# Shear stress, arterial identity and atherosclerosis.

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In the developing embryo, the vasculature first takes the form of a web-like network called the vascular plexus. Arterial and venous differentiation is subsequently guided by the specific expression of genes in the endothelial cells that provide spatial and temporal cues for development. Notch1/4, Notch ligand delta-like 4 (Dll4), and Notch downstream effectors are typically expressed in arterial cells along with EphrinB2, whereas chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and EphB4 characterize vein endothelial cells. Hemodynamic forces (blood pressure and blood flow) also contribute importantly to vascular remodeling. Early arteriovenous differentiation and local blood flow may hold the key to future inflammatory diseases. Indeed, despite the fact that atherosclerosis risk factors such as smoking, hypertension, hypercholesterolemia, and diabetes all induce endothelial cell dysfunction throughout the vasculature, plaques develop only in arteries, and they localize essentially in vessel branch points, curvatures and bifurcations, where blood flow (and consequently shear stress) is low or oscillatory. Arterial segments exposed to high blood flow (and high laminar shear stress) tend to remain plaque-free. These observations have led many to investigate what particular properties of arterial or venous endothelial cells confer susceptibility or protection from plaque formation, and how that might interact with a particular shear stress environment.

## Introduction

Atherosclerosis is a complex chronic disease of the vasculature. Atherosclerotic plaque formation is initiated by the accumulation of lipoproteins in the arterial wall which trigger a persistent inflammatory response. Locally, endothelial cells (ECs) and vascular smooth muscle cells become activated. The ensuing expression of adhesion molecules and chemokines stimulates the recruitment of monocytes and T lymphocytes which adhere to the endothelium and transmigrate into the underlying intima. The monocyte-derived macrophages accumulate lipids and become trapped in the form lipid-laden foam cells (1, 2). Dendritic cells, originating in arterial intima or derived from monocytes, also differentiate into foam cells (3, 4). The resulting accumulation of cells, coupled with continuous inflammation, drive the chronic recruitment and activation of leukocytes, perpetuating plaque growth and encroachment in the vascular lumen (1, 2, 5). Severe vascular narrowing, along with plaque erosion and rupture, are intimately linked to clinical events (6).

It is clear that both a lipid and an inflammation component contribute significantly to the progression of atherosclerotic lesions and associated clinical outcomes. On the one hand, current and emerging therapeutic agents for this disease are primarily focused on modulating lipids, and lipid-lowering statin therapies have proven to be highly effective in reducing the cardiovascular events and improving the quality of life for patients with coronary heart disease (7). On the other hand, both preclinical and clinical research has provided multiple lines of evidence that inflammation and immune responses are integral components of the pathogenesis of atherosclerosis (8, 9). Nevertheless, these approaches do not address the fundamental question regarding the specificity of atherosclerotic plaque localization, which itself may hold clues to how plaque formation may be prevented or reversed.

## Shear stress and atherosclerosis

It is well established that atherosclerosis is not distributed evenly in the vasculature. Despite the fact that atherosclerosis risk factors such as smoking, hypertension, hypercholesterolemia, and diabetes all induce endothelial cell (EC) dysfunction throughout the vasculature (10-12), plaques develop essentially in arterial branch points, curvatures and bifurcations. These sites are characterized by low, disturbed or oscillating blood flow. In comparison, straight segments of arteries, exposed to laminar blood flow, remain consistently lesion-free. Differences in shear stress, the frictional force due to blood flow, form the basis of these differences in atherosclerosis predilection (13-17). Shear stress is sensed essentially by ECs. Slow and oscillatory blood flow induces the expression of endothelial adhesion molecules, chemokines, and growth factors that are

important for leukocyte recruitment and extravasation. Increased activity of the proinflammatory transcription factor NFκB by oscillatory flow (18) is likely to contribute to these effects. Conversely, high pulsatile shear stress decreases EC turnover and promotes anti-oxidative and anti-inflammatory processes. Activation of endothelial NO synthase (eNOS) appears to be a key mediator of these atheroprotective effects. NO reduces endothelial permeability, migration of leukocytes, and vascular smooth muscle cell proliferation while simultaneously promoting EC survival (19, 20).

**Arteriovenous identity**

Another particularity of atherosclerosis is that it develops essentially in arteries. Although arteriovenous identity is ambiguous in the early embryo, arteries and veins gradually differentiate into distinct vessels that have characteristic endothelial cells. EC properties were originally believed to derive from the milieu where they evolved, dependent on local blood pressure, shear stress, blood oxygenation, or pH (21). However, this notion was challenged by the discovery that even before the onset of blood flow, endothelial cells express arteriovenous differentiation markers (22, 23). Therefore, although full differentiation depends on external cues such as flowing blood, early arterial specification occurs intrinsically (24). From hence, it was appreciated that the identity of artery and vein endothelium is genetically determined. This did not preclude a potential change in EC fate. If for example the local blood flow was reversed in the developing embryo, arteriovenous differentiation would similarly be reset according to the new conditions (25). Nevertheless, this line of work established a number of genes whose expression characterize and, in some cases serve to maintain, arterial and venous identity (26). These form a coherent set of interacting molecules (Figure 1). In arteries, high levels of vascular endothelial growth factor (VEGF), acting on its co-receptor neuropilin 1 (Nrp1), leads to Delta-like 4 (Dll4)-dependent activation of Notch1/4 and downstream expression of hairy and enhancer of split (Hes), and hairy and enhancer of split with YRPW motif (Hey1/2). In veins, lower levels of VEGF interacting with the Nrp2 co-receptor induce the expression of chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), which inhibits Notch signaling (27-29). Disparities in Notch signaling also account for greater expression of EphrinB2 in arteries, and enhanced expression of its receptor EphB4 in veins. Hence, differential expression of Notch ligands, receptors and effectors provides temporal and spatial cues critical for embryonic development (30). Furthermore, Notch components continue to be expressed across the body in a tissue-dependent manner, and Notch signaling continues to play a vital role in regulating differentiation, proliferation, and survival in adult tissues including the hematopoietic system (31, 32).

**Shear stress and arteriovenous differentiation markers**

In an effort to reconcile shear stress-dependent plaque localization and the arterial-specific nature of the disease, it is interesting to investigate how these factors interact. Although there is consensus on the mechano-sensitivity of arteriovenous differentiation markers in general, in vitro experiments have yielded diverse findings, which might depend on the type of endothelial cell used or the specific culture conditions.

Arterial levels of shear stress applied to ECs derived from pluripotent stem cells upregulated mRNA levels of Notch1, as well protein-level expression of Notch1 intracellular domain (NICD)(33). In embryonic stem cells, Notch1 activation and NICD translocation were likewise observed within 30 min and augmented with time in response to shear stress, increasing EphrinB2 expression dose-dependently (34). In comparison, early outgrowth cells (also called endothelial progenitor cells) were more responsive to low shear stress (1-5 dynes/cm2), producing increased mRNA levels of Notch1/3, Hey1/2, and activin receptor-like kinase 1 (Alk1)(35). Comparable shear stress levels were also found to upregulate Notch1 expression in human abdominal aortic endothelial cells, whereas higher shear stress levels ($\geq$10 dynes/cm$^2$) did not (36). Notch1 expression was required for upregulation of other arterial markers Dll4, Hey1, and Nrp1 by shear stress in aortic ECs (36). Similarly, Notch1/4 upregulation was found to precede increased Dll1/4, Jagged1, and Hes expression in a rat model of arteriovenous malformation, in correlation with increased wall shear stress (37). Shear stress-induced arterial specification also required the activation of the Notch pathway through a mechanism that involved upregulation of Notch ligands Dll1/4 (38). Interestingly, the Notch inhibitor DAPT phenocopied the effects of lowering shear stress on mouse embryo, preventing vascular remodeling. The effect of DAPT treatment could be partially rescued by injection of starch to increase shear stress levels (39). In a similar fashion, optimal Notch1 levels were required for optimal endothelial progenitor cell proliferation, migration and adhesion, and consequently re-endothelialization capacity of these cells after arterial denudation (40). Collectively, these studies suggest that Notch1 is an important regulator of endothelial identity and function, which is preferentially induced by low shear stress. Nevertheless, the degree to which cells respond to the shear stress stimulus may reflect the development stage of the cells from which ECs are derived or the vessels from which they are isolated.

Results have not been as clear-cut when considering the effects of shear stress on the EphrinB2-EphB4 balance. The mRNA levels of the arterial EC marker EphrinB2 increased in response to low shear stress (1-5 dynes/cm$^2$) in early outgrowth cells, but levels of the venous endothelial cell markers EphB4 and Nrp2 decreased (35). The same expression pattern was observed in embryonic stem cells (34). In response to arterial shear stress, early outgrowth cell expression of EphrinB2 also augmented, but EphB4 remained unchanged (41). Likewise, arterial levels of shear stress were found to

upregulate mRNA levels of EphrinB2, when applied to ECs derived from pluripotent stem cells (33). However, another study found that EphrinB2 expression was enhanced in ECs exposed to oscillatory flow rather than laminar flow (42). Furthermore, arterial shear stress downregulated EphrinB2 mRNA in coronary endothelial cells and umbilical vein endothelial cells, but conditions mimicking either venous or arterial shear stress did not affect EphB4 expression (43). Finally, in isolated perfused veins, the opposite was observed. Levels of EphrinB2 were unaffected by arterial shear stress, whereas EphB4 was decreased under such flow (44). Hence, although there is some discrepancy regarding the extent to which EphrinB2/EphB4 are affected by shear stress, there is a trend towards upregulation of EphrinB2 in cells exposed to shear stress, whereas EphB4 tends to remain unchanged or is reduced in such conditions.

**Atherosclerosis and arteriovenous differentiation markers**

It is interesting to note the loss of venous identity without gain of arterial identity in veins exposed to arterial magnitudes of shear stress *ex vivo* (44). There was even a loss of EphB4 in vein grafts placed in the arterial circulation *in viv*o (45, 46). Since vein grafts used as coronary artery bypass can develop atherosclerosis (47), these observations suggest that venous identity is protective rather than arterial identity harmful. In the setting of venous bypass grafts, it is important to remember that imposing arterial blood pressure produces a high tensile stress that could itself instigate pro-inflammatory processes consistent with atherosclerosis formation (48). Moreover, the vein diameter increases in this context, lowering shear stress; placing a reinforcing mesh around a vein graft in bypass conditions lowers remodeling and maintains high shear stress (49). Nevertheless, venous ECs were found to be less thrombogenic than arterial ECs (50). Moreover, COUP-TFII knockdown in venous ECs resulted in the expression of Notch1 and Jagged1, which are normally expressed only in arterial ECs. This led to enhanced expression of atherogenic genes after stimulation with angiotensin II, and increased angiotensin-induced cell adhesion (51). Interestingly, endothelial expression of the angiotensin II AT1 receptor is shear stress-sensitive and expressed essentially in athero-prone arterial sites (52). Inversely, attenuation of Notch1 signaling by application of soluble jagged1 (53) or by Notch1 inhibition with DAPT (54) suppressed intimal hyperplasia in a model of rat vein graft. In summary, there is evidence vein graft disease, which occurs when veins are transposed in an arterial context, may be attenuated by modulating arteriovenous differentiation in the favor of venous markers.

Although vein grafts can develop atherosclerosis, the disease is primarily found in arteries, at sites of predilection determined by the shear stress environment (Figure 2). A comparison of patient atherosclerotic arteries with healthy controls revealed aberrantly low methylation of Notch1 in diseased vessels, and an accompanying increased Notch1

expression (55). Another study noted increased expression of Notch1/4 and Hey1 at atherosclerotic sites of both human and mouse aortas (56). In mice, no differences in the expression of EphrinB2 mRNA were detected between the plaque-prone inner curvature and athero-protected outer curvature of the aorta. However, endothelial staining for EphrinB2 was detected in the inner aortic curvature but not in the outer curvature (42). Strong expression of Notch1 and Hes1 was also noted in aortas of hypercholesterolemic apolipoprotein E (ApoE)-/- mice, compared with wild-type controls, being particularly abundant in plaque macrophages. Fittingly, treatment of with a Notch1 inhibitor reduced plaque size and lowered macrophage infiltration in these mice (57). Along the same lines, administration of a Dll4 neutralizing antibody reduced NFκB activity, decreased macrophage accumulation, and abated the development of atherosclerosis in low density lipoprotein receptor (LDLR)-/- mice (58).

Atherosclerosis predisposes to formation of aneurysm both in patients and in mice. Accordingly, activation of Notch1 signaling was observed in the aortic aneurysmal tissue of ApoE-/- mice, and a similar activation of Notch1 was observed in aneurysms of humans undergoing abdominal aortic aneurysms (AAA) repair (59). Notch1 haploinsufficiency or pharmacological inhibition of Notch1 significantly reduced the occurrence of AAA in response to angiotensin II in ApoE-/- mice (59). These findings were confirmed in a second study showing that inhibition of Notch1 signaling reduces macrophage accumulation and AAA formation in mice (60). Interestingly, Notch intracellular domain (NICD) and Hes1 were detected predominantly in aneurysmal fibroblasts and macrophages (61), suggesting that Notch1 signaling in inflammatory cells contributes importantly to progression of the disease. This concept also held in a wound healing model mice, where myeloid-specific Notch1 deletion decreased tumor necrosis factor (TNF)-α expression and macrophage recruitment (62). Finally, flow cytometry and immunohistochemistry demonstrated that Notch1 haploinsufficiency prevented the influx of inflammatory macrophages at the aneurysmal site by causing defects in macrophage migration and proliferation (59). Hence expression of Notch1 in leukocytes is paramount to the normal function and the inflammatory response in these cells.

In fact, Notch1 signaling is both activated by and stimulator of inflammatory signals. For the most part, these processes have been studied in macrophages, a predominant cell type in atherosclerotic plaques. Macrophage function is strongly influenced by exposure to cytokines; this polarization process generates two macrophage subtypes broadly referred to as M1 and M2. M1 “classically activated” macrophages are induced by interferon (IFNγ) and drive pro-inflammatory responses (63, 64). M2 ”alternatively activated” macrophages differentiate in the presence of interleukin (IL)-10, IL-4 or IL-13 and express anti-inflammatory mediators (65), associated with wound healing. The progression and exacerbation of atherosclerosis are propelled by gradual lesional accumulation of M1 macrophages which secrete pro-inflammatory cytokines (66).

Conversely, the clearance of apoptotic cells by phagocytosis and plaque regression are associated with M2 macrophage function (67, 68). Notch1 expression is enhanced during cellular stress in macrophages, and Notch1 inhibition reduces inflammatory cytokine secretion and promotes the M2 macrophage phenotype in these conditions (69). In agreement, Dll4 skews macrophages towards an M1 phenotype (58). In LDLR-/- mice, administration of a Dll4-blocking antibody suppressed macrophage M1 pro-inflammatory gene expression, prevented vein graft macrophage accumulation, and diminished lesion development (70).

Beyond M1/M2 conversion, Notch1 has been found to influence macrophage response to toll-like receptor (TLR) agonists such as bacterial lipopeptide, polyI:C, lipopolysaccharide (LPS) and unmethylated CpG DNA. These agonists all induced up-regulation of Notch1 in primary and macrophage-like cell lines (71). Moreover, Notch and TLR pathways cooperated to activate Notch target genes, including Hes1 and Hey1, and to increase production of canonical TLR-induced cytokines TNF, IL-6, and IL-12 (72). In fact, Notch signaling was found to increase both basal and LPS-induced NFκB activation, favoring the expression these cytokines (73). In return, inhibition of Notch signaling decreased induction of the inflammatory cytokines in macrophages stimulated with LPS (62, 71). Additionally, macrophages from Notch1 (+/-) mice demonstrated decreased induction of IL-6, IL-12, and TNF-α in response to LPS, compared with wild-type mice (62). Although LPS was an efficient stimulator of Notch1, Dll4 was the most effective ligand to induce Notch activation and increase NFκB transcriptional activity in macrophages (73). Keeping the context of atherosclerosis in mind, it is interesting to note that not only LPS, but also low density lipoproteins can enhance Dll4 expression in macrophages (74). Finally, although there is a preponderance of experiments investigating pro-inflammatory effects of Notch1 in leukocytes, it is clear that other cells within the plaque have influence on or are impacted by Notch signaling. For example, smooth muscle cells were found to highly express Jagged1, which subsequently activated Notch1 in the transmigrated endothelial progenitors to promote their differentiation into macrophages (75). More importantly, when considering the shear stress-specific localization of atherosclerotic plaques and shear-dependent Notch1 regulation, Notch1 activation inhibited EC growth and increased EC senescence, and it enhanced leukocyte transendothelial migration *in vitro*, at least in part through IL-6 (76).

Despite the fact that the majority of studies report a benefit of Notch1 inhibition in reducing inflammatory, there is some evidence to the contrary. In ECs derived from stem cells, it was revealed that Notch1 expression is necessary for the activation of anti-inflammatory networks by shear stress (77). In agreement, expression of the Notch1 inhibitor delta-like 1 homolog (Dlk1) was accentuated in response to reduced flow and coincided with poor EC turnover and enhanced atherosclerosis (78). Endothelial Notch1 haploinsufficiency was also found to enhance leukocyte adhesion and atherosclerotic

lesions in mice (79), suggesting that an optimal expression of Notch1 may be necessary for ideal EC function. In cultured human monocytes, Dll4 induced the transcription of Notch target gene Hes1 and inhibited the basal and TNF-α-stimulated production of IL-8 (80). Furthermore, compared to LPS alone, simultaneous stimulation of dendritic cells with Jagged1 fusion protein and LPS resulted in significantly enhanced expression of the anti-inflammatory cytokine IL-10, whilst secretion of pro-inflammatory IL-12 was significantly inhibited (81). Finally, LPS was found to inhibit Notch1 intracellular domain transcription activity (82). Activation of a Hes1- and Hey1-mediated inhibitory feedback loop could very well account for some protective effects of Notch1 (72). Moreover, both pro- and anti-inflammatory cytokines were shown to alter Notch expression levels or elicit a switch in Notch expression, with corresponding impact on EC function (83, 84). Further work will be needed to more precisely define which Notch ligands and isoforms contribute to inflammation, and how this might be influenced by the pathophysiological setting.

To further complicate matters, EphrinB2/EphB4 differention markers also play a role in the inflammatory response. In principle, Eph receptors are activated by membrane-bound Ephrins, such that direct cell-to-cell contact is required for receptor activation. Moreover, Ephrin-Eph binding triggers signaling in both the ligand-bearing and the receptor-bearing cell. This bi-directional signaling tends to result in the repulsion of the cells involved. Hence, Ephrin-Eph interaction between ECs and leukocytes would tend to limit their interaction. Conversely, decreased expression of Ephrin receptors in inflamed vasculature would promote leukocyte adhesion (85). Human CD4+ T cells and polymorphonuclear cells demonstrated increased expression of EphrinB2 mRNA in response to TNFα (86). In addition, monocytes were found to express EphB2, one of the possible receptors for EphrinB2, and the expression of EphB2 in monocytes was increased upon their adhesion (87). These observation would suggest that Ephrin-Eph expression would limit interaction between leukocytes. High EphB2 in monocytes could also explain their lower adhesion to ECs exposed to shear stress, since these conditions tend to increase EphrinB2 expression, as noted above. Nevertheless, the activation of EphrinB ligands was found to lower the integrity of EC junctions and enhance the pro-inflammatory phenotype of the endothelium, facilitating the through of extravasation of EphB2 positive leukocytes (88).

## Conclusion

Atherosclerotic plaque localization is generally restricted to arterial segments that are exposed to low or oscillatory shear stress. Because endothelial cells are the primary sensors of shear stress, it stands to reason that they hold a key to the processes that allow for local plaque development. Even in the developing embryo, arterial endothelial cells express specific differentiation markers that may hold the key to understanding why this vasculature is uniquely susceptible to plaque formation. There is accumulating evidence that Notch1 may be a promising target in the fight against this disease, both because Notch1 tends to amplify inflammatory signaling and because there is evidence that at least in some contexts, endothelial Notch1 expression is enhanced by low shear stress typical of athero-prone sites. Nevertheless, it appears that an optimal levels of Notch1 are required for proper endothelial cell function, such that specific targeting of macrophage Notch1 may prove to be the better therapeutic choice. As regards the EphrinB2-EphB4 balance, adequate expression of the arterial marker may be protective, but only to a certain extent. As is the case with Notch1, EphrinB2 overexpression may tip ECs towards a pro-inflammatory phenotype. Finally, although little studied, it appears that the expression of the venous marker COUP-TFII protects vessels from inflammatory cell infiltration.

**Figure 1. Signaling pathways involved in arteriovenous differentiation.**

In arteries, high VEGF levels acting on the NRP1 co-receptor activate the Dll4/Notch pathway, leading to activation of Hey and Hes1/2. This pathway is further induced by Wnt signaling. As a result, EphrinB2 expression is enhanced whereas EpbB4 and COUP-TFII are repressed. In veins, lower VEGF levels acting on the NRP2 co-receptor produce an abated Dll4/Notch response, lifting the repression of EphB4 and COUP-TFII. Brahma-related gene 1 (BRG1) contributes to COUP-TFII activation.

**Figure 2. Interaction between shear stress, arteriovenous differentiation markers, and cells that make up the atherosclerotic plaque.**

**A.** The distribution of atherosclerotic lesions (yellow) within arteries follows a distinct pattern based on local blood flow. **B.** Arterial sections exposed to high laminar shear stress, such as the outer wall of the aortic arch, are typically devoid of plaques. Endothelial cells (EC) in such regions express optimal levels of Notch1, which are associated with pro-survival and anti-inflammatory responses. Moreover, overexpression of EphrinB2 in ECs exposed to high shear stress acts as a repellent for monocytes that express EphB2. **C.** Plaques typically form in regions where blood flow is low or oscillatory, such as the inner curvature of the aortic arch. ECs exposed to low flow tend to express high levels of Notch1 and EphrinB2, which induce endothelial dysfunction and leukocyte extravasation. In the plaque, oxidized LDL accumulation and subsequent chemokine release further stimulate monocyte influx and induce Notch1 expression macrophages. This skews macrophages towards the pro-inflammatory M1 type, and further stimulates pro-inflammatory signaling (NFκB) and cytokine production. On the contrary, if Notch1 is inhibited in this context, M2 macrophages arise and anti-inflammatory cytokines are expressed.

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