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DOI:

[10.1016/j.pharmthera.2017.09.003](https://doi.org/10.1016/j.pharmthera.2017.09.003)

*Document Version*

Peer reviewed version

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*Citation for published version (APA):*

Jain, T., Nikolopoulou, E. A., Qu, A., & Xu, Q. (2017). Hypoxia inducible factor as a therapeutic target for atherosclerosis. *Pharmacology and Therapeutics*. Advance online publication.

<https://doi.org/10.1016/j.pharmthera.2017.09.003>

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## Accepted Manuscript

Hypoxia inducible factor as a therapeutic target for atherosclerosis

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PII: S0163-7258(17)30237-1  
DOI: doi:[10.1016/j.pharmthera.2017.09.003](https://doi.org/10.1016/j.pharmthera.2017.09.003)  
Reference: JPT 7128

To appear in: *Pharmacology and Therapeutics*



Please cite this article as: Jain, T., Nikolopoulou, E.A., Qu, A. & Xu, Q., Hypoxia inducible factor as a therapeutic target for atherosclerosis, *Pharmacology and Therapeutics* (2017), doi:[10.1016/j.pharmthera.2017.09.003](https://doi.org/10.1016/j.pharmthera.2017.09.003)

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P&amp;T 23149

**Hypoxia Inducible Factor as a Therapeutic Target for Atherosclerosis**Tanmay Jain<sup>1\*</sup>, Eleni Aliko Nikolopoulou<sup>1\*</sup>, Aijuan Qu<sup>2</sup> and Qingbo Xu<sup>1</sup><sup>1</sup>Cardiovascular Division, King's College London BHF Centre, London, UK<sup>2</sup>Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Capital Medical University; Key Laboratory of Remodelling-Related Cardiovascular Diseases, Ministry of Education, Beijing, China**Word count:** 7,587**Running title:** HIF and atherosclerosis

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## Abstract

Atherosclerosis is a highly prevalent disease that can significantly increase the risk of major vascular events, such as myocardial or cerebral infarctions. The anoxemia theory states that a disparity between oxygen supply and demand contributes to atherosclerosis. Hypoxia inducible factor-1 (HIF-1) is a heterodimeric protein, part of the basic helix-loop-helix family and one of the main regulators of cellular responses in a low-oxygen environment. It plays a key role in the development of atherosclerosis through cell-specific responses, acting on endothelial cells, vascular smooth muscle cells (SMCs) and macrophages. Through the upregulation of VEGF, NO, ROS and PDGF, HIF-1 is able to cause endothelial cell dysfunction, proliferation, angiogenesis and inflammation. Activation of the NF- $\kappa$ B pathway in endothelial cells is an important contributor to inflammation and positively feeds back to HIF-1. HIF-1 also plays a significant role in both the proliferation and migration of smooth muscle cells – two important features of atherosclerosis, while the formation of foam cells (lipid-laden macrophages) is also a critical step in atherosclerosis and mediated by HIF-1 through various mechanisms such as dysfunctional efflux pathways in macrophages. Overall, HIF-1 exerts its effect on the pathogenesis of atherosclerosis via a variety of molecular and cellular events in the process. In this review article, we examine the effects of HIF-1 on vascular cells and macrophages in the development of atherosclerosis, highlighting the environmental cues and signalling pathways that control HIF-1 expression/activation within the vasculature. We will highlight the potential of HIF-1 as a therapeutic target on the disease development.

**Keywords:** HIF, endothelial cells, smooth muscle cells, macrophages, atherosclerosis

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## 1. Introduction

Atherosclerosis is a chronic inflammatory disorder that affects the arteries in the body and has a high association with comorbidities and mortality (Gao et al., 2012). It is the primary cause of coronary artery disease and stroke in the developing world (Jaipersad et al., 2014b) and most common cause of coronary artery disease and peripheral arterial disease worldwide (Sanchis-Gomar et al., 2016). The development of atherosclerosis is initiated by endothelial activation and involves lipid accumulation within the layers of the arterial wall, monocyte adherence, infiltration and the formation of a lipid core. Foam cell (lipid-laden macrophages) and smooth muscle cell proliferation are characteristic features of atherosclerosis. Monocytes migrate into the arterial intima where they proliferate and differentiate into macrophages. These macrophages proceed to take up oxidised LDL, forming foam cells. As a consequence of cell aggregation, initial lesions progress to advanced plaques. Production of reactive oxygen species (ROS) and ongoing inflammation leads to matrix degradation and a combination of cellular apoptosis and necrosis. This can cause eventual thinning of the fibrous cap on the plaque. Complications of atherosclerosis can include occlusion of the vascular lumen due to plaque growth or rupture of vessel wall and formation of a thrombus (Hansson, 2005).

As the plaque develops, the arterial wall thickens, significantly reducing oxygen diffusion into the intima. There is increasing evidence to suggest hypoxia and oxygen disruption in the pathogenesis of atherosclerosis. The anoxemia theory of atherosclerosis states that a mismatch between oxygen supply and demand in the arterial wall leads to the development of lesions and plaques (Björnheden et al., 1999). This is thought to occur by a number of potential mechanisms, such as promoting lipid accumulation, increasing inflammation and angiogenesis (Hultén and Levin, 2009). However, despite the plethora of research, money and time invested in this field, there has been limited development of successful preventative and therapeutic treatments (Duguid, 1946; Grotel, 1945). In order for such treatments to be generated a greater understanding of the molecular events leading up to inflammation is required. One protein family of interest is hypoxia inducible factor (HIF).

HIF is a known vascular transcription factor. It is heterodimeric and consists of two main subunits,  $\alpha$  and  $\beta$ , but also has three forms, HIF-1, HIF-2 and HIF-3. It has been shown that in low oxygen concentrations HIF functions to a greater extent than in normal or high oxygen conditions. Hypoxia, decreases the usual hydroxylation of HIF, limiting its proteolytic degradation. HIF enhances the compliance of the both cells and tissue in hypoxic states; it affects mitochondrial oxygen consumption, regulates mitochondrial autophagy, increases serum erythropoietin (a protein coding for increased production of red blood cells that carry oxygen to the tissues), and more crucially, controls angiogenesis. A hot topic, attracting interest in the past decade, is the role of HIF in the progression of vascular diseases and how changes in its expression levels affect the resolution of such conditions. In addition to the known link between HIF and oncogenesis, HIF is also associated to angiogenesis occurring in vascular diseases, such as angiogenesis after thrombosis and stenosis, or the weakened vessel formation in atherosclerosis, which increases the risk of plaque rupture or total arterial occlusion. Although it is known that HIF plays a role in the progression of atherosclerosis, it is important to note that in some cases HIF may

play a beneficial role. As demonstrated by revascularization and collateral circulation in ischaemic brains; HIF appears to be primarily induced in the penumbra – the salvageable tissue post ischaemia (Shi, 2009). Similar evidence of revascularization has also been found in non-ischemic tissue, such as corneal revascularization, which may increase the viability of the cornea in degenerative conditions or abrasions (Kelly et al., 2003). This review aims to evaluate the role of HIF in atherosclerosis and to highlight its potential as a therapeutic target for treatment of atherosclerosis.

## 2. HIF family

HIFs are critical mediators of the cellular response to oxygen. They belong to the Per-ARNT-SIM subfamily of the basic helix-loop-helix (bHLH PAS) protein family that can regulate cells in low oxygen environments. To date, 3 transcription factors have been discovered (HIF-1, 2, 3; Figure 1). As HIF is a transcription factor, it determines angiogenesis and thus oxygen supply to tissues under hypoxic conditions, through the regulation of transcription of genes including Vascular Endothelial Growth Factor (VEGF) and Erythropoietin (EPO). VEGF is the major protein involved in promoting vessel formation throughout the organism life, whereas EPO controls erythropoiesis, the production of red blood cells. (Benizri et al., 2008; Formenti et al., 2010; Smith et al., 2008; Wilkins et al., 2016)

The key regulator of hypoxia, HIF-1 is a ubiquitous transcription factor able to control the responses of nucleated cells in hypoxia. It is a heterodimer protein made up of HIF-1 $\beta$  (its activity is unaffected by hypoxia) and HIF-1 $\alpha$  - the active subunit, that has a half-life of 5 minutes and is highly regulated by oxygen (Salceda and Caro, 1997). HIF-1 $\alpha$  is expressed in all nucleated cells (Lim et al., 2013) and enables a rapid response to hypoxia. A significant amount of energy is devoted to the continuous turnover of HIF-1 $\alpha$  in normoxic conditions. HIF-1 $\alpha$  allows cells to adapt to low oxygen environments by activating genes that alter energy metabolism (Semenza et al., 1994), cell proliferation (Carmeliet et al., 1998b), angiogenesis and vascular remodelling (Rose et al., 2002).

In non-hypoxic conditions, two specific proline residues on HIF-1 $\alpha$  protein are hydroxylated in oxygen-dependent degradation domains by prolyl hydroxylase domain proteins (PHD). In addition to that, acetylation of a lysine residue allows for the binding of von Hippel-Lindau, which interacts with the elongin C protein. This facilitates the recruitment of an ubiquitin ligase complex, marking HIF-1 $\alpha$  for proteasomal degradation. FIH-1 (Factor inhibiting HIF-1) is also able to inhibit HIF-1 through the hydroxylation of asparagine residue 803. This blocks the transactivation activity, thus enabling the negative regulation of HIF-1. During hypoxia, the oxygen dependent activity of prolyl hydroxylase domain enzymes and FIH-1 are inactivated. Oxygen is required as a substrate for the enzymes to hydroxylate HIF-1 proteins, but as it is unavailable, it causes HIF-1 $\alpha$  to accumulate and translocate to the nucleus (Wang et al., 1995). Here, it dimerises with the HIF-1 $\beta$  subunit through a common helix-loop-helix bonding (Jiang et al., 1996), to form an active transcription factor and proceeds to form a complex with the hypoxia-responsive element (HRE) of target genes.

HIF-2 is similarly made up of 2 subunits and structurally, is approximately 48% homologous to HIF-1. It therefore shares some properties with HIF-1 $\alpha$ , such as

binding with HIF-1 $\beta$ . HIF-2 $\alpha$  plays an important role in development of the embryonic heart and provides essential protection against heart failure via catecholamine homeostasis in utero (Sun et al., 2015). Although HIF-1 is ubiquitous, HIF-2 appears to be cell type specific, including, but not limited to endothelial cells, cardiomyocytes, glial cells and hepatocytes (Wiesener et al., 2003). The involvement of HIF-2 in cancers through cell proliferation, angiogenesis, metastasis and resistance to chemotherapy, has been well established (Zhao et al., 2015).

The role of HIF-3 is less understood. The gene is able to give rise to several variants of HIF-3 $\alpha$  through the use of different promotor regions, different transcription initiation sites and varied splicing. Thus, these variants are expressed in different tissues at different development stages, with varied responses to hypoxia. There is strong evidence to suggest that some variants have contrasting functions (Rankin and Giaccia, 2008). HIF-3 plays an important role in the regulation of HIF-1 and HIF-2. While some HIF-3 variants are able to act as dominant-negative regulators, other variants can inhibit HIF-1/2 (by competing for the binding of HIF-1 $\beta$ ) (Duan, 2016). As HIF-1 $\alpha$  has been the focus of most studies, any reference to HIF will be regarding the HIF-1 $\alpha$  subunit, unless otherwise stated.

HIF activates gene transcription mainly by binding to a cis-acting regulatory element named “hypoxia response element (HRE)” as defined by the core DNA sequence 5'-A/GCGTG-3'. Genome-wide chromatin immunoprecipitation (ChIP) assays have identified that HIF directly regulates the expression of more than 1,000 genes in human. Some examples of adaptive responses initiated under hypoxia include matrix metabolism (MMP-1, MMP-2, MMP-3), angiogenesis (VEGF, ENG, LEP), erythropoiesis (EPO) and cell proliferation (Cyclin G2, IGF2). Other than hypoxia, PHD enzymes can also be inhibited through other mechanisms, such as reactive oxygen species (Fe<sup>2+</sup> is oxidized to Fe<sup>3+</sup> – the depletion of the essential Fe<sup>2+</sup> cofactor inactivates the enzymes) (Niecknig et al., 2012).

Recent studies show that less than 2% of human DNA codes for protein and 98% is composed of various classes of non-coding RNAs, such as microRNA (miRNA), tRNA, snRNA and long non-coding RNAs (lncRNAs). Some of these have the potential to control gene expression via transcription, post-transcription and epigenetic regulation, and can often become dysfunctional in disease processes. MiRNAs stabilise and translate mRNA; long-non-coding RNAs have a more varied function, such as chromatin modification and have been reported to play a key role in HIF-1 regulation (Choudhry et al., 2016). The action of HIF-1 can be modulated in hypoxic condition. This may occur through both the upregulation and downregulation of specific non-coding RNA molecules. miR-210 has been studied in cells affected by tumours and cardiac disease. It plays a critical role in cell differentiation, proliferation and angiogenesis in hypoxic environments. When studied on murine models, it was able to improve angiogenesis and cardiac function in the treatment of heart disease (Hu et al., 2010). Although they play a major regulatory role, it is important to note that a direction relationship between non-coding RNA molecules and HIF transactivation was not found. Furthermore, much of the research between non-coding RNA and HIF exists in the context of cancer and oncogenesis, as opposed to atherosclerosis (Shih et al., 2017).

The role of these HIF-1-dependent homeostatic mechanisms in the pathophysiology of atherosclerosis is described in greater detail below.

### 3. HIF and endothelial cells

As endothelial cells are the first layer in contact with blood, it is important they are able to regulate vascular homeostasis. This is maintained through various mechanisms such as anti-thrombotic functions, recruitment of inflammatory mediators and the endothelial cell effect on vascular tone. These mechanisms are altered when the vasculature experiences hypoxia (Ten and Pinsky, 2002). Through the activation of HIF-1 $\alpha$ , the cell is able to adapt to lower metabolic needs in a low oxygen environment. Both directly and indirectly, more than 2% of all human genes are regulated by HIF-1 in endothelial vascular cells (Manalo et al., 2005).

HIF-1 $\alpha$  causes major alteration within endothelial cells, through 3 main ways: vascular endothelial growth factor (VEGF), nitric oxide (NO) and reactive oxygen species (ROS). An upregulation of VEGF, free radicals and nitric oxide production, along with platelet-derived growth factor (PDGF) promote the development of atherosclerosis. This is worsened by endothelial cell dysfunction, endothelial cell proliferation, angiogenesis and inflammation (Gao et al., 2012). Overproduction of free radicals and ROS lead to a large disruption of endothelial cell function (Madamanchi et al., 2005). In hypoxia, two major responses have been observed in endothelial cells, which depend on the severity and duration of oxygen loss. Acute hypoxia leads to activation of endothelial cells, causing them to release inflammatory mediators and growth factors that promote the adherence of phagocytes (such as neutrophils and macrophages) to the vascular wall. Over a longer period of time, hypoxia causes transcriptional induction of genes such as Glut-1 (involved in transendothelial glucose transport), VEGF and iNOS (inducible nitric oxide synthase), enabling cells to survive in low oxygen environments (Harris et al., 2014) (Figure 2).

HIF-1 upregulates the production of VEGF by binding to the hypoxia response element (HRE) in the VEGF promoter region. VEGFR-1 and VEGFR-2 are the two main receptors expressed on endothelial cells, both mediated by HIF-1 (Kaur et al., 2005). The upregulation of VEGFR-1 is directly induced by HIF binding to the enhancer element within the VEGFR-1 promoter region, whereas the expression of VEGFR-2 during hypoxia is via post-transcriptional regulation. VEGF acts as a chemotactic and mitogenic factor on endothelial cells via both VEGFR-1 and VEGFR-2 causing cell proliferation, migration, enhancing cell permeability and angiogenesis (Tang et al., 2004).

The production of NO is regulated by HIF-1 through 2 major mechanisms: increasing iNOS and subunit 4-2 of cytochrome c oxidase (COX4-2). In order to increase perfusion, HIF-1 activates iNOS which increases NO production and leads to vascular vasodilation, increasing blood and oxygen supply to cells (Hendrickson MD, 2015). Interestingly, some studies have reported that iNOS is able to positively regulate VEGF expression, however this was seen specifically in tumorigenesis (Van der Wall, 2006; Wang, 2001).

Several studies have been done to assess the individual phenotypic processes involved in angiogenesis such as cell migration or cell proliferation of endothelial cells. There have been conflicting results with some studies showing hypoxia activating endothelial cell proliferation (through stimulation by adenosine) (Meininger



et al., 1988) and others showing a reduction in proliferation (Shreeniwas et al., 1991; Tucci M, 1997). However, the exact reason for these differences is unclear, due to the nature of *in vitro* testing and isolated system use, it could be caused by varying severity of hypoxia, type of endothelial cell used (human vs bovine) or other factors, such as integrated counter-regulatory controls.

One study concluded that although endothelial cells were able to grow and survive in hypoxia, they had altered function. Their growth was slower in hypoxia, and despite a confluent monolayer being achieved *in vitro*, the cells were larger in size. That in addition to an altered actin-based cytoskeleton seen post-hypoxia, meant that adjacent cells had small gaps, leading to increased permeability of the vasculature (Meininger et al., 1988).

It has been well recorded that HIF-1 activation correlates with atherosclerotic disease progression and inflammation (Sluimer et al., 2008; Vink et al., 2007). In atherosclerotic endothelial cells, CXCL1 and TNF $\alpha$  were found to be the two most significant pro-inflammatory cytokines released. Mildly oxidized-LDL (and its derivative LPA) induced pro-inflammatory status in endothelial cells through the upregulation of HIF-1 $\alpha$ . An increased expression of miR-19a, a non-coding RNA molecule that regulates gene expression, led to the activation of the NF- $\kappa$ B inflammatory pathway and pro-inflammatory cytokine release. Endothelial NF- $\kappa$ B activation also upregulates HIF-1 expression via creating a positive feedback loop. Alternatively, endothelial cells deficient in HIF-1 had a downregulation of miR-19a in atherosclerotic arteries (Akhtar et al., 2015). Thus, they were able to conclude that hyperlipidemia caused an increase in HIF-1 production and endothelial cell activation. HIF-1 accumulates in cells primarily due to protein stabilisation in hypoxia, however glycolysis (an anaerobic process) is the main form of energy in endothelial cells and oxygen supply is not limited to endothelial cells via the blood stream, at least in the primary stages of the disease. Thus, non-hypoxic stimuli such as angiotensin II, TNF $\alpha$  or MoxLDL may be more important for the activation of HIF in endothelial cells, at least in the initial stages of atherosclerosis.

#### 4. HIF and smooth muscle cells

The proliferation and migration of smooth muscle cells from the media to the intima is one of the key features of the atherosclerotic process. One of the first signs of atherosclerosis is a pathological thickening of the intima (de Groot et al., 2008). It has been documented that hypoxia, more specifically HIF-1 $\alpha$ , is a stimulus of vascular remodelling – ultimately leading to a narrowing of the arterial lumen (Diebold et al., 2008; Schultz et al., 2009). Migration Inhibitory Factor (MIF) has been described as an important mediator of this process. Under hypoxic conditions, HIF-1 $\alpha$  is upregulated, causing an overexpression of the MIF gene (alternatively, a knockout of the HIF-1 $\alpha$  gene in human umbilical artery smooth muscle cells - HUASMCs - inhibited MIF production) (Fu et al., 2010). MIF is an important upstream product of the inflammatory cascade and has been associated with foam cell transformation along with vascular remodelling. Hypoxia led to the activation of several mediators such as ROS and extracellular signal-regulated kinase (ERK). Blocking both (with an antioxidant and PD98059 respectively), caused a decrease in hypoxia induced HIF-1 expression, indicating their importance in the process. HUASMCs were cultured in hypoxia (3% oxygen) and proliferated 113.6% more than

cells cultured in normoxia. Furthermore, there was only partial proliferation in MIF-siRNA blocking cells and no proliferation seen in HIF-1 knockout cells, suggesting that MIF is involved in HUASMC proliferation. The effect of MIF in cell migration was assessed in a similar manner, resulting in identical findings. MIF-siRNA partly prevented migration in both hypoxia and normal conditions. Over expression of HIF caused increased HUASMC proliferation, even in normoxia and knockdown of HIF expression prevented any hypoxia induced migration (Fu et al., 2010)

Thrombospondin-1 (THBS1) expression has been associated with migration of human vascular smooth muscle cells (VSMC) (Ichii et al., 2001; Patel et al., 1997). However, the factors that regulate the migration of VSMCs in hypoxia are still being explored. It has been found that THBS1 is upregulated in hypoxic conditions via the HIF-1 $\alpha$  pathway (Phelan et al., 1998). Hypoxia-induced migration of VSMCs was inhibited by a neutralising antibody against THBS1 and by a RDG peptide binding to integrin  $\beta$ 3. Supporting previous evidence of the relationship between integrin  $\beta$ 3 and THBS1 in migration (Sajid et al., 2000). Neutralising antibodies to THBS1 did not affect hypoxia-induced DNA synthesis, suggesting that THBS1 is unlikely to be involved in VSMC proliferation, but rather migration. It is more likely that proliferation is due to a different HIF-dependent pathway such as PDGF or fibroblast growth factor (FGF) (Schultz et al., 2006). HIF-1 knockdown led to a decrease in hypoxia induced THBS1 and subsequent reduction in cell migration. Simultaneously, an overexpression of HIF-1 led to increased migration compared to control cells (Osada-Oka et al., 2008).

It is well known that PDGF is involved in proliferation and migration of VSMCs, however its exact relationship in hypoxia is less well known. PDGF-dependent proliferation and migration of smooth muscle cells is increased in hypoxia, with PI3K and PLC $\gamma$  important mediators of the pathway. Chronic hypoxia leads to an accumulation of HIF-1 in VSMCs, causing a decrease in expression of PDGFR-antagonising protein tyrosine phosphatases (PTPs). These PTPs are important negative regulators and in their absence, ligand-induced PDGFR phosphorylation is enhanced, along with cellular proliferation and migration. Pharmacological inhibition of PI3K and PLC $\gamma$  prevented PDGF-dependent migration and proliferation in both hypoxic and normal oxygen environments, suggesting that the hypoxia sensitive signalling molecule is proximal in the signalling pathway, although the exact mechanism is unclear (Freyhaus et al., 2011). One study did hypothesise that cell proliferation was linked to the stimulation of cyclin A gene transcription (Schultz et al., 2006). It is also important to mention that hypoxia alone (without ligand binding) did not lead to PDGF-dependent proliferation or migration, eluding to an indirect hypoxia induced receptor activation and phosphorylation (Freyhaus et al., 2011).

Similar mitogenic cellular responses (increased migration and proliferation of VSMCs) were seen when using fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) - also 2 tyrosine kinase-receptor activating growth factors. Interestingly, hypoxia did not induce mitogenic effects in the presence of mitogens that signal via serine/threonine kinase receptors (such as TGF- $\beta$ ) or via G protein coupled receptors (thrombin and angiotensin II). It is not clear whether the reason for this is the selective influence of hypoxia on mitogens or whether it is due to the level of hypoxia or inadequate expression of receptors/downstream signalling molecules.

## 5. HIF and macrophages

An accumulation of macrophages and their uptake of oxidised LDLs is an important feature in atherosclerosis. It is widely known that macrophages are major contributors to atherosclerotic progression (Nakashima et al., 2007). HIF-1 $\alpha$  is able to activate pro-angiogenic genes in macrophages, such as TNF $\alpha$  and VEGF. It also appears to play a critical role in the maturation of macrophages (Fang et al., 2009). Monocytes produce PDGF that causes mitotic division of both vascular smooth muscle and endothelial cells, contributing to the vascular remodelling process in disease progression. Once activated, the monocytes and macrophages that have infiltrated the plaque, as a result of increased permeability and local inflammation, take up oxidized lipids and become foam cells (Figure 3). Foam cells have a similar contribution of vascular remodelling by stimulating migration in smooth muscle cells (Kruth, 2001). Although macrophages are derived from the monocyte lineage, once monocytes have infiltrated the tissue layers of the blood vessel, a considerable number of them will differentiate into dendritic cells. This leads to a subsequent activation of T lymphocytes via specific antigens contributing to local inflammation (Elsheikh et al., 2005).

The ability of cells to remove excess cholesterol is important for survival and most of them do this via efflux pathways. For the efflux of cholesterol, both a plasma membrane transporter and cholesterol acceptor are required. Under hypoxic conditions cholesterol and triglyceride levels were significantly higher in human macrophages. Interestingly, the cholesterol content was different as well. Macrophages cultured in hypoxia showed a higher percentage of unesterified cholesterol, which can be toxic to human macrophages (Liu et al., 2007). Normally macrophages are able to esterify free cholesterol into cholesteryl esters for storage as lipid droplets, however, as expected, cholesteryl esters were significantly lower in hypoxic macrophages. Under hypoxic conditions, the overexpression of HIF-1 caused a decrease in cholesterol efflux in macrophages, leading to the formation of foam cells. The ability of ABCA1, the major cholesterol efflux transporter in macrophages, had a substantially reduced function (84% decrease) compared to macrophages in normoxia (Parathath et al., 2013). Alternatively, HIF-1 knockdown cells in hypoxic conditions showed decreased HMG CoA reductase mRNA levels, higher efflux of cholesterol, reduced triglyceride levels and lower total cholesterol content.

A lack of HIF-1 $\alpha$  in macrophages led to a reduced inflammatory phenotype compared to control cells. mRNA expression of osteopontin and MCP-1 (monocyte chemoattractant protein – 1) was decreased under normoxic conditions in HIF-1 knockout macrophages. The expression of iNOS was also reduced in HIF-1 knockdown cells compared to the wild type. In addition, HIF-1 was able to regulate the polarisation of macrophages into the M1 or M2 phenotype. Upon differentiation into the pro-inflammatory M1 phenotype, through interferon  $\gamma$  and lipopolysaccharide, HIF-1 $\alpha$  deficient cells showed a lower expression of M1-typical genes compared to control cells. However, in M2 polarisation by interleukin-4, HIF-1 $\alpha$  knockdown macrophages showed no significant difference. Thus the elimination of HIF-1 $\alpha$  from macrophages blunts the pro-inflammatory phenotype of M1 macrophages (Aarup et al., 2016b).

The retention of apoB-100-containing lipoproteins within the arterial wall seems to be a critical step in the development of plaques. This process is mediated by the negatively charged glycosaminoglycan (GAG) chains on proteoglycans in the intima and the positively charged parts of apoB-100. HIF-1 $\alpha$  has been associated with the production of proteoglycans by macrophages, such as versican. Furthermore, knockdown of HIF-1 $\alpha$  inhibited hypoxia induced production of versican (Heikal et al., 2016). It was also found that human monocyte derived macrophages exposed to a hypoxic environment for longer, secreted GAGs that were more sulphated and displayed a higher affinity for LDL. Indicating that changes in the biosynthesis of macrophages induced by hypoxia may play an important role in the development of atherosclerosis (Asplund et al., 2011).

The contribution of macrophages and macrophage-derived HIF-1 to arterial remodelling has been recorded both *in vitro* and *in vivo* models. There is a significant contribution from HIF-1 to local inflammation due to the production of pro-inflammatory cytokines and chemokines, such as IL-6 and TNF- $\alpha$ . HMGB-1 is a nuclear protein present after arterial injury that contributes to the inflammatory cascade (increased HMGB-1 expression has been seen in necrotic tissues and atherosclerotic plaques). To determine the role of HIF-1 deficient macrophages in secreting pro-inflammatory cytokines, HMGB-1 was used as a stimulant. Under both hypoxic and normal conditions, HMGB-1 led to an increased level of HIF-1 and subsequent elevation of HIF-1 responsive genes (such as Glut-1 and VEGF). However, in HIF-1-deficient macrophages, there was a significantly blunted response to HMGB-1 stimulation, indicating the importance of the HMGB-1/HIF pathway in vascular remodelling.

There was no significant difference found in macrophage proliferation in hypoxia compared to normal conditions. HMGB-1 was similarly used for stimulation in both normal macrophages and HIF-1 deficient macrophages, with no statistical difference being noted. Suggesting HIF-1 may not play a major role in proliferation of macrophages. The migration of macrophages however, was influenced by HIF-1. After MCP-1 stimulation, HIF-1 deficient macrophages showed reduced migration, both in normoxia and hypoxia (Nakayama et al., 2013).

## 6. HIF and atherogenesis

The final stage of plaque development inflammatory cytokines and chemoattractants produced within the plaque, including IL-1 $\beta$  produced by the enlarging foam cells and PDGF, induce the infiltration of proliferating SMCs into the plaque. Collagen production is also stimulated. This limits the vessel compliance and increases the stiffness of the arterial wall. Additional cells are subsequently recruited, including T cells and dendritic cells. Vessel wall rigidity predisposes the wall segment to rupture whereby platelets will then accumulate, in response to exposed collagen and tissue factor, and form a fragile fibrous cap. This further reduces elasticity and risks the development of thrombosis, which could fully occlude the vessel or embolise to a distal area. Parallel to these processes, a major risk factor for the development of the plaque and the progression of complications is the formation of new weak vessels. HIF, which is expressed within the core of the atherosclerotic plaque, induces the expression of VEGF and is associated with angiogenesis. This angiogenesis involves the invasion and formation of new vessels sprouting from the vasa vasorum;

the arterial network supplying the adventitia – the most external of the three layers of the vessel wall. This not only increases the haemodynamic instability and fragility of the plaque, but also allows further infiltration of inflammatory cells, thus additionally increasing the inflammation in the atherosclerotic lesion (Libby et al., 2011; Sluimer and Daemen, 2009). There is a clear correlation between the growth and potential rupture of the atherosclerotic plaque and the expression of VEGF and HIF. Research indicates that adventitial microvessels are more numerous in vulnerable and older plaques, implying that the HIF and the angiogenic pathway is key in atherosclerosis and the progression to its life-threatening complications (Kamat et al., 1987; Lappalainen et al., 2004; Mofidi et al., 2001; Moreno et al., 2004; Sluimer and Daemen, 2009).

Nuclear HIF-1 $\alpha$  has been found to be present in atherosclerotic plaques, suggesting that HIF-1 $\alpha$  was in its active form in these areas, and translocated to the nucleus in order to induce transcription of genes. This was observed in human carotid and femoral endarterectomy specimens, of which about half showed a positive staining for macrophages. (Vink et al., 2007) Furthermore, pimidazole, a stain indicating hypoxia, showed increased hypoxic regions in the atherosclerotic core, thus implying the association of hypoxia with the development of atherosclerosis. In addition, pimidazole was also co-localised with CD163, a specific marker of macrophage receptor. In measuring the expression of HIF-1 $\alpha$  of these cells, it was found to be augmented compared to normal vessel wall segments, inferring the role of macrophage HIF in the progression of atherosclerosis (Aarup et al., 2016a). Co-localisation of macrophages and HIF1 $\alpha$  within the plaque is supported by additional studies, (Luque et al., 2008) including data produced by the use of nuclear staining and CD68+, another known macrophage marker (Higashida et al., 2008; Vink et al., 2007). Findings suggested that the plaque haemorrhage is a common phenomenon in plaques with increased numbers of macrophages, underlining their importance in atherosclerosis. Another important finding is that the expression of PR39, a macrophage derived protein known to inhibit the degradation of HIF-1 $\alpha$ , was greatly increased under hypoxia, in both human and animal cells. Cells secreting this protein are also found in the coronary vasculature. PR39 may be important in explaining the connection between inflammatory cells, HIF-1 $\alpha$  and atherosclerosis, however, further work is required (Li et al., 2000).

HIF1 $\alpha$  is known to control the recruitment of progenitor cells that attempt to repair the ischaemic tissue by inducing the expression of stromal cell-derived factor (SDF-1 $\alpha$ ) (Marsch et al., 2013). However, it is thought that the increase in progenitor cells in the necrotic plaque area may additionally lead to a rise in inflammation and in the vulnerability of the lesion. Research studying the exposure of vascular and perivascular areas to hypoxia, indicated a 7 and 12-fold increase in the expression of SDF-1 after 6 and 9 hours of 1% oxygen exposure respectively. Furthermore, HIF-1 $\alpha$  and SDF-1 were co-localised in approximately 75% of endothelium of arteriole and capillary lining, a finding which was absent in the lack of HIF oxygen dependent degradation domain, induced by genetic modification, signifying the hypoxic element of the process. It is hence proposed that a pharmacological blocker of HIF targeting endothelial cells would decrease inflammation and result in a less fragile plaque, decreasing the risk of complications (Ceradini et al., 2004).

Similarly, it has been suggested that SMCs are also involved in the function of HIF in the atherosclerotic progression. The genetic knockout of HIF-1 $\alpha$  in SMCs in atherosclerosis predisposed apolipoprotein E knockout mice causing a decrease in the plaque size, but also reduced the inflammation of the site. Therefore, it can be assumed that HIF expression in SMCs guides the worsening progression of atherosclerosis (Liu et al., 2016). However, there is opposing evidence advocating that SMC derived HIF has a protective role in aneurysms, another disease process involving atherosclerosis. Here it was found that the deficiency of HIF-1 $\alpha$  in SMCs augmented aortic aneurysms, by disturbing the formation of elastic fibres, but not by effecting the pre-existing arterial fibres. The difference may be explained by the fact that aneurysms are mostly caused by the change in metalloproteinases and compliance of the weakening arterial wall, whereas stenosis due to atherosclerosis leads to fragile, harden walls. Further work is necessary to clearly establish the protective role of HIF (Imanishi et al., 2016).

## 7. HIF and angiogenesis

As mentioned above, angiogenesis occurs in late stage atherosclerosis, i.e. vasa vasorum formation, in which HIF-1 plays a major role. This factor is known to mediate apoptosis and increase vessel formation through controlling the expression of VEGF, a major angiogenic factor. The link between angiogenesis and HIF was made clear through experimentation with transgenic mice. These mice expressed constitutively active HIF-1 $\alpha$  in the epidermis, which led to approximately a 65% increase in the dermal vascularity, as measured by quantification of microvasculature vessel length and density. In addition to this, PCR measurement of VEGF expression in these mice showed a 13-fold increase in total VEGF expression when compared to wild-type controls (Elson et al., 2001). In situations where the loss of HIF-1 was induced, VEGF expression was reduced, again suggesting a major link between the two factors (Role of HIF-1 in hypoxia mediated apoptosis, cell proliferation and tumour angiogenesis) (Carmeliet et al., 1998a).

The importance of VEGF is clear in the embryonic stages of vessel development. Research done, involving the study of VEGF mutant embryos found that heterozygous mutants presented with a great number of vascular anomalies, when compared to wild type embryos, while homozygous VEGF mutants were difficult to study as many died. The defects seen in the heterozygous VEGF mutants resemble the vessel abnormalities in HIF-1 mutant embryos. The link between the two factors was confirmed in this HIF-1 mutant model, as co-transfection of the embryonic cells with HIF-1 $\alpha$  and HIF-1 $\beta$  activated VEGF expression in a 5-fold increased manner under normoxic conditions and in approximately 77-fold increase under hypoxic conditions, when compared to cells with no cotransfection (Carmeliet et al., 1996). Additionally, evidence suggests that neovascular formation requires the inflammatory process of endothelial activation, which involves VEGF interaction with its receptors. This is thought to alter the interactions between endothelial cells and SMCs (Thurston et al., 1999).

The atherosclerotic core consists of an inflammatory, highly cellular environment with elevated metabolic stresses. The resulting imbalance of oxygen supply versus

oxygen demand causes the impression of a hypoxic setting, which induces the expression of HIF. This is followed by the production of VEGF, which as discussed above increases the vascularity of the area. The inflammatory evolution of the region is progressed, more cells reach the necrotic core, intensify the metabolic demand and severity of the hypoxic state and thus further increase the HIF and VEGF expression (Libby et al., 2011).

Long-term adaptation to hypoxia is primarily achieved through the process of angiogenesis. It is a major consequence of hypoxia as a result of various HIF dependent genes such as VEGF, endothelin-1 and matrix-metalloproteinase-2 (Semenza, 2003). HIF-1 $\alpha$  is able to directly activate VEGF by binding to HRE, allowing for transcription of the protein. Many studies have been able to establish the role between HIF-1 and VEGF expression. VEGF is a pro-angiogenic growth factor, mainly responsible for the formation of new capillaries. Developmentally, it is required in embryonic angiogenesis, but pathologically it is seen in many diseases such as tumours, atherosclerosis and retinal eye disease. It mainly acts through the binding of tyrosine-kinase receptors (Forsythe et al., 1996). As previously mentioned, the process of angiogenesis and neovascularisation is thought to be a key contributor to the progression of a stable asymptomatic plaque to an unstable and rupture prone one. A 2-4 fold increase was observed in the number of vasa vasorum in ruptured or vulnerable plaques compared to stable ones (Virmani et al., 2005). A clear association between the angiogenesis and the presence of new vessels in a plaque has been linked to its rupture (Kumamoto et al., 1995).

In hypoxia, the accumulation of HIF activates gene transcription for VEGF and VEGFR. VEGF produces a pro-angiogenic environment through mobilising endothelial cells (Inoue et al., 1998). Aggressive plaque development and accelerated angiogenesis was seen in rabbit models when administered VEGF (Celletti et al., 2001). VEGF is expressed by many cells including macrophages, T lymphocytes and endothelial cells and causes production of chemoattractant proteins such as MCP-1 that causes cell migration and increases the permeability of the endothelial layer. The basement membrane is partly degraded by digestive enzymes such as matrix-metalloproteinases, allowing a fibrin matrix of plasma proteins to form with a lumen, where endothelial cells migrate. Physiological angiogenesis has sprouts that are supported with basement membrane formation and smooth muscle (and pericyte) cell recruitment (Dvorak et al., 1995). Pathological angiogenesis, as seen in atherosclerosis and tumours, is characterised by extensive, thin-walled vasculature that is prone to collapse. The endothelium is unable to control leakage adequately in pathological angiogenesis due to disruption of the intercellular junctions, a major contributor to the cause of intraplaque haemorrhage in atherosclerosis. It is thought the genes responsible for angiogenesis are similarly responsible for atherosclerotic development through plaque destabilisation, inflammation and plaque haemorrhage (Michel et al., 2011).

VEGF expressed by macrophages activates MCP-1 in endothelial cells and increases the permeability of the endothelial layer, leading to chronic low level inflammation and monocyte infiltration. The monocytes and macrophages in the lesions express proteases such as urokinase plasminogen activator. This enables them to interact with substances in the extracellular matrix such as proteoglycans and collagen. Urokinase plasminogen activator initiates plasmin in the breakdown of

the extracellular matrix; an important step in angiogenesis and vascular remodelling (Menashi et al., 1993). The expression of E26 transformation-specific sequence 1 (Ets-1) is induced by HIF1 directly and indirectly via VEGF (Oikawa et al., 2001). Ets-1 contributes to angiogenesis through the proliferation of endothelial cells and converting them to the angiogenic phenotype (Oda N, 1999). Ets-1 and VEGF are able to enhance the expression of the other and create a positive feedback loop to facilitate angiogenesis.

Angiogenesis is a complex process and although the majority of the discussion has focussed on VEGF, several other genes regulated by HIF-1 are also involved, such as Angiopoietin 1 (ANGPT1), ANGPT2, placental growth factor (PLGF) and Platelet-derived growth factor b (PDGFB). Although HIF-1 directly regulates the transcription of VEGF, it is unclear whether the regulation of ANGPT1, ANGPT2, PLGF and PDGFB is direct or indirect (Hirota and Semenza, 2006). HIF-1 mediated VEGF response does not appear to be cell type specific, that is to say, whether endothelial cells, VSCMs or macrophages are concerned, hypoxia leads to a similar response. However, depending on the cell type, expression of ANGPT1 and ANGPT2 was either activated or suppressed in response to hypoxia. In arterial endothelial cells, hypoxia-induced ANGPT2 expression was suppressed in VSMCs and unchanged in cardiac myocytes and fibroblasts. The underlying molecular mechanism remains to be established (Kelly et al., 2003). ANGPT2 seems to be implicated in the angiogenic process and is upregulated in ischemic tissue (Jaipersad et al., 2014a). The binding of ANGPT2 is via a receptor called TIE-2, expressed largely in endothelial cells. TIE-2 is involved in the stabilisation of mature blood vessels and promotes the relationship between endothelial cells and periendothelial cells (Thurston, 2003).

Interestingly, the adrenergic system has been shown to be involved in angiogenesis and the regulation of pro-angiogenic factors. Although the exact role of noradrenaline-mediated VEGF upregulation in atherosclerosis is unclear, it was found that a post-transcriptional mechanism was involved in noradrenaline mediated HIF-1 production and VEGF modulation (Park et al., 2011).

PHDs serve as an oxygen sensor to regulate HIF hydroxylation and subsequent adaptive cellular response under different oxygen tension. The studies from Mazzon's group (Hamm et al., 2013; Takeda et al., 2011) showed that hind limb ischemia induced ANGPT1 dependent PHD2 repression and ANG receptor TIE-2 induction to form a feed-forward loop in macrophages. PHD2 haploinsufficiency skewed macrophage polarization towards a pro-arteriogenic phenotype, hence, prevented ischemia by inducing arteriogenesis.

## **8. Summary and perspectives**

Atherosclerosis is the principal process that results in many of the cardiovascular events, including coronary heart disease, critical limb ischaemia, and formation of emboli leading to strokes and myocardial infarctions. HIF is known to be a chief element in the induction of angiogenesis. The destabilized angiogenesis occurring in the ischaemic and necrotic atherosclerotic lesion is stimulated by HIF expression. This is one of the main potentiators of plaque rupture, which then leads to complications and the development of the mentioned diseases. Studies show that



the expression of HIF within the plaque is detrimental, specifically expression in SMCs, ECs and even more importantly macrophage and foam cells lead to a worsened outcome. However, there is little data concerning the targeting of HIF in order to pursue and seek the effects of its pharmacological inhibition.

Predominantly through transcriptional activation, HIF-1 $\alpha$  is able to regulate the response of endothelial cells, macrophages and smooth muscle cells to promote the development of atherosclerosis. Cell proliferation, migration, angiogenesis, foam cell formation and a pro-inflammatory environment are the major consequences. However, to say that HIF-1 $\alpha$  is an atherogenic factor would be inappropriate, as the response it induces is cell-type specific. Indeed, one study found that the overexpression of HIF-1 $\alpha$  in lymphocytes, led to inhibition of plaque progression in murine (Ben-Shoshan et al., 2009). Given the role of HIF-1 $\alpha$  as the main regulator of adaptive responses to hypoxia, it presents as a key therapeutic target. Much research has gone into the regulation of HIF-1 $\alpha$ , through pharmacological intervention to slow or inhibit disease progression, however numerous problems have been faced. Firstly, the hypoxia-signalling pathway is imperative for several physiological processes and thus systemic targeting could cause undesirable effects. Furthermore, as HIF-1 $\alpha$  plays a major role in angiogenesis, upregulation may actually be beneficial for patients with ischaemic diseases. Targeting HIF-1 $\alpha$  too late in the atherosclerotic process could trigger the rupture of fragile plaques, leading to disastrous consequences (Figure 4).

Furthermore, the research conducted tends to examine primarily one of the two subunits of HIF-1: HIF-1 $\alpha$ , and there is little data available focusing on the role of HIF-1 $\beta$  or on HIF-2, another factor subtype. Hence, further research differentiating the factor roles may be useful in acquiring a clear picture. Additionally, as highlighted by the literature, there is a great difference between local and systemic effects of HIF action, and similarly there are differences in the effects of its inhibition. There is much debate regarding the available literature, as much data suggests that on a systemic level HIF improves the outcome of cardiovascular diseases as it stimulates the formation of collateral vascular networks. The local effect, the expression of HIF within the plaque itself and not wholly in the organism, is what this report has focused on and given that there is little accessible research specifically regarding the local consequences, the idea of HIF being a potential therapeutic target is supported but has yet to be proven.

Although no clinical trials have been directly focused on the hypoxia signalling pathways, findings in animal models have shown promising results. Local inhibition of HIF-1 signalling in ApoE<sup>-/-</sup> mice significantly suppressed atherosclerotic plaque growth (Christoph et al., 2014). HIF-1 shows greatly versatility as a therapeutic target and some studies have raised the idea of altering the HIF-1 pathway to increase the bactericidal capacity of phagocytes, for infectious diseases (Zarembler and Malech, 2005). An advantage of targeting host molecules that encourage macrophage recruitment against pathogens, is the lack of selective pressure for resistance. Future studies will be most beneficial by focusing on the HIF-1 $\alpha$  inhibition in specific cell types and investigating the outcome in both ischaemic and atherosclerotic patients.

In terms of therapeutic studies published, there is a niche to be covered with regards to the local targeting of HIF in atherosclerosis. Most models studied relative to HIF in cardiovascular conditions concentrated on the administration of the factor itself, or the induction of its expression and the subsequent development of collateral vessels in ischaemic situations. The concept of atherosclerosis and the local outcome of the factor action were not examined (Benizri et al., 2008; Vincent et al., 2000). Moreover, research examining the antiangiogenic treatment used in the case of thrombosis showed that the therapy led to limited thrombosis resolution. Despite the discrepancy that this finding proposes with regards to this review, the development and resolution of thrombosis differs greatly compared to atherosclerosis, in terms of cells, inflammatory mediators and initiation of the processes. In addition, this study focused on venous thrombosis, which is much different to any arterial event (Evans et al., 2014). Evidence regarding *in vivo* studies as well, implies that the role of HIF is of great significance in the inflammatory process of atherosclerosis but the use of its pharmacological blockade in atherosclerosis has yet to be recorded and published. As the factor has a very particular and local role in atherosclerosis, one of the issues faced is the difficulty in assuring that the mediator administered for its inhibition will reach the atherosclerotic site, without its metabolism or reach at other areas of hypoxia or inflammation. Perhaps an idea to be implemented could be to target the specific cells, which actively express HIF in atherosclerosis, urging its development. HIF derived from macrophages, SMCs and progenitor endothelial cells, involved in the process by using additionally cell markers, may consist of convenient and effective targets. In the hope that such an agent is created, it is important to block the inflammatory aspect of the process, which has a detrimental effect on atherosclerosis progression. This aspect calls for greater research.

In summary, HIF plays a key role in the complex progression of atherosclerosis, and greater HIF expression and consequent intraplaque vascularization development is associated with increased plaque instability and worsened outcomes. The therapeutic role of HIF in atherosclerosis has been suggested, although there has been limited investigation into its potential. Further research in this field would provide a great potential for treatment of patients with severe atherosclerosis in clinic.

#### **Funding sources**

This work was supported by the British Heart Foundation and the Oak Foundation, National Natural Science Foundation of China (81370521, 81320157, and 81670400), The Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions (CIT&TCD20150325), The Key Science and Technology Project of Beijing Municipal Institutions (KZ201610025025), The Fok Ying-Tong Education Foundation (151041).

#### **Conflict of interest statement**

The authors declare that there is no conflict of interest including any financial, personal or other relationships with individuals or organizations within three years of initiating the work that could inappropriately influence, or be perceived to influence, the study design or data interpretation.

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## Figure legends

**Figure 1. The domain structure for HIF-1 $\alpha$ , HIF-2 $\alpha$ , HIF-3 $\alpha$  and HIF-1 $\beta$ .** The bHLH and PAS regions are required for the heterodimerisation of the  $\alpha$  and  $\beta$  subunits. The oxygen dependent degradation domain (ODDD) region mediates the stability of the molecule through the hydroxylation of the two proline (P) residues and acetylation of lysine (K). Both HIF-2 $\alpha$  and HIF-3 $\alpha$  are also regulated in a similar oxygen dependent manner through P and K residues. HIF-1 $\alpha$  has 2 trans-activating domains (N-TAD and C-TAD), similar to HIF-2 $\alpha$ , but HIF-3 $\alpha$  and HIF-1 $\beta$  only have one TAD. The N terminus is a bHLH that allows for DNA binding, and the C terminus recruits transcriptional regulatory proteins. The number at the end represents the number of amino acids in the subunit.

**Figure 2. The regulation of endothelial cells by HIF-1** – In hypoxic conditions, HIF-1 $\alpha$  and HIF-1 $\beta$  dimerise into the active form of HIF-1. Through the up regulation of nuclear factor kB (NF-kB), vascular endothelial growth factors (VEGF), their receptors (VEGFR), reactive oxygen species (ROS), free radicals (FR) and nitric oxide synthase (iNOS) leads to endothelial cell (EC) dysfunction, migration and proliferation contributing to the progression of atherosclerosis.

**Figure 3. The regulation of macrophages via HIF-1 $\alpha$**  – HIF-1 promotes inflammation and migration of macrophages by encouraging M1 phenotype differentiation and upregulation of MCP-1, osteopontin, TNF $\alpha$ , iNOS, IL-6 and VEGF. HIF-1 $\alpha$  also promotes the formation of foam cells by reducing cholesterol efflux mechanisms, specifically the ABCA1 cholesterol efflux protein, and higher affinity of extracellular molecules for LDL. The increased level of cholesterol and triglycerides in the macrophages is toxic and more likely to cause cell death. This along with smooth muscle migration and proliferation is contributing to plaque growth and atherosclerosis.

**Figure 4. The involvement of HIF in atherosclerosis** – At the early stages, HIF leads to the expression of reactive oxygen species (ROS) as well as iNOS, VEGF and NF-kB which promote increased inflammation, and then leading to endothelial cell dysfunction and leukocyte attachment. The accumulation of lipids and macrophages result in foam cell formation, and smooth muscle cell migration and proliferation, on which HIF exerts its effect. Finally atherosclerotic plaque forms a complicated lesion where hypoxia amplifies HIF expression and activation.

Table: Reagents displaying therapeutic potential related to HIF1 $\alpha$ .

Name of compound	Inhibitor/Activator of HIF activity	Mechanism of action	Model of study	Effect	Reference
Ad2/HIF1 $\alpha$ /VP16	Activator	Acts as HIF1 $\alpha$ subunit dimerization domains and binds onto DNA to induce transcription	No-Option Critical Limb Ischaemia Patients (Phase I)	No- large improvements seen as suggested by the mortality and amputation percentage a 6months and 1 year in treatment group versus control	<b>(Rajagopalan et al., 2007)</b>
HIF1 $\alpha$ /VP16	Activator	Acts as HIF1 $\alpha$ subunit dimerization domains and binds onto DNA to induce transcription	Rabbit Model hind limb ischaemia ( <i>in vivo</i> )	Increased EPO and VEGF expression, increased lactate dehydrogenase expression and increased vascularity in treatment group vs control	(Vincent et al., 2000)
Dimethylxalylglycine (DMOG)	Activator	Inhibits the oxoglutarate-dependent dioxygenase which normally causes inactivation of HIF1 $\alpha$	Ischaemic Skeletal Muscle Mouse model ( <i>in vivo</i> )	Increased VEGF expression and vascularization of the muscles treated in the treatment group vs control	(Milkiewicz et al., 2004)
Adenoviral PR39	Activator	Inhibits HIF1 $\alpha$ breakdown by inhibiting the proteasome	Pig Model Chronic Myocardial Ischaemia ( <i>in vivo</i> )	Increased expression of VEGF and its receptors, enhanced myocardial blood flow, collateral vessel formation in treatment group vs control	(Post et al., 2006)
FG-4592 (Roxadustat)	Activator	Stabilize HIF via inhibition of PHD	Correction of anemia in patients with chronic kidney disease (Phase III)	Erythropoiesis by increasing translation of EPO in the kidneys, EPO receptor in	(Besarab et al., 2015; Provenzano et al., 2016)

				erythroid progenitors, divalent metal transporter 1 in duodenal enterocytes, transferrin receptor in hepatocytes	
N-acetylcysteine amide thiol: AD4	Inhibitor	Inhibits the expression of HIF1 $\alpha$	Mouse Allergic Airway disease (Asthma) ( <i>in vivo</i> )	Reduced Th2 cytokine production, VEGF expression, mucus production and airway resistance caused in allergic airway disease in treatment group vs control	(Lee et al., 2007)
HIF-1a shRNA	Inhibitor	Knockdown (KD) of HIF1 $\alpha$ expression	Triple negative breast cancer (TNBC) cell lines - MDA-MB-231	Increased early and late cancer cell apoptosis	(Li et al., 2015)
Bortezomib (PS-341)	Inhibition	Proteasome inhibitor, inhibits p300-HIF1 $\alpha$ binding	Pancreatic and lung cancers, multiple myeloma (Phase I)	Inhibits angiogenesis, increases remission period	(Befani et al., 2012; Chen et al., 2011; Cortes et al., 2004; Iskandarani et al., 2016; Kontopodis et al., 2016; Mian et al., 2016)
PX-478	Inhibition	Inhibition of HIF1 $\alpha$ deubiquitination, increased polyubiquitinated HIF1 $\alpha$ levels, decreased HIF1 $\alpha$ mRNA expression, inhibition of HIF1 $\alpha$ translation	Human lung adenocarcinoma cell and small lung cancer cell (SCLC) injected into mice model ( <i>in vivo</i> )	Decreased mediastinal metastasis, survival time; decreased primary lung tumour volume in treatment groups vs control, results were more prominent in SCLC	(Jacoby et al., 2010; Jordan et al., 2005)

Figure 1

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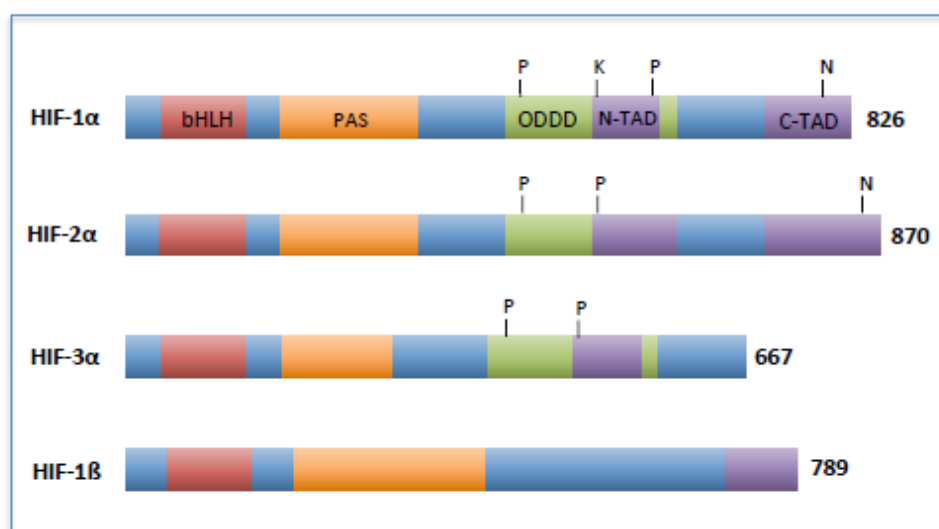


Figure 2

Jain et al.

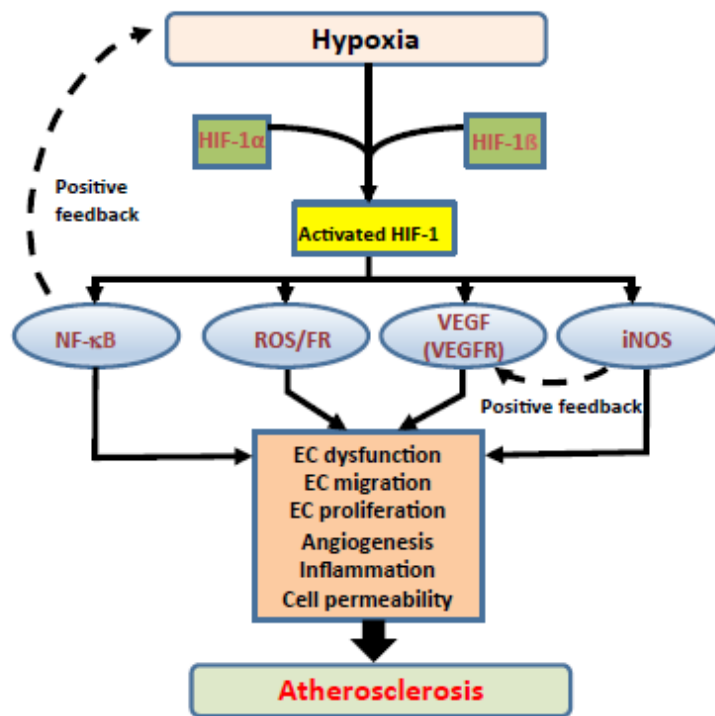
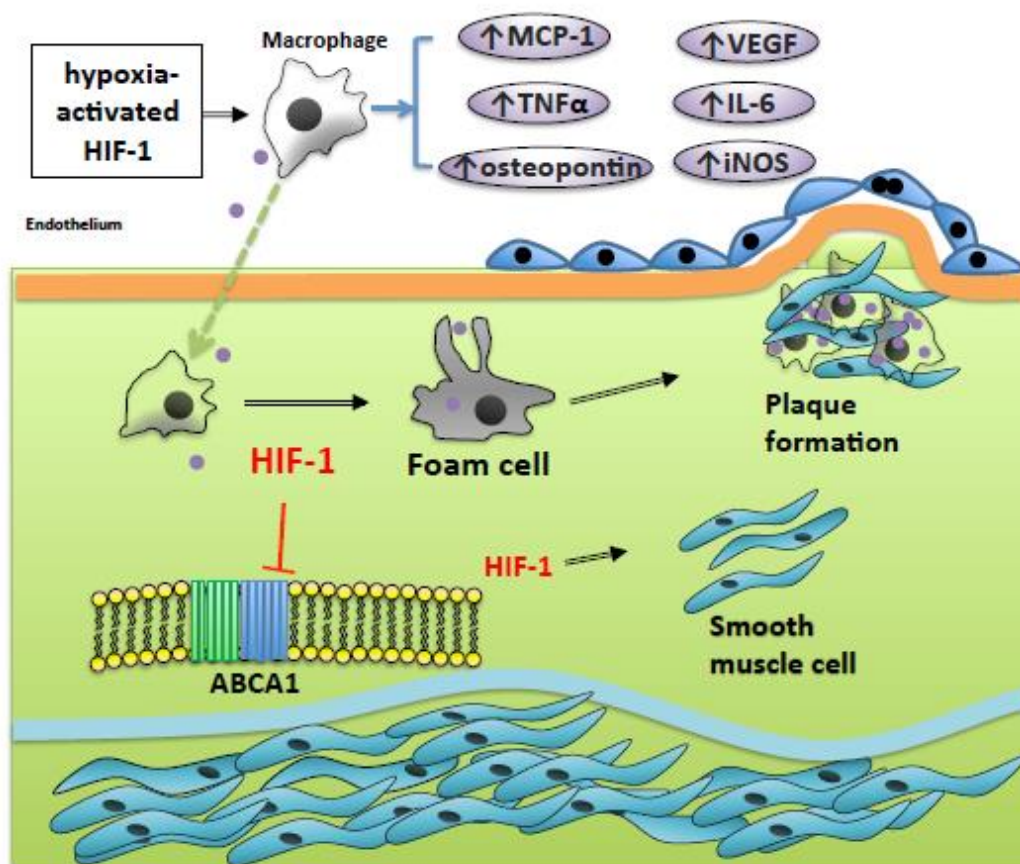


Figure 3

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Figure 4

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