Effects of Oscillatory Blood Flow on Atherosclerosis

Chia-Pei (Denise) Hsu
May 27th, 2019

Advisors:
Dr. Joshua Hutcheson
Dr. Sharan Ramaswamy

FIU | Biomedical Engineering
FLORIDA INTERNATIONAL UNIVERSITY

Table of Contents

SUMMARY

What is atherosclerosis?

Atherosclerosis is a disease involving narrowing of the arteries due to plaque buildup. It is a major underlying cause for strokes and heart attacks, which are leading causes of morbidity and mortality worldwide [1]. These plaques mainly consist of cholesterol, fatty substances, cellular waste products, calcium, and fibrin. They can occlude blood supply to the distal bed, and plaque rupture can activate unnecessary thrombus deposition that further interrupts blood flow in smaller vessels. Significant blood blockage in the coronary artery leads to heart attacks, while blockage in the cerebral circulation results in strokes. Plaque deposition begins with endothelial dysfunction and unusual smooth muscle cell behavior due to environmental cues [2]. Vascular remodeling often involves paracrine signaling between endothelial and smooth muscle cells, and diseases such as atherosclerosis can result from improper communication between these cells.

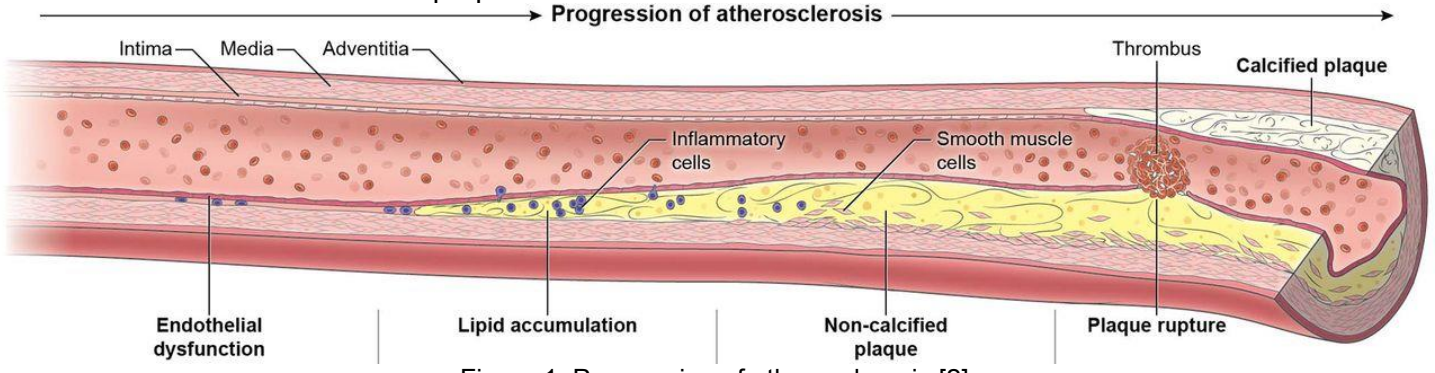

Figure 1: Progression of atherosclerosis [2]

What are vascular endothelial cells (VasEC) and vascular smooth muscle cells (VasSMC)?

VasECs are cells that form the inner lining of blood vessels. They play critical roles in maintaining vascular homeostasis in thrombosis, inflammation, as well as vascular remodeling [3]. VasSMCs are the most abundant cell type in blood vessels. They maintain vascular homeostasis via active contractions and relaxations. Loss of contractile function in VasSMCs due to environmental changes may lead to cell migration, atherosclerotic plaque formation, and vascular calcification [4].

What is fluid shear stress? What is oscillatory shear index (OSI)?

Shear stress in a fluid is the tangential force per area needed to move a layer of fluid over another in response to friction from fluid viscosity. OSI is a parameter between 0 and 0.5, which quantifies the disturbance of flow characterized by change in direction of wall shear stress [5]. Shear stress and OSI equations are defined as:

$$\tau_w = \mu \frac{du}{dy} \ , \ OSI = \frac{1}{2}\left(1 - \frac{\left|\int_0^T \tau_w dt\right|}{\int_0^T |\tau_w| dt}\right)$$

Where $\tau_w =$ wall shear stress, $\mu =$ dynamic viscosity, $u =$ flow velocity, $y =$ distance from wall, $T =$ duration of cycle, $t =$ time [5]

What are the clinical/research needs in this study?

Magnitude and direction of shear stress on the endothelium has a significant impact on vasoconstriction and vasodilation. Excessively high shear stresses may further damage red blood cells and the endothelium in blood vessel linings. Other flow patterns such as oscillatory shear may activate platelets, resulting in thrombus formation [6]. Studies have shown increased blood flow disturbances at bifurcation sites. Flow in these regions exhibit turbulence, separation, recirculation, and oscillation. Cell alignment in response to flow also show a significant impact on endothelium morphology, as shown in figure to the right [6].

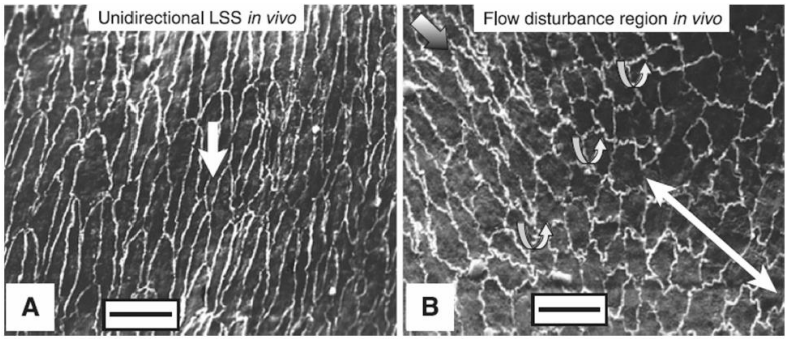

Figure 2: Endothelium morphology in regions of laminar flow (A) and regions of disturbed flow (B) [6]

As blood flow involves laminar and disturbed shear stresses on cardiovascular tissues, cellular responses due to various levels of shear and oscillation are not yet well investigated. Understanding the behavior and signaling of the endothelium due to flow environments can serve as high potential for diagnostics and therapeutics. This research project will help us identify different OSI values and their effects on VasEC and VasSMC phenotypes that are associated with atherosclerosis development. Four conditioning groups covering the full range of no oscillation (0 OSI) to full oscillation (0.50 OSI) will be studied: static, steady flow (0 OSI), 0.25 OSI, and 0.5 OSI.

NARRATIVE

The vascular system consists of blood vessels that circulate blood throughout the entire body, delivering nutrients and removing waste products to maintain regular body functions.

Vascular diseases are abnormal conditions of the blood vessels (arteries, veins, and capillaries) that prevent blood circulation, in which the causes may be associated with loss of proper function and communication amongst vascular cell types due to environmental cues.

Given that blood flow throughout the circulatory system induces shear stress on the inner lining of vessel walls, cellular responses due to oscillatory shear can be further investigated to understand the relation between flow environment and blood vessel integrity, specifically in assessing the risk for developing atherosclerosis.

SPECIFIC AIMS

Atherosclerosis is an important factor to myocardial infarction and strokes, which are leading causes of morbidity and mortality. Currently, the risk for developing atherosclerosis is difficult to assess. Medium and large arteries including the coronary, carotid, cerebral, their respective branches, and other major arteries can all be affected by atherosclerosis. In 2015, coronary and cerebrovascular atherosclerosis caused up to 15 million deaths worldwide and is increasing in prevalence in many countries [1]. Previous studies have shown alterations in vascular endothelial cell functions due to various hemodynamic forces, specifically promotion of atherogenesis associated with disturbed flow patterns [6]. Other studies have shown increase in gene expression related to cardiovascular maintenance when cells were conditioned under native human aortic pulsatile flow environments [7]. Despite the various outcomes of cell behavior under different flow profiles, the atherosclerotic mechanisms associated with communication between vascular endothelial cells (VasEC) and vascular smooth muscle cells (VasSMC) due to oscillatory flow environments remain unclear. The objective of this study is to understand the relation between various degrees of flow oscillation in modulating VasEC phenotypes and their subsequent paracrine regulation of VasSMC in development of atherosclerosis. The findings of this research will help us determine an oscillation range that promotes atherogenesis, and a range that prevents it.

Hypothesis: Using oscillatory shear index (OSI) to quantify disturbance of flow [5], we hypothesize that vascular regions exposed to low OSI (OSI ≤ 0.25) will lead to low expression of atherogenic genes, in which attenuates plaque formation and therefore preventing atherosclerosis.

Aim 1: To test if high OSI environment (OSI = 0.50) causes VasEC injury that leads to endothelium dysfunction, promoting atherogenesis. To test if low OSI (OSI ≤ 0.25) results in low expression of endothelial injury, reducing the risk for atherosclerosis.

Experimental Strategy: Using the BioFlux 200 System (Fluxion Biosciences, San Francisco, CA) consisting of a microchannel plate connected to a pneumatic pump that delivers shear flow, VasECs will be exposed to various unidirectional and bidirectional flow profiles. Four OSI conditions to be tested include static (control), unidirectional steady flow (0 OSI), 0.25 OSI, and 0.50 OSI. VasEC integrity and phenotype will be assessed after the 48-hour conditioning period via gene expression, protein analyses, and nitric oxide (NO) assay on the conditioned media.

Expected Outcome: We hypothesize that high oscillation shear flow will result in low nitric oxide production due to loss of endothelium, in addition to higher gene and protein expressions in cytokine monocyte attraction, wound healing, inflammation, and cell adhesion due to endothelial injury.

Aim 2: To determine whether paracrine regulation from VasECs previously conditioned in high OSI (OSI = 0.50) leads to increased atherosclerotic expression and calcification of VasSMCs. To compare VasSMCs integrity and behavior after paracrine regulation from VasECs previously conditioned in high OSI (OSI = 0.50) and low OSI (OSI ≤ 0.25) groups.

Experimental Strategy: To expose the biochemical end-products generated by VasECs under different OSIs, conditioned media from VasECs in Bioflux plates will be collected and re-used for subsequent VasSMC culture in static. VasSMCs will be cultured with 50% VasEC conditioned media and 50% fresh media. To further induce calcification, a separate experimental group with osteogenic media will also be used instead of fresh media.

Expected Outcome: We hypothesize that VasSMC's exposure to biochemical end-products secreted by VasECs conditioned under high OSI (OSI = 0.50) will result in upregulation of atherogenic genes and proteins, in addition to loss of VasSMC contractility and integrity. We also expect to see increased alkaline phosphatase (ALP) and calcification activities in VasSMCs cultured in high OSI conditioned media with osteogenic media.

Aim 3: To assess vascular atherogenic and calcification activities in *ex vivo* mice arterial tissues conditioned under different OSIs with high phosphate media in a bioreactor system.

Experimental Strategy: Thoracic aortas from wildtype male mice (C57BL/6J, *Mus musculus*) will be explanted and conditioned in a bioreactor system with high phosphate media under these flow conditions: Steady flow (0 OSI), 0.25 OSI, and 0.50 OSI. Static tissue culture will be used as control. Given the geometries of bioreactor chamber, tubes, and explanted tissue samples, computational fluid dynamics (CFD) will be used to determine waveforms that deliver the desired OSIs. Volumetric flow and pressure values will be measured with flow probe and pressure transducers for validation. Assessments of the conditioned *ex vivo* arterial tissue include histology, intimal thickness measurement, immunohistochemical staining, gene expression, protein analyses, and ALP activity.

Expected Outcome: We hypothesize that tissues exposed to high OSI (OSI = 0.50) will have larger intimal thickness and lumican protein found in the inner medial layer, indicating development of atherogenesis. In addition, we expect high ALP and calcification activity to be present in the high OSI group.

RESEARCH STRATEGIES

Cell Culture and Cell Expansion *(Aims 1 and 2)*

VasEC and VasSMC cell types to be used for this study will be primary Human Aortic Endothelial Cells (HAoEC) and primary Human Aortic Smooth Muscle Cells (HAoSMC), respectively. HAoEC will be purchased (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in 6-cm dishes in Endothelial Cell Growth Medium (Cell Applications, Inc., San Diego, CA, USA) with 10% fetal calf serum (Atlanta Biologicals, Flowery Branch, GA, USA) and 1% penicillin/streptomycin at 5,000 units/mL (Gibco, Thermo Fisher Scientific) for expansion. To enhance HAoEC cell attachment during expansion, culture dishes will be coated with 2% (w/v) type B gelatin solution and washed with phosphate buffered saline (1x PBS, Gibco) prior to seeding. HAoSMC will be purchased (Thermo Fisher Scientific) and cultured in 10-cm dishes in Smooth Muscle Cell Growth Medium 2 (PromoCell GmbH, Heidelberg, Germany) with 10% Supplement Mix (PromoCell) and 1% penicillin/streptomycin at 5,000 units/mL (Gibco) for expansion. All cells will be cultured in sterile environment and incubated at 37°C in 5% CO_2 in humidified air, and media will be changed every 2 – 3 days. Cells at 80%-90% confluency are to be washed in PBS before trypsinizing (Trypsin-EDTA Solution, 0.05% 1x, Caisson Laboratories, Inc., Smithfield, UT, USA) and centrifuged at 1000 rpm for passaging. Experiments will be run between cell passages 5 – 10 for optimal *in vitro* studies [8].

Conditioning HAoECs in Bioflux Shear Assay Plates *(Aim 1)*

The endothelium consists of a monolayer of endothelial cells [9], hence we can simulate the endothelium by conditioning HAoECs in a two-dimensional environment. To generate flow, Bioflux (Fluxion Biosciences, San Francisco, CA) microchannel plates consisting of eight channels per plate will be used, in which require low priming and media volumes. With dimensions of width at 350 µm and height at 75 µm, the system uses Hagen-Poiseuille to apply shear force in the channels. Prior to cell seeding, channels will be primed with PBS and coated with 2% (w/v) gelatin solution, a protein derived from collagen that improves attachment of endothelial cells. Once seeded, the cells remain in static for 24 hours before shear is applied. A pneumatic pump that provides forward and reverse flow profiles over 48 hours of conditioning time [10] will be connected to the plate. Oscillatory shear indices (OSI) will be determined by the ratio of forward versus reverse flow directions. The four shear stress conditions to be tested on HAoECs are static, steady flow (0 OSI unidirectional), 0.25 OSI, and 0.50 OSI, as summarized in Figure 3.

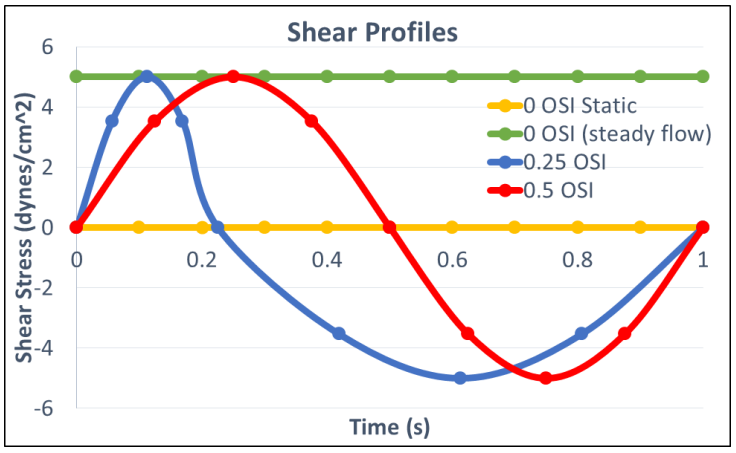

Figure 3: HAoEC Shear Profiles

Hagen Poiseuille Equation:

$\Delta P = \frac{8\mu LQ}{A}$, where $\Delta P =$ pressure drop, $\mu =$ dynamic viscosity, $L =$ length of channel, $Q =$ volumetric flow rate, $A =$ cross-sectional area of microfluidic channel

$\tau_\omega = \frac{6\mu Q}{h^2 W}$, where $\tau_\omega =$ wall shear stress, $\mu =$ dynamic viscosity, $Q =$ volumetric flow rate, $h =$ height of microfluidic channel, $W =$ width of microfluidic channel

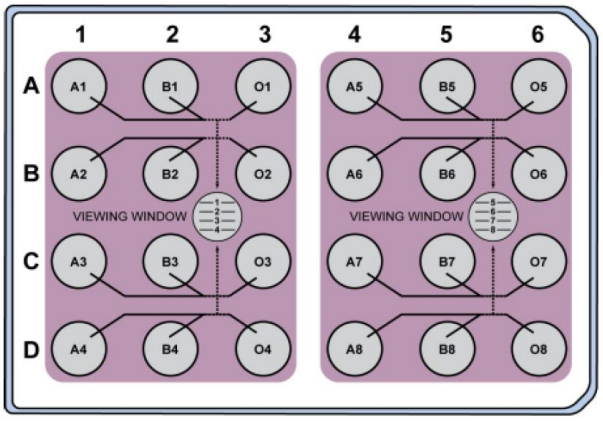

Figure 5: 24-Well Bioflux Plate

Figure 4: Bioflux System [40]

Nitric Oxide Assessment in Conditioned Media from HAoEC *(Aim 1)*
Nitric oxide (NO) is known to inhibit the development of atherosclerosis via vasodilation and anticoagulation [11]. It is synthesized from L-arginine under the presence of nitric oxide synthase enzyme (NOS), which requires tetrahydrobiopterin to facilitate production of NO [12]. Blood vessel vasodilation in response to shear forces require intact endothelium [13]. Therefore, assessment of NO level can provide information on function, viability, and integrity of the vascular endothelium, in which low presence of NO may indicate endothelial dysfunction. As NO is oxidized to nitrites and nitrates (NO_2^-/NO_3^-) by water and oxygen, NO level can be measured by quantitating total NO_2^-/NO_3^- in the system. QuantiChrom™ Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA, USA) will be used to measure NO concentration in the HAoECs' conditioned media from all flow groups at a minimum of n=3 per group. The concentrations will be statistically compared using methods under the "Statistical Analysis" section.

HAoEC-HAoSMC Paracrine Regulation *(Aim 2)*
HAoSMC seeding density and media volume per well in a 6-well plate will be 0.3 x 10^6 cells and 2 mL, respectively [14]. Of which 1 mL will be conditioned media from HAoEC and 1 mL will be fresh Dulbecco's Modified Eagle Medium (DMEM, 4.5g/L glucose, high L-glutamine, no sodium pyruvate, Gibco) with 10% fetal calf serum (Atlanta Biologicals) and 1% penicillin/streptomycin at 5,000 units/mL (Gibco). Conditioning time for HAoSMCs will be divided into two groups: 7 days and 14 days, and media will be changed every 3 days. All HAoSMC groups are to be cultured in static with flow conditioned media from HAoECs. To induce calcification and further study the effects of flow on calcific activities via paracrine signaling, fresh osteogenic media will be used in replacement of regular DMEM. The osteogenic media [15] consists of DMEM, 10% fetal calf serum, 1% penicillin/streptomycin, 50 µg/mL L-Ascorbic acid (Sigma Aldrich, St. Louis, MO, USA), 10 mM β-glycerophosphate (MilliporeSigma, Burlington, MA, USA), and 10 nM dexamethasone (Sigma Aldrich).

Table 1: Summary of Flow Conditions, Media, and Conditiong Time for each Cell Type

Cell Type	Flow Group	Media		Conditioning Time	
HAoEC	Static	Endothelial Growth Media		48 Hours	
	Steady Flow (0 OSI)				
	0.25 OSI				
	0.50 OSI				
HAoSMC	Static	50% flow conditioned media from HAoEC + 50% regular DMEM	50% flow conditioned media from HAoEC + 50% osteogenic media	7 days	14 days
	Steady Flow (0 OSI)				
	0.25 OSI				
	0.50 OSI				

Total RNA Isolation and RT-qPCR *(Aims 1, 2, and 3)*
Cellular responses to environmental cues involve conversion of DNA into working proteins, and this process of translation utilizes messenger RNA (mRNA) transcription as well as transfer RNA (tRNA). To evaluate the gene expression step that precedes protein translation, RNA will be extracted from the cells followed by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR).

Upon termination of the 48-hour flow conditioning on the HAoECs, conditioned media will be collected to further culture HAoSMCs, in addition to nitric oxide assessment. 250 µL of TRIzol Reagent (Invitrogen, Thermo Fisher Scientific) will be pumped into each microfluidic channel to lyse cell membranes and expose the mRNA. The RNA-rich TRIzol solution will be collected into Eppendorf tubes at 1 mL per tube for isolation. Total RNA isolation will be performed using the reagent protocol [16]. RNA concentration will be quantified using NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). Using the extracted RNA as templates, we will use Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, CA, USA) with primers to amplify and quantify our genes of interest. Forward and reverse primers will be sequenced in the NCBI GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) and purchased from IDT PrimerQuest® Tool (Integrated DNA Technologies, Coralville, IA, USA). C_T, cycle threshold, in polymerase chain reaction is defined as the number of temperature cycles needed for fluorescent signal to cross the threshold, or the background level. C_T values obtained from RT-qPCR using "static" group as control allows us to determine upregulated and downregulated genes amongst flow groups. Housekeeping genes are constitutive genes uniformly expressed with low variance amongst sample groups and are used as reference points for analyses. For HAoEC and HAoSMC, they will be hypoxanthine phosphoribosyltransferase

1 (HPRT1) and peptidylprolyl isomerase A (PPIA), respectively [17]. Key phenotypic markers of respective cell types are summarized in subsections below. By applying the Livak method [18], or $\Delta\Delta C_T$, we will compute fold change, or the increase in ratio between final and initial concentration values, across all flow groups in HAoEC as well as HAoSMC groups after paracrine communication. Genes of interest include endothelium and smooth muscle cell markers, in addition to activities of inflammation, collagen production, cell adhesion, dysfunction, and calcification.

Markers Associated with Endothelial Integrity or Dysfunction

- Nitric oxide synthase 3 (NOS3)

  Upregulation of NOS3 is indicative of angiogenesis and high concentration of endothelial cell activity, and downregulation suggests decrease in angiogenesis and indication of endothelial cell apoptosis [13].

  Fwd: TGAAGGCGACAATCCTGTATG, Rev: CTGCAAAGCTCTCTCCATTCT
- Von Willebrand factor (vWF)

  vWF is a glycoprotein related to endothelial activation in blood coagulation [13]. It is synthesized exclusively in endothelial cells, therefore loss of vWF expression is correlated to loss of endothelial cell function or viability.

  Fwd: AAGCAGACATACCAGGAGAAAG, Rev: CAGGAGCAGGTGTCGTAAAT
- Bone morphogenetic protein 2 (BMP2)

  BMP2 is a subgroup of BMPs that stimulate differentiation of myoblasts into osteoblasts. Upregulation is an indication of increase in calcification and mineralization via TGFβ1 pathway [19].

  Fwd: GGGTGGAATGACTGGATTGT, Rev: GTCAAGGTACAGCATCGAGATAG
- Matrix metalloproteinase-2 and -9 (MMP-2, MMP-9)

  MMPs are involved in remodeling of the extracellular matrix (ECM). They play important roles in angiogenesis, morphogenesis, remodeling, and wound healing [20]. Upregulation of the gene may indicate cellular responses to loss of ECM or endothelial integrity, and that remodeling would be needed. MMP-2 is associated with increased angiogenic phenotype, and MMP-9 is associated with release of vascular endothelial growth factor (VEGF) [21].

  Fwd: CTACGATGGAGGCGCTAATG, Rev: GCGAGGGAAGAAGTTGTAGTT (MMP-2)

  Fwd: CTTCCAAGGCCAATCCTACTC, Rev: GCCATTCACGTCGTCCTTAT (MMP-9)
- Vascular endothelial growth factor (VEGF)

  VEGF is associated with promotion of endothelial cell adhesion, migration, and proliferation. It also serves as a potent vascular regulator responsible for vasculogenesis [3]. Loss of VEGF expression may indicate dysfunction of the endothelium.

  Fwd: CCATGAACTTTCTGCTGTCTTG, Rev: ACTCGATCTCATCAGGGTACT
- Selectin E (SELE, CD62E)

  Selectin E, or E-selectin, is a leukocyte adhesion molecule expressed by endothelial cells when activated by inflammatory cytokines such as IL-1 and TNF-α [13]. This gene will be assessed in comparison to vWF and TNF-α proteins, as they are often elevated in ischemic heart diseases. However, controlled blood pressure tend to reduce only the expression of vWF during hypertension and hypercholesterolemia [13].

  Fwd: GTATGTTAGGGTGCTCTGGAAG, Rev: CCTCTGCTGTTCTGATCCTTATC
- Vascular cell adhesion molecule 1 (VCAM-1)

  Expression of VCAM-1 is elevated during endothelial injury and inflammation, in which similar to Selectin E, VCAM-1 functions as leukocyte migration [22]. Increased VCAM-1 is also found in sites predisposed to formation of atherosclerotic lesions, suggesting an early role of VCAM-1 in atherosclerosis [23].

  Fwd: TGGGAATCTACAGCACCTTTC, Rev: CACAGCCCATGACACTACAT

Markers Associated with Smooth Muscle Cell Contractility, Phenotype Change, and Atherogenesis

- Alpha smooth muscle actin (α-SMA)

  Upregulation of α-SMA indicates an increase in vascular contractility and blood pressure homeostasis. Downregulation may indicate loss of smooth muscle cell contractility and therefore loss of blood pressure homeostasis [24].

  Fwd: ACACTGGGCTGGACAATAAC, Rev: GTGACTGAGCAGAATCCCTAAC
- Collagen type I alpha chain (Col1a1)

  Type I collagen is the most abundant collagen protein in vertebrates. It is also the key composition of vascular walls and connective tissues. Regulation of collagen production suggests vascular integrity, in which loss of collagen may indicate vascular injury [25].

  Fwd: CCAAGGGTTTCCTCCGTATTT, Rev: CCTTCTTCTTCCCTCGCTTTAG
- Bone morphogenetic protein 4 (BMP4)

  One of the bone morphogenetic family members that is associated with differentiation of osteoblasts.

Upregulation may be an indication of increase in calcification and mineralization. BMP4 expression is also observed in promotion of vascular remodeling, in which suggests presence of vascular injury [26].
Fwd: GGAGATGGTAGTAGAGGGATGT, Rev: AGGTCAAGGTGAATGTTTAGGG

- Runt-related transcription factor 2 (RUNX2)
  RUNX2 is associated with osteoblast differentiation and transition of smooth muscle cells into osteogenic phenotype [27], therefore upregulation may be indicative of calcification.
  Fwd: CACTATCCAGCCACCTTTACTT, Rev: GAGCCTTCTGCTTCTCAATATCT
- Bone gamma-carboxyglutamate protein (BGLAP)
  BGLAP is a precursor for production of osteocalcin, which is a non-collagenous protein secreted by osteoblasts. Presence of this gene may suggest a possible osteoblast phenotype and indication of mineralization. Expression of osteocalcin protein was found in atherosclerotic plaques [28].
  Fwd: AGCCTTTGTGTCCAAGCA, Rev: CTAGACCGGGCCGTAGAA
- Wnt family member 4 (WNT4)
  Wnt is associated with increase in smooth muscle proliferation and intimal thickening via the β-catenin pathway, in which is an aspect of atherosclerosis formation [29].
  Fwd: AACTCTCCAGCACACATACAC, Rev: GACTTTATTGCACAGCCCTTTC

Protein Extraction, Bicinchoninic Acid (BCA) Protein Assay, and Western Blot *(Aims 1, 2, and 3)*
Proteins formed from DNA after mRNA transcription and tRNA translation can be extracted with RIPA Lysis and Extraction Buffer (Thermo Fisher) mixed with protease and phosphatase inhibitors. Pierce™ BCA Protein Assay Kit (Thermo Fisher) will be used to quantify protein concentration. The results from BCA assay will be used to normalize the volume of proteins for Western Blot. The goal of the Western Blot is to identify an antigen on the protein of interest with specific antibodies. The proteins will travel various distances through an electrophoresis gel depending on molecular weight. Each western blot will be run with one negative control. Once protein movement is stabilized, they will be transferred to a membrane to be probed with antibodies. The fluorescently labeled antibodies attached to the membrane will then be imaged using the Li-COR Odyssey CLx Imaging System and software (LI-COR Biosciences, Lincoln, NE, USA) for detection at 680 nm or 800 nm. All equipment used for Western blotting will be purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Proteins of interest for the respective cell types are summarized below.

<u>Proteins Associated with Endothelial Integrity or Dysfunction</u>

- Nitric oxide synthase 3 (eNOS), Molecular weight: ~140 kDa
  This protein is associated with synthesizing nitric oxide, which is responsible for vasodilation, anticoagulation, and inhibition of atherogenesis. Absence of eNOS may be an indication of endothelial dysfunction [12].
  Primary antibody: Mouse monoclonal anti-eNOS (ab76198)
  Secondary antibody: Goat Anti-Mouse LI-COR IRDye 800 RD
- Phosphorylated eNOS (p-eNOS), Molecular weight: ~133 kDa
  Phosphorylated eNOS is the activated version of nitric oxide synthase 3
  Primary antibody: Rabbit polyclonal anti-eNOS (phospho T494) (ab138430)
  Secondary antibody: Goat Anti-Rabbit LI-COR IRDye 680 RD
- Matrix metalloproteinase-2 and -9 (MMP-2, MMP-9), Molecular weight: ~ 72 kDa and ~ 92 kDa
  In addition to gene expression of MMP-2 and MMP-9 mentioned above, further observation (if any) of protein from western blots adds rigor to loss of ECM and endothelial integrity.
  Primary antibody (MMP-2): Rabbit polyclonal MMP2 antibody (ab37150)
  Secondary antibody (MMP-2): Goat Anti-Rabbit LI-COR IRDye 680 RD
  Primary antibody (MMP-9): Rabbit polyclonal MMP9 antibody (ab38898)
  Secondary antibody (MMP-9): Goat Anti-Rabbit LI-COR IRDye 680 RD

<u>Proteins Associated with Smooth Muscle Cell Contractility, Phenotype Change, and Atherogenesis</u>

- Bone morphogenetic protein 2 (BMP2), Molecular weight: ~45 kDa
  Bone morphogenetic protein induces calcification from TGF-β signaling pathway [19].
  Primary antibody: Rabbit polyclonal anti-BMP2 (ab14933)
  Secondary antibody: Goat Anti-Rabbit LI-COR IRDye 680 RD
- Tumor necrosis factor alpha (TNF-α), Molecular weight: ~17 kDa
  Tumor necrosis factor alpha is an inflammatory cytokine, indicating potential loss of cell function and increase in inflammatory response.
  Primary antibody: Mouse monoclonal anti-TNF-α (ab1793)
  Secondary antibody: Goat Anti-Mouse LI-COR IRDye 800 RD
- Osteocalcin (BGLAP), Molecular weight: ~ 12 kDa

In addition to gene expression of BGLAP mentioned above, further observation (if any) of osteocalcin protein suggests precursors for initial formation of atherosclerotic plaques [28].
Primary antibody: Mouse monoclonal Osteocalcin antibody (ab13420)
Secondary antibody: Goat Anti-Mouse LI-COR IRDye 800 RD

- β-catenin, Molecular weight: ~ 94 kDa
  This protein is responsible for smooth muscle cell proliferation and intimal thickening [29].
  Primary antibody: Rabbit polyclonal beta -catenin antibody (ab6302)
  Secondary antibody: Goat Anti-Rabbit LI-COR IRDye 680 RD
- Calponin, Molecular weight: ~ 34 kDa
  Calponin is specific to mature smooth muscle cells and regulates smooth muscle cell contractility [30].
  Loss of calponin may indicate change in smooth muscle cell phenotype from contractile to synthetic [31].
  Primary antibody: Rabbit monoclonal [EP798Y] to Calponin 1 (ab46794)
  Secondary antibody: Goat Anti-Rabbit LI-COR IRDye 680 RD

Alkaline Phosphatase Activity Assay and Alizarin Red Staining *(Aims 2 and 3)*
Alkaline phosphatase (ALP) is an enzyme involved in bone formation by increasing the availability of phosphates used for hydroxyapatite synthesis [32]. The increase in availability of phosphates also increases formation of calcium phosphates, or the mineral commonly found in vascular calcification [33]. By measuring ALP level, we would be able to compare the probability of calcium phosphate formation amongst different flow groups. Alkaline Phosphatase Activity Colorimetric Assay Kit (BioVision, Inc., Milpitas, CA, USA) will be used to measure ALP activity level in the cell culture media collected from HAoSMCs from days 7 and 14, in addition to tissue sample media from bioreactor chambers after 14 days. Media from all flow groups will be homogenized in the assay buffer to prepare a lysate for ALP activity assay. To detect presence of calcification, HAoSMCs from days 7 and 14 will be fixed in 10% formaldehyde and stained with Alizarin Red S Staining Quantification Assay Kit (ScienCell Research Laboratories, Carlsbad, CA, USA). The assay will quantify level of calcium deposits in each of the samples detected by a spectrophotometer at 405 nm. ALP activity and alizarin red staining will also be performed on the bioreactor-conditioned *ex vivo* mice aortic samples.

Mice Aorta Tissue Harvest and Conditioning *Ex Vivo* Tissue Samples in Bioreactor *(Aim 3)*
Twenty-five C57BL/6J wildtype male mice (Mus musculus) at 20 weeks will be purchased from the Jackson Laboratory (Bar Harbor, ME, USA). As mice reach mature adulthood at 6 months [34], to ensure that adult mice tissues are conditioned in flow samples, all mice will be fed with the same diet and food volume consecutively for 8 weeks before tissue explant. Thoracic aortas will be dissected from mice and cut into rectangular strips before conditioning in bioreactor under steady flow (0 OSI), 0.25 OSI, and 0.50 OSI. Tissue samples will be conditioned for 14 days in incubator at 37°C and 5% CO_2. Two bioreactor chambers will be used per flow group, with each chamber consisting of three samples. To induce calcification, conditioning media will be regular DMEM (4.5g/L glucose, high L-glutamine, no sodium pyruvate, Gibco) supplemented with 10% calf serum, 1% penicillin/streptomycin, and 3.0 mmol/L inorganic phosphate (Pi, Na2H2PO4/NaH2PO4, pH7.4) [35]. A peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) and a programmable pulse duplicator pump (Vivitro Labs, Victoria, Canada) will be used to deliver steady and oscillatory flows, respectively.

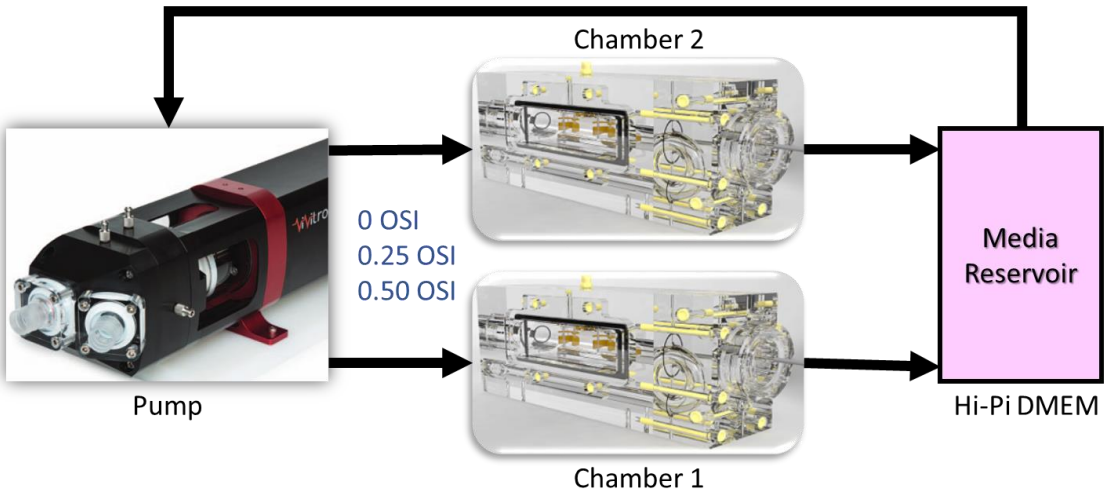

Figure 6: Bioreactor Schematic [36]

Computational Fluid Dynamics *(Aim 3)*

Computational fluid dynamics (CFD) modeling will be carried out using commercially available software MeshLab (ISTI-CNR, Rome, Italy) to create the 3-dimensional geometry and COMSOL Multiphysics® (COMSOL Inc., Stockholm, Sweden) for fluid flow simulations within Windows 10, 64-bit operating system (Microsoft, Inc., Redmond, WA, USA). The workstation will include dual processors Intel Core™ i7-6700HQ CPU at 2.60GHz/2.60GHz (Santa Clara, CA, USA) with 40GB RAM and 500GB SSD hard drive or similar. Bioreactor geometries of chambers and tubes will be generated in MeshLab. Geometries will then be imported into COMSOL Multiphysics® for flow simulation. The inlet pulsatile velocity boundary conditions will be set to 20.4 mL/min [37], to examine flow profiles and shear stresses on samples. Flow simulations will be run at an average mesh quality of 0.7 or higher in COMSOL Multiphysics®.

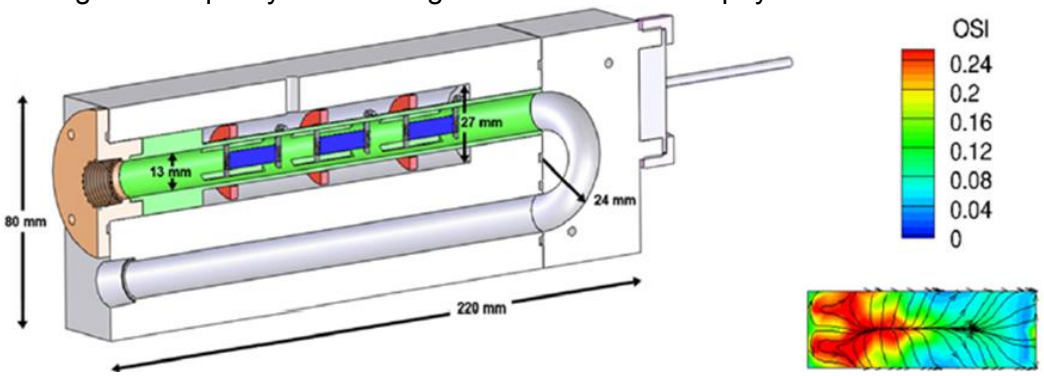

Figure 7: CFD of Bioreactor and OSI [39]

The CFD model will determine and validate waveforms that deliver the desired OSI values to the samples in the chamber. Once flow profile waveforms are programmed into the pump, volumetric flow and pressure values will be measured with flow probe and pressure transducers to validate the system.

Histology, Immunohistochemistry, and OsteoSense *(Aim 3)*

The samples conditioned in the bioreactor will be processed for histology, immunohistochemistry, RT-qPCR, western blot, and fluorescent detection of calcification with OsteoSense. To highlight important features of cells and tissue samples, histological stains will be performed. Histological samples will be fixed in 10% formalin for 24-36 hours and embedded with optimal cutting temperature (OCT) compound in -80°C freezer for an additional 24 hours before sectioning with cryostat. The embedded tissue will be sectioned at 10 microns per slice, which will be melted on glass slides for hematoxylin and eosin (H&E) staining. Intimal thickness will be measured to determine thickening across flow groups, in which is a critical event in development of atherosclerosis [38]. To detect presence of lumican protein in the intima, media, and adventitia, immunohistochemical staining with rabbit polyclonal antibody against lumican protein will be performed. In normal artery without intimal thickening, minimal or no lumican protein should be found in the inner layer of medial smooth muscle layer. Presence of lumican protein in the inner medial smooth muscle layer indicates migration of VasSMCs toward the intima, therefore increasing intimal thickness [38]. To detect presence of calcification, tissue samples will be immersed in OsteoSense 680EX, which binds to calcium nodules. The samples will then be washed with PBS and placed in Li-Cor Odyssey System to be imaged at 680 nm.

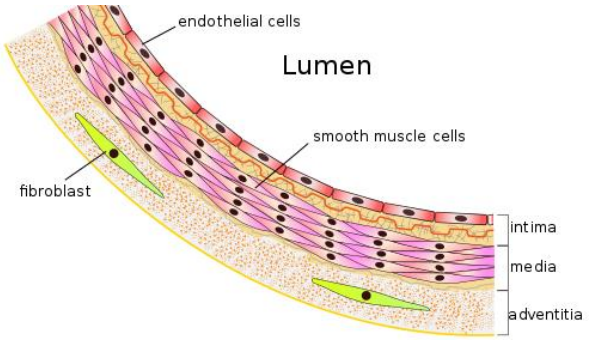

Figure 8: Tunica adventitia [41]

Statistical Analysis

Each flow group experiment will be conducted at minimum of n=3. All graphs and numerical values will be presented as the mean ± standard error of the mean. Data will be evaluated using analysis of variance (ANOVA) in SPSS Software (IBM Corporation, Armonk, New York, USA) to analyze variations within the group and between the groups. Probability values less than 0.05 ($p<0.05$) will be considered significant and the null hypothesis will be rejected. To achieve a high confidence interval (≥95%) and low type II error (≤0.20), a reasonable sample size will be determined per conditioning group.

By understanding the effects of oscillatory flow on atherogenesis, the long-term goal is to identify a flow profile that promotes vascular maintenance. The findings of this research will also help us better assess the risk for developing atherosclerosis by measuring blood oscillation patterns. This grant was prepared for a timeline of 3 years. See Exhibit A and Exhibit B for Budget Sheet and Timeline/Milestones, respectively.

REFERENCES

[1] G. Thanassoulis and M. Afshar, "Atherosclerosis," Merck Manual Professional Version, March 2017. [Online]. Available: https://www.merckmanuals.com/professional/cardiovascular-disorders/arteriosclerosis/atherosclerosis.

[2] J. Groenendyk and N. Mehta, "Applying the ordinal model of atherosclerosis to imaging science: a brief review," *Open heart,* vol. 5, no. 2, 2018.

[3] C. Michiels, "Endothelial cell functions," *Journal of cellular physiology,* pp. 430-443, 2003.

[4] K. Steucke, P. Tracy, E. Hald, J. Hall and P. Alford, "Vascular smooth muscle cell functional contractility depends on extracellular mechanical properties," *Journal of biomechanics,* vol. 48, no. 12, pp. 3044-3051, 2015.

[5] X. He and D. Ku, "Pulsatile Flow in the Human Left Coronary Artery Bifurcation: Average Conditions," *ASME. Journal of Biomechanical Engineering,* vol. 118, no. 1, pp. 74-82, 1996.

[6] J. Chiu and S. Chien, "Effects of Disturbed Flow on Vascular Endothelium: Pathophysiological Basis and Clinical Perspectives," *Physiological reviews,* vol. 91, no. 1, pp. 327-387, 2011.

[7] A. Williams, S. Nasim, F. Salinas, A. Moshkforoush, N. Tsoukias and S. Ramaswamy, "A "sweet-spot" for fluid-induced oscillations in the conditioning of stem cell-based engineered heart valve tissues," *Journal of biomechanics,* vol. 65, pp. 40-48, 2017.

[8] H. Liao, H. Hui, Y. Chen, F. Zeng, J. Huang and L. Wu, "Effects of long-term serial cell passaging on cell spreading, migration, and cell-surface ultrastructures of cultured vascular endothelial cells," *Cytotechnology,* vol. 66, no. 2, 2014.

[9] M. Feletou, "The Endothelium: Part 1: Multiple Functions of the Endothelial Cells—Focus on Endothelium-Derived Vasoactive Mediators," in *Integrated Systems Physiology: from Molecule to Function to Disease*, San Rafael (CA), Morgan & Claypool Life Sciences, 2011, p. 306.

[10] A. Co, M. Lagomarsino, M. Caselle and M. Osella, "Stochastic timing in gene expression for simple regulatory strategies," *Nucleic acids research,* vol. 45, no. 3, pp. 1069-1078, 2017.

[11] A. Flammer, T. Anderson, D. Celermajer, M. Creager, J. Deanfield, P. Ganz, N. Hamburg, T. Luscher, M. Shechter, S. Taddei, J. Vita and A. Lerman, "The Assessment of Endothelial Function," *Circulation,* vol. 126, no. 6, pp. 753-767, 2012.

[12] S. Verma, M. Buchanan and T. Anderson, "Endothelial Function Testing as a Biomarker of Vascular Disease," *Circulation,* vol. 108, no. 17, pp. 2054-2059, 2003.

[13] A. Chong, A. Blann and G. Lip, "Assessment of endothelial damage and dysfunction: observations in relation to heart failure," *QJM: An International Journal of Medicine,* vol. 96, no. 4, pp. 253-267, 2003.

[14] "Useful Numbers for Cell Culture," ThermoFisher Scientific, [Online]. Available: https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html.

[15] H. Lee, J. C. L. T. K. Tang, C. Jiang, P. Yang, P. Yang and G. Huang, "Osteogenic differentiation of mesenchymal stem cells derived from bone marrow of patients with myeloproliferative disorders," *Journal of the Formosan Medical Association,* vol. 101, no. 2, pp. 124-128, 2002.

[16] "TRIzol Reagent User Guide," Invitrogen, [Online]. Available: http://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf.

[17] B. Zyzynska-Granica and K. Koziak, "Identification of Suitable Reference Genes for Real-Time PCR Analysis of Statin-Treated Human Umbilical Vein Endothelial Cells," *PLoS One,* vol. 7, no. 12, 2012.

[18] K. Livak and T. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method," *Methods,* vol. 25, pp. 402-408, 2001.

[19] M. Rahman, N. Akhtar, H. Jamil and R. Banik, "TGF-b/BMP signaling and other molecular events: regulationof osteoblastogenesis and bone formation," *Nature,* vol. 3, no. 15005, 2015.

[20] R. Wan, Y. Mo, X. Zhang, S. Chien, D. Tollerud and Q. Zhang, "Matrix metalloproteinase-2 and -9 are induced differently by metal nanoparticles in human monocytes: The role of oxidative stress and protein tyrosine kinase activation," *Toxicology and APplied Pharmacology,* vol. 233, pp. 276-285, 2008.

[21] M. Rojiani, J. Alidina, N. Esposito and A. Rojiani, "Expression of MMP-2 correlates with increased angiogenesis in CNS metastasis of lung carcinoma," *International Journal of Clinical and Experimental*

Pathology, vol. 3, no. 8, pp. 775-781, 2010.

[22] J. Cook-Mills, M. Marchese and H. Abdala-Valencia, "Vascular Cell Adhesion Molecule-1 Expression and Signaling During Disease: Regulation by Reactive Oxygen Species and Antioxidants," *Antioxidants & Redox Signaling,* vol. 15, pp. 1607-1638, 2011.

[23] J. Cook-Mills, "VCAM-1 signals during lymphocyte migration: role of reactive oxygen species," *Molecular immunology,* vol. 39, no. 9, pp. 499-508, 2002.

[24] S. Allahverdian, C. Chaabane, K. Boukais, G. Francis and M. Bochaton-Piallat, "Smooth Muscle Cell Fate and Plasticity in Atherosclerosis," *European Society of Cardiology,* vol. 144, pp. 540-550, 2018.

[25] K. Henriksen and M. Karsdal, "Type I Collagen," in *Biochemistry of Collagens, Laminins and Elastin*, Academic Press, 2016, pp. 1-11.

[26] D. Frank, A. Abtahi, D. Yamaguchi, S. Manning, Y. Shyr, A. Pozzi, H. Baldwin, J. Johnson and M. de Caestecker, "Bone morphogenetic protein 4 promotes pulmonary vascular remodeling in hypoxic pulmonary hypertension," *Circulation research,* vol. 97, no. 5, pp. 496-504, 2005.

[27] M. Lin, T. Chen, E. S. M. Leaf and C. Giachelli, "Runx2 Expression in Smooth Muscle Cells Is Required for Arterial Medial Calcification in Mice," *The American Journal of Pathology,* vol. 185, no. 7, pp. 1958-1969, 2015.

[28] R. Alves, M. Eijken, J. van de Peppel and J. van Leeuwen, "Calcifying vascular smooth muscle cells and osteoblasts: independent cell types exhibiting extracellular matrix and biomineralization-related mimicries," *BioMed Central Genomics,* vol. 15, no. 1, 2014.

[29] A. Tsaousi, H. Williams, C. Lyon, V. Taylor, A. Swain, J. Johnson and S. George, "Wnt4/β-catenin signaling induces VSMC proliferation and is associated with intimal thickening," *Circulation Research,* vol. 108, no. 4, pp. 427-436, 2011.

[30] J. Carmichael, S. Winder, M. Walsh and G. Kargacin, "Calponin and smooth muscle regulation," *Canadian Journal of Physiology and Pharmacology,* vol. 72, pp. 1415-1419, 1994.

[31] N. Latif, P. Sarathchandra, A. Chester and M. Yacoub, "Expression of smooth muscle cell markersand co-activators in calcified aortic valves," *European Society of Cardiology,* vol. 36, pp. 1335-1345, 2015.

[32] K. Holtz and E. Kantrowitz, "The mechanism of the alkaline phosphatase reaction: insights from NMR, crystallography and site-specific mutagenesis," *FEBS Letters,* vol. 462, no. 1-2, pp. 7-11, 1999.

[33] H. Orimo, "The mechanism of mineralization and the role of alkaline phosphatase in health and disease," *Journal of Nippon Medical School,* vol. 77, no. 1, pp. 4-12, 2010.

[34] C. Hagan, "When are mice considered old?," The Jackson Laboratory, [Online]. Available: https://www.jax.org/news-and-insights/jax-blog/2017/november/when-are-mice-considered-old.

[35] T. Akiyoshi, O. Hidetaka, K. Iijima, B. Son, T. Kahyo, M. Setou, S. Ogawa, Y. Ouchi and M. Akishita, "A novel organ culture model of aorta for vascular calcification," *Atherosclerosis,* vol. 244, pp. 51-58, 2016.

[36] "SUPERPUMP," Vivitro Labs, [Online]. Available: https://vivitrolabs.com/wp-content/uploads/2016/02/SuperPump-and-Accessories-2016-VIVI-MKT-045.pdf.

[37] M. Kreissl, H. Wu, D. Stout, W. Ladno, T. Schindler, X. Zhang, J. Prior, M. Prins, A. Chatziioannou, S. Huang and H. Schelbert, "Noninvasive measurement of cardiovascular function in mice with high-temporal-resolution small-animal PET," *Journal of Nuclear Medicine,* vol. 47, no. 6, pp. 974-980, 2006.

[38] M. Onda, T. Ishiwata, K. Kawahara, R. Wang and Z. S. Y. Naito, "Expression of Lumican in Thickened Intima and Smooth Muscle Cells in Human Coronary Atherosclerosis," *Experimental and Molecular Pathology,* vol. 72, pp. 142-149, 2002.

[39] S. Ramaswamy, S. Boronyak, T. Le, A. Holmes, F. Sotiropoulos and M. Sacks, "A Novel Bioreactor for Mechanobiological Studies of Engineered Heart Valve Tissue Formation Under Pulmonary Arterial Physiological Flow Conditions," *Journal of Biomechanical Engineering,* vol. 136, no. 12, 2014.

[40] "BioFlux 200," [Online]. Available: https://il-biosystems.com/fileadmin/_processed_/6/5/csm_Bioflux200_8f5159e2bb.png.

[41] Lakna, "Difference Between Serosa and Adventitia," PEDIAA, 24 December 2017. [Online]. Available: https://pediaa.com/difference-between-serosa-and-adventitia/.

EXHIBIT A: Budget Sheet

ITEM	SOURCE / BRAND	CATEGORY	COST (USD)	LINK
Bioflux				
Bioflux Plates	Fluxion	Consumption	284.70	https://www.fishersci.com/shop/products/NC0580959/nc0580959#?keyword=bioflux
Bioflux 200 Shear Assay System	Fluxion	Equipment	41590.01	http://finance.fiu.edu/purchasing/ss/Fluxion_Biosciences.pdf
Cell Culture and Expansion				
Primary Human Aortic Endothelial Cells (Cryopreserved)	PromoCell	Consumption	748.00	https://www.promocell.com/product/human-aortic-endothelial-cells-haoec/
Endothelial Cell Growth Medium	Cell Applications, Inc	Consumption	142.30	https://www.fishersci.com/shop/products/endo-cell-growth-medium-500ml/50608999#?keyword=endothelial+cell+growth+medium+cell+applications
Primary Human Aortic Smooth Muscle Cells	PomoCell	Consumption	733.00	https://www.promocell.com/product/human-aortic-smooth-muscle-cells-haosmc/
Smooth Muscle Cell Growth Medium 2 (Ready-to-use)	PomoCell	Consumption	144.00	https://www.promocell.com/product/smooth-muscle-cell-growth-medium-2/
Fetal Calf Serum	Atlanta Biologicals	Consumption	62.50	https://www.atlanta-biologicals.com/shop/cell-culture-sera/calf-serum/
Penicillin/Streptomycin	Gibco	Consumption	22.36	https://www.thermofisher.com/order/catalog/product/15140122
Phosphate Buffered Saline (PBS) 1x	Gibco	Consumption	182.00	https://www.thermofisher.com/order/catalog/product/10010023
Dulbecco's Modified Eagle Medium (DMEM) (pack of 10)	Gibco	Consumption	222.00	https://www.thermofisher.com/order/catalog/product/11965118
L-Ascorbic Acid	Sigma Aldrich	Consumption	66.60	https://www.sigmaaldrich.com/catalog/product/sigma/a4403?lang=en&region=US&gclid=CjwKCAjw1dzkBRBWEiwAROVDLBvzvZSj9lcPIn2kCsB41XAwbCfY88I23j8VxwlORXhS8zYCurP_MRoC0QYQAvD_BwE
Beta-Glycerophosphate	MilliporeSigma ™	Consumption	67.00	https://www.fishersci.com/shop/products/calbiochem-atr-kinase-inhibitor-ii-1/35675100gm?searchHijack=true&searchTerm=35675100GM&searchType=RAPID&matchedCatNo=35675100GM
Dexamethasone	Sigma Aldrich	Consumption	34.90	http://www.sigmaaldrich.com/catalog/product/SIGMA/D4902?lang=en&region=US
Trypsin-EDTA Solution 0.05% 1x	Caisson Labs	Consumption	9.85	https://www.caissonlabs.com/product-TRL01-100ML-Trypsin-EDTA-Solution-(1X).php?id=266
Type B Gelatin powder	Sigma-Aldrich	Consumption	28.10	https://www.sigmaaldrich.com/catalog/product/sigma/g9391?lang=en&region=US
6-cm culture dishes, airvent lid (case of 400)	ThermoFisher	Consumption	325.00	https://www.thermofisher.com/order/catalog/product/172931
10-cm culture dishes, airvent lid (case of 150)	ThermoFisher	Consumption	227.00	https://www.thermofisher.com/order/catalog/product/172931
6-well culture plates (case of 50)	Corning	Consumption	124.27	https://www.thomassci.com/Laboratory-Supplies/Plates/_/Culture-Plate-6-Well-Non-Treated?q=6%20well%20plate
37 degC Water Bath	Cole-Parmer	Equipment	2200.00	https://www.coleparmer.com/i/polyscience-sd07h170-7l-standard-digital-heated-circulating-bath-120vac/1211610?PubID=UX&persist=true&ip=no&gclid=EAIaIQobChMIg-6ajImz4QIVzLrACh0toQSgEAQYASABEgIEiPD_BwE
Hemocytometer w/standard lines	Weber Scientific	Equipment	125.13	https://www.weberscientific.com/hemocytometer-for-cell-counting?gclid=EAIaIQobChMI3ZK8k-SN4QIVeh-tBh3-kQi4EAYYASABEgKtkvD_BwE
Incubator	Sheldon Manufacturing	Equipment	5642.00	https://labincubators.net/collections/co2-incubators/products/sco-air-jacket-co2-incubators

ITEM	SOURCE / BRAND	CATEGORY	COST (USD)	LINK
Benchtop Centrifuge	Beckman Coulter	Equipment	6060.82	https://www.gmi-inc.com/product/beckman-x-14r-benchtop-centrifuge/?keyword=&gclid=EAIaIQobChMIjZjsp4qz4QIVDdvACh11PQhkEAQYBSABEgKUKfD_BwE
Microscope	AmScope	Equipment	2619.99	https://www.amscope.com/40x-1000x-long-distance-plan-optics-inverted-microscope-8mp-camera.html?medium=tsa&gclid=EAIaIQobChMImbTPy4qz4QIVENvACh0X_QLEEAYYBSABEgJckvD_BwE&gclsrc=aw.ds
Cell Culture Sterile Hood	Biotect Elite	Equipment	3901.00	https://www.globallabsupply.com/6-NSF-Biological-Safety-Cabinet-Class-II-A2-p/bsc-a2-nsf72.htm?gclid=EAIaIQobChMIj6bH_42z4QIVjIbACh3GxQcDEAQYHCABEgKYQvD_BwE
Serological Pipette Gun	Scilogex	Equipment	195.00	https://www.pipettes.com/levo-plus-pipette-filler-motorized?fee=1&fep=4201&utm_source=Google+Shopping&utm_medium=ads&utm_campaign=feed&gclid=EAIaIQobChMInparx46z4QIVQZ7ACh0SkQrpEAYYASABEgKjqfD_BwE
Ethanol 95% (1 gallon)	Carolina	Consumption	28.60	https://www.carolina.com/specialty-chemicals-d-l/ethanol-95-laboratory-grade-4-l/861283.pr?question=ethanol+95%25
Reverse Osmosis Water System	3M	Equipment	1250.00	https://www.consolidatedfoodservice.com/cuno-tfs450-ro-system?matchtype=&network=g&device=c&keyword=&campaign=1466823336&adgroup=pla-298456124795&gclid=EAIaIQobChMI77i8rI-z4QIVhLrACh0MnQTGEAYYASABEgKq8vD_BwE
5mL Serological Pipettes (case of 200)	USA Scientific	Consumption	46.10	https://www.usascientific.com/5ml-serological-pipet-sterile.aspx
10mL Serological Pipettes (case of 200)	USA Scientific	Consumption	49.15	https://www.usascientific.com/10ml-serological-pipet-sterile.aspx
25mL Serological Pipettes (case of 200)	USA Scientific	Consumption	109.00	https://www.usascientific.com/25ml-serological-pipet-sterile.aspx
Micropipettes (full set)	Fisherbrand	Equipment	1086.64	https://www.fishersci.com/shop/products/fisherbrand-elite-pipetter-kit/14388100
Micropippette tips (1000 uL) (case of 600)	Greiner Bio-One	Consumption	109.00	https://www.fishersci.com/shop/products/universal-sterile-filter-tips-gilson-p1000/07000822#?keyword=Micropippette+tips+1000+uL
Micropippette tips (200 uL) (case of 960)	Fisherbrand	Consumption	126.50	https://www.fishersci.com/shop/products/fisherbrand-sureone-aerosol-barrier-pipette-tips-14/02707430
Micropippette tips (10 uL) (case of 960)	Sartorius	Consumption	97.00	https://www.fishersci.com/shop/products/sartorius-biohit-optifit-0-1-10-l-tips-single-tray-2/14559460?keyword=true
15 mL sterile conical tubes (case of 500 racked)	Nunc	Consumption	266.00	https://www.thermofisher.com/order/catalog/product/339651?SID=srch-srp-339651
50 mL sterile conical tubes (case of 300 racked)	Nunc	Consumption	222.00	https://www.thermofisher.com/order/catalog/product/339651?SID=srch-srp-339651
Beakers (all sizes, case of 5)	Pyrex	Equipment	40.00	https://www.coleparmer.com/i/pyrex-1000-pack-brand-1000-low-form-glass-beaker-starter-set-50-to-1000-ml-5-pk/3450290?PubID=UX&persist=true&ip=no&gclid=EAIaIQobChMI0Y6HpJKz4QIVBLXACh2h8QcQEAYYASABEgLf8vD_BwE
Total RNA Isolation				
TRIzol Reagent (100 mL)	Invitrogen	Consumption	194.00	https://www.thermofisher.com/order/catalog/product/15596026
Chloroform (500 mL)	Ricca Chemical	Consumption	102.40	https://www.coleparmer.com/i/ricca-chemical-company-hydrospec-coulometric-ag-c-anolyte-solution-with-chloroform-cells-with-without-diaphragm-500-ml/8699812?PubID=UX&persist=true&ip=no&gclid=EAIaIQobChMIz5O305Kz4QIVWLbACh1ZjQhgEAQYAiABEgJg8_D_BwE

ITEM	SOURCE / BRAND	CATEGORY	COST (USD)	LINK
Isopropanol 99% (1 gallon)	ULINE	Consumption	122.00	https://www.uline.com/Product/Detail/S-17475/First-Aid/99-Isopropyl-Alcohol-1-Gallon-Bottle?pricode=WB0981&gadtype=pla&id=S-17475&gclid=EAIaIQobChMI9ZSU5ZKz4QIVWrnACh3TnwzJEAQYASABEgI_0_D_BwE&gclsrc=aw.ds
DEPC-Treated and Nuclease-free Water (1L)	Fisher Bioreagents	Consumption	71.00	https://www.fishersci.com/shop/products/water-sterile-for-rna-work-depc-treated-dnase-rnase-free-mol-biol-fisher-bioreagents/BP5611
Quantabio 5 PRIME Phase Lock Gel Tubes (case of 200)	Quantabio	Consumption	189.33	https://us.vwr.com/store/catalog/product.jsp?catalog_number=10847-800
Eppendorf Tubes 1.5mL (case of 250)	Eppendorf	Consumption	48.45	https://www.fishersci.com/shop/products/eppendorf-dna-lobind-microcentrifuge-tubes-6/p-4909334#?keyword=Eppendorf+Tubes
Micro Centrifuge	Southwest Science	Equipment	649.00	http://www.southwestscience.com/SCARD12_Microcentrifuge.html
Vortex	Labnet	Equipment	140.94	https://www.labwholesale.com/vx200-vortex-mixer-by-labnet-1-ea.html?___store=lab&gclid=EAIaIQobChMI6YzIjZSz4QIVFrjACh0NAwkBEAQYAyABEgKSiPD_BwE
Nanodrop for RNA quantification	ThermoFisher	Equipment	10300.00	https://www.fishersci.com/shop/products/nanodrop-one-spectrophotometer/13400518
qPCR and Primers				
Power SYBR Green RNA-to-CT 1-Step Kit (40 reactions)	ThermoFisher	Consumption	185.00	https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-reagents/one-step-real-time-rt-master-mix/power-sybr-rna-to-ct-1-step.html
Step-One Software for RT-PCR	ThermoFisher	Equipment	0.00	https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/StepOne-and-StepOnePlus-Real-Time-PCR-System.html
Primers (FWD + REV pair)				
NOS3	IDT Technologies	Consumption	15.54	https://www.idtdna.com/site/order/cart
vWF	IDT Technologies	Consumption	15.54	https://www.idtdna.com/site/order/cart
VEGF	IDT Technologies	Consumption	15.91	https://www.idtdna.com/site/order/cart
Col1a1	IDT Technologies	Consumption	15.91	https://www.idtdna.com/site/order/cart
BMP2	IDT Technologies	Consumption	15.91	https://www.idtdna.com/site/order/cart
Alpha-SMA	IDT Technologies	Consumption	15.54	https://www.idtdna.com/site/order/cart
PPIA	IDT Technologies	Consumption	15.54	https://www.idtdna.com/site/order/cart
HPRT1	IDT Technologies	Consumption	17.02	https://www.idtdna.com/site/order/cart
MMP-2	IDT Technologies	Consumption	15.17	https://www.idtdna.com/site/order/cart
MMP-9	IDT Technologies	Consumption	15.17	https://www.idtdna.com/site/order/cart
Selectin E	IDT Technologies	Consumption	16.65	https://www.idtdna.com/site/order/cart
VCAM1	IDT Technologies	Consumption	15.17	https://www.idtdna.com/site/order/cart
BMP4	IDT Technologies	Consumption	16.28	https://www.idtdna.com/site/order/cart
RUNX2	IDT	Consumption	16.65	https://www.idtdna.com/site/order/cart

ITEM	SOURCE / BRAND	CATEGORY	COST (USD)	LINK
	Technologies			
BGLAP	IDT Technologies	Consumption	13.32	https://www.idtdna.com/site/order/cart
WNT4	IDT Technologies	Consumption	15.91	https://www.idtdna.com/site/order/cart
BCA, Western Blot, and Antibodies				
Pierce Bicinchoninic Acid (BCA) Assay Kit	Thermo Scientific	Consumption	130.00	https://www.thermofisher.com/order/catalog/product/23227
96-well Microplates (case of 40)	Sigma Aldrich	Consumption	279.40	http://www.sigmaaldrich.com/catalog/product/SIGMA/M5686?lang=en&region=US
Synergy HTX Multi-Mode Microplate Reader	BioTek	Equipment	16815.37	https://www.fishersci.com/shop/products/biotek-synergy-htx-multi-mode-microplate-reader-5/p-4911692
Western Blot Bio-Rad PowerPac Basic Mini Electrophoresis System	Bio-Rad	Equipment	392.00	http://www.bio-rad.com/en-us/product/powerpac-basic-power-supply?ID=bea5dea1-cef0-43ad-8af5-b2c0287f6e07
Western Blot Gel Tetra Handcast System	Bio-Rad	Equipment	1024.00	http://www.bio-rad.com/en-us/product/mini-protean-tetra-handcast-systems?ID=N3F2W9KG4
Western Blot Trans-Blot Turbo Blotting System	Bio-Rad	Equipment	3284.00	http://www.bio-rad.com/en-us/sku/1704150-trans-blot-turbo-transfer-system?ID=1704150
Trans-Blot Turbo Mini Format, 0.2 microm Nitrocellulose Transfer Pack	Bio-Rad	Consumption	170.00	http://www.bio-rad.com/en-us/sku/1704158-trans-blot-turbo-mini-nitrocellulose-transfer-packs?ID=1704158
RIPA Lysis and Extraction Buffer	Thermo Fisher	Consumption	97.25	https://www.thermofisher.com/order/catalog/product/89900
Protease Inhibitor	Fisher Scientific	Consumption	293.00	https://www.fishersci.com/shop/products/pierce-protease-inhibitor-tablets-1/pia32963
Phosphatase Inhibitor	Thermo Fisher	Consumption	174.00	https://www.thermofisher.com/order/catalog/product/A32957
Acrylamide/Bisacrylamide (1L)	Sigma Aldrich	Consumption	278.00	https://www.sigmaaldrich.com/catalog/product/sigma/a2917?lang=en&region=US
Tris (500 g)	Sigma Aldrich	Consumption	94.50	https://www.sigmaaldrich.com/catalog/product/SIGMA/GE17132101?lang=en&region=US&cm_sp=Insite-_-prodRecCold_xviews-_-prodRecCold5-5
HCl (500 mL)	Fisher Chemical	Consumption	103.00	https://www.fishersci.com/us/en/products/I9C8K49K/hydrochloric-acid.html
Glycine (500 g)	Fisher BioReagents	Consumption	78.00	https://www.fishersci.com/shop/products/glycine-white-crystals-crystalline-powder-fisher-bioreagents-4/BP381500
NaCl (500 g)	Fisher Chemical	Consumption	64.75	https://www.fishersci.com/shop/products/sodium-chloride-crystalline-certified-acs-fisher-chemical-6/S271500#?keyword=NaCl
Sodium Dodecyl Sulphate (SDS) (25 g)	Sigma Aldrich	Consumption	45.80	https://www.sigmaaldrich.com/catalog/product/sial/436143?lang=en&region=US
TEMED (26 mL)	Fisher BioReagents	Consumption	73.50	https://www.fishersci.com/shop/products/temed-electrophoresis-fisher-bioreagents-2/BP15020#?keyword=TEMED
Ammonium persulphate, APS (case of 4)	Thermo Scientific	Consumption	54.20	https://www.fishersci.com/shop/products/pierce-ammonium-persulfate-aps/pi17874#?keyword=Ammonium+persulphate
Plate Heater	Benchmark Scientific	Equipment	200.00	https://www.pipettes.com/myblock-mini-dry-bath-100-240v-us-plug?fee=1&fep=4495&utm_source=Google+Shopping&utm_medium=ads&utm_campaign=feed&gclid=EAIaIQobChMIsemD6Zaz4QIVhsDACh3jbwiJEAQYAiABEgKq-fD_BwE
Shaker	Boekel Scientific	Equipment	404.07	https://www.boekelsci.com/mini-orbitron.html?keyword_session_id=vt~adwords|kt~|mt~|ta~327060107999&_vsrefdom=wordstream&gclid=EAIaIQobChMIo

ITEM	SOURCE / BRAND	CATEGORY	COST (USD)	LINK
				7Tr_5az4QIVS7nACh02Ug0GEAQYFSABEgLBa_D_BwE
TBS-Tween-20 (1x) (4L)	Boston Bioproducts	Consumption	50.00	https://bostonbioproducts.com/products/tbs-tween-20-1x/
Casein Blocking Buffer (1L)	Thermo Scientific	Consumption	144.00	https://www.thermofisher.com/order/catalog/product/37528
Li-COR Odyssey blocking buffer (500 mL)	LI-COR	Consumption	130.00	https://www.licor.com/bio/reagents/odyssey-blocking-buffer-in-pbs
Li-COR Odyssey CLx Imaging system and software	LI-COR Biosciences	Equipment	11995.00	https://www.ebay.com/i/173849884977?chn=ps
WB Antibodies				
Mouse monoclonal anti-eNOS [M221] (ab76198)	abcam	Consumption	449.00	https://www.abcam.com/enos-antibody-m221-ab76198.html
Rabbit polyclonal to anti-eNOS (phospho T494) (ab138430)	abcam	Consumption	419.00	https://www.abcam.com/enos-phospho-t494-antibody-ab138430.html
Rabbit polyclonal MMP2 antibody (ab37150)	abcam	Consumption	455.00	https://www.abcam.com/mmp2-antibody-ab37150.html
Rabbit polyclonal MMP9 antibody (ab38898)	abcam	Consumption	459.00	https://www.abcam.com/mmp9-antibody-ab38898.html
Rabbit polyclonal to anti-BMP2 (ab14933)	abcam	Consumption	445.00	https://www.abcam.com/bmp2-antibody-ab14933.html
Mouse monoclonal anti-TNF-alpha (ab1793)	abcam	Consumption	459.00	https://www.abcam.com/tnf-alpha-antibody-52b83-ab1793.html
Mouse monoclonal Osteocalcin antibody (ab13420)	abcam	Consumption	449.00	https://www.abcam.com/osteocalcin-antibody-ocg3-ab13420.html
Rabbit polyclonal beta-catenin antibody (ab6302)	abcam	Consumption	459.00	https://www.abcam.com/beta-catenin-antibody-ab6302.html
Rabbit monoclonal [EP798Y] to Calponin 1 (ab46794)	abcam	Consumption	505.00	https://www.abcam.com/calponin-1-antibody-ep798y-ab46794.html
Goat Anti-Rabbit LI-COR IRDye 680 RD	LI-COR	Consumption	99.00	https://www.licor.com/bio/products/reagents/secondary_antibodies/irdye_680rd.html
Goat Anti-Mouse LI-COR IRDye 800 RD	LI-COR	Consumption	99.00	https://www.licor.com/bio/products/reagents/secondary_antibodies/irdye_800cw.html
Bioreactor				
C57BL/6 Wildtype Male Mice Twenty-week old (x25)	The Jackson Laboratory	Consumption	1680.50	https://www.jax.org/strain/000664
Forceps	MilliporeSigma	Equipment	54.75	https://www.fishersci.com/shop/products/emd-millipore-filter-forceps/xx6200006p#?keyword=tweezer
Surgical disecting scissors (curved)	Fisherbrand	Equipment	14.75	https://www.fishersci.com/shop/products/fisherbrand-sharp-pointed-dissecting-scissors-2/p-60770#?keyword=scissors
Surgical disecting scissors (straight)	Fisherbrand	Equipment	23.00	https://www.fishersci.com/shop/products/fisherbrand-sharp-pointed-dissecting-scissors-2/p-60770#?keyword=scissors
Peristaltic Pump	Cole-Parmer	Equipment	2665.00	https://www.coleparmer.com/i/ismatec-reglo-digital-2-channel-6-roller-variable-speed-pump-0-005-to-59-ml-min-115-230-vac/7801642
Pulse Duplicator SuperPump	Vivitro Labs	Equipment	13800.00	http://www.mechanobiology.uct.ac.za/sites/default/files/image_tool/images/31/Pitiri_BSc_2003.pdf
Pump head	Vivitro Labs	Equipment	5999.99	https://vivitrolabs.com/wp-content/uploads/2016/02/Pump-Head-Brochure-2016-VIVI-MKT-055.pdf
ViVitest/ViVigen software	Vivitro Labs	Equipment	2400.00	http://www.mechanobiology.uct.ac.za/sites/default/files/image_tool/images/31/Pitiri_BSc_2003.pdf

ITEM	SOURCE / BRAND	CATEGORY	COST (USD)	LINK
Histology				
Cryostat	R.Kay Traders	Equipment	16639.99	https://spwindustrial.com/cryostat-microtome-fully-automatic-cryostat-new/?gclid=EAIaIQobChMIrKfCoey4gIV-SCtBh3QsAWrEAQYAiABEgK6PfD_BwE
Microscope slides	Fisher Scientific	Consumption	86.50	https://www.fishersci.com/shop/products/shandon-polysine-slides/p-4531214
OCT compound	Fisher Scientific	Consumption	15.00	https://www.fishersci.com/shop/products/fisher-healthcare-tissue-plus-o-c-t-compound-2/p-4753295
Formalin (10%, buffered)	Fisher Scientific	Consumption	303.00	https://www.fishersci.com/shop/products/formalin-buffered-10-phosphate-buffer-certified-fisher-chemical-3/p-213368
H&E histology staining kit	Vector Laboratories	Consumption	90.00	https://vectorlabs.com/hematoxylin-and-eosin-stain-kit.html
Computation Fluid Dynamics				
Desktop HP Workstation Windows 10 Intel Core i7 @ 2.60 GHz 2.60 GHz Windows 64-bit 40GB RAM 500GB SSD HD (or similar)	HP	Equipment	1149.99	https://www.amazon.com/HP-Pavilion-590-Desktop-Graphics/dp/B07FM7J859/ref=sr_1_1?qid=1554264238&refinements=p_n_feature_five_browse-bin%3A13580787011%7C13580788011%2Cp_89%3AHP%2Cp_n_feature_four_browse-bin%3A2289792011%2Cp_n_condition-type%3A2224371011&rnid=2224369011&s=pc&sr=1-1
Meshlab	ISTI-CNR	Equipment	0.00	http://www.meshlab.net/#download
COMSOL Multiphysics (R)	COMSOL, Inc	Equipment	1695.00	http://collaborate.bu.edu/engit/Comsol?action=AttachFile&do=get&target=comsol-pricelist.pdf
Computer Monitor	HP	Equipment	139.99	https://www.amazon.com/HP-Premium-Business-1920x1080-Backlight/dp/B01N80X2XK/ref=sr_1_2?hvadid=323398757692&hvdev=c&hvlocphy=9060014&hvnetw=g&hvpos=1t1&hvqmt=b&hvrand=3045395035205353 27&hvtargid=kwd-303049990536&keywords=hp+hd+computer+monitor&qid=1554264816&s=gateway&sr=8-2
Other Assay Kits and Components				
QuantiChrom Nitric Oxide Assay Kit	BioAssay Systems	Consumption	259.00	https://www.bioassaysys.com/Nitric-Oxide-Assay-Kit.html
Alkaline Phosphatase (ALP) Activity Colorimetric Assay Kit	BioVision	Consumption	415.00	http://www.biovision.com/alkaline-phosphatase-activity-colorimetric-assay-kit.html
Alizarin Red S Staining Quantification Assay	ScienCell Research Laboratories	Consumption	156.00	https://www.sciencellonline.com/alizarin-red-s-staining-quantification-assay.html
Formaldehyde	Sigma Aldrich	Consumption	29.40	https://www.sigmaaldrich.com/catalog/product/sigma/f8775?lang=en&region=US
Ammonium hydroxide solution	Sigma Aldrich	Consumption	52.60	https://www.sigmaaldrich.com/catalog/product/sial/09859?lang=en&region=US
OsteoSense 680EX	Perkin Elmer	Consumption	607.00	http://www.perkinelmer.com/product/osteosense-680ex-24-nmol-nev10020ex
		TOTAL EQUIPMENT	154497.43	
		TOTAL CONSUMPTION	15890.74	
		TOTAL ALL (USD)	170388.17	

EXHIBIT B: Timeline/Milestones

TASK	YEAR 1		YEAR 2		YEAR 3	
Aim 1: OSI range that causes EC injury						
EC culture and expansion						
Bioflux conditioning of ECs						
EC assessments						
Aim 1 statistical analysis						
Aim 2: Paracrine regulation of SMC with different OSI conditioned EC media						
SMC culture and expansion						
Conditioning of SMCs with spent EC media						
SMC assessments						
Aim 2 statistical analysis						
Aim 3: OSI effects on explanted animal tissue						
Mice maintenance and dissection						
Computational fluid dynamics of bioreactor						
Conditioning dissected tissues in bioreactor						
Tissue assessments						
Aim 3 statistical analysis						