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CHARACTERIZING ENDOTHELIAL NOTCH1 RELEASE IN RESPONSE TO CHANGES IN VASCULAR WALL SHEAR STRESS

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By

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A Thesis

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ABSTRACT

Murine and cell culture models have identified Notch1 as a novel endothelial mechanosensor that may exert a protective role for vascular adjustments in response to changes in vascular wall shear stress. However, *in vivo* studies in humans are lacking. Accordingly, we sought to characterize the concentrations of Notch1 extracellular domain (ECD) prior to, during, and following 20-min of altered shear stress in the brachial artery of ten young and healthy adults (6M/4F). Alterations in shear were induced by placing a pneumatic cuff inflated to 220mmHg around the left wrist. Cuffs were also placed below the axilla of both arms and inflated to 40mmHg to trap the released Notch1 ECD. The right arm (no wrist cuff) was treated as a time control. Blood samples were collected from a superficial antecubital vein of both arms at baseline, 20-min of wrist cuff inflation, as well as 1-min and 15-min following wrist cuff release. The Notch1 ECD was quantified from plasma using a commercially available ELISA kit. Duplex ultrasound was used to confirm alterations in shear stress. In the experimental arm, concentrations of Notch1 ECD remained statistically similar to baseline values following 20-min of reduced antegrade shear stress, but were significantly elevated by ~50% (P=0.033) immediately following cuff release, coinciding with a ~100% increase in antegrade shear. Concentrations of Notch1 ECD remained unchanged in the control arm and were statistically similar to baseline values at 15 min recovery in both the control and experimental arm. These data indicate that Notch1 may be an important mechanistic regulator of vascular function, particularly in response to increased antegrade shear.

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cGMP	cyclic 3', 5'-Guanosine Monophosphate
CSL	CBF1/RBPJ-k, Suppressor of Hairless, Lag-1
CVD	Cardiovascular Disease
Dll4	Delta-like 4
DSL	Delta, Serrate, LAG-2
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
FMD	Flow-Mediated Dilation
GTP	Guanosine Triphosphate
L-NMMA	N(G)-monomethyl L-arginine
NO	Nitric Oxide
NECD	Notch Extracellular Domain
NICD	Notch Intracellular Domain
PECAM-1	Platelet and Endothelial Cellular Adhesion Molecule-1
PI3-K	Phosphoinositide 3-Kinase
RBPJ	Recombination Signal Binding Protein for Immunoglobulin Kappa J Region
VEGFR2	Vascular Endothelial Growth Factor Receptor-2

1. REVIEW OF THE LITERATURE

Introduction

Cardiovascular disease (CVD) is the leading cause of death globally, taking ~18 million lives each year (World Health Organization). The progression of CVD begins with the disruption of vascular homeostasis – the balance between vascular repair and injury – such that more cells are being damaged/destroyed than repaired/replaced (Halcox et al, 2002). Of particular importance is the damage and death of endothelial cells that line the vasculature as they function to sense and respond to alterations in the hemodynamic environment (Widlansky et al, 2003). Under normal conditions, the endothelium secretes several factors that promote vasodilation and prevent the formation of fatty plaque build-up in blood vessels; however, alterations in the hemodynamic environment correspond with an increased release of atheroprone and vasoconstricting factors such as endothelin-1 (ET-1) (Galley & Webster, 2004).

Atherosclerosis is a disease of the vasculature manifested through endothelial dysfunction which stems from altered endothelial gene expression as a result of altered blood flow dynamics. Blood vessel narrowing, stiffening and plaque formation are characteristic of overt atherosclerosis, but it is always preceded by a silent period of progressive vascular endothelial dysfunction (Lüscher & Barton, 1997). The maintenance of vascular homeostasis is therefore critical to the prevention of atherosclerotic CVD. Vascular homeostasis is dependent on a complex interaction between multiple signaling pathways and downstream effectors. However, recent interest, and in turn focus of this thesis has been directed at the interplay between Notch1 activation in response to alterations in vascular wall shear stress.

The Notch signaling pathway is a highly conserved mediator of cell-fate decisions in mammals that assembles a transcriptional complex essential for the expression of endothelial phenotypes (Ding-Yuan Tian, 2017; Kopan & Ilagan, 2009). Dysregulation of the Notch signaling pathway may serve a key role in endothelial apoptosis, misalignment, and proliferation which has led to its consideration as a target for therapeutic strategies in vascular disease.

Progressive vascular dysfunction that develops into atherosclerosis is initiated by impairments in the endothelium which involves the combined influence of the hemodynamic environment and the Notch1 signaling pathway (Cunningham & Gotlieb, 2005; Vion et al, 2013; Mack et al, 2017). However, to date, the bulk of data is limited to animal and *in vitro* study (Mack et al, 2017). There is a clear need to explore Notch1 *in vivo,* in humans. As such, the overarching goal of this thesis is to describe alterations in Notch1 activity in humans following experimental manipulation of endothelial shear stress in the forearm. Characterizing the Notch1 dynamics in humans under conditions known to impair vascular endothelial function (i.e., changes in vascular wall shear stress) is the first step in determining its functional role for vascular homeostasis, and in turn potential utility as a target for vascular therapy.

The endothelium: Overview and function

The endothelium is the monolayer of ~10 trillion cells lining the apical surface of blood vessels comprised of interlinking endothelial cells of varying permeability (Galley & Webster, 2004; Furchgott & Vanhoutte, 1989). In the 1900s, it was known that the structure of the endothelial surface itself prevented atherosclerotic build-up, but its role was believed to be passive in nature (Rubanyi, 1991). The endothelium's function as a barrier is essential for the selective restriction of bloodborne molecule passage to the subendothelial space and neighbouring smooth muscle. In the 1970s it was revealed that the endothelium also secretes a variety of

substances/enzymes that regulate blood clotting, immune function, platelet adhesion and vascular tone (Moncada et al, 1976; Furchgott, & Vanhoutte, 1989; Galley & Webster, 2004). It is now well known that the endothelium serves an active and integral role in vascular homeostasis by regulating vascular tone through the release of vasoactive factors (e.g., nitric oxide, endothelin-1) in response to alterations in the local shear stress profile (Lüscher & Barton, 1997).

Endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO) by converting L-arginine to L-citrulline and NO. NO diffuses into the surrounding smooth muscle where it binds to nitric oxide receptors (soluble guanylate cyclase) facilitating guanosine triphosphate (GTP) conversion into cyclic 3', 5'-guanosine monophosphate (cGMP). The secondary messenger cGMP then binds and activates protein kinase G, resulting in smooth muscle relaxation by phosphorylating downstream targets to prevent smooth muscle myosin crossbridge formation (Denninger & Marletta, 1999). When NO production is attenuated, a reduction in smooth muscle vasodilation is observed which – by means of tonic vasoconstriction – increases vascular resistance to blood flow (Cunningham & Gotlieb, 2005; Cines et al, 1998). Accordingly, inhibition of eNOS in mice via the non-specific NOS inhibitor N(G)-monomethyl L-arginine (L-NMMA) which competes for the same precursor as L-arginine, promotes an atheroprone phenotype in endothelial cells (Lüscher & Barton, 1997; Miller & Burnett, 1992). Chronic reductions in NO bioavailability have been observed to reduce vasodilatory capacity in obese Zucker rats, promoting the pathological manifestations observed in vascular disease (Frisbee, 2005). Moreover, human studies in hypertension (Weil et al, 2011), obesity (Higashi et al, 2001), aging (Taddei et al, 2001), and dyslipidemia (Dow et al, 2015), among others (Chen et al, 2017), all demonstrated decreased bioavailability of NO. Ultimately it is clear that disruption of vascular homeostasis involves the

under-expression of NO and over-expression of endothelial derived contracting factors which then contributes to an increased mechanical stress on the vascular network (Sessa et al, 2019).

Shear stress and vascular homeostasis

Shear stress is the frictional and tangential force of the blood exerted on the vessel walls (Malek et al, 1999). Blood does not flow uniformly throughout the body as a consequence of normal vascular geometry and/or thrombi formation which can be the result of endothelial dysfunction (Givens & Tzima, 2016). Therefore, the shear stress exerted on the vessels varies throughout the vascular network and is described as either laminar or oscillatory in nature (Vion et al, 2013). Laminar shear stress is considered atheroprotective while oscillatory shear stress is deemed to be atheroprone, contributing to vascular dysfunction (Kim et al, 2015). Oscillatory shear stress is described as turbulent flow and is most prevalent immediately distal to bifurcations, curvatures, and branches in vessels, such as the descending thoracic aorta (Lüscher & Barton, 1997; Mack et al, 2017). In turn, atherosclerosis is more likely to develop following a branch-off point in the vessels where oscillatory flow is common. These regions of greater susceptibility where oscillatory flow is more common are typically referred to as flow separation zones (Lüscher & Barton, 1997). The endothelium requires laminar shear stress to maintain endothelial cell function and structural/positional integrity. Without laminar shear stress, endothelial cells adopt a pathological phenotype that promotes cell proliferation, platelet aggregation, thrombi formation, inflammation, and disruption of cell organization. In the presence of laminar shear stress, endothelial cells elongate, are arranged parallel to flow, and maintain a state of quiescence (Topper & Gimbrone, 1999).

Thijssen and colleagues (2009) demonstrated that retrograde/oscillatory shear stress has a profound impact on endothelial function in humans *in vivo*. The authors artificially induced

retrograde shear stress by inflating a pneumatic cuff on the forearm of participants for 30 minutes. Thereafter, vascular endothelial function using flow-mediated dilation (FMD), a protocol that is used to approximate the NO-mediated vasodilatory capacity of an artery (Green et al, 2014), was observed to be impaired (Thijssen et al, 2009). Thijssen et al, observed that the FMD response following 30 minutes of cuff inflation was reduced compared to no cuff inflation, suggesting detriments to NO-induced vasodilatory function. This was the first *in vivo* study performed on humans to explore the deleterious impact of induced retrograde/oscillatory shear stress on the endothelium using FMD (Thijssen et al, 2009). Since then, several other studies have been able to replicate these findings in humans using similar distal blood flow occlusion techniques (Thijssen et al, 2011; Jenkins et al, 2013; Dawson et al, 2021).

Notch signaling pathway: Notch1 as a mechanosensor

Notch and its ligands are part of an evolutionarily conserved signaling pathway that is essential for endothelial cell-fate determination (Artavanis-Tsakonas, et al, 1999; Sheldon et al, 2010). The Notch signaling pathway is implicated in a variety of roles in development and adulthood including angiogenesis, neurogenesis, limb development, hematopoiesis and most recently, mechanosensing (Artavanis-Tsakonas et al, 1999; Shawber & Kitajewski, 2004; Mack et al, 2017). There are 5 DSL (Delta, Serrate, LAG-2) ligands in humans that bind to Notch receptors: Delta-like 1, 3 and 4, jagged-1 and -2 (Dll1, Dll3, Dll4, Jag1, Jag2). Canonical Notch signaling occurs via cell-cell interaction where a DSL signal-sending cell interacts with the Notch extracellular domain (NECD) of a signal-receiving cell (D'Souza et al, 2010). Following NECD binding, a series of proteolytic cleavages are triggered. The initial cleavage of the NECD begins with the disintegrin and metalloproteinase, ADAM10 (S1) and is followed by y-secretase cleavage (S2) where the NECD is then internalized within the signal sending cell (Kopan & Ilagan, 2009;

Phng & Gerhardt, 2009). A final cleavage (S3) allows the Notch intracellular domain (NICD) to disassociate from the membrane and translocate to the nucleus where it directly interacts with RBPJ (Recombination signal binding protein for immunoglobulin kappa J region), a DNA-binding protein, and coactivator mastermind-like 1 (MAML1). RBPJ, also referred to as CSL (CBF1/RBPJ-k, Supressor of Hairless, Lag-1), is a complex that regulates transcription through its interaction with a corepressor complex (Kopan & Ilagan, 2009). Binding of NICD to CSL/RBPJ results in disassociation from the corepressor complex and subsequent association with a transcriptional activation complex allowing the activation of downstream Notch target genes (Phng & Gerhardt, 2009). See Figure 1 for an illustration of the canonical Notch signaling pathway.

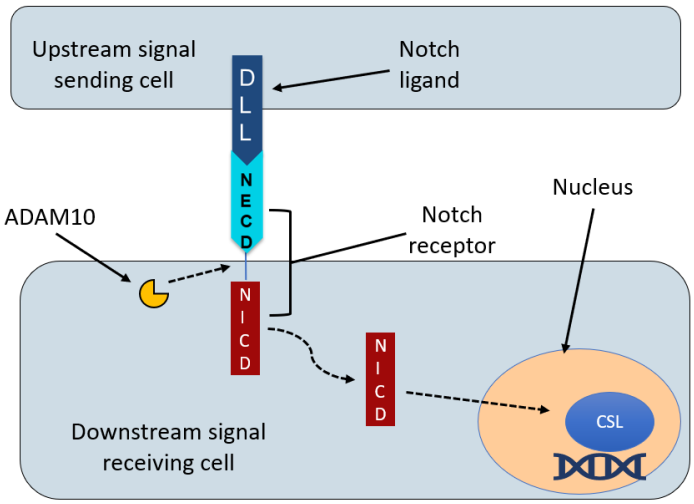

Figure 1: *A basic outline of the Notch signaling pathway. Notch signaling is initiated when an upstream signal-sending cell containing a Notch ligand interacts with a downstream cell containing the Notch receptor. This interaction leads to the cleavage of the Notch intracellular domain from the receptor complex by ADAM10/y-secretase. The Notch intracellular domain then translocates to the nucleus, leading to downstream activation of Notch targets.*

Endothelial cells transduce the mechanical force of shear stress acting on the vessel walls although they require an intermediate that has the capacity to sense alterations in flow patterns. There are several known mechanosensors located on the endothelium that include platelet endothelial cell adhesion molecule-1 (PECAM-1), the apical glycocalyx, basal integrins and most

recently, Notch1 (Givens & Tzima 2016; Mack et al, 2017). Mechanosensors are proteins/complexes that register the physical force of shear stress and subsequently initiate a biochemical response via signaling pathways to regulate endothelial cell phenotype. Importantly, Notch1 signaling appears to be disrupted under oscillatory shear stress thereby contributing to vascular dysfunction.

In a seminal study, Mack et al (2017), observed robust Notch1 signaling on the endothelium of non-angiogenic adult mouse aorta, indicating its continuous expression in adult arteries. They then transfected human aortic endothelial cells with a reporter construct to determine the effect of shear stress on Notch1 transcripts by subjecting them to both static and flow conditions. Under laminar flow, they found that Notch1 transcription was upregulated – an increase that was similarly observed in PECAM-1, a known flow-sensitive mechanosensor of the endothelium. Active NICD was observed to have a 1.7-fold increase under flow conditions and transcription was also upregulated 1.6-fold, implicating that Notch is activated under laminar flow conditions and that there is a positive feedback mechanism of Notch in response to such flow conditions.

In the same study (Mack et al, 2017) using a fluidic chamber, the authors observed endothelial cell elongation and alignment with high laminar flow (26 dynes/cm^2) that corresponded with a marked increase in the presence of Notch1 nuclear translocation. Under low flow (10 dynes/cm^2) the opposite was observed. To determine whether Notch1 is necessary for endothelial phenotype the authors removed Notch1 protein via siRNA knockdown using a y-secretase inhibitor (DAPT) and analyzed endothelial cells under flow. Compared to control monolayers, the knockdown monolayer was observed to have gaps between endothelial cells, a reduction in flow orientation, discontinuous cell-cell junctions and reduced endothelial cell elongation. These

experimental results provided evidence that Notch1 is responsive to flow and that Notch1 is required for endothelial cell responses to flow such as elongation, alignment, junctional integrity, and endothelial quiescence (Mack et al, 2017).

Dll4 is the DSL ligand responsible for regulating the differentiation of tip and stalk cells during angiogenesis to control vascular sprouting and branching (Phng & Gerhardt, 2009). Tip cells are on the front line of the new vessel sprouting process and have been observed to be saturated in Dll4 while stalk cells form the vascular wall and do not generally have much Dll4 present on their surface (Hellström et al, 2007). It is well-known that the Notch signaling pathway plays an essential role in vascular morphogenesis; however, its role as a mechanosensor of the endothelium is just being uncovered. In the presence of laminar shear stress, Dll4 ligand trans-interaction with the Notch1 receptor of a signal-receiving cell leads to the trans-activation of Notch signaling and subsequent activation of the atheroprotective NO-secreting endothelial phenotype. Dll4-Notch1 interaction is also known to exist within a cell. These cis-interactions reduce the ability of the cell to receive the signal from neighboring cells by a process known as cis-inhibition of the receptor by the ligand (Del Álamo et al, 2011). The ratio between Notch receptors and ligands within a cell is therefore important in determining whether Notch activation can occur. Over-expression of Dll4 may cis-inhibit the Notch1 receptor on the signal receiving cell, which would lead to the deactivation and subsequent removal of the receptor from the cell surface (Del Álamo et al, 2011). There is little to no evidence of how Notch1 functions as a mechanosensor in humans. Thus, we must first examine the dynamics of Notch1 activation under high and low antegrade shear before attempting to understand Notch1s role in endothelial function.

2. AIMS AND HYPOTHESES

Aim:

To characterize the dynamics of Notch1 ECD following altered shear stress patterns in the brachial artery of young healthy adults.

Hypotheses:

1) Notch1 ECD will be reduced from baseline values in conditions of decreased antegrade shear
2) Notch1 ECD will be increased from baseline values in conditions of increased antegrade shear

3. METHODOLOGY

Ethical Approval

The Research Ethics Board at the University of Windsor (REB# 36466) as well as the Research Health and Safety Board approved all experimental procedures and protocols in adherence with the principles of the Tri-Council Policy Statement and the University of Windsor Guidelines for Research Involving Human Participants.

Participants

Of the 15 participants recruited, 5 participants were excluded because of several failed attempts to establish a venous catheter in either or both arms. As such, data are presented as n=10 (6 M) healthy young adults (18-24 years of age) recruited from the University of Windsor through email campaigns. Participants included in the study were free from any known cardiovascular abnormalities/risk factors: hypertension/hypotension, presence of obesity and history of smoking/nicotine use in the past twelve months. A medical pre-screening was conducted over Microsoft Teams to identify any potential exclusion factors and included a COVID-19 self-screen. Both written and verbal consent were obtained from each subject following a briefing of the experimental protocol.

Table 1*: Select participant characteristics. ID = participant identification number; BMI = body mass index; SBP/DBP = resting systolic and diastolic blood pressure, respectively.*

ID	BMI (kg/m^2)	SBP (mmHg)	DBP (mmHg)	Sex (M/F)	Age (y)
1	24.1	121	70	M	23
2	22.3	108	70	F	20
3	26.6	121	78	F	24
4	24.8	126	89	M	20
5	25.1	130	80	M	24
6	21	112	85	F	23
7	19.1	133	77	M	20
8	19.5	112	80	F	19
9	24.4	110	74	M	24
10	25.1	119	78	M	21
Mean±SD	23.2±2.6	119.2±8.2	78.1±5.7		21.8±1.9

Experimental Design

We investigated the release of endothelial Notch1 ECD under differing conditions of vascular wall shear stress in 10 (6M/4F) young healthy adults *in vivo* using a repeated measures time controlled experimental design. See figure 2 for illustration of the experimental timeline. Participants reported to the lab (HK 221) at the University of Windsor having fasted overnight and abstained from any caffeine, alcohol, or vigorous physical activity 24 hours prior to visit. On testing day, venous catheters were placed in superficial antecubital veins of participants left and right arms and secured for the duration of the study. Following catheterization, two pneumatic cuffs were secured around the experimental arm: One on the wrist inflated to 220mmHg and one on the upper arm just below the axilla inflated to 40mmHg. An upper cuff was also placed on the contralateral 'control' arm and inflated to 40mmHg (figure 3). Before cuff inflation, blood samples were taken from both arms to establish Notch1 ECD baseline concentrations Thereafter, all cuffs were simultaneously inflated. The wrist cuff was inflated for 20 minutes – a method which

has previously been demonstrated to acutely induce disturbed blood flow in the arm (Jenkins et al, 2013). The upper cuffs at 40 mmHg was inflated for the entirety of the experiment (~35 minutes) for the purpose of 'trapping' circulating Notch1 extracellular domain (NECD) in the arm. Blood draws were taken while cuffs were inflated (at 10- and 20-minutes) and following deflation of the wrist cuff (immediately at cuff release, and 15 minutes post) (figure 2).

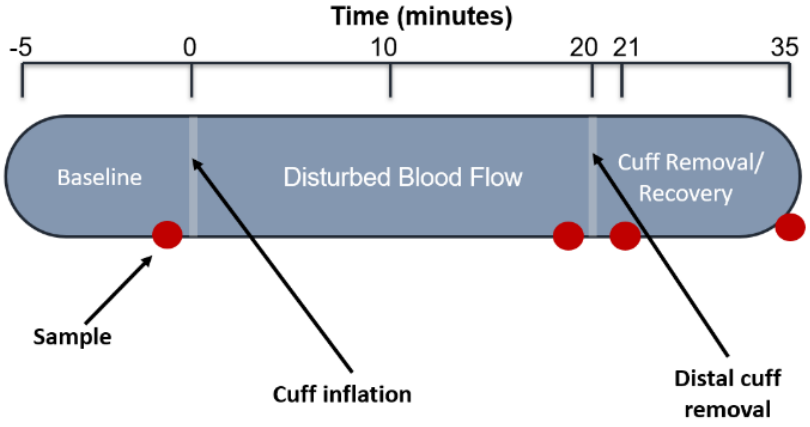

Figure 2: *Timeline of the experimental design. Filled red circles represent sampling periods where both vascular ultrasound and blood draws were performed. At cuff inflation, both the 220mmHg and the 40mmHg cuffs were simultaneously inflated.*

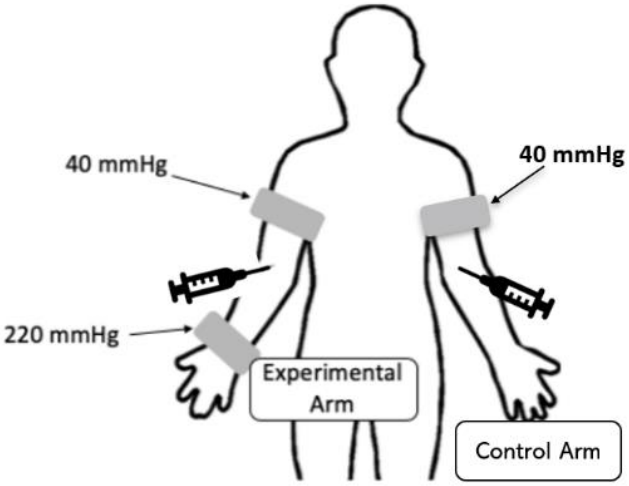

Figure 3: *Animation of the experimental setup. Grey bands represent pneumatic cuff placements while syringes represent insertion points for venous catheters.*

Measurement Techniques

Shear stress was manipulated in the experimental arm by inflation/deflation of the distal cuff and was quantified via ultrasound (Terason T3200) of the brachial artery for ~30 seconds around each blood draw. Ultrasound videos were screen captured and shear was analyzed offline using custom-designed edge detection software. Shear rate was calculated as 4 X the ratio between mean blood velocity (in cm/s) and artery diameter (in cm) [Shear Rate = 4 × (mean velocity/diameter)].

Blood samples were collected in sodium citrate tubes (BD Vacutainer, Cat # 363080) and centrifuged to collect platelet-poor plasma and subsequently stored at -80°C. Notch1 activity (circulating Notch1 ECD) was quantified from the plasma using commercially available immunosorbent assays (RayBio, Cat # ELH-NOTCH1), with an intra- and inter-assay CV of <10% and <12%, respectively. The detection limit of the assay is 20 pg/ml to 7000 pg/ml. ELISA analysis was conducted according to manufacturers instructions, and all samples and reagents were brought to room temperature before mixing. Samples were read at 450nm on a microplate reader (BioTek Synergy HT) using the Gen5 software (Version 1.11).

Statistical Analysis

Concentrations of Notch1 extracellular domain were determined by a 2x4 two-way repeated measures ANOVA using the factors of condition (control vs. experimental) and time point (BL, 20-min, cuff release, 15-min recovery). Tests for normality were confirmed by a Shapiro-Wilk test. When a significant interaction was found, post-hoc tests were performed using two-tailed repeated measures Student's T-tests, corrected for multiple comparisons using the Bonferroni

correction. Differences in shear on the experimental arm were determined by a one-way repeated measures ANOVA and Bonferroni corrected two-tailed T-tests from baseline. All statistical analyses were performed in SPSS 26 (IBM). Significance was determined at an alpha of <0.05.

4. RESULTS

Shear rate: Absolute shear rate values (arbitrary units expressed as 1/s) of the experimental arm at baseline (BL), 20-min (20), 1-2 minutes following distal cuff release (Release), and 15-min following distal cuff release (post) are depicted in Figure 4. On the experimental arm there was a main effect of time (P<0.001). Compared to baseline (121±114), shear rate was reduced at 20 minutes by 81% (P=0.037). Immediately following deflation of this pneumatic cuff, shear rate was significantly elevated by 106% (P=0.022). At 15 minutes following cuff release (post), shear rate was statistically similar to baseline values (P=0.157), albeit was on average reduced by 38%.

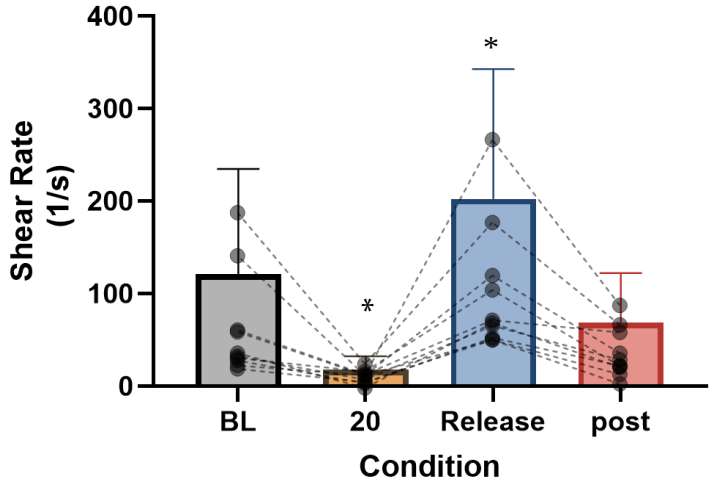

Figure 4: *Changes in brachial artery shear rate of the experimental arm. Shear rate on cuff release was significantly higher than baseline. Asterix (*) denote significantly different (P<0.05) from baseline. Connecting lines denote individual data. Filled bars and error bars denote mean±SD.*

Notch1 ECD: The concentration of Notch1 ECD in the control and experimental arm is shown in Figure 5, panels A and B, respectively. There was a significant interaction between the experimental and control arm (condition x time, P=0.008). In the control arm, there was no significant difference at any time point compared to baseline (P all > 0.05). However, in the experimental arm, the concentration of Notch1 ECD was significantly elevated by an average of

52% (P=0.033) immediately following cuff release (Figure 5), where the average increase in antegrade shear was 106% (Figure 4). Compared to baseline, there was no other difference in Notch1 ECD at any other time point, albeit it trended to be lower (by ~30%) at 15 minutes following cuff release (post, P=0.133). In contrast to the first hypothesis, at 20 minutes of reduced shear, the Notch1 ECD was not lower from baseline (P=1.00), despite the average reduction in antegrade shear by 81%, indicating that increases, but not decreases in antegrade shear, acutely alter the Notch1 ECD profile.

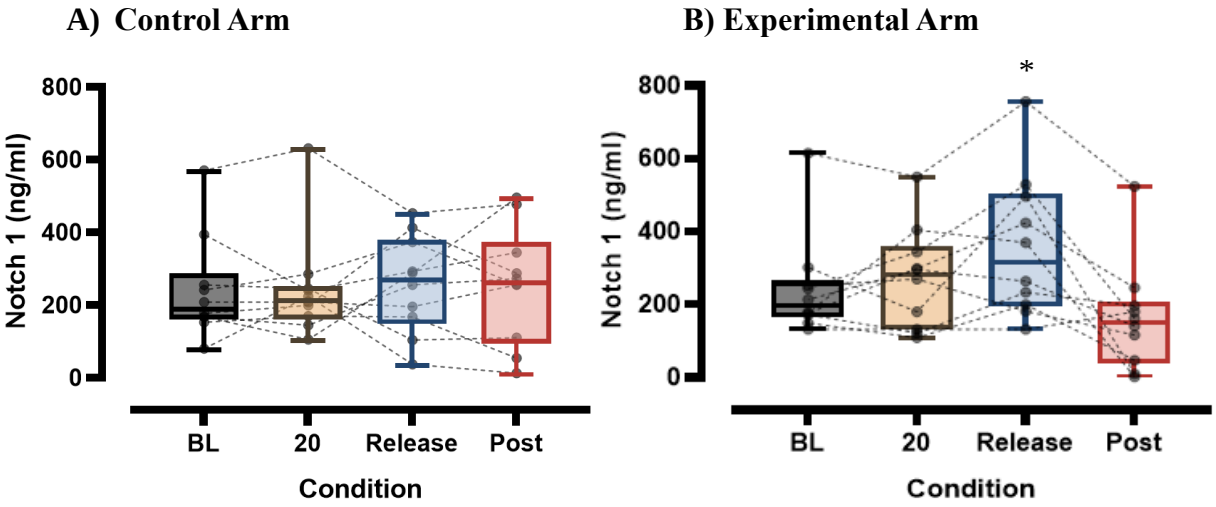

Figure 5: *Notch1 extracellular domain concentration (ng/ml) in the control arm (Panel A) and experimental arm (Panel B). Notch1 ECD was significantly increased (P=0.033) from baseline following distal cuff removal in the experimental arm. Connecting lines denote individual data. Filled bars and error bars denote mean±SD.*

5. DISCUSSION

To our knowledge, these are the first data to demonstrate *in vivo* alterations of Notch1 in response to alterations of endothelial shear stress in healthy adults. We show that in the healthy human forearm, acute increases but not decreases in brachial shear stress alter the venous Notch1 ECD profile, indicative of endothelial nuclear Notch1 translocation. Specifically, a ~100% increase in antegrade shear over only a few minutes increased the concentration of venous Notch1 ECD by ~50%. In contrast, 20 minutes of reduced antegrade shear, by an average of 80% from distal forearm blood flow occlusion, had no significant impact on the concentration of Notch1 ECD. These data provide novel information on the *in vivo* dynamics of Notch1 and its activity as an endothelial mechanosensor, and provide a novel mechanistic target for understanding the mechanisms of improved endothelial function following increases in antegrade (laminar) shear stress.

Notch1 as an endothelial mechanosensor

It is well known that laminar shear stress (high antegrade shear) induces an atheroprotective phenotype in endothelial cells, that is, the hemodynamic environment is the stimulus for endothelial health. (Davies, 2008). The endothelium's ability to secrete NO is dependent on the registration and subsequent biochemical translation of shear forces into cellular responses that favor atheroprotective phenotypic traits of endothelial cells (Fels & Kusche-Vihrog, 2020). Without mechanosensors, the endothelium consequentially loses its vasodilatory capacity in response to changes in blood flow – i.e. the flow-mediated dilation.

There are many known endothelial mechanosensors in humans. For example, the mechanosensory complex involving platelet and endothelial cellular adhesion molecule (PECAM-

1), vascular endothelial growth factor receptor 2 (VEGFR2) and vascular endothelial-cadherin is also an important regulator of endothelial cell function. In this complex, antegrade shear induces tension on PECAM-1 which triggers the activation of Src kinases. Vascular endothelial-cadherin links PECAM-1 to VEGFR2 allowing the Src-dependent trans-activation of VEGFR2 which in turn activates phosphoinositide 3-kinase (PI3-K) (Givens and Tzima, 2016). PI3-k activation then stimulates protein kinase-B (PKB) which is not only important for endothelial cell survival but also for eNOS-induced production of NO (Tzima et al, 2005). In this complex, PECAM-1 is directly responsible for registering shear stress, while VEGFR2 is required for the mobilization/activation of the biochemical signals that lead to atheroprotective phenotypes (Maringanti et al, 2021).

Although not previously considered to function as an endothelial mechanosensor, it is now evident that Notch1 should be added to the important endothelial complex that responds to alterations in shear. Importantly, Notch1 was found to be an indispensable factor for endothelial cell elongation, alignment with flow, secretion of NO, and suppression of proliferation (Mack et al, 2017). Mack et al, 2017, demonstrated in culture and rodent models that Notch1 was required to establish these phenotypes through genetic silencing experiments. Other putative mechanosensors such as Krüppel-like Factor 2 (KLF-2) - an endothelial mechanosensor in part responsible for regulating junctional stability - were upregulated in these experiments in response to flow but could not compensate for the losses incurred from Notch1 deletion. Thus, Notch1 was demonstrated to function as a potent mechanistic regulator of vascular function in these models. In our experiment, we took the first step to translating these experimental results to humans, which was characterizing how Notch1 responds to alterations in shear conditions in young, healthy adults. In congruence with Mack et al., 2017, we observed Notch1 activation to increase alongside a robust

increase in antegrade shear rate induced by the removal of a pneumatic cuff. Our results suggest that Notch1 is rapidly activated in response to increases in antegrade shear in adults. Given these novel data, a logical extension of this study is to quantify the actual endothelial function (e.g. flow-mediated dilation) alongside Notch1 activation.

It is interesting that the release of Notch1 ECD was not reduced in response to 20-min of decreased antegrade shear, and in some participants even increased (on average from 244 pg/ml at baseline to 271 pg/ml at 20-min; P=1.00; Figure 5). Several explanations could explain these findings. Foremost, the increased variability could be related to the increase in oscillatory shear (turbulent flow) that occurred with the distal cuff inflation, which may have mechanically cleaved the Notch1 ECD in some participants. Secondly, local hypoxia could have also caused Notch1 ECD cleavage. Indeed, Notch1 ICD is upregulated in hypoxic tissue (Gao et al, 2012), likely through HIF-1 – VEGF interactions that is responsible for revascularization in ischemic tissue (Hyun et al, 2021).

Notch1 and atherosclerosis

In a study that examined fibrous cap formation in atherosclerotic murine and human EC's, Notch1 blockade led to the observation that Notch1 was important in the re-acquisition of smooth muscle cell (SMC) identity in the cap. Forced Notch1 signaling, on the other hand, prevented lesion development and medially-derived SMC from contribution to plaque caps (Martos-Rodríguez et al, 2021). In this study they used conditional knockout of RBPJ and overexpression of NICD to block and force Notch signaling respectively. In another study, EC monolayers that were treated with soluble Dll4 were found to have positive influences on barrier protein expression and integrity through Notch1 signaling activation (Boardman et al, 2019). Barrier permeability was then examined using y-secretase inhibitor to confirm Notch involvement in the bolstering EC

junctional integrity. This reversed the reduction of solute flux experienced by Dll4-treated monolayers. Ultimately, there is a complex web of endothelial proteins responsible for the maintenance of an atheroprotective endothelium. Accumulating evidence suggests that these endothelial factors are not operating individually but that they interact and act in parallel (Maranganti, 2021). Nonetheless, Notch1 appears to function as a key regulator of endothelial health in respect of both vasodilatory ability and an antithrombotic phenotype (Mack et al, 2017).

Study considerations

Notch1 ECD is the extracellular component of the transmembrane receptor that is released following the ADAM 10 cleavage event. Following this event, the Notch1 ICD is also released so it can translocate to the nucleus. Although we did not analyze Notch1 ICD which would be the most direct method for quantifying activation of the protein, nuclear translocation is indicative of Notch1 activation and ECD cleavage occurs alongside ICD translocation. Thus, the ECD provides accurate analysis of local Notch1 activation from ECD which can be found in the circulation (Mack et al, 2017). Indeed, while quantification of Notch1 ICD would provide a better marker of true Notch activation, it is unfeasible in an *in vivo* human model. EC extraction is possible; however, the endothelial phenotype is markedly altered when examined under subsequent culture, and would no longer reflect the true *in situ* response.

Contrary to our hypothesis, we did not observe a reduction in Notch1 ECD following 20-min of reduced antegrade shear. This may have been a consequence of our experimental design. 20 minutes of reduced antegrade shear may not be sufficient to elicit an inhibitory Notch1 response in the brachial artery of young and healthy adults. Indeed, while loss of Notch1 has been observed in human biopsies of atherosclerotic regions prone to decreased shear (Mack et al., 2017), downregulation may only be secondary to a more chronic and pro-inflammatory environmental

milieu. Alternatively, and as discussed earlier, the ischemic environment may have upregulated the Notch1 pathway in some participants (Hyun et al, 2021). Future research is thus necessary to glean *in vivo* Notch1 activity in more chronic states of ischemia and hypoxia.

For quantification of Notch1 ECD, we used a commercially available human Notch1 ECD ELISA kit with the lower limits of detection at 20pg/ml. We chose a plasma dilution of 2x, which, in some cases required us to read at the lower limits of detection. As such we ran a separate ELISA on a subset of samples with no plasma dilution. However, this led to over-saturation in several wells. This was most likely attributable to an excessive amount of non-specific binding. To address concerns of non-specific binding in the ELISA, future work should consider confirmation of Notch1 activity by combining Western blot density bands to aid in the accuracy of detecting Notch1 ECD.

Another consideration is the experimental vascular bed used in the current study – i.e., the forearm. The brachial artery itself is highly resistant to plaque formation and physiologically sustained oscillatory shear stress (Bentzon et al, 2014). As such, it may be more clinically applicable to observe more susceptible arteries (e.g. coronary arteries). Moreover, we were underpowered to detect any sex differences in the current study. Future study is required to determine whether there are sex differences in the Notch1 response, or whether the Notch1 activity varies throughout the menstrual cycle.

Lastly, it is important to note that while 20-min of reduced antegrade shear is a known model that acutely reduces vascular dilatory function (Jenkins et al, 2013), functional measures were not performed in the current study. Quantifying endothelial function in response to increased/reduced Notch1 signaling may provide valuable insight into its importance as a rapidly acting mechanosensory complex. Future research on characterizing the Notch1 response to shear

stress in humans may seek to clarify the many uncertainties that exist such as the Notch1 response to sustained vascular insults like atherosclerosis, CVD and aging populations compared to healthy participants.

6. CONCLUSION

Vascular dysfunction is the common etiologic pathway of all CVD and related events - the number one cause of morbidity and mortality worldwide (Barthelmes et al, 2017). Chronic disruption of the vascular environment initiates a multi-factorial catalytic response that leads to a vicious cycle of endothelial damage, apoptosis and proliferation. It is clear that endothelial dysfunction is an indicator of vascular impairment, preceding atherosclerosis; however, the precise mechanisms through which endothelial dysfunction stems remains unclear. Notch1 has been identified to function as an endothelial mechanosensor in culture and murine models in 2017. However, there has not been much attention given to the Notch1 signaling pathway and its translatability to humans. In this study, using an *in vivo* model in healthy adults, we observed that Notch1 is notably upregulated in response to acute elevations in antegrade shear, but not 20-min of sustained reduced shear. As Notch1 is suggested to be necessary in the regulation of vasodilatory function, endothelial proliferation, orientation with flow, junctional integrity, and endothelial cell elongation (at least in animal and *in vitro* models), identifying the unique conditions that alter Notch1 activity in humans may yield an important step for improvements in targeted vascular therapy.

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