C or the T allele at position 665 in the NOS3 gene. The eNOS gene's 4b/4a VNTR polymorphism in intron 4 (varying number of tandem repeats on intron 4) controls eNOS after transcription by modifying the production of a small interfering ribonucleic acid (siRNA). The most frequently identified alleles of this polymorphism are those containing four (variant 4a) or five (variant 4b) copies of the 27 bp siRNA sequence (42). According to research by Zhang et al. (43), endothelial cells with variant 4b exhibit higher amounts of sirRNA than cells with variant 4a, which results in a decrease of NOS3 mRNA.

**Oxidative stress and HIF1 bioavailability**

When oxygen levels in the cell microenvironment are lower than the usual physiological condition, numerous physiological and pathological processes happen (44). HIF is an essential regulatory protein that maintains oxygen homeostasis and is primarily responsible for maintaining the equilibrium between oxygen demand and supply (18). A constitutive β subunit plus one of the three oxygen-dependent α subunits, HIF-1α, HIF-2α, or HIF-3α, constitute HIFs (45). In normoxia, certain prolyl hydroxylases (PHDs) utilize $O_2$ as a substrate to hydroxylate HIF-α. When HIF-α is hydroxylated, it can connect with von Hippel–Lindau protein and attract ubiquitin ligase, which causes HIF-α to be degraded by proteases. When PHD activity is suppressed in hypoxic environments, HIF-α builds up, diffuses to the nucleus, and combines with the HIF-β subunit to produce HIF-1, HIF-2, or HIF-3 (46). Factors inhibiting HIF are additional HIF-α controlling enzymes that facilitate an asparagine hydroxylation process that inhibits the binding of HIF-α with CBP/p300 transcriptional co-activating factors (47) (Fig.3).

After that, HIF promoting angiogenesis by upregulating growth factors like VEGF and enabling an energy-saving transition from aerobic to anaerobic metabolism by upregulating important glycolytic enzymes (48). Although HIF activation increases tissue oxygenation and angiogenesis, during chronic ischemia its adaptive function becomes inappropriate, worsening tissue damage by producing more ROS. The cytochrome chain, which is primarily controlling mitochondrial oxidative phosphorylation, may be altered by HIF-1, leading to decreased adenosine triphosphate synthesis and increased ROS production (49, 50). Furthermore, HIF-1 increases the production of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) class, especially NOX1 and NOX4. These NOX subtypes increase oxidative stress by producing ROS (51).

Due to its primary function in many cells, the research specifically focused on HIF1α polymorphism and concluded that polymorphisms could impact the HIF1α mRNA or protein expression and stability (52, 53). Chromosome 14 (q21-24) encodes the human HIF-1α gene, which is subject to several single-nucleotide polymorphisms; thereby, the structure and biological activity of the HIF-1α gene can be affected (54). C1772T (rs11549465), G1790A (rs11549467), C111A, and rs2057482 in UTR are considered the most widely researched SNPs (55). For example, Kim et al. (56) reported that the C1772T polymorphism had been associated with elevated expression of HIF-1α. According to Fraga et al. (57), higher production of the HIF-1α protein is linked to the C1772T and G1790A SNPs of the HIF-1α gene.

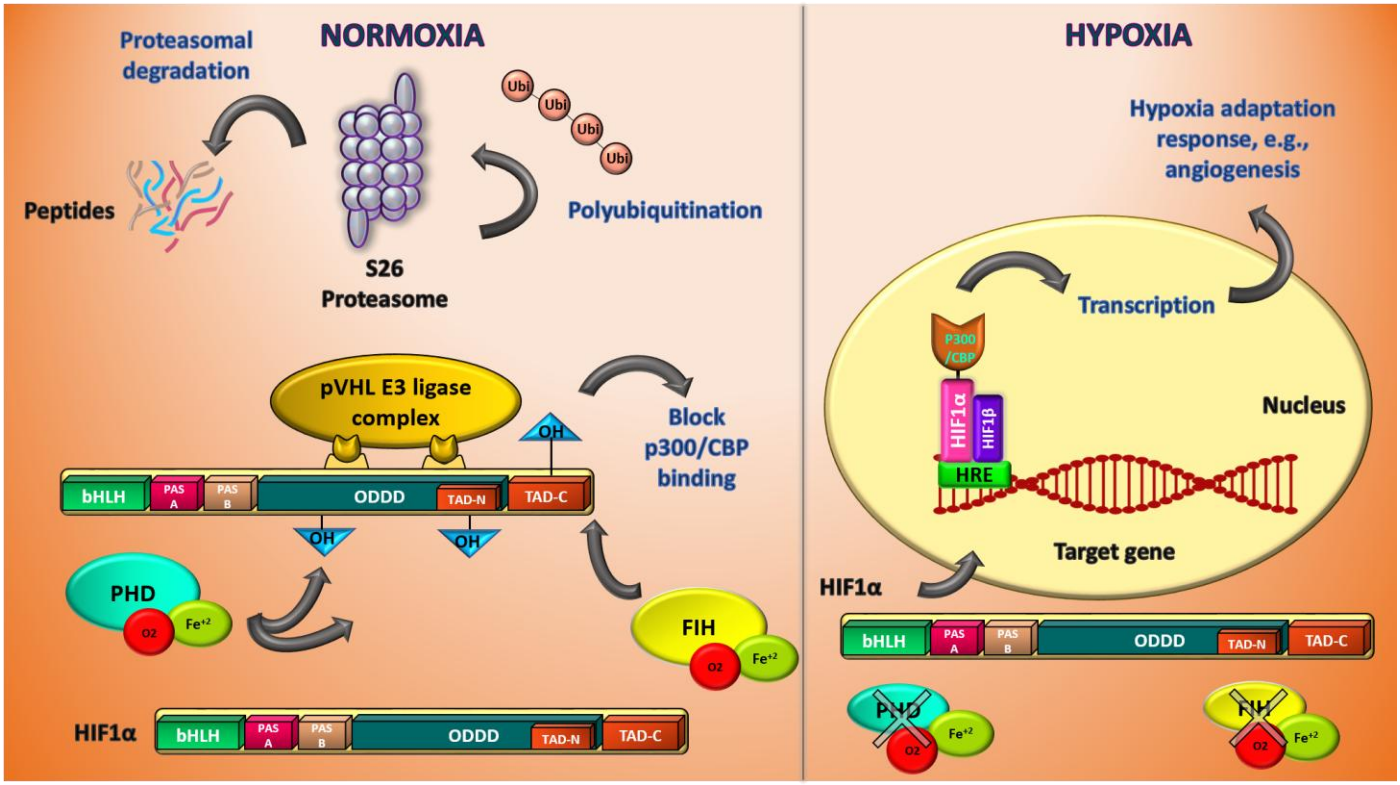



**Figure 3. Correlation between oxygen status and hypoxia inducible factor 1 (This figure was created by Canva.com)**
FIH -factors inhibiting hypoxia inducible factor, HIF- hypoxia inducible factor, PHD- prolyl hydroxylases, pVHL- von Hippel–Lindau protein

Koukourakis et al. (58) investigated the impact of the C2028T polymorphism of the HIF-1α on the protein expression and found a positive correlation.
Additionally, several HIF-1α genetic polymorphisms, including rs10873142 (C>T) in intron 8, rs41508050 (C>T) in exon 10, rs2783778 (C>T) and rs7148720 (T>C) in 5'-flanking, rs11549465, (C>T), and rs11549467 (G>A) in exon 12, and rs2057482 (T>C) 3'in UTR region, have been linked to cardiovascular diseases such as ischemic heart disease, acute myocardial infarction, and coronary artery disease (59).

**Oxidative stress risk factors**
Numerous earlier studies have shown that conditions including diabetes mellitus, high blood pressure, and high cholesterol increase the production of ROS, which causes oxidative stress (60), which will be clarified in the following section (Fig. 4).
**Diabetes mellitus**
In hyperglycemia, nonenzymatic interactions between monosaccharides, such as glucose, glyceraldehyde, and fructose, and amino groups of proteins, lipids, and nucleic acids can produce advanced glycation end products (AGEs) (61). ROS are produced when these AGEs bind with their receptors, causing oxidative stress via triggering of protein kinase C (PKC), which increases NADPH oxidase and lipoxygenase (62). As a result of the inhibition of antioxidant systems caused by this oxidative stress, DNA damages induce repair enzymes like poly-ADP ribose polymerase-1, which inactivate glyceraldehyde-3-phosphate dehydrogenase. This inactivation leads to glyceraldehyde-3-phosphate, glucose 6-phosphate, and fructose 6-phosphate accumulation (63). This accumulation results in activation of alternative undesirable pathways, such as the polyol pathway, where the enzyme aldose reductase breaks down excess glucose into sorbitol and fructose, which worsens oxidative stress by reducing NADPH, which is an essential ingredient for the production of reduced glutathione (GSH) (64). All of these mechanisms lead to boosting oxidative stress state.

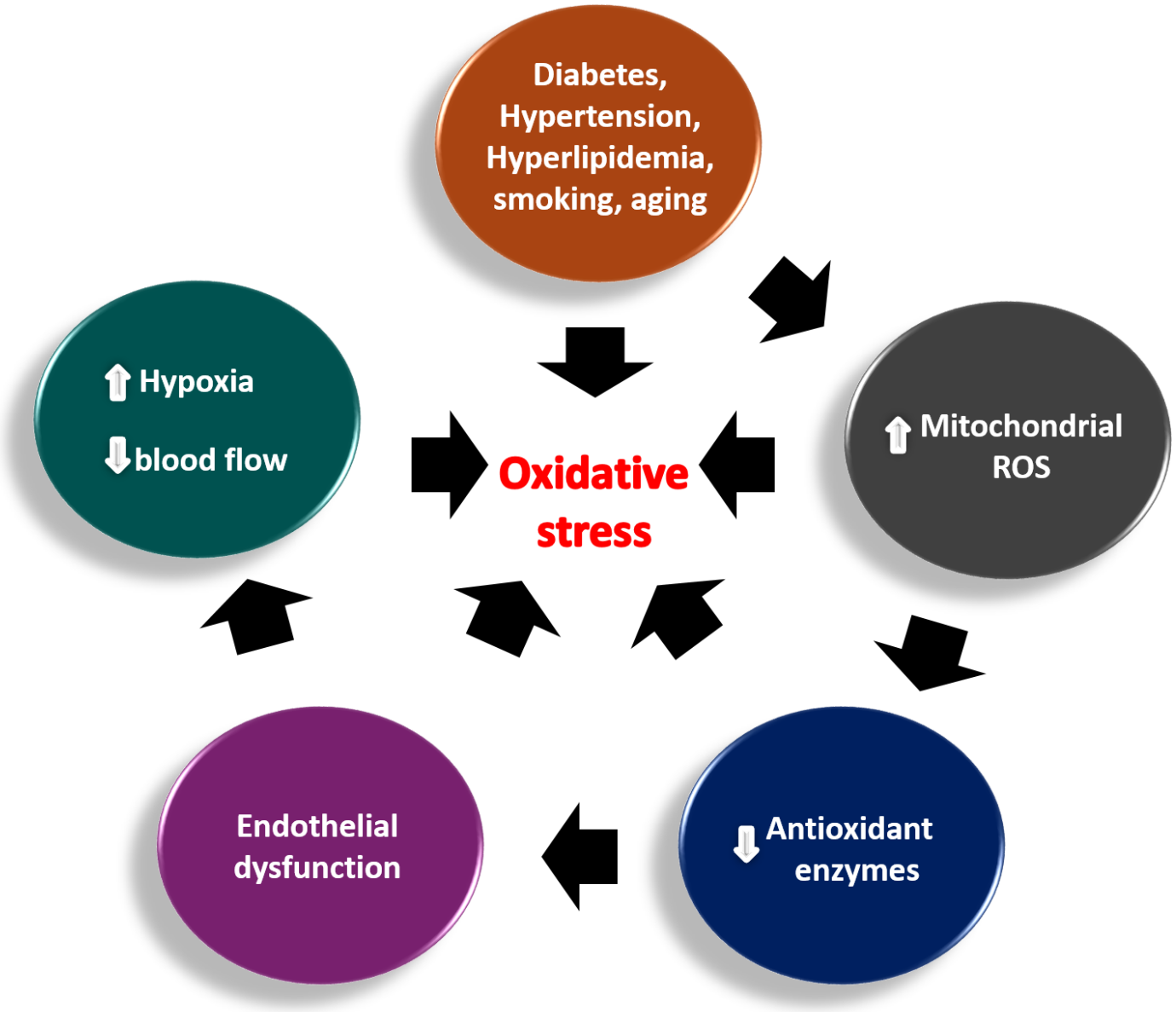



**Figure 4. Oxidative stress risk factors associated with CLTI (This figure was created by Canva.com)**
CLTI - chronic limb-threatening ischemia

**Hyperlipidemia**

Increased cholesterol results in modifications to the cell membrane's structure, which may make it easier for ROS to escape from the mitochondrial electron chain and activate NADPH oxidase. Lipid peroxidation in the cell membrane is caused by these reactive free radicals, which also produce additional free radicals (65). oxLDL suppresses mitochondrial activity and increases the production of mitochondrial ROS, which is implicated in LDL oxidation, leading to a continuous cycle (66). Excess LDLs cause oxidative stress by lowering antioxidant systems like superoxide dismutase, glutathione, and catalase , and elevating the activity of ROS-producing enzymes such as myeloperoxidase (67). High-density lipoprotein (HDL) cholesterol is known to have strong anti-inflammatory and antioxidant effects, as well as the potential to inhibit cholesterol transport and encourage cholesterol efflux from macrophages. HDL function depends on the phospholipid monolayer on its surface. OxLDLs are an effective provider of phospholipid oxidation, which leads to HDL inactivation (68). HDL levels are frequently lower in hyperlipidemia, and they also exhibit altered structure and decreased efficiency (69).

**Hypertension**

An elevation in blood pressure within the blood vessels induces a biological stress response, which is transferred to the intracellular region via different sensor systems, and it has been contributed to the elevated generation of ROS, which serves as the cause for vascular dysfunction (70). Angiotensin II (Ang II) significantly increases the function of the NADPH oxidase enzyme, which leads to the generation of superoxide anion via the AT1 receptor. Long-term Ang II activation also increases the production of certain NOX components, such as p22 $^{phox}$ and p47 $^{phox}$, and reduces the expression of several scavenging proteins in the cell, which raises intracellular ROS levels. Also, elevated ROS levels may promote the expression of renin-angiotensin-aldosterone system factors that prefer Ang II, creating a potentially damaging feedback loop (71, 72). Furthermore, PKC and Rho family guanosine triphosphatases (GTP), such as Rac1, are phosphorylated and activated during Ang II stimulation. These processes are necessary for the formation of the functional NOX molecule at the cell membrane (73). Elevated aldosterone can also cause oxidative stress through a variety of mechanisms, such as activating NADPH oxidase and uncoupling of NOS (74). An overproduction of unfolded and misfolded proteins in the endoplasmic reticulum (ER) lumen, known as ER stress, is becoming recognized as a potential contributor to hypertension, which leads to the accumulation of ROS and oxidative stress. Additionally, mitochondrial malfunction and an increase in mitochondrial ROS production can be driven by ER stress (75). Additionally, decreases in glutathione peroxidase and superoxide dismutase activity have been reported in hypertensive people, increasing the oxidative stress state (76).

**Aging**

The oxidative stress theory of aging relies on the structural damage due to the generation of oxidative degradation of macromolecules such as DNA, lipids, and proteins via RNS/ROS, resulting in age-related biological deficits (77). Oxidative stress is a result of a variety of modifications caused by age, including mitochondrial malfunction and a decline in the effectiveness of different physiological antioxidant defense mechanisms (78). The existence of a smaller percentage of l-arginine in the cytoplasm of endothelial cells to be used as an eNOS substrate is one of the mechanisms causing the uncoupling of eNOS and diminished NO release with aging. This is because arginase, an enzyme that breaks down L-arginine, is more expressed and active in elderly individuals (79). Aging-related insufficient autophagy is thought to be another factor contributing to a rise in oxidative stress, which builds up damaged proteins and encourages more oxidative stress along with inflammation (80). Telomere shortening is a significant biomarker of aging that activates the DNA damage response. The continuous triggering of these damage pathways causes cells to enter senescence or apoptosis, as well as inducing a pro-inflammatory response, which exacerbates oxidative stress within the cell. It can often be associated with mitochondrial malfunction, leading to the overproduction of ROS (81).

**Smoking**

Numerous smoking-related illnesses are attributed to systemic oxidative stress, and smoking is recognized to be a cause and a promoter of oxidative stress (82). Cigarette smoke contains thousands of compounds that can induce cellular oxidative stress, including quinones, reactive aldehydes, polycyclic hydrocarbons, and reactive oxygen and nitrogen species (ROS/RNS) (83). It has been demonstrated that smoking tobacco cigarettes significantly raises NOX2 activity, which is responsible for the increased production of superoxide in the vasculature (84). Cigarette smoking partially depletes cellular BH4 by i mpeding its de novo production via degradation of its rate-limiting enzyme GTP cyclohydrolase (GTPCH) by the ubiquitin proteasomal system (UPS). This leads to eNOS uncoupling and subsequently the generation of peroxynitrite anion (83, 85). The tobacco smoke tar phase compounds reduced molecular oxygen to produce $O^{-}_{2}$, which can then be converted into other free radicals such as $H_2O_2$ and OH. In addition, metals in cigarettes, such as chromium, nickel, iron, and copper, generate ROS via Fenton-like reactions, causing oxidative stress, whereas metals such as arsenic, cadmium, and mercury have an indirect effect on the antioxidant response by hindering GSH function, lowering its availability for the cellular antioxidant system. This creates an additional load of ROS (86). In addition to GSH, important endogenous and exogenous antioxidants like vitamin C (ascorbic acid), carotene, glutathione peroxidase, and superoxide dismutase are all downregulated by cigarette smoke (87). Furthermore, in the vapor part, Acrolein can stimulate the development of free radicals in endothelial cells (82).

**Therapeutic potential and clinical implications**

While conventional management options, such as endovascular or open surgical revascularization and angioplasty, are established treatments for CLTI (88, 89), adjunctive therapies targeting underlying mechanisms are warranted.

Focusing on reducing oxidative damage, enhancing NO-driven angiogenesis, and leveraging HIF-1's role in promoting tissue oxygenation and vascular growth may have a potential outcome for improvement of the CLTI clinical outcomes.

Numerous antioxidants have shown encouraging results in lowering oxidative stress and enhancing CLTI outcomes. For instance, Lejay et al. (90) showed that N-acetyl cysteine therapy, which is thought to be a free radical scavenger by preserving an abundance of the rate-limiting cysteine precursor for glutathione antioxidant, permitted functional enhancement and restored mitochondrial activity in CLTI. Kuroda et al. (91) indicated that the application of Edaravone decreased oxidative stress in the ischemia-reperfusion limb. Furthermore, prior research has shown that therapy for hind limb ischemia with the significant antioxidants ellagic acid, berberine, and coenzyme Q10 can reduce lipid peroxidation byproducts and boost antioxidant enzyme activity (92, 93). Furthermore, Karas et al. (94) demonstrated that selenium nanoparticles have a significant capacity for capturing free radicals, resulting in faster wound healing. They also increase endogenous antioxidant enzyme activity by raising GSH and the mRNA expression of their associated enzymes, superoxide dismutase, and catalase.

The use of exogenous NO and activating eNOS has proved to be useful in increasing angiogenesis and arteriogenesis under critical ischemia conditions (95). According to Lessiani et al. (96), individuals with CLTI who use Iloprost had considerably higher levels of plasma nitrate and nitrite as well as significantly lower levels of endothelial dysfunction and oxidative stress. Moreover, as reported by Morita et al. (97), supplementing with l-arginine and l-citrulline increased plasma l-arginine levels more quickly and significantly improved NO bioavailability and plasma cGMP level in atherosclerotic rabbits. Statins were indicated to enhance the expression of the NOS3 gene and eNOS activity by enhancing the levels of $BH_4$ via activation of its rate-limiting enzyme GTP cyclohydrolase 1 or inhibiting its oxidation (98). Additionally, naftidrofuryl, a 5-hydroxytryptamine type 2inhibitor, and cilostazol, a phosphodiesterase III inhibitor, are regarded as NO donors, as both can raise NOS expression, which triggers NO production in PADs (99). Efficient NO-releasing systems have been used to transport NO donors to particular areas, bypassing the limitations of NO's short lifespan and its random delivery. For example, Wijaya et al. (100) developed PLGA nanomaterial loaded with L-arginine and lovastatin to accomplish long-term release and increase NO production, exhibiting effective outcomes for atherosclerosis treatment. Ma et al. (101) constructed a nanosystem with porous cerium oxide nanoparticles loaded with L-arginine. The released L-arginine has been transformed into NO, which has a high potential for improving wound healing (101). Furthermore, Hou et al. (102) developed a NO delivery system relying on an enzyme-prodrug pair, galactosidase-galactosyl-NO, which has significantly boosted its therapeutic efficiency in the management of ischemic disorders and eliminated adverse effects related to the systemic release of NO. Cell therapy can also be employed to enhance NO production; for example, Madaric et al. (103) employed stem cell therapy in patients with CLTI and showed that the implemented bone marrow-derived mononuclear cells were responsible for decreased levels of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, resulting in a rise of NO and a reduction of oxidative stress. Using tissue engineering techniques, Zhang et al. (104) constructed vascular grafts using eNOS-modified MSCs implanted and grown on electrospun tubular scaffolds to generate artificial vessels that generated high eNOS enzyme and NO and might be effective in blood vessel regeneration.

Because HIF-1α plays a fundamental role in stimulating angiogenesis during hypoxic circumstances, it has been thoroughly investigated as a potential therapy for CLTI (48). Botusan et al. (105) found that dimethyloxalylglycine, a competitive antagonist of α-ketoglutarate, a cofactor for α-KG-dependent dioxygenases, including prolyl hydroxylase, can inhibit prolyl hydroxylase function and enhance HIF-1α stabilization, implying tissue regeneration and wound healing. Furthermore, several recent studies suggest that gene therapy might be utilized to boost HIF-1 levels and alleviate ischemia effects. For example, Vincent et al. (106) found that injecting a rabbit model of hindlimb ischemia with the HIF-1α/VP16 hybrid gene resulted in improved blood flow to affected regions. In a study by Rajagopalan et al. (107), individuals with PAD and CLTI had pain reduction and ulcer healing after receiving a single inramuscular injection of an adenoviral vector of HIF-1α.

In a diabetic model of CLTI, Sarkar et al. (108) demonstrated that an adenovirus encoding a constitutively active form of HIF-1α improved the recovery of limb perfusion and performance, restored the function of angiogenic cells, enhanced eNOS activity, and increased vessel density in the ischemic limb. HIF-α activity can also be reestablished through cell-based therapy.

For instance, in a mouse hind limb ischemia model, Pei et al. (109) indicated that transfecting HIF-1α into scaffold-based cardiac stem cells (CSCs) and introducing them to ischemic tissue resulted in enhanced circulation with muscle regeneration, suggesting that HIF-CSCs may be a potential alternative treatment for ischemic tissue reconstruction.

**Gaps in current knowledge**

There are few studies that explicitly look at the oxidative stress-NO-HIF tripartite interaction in human CLTI tissues; the majority of the information comes from general PAD or animal models of hind limb ischemia. Therefore, there is a need for models that replicate the complicated, contemporaneous interactions of ROS, NO, and HIF in order to create successful multi-target treatments. In addition, gaps include revascularization testing with NO donors, HIF stabilizers, or oxidative stress scavengers. Furthermore, multivariate research is necessary since comorbidities like diabetes influence results.

**Future perspectives**

CLTI patients show a wide range of differences in oxidative stress levels, microvascular dysfunction, and hypoxia signaling, making their condition highly heterogeneous. Incorporating advanced omics technologies such as transcriptomics, metabolomics, and redox proteomics, and integrating cutting-edge experimental platforms such as organoids and organ-on-chip technologies can help create personalized disease profiles for each patient. Combining these molecular insights with biochemical markers, imaging results, and clinical data through predictive algorithms can enhance diagnostic precision and treatment planning. Such strategies hold the promise of transforming CLTI management into a more personalized, effective approach tailored to individual patient needs.

**Conclusion**

In summary, oxidative stress, nitric oxide insufficiency, and hypoxia-inducible factor signaling interact intricately to cause chronic limb-threatening ischemia. Increased oxidative stress reduces the bioavailability of nitric oxide, thereby worsening endothelial dysfunction. Despite stabilizing HIF in this hypoxic environment, which encourages compensatory mechanisms like angiogenesis, these adaptations are not enough to mitigate ischemic tissue damage. Comprehending and focusing on these oxidative pathways, reestablishing nitric oxide signaling, and adjusting HIF activity have considerable therapeutic promise for enhancing CLTI results in clinical trials.

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