ADENOSINE AND VASCULAR HOMEOSTASIS

by

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Abstract

Despite advancements in percutaneous coronary intervention, stents are still limited by a 2% annual rate of in-stent restenosis (ISR) related to neointimal (NI) tissue proliferation. Efforts to prevent ISR formation remain the focus of ongoing work. Adenosine (ADO) is a purine nucleoside with integral roles in vascular homeostasis, though it has limited clinical application. ADO signals primarily via four receptors with ADO receptor-A2B (ADOR-A2B) considered to play an integral role in vascular healing. Dipyridamole (DP) is a commercially approved therapy known to improve vascular events and modulate adenosine biology. Our objectives with this study included (i) assessing whether ADO could serve as a biomarker of cardiac events; (ii) determine if DP could mitigate NI formation in a pre-clinical stent model; and, (iii) quantify the mechanisms of DP-related vasculoprotection, specifically related to ADOR-A2B.

We assessed the analytic and biologic variability of circulating ADO levels in humans and demonstrated that circulating ADO was not predictive of cardiac events at one year following invasive coronary angiography. We then assessed whether modulation of adenosine biology with DP had therapeutic efficacy in a pre-clinical model. Utilizing meta-analysis, we confirmed the sustained effects of DP on vascular patency rates in both pre-clinical and clinical studies. We refined a pre-clinical rabbit model of stent implantation with assessment of stent healing by intravascular optical coherence tomography – with excellent translation to clinical observations. We then assessed DP in a pre-clinical model, demonstrating reduction in ISR and improved stent healing with DP compared to control. Last, we sought to elucidate the mechanisms behind the observed DP effects, specifically related to ADOR-A2B. *In vivo,* DP therapy demonstrated reduced NI smooth muscle cell (SMC) content. *In vitro* assessment of DP demonstrated dose-

dependent inhibition of SMC proliferation and migration with alteration of SMC phenotypic switching, while selective modulation of ADOR-A2B and ADOR-A2B knockdown support an ADOR-A2B-mediated component to the observed DP effects.

Adenosine biology is integral to vascular homeostasis. In humans, circulating adenosine levels in humans are not predictive of one year cardiovascular events. However, DP may improve vascular healing post stent implantation and warrants clinical evaluation for stent healing. The observed DP benefits may, in part, stem from ADOR-A2B modulation. ADOR-A2B is a viable target for assessment of small molecule modulation as a novel therapeutic target to improve vascular outcomes.

Overview of Hypotheses/Objectives/Chapters:

1) **Introduction**
    a. Describe adenosine biology and the implications for vascular homeostasis ***(Chapter 1)***
    b. Demonstrate the scope of the clinical problem to be addressed ***(Chapter 2)***

2) **Hypothesis #1 – Clinical – Adenosine as a biomarker of cardiovascular events**
    a. Hypothesis
        i. Plasma adenosine is a predictor of major adverse cardiovascular events
    b. Aims/plan
        i. Establish the analytical and biological variability of circulating adenosine levels in humans ***(Chapter 3)***
        ii. Perform a prospective, observational cohort study quantifying adenosine levels in humans to determine their association with adverse cardiac events. ***(Chapter 4)***

3) **Hypothesis #2 – Pre-Clinical – Adenosine as a therapeutic target for vascular healing**

   a. Background

      i. Dipyridamole has documented vasculoprotective effects and is known to modulate adenosine signaling.

   b. Hypothesis

      i. Dipyridamole will reduce NI Formation in a pre-clinical rabbit stent model

   c. Aims/plan

      i. Quantify the impact of dipyridamole on NI formation in both clinical and pre-clinical studies to date. ***(Chapter 5)***

      ii. Develop and standardize a rabbit pre-clinical model of stent implantation utilizing virtual histology (OCT) for assessment of stent healing

         1. Evaluation of non-contrast flush media for OCT imaging ***(Chapter 6)***
         2. Evaluation of a rabbit model of stent healing: application of OCT ***(Chapter 7)***

      iii. Utilizing this validated rabbit stent model, assess the therapeutic effect of dipyridamole for reduction in NI proliferation and improved stent healing assessed by OCT. ***(Chapter 8)***

         1. Assess dipyridamole's therapeutic efficacy in the absence of atherosclerosis
         2. Assess dipyridamole's therapeutic efficacy in the presence of atherosclerosis

4) **Hypothesis #3 – *In vitro* – Dipyridamole, vascular homeostasis and adenosine receptor-A2B**

   a. Background

i. Existing data suggests that dipyridamole may enact its vasculoprotective benefits via adenosine-receptor A2B modulation.

b. Hypothesis

i. Dipyridamole modulates its vasculoprotective effects via ADOR-A2B

c. Aims/plan: Using *in vitro* models, gain mechanistic insights into dipyridamole's effects on human coronary smooth muscle cells (SMCs) to further refine our mechanistic understanding and therapeutic approaches. ***(Chapter 8)***

i. Assess coronary SMC proliferation, apoptosis, migration and phenotypic switching in response to treatment with dipyridamole and selective adenosine receptor A2B agonists and antagonists.

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To my supervisor and mentor, Dr Hibbert, you have proven what a truly altruistic leader can achieve, while uniting individuals into a synergistic team of colleagues and friends. Our success is your success. Thank you.

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List of Abbreviations

- AC – adenylate cyclase
- ACEi – angiotensin converting enzyme inhibitor
- ACS – acute coronary syndrome
- ACS – acute coronary syndrome
- ACTA2 – smooth muscle actin alpha 2
- ADA – adenosine deaminase
- ADO – adenosine
- ADOR – adenosine receptor
- ADOR-A2B – adenosine receptor A2B
- ADP-adenosine diphosphate
- AMP-adenosine monophosphate
- AMPD1 – adenosine monophosphate deaminase locus 1
- ARB – angiotensin receptor blocker
- ASA - acetylsalicylic acid
- ASA – acetylsalicylic acid
- ATP- adenosine triphosphate
- BMS – bare metal stent
- BVS – bioresorbable vascular scaffold
- CABG – coronary artery bypass grafting
- CAD – coronary artery disease
- CAD – coronary artery disease
- cAMP – cyclic adenosine monophosphate
- CAPITAL – Cardiovascular And Percutaneous clInical TriALS
- CBF – coronary blood flow
- cGMP – cyclic guanosine monophosphate
- CGS 21680- 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamido-adenosine
- CHF – congestive heart failure
- COX-2 - cyclooxygenase-2
- CRP – C-reactive protein
- CV – coefficient of variation
- CVa – analytical coefficient of variation
- CVg – inter-subject coefficient of variation
- CVi – intra-subject coefficient of variation
- DAPT – dual antiplatelet therapy
- DES – drug eluting stent
- DM – diabetes mellitus
- DMSO - dimethyl sulfoxide
- DP – dipyridamole
- EC – endothelial cell
- ECM – extracellular matrix
- EHNA- erythro-9-(2-hydroxy-3-nonyl)adenine

- E-NPP - ecto-nucleotide pyrophosphatase/phosphodiesterase
- ENT – equilibrative nucleoside transporter
- GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
- GPCR – G-protein coupled receptor
- HbA1c – hemoglobin A1c
- HDL – high-density lipoprotein
- HF – heart failure
- HIF1 – hypoxia inducible factor-1
- HPLC – high performance liquid chromatography
- HR – hazard ratio
- IC – intracoronary
- IQR – interquartile range
- ISR – in-stent restenosis
- IV – intravenous
- K/D – knockdown
- KLF4 - Krüppel-like factor 4
- KLF5 - Krüppel-like factor 5
- LA – luminal area
- LDL – low density lipoprotein
- LDL – low-density lipoprotein
- LMWH – low-molecular weight heparin
- MA - Medial area
- MACE – major adverse cardiac events
- MBF – myocardial blood flow
- MCX – Mixed-mode, strong Cation-eXchange
- MI – Myocardial infarction
- MLA - minimal luminal area
- MLR – multivariable linear regression
- MSA – minimal stent area
- NECA - 5'-N-ethylcarboxyamidoadenosine
- NF-KB - nuclear factor-KB
- NI – neointima
- NSTEMI – non-ST-elevation myocardial infarction
- NZW rabbit – New Zealand White rabbit
- OCT – optical coherence tomography
- OR – odds ratio
- OSH – optimal strut healing
- PAC – plasma adenosine concentration
- PBS – phosphate buffered saline
- PCI – percutaneous coronary intervention
- PDE – phosphodiesterase
- PDGF – platelet derived growth factor
- PET – positron emission tomography

- PKA – protein kinase A
- PMN – polymorphonuclear neutrophils
- PTCA – percutaneous transluminal coronary angioplasty
- PTFE - Polytetrafluoroethylene
- RBC – red blood cell
- RSD – relative standard deviation
- SC – subcutaneous
- siRNA – small interfering RNA
- SMC – smooth muscle cell
- STEMI – ST-elevation myocardial infarction
- T2DM – type 2 diabetes mellitus
- TLF – target lesion failure
- TLR – target lesion revascularization
- TxA2 - thromboxane A2
- UA – unstable angina
- UFH – unfractionated heparin
- ULR – univariable linear regression

Chapter 1

Introduction – Adenosine as a Marker and Mediator of Cardiovascular Homeostasis: A Translational Perspective

1.1 Preface

This chapter has been previously published as a review article in *Cardiovascular & Hematological Disorders-Drug Targets*

Simard T, Jung R, Labinaz A, Faraz MA, Ramirez FD, Di Santo P, Pitcher I, Motazedian P, Gaudet C, Rochman R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo JJ, Couture E, Beanlands RS, Hibbert B. Adenosine as a Marker and Mediator of Cardiovascular Homeostasis: A Translational Perspective. Cardiovasc Hematol Disord Drug Targets. 2019;19(2):109-131. doi: 10.2174/1871529X18666181011103719. PMID: 30318008.

All authors made substantial contributions to the conception/ design of the work; and/or the acquisition, analysis, or data interpretation; and, (ii) drafted and/or revised the content critically; and, (iii) provided final approval of the published version; and, (iv) agree to be accountable for the work.

1.2 Abstract

Adenosine, a purine nucleoside, is produced broadly and implicated in the homeostasis of many cells and tissues. It signals predominantly via 4 purinergic adenosine receptors (ADORs) – ADORA1, ADORA2A, ADORA2B and ADORA3 in addition to non-ADOR mediated effects. Through these signaling mechanisms, adenosine exerts effects on numerous cell types crucial to maintaining vascular homeostasis, especially following vascular injury. Both *in vitro* and *in vivo* models have provided considerable insights into adenosine signaling and identified targets for therapeutic intervention. While numerous pharmacologic agents have been developed that modulate adenosine signaling, both through design as specific ADOR agonists and antagonists and as off-target effects of

existing anti-platelet medications. However, adenosine has yet to be firmly established as either a therapeutic or a prognostic tool in clinical medicine to date. Herein, we provide a bench-to-bedside review of adenosine biology, highlighting the key considerations for further translational development of this promising molecule.

1.3 Adenosine Biology

1.3.1 Metabolism

Adenosine is a purine nucleoside existing in a metabolic continuum with adenine nucleotides. Given its broad production, adenosine was thought to serve as a general regulatory molecule in the body, both intracellularly and extracellularly. However, unlike traditional endocrine hormones, adenosine lacks any mechanism of storage and release from any particular cell type.(1) Accordingly, the levels of intracellular and extracellular adenosine are tightly regulated to ensure ranges typically remain below the 1000-2000nM range.(1,2) Cellular uptake and export of adenosine are primarily mediated by membrane-bound equilibrative nucleoside transporters (ENT-1 and 2) based on concentration gradients.(3) These transporters, and an array of associated signaling pathways, facilitate a fine balance of adenosine production and degradation to maintain this delicate equilibrium.

Adenosine production is based upon both intracellular and extracellular pathways. Intracellular adenosine is generated via degradation of AMP via 5'-nucleotidase (5'-NT) and S-adenosyl homocysteine via S-adenosyl homocysteine hydrolase into adenosine.(4) Intracellular adenosine can then be exported from the cell via ENTs to contribute to the extracellular adenosine pool. However, adenosine is also generated in the extracellular

space. First, release of ATP into the extracellular space is mediated via Pannexin-1 (Panx-1) hemichannels on activated inflammatory and endothelial cells in both physiologic and pathophysiologic states (hypoxia, cellular injury etc.). (5,6) The presence of ATP in the extracellular space serves as a substrate for ectonucleotidases resulting in degradation to ADP, AMP and adenosine. (5) These ectonucleotidases include E-NPP (ecto-nucleotide pyrophosphatase/phosphodiesterase) which degrades ATP to AMP, while CD39, an ecto-apyrase (E-NTDPase, ecto-nucleoside triphosphate diphosphydrolase) degrades ATP to ADP and AMP. Lastly, CD73, an ecto-5' nucleotidase (5'NT) will degrade AMP to adenosine.(7,8) Hence, the balance between ATP and adenosine in the extracellular space is tightly controlled by the presence of CD39 and CD73.(2) **(Figure 1)**

Removal of extracellular adenosine occurs via two processes – degradation and uptake. Degradation in the extracellular space is predominately mediated by deamination to inosine by membrane-bound ecto-adenosine deaminase (ADA), thereby reducing the extracellular adenosine pool. Similarly, cellular uptake, facilitated by ENTs functioning based on a concentration gradient, further reduce extracellular adenosine. This rapid adenosine transport system is the predominate mechanism behind the swift decline in plasma adenosine levels following IV adenosine administration, and subsequent rapid relief of its clinical effect.(3) Hence, pharmacological inhibition (i.e. dipyridamole) of adenosine transport or transcriptional modulation to reduce ENT expression will prolong the effect of extracellular adenosine.(9) Once adenosine is transported to the intracellular space it is typically degraded to inosine via intracellular ADA or phosphorylated to AMP

via adenosine kinase where it can be further metabolized back to ATP.(4,10) (4) Modulation of the intracellular metabolism of adenosine has a direct influence on extracellular levels. For example, inhibition of adenosine kinase will augment extracellular adenosine resulting in protective effects in response to hypoxia.(11) However, excessive extracellular adenosine, generated via genetic deletion of ADA in murine models results in drastic increases in extracellular adenosine, culminating in pulmonary toxicity and subsequent fatality.(12) Thus, the strict regulation of adenosine metabolism must include both intracellular and extracellular adenosine pools to modulate cellular homeostasis, while highlighting potential targets for therapeutic intervention **(Figure 1)**

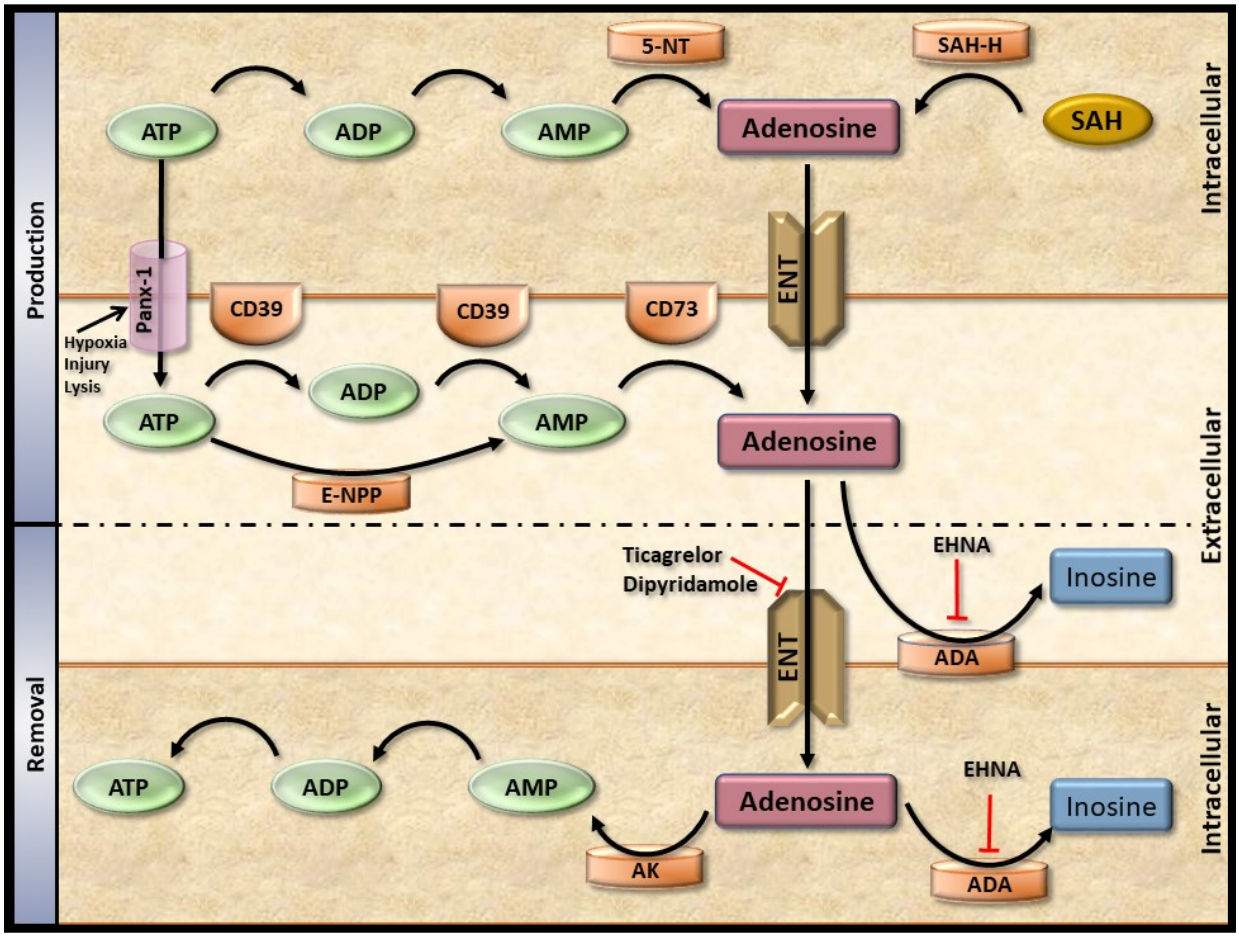

Figure 1 Production and removal of extracellular adenosine.

Intracellular production highlights both 5-NT and SAH-H mediated generation of intracellular adenosine with subsequent transport via ENT. Extracellular production via Panx-1 mediated ATP release in response to stressors (hypoxia, trauma etc) with subsequent ectoenzyme degradation to adenosine. Removal includes extracellular degradation via membrane bound ADA to inosine and derivates, as well as ENT-mediated uptake into cells with subsequent breakdown to inosine and adenine nucleotides. ATP- adenosine triphosphate, ADP-adenosine diphosphate, AMP-adenosine monophosphate, 5-NT-5'nucleotidase, SAH - S-adenosyl homocysteine , SAH-H – S-adenosyl homocysteine hydrolase, ENT – equilibrative nucleoside transporter, Panx-1 – Panx-1 hemichannel, CD39 – E-NTDPase, CD73 – 5'nucleotidase, E-NPP - ecto-nucleotide pyrophosphatase/phosphodiesterase, AK – adenosine kinase, EHNA- erythro-9-(2-hydroxy-3-nonyl)adenine, ADA -adenosine deaminase.

1.3.2 Adenosine Receptors and Signaling

Adenosine remains in a continuum with AMP, ADP and ATP within the extracellular space, with each of these molecules signaling via purinergic receptors on the cellular membrane. This is well demonstrated by the process of cardiac, skeletal or smooth muscle contraction.(13,14) A crucial step involves the hydrolyzation of ATP to ADP enabling the re-activation of myosin and subsequent contraction while releasing adenosine in the process.(13,15,16) Adenosine then binds to adenosine receptors leading to muscle relaxation via modulation of cAMP, thereby completing a homeostatic loop.(1,13,15,17) This signaling is facilitated by two classes of purinergic receptors on the plasma membrane – P1 and P2 receptors.

P2 receptors, preferentially bind ATP/ADP, and include two types of channels – P2X and P2Y channels. There are a total of seven P2X receptors (P2X1-7) that are ATP ligand-gated ion channels which gate extracellular cations in response to ATP binding.(8) Whereas the metabotropic P2Y receptors are G-protein coupled and include 8 subtypes that respond to adenine nucleotides (ATP/ADP preferentially). They are further subclassified into those that couple to Gq (P2Y1,2,4,6,11) and activate phospholipase C-beta versus those that are Gi-coupled (P2Y11, 12, 13, 14) and hence inhibit adenylyl cyclase and regulate ion channels.(18) One particular subtype is P2Y12, which responds to ADP and is the target of a number of antiplatelet medications capitalizing on its effects in modulating ADP-mediated platelet aggregation.(19) **(Figure 2)**

P1 receptors, known as adenosine receptors, are preferentially receptive to adenosine itself and signal via G-protein coupled receptors (GPCRs). There are 4 sub-types of adenosine receptors (A1, A2A, A2B and A3) which are aptly designated as ADORA1, ADORA2A, ADORA2B and ADORA3.(20) (Figure 2) These subtypes exhibit differential responses to adenosine binding in keeping with their respective GPCR subunits. ADORA1 and ADORA3 are associated with the G_i alpha subunit that inhibits intracellular adenylate cyclase (AC) and thus cAMP generation from ATP. Conversely, ADORA2A and 2B are associated with G_S alpha subunit which stimulates AC thereby activating the cyclic adenosine monophosphate (cAMP) pathway. Studies in brain slices demonstrated that A2A is stimulated by low (0.1-1uM) adenosine concentrations, while A2B requires high dose (>10uM) adenosine concentrations – resulting in A2A being classed a high affinity receptor and A2B a low affinity receptor.(21,22) Indeed, A1, A2A and A3 typically have an EC50 for adenosine of 10nM-1000nM, while A2B exceeds 10,000nM. Given circulating adenosine levels typically remain below 1000-2000nM, A2B is thought to be activated only in pathologic states with significant adenosine elevation, while the others can be activated within the regular physiological range of signaling.(23) As well, prolonged exposure to agonists is known to cause ADORA1 desensitization, though ADORA2A receptors do not desensitize with prolonged stimulation in porcine models.(24) Hence, purinergic signaling represents a complex and inter-related sequence of metabolism and cell surface signaling, maintaining a cohesive balance between both intracellular and extracellular adenosine levels.

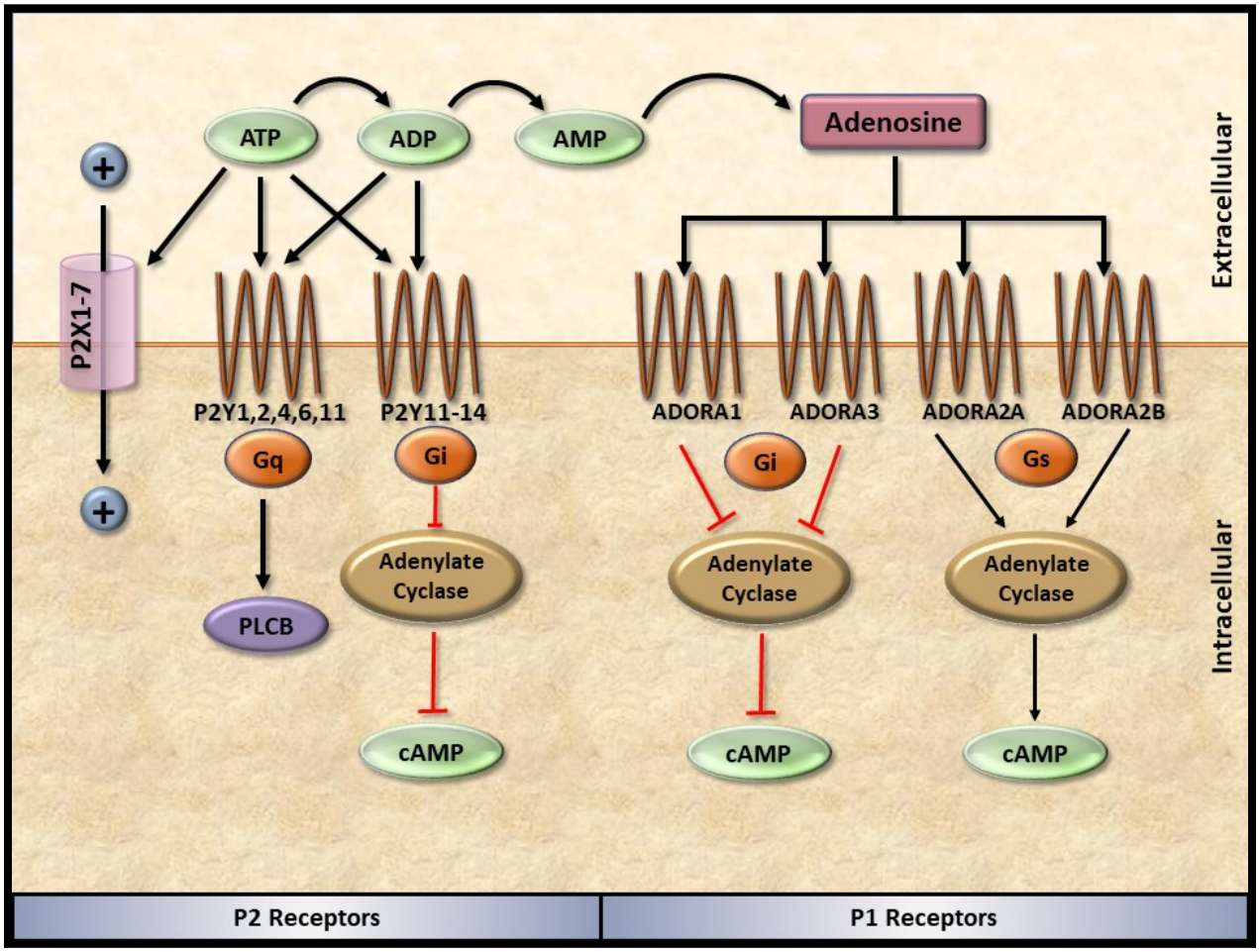

Figure 2. Extracellular purinergic signaling pathways.

Extracellular adenosine and its adenine nucleotides interact with a variety of membrane-bound purinergic receptors to enact intra-cellular effects. P2X receptors respond to ATP and facilitate cation transfer. P2Y receptors are G-protein coupled receptors that bind ATP and ADP to facilitate intracellular signaling. P1 receptors, or adenosine receptors, are also G-protein coupled receptors and include 4 subtypes which differentially regulate adenylate cyclase leading to changes in intracellular cAMP. ATP- adenosine triphosphate, ADP-adenosine diphosphate, AMP-adenosine monophosphate, cAMP-cyclic adenosine monophosphate, ADOR – adenosine receptor, PLCB – phospholipase C-beta, + - cation.

1.4 Impact of Adenosine on Vascular Cells

1.4.1 Inflammatory Cells

Inflammatory cells are key mediators in the pathogenesis of atherosclerosis and neointima formation. Inflammatory cells are known to migrate to areas of need based upon a cytokine stimuli and a fine balance of excitatory signals at the leading edge and inhibitory signals at the trailing edge of the cell – facilitating motion towards the area of interest.(25) As well, ATP release by the inflammatory cells themselves leads to autocrine stimulation of P2Y2 receptors directing orientation. This is further augmented by degradation to adenosine where it signals via ADORA3 on the leading edge – promoting gradient sensing and cell migration. Moreover, ADORA2A receptors have been demonstrated to have a uniform distribution in resting neutrophils, while polarized neutrophils demonstrated ADORA2A predominance on the trailing edge where stimulation leads to increased cAMP and protein kinase A (PKA) activation, ultimately reducing chemoattractant receptor signaling.(26) The extracellular ATP milieu also impacts regulatory T cells (Treg), which are implicated in modulating the immune response and are known to express the ectoenzymes CD39 and CD73, responsible for generating adenosine, on their surfaces.(27) Interestingly, adenosine derived from CD39 and CD73 breakdown were noted to influence a large proportion of the anti-inflammatory activities of regulatory T cells.(27) Specifically, endogenous adenosine generated by these ectoenzymes led to reduced T effector (Teff) cell activity due to reduced nuclear factor-KB (NF-KB) activity and diminished proinflammatory cytokine release – all mediated via ADORA2A signaling.(28) Moreover, stimulation of ADORA2A has been shown to reduce neutrophil and macrophage recruitment as well as cytokine expression

and neointima formation.(29) Conversely, knockout of ADORA2B resulted in augmented proinflammatory cytokines, including TNF-alpha, leading to increased expression of P-selectin, E-selectin, and ICAM-1 and amplifying leukocyte adhesion to the vascular wall.(30) Interestingly, this effect was thought to be related to hematopoietic ADORA2B, as wild type bone marrow transplantation abrogated this response, and less so vascular ADORA2B.(30) **(Table 1)**

1.4.2 Smooth Muscle Cells

Smooth muscle cells play vital roles in vascular homeostasis, forming a large component of the vessel wall, while also migrating and infiltrating in pathologic states to form fibrotic lesions and neointima in response to vascular injury.(31) Adenosine is implicated in antispasmodic and vasodilatory effects on the vasculature via depression of vascular smooth muscle cell (SMC) activity.(1) As well, stimulation of ADORA2B, via adenosine or injury, is known to augment cAMP and inhibit SMC proliferation. Vascular injury is known to cause upregulation in ADORA2B, specifically within SMCs – supporting a vasculoprotective role for ADORA2B.(32) Indeed, primary culture of SMCs derived from ADORA2B K/O mice demonstrate increased secretion of inflammatory cytokines, specifically IL-6.(30) Similarly, aortic SMCs derived from ADORA2B knockout mice demonstrate increased proliferation over wild-type controls, while bone marrow transplant studies support a hematopoietic source for the observed effect.(32) Furthermore, downstream signaling of A2B/cAMP via protein kinase A (PKA) is thought to inhibit multiple pathways which converge at cyclin D, a G1 phase cyclin, resulting in reduced expression and function of cyclin D and ultimately cell cycle arrest in G1.(33) Conversely, ADORA1 activity is proportional to the degree of vascular injury, with

ADORA1 antagonists known to inhibit SMC proliferation while not adversely impacting re-endothelialization in swine models.(34) Mechanistically, ADORA1 is thought to lead to coronary SMC proliferation via activation of ERk, JNK, PO5-kinase/AKT pathways resulting in proliferation.(35)Taken together, these findings support adenosine signaling as a mediator of vascular repair post-injury – specifically modulating SMC proliferation – a key process known to contribute to neointima formation. **(Table 1)**

Extracellular adenosine has been shown to stimulate apoptosis of human arterial SMCs, a process mediated via A2B receptors via a cAMP-dependent pathway.(36) This effect is not related to adenosine degradation by-products, as co-administration of EHNA (ADA inhibitor) did not alter the apoptotic rate. Similarly, co-administration of dipyridamole (ENT1 inhibitor) did not change the apoptotic response, suggesting that cellular uptake was not required and the effect on SMC apoptosis was most likely mediated via adenosine cell surface receptor signaling. This was confirmed via specific receptor antagonists which abolished the apoptotic response when co-administered with adenosine, suggesting that predominately A2B is responsible for adenosine-mediated SMC apoptosis via a cAMP-dependent pathway.(36) The ultimate phenotypic effects of this observation are controversial with some postulating that augmented apoptosis could favor propagation of atherosclerosis, while others note the favorable effect this could have on mitigating neointima formation post vascular injury.(37) However, in human coronary artery SMCs, adenosine has been shown to inhibit proliferation and migration while reducing rat carotid neointimal hyperplasia.(33) Similarly, human aortic SMCs demonstrate reduced proliferation in response to adenosine, mediated via A2B

receptors.(38) Overall, SMCs are intimately involved in number of physiologic and pathologic processes in vascular biology, with clear modulation by adenosine and its cell surface receptors.

1.4.3 Endothelial Cells

Endothelial cells (ECs) form the physical interface between the vessel wall and the lumen, mediating many key processes in the vascular response to injury, including endothelial activation and leukocyte recruitment.(29,31) The predominant adenosine receptors expressed in ECs from multiple origins are ADORA2A and ADORA2B, while ADORA2B may be preferentially found in ECs of microvascular origin.(39,40) Moreover, ECs also house the ecto-nucleotidases – crucial for metabolizing adenosine and its precursors to maintain homeostasis.(41) These ectoenzymes are present at basal conditions, but can be modulated in response to various stimuli.(41-43) Human aortic endothelial cells are known to contain both A2A and A2B receptors which regulate intracellular cAMP levels, providing endothelium-dependent vasodilatory actions.(44) Adenosine administration in ECs leads to reduced leukocyte-EC interaction, via reduced IL-6 and IL-8 release from activated ECs.(45) Specifically, treatment of ECs with A2 receptor agonists generated a dose-dependent increase in cAMP generation, which was blocked by co-administration of theophylline – supporting an adenosine receptor dependent effect. As well, the vasodilatory effect was lost if the endothelial cell layer was removed.(44) Further work specifically in human and porcine coronary arteries confirmed the presence of both A2A and A2B receptors with key roles in cAMP signaling.(46) Adenosine receptors have also been implicated in modulating the expression of angiogenic factors and contributing to endothelial cell heterogeneity. In

fact, differential adenosine receptor stimulation in cultured endothelial cells demonstrated varying effects on the secretion of interleukin-8 (IL-8), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) with ADORA2B activation serving a crucial role in angiogenesis.(39,47) Accordingly, hypoxia leads to upregulation of ADORA2B and suppression of ADORA2A.(47,48) Pulmonary endothelial cells activate their cytoskeleton in response to adenosine signaling via ADORA2A in an effort to augment their barrier effects.(49) In hypoxic states, adenosine is known to provide an adaptive response, limiting hypoxia induced vascular leakage. Deletion of CD73, preventing production of extracellular adenosine, results in baseline vascular leakage and, following hypoxic insult, fulminant vascular leakage – particularly in the lung.(50) Logically, the expression of CD39 and CD73 are strongly upregulated with the onset of hypoxia, facilitating adenosine signaling and ensuring close regulation in this setting to maintain endothelial integrity.(41)

Similarly, during inflammation, the interaction between polymorphonuclear leukocytes (PMNs) and the EC barrier is crucial to facilitate transendothelial migration while still maintaining wall integrity to limit fluid extravasation and permeability. PMN release of 5'-AMP and adenosine augment endothelial barrier function, facilitating endothelial resealing following transendothelial migration and limiting excessive permeability during inflammatory states.(51) In essence, PMNs establish a paracrine signaling loop via release of 5'-AMP, metabolized by EC-based CD73 into adenosine, which via ADORA2B receptors, results in endothelial junction reorganization and augmented barrier function.(42) This process is thought to facilitate PMN migration while

maintaining EC wall integrity. The specific mechanism by which adenosine enacts its anti-inflammatory effects within ECs remains unclear – though, suppression of TNF-induced activation of the NF-kappaB pathway appears central to this process.(52,53) Interestingly, while adenosine-induced AR activation prevents the pro-inflammatory response, presumably via cAMP generation, this effect cannot be mimicked simply by augmenting intracellular cAMP – supporting adenosine activation of alternative pathways within ECs.(41) Hence, endothelial cells play a crucial role in maintaining vascular homeostasis, with both autocrine and paracrine adenosine signaling playing a substantial role in this balance. **(Table 1)**

1.4.4 Platelets

Platelets accumulate at sites of vascular injury and are crucial initiators of vascular healing, while also interacting with ECs and leukocytes to promote inflammation.(54) Platelet hyper-reactivity is associated with cardiovascular risk factors including dyslipidemia, hypertension, and diabetes.(55) Indeed, patients with acute coronary syndrome (ACS) have 5-fold elevated expression of the platelet-activation epitope CD63 than those with stable CAD.(56) Platelet activation is a complex process involving the stimulation of many receptors which signal via GPCRs. These interactions include thromboxane A2 (TxA2) to the TxA2-receptor, adenosine diphosphate (ADP) to P2Y1 or P2Y12 receptors, collagen to glycoproteins, and thrombin to PAR-1 and PAR-4 receptors (a pathway that does not affect hemostasis) amongst others.(54,57) Strategies to mitigate platelet activation and alleviate thrombotic risk in ACS are abundant and include targeting the purinergic P2Y12 receptor of platelets. While these agents are efficacious,

the multiple pathways involved in platelet activation still present challenges for achieving optimal anti-platelet effects.(58)

Adenosine has long been implicated in platelet function via modulation of adenylate cyclase through purinergic receptor signaling.(59,60) Notably, ADORA1, 2A, and 2B have all been identified on platelets. Specifically, the importance of ADORA2A for adenosine signaling is well documented, inhibiting platelet aggregation via augmenting cAMP levels. While other small molecule studies revealed that A1 was not a potent mediator of platelet aggregation.(61) The role of ADORA2B is somewhat less well characterized, though its effects appear to be similar, albeit with a reduced affinity.(62) AR stimulation leads to activation of adenylyl cyclase and cAMP generation.(63) To counter synthesis, the rate of cAMP hydrolysis is regulated by phosphodiesterase(64). Increased cAMP will activate protein kinase A which phosphorylates downstream molecules including vasodilator-stimulated phosphoprotein (VASP), inhibiting platelet activation(65). Another marker of interest is PAI-1, an anti-fibrinolytic protein, which is elevated in diabetics.(66) The relationship between PAI-1 and adenosine has been reported in human mast cells with conflicting results. Some suggest that cAMP positively regulates PAI-1 expression by binding to the hypoxia response element at -158 to -153 upstream of the human PAI-1 promoter in mast cells(67), while others show no relationship between adenosine and PAI-1 transcription nor activity levels.(68) Further work is needed to define the role, if any, of adenosine and PAI-1 biology.

Regardless, adenosine is believed to modulate platelet activation and aggregation. Indeed, incubation of adenosine with human blood inhibited platelet ATP release, aggregation and activation when stimulated by both ADP and collagen.(63). As well, activated platelets are known to release ADP from intracellular stores thereby creating an additional extracellular source of nucleotides which can be metabolized to adenosine – forming a regulatory loop.(69) Addition of 5'-nucleotidase (5'-NT) to blood results in augmented extracellular adenosine levels and complete inhibition of both ADP and collagen-mediated platelet aggregation, with addition of 5'-NT inhibitor restoring full aggregation. (70) Hence, adenosine and its precursors appear intricately linked to the delicate balance of platelet activation, a key component of vascular healing. **(Table 1)**

Table 1. Differential effects of adenosine receptor signaling

ADOR	GPCR	cAMP	Clinical	Platelets	Endothelial Cells	Inflammatory Cells	Smooth Muscle Cells
A1	**Gi**	↓	Atrioventricular block Bronchoconstriction Coronary vasodilation ↑ neointima formation	Minimal effect	No effect	↓ inflammation	↑ proliferation
A2A	**Gs**	↑	↑↑Coronary vasodilation ↑ aortic atheroma ↓ neointima formation	↓↓ aggregation	↑ barrier effects ↓ adhesion ↓ cytokines	↓ attractant signaling ↓ cytokine release ↓ inflammation ↓ recruitment	↑ proliferation
A2B	**Gs**	↑	↑Coronary vasodilation Bronchoconstriction ↓ aortic atheroma ↓ neointima formation	↓ aggregation	↑ barrier effects ↓ adhesion ↓ cytokines ↑ angiogenesis	↓ leukocyte adhesion ↓ cytokine release ↓ leukocyte adhesion	↓ proliferation ↓ migration ↓ inflammatory cytokines ↑ apoptosis
A3	**Gi**	↓	Mast cell degranulation Bronchoconstriction Foam cell formation	Minimal effect	No effect	↑ gradient sensing ↑ cell migration ↑ inflammation	No effect

1.5 Pre-clinical Models

Animal models are the cornerstone for evaluation of physiology and biomedical therapeutics in a robust manner.(71) Mice, rabbits and swine are the predominant models in which vascular biology is validated for preclinical application, with a focus on adenosine biology. Each of these models are akin to humans with plasma adenosine levels in the venous and arterial blood of mice, pigs and rabbits ranging from 500-2000nM, providing comparative extracellular levels to humans suitable for pre-clinical assessment of adenosine and vascular disease.(72,73)

1.5.1 Mice

Murine models facilitate receptor and enzymatic knockout models and have formed the dominant small animal model with which insights into adenosine biology have been gained. At a receptor level, knockout of ADORA1A in mice resulted in greater plasma renin activity and elevated blood pressure compared to wild-type mice.(74) Others demonstrated a diminished protective effect of adenosine against hypoxic/ischemic injury, in addition to augmented anxiety and hyperalgesia following knockout of ADORA2A.(75) These knockout mice also exhibit reduced exploratory activity, increased aggression and anxiety with augmented blood pressure and platelet aggregation.(76) Despite the relative hypertension in ADORA2A knockouts they demonstrate unchanged basal coronary flow, though their ability to dilate in response to adenosine is reduced. However, use of NECA (nonselective adenosine analog) did induce coronary dilation in ADORA2A knockout hearts suggesting other receptor subtypes may also contribute to this role, specifically ADORA2B given that alloxazine (ADORA2B antagonist) attenuated this response. (77) ADORA3 knockout mice demonstrated no

difference in heart rate or blood pressure and fail to elicit antigen-mediated degranulation of mast cells.(78) Interestingly, ADORA3-/- mice demonstrated reduced infarct size post ischemic injury than their wild type colleagues – suggesting an injurious role during ischemia.(79) While receptor knockout models are most common, others have selectively removed enzymes in the metabolic pathway of adenosine, including the ectonucleotidases. Indeed, knockout of CD39 with $cd39^{-/-}$ mice is reported. However, a major limitation of this model is the dual impact such a deletion has by augmenting extracellular nucleotides (ATP/ADP) via reduced CD39-mediated catabolism, while also reducing extracellular adenosine production. Similarly, $cd73^{-/-}$ models exhibit augmented AMP and attenuated adenosine levels, complicating a definitive assessment of the causative factors behind the observed biological response.(2) Hence, murine models have provided fundamental insights into adenosine and adenosine receptor biology, though they are limited in their clinical applicability.

1.5.2 Rabbits

Rabbits serve as a key intermediate animal model for advancing our understanding of adenosine biology. Local adenosine administration intravitreally to optic nerves demonstrated enlarged retinal capillaries with augmented capillary blood flow within the optic nerve head, an effect not seen when adenosine was administered intravenously.(80) Use of DPCPX, an ADORA1 antagonist, neutralized the vasodilatory effect of adenosine without affecting the capillary blood flow velocity in the optic nerves of rabbits, while CPA, an ADORA1 agonist, augmented capillary blood flow supporting the role of ADORA1. Similarly, CSC, a ADORA2A antagonist, did not alter blood flow while CGS-21680, a ADORA2A agonist, increased blood flow velocities. Taken together, they

concluded that adenosine augments capillary blood flow via local effect on ADORA1A and 2A and signals via alternative pathways than adenylate cyclase given the lack of effect with cAMP analog administration.(80) Others evaluated the effect of adenosine on rabbit's pulmonary circulation, demonstrating adenosine-induced pulmonary dilation even following endothelial denudation, while highlighting the key roles that guanylate cyclase and cGMP play in this process.(81) While others have shown reduced heart rate and altered right atrial conduction patterns with a shift towards the crista terminalis (less adenosine sensitive) following administration of adenosine.(82) New Zealand White rabbits have been used to assess platelet aggregation utilizing small molecule agonists to ADORA1 and ADORA2A, demonstrating that A2A agonism was more potent for inhibiting platelet aggregation, while A1 was not. (61) Rabbits have also been used to demonstrate the effect of dipyridamole administration on circulating adenosine levels and cAMP, demonstrating similar physiologic adenosine levels to humans.(72) Given their size, rabbits also have abdominal aortas with diameters similar to human coronary arteries – enabling assessment of arterial healing post stent implantation.(83) Moreover, rabbits models are susceptible to diet-induced atherosclerosis, enabling serial assessments by virtual histology.(84) Hence, while the rabbit model affords many advantages for assessment of vascular biology, the modulation of adenosine biology in this setting is limited to predominantly costly small molecule inhibitors – though continued advancements are moving genetically modified rabbit models closer to reality.(85)

1.5.3 Swine

Swine is a large animal model that is commonly used in the development of intravascular interventions given the similar heart and blood vessel size to humans.(86) Ossabaw swine

have been used to assess the effect of adenosine on coronary stents and coronary blood flow (CBF) utilizing a coronary flow wire similar to that used in humans.(87) In this study, dyslipidemic swine, compared to the control group, demonstrated greater expression of ADORA1, 2A and 2B mRNA in the coronary microvessels distal to the site of stent implantation. Moreover, ADORA2B and ADORA3 antagonists had a relatively small impact on coronary blood flow while ADORA2A antagonists significantly reduced coronary blood flow, thus suggesting that ADORA2A is responsible for adenosine's vasodilatory effects. Interestingly, this study discovered that in the absence of adenosine, the ADORA1 antagonist DPCPX, unlike ADORA2 antagonists, increases the average peak basal velocity of coronary blood flow in healthy swine. In contrast however, ADORA1 agonists had no effect on the average basal peak velocity of coronary blood flow regardless of the presence of adenosine. They concluded that the post-stenting alteration to adenosine-mediated CBF in a dyslipidemic model is likely related to a differential balance between A1 and A2A signaling rather than changes in physical receptor expression.(87) Others implanted coronary stents in Ossabaw swine to assess the relationship between ADORA1 receptors and neointimal growth. They noted that as the degree of arterial injury increased via increasing stent overexpansion, there was a proportional increase in ADORA1 receptor mRNA – suggesting that ADORA1 receptors were involved in neointimal development. Moreover, treatment with ADORA1 agonist, CCPA, promoted the SMC proliferation while the ADORA1 antagonist, DPX, inhibited SMC proliferation, while not inhibiting re-endothelization. Taken together, ADORA1 receptor antagonism may represent a therapeutic target for reducing in-stent restenosis.(34) Overall, while limited due to cost and housing considerations, swine

provides a viable large animal model for assessing arterial healing while also modulating adenosine biology via small molecule inhibitors – providing a true translational model for pre-clinical research.

1.6 Pharmacological Modulation of Adenosine Biology

Our ever-advancing understanding of adenosine biology fuels progressively more sophisticated approaches to alter this biology to achieve therapeutic benefit. The breadth and number of medical conditions for which purinergic signaling is being explored as a therapeutic option continues to rise, spanning multiple organ systems and disease pathologies.(88) Specifically, adenosine receptor agonists and antagonists are the focus of a number of dedicated agents being explored by over 10 companies with over 100 patent applications related to this field.(89) While a number of agents targeting adenosine signaling are currently undergoing clinical trials, only adenosine, some methylxanthines, regadenoson and dipyridamole have garnered regulatory body approval for clinical use so far – though this is expected to expand in the near future.(90)

1.6.1 Adenosine and Adenosine Analogs

Adenosine itself has had a relatively small impact in clinical application owing largely to its short half-life. It is predominately used clinically for the termination of supraventricular tachycardia via its ADORA1-mediated AV node suppression effects, and as a pharmacologic stress agent for myocardial perfusion studies via its predominately ADORA2A-mediated effects.(91) Given its short half-life, the use of adenosine directly for mechanistic studies has been limited and instead led to the development of a number of adenosine analogs.(92) 5’-N-ethylcarboxyamidoadenosine

(NECA) is an adenosine analog that was first described in 1977 and demonstrated to be over 20,000 times more potent than endogenous adenosine with regards to coronary dilatation.(93) Subsequent work demonstrated 5-10-fold greater inhibition of ADP and adrenaline-induced platelet aggregation via augmentation of cAMP, an effect that was abolished with theophylline administration.(59) Interestingly, while the aggregation inhibition was 5-10-fold greater, the platelet cAMP levels were only 1.3-fold greater. Previous adenosine analogs enacted their effect via inhibiting other activating pathways such as competitive inhibition of ADP for ADP receptors. NECA uniquely establishes platelet inhibition independent of other pathways examined, providing an ADOR specific platelet inhibition coupled with activation of adenylate cyclase.(59) Another approach is to add a chlorine moiety to adenosine generating 2-chloroadenosine and 5'-chloro-5'deoxyadenosine with significantly improved stability, enabling their use as a therapeutic agent.(94) In fact, 2-choloroadenosine has been employed in *in vitro* studies where it led to reduced inflammatory cytokine release in activated ECs to the same degree or even better than adenosine **(Figure 3)**.(45)

1.6.2 Adenosine Receptor Agonists and Antagonists

Given adenosine's relative instability and the differential effects it causes depending on specific receptor subtype stimulated, considerable efforts have focused on generating small molecule agents to stimulate or inhibit each specific adenosine receptor **(Figure 3)**.There is an extensive array of molecules currently under development, the details of which are beyond the scope of this review, but has been reviewed previously, with a variety of clinical applications.(88-91,95) One agent is ATL146, an ADORA2A selective agonist, that reduces upregulation of VCAM-1, P-selectin and ICAM-1 and

neointima formation following vascular injury, an effect abrogated by co-administration of the selective ADORA2A antagonist ZM241385 – suggesting an ADORA2A-dependent effect.(29) Another agent is CPA, which has been shown to be 15-fold selective for ADORA1 while CGS21680 is 170-fold more selective for ADORA2A. (96) Specifically, ADORA2A and not ADORA1 receptors are thought to be the driving factors behind coronary dilation. Only ADORA2A receptors are present in the microvasculature, with microvascular dilation occurring independent of cAMP signaling.(97) The ADORA2 agonist 2-hexynyl-NECA, could more effectively inhibit platelet aggregation in rabbits compared to NECA, a pharmacologically recognized ADORA2 agonist. Similarly, the ADORA2A agonist, CGS21680, had comparable properties to adenosine but had a weaker inhibitory effect on platelet aggregation in rabbits compared to NECA. Finally, ADORA1 agonists appeared to have no effect on preventing platelet aggregation, suggesting that adenosine compounds with high affinity for ADORA2 receptors are more potent in inhibiting platelet aggregation in rabbits compared to adenosine compounds attracted to ADORA1 receptors.(61)

ADORA2A receptor specific agonists have been a particular focus, owing to the beneficial effects of coronary vasodilation for perfusion studies, while attempting to avoid the off-target effects on ADORA1, A2B and A3 which are known to lead to atrioventricular block, bronchoconstriction and mast cell degranulation amongst others.(91) Regadenoson, an A2A receptor agonist, was specifically developed with this in mind and became the first A2A receptor agonist approved by the FDA.(98) Initial placebo-controlled randomized safety trials in asthmatic patients demonstrated that

regadenoson was safe and well tolerated with the most common adverse effects being tachycardia, dizziness, headache and dyspnea with a mean heart rate increase of 10.5 beats/min and no difference in bronchospasm.(99) A similar safety profile was achieved in healthy subjects submitted to repeated dosing of regadenoson – demonstrating transient dose-dependent increases in heart rate in all groups.(100) Regadenoson demonstrated similar augmentation of myocardial blood flow (MBF) compared to adenosine, while not requiring a continuous infusion as adenosine given its longer systemic half-life.(101) As well, regadenoson demonstrated a dose-dependent increase in MBF, attenuated by aminophylline, with persistent tachycardia, further supporting a sympathoexcitatory mechanism.(102) Ultimately, FDA approval was based upon two phase 2 randomized trials in which patients underwent baseline adenosine MPI followed by randomization to either regadenoson or adenosine, meeting the pre-specified non-inferiority criteria with a reduced side effect profile in the regadenoson group.(103,104) Hence, regadenoson provides an ideal agent for pharmacologic stress testing, being administered as a fixed bolus dose which affords similar hyperemia to adenosine with rapid onset, short duration and ready reversibility, while also being safe in mild-moderate airway disease. (98) It is now being assessed for novel indications including perfusion assessment for cardiac allograft vasculopathy.(105) Accordingly, regadenoson is seeing a steady increase in market share, surpassing that of dobutamine (11% vs 7%) as of 2008 and rising. As well, additional A2A selective agonists, binodenoson and apadenoson, are currently in development. (98)

1.6.3 Methylxanthines

Methylxanthines and their derivatives include caffeine, theophylline, aminophylline and doxofylline – molecules known to function as broad adenosine receptor antagonists. These agents were long known to induce hypertension following administration.(1) Even in patients with autonomic dysfunction who had not consumed any methylxanthines for the preceding three days, the acute administration of caffeine led to a significant increase in blood pressure, akin in both normal and hypertensive patients.(106) This effect appeared to occur independently of both the renin-angiotensin and sympathetic nervous systems.(106) As well, administration of caffeine has been employed as a therapy for reducing post dural puncture headaches, mediating its effects potentially via inhibition of central ADORA2B – though the effects remain controversial.(107)

Theophylline, via many effects including antagonism of ADORA2, found a role for reducing chronic bronchoconstriction in asthma. Adenosine is known to cause bronchoconstriction in asthmatic patients via ADORA2B-mediated mast cell histamine release.(108) However, theophylline carried serious side effects owing to its ADORA1 antagonism effects including seizures and cardiac arrhythmias at toxic doses. Accordingly, it has largely been replaced by long acting beta agonists which carry an improved side effect profile.(108) Aminophylline offers improved solubility and reduced potency via combination of theophylline with a ethyledendiamine salt in a 2:1 ratio enabling both oral and intravenous administration. It was previously used an acute bronchodilator for asthma exacerbations, though it is now being replaced by beta-agonists, Accordingly, the primary use now is in nuclear stress testing as a reversal agent for dipyridamole, regadenoson or other adenosine-mediated stress protocols.(108)

Doxofylline, a novel methyxanthine derivate, offers an improved pharmacologic profile over theophylline, with reduced risk of neurological and cardiac side effects owing to its reduced potency.(109)

1.6.4 Antiplatelet Agents

Antiplatelet medications are intended to reduce platelet activation, aggregation and thrombus formation. The signaling involved in platelet activation is complex involving multiple receptors and pathways.(66) Aspirin, or acetylsalicylic acid (ASA), irreversibly inhibits cyclooxygenase-2 (COX-2) preventing production of thromboxane A2 (TxA2) and inhibiting platelet aggregation.(110) Aspirin does not impact the intracellular uptake of adenosine from the extracellular space, though dipyridamole was shown to augment extracellular adenosine.(111) Moreover, anti-platelet trials have noted clinical benefits beyond those expected solely from the primary anti-platelet effects of these medications.(112) Taken together, these observations have spurned further work to identify the origins of the pleiotropic effects of these antiplatelet medications – potentially via modulation of adenosine signaling.

Dipyridamole

Dipyridamole is a phosphodiesterase inhibitor which prevents the breakdown of cAMP to AMP, augmenting intracellular cAMP levels and reducing intracellular calcium resulting in the inhibition of platelet aggregation. In addition, it also prevents the breakdown of cGMP, augmenting downstream SMC relaxation and subsequent vasodilation. Lastly,

dipyridamole is known to inhibit adenosine uptake via ENT-1 resulting in augmented extracellular adenosine levels and also contributing to vasodilation.(113) Given these effects, dipyridamole has been use for two predominant clinical indications – an antiplatelet for stroke prevention and vasodilator for pharmacological stress perfusion imaging. For stroke prevention, trials of ASA versus dipyridamole demonstrated greater bleeding in the ASA group than the placebo or dipyridamole groups – supporting the relative safety of dipyridamole.(114,115) Ultimately, large studies and meta-analyses demonstrated benefit in composite endpoints including cardiovascular death, stroke and myocardial infarction.(116) The predominant side effect noted with dipyridamole was headache – leading to increased dipyridamole cessation.(116) Another role in which dipyridamole is used clinically is in myocardial perfusion imaging, where IV dipyridamole is commonly administered as a stress agent with a relatively benign side effect profile including chest pain (20%), headache (12%), dizziness (12%), ST changes (8%).(117)

Extensive studies have further explored the impact of dipyridamole on adenosine biology. In rabbit studies, intravenous administration of dipyridamole resulted in hypotension and augmented cerebral perfusion pressure given the vasodilatory and neuroinhibitory effects. Interestingly, administration of 0.7 mg/kg dipyridamole inhibited adenosine uptake by only 25%, an effect that was no longer detectable 10 minutes following dipyridamole administration.(72) A biphasic increase in plasma adenosine was demonstrated with an initial peak noted at the end of the dipyridamole infusion, rapidly dropping only 1 minute later to return to baseline levels (or even below at a dose of 1.4 mg/kg) followed by an

additional peak 20 minutes later which was approximately doubled at a 1.4 mg/kg dosing. This was mirrored by a similar profile in the circulating cAMP levels.(72) In humans, IV dipyridamole infusion (0.56mg/kg for 4min, 4 min rest, then 0.28mg/kg for 2 minutes) in 15 patients demonstrated an ~4-fold increase in plasma adenosine levels (213nM to 776nM post infusion) without a bimodal distribution as noted in rabbits.(118) Similarly, continuous administration of IV dipyridamole yielded an augmented hemodynamic influence of adenosine administration of approximately 4-fold.(119) Low dose IV dipyridamole administered as serial boluses demonstrates an attenuated augmentation of plasma adenosine though with a similar pattern during dose-finding studies.(120) Oral dipyridamole has also been shown to augment plasma adenosine levels (average increase of 0.133uM, range 0.063uM-0.197uM 37%-212% increase) in 5 subjects who received 5 days of oral dipyridamole when compared to 5 days without therapy. The most significant enhancement in adenosine levels was 48hrs following initiation of therapy, while a positive correlation between plasma adenosine levels and dipyridamole levels was also demonstrated.(121) Dipyridamole is also known to be a markedly more potent ENT1 inhibitor than ticagrelor. When adenosine is added to whole blood, it remains detectable for 3-6 minutes in the presence of ticagrelor, but over 60 minutes in the presence of dipyridamole.(122) Dipyridamole has also been demonstrated to improve exercise tolerance in those with stable CAD, ostensibly via augmentation of endogenous adenosine and resultant pre-conditioning and optimizing coronary flow.(123) The combination of adenosine with dipyridamole also prolongs the conduction effects of adenosine.(124,125)

Dipyridamole's augmentation of adenosine, together with prior documentation of ADORA2B-mediated inhibition of SMC proliferation, led to further work examining its potential role as a neointimal suppressant.(38) Indeed, dipyridamole showed considerable promise in reducing intimal thickening following balloon arterial injury in animal models.(126,127) In humans, clinical trials have demonstrated improved hemodialysis graft patency, purportedly via inhibition of neointimal hyperplasia and/or thrombosis.(128,129) This effect remains controversial, with a larger systematic review and meta-analysis not demonstrating any clear benefit of dipyridamole in improving graft patency.(130) Similarly, canine studies of coronary artery bypass grafting (CABG) demonstrated reduced intimal hyperplasia (131) and thrombosis(132) following administration of ASA and dipyridamole. This was followed by clinical trials demonstrating improved vein graft patency following CABG with administration of dipyridamole plus aspirin versus placebo at both early and late follow-up.(133,134) Despite these promising trials, dipyridamole has not been firmly established as a therapeutic agent for neointimal suppression.

Clopidogrel

Clopidogrel, a thienopyridine, is a pro-drug that is hepatically metabolized (via a cytochrome P450-dependent pathway) to its active metabolite. Its anti-platelet effects stem from irreversible inhibition of ADP binding to platelet P2Y12 receptors, preventing downstream signaling leading to activation.(135) With respect to pleiotropic effects, clopidogrel has been shown to improve microvascular endothelial function via reactive hyperemia testing in those with stable CAD independent of its platelet inhibition

effects.(136) Reactive hyperemia testing has been associated with adverse cardiovascular events.(137) Others have demonstrated a benefit of pleiotropic effects of clopidogrel in improving endothelial NO bioavailability and reducing inflammation and CRP levels post PCI.(138,139) The PROMICRO-2 (PROtecting MICROcirculation during coronary angioplasty) study demonstrated improved microvascular function with prasugrel over clopidogrel, presumably due to greater platelet inhibition and reduced microvascular obstruction, quantified by index of microvascular resistance (IMR).(140) However, studies assessing the impact of clopidogrel on circulating adenosine levels failed to show any difference in adenosine levels or reactive hyperemia index (RHI) in post-ACS patients on 30 days of therapy, though ticagrelor did demonstrate some benefit.(141) Hence, while clopidogrel may afford some pleiotropic benefits, it does not impact adenosine levels.

Ticagrelor

Ticagrelor directly acts via reversible-antagonism of P2Y12 inhibiting ADP-induced platelet aggregation, while not competing with ADP binding.(142) However, unlike the thienopyridines, ticagrelor binds reversibly and demonstrates rapid onset/offset effects which mirror drug levels.(143) While ticagrelor demonstrated more potent platelet inhibition than clopidogrel, it also provided improved clinical outcomes, specifically cardiovascular mortality, in patient's presenting with acute coronary syndrome (ACS).(144) In comparison, prasugrel, despite similar platelet inhibition attributes(145), failed to demonstrate a mortality benefit compared to clopidogrel – suggesting ticagrelor's noted effect stemmed from an effect aside from platelet inhibition.(146)

This discrepancy spurned efforts to identify the mechanism(s) by which ticagrelor yielded these beneficial effects – specifically via adenosine.(147) Ticagrelor was noted to dose-dependently inhibit adenosine uptake predominately via inhibition of ENT-1, albeit with 10-fold less potency than dipyridamole.(148) ACS patients also demonstrate significant ticagrelor-induced augmentation of plasma adenosine concentration over clopidogrel (1.5uM (0.98-1.7) versus 0.68uM(0.49-0.78)) via inhibition of adenosine uptake by RBCs.(149) Moreover, post-ACS patients administered 30 days of ticagrelor demonstrate an augmented reactive hyperemia index (RHI) which correlates with elevated adenosine levels, an effect not seen in those on clopidogrel, and known to be associated with improved cardiovascular event rates.(137,141) Ticagrelor has also been shown to augment cAMP while not increasing ADA levels in ACS patients.(150) As well, ticagrelor has been shown to induce significant dose-dependent ATP release from RBCs, which is then degraded to adenosine, further augmenting the extracellular adenosine pool. While the IC50 to trigger ATP release (14umol/L) is higher than that of ENT1 inhibition (0.1umol/L), the ability for partial release is still felt to contribute to the extracellular adenosine pool. (151) Moreover, the augmented adenosine levels themselves are believed to provide further antiplatelet effects, over and above the traditional P2Y12 mediated effects.(122) Ticagrelor clearly demonstrates a reliable augmentation of extracellular adenosine, the clinical implication of which remains to be defined.

Administration of adenosine is beneficial in limiting infarct size in the setting of acute myocardial infarction, though improved clinical outcomes remains controversial, limiting its use.(152,153) Co-delivery of ticagrelor and dipyridamole in canine models demonstrated dose-dependent augmentation of blood flow with both reactive hyperemia post-occlusion and following adenosine infusion.(148) In healthy volunteers, ticagrelor was associated with enhanced dyspnea sensation and augmented adenosine-induced coronary blood flow velocity as assessed by transthoracic echocardiography.(154) While in stable CAD patients, those receiving ticagrelor for 10 days show augmented adenosine-induced myocardial blood flow (MBF) compared to clopidogrel following intermediate-dose (80ug/kg/min), but not with high-dose (140ug/kg/min) adenosine infusions in positron-emission tomography (PET)-based studies. This study also demonstrated a non-significant difference of 8% higher MBF at baseline in ticagrelor versus clopidogrel groups – this may indicate baseline ticagrelor-induced augmentation of MBF, though this remains to be proven. (155) In patients with non-ST-elevation ACS, a greater adenosine-induced augmentation of coronary blood flow was noted at peak hyperemia in those on ticagrelor versus prasugrel without any difference noted in blood pressure and heart rate.(156) Taken together, the clinical benefits and side effects noted with ticagrelor are thought to be related, at least in part, from modulation of adenosine biology.(112) Given that ticagrelor augments plasma adenosine into the range of activation of A2A receptors, this may provide a mechanistic means for this observation.(37)

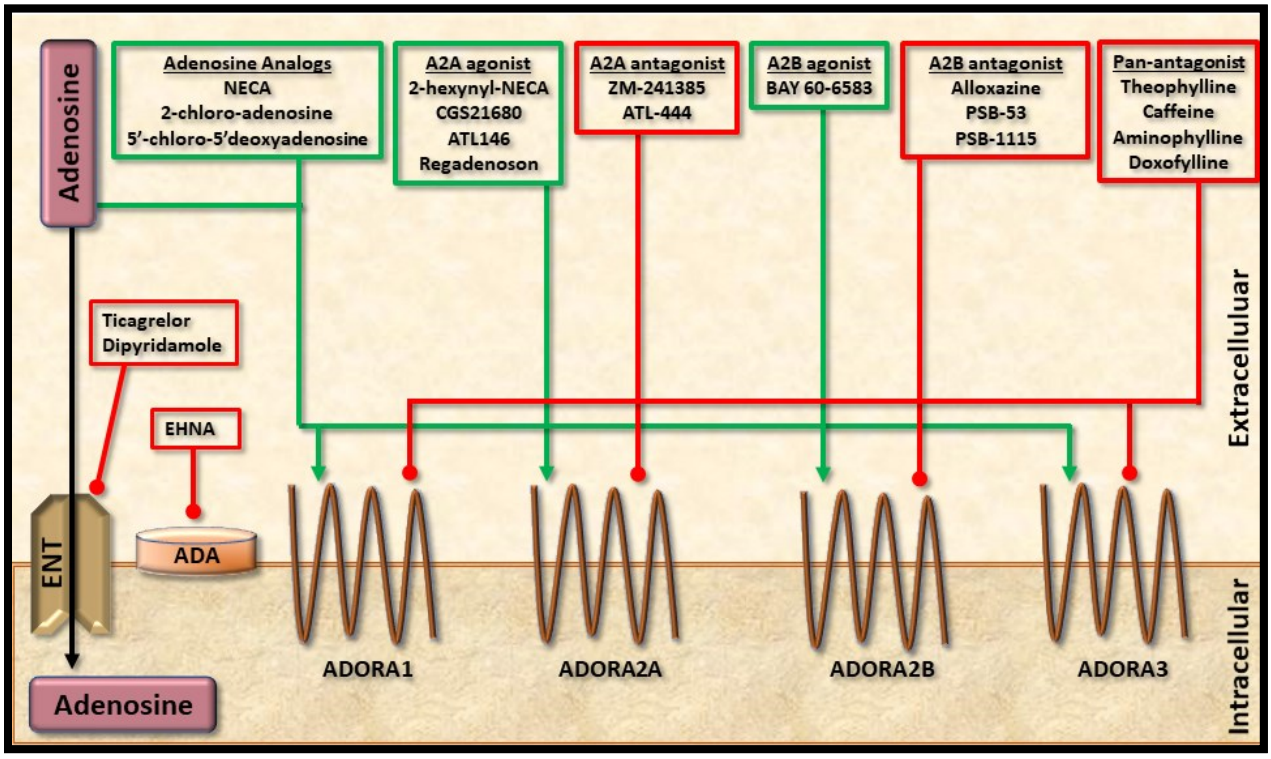

Figure 3. Pharmacological Modulation of Adenosine Biology

Adenosine uptake via ENT inhibited by ticagrelor and dipyridamole, while EHNA inhibits ADA-mediated adenosine breakdown. Adenosine and adenosine analogs serve as pan-agonists of all adenosine receptor sub-types, while pan-antagonists broadly inhibit all receptor sub-types. Receptor-specific small molecule agonists and antagonists directed at ADORA2A and ADORA2B. ENT – equilibrative nucleoside transporter, EHNA-erythro-9-(2-hydroxy-3-nonyl)adenine, ADA – adenosine deaminase, CGS 21680- 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamido-adenosine, NECA - 5'-N-ethylcarbox-amidoadenosine.

1.7 Clinical Implications of Adenosine Biology

1.7.1 Physiologic Effects of Adenosine

The clinical use of adenosine requires either rapid boluses or continuous infusions to manifest any effects. The physiologic effects of adenosine administration are closely linked to the differential influence that stimulation of the specific adenosine receptor subtypes have on intracellular cAMP, as reviewed above, as well as off-target effects.(1)

(Table 1) ADORA1 stimulation leads to negative chronotropic, dromotropic, inotropic and anti-beta-adrenergic effects, including slowed AV conduction and even chest pain.(157) Interestingly, despite these ADORA1 effects, adenosine is known to cause tachycardia, believed to be related to sympathoexcitation effects and independent of the changes noted in blood pressure.(158) This is supported by physiologic studies assessing those with innervated and denervated hearts.(159) ADORA 2 receptors, specifically ADORA2A and to a lesser extent ADORA2B, lead to vasodilation in the coronary and peripheral arterial beds, augmenting myocardial blood flow. (160) In humans, ADORA2A receptors are thought to be present in large reserves in the coronary vasculature where they potentiate significant coronary dilation and augment flow.(161) Moreover, A2A receptors are found in the microvasculature, though studies have demonstrated that microvascular dilation can occur independent of cAMP signaling.(97) Lastly, ADORA3, and to some extent 2B, are known to cause to mast cell degranulation and bronchial constriction.(98) Administration of IV adenosine is known to induce transient dyspnea, in the absence of bronchoconstriction or alteration in pulmonary function, ostensibly due to ADORA1-mediated pulmonary vagal C fiber stimulation.(162,163) In PLATO, 14.5% of patients receiving ticagrelor demonstrated similar dyspnea symptoms versus only 8.7% of patients receiving clopidogrel – suggesting an adenosine-related influence given ticagrelor's modulation of adenosine levels.(164) Tachypnea itself is also common after an adenosine infusion but is not related to changes in airway resistance whatsoever, instead thought to be related to carotid chemoreceptors.(165,166)

Capitalizing on the ADORA1 effects, adenosine was first utilized in humans to treat cardiac arrhythmias in the 1930s.(167,168) In this sentinel work, adenosine was administered either via intravenous, subcutaneous or intramuscular routes in doses ranging from 5-50mg, though only the intravenous route appeared to provide therapeutic benefit from a tachycardia perspective.(168) This work focused on auricular (atrial) fibrillation (AF) and flutter, demonstrating some slowing of the atrial rate in atrial flutter, with reduction in the atrioventricular conduction, enabling improved visualization of the underlying atrial rhythm – a key role for adenosine's use clinically today.(168) This effect is mediated predominately via ADORA1 receptors and necessitates rapid administration of adenosine via bolus and flush to ensure rapid delivery to the heart prior to being degraded – without this approach, adenosine is not effective.(98) Adenosine is also utilized during catheter ablation for AF via pulmonary vein isolation (PVI) – a common practice that continues to be challenged by high rates of recurrence.(169) Recurrence is, at least in part, related to reconnection of the previously ablated pulmonary vein tissue to the left atrium resulting in the return of AF.(170) During ablation, adenosine is employed to unmask dormant conduction tissue and facilitate additional focused ablation to reduce the risk of reconnection and AF recurrence.(171) However, the incremental reduction in AF recurrence using adenosine-guided ablation remains the subject of debate.(172-176) Moreover, adenosine is also involved in the evaluation of syncope,(177,178) with circulating adenosine levels, specifically "low adenosine syncope" being defined as a distinct clinical entity.(179-181) Adenosine administration also serves as a diagnostic tool for assessing those with syncope. Indeed, the adenosine, or ATP test, employs a bolus dose to cause a cardiac pause, the duration of

which aids in identifying patients with unexplained syncope that may benefit from pacemaker insertion.(182) This test has been extensively explored(183) and noted in recent guidelines(184), though defining a specific role for this test in the diagnostic evaluation of syncope remains the subject of debate.(185)

The other predominant clinical role for adenosine is focused upon its ADORA2-mediated effects – specifically coronary vasodilation and myocardial blood flow. Indeed, stimulation of ADORA2 is achieved with exogenous adenosine administration at 10-fold lower doses than that required to elicit ADORA1-mediated chronotropic, inotropic or dromotropic changes, including peripheral vasodilation. (160,186) Accordingly, continuous IV infusions of adenosine are utilized to stimulate maximal hyperemia via coronary dilation, enabling physiologic flow testing during both noninvasive myocardial perfusion imaging and invasive angiography – a process known as fractional flow reserve (FFR).(187) Intravenous infusion of adenosine (mean 130ug/kg/min) has been demonstrated to augment mean plasma adenosine concentration measured via cannulas in the aortic arch from 70nM to 1200nM.(188) As well, a solution of 2mg/ml adenosine remains stable in a saline solution at both room temperature and under refrigeration for up to two weeks, facilitating its use as a continuous infusion.(189) Adenosine-mediated flow dilation also forms the basis of pharmacologic myocardial perfusion testing with adenosine and dipyridamole forming the predominant agents utilized to date. However, these agents also lead to activation of ADORA1 and ADORA3, causing reduction in flow and counteracting the effects on ADORA2A and ADORA2B.(190) To this point, use of ADORA2A specific agonists enables replication of coronary dilation effects without the

concern for other systemic effects.(161) Accordingly, considerable efforts have focused on generating ADORA2A specific agents – leading to the development of regadenoson and others, as reviewed earlier.(98,157)

1.7.2 Diabetes Mellitus

Adenosine plays a crucial role in managing glucose homeostasis with signaling known to modulate the uptake of glucose into the tissues.(191) Specifically, studies in rat skeletal muscle have demonstrated a role for ADORA1 receptors in regulating insulin-mediated glucose transport.(192) ADORA1 knockout models also demonstrate augmented insulin and glucagon secretion in murine models,(193) while overexpression of ADORA1 confers protection from obesity-related insulin resistance.(194) Adenosine's effect on glucose transport is noted in the absence of any impact on insulin binding, suggesting adenosine impacts insulin action distal to the insulin receptor itself, thereby reducing blood glucose levels.(195) ADORA2B has also been implicated in glucose homeostasis, with A2B antagonists (PSB-53 and PSB-1115) augmenting plasma insulin levels.(196) Interestingly, others have postulated a potential therapeutic role for adenosine stimulation in treating type 1 diabetes.(197)

In humans, diabetes is known to impact another key component of adenosine signaling – adenosine deaminase (ADA) – responsible for degrading adenosine in both the intracellular and extracellular spaces. One study demonstrated increased ADA activity in overweight and obese individuals, suggesting a possible role in insulin resistance and diabetes.(198) Indeed, one study examined 56 patients with type 2 diabetes mellitus

(T2DM) compared to 45 age and sex matched healthy controls. The T2DM group demonstrated higher BMI than the controls, but no correlation between BMI and ADA levels were reported. Ultimately, they demonstrated elevated ADA levels among patients with T2DM and a positive correlation between fasting plasma glucose and ADA levels in non-obese T2DM patients, an effect not seen in controls.(199) Similarly, another study confirmed elevated ADA activity in T2DM patients with a positive correlation between ADA and blood glucose levels. Interestingly, they demonstrated that poorly controlled diabetics (HbA1c >9%) had higher ADA activity levels than well controlled diabetics.(200) They also examined the impact of dipeptidyl peptidase 4 (DPP-4), an agent known to bind with ADA, but failed to show any additional specific activity on ADA above that of other glycemic agents.(200) Taken together, elevated ADA levels would reduce circulating adenosine, which via its impact on glucose transport, result in reduced tissue uptake of glucose and increased plasma glucose – highlighting a key interplay of adenosine and diabetes.

1.7.3 Hypertension and Angiogenesis

Systemic hypertension is a known cardiovascular risk factor leading to end organ damage and a known association with adenosine signaling.(201,202) In humans, studies have assessed normotensive and hypertensive patients demonstrating diminished adenosine levels in the microcirculation of hypertensive patients, ostensibly contributing to the increased peripheral vascular resistance noted. Moreover, vasodilation can be induced by arterial infusion of adenosine following aerobic training, demonstrating no change in vasodilation in the hypertensive cohort, whereas the normotensive did show reduced vascular conductance.(203) One study assessing innervated and denervated hearts,

administered intracoronary adenosine and demonstrated an increase in blood pressure and catecholamines, while no changes were seen in the denervated cohort – supporting the role of the sympathetic pathway in this phenomenon.(159) However, adenosine receptor signaling is still thought to be related to hypertension. Some have implicated ADORA3 in the mediation of vasodilation in spontaneously hypertensive rats.(204) Loss of ADORA2A results in reduced adenosine-mediated aortic relaxation – supporting the importance of ADORA2A for vascular tonicity and providing a potential role for its involvement in systemic hypertension.(205) Moreover, in hypertensive patients, ADORA2A is known to have reduced function, normalized by treatment with doxazosin, but not propranolol.(206) ADORA2A specifically has been the focus of a number of novel agents with the potential to act as a therapy for hypertension given its effects in the peripheral vessels and kidneys.(207)

Adenosine has also been implicated in the pathogenesis of pulmonary arterial hypertension. Plasma adenosine concentration has been quantified in patients with pulmonary hypertension, demonstrating elevated levels in the pulmonary circulation when compared to the systemic circulation. At room air, pulmonary adenosine was lower in those with pulmonary hypertension (450nM vs 1260nM in controls), and following oxygen administration diminished further – suggesting adenosine may play a role in pulmonary hypertension.(208) Indeed, adenosine has also been employed as an agent for vasoreactivity testing in pulmonary hypertension with promising results following intra-arterial infusion into the pulmonary arteries.(209) As well, murine models with ADORA2A knockout lead to development of pulmonary arterial hypertension.

Assessment of the pulmonary arteries in this model demonstrates augmented proliferation of endothelial and smooth muscle cells with fibroblast activation and collagen deposition within the arterial walls – supporting to importance of adenosine in the pathogenesis of pulmonary arterial hypertension.(210)

Adenosine plays an important role in angiogenesis(211) and lymphangiogenesis – supporting the role for adenosine antagonists as potential anti-cancer agents.(212) Indeed, adenosine has been implicated in the pathogenesis of many cancers, including head and neck cancer – providing a focus of novel therapeutic approaches.(213) As well, ADORA2B stimulation has been implicated in vascular endothelial growth factor (VEGF)-mediated wound angiogenesis, facilitating repair in human endothelial cells.(214) Moreover, adenosine plays a role in disorders characterized by pathological angiogenesis. For example, patients with proliferative retinopathies, have demonstrated markedly elevated levels of ADORA2A, with receptor-specific antagonists serving as therapeutic options in these cases.(215) ADORA2A has also been implicated in the pathogenesis of abdominal aortic aneurysms. Specifically, receptor activation leads to diminished inflammation and elastin fragmentation translating to reduced aneurysm formation.(216) Interestingly, from a therapeutic perspective, adenosine is also utilized during intervention on cerebral aneurysms to induce transient cardiac standstill and provide a stable operative field facilitating reliable clip placement.(217,218)

1.7.4 Sepsis, Heart Failure and Critical Illness

Most literature exploring adenosine is focused on its signaling mechanisms and assessing means of modulating it signaling pathways. However, the endogenous levels of

circulating adenosine are also felt to portend clinical importance. Indeed, circulating adenosine is known to be elevated in settings of stress and hypoxia serving as a modulator of inflammation and facilitating healing. (219-221) Accordingly, preclinical sepsis models suggested that antagonism of ADORA2A may have benefit and improve survival in sepsis, (222,223) while others suggested that inhibition of ADA led to improved outcomes in rat models of sepsis.(224) However, subsequent clinical trials with induced endotoxemia in humans confirmed elevated circulating adenosine levels, but were unable to demonstrate any difference with caffeine co-administration.(225) Other clinical trials have suggested that circulating adenosine levels are predictive of survival in septic shock, with higher adenosine levels occurring at each timepoint in patients that do not survive.(226) However, this remains controversial as other trials have failed to show prognostic benefit of adenosine levels in critically patients.(227) Adenosine is also implicated in pathophysiology of CHF(228) with elevated adenosine levels noted in those with CHF.(229) Indeed, CHF patients with AMPD1 variations with reduced function, theoretically resulting in elevated circulating adenosine levels, demonstrate improved clinical outcomes.(230,231) Hence, these studies demonstrate the potential role for adenosine as either a therapeutic agent or biomarker of clinical outcomes.

1.7.5 Atherosclerosis

Aside from hypertension and diabetes, dyslipidemia is another established cardiovascular risk factor, with a clear correlation with adverse cardiovascular events.(232) Adenosine, specifically ADORA2B, has been demonstrated to regulate dyslipidemia and, ultimately, atherosclerosis.(233,234) One sentinel event is the presence of oxidized-LDL within the

vessel wall.(235) This facilitates the inwards migration of inflammatory cells which engulf oxidized LDL, thereby becoming foam cells and generating a fatty streak.(236) Next, SMC migration and ECM deposition leads to fibrous plaque formation, while cellular necrosis and neovascularization later result in plaque rupture and thrombus formation.(234,237) The numerous steps and cell types involved in this pathogenesis afford several roles for adenosine modulation, including the honing, migration and activation of inflammatory cells.(238)

Adenosine is known to inhibit IFN-γ induced macrophage activation, crucial to lesion development.(239) Adenosine has also been implicated in the formation of foam cells – a key step in this process. Treatment with adenosine facilitated development of foam cells in part due to modulation of hypoxia-inducible factor-1 (HIF-1). Moreover, sequential knock outs of each adenosine receptor demonstrated key roles for adenosine receptors in major steps of plaque development, specifically ADORA2B and ADORA3.(240) As well, ADORA2A knockout mice demonstrate elevated plasma lipid and inflammatory cytokine levels, with those lacking ADORA2A especially in bone-marrow derived cells showing reduced atherosclerotic plaques – supporting ADORA2A inhibition as a potential therapeutic approach.(241) Interestingly, clinical trials have demonstrated that dipyridamole, an ENT-1 inhibitor known to augment circulating adenosine levels, can reduce the progression of atherosclerotic peripheral arterial disease over placebo controls.(242) In humans, studies have assessed outcomes in patients with genetic variations causing reduced adenosine monophosphate deaminase (AMPD) activity, believed to lead to increased persistence of circulating adenosine, showing improved

cardiovascular survival in those with CAD (243) Taken together, adenosine biology is implicated in many crucial steps of atherosclerosis progression and may serve as both a viable therapeutic target and possible marker of vascular health and outcomes .

1.7.6 Revascularization and Restenosis

Restenosis refers to the re-narrowing of a previously re-vascularized vessel, typically via the excessive formation of neointimal tissue resulting in luminal loss.(31) In-stent restenosis (ISR) specifically refers to re-narrowing of an artery following percutaneous stenting, the primary conventional means of revascularization for atherosclerotic disease.(244) The pathophysiology of this process is incompletely understood, but known to include a cascade of events including inflammatory cell activation, SMC migration and ECM deposition each of which have potential implications for adenosine modulation.(31)

Adenosine

Both *in vitro* and *in vivo* models have demonstrated numerous roles for adenosine in the modulation of neointima formation. As reviewed above, ADORA1 stimulates neointima formation(34,35), while ADORA2B (predominately vascular distributed), reduces inflammation, leukocyte vascular adhesion, SMC proliferation and neointima formation, ostensibly via a bone-marrow mediated effect.(30) Similarly, ADORA2A activation leads to reduced inflammation, leukocyte honing and neointimal formation following vascular injury.(29,245) Hence, antagonism of ADORA1 and agonism of ADORA2A and ADORA2B may serve as viable targets to mitigate neointimal formation following vascular injury. Intracoronary infusion of adenosine prior to PTCA has been shown to attenuate ischemic injury during revascularization.(246) One small study was performed

in ten patients with one-vessel CAD that was >70% stenosed undergoing percutaneous transluminal coronary angioplasty (PTCA). They demonstrated a reduced intracoronary adenosine level in all patients post PTCA from a mean of 736nM pre-PTCA to 109 nM post-PTCA and proposed that this may predict restenosis rates. (247) To this end, a small study in patients with reduced AMPD activity, and theoretically increased adenosine levels, failed to show any clear restenosis benefit at 7 months post coronary revascularization.(248) To date, no studies have definitively linked adenosine at the time of revascularization as either a therapeutic agent or predictor of clinical outcomes.

Dipyridamole

Dipyridamole, an ENT-1 inhibitor known to augment circulating adenosine and inhibit SMC proliferation amongst other effects, has also drawn considerable interest for reducing restenosis rates. Rabbit models demonstrated reduced platelet deposition in PTFE grafts with dipyridamole.(249) PTFE graft models demonstrated no impairment to re-endothelialization with intimal formation arising from the anastomotic sites and islands in the center of the graft.(250) Early rabbit work post PTCA revealed reduced intraluminal clot and intimal thickening in those treated with ASA/dipyridamole, suggesting perhaps platelet aggregation may contribute to restenosis.(126) Subsequent work demonstrated that dipyridamole inhibited SMC proliferation *in vitro*, independent of its anti-platelet effects.(127) This group raised concern regarding the high degree of serum protein binding with over 99% of dipyridamole being bound, which following typical physiologic dosing of 0.5-1ug/ml would translate to a free dose of 0.005-0.01ug/ml - ~500-1000-fold lower than expected to inhibit SMC proliferation. Hence,

adventitial dipyridamole therapy was pursued following arterial injury in rabbits, demonstrating a 63% reduction in proliferation and 20% reduction in neointimal formation.(127) Similarly, canine models of CABG demonstrated reduced intimal thickening following ASA/dipyridamole administration.(131)

In humans, dipyridamole has been assessed in many trials focused on restenosis rates in hemodialysis grafts, coronary-artery bypass grafts and following PTCA. ASA and dipyridamole have been employed in a number of large clinical trials demonstrating improvements in hemodialysis graft patency via inhibition of neointimal hyperplasia and thrombosis, though the benefits remain controversial.(128-130) Similarly, ASA/dipyridamole has demonstrated improved vein graft patency post CABG at both early and late follow-up.(133,134) Vein graft restenosis also occurs post stent placement via progression of atherosclerosis, neointima formation or late thrombus formation.(251) Dipyridamole has also been the focus of a few trials in the setting of PTCA. One RCT randomized patients to ASA versus ASA and dipyridamole prior to elective PTCA, demonstrating no significant differences in acute complications.(252) A retrospective analysis administering IV dipyridamole pre-PTCA established reduced acute thrombosis and complication rates in the 24 hour period post PTCA.(253) A larger RCT involved 550 patients with the addition of intracoronary dipyridamole in the setting of PTCA, with only bailout stenting being performed, demonstrating reduced abrupt vessel closure in both stable CAD and ACS populations.(254) Others have shown improved systolic and diastolic ventricular function with intracoronary dipyridamole during PTCA.(255) One trial assessed both early and delayed (6 month) outcomes following intracoronary

dipyridamole administration in coronaries <2.75mm, with only bailout stenting being performed. They similarly showed reduced acute complications, and improved net gain (acute gain minus late loss), but did not show any difference in angiographic restenosis or target-vessel revascularization rates at 6 month follow-up.(256) The major limitation to this work is that it precedes the widespread use of conventional stents which significantly reduce acute complications and provide limited insights into long term outcomes. Hence, dipyridamole, potentially via a combination of platelet inhibition, SMC suppression and adenosine-mediated effects, appears to be a viable therapy for reducing neointimal progression – though to date it has yet to be used broadly for this indication.

1.8 Conclusion

Adenosine is ubiquitous throughout the entire body, playing a crucial role in regulating homeostasis across multiple cell types and pathways. Following vascular injury, adenosine modulates several cells critical to vascular repair and restoration. Animal models provide insights into the mechanisms underlying adenosine signaling and have highlighted numerous potential therapeutic targets. Despite this, the clinical use of adenosine and its ligands have remained relatively limited, focusing predominately on its vasodilatory properties. However, preclinical and early clinical trials strongly suggest that modulation of adenosine may provide therapeutic benefit and warrant additional efforts to better elucidate adenosine's role as a mediator of vascular repair. In addition, while preliminary studies have explored the role for adenosine as a biomarker or predictor of outcomes in patients with sepsis(225,226), CHF(230,231), CAD(243) and post-revascularization(248) – considerable work remains to establish circulating adenosine as

a possible predictor of clinical outcomes. Taken together, adenosine is a promising agent to serve as a possible marker and/or mediator cardiovascular health.

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1.10 Conflicts of Interest

None declared

Chapter 2

Introduction – Modifiable Risk Factors and Residual Risk Following Coronary Revascularization: Insights From a Regionalized Dedicated Follow-Up Clinic

2.1 Preface

This chapter has been previously published in *Mayo Clinic Proceedings: Innovations, Quality & Outcomes:*

Simard T, Jung RG, Di Santo P, Harnett DT, Abdel-Razek O, Ramirez FD, Motazedian P, Parlow S, Labinaz A, Moreland R, Marbach J, Poulin A, Levi A, Majeed K, Boland P, Couture E, Sarathy K, Promislow S, Russo JJ, Chong AY, So D, Froeschl M, Dick A, Labinaz M, Le May M, Holmes DR Jr, Hibbert B. Modifiable Risk Factors and Residual Risk Following Coronary Revascularization: Insights From a Regionalized Dedicated Follow-Up Clinic. Mayo Clin Proc Innov Qual Outcomes. 2021 Dec 4;5(6):1138-1152. doi: 10.1016/j.mayocpiqo.2021.09.001. PMID: 34934904; PMCID: PMC8654638.

All authors made substantial contributions to the conception/ design of the work; and/or the acquisition, analysis, or data interpretation; and, (ii) drafted and/or revised the content critically; and, (iii) provided final approval of the published version; and, (iv) agree to be accountable for the work.

2.2 Abstract

Objective:

To ensure compliance with optimal secondary prevention strategies and document the residual risk of patients following revascularization we established a post-revascularization clinic for risk factor optimization at one-year with outcomes recorded in a web-based registry. Although coronary revascularization can reduce ischemia – medical treatment of coronary artery disease (CAD) remains the cornerstone of ongoing

risk reduction. While standardized referral pathways and protocols for revascularization are prevalent and well-studied, post-revascularization care is often less formalized.

Patients and Methods:

The University of Ottawa Heart Institute is a tertiary care center providing coronary revascularization services. From 2015-2019, data was prospectively recorded in the CAPITAL revascularization registry and patient-level procedural, clinical and outcome data are collected in the year following revascularization. Major adverse cardiac event (MACE) was defined as death, myocardial infarction, unplanned revascularization, or cerebrovascular accident. Kaplan-Meier curves were generated to evaluate time-to-event data for clinical outcomes by risk factor management, comparisons were performed using log-rank tests and reported by hazard ratio (HR) and 95% confidence intervals.

Results:

4,147 patients completed one-year follow-up after a revascularization procedure that included 3,462 undergoing PCI,589 undergoing CABG, and 96 undergoing both PCI and CABG. In the year following revascularization (median follow-up 13.3 months [IQR,11.9-16.5]), 11% of patients experienced a MACE with females being disproportionately at risk. Moreover, 47.7% of patients had $\geq$2 risk factors (diabetes, dyslipidemia, overweight, active smoker) at the time of follow-up with 45.0% of diabetic patients failing to achieve target HbA1c, 54.8% of smokers continuing to smoke and 27.1% of patients failing to achieve guideline-directed lipid targets.

Conclusion:

Patients who have undergone revascularization procedures remain at elevated risk for MACE and inadequately controlled risk factors are prevalent in follow-up. This

highlights the need for aggressive secondary prevention strategies and implementation of programs to optimize post-revascularization care.

2.3 Introduction

Risk factors for adverse cardiovascular events are well established. Conversely, protective factors including healthy diet and exercise are known to mitigate risk.(257) These form the baseline of care irrespective of whether the patient has undergone revascularization or not. Accordingly, secondary prevention strategies are vital to optimize a patient's risk profile and to minimize the risk of adverse events following coronary revascularization. Despite these efforts, coronary artery disease (CAD) continues to be a leading cause of morbidity and mortality.(258,259)

Advances in revascularization care include both changes in medical therapy(260-262) and procedural technology/technique.(244,263-266) Contemporary revascularization is more commonly performed via percutaneous coronary intervention (PCI) than coronary artery bypass grafting (CABG) – with the mode of revascularization selected based on clinical presentation, disease complexity and comorbidity burden.(267,268) For PCI, improvements in stent design and techniques (e.g. imaging/fractional flow reserve) has reduced repeat revascularization rates(269-273) though this has not translated to reduced rates of death or myocardial infarction (MI).(270) Similarly, CABG reports annualized graft failure rates of <5% for arterial and up to 25% for venous conduits, with pooled data suggesting a benefit of arterial conduits to reduce MI.(274) However, irrespective of the mode of revascularization, long-term outcomes are most impacted by risk factor modification and medical therapy. Indeed, cumulative rates of death or non-fatal MI post

PCI approach 17% at 6 years post-revascularization without plateauing.(270) Indeed, following CABG or PCI MACE (death, myocardial infarction, stroke or repeat revascularization) rates approach 20-28% at 3 years with a 24-27% mortality rate at 10 years(275) – highlighting the need for ongoing risk factor control.(276)

The first year post-revascularization represent the highest risk period for patients with coronary artery disease (CAD).(276) While considerable resources and research have established optimal pathways to enable patients to achieve timely revascularization,(277) protocols for optimal care thereafter are not as well established. Accordingly, we established a standardized post-revascularization clinic, whereby all patients undergoing a revascularization procedure undergo a protocolized assessment in the year following their revascularization procedure. The purpose of this program is to uniformly assess their risk factor management and to implement optimal secondary prevention strategies. Herein, we evaluate the effectiveness of currently established care pathways on risk factor management during the first 12 months after coronary revascularization.

2.4 Methods

Study population and data collection

The University of Ottawa Heart Institute is a large tertiary care center providing the sole coronary revascularization services to over 1.2 million people in the capital region of Canada, including an established primary PCI program for patients with ST-elevational myocardial infarction (STEMI) with a hub-and-spoke model for peripheral community centers.(277) Our center includes an established cardiac rehabilitation program with

integrated physical therapy, dietary, psychosocial and smoking cessation programs offered to all revascularization patients.(278-281) All patients undergoing revascularization have their data prospectively recorded in the Cardiovascular And Percutaneous clinical TriALs (CAPITAL) revascularization registry, a web-based registry developed in-house that captures over 1,200 clinical data points on background and procedural factors related to revascularization. This registry also includes a subset of patients with samples collected in the CAPITAL Biobank to gain insights into novel biomarkers in the post-revascularization setting.(282-285) Comorbidities are documented at the time of pre-procedural assessment by the clinician with hypertension and dyslipidemia determined based on existing diagnosis using guideline recommendations and/or presence of dedicated medical therapy.(286,287) Diabetes mellitus (DM) was determined from previous history, presence of DM agents, or a hemoglobin A1c (HbA1c) ≥6.5% a presentation with types delineated as type I, type II – non-insulin-dependent, type II-insulin-dependent. Medications were recorded from medical reconciliation lists. Acute coronary syndrome (ACS) was subclassified as STEMI, non-ST-segment elevation myocardial infarction (NSTEMI) and unstable angina (UA).(288) CAD was defined as ≥50% visual stenosis of an epicardial artery documented at the time of invasive angiography. Revascularization procedures include patients who underwent angiography at our center and subsequently underwent PCI or CABG. Subjects with multiple invasive angiograms were included once for the purposes of analysis with their first invasive angiogram representing the index event and subsequent invasive angiograms recorded and used to identify revascularization events

The study was approved by Ottawa Health Science Network Research Ethics Board (OHSN-REB #20190224-01H) to evaluate clinical outcomes following revascularization.

Follow-up protocol

After revascularization, per our local process, patients assumed established, pre-defined, cardiac rehabilitation protocols and follow-up with primary care physicians. At one-year post-revascularization, they were contacted to return for clinical follow-up with reassessment of lipid profile and glycemic control at that time. Patients who were unable to physically return for in-person follow-up underwent telephone follow-up. In-person follow-ups were completed by physicians performing standardized assessments with a focus on cardiovascular risk factor management and optimization of relevant medical therapy **(**Figure 4**)**.

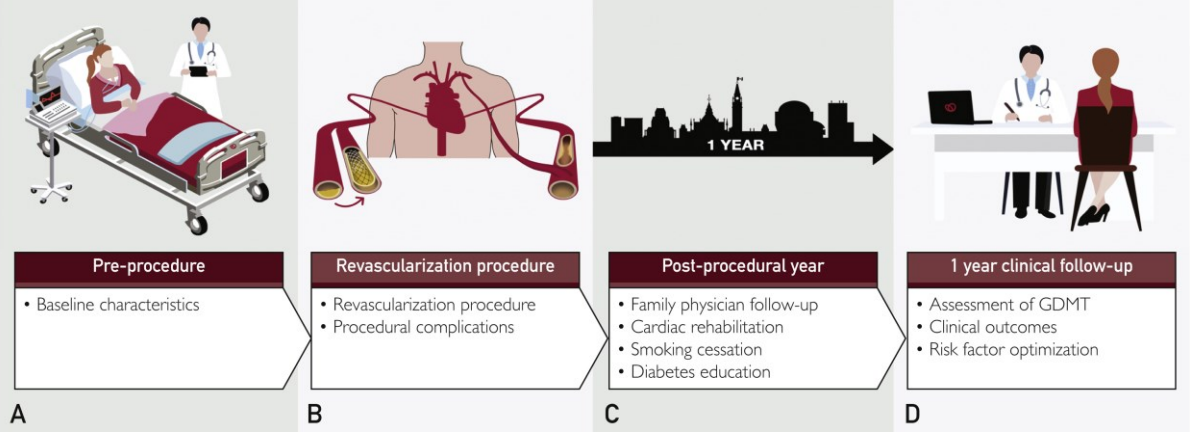

Figure 4. Post-revascularization clinic workflow

(A) Prior to the procedure, all baseline characteristics are recorded in the CAPITAL revascularization registry. **(B)** Procedural data and complications are recorded following the completion of revascularization. **(C)** Following revascularization, patients undergo primary care physician follow-up, cardiac rehabilitation, smoking cessation, and diabetes management as appropriate. **(D)** One-year clinical follow-up performed with assessment of guideline-directed medical therapy, risk factor optimization and clinical outcomes.

Clinical outcomes

Pre-defined clinical parameters were recorded at the time of angiography and repeated at the time of follow-up. Risk factor management was dichotomized following clinical guidelines. Modifiable risk factors available were pre-defined as diabetes, smoking, lipid levels and BMI status. Glycemic control was defined as HbA1c$\leq$7.0% in those with DM. Smoking status was dichotomized as active or not active at the time of follow-up with subsets including "quit but relapsed" and "never quit since index case" to reflect changes in the year following revascularization. Low density lipoprotein (LDL) levels were reassessed with adequate levels set as per guidelines targets of <1.8-2.0 mmol/L.(289-291) Patient body-mass indices (BMI) were recorded at the time of angiography and again at one-year follow-up. Baseline and follow-up BMIs were grouped into underweight (<18.5 kg/m^2), normal (18.5-24.9 kg/m^2), and overweight/obese ($\geq$25.0kg/m^2). Significant weight loss was defined as a follow-up weight that was $\geq$10% less than the body weight at the time of index case.(292) MACE was assessed at 1 and 12 months, defined as a composite of death, MI, stroke (as per neurologist assessment or hemorrhagic cerebrovascular event with confirmatory imaging), or any repeat unplanned revascularization procedure, individual components of this outcome are reported separately. Patients who died prior to follow-up assessment were excluded from risk factor analysis.

Statistical analysis

Continuous variables are reported as mean± standard deviation or median ± interquartile range (IQR). Categorical variables were compared using the chi-squared or Fisher's exact tests and continuous variables were compared by Student *t*-tests or Mann-Whitney *U* tests, as appropriate. Kaplan-Meier curves were generated to evaluate time-to-event data for clinical outcomes by risk factor management; comparisons were performed using log-rank tests. Patients were censored after the first occurrence of MACE. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using Cox regression. Odds ratios (OR) with 95% CI were calculated to evaluate the association between modifiable risk factors at time of follow-up. All statistical analyses were performed using SAS v9.4 (SAS Institute, Inc.,Cary,NC,USA) and all figures were created using GraphPad Prism v8 (GraphPad Software, La Jolla,CA,USA). $P<0.05$ was considered statistically significant.

2.5 Results

From August 2015- October 2019, 18,210 coronary angiograms were performed. 1,234 were repeat procedures and 6,717 did not undergo revascularization. 10,259 patients went on to revascularization, of which 2,987 were excluded as 12 months had not elapsed since their procedure at the time of analysis. 3,125 patients elected for routine follow-up outside of the revascularization clinic. Thus, outcome data of interest were available for 4,147 patients, of which 3,462 patients underwent PCI, 589 underwent CABG, and 96 had staged procedures with both PCI and CABG **(Figure 5).**

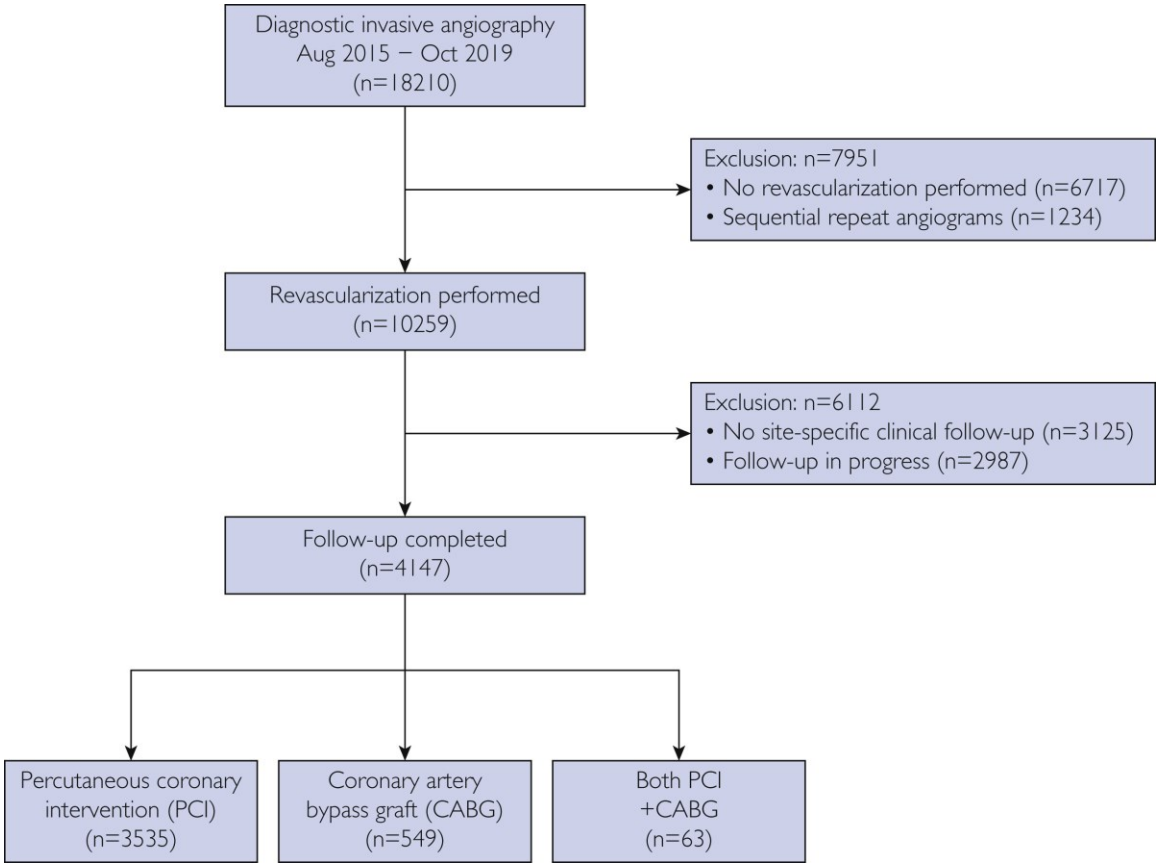

Figure 5. Flow diagram

Regional follow-up clinic patient flow following revascularization.

Patient characteristics

The baseline characteristics of patients are summarized in **Table 2**. The mean age was 65.8±11.8 years and 1,068 patients (25.8%) were women. The mean BMI was 29.0±5.7 kg/m^2. Risk factors included type 2 DM (25.7%), active smoking (19.9%), dyslipidemia (57.4%), hypertension (60.3%), and family history of CAD (14.3%). At baseline, medical therapy in the cohort included aspirin (90.8%), $P2Y_{12}$ inhibitors (91.1%), statins (69.6%), angiotensin converting enzyme inhibitor or angiotensin receptor blocker (ACEi/ARB) (41.5%), and beta-blockers (45.6%). The indication for revascularization varied, 64.4%

presented with ACS [28.0% STEMI, 36.4% NSTEMI/UA] whereas 24.2% of patients had stable CAD/chronic coronary syndrome.(293) Females were older with a higher incidence of hypertension, diabetes and smoking while being less likely to have had prior PCI or MI. Females presented more commonly with ACS, underwent more femoral access and were more likely to be revascularized by PCI and less likely CABG compared to males. In follow-up, females were less likely to be taking ASA, ACEi/ARB and statins.

Table 2. Baseline characteristics

	Total (n=4147)	**Male (n=3079)**	**Female (n=1068)**	**P-value**
Age - years, mean ± SD	65.8 ± 11.8	64.5 ± 11.5	69.5 ± 11.8	**<0.0001**
Sex (female) - no. (%)	1068 (25.8)	-	-	-
BMI - kg/m^2, mean ± SD	29.0 ± 5.7	28.9 ± 5.2	29.2 ± 7.0	0.17
Hypertension - no. (%)	2502 (60.3)	1763 (57.3)	739 (69.2)	**<0.0001**
Dyslipidemia - no. (%)	2381 (57.4)	1759 (57.1)	622 (58.2)	0.53
Diabetes - no. (%)				
Type I	26 (0.6)	16 (0.5)	10 (0.9)	0.14
Type II	1065 (25.7)	756 (24.6)	309 (28.9)	**0.004**
Smoking - no. (%)				**0.0005**
Never	2402 (57.9)	1731 (56.2)	671 (62.8)	
Remote (quit >1 month ago)	920 (22.2)	701 (22.8)	219 (20.5)	
Active	825 (19.9)	647 (21.0)	178 (16.7)	
Previous History - no. (%)				
PCI	745 (18.5)	601 (20.1)	144 (13.9)	**<0.0001**
MI	635 (15.8)	500 (16.7)	135 (13.0)	**0.005**
CABG	210 (5.2)	166 (5.5)	44 (4.2)	0.1
PAD	193 (4.7)	134 (4.4)	59 (5.5)	0.12
CVA	178 (4.3)	122 (4.0)	56 (5.2)	0.08
Heart failure	138 (3.3)	96 (3.1)	42 (3.9)	0.2
Medications - Baseline - no. (%)				
ASA	3767 (90.8)	2779 (90.3)	988 (92.5)	**0.03**
P2Y12	3776 (91.1)	2780 (90.3)	996 (93.3)	**0.003**
ACEi/ARB	1722 (41.5)	1265 (41.1)	457 (42.8)	0.33
B-blocker	1889 (45.6)	1390 (45.1)	499 (46.7)	0.37
Calcium Channel Blocker	403 (9.7)	288 (9.4)	115 (10.8)	0.18
Statin	2888 (69.6)	2136 (69.4)	752 (70.4)	0.52
PPI	492 (11.9)	319 (10.4)	173 (16.2)	**<0.0001**
Investigations – Baseline				
Creatinine – mean +/- SD (mmol/L)	93.7 ± 68.1	96.4 ± 67.1	86.2 ± 70.3	**<0.0001**
CrCl – mL/min – mean +/- SD	91.1 ± 40.2	96.2 ± 39.5	77.0 ± 38.8	**<0.0001**
LVEF [n=1138]				0.06
Normal	813 (71.4)	564 (69.4)	249 (76.6)	
>45%	134 (11.8)	104 (12.8)	30 (9.2)	
30-45%	139 (12.2)	109 (13.4)	30 (9.2)	
<30%	52 (4.6)	36 (4.4)	16 (4.9)	
Mitral valvulopathy (≥moderate)	53 (1.3)	30 (1.0)	23 (2.2)	0.003
Aortic valvulopathy (≥moderate)	108 (2.6)	72 (2.3)	35 (3.4)	0.07
Procedural details				
Indications - no. (%)				
Acute coronary syndrome	2670 (64.4)	1949 (63.3)	721 (67.5)	**0.01**
STEMI	1160 (28.0)	863 (44.3)	297 (41.2)	0.15
NSTEMI/Unstable Angina	1510 (36.4)	1086 (55.7)	424 (58.8)	
Staged PCI	311 (7.5)	242 (7.9)	69 (6.5)	0.13
Stable CAD	1005 (24.2)	757 (24.6)	248 (23.2)	0.37
Shock	53 (1.3)	34 (1.1)	19 (1.8)	0.09
Access - no. (%)				**<0.0001**
Radial	3231 (77.9)	2454 (79.7)	777 (72.8)	
Femoral	910 (21.9)	621 (20.2)	289 (27.1)	

Revascularization method - no. (%)				
PCI	3462 (83.5)	2560 (83.1)	935 (87.6)	**0.001**
CABG	589 (14.2)	437 (14.2)	112 (10.5)	**0.002**
Both	96 (2.3)	51 (1.7)	12 (1.1)	0.22
Medications - Follow-up - no. (%)				
ASA	3393 (81.8)	2579 (83.8)	814 (76.2)	**<0.0001**
P2Y12	2360 (56.9)	1749 (56.8)	611 (57.2)	0.82
ACEi/ARB	2529 (61.0)	1913 (62.1)	61 (57.7)	**0.01**
B-blocker	2678 (64.6)	1998 (64.9)	680 (63.7)	0.47
Calcium Channel Blocker	518 (12.5)	372 (12.1)	146 (13.7)	0.18
Statin	3484 (84.0)	2619 (85.1)	865 (81.0)	**0.002**
DAPT score ≥ 2 - no. (%)	1354 (32.7)	1039 (33.7)	315 (29.5)	**0.01**

BMI - body mass index, PCI - percutaneous coronary intervention, MI - myocardial infarction, CABG - coronary artery bypass graft, PAD - peripheral arterial disease, CVA - cerebrovascular accident, ASA - acetylsalicylic acid, ACEi/ARB - angiotensin-converting enzyme inhibitor/angiotensin-receptor blocker, CAD - coronary artery disease

Clinical outcomes

The median follow-up period for the full cohort was 13.3 months (IQR, 11.9-16.5 months). During this study period, MACE occurred in 11.0%, death in 5.6%, MI in 1.7%, unplanned revascularization in 4.2%, and cerebrovascular accidents in 1.4%. MACE occurred in 3.9% of patients at 30 days. **(Figure 6)**. Females demonstrated higher MACE rates than males driven primarily by greater rates of death (HR 1.9,95%CI 1.46-2.47,p<0.0001). No differences in MACE were observed between patients undergoing PCI or CABG [11.3% versus 9.8%,HR 1.20 (0.92-1.58),p=0.18]. Subgroup analysis of patients who presented as ACS versus stable CAD demonstrated a higher proportion of MACE in the ACS cohort [12.5% versus 7.5%,HR 1.73 (1.34-2.24),p<0.0001] **(Figure 7)**. Patients with three-vessel disease accrued higher MACE rates in follow-up, an effect that remained consistent in both males and females, though with a trend towards females experiencing greater MACE rates in the setting of three-vessel disease **(Figure 8).** Similarly, worsening left ventricular function also portended greater rates of MACE in follow-up **(Figure 9).** Unadjusted and adjusted analysis did not suggest that female sex

was associated with MACE [HR 1.21(0.97-1.52)] or death [HR 1.30(0.96-1.75)] in the year following revascularization **(Table 3).**

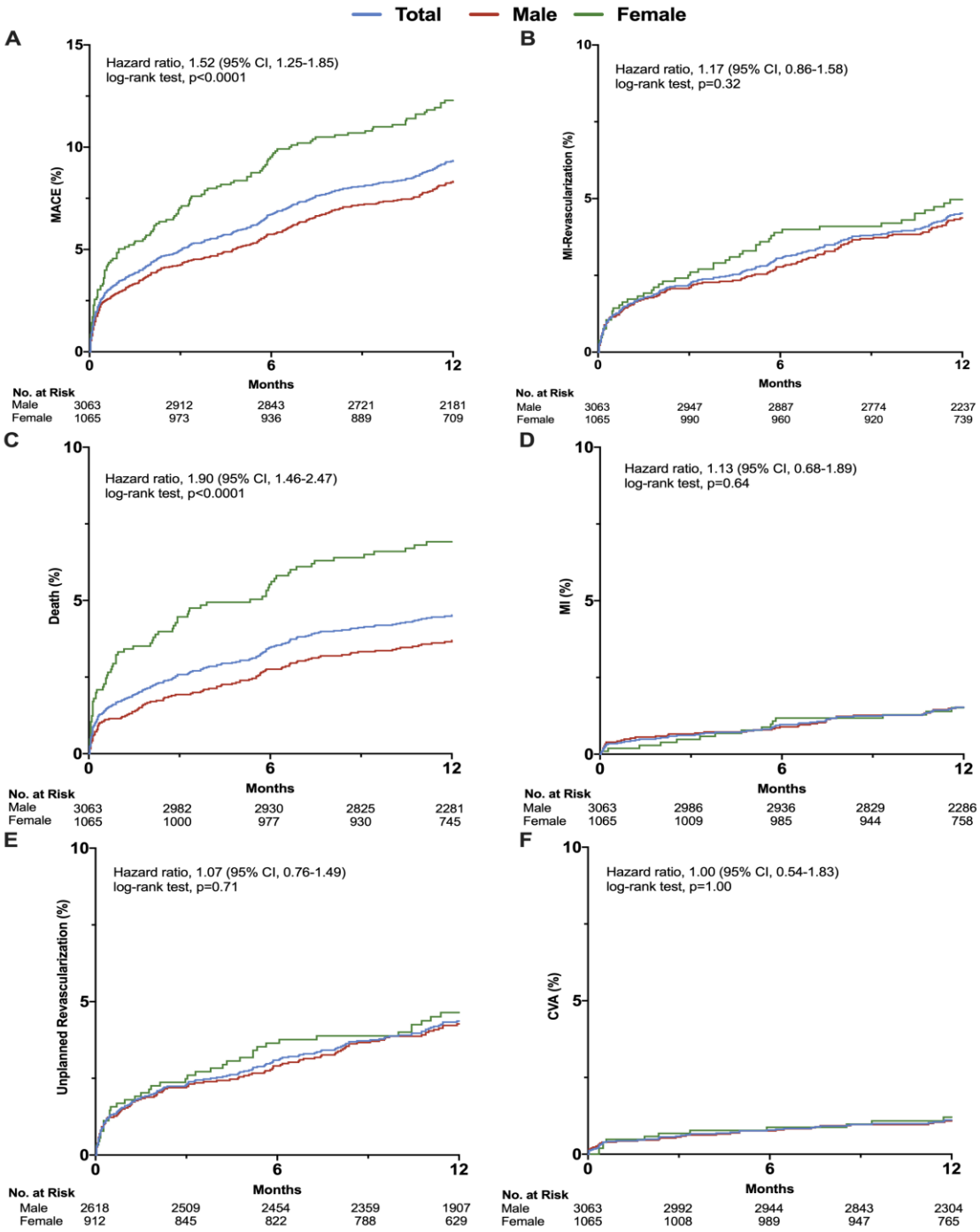

Figure 6. Sex-based cardiovascular outcomes post-revascularization

(A) Cumulative incidence of major adverse cardiac events (myocardial infarction[MI], unplanned revascularization, death, cerebrovascular accident[CVA]). Subsequent panels demonstrating cumulative incidence of individual components including **(B)** MI and unplanned revascularization, **(C)** mortality, **(D)** myocardial infarction, **(E)** unplanned revascularization and **(F)** CVA in the year following revascularization. Total cohort (blue), males (red), females (green) with hazard ratios (HR) and 95% confidence intervals (CI) presented for outcomes of females compared to males.

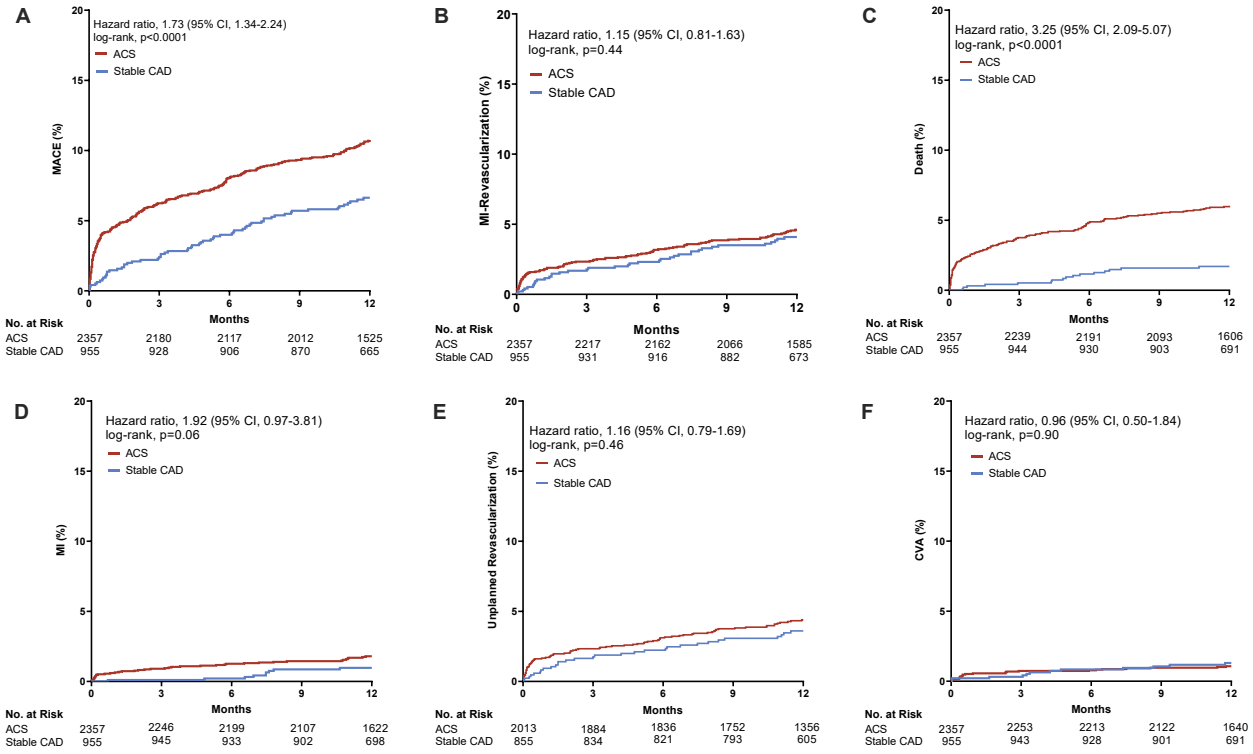

Figure 7. Impact of acute coronary syndrome on clinical outcomes.

ACS patients noted to have elevated rates of MACE (A) and death (C) when compared to stable CAD controls, no differences in MI-revascularization (B), MI (D), revascularization (E), or CVA (F).

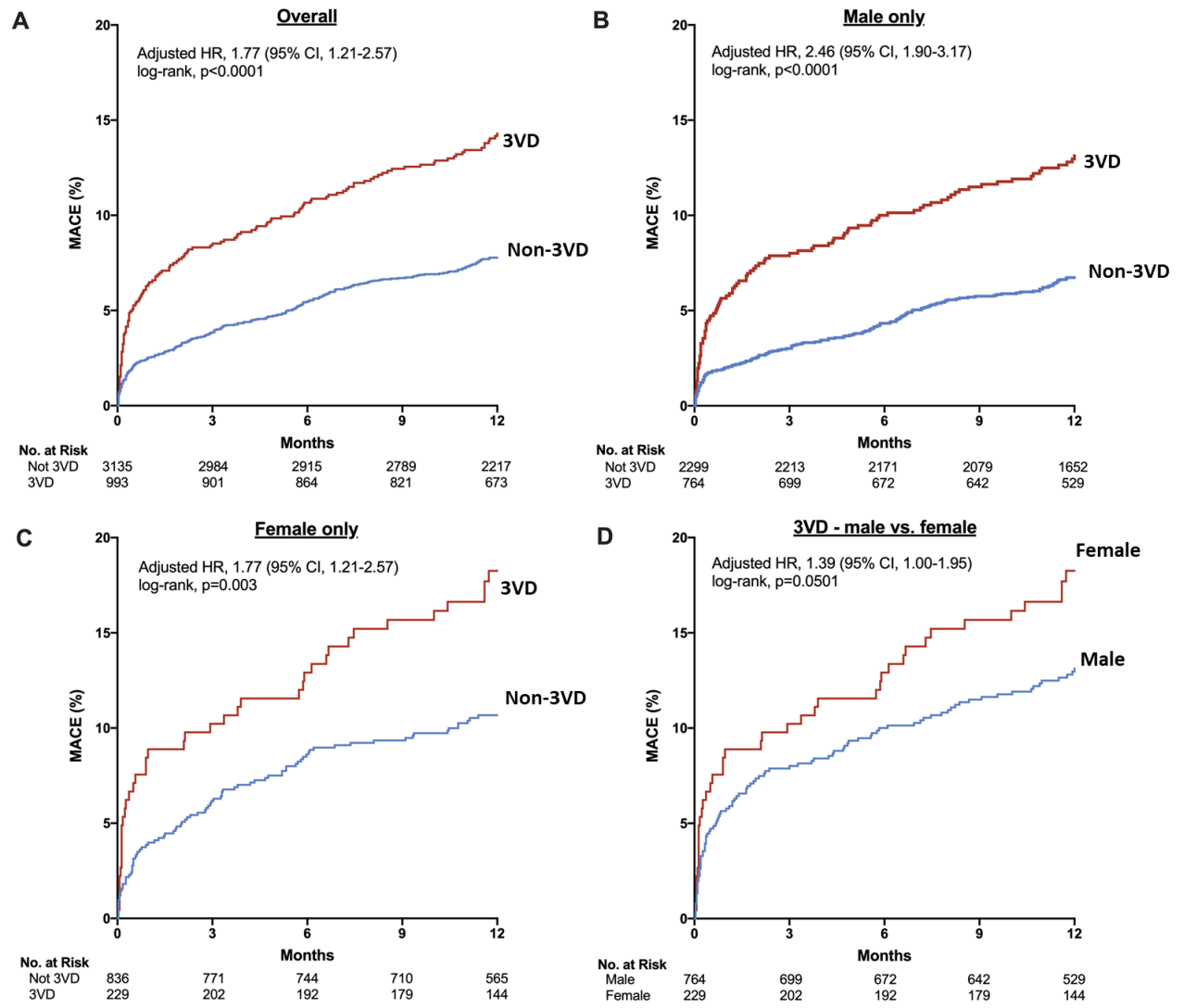

Figure 8. Sex-based impact of coronary artery disease burden on clinical outcomes

(A) Total cohort dichotomized into those with (3VD) or without three-vessel CAD (non-3VD). Sex-based differences of the impact of 3VD vs non-3VD assessed in males **(B)** and females **(C). (D)** When assessing strictly those with 3VD females demonstrate more adverse events than males. MACE – major adverse cardiac events - death, myocardial infarction, cerebrovascular accident, unplanned revascularization. Hazard ratios (HR) and 95% confidence intervals (CI) presented for outcomes of 3VD compared to non-3VD and females compared to males.

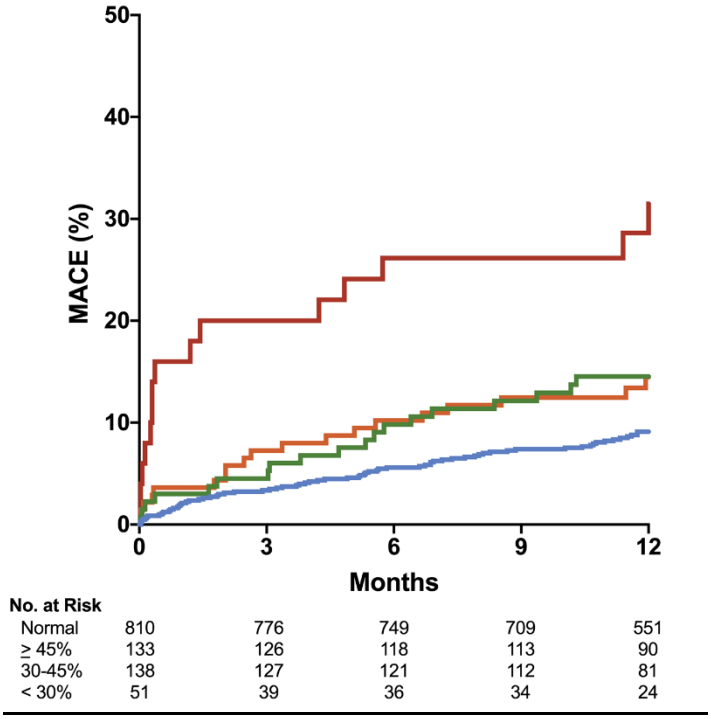

Figure 9. Impact of left ventricular function on clinical outcomes

Clinical outcomes stratified by left ventricular function on presentation (<30% -red, 30-45%-orange, ≥45%-orange**,** ≥55%-blue) demonstrating deteriorating clinical outcomes with worsening LV function. MACE – major adverse cardiac events - death, myocardial infarction, cerebrovascular accident, unplanned revascularization.

Table 3. Clinical outcomes associated with gender

	Unadjusted HR (95% CI)	**Adjusted HR (95% CI)***
MACE	1.52 (1.25-1.85)	1.21 (0.97-1.52)
Death	1.90 (1.46-2.47)	1.30 (0.96-1.75)

Reference for gender is male

*Adjusted for age, acute coronary syndrome, type 2 diabetes, active smoking, dyslipidemia, hypertension, congestive heart failure, and obesity

Risk factor management

HbA1c was available in 745 (68.3%) patients with DM at one-year follow-up with 410 patients (55.0%) achieving adequate glycemic control (HbA1c≤7.0%). Of the 335 patients with DM who failed to achieve target HbA1c at 1 year (45.0%), 14(1.9%) had type I diabetes, 202(27.1%) type II diabetes (non-insulin-dependent) patients, and 119 (16%) had type II diabetes (insulin-dependent) patients **(Figure 10a**). Smoking status was assessed and documented in 4,004 patients, of which 3,574 patients (89.3%) were not smoking at the time of clinical follow-up. Among the non-smokers, 1,889 patients (47.2%) were lifelong non-smokers and 1,286 patients (32.1%) quit prior to the index procedure. Among active smokers at the time of angiography, 45.2% had quit smoking in follow-up, while 54.8% continued to smoke (24.8% having quit but relapsed, 30.0% having never quit). **(Figure 10b)**. Lipid levels were available in 1,955 patients (47.1%). A total of 1,425 patients (72.9%) achieved an LDL≤1.8 mmol/L, 137 patients (7.0%) had an LDL level between 1.8-2.0mmol/L, and 393 patients (20.1%) had an LDL>2.0mmol/L **(Figure 10c)**. In follow-up, 84% of all patients were on a statin with females being less likely than males to be on a statin (81% vs 85%, p=0.002) **(Table 2)**

Table 2. Baseline characteristics

	Total (n=4147)	**Male (n=3079)**	**Female (n=1068)**	**P-value**
Age - years, mean ± SD	65.8 ± 11.8	64.5 ± 11.5	69.5 ± 11.8	**<0.0001**
Sex (female) - no. (%)	1068 (25.8)	-	-	-
BMI - kg/m^2, mean ± SD	29.0 ± 5.7	28.9 ± 5.2	29.2 ± 7.0	0.17
Hypertension - no. (%)	2502 (60.3)	1763 (57.3)	739 (69.2)	**<0.0001**
Dyslipidemia - no. (%)	2381 (57.4)	1759 (57.1)	622 (58.2)	0.53
Diabetes - no. (%)				
Type I	26 (0.6)	16 (0.5)	10 (0.9)	0.14
Type II	1065 (25.7)	756 (24.6)	309 (28.9)	**0.004**
Smoking - no. (%)				**0.0005**
Never	2402 (57.9)	1731 (56.2)	671 (62.8)	
Remote (quit >1 month ago)	920 (22.2)	701 (22.8)	219 (20.5)	
Active	825 (19.9)	647 (21.0)	178 (16.7)	
Previous History - no. (%)				
PCI	745 (18.5)	601 (20.1)	144 (13.9)	**<0.0001**
MI	635 (15.8)	500 (16.7)	135 (13.0)	**0.005**
CABG	210 (5.2)	166 (5.5)	44 (4.2)	0.1
PAD	193 (4.7)	134 (4.4)	59 (5.5)	0.12
CVA	178 (4.3)	122 (4.0)	56 (5.2)	0.08
Heart failure	138 (3.3)	96 (3.1)	42 (3.9)	0.2
Medications - Baseline - no. (%)				
ASA	3767 (90.8)	2779 (90.3)	988 (92.5)	**0.03**
P2Y12	3776 (91.1)	2780 (90.3)	996 (93.3)	**0.003**
ACEi/ARB	1722 (41.5)	1265 (41.1)	457 (42.8)	0.33
B-blocker	1889 (45.6)	1390 (45.1)	499 (46.7)	0.37
Calcium Channel Blocker	403 (9.7)	288 (9.4)	115 (10.8)	0.18
Statin	2888 (69.6)	2136 (69.4)	752 (70.4)	0.52
PPI	492 (11.9)	319 (10.4)	173 (16.2)	**<0.0001**
Investigations – Baseline				
Creatinine – mean +/- SD (mmol/L)	93.7 ± 68.1	96.4 ± 67.1	86.2 ± 70.3	**<0.0001**
CrCl – mL/min – mean +/- SD	91.1 ± 40.2	96.2 ± 39.5	77.0 ± 38.8	**<0.0001**
LVEF [n=1138]				0.06
Normal	813 (71.4)	564 (69.4)	249 (76.6)	
>45%	134 (11.8)	104 (12.8)	30 (9.2)	
30-45%	139 (12.2)	109 (13.4)	30 (9.2)	
<30%	52 (4.6)	36 (4.4)	16 (4.9)	
Mitral valvulopathy (≥moderate)	53 (1.3)	30 (1.0)	23 (2.2)	0.003
Aortic valvulopathy (≥moderate)	108 (2.6)	72 (2.3)	35 (3.4)	0.07
Procedural details				
Indications - no. (%)				
Acute coronary syndrome	2670 (64.4)	1949 (63.3)	721 (67.5)	**0.01**
STEMI	1160 (28.0)	863 (44.3)	297 (41.2)	0.15
NSTEMI/Unstable Angina	1510 (36.4)	1086 (55.7)	424 (58.8)	
Staged PCI	311 (7.5)	242 (7.9)	69 (6.5)	0.13
Stable CAD	1005 (24.2)	757 (24.6)	248 (23.2)	0.37
Shock	53 (1.3)	34 (1.1)	19 (1.8)	0.09
Access - no. (%)				**<0.0001**
Radial	3231 (77.9)	2454 (79.7)	777 (72.8)	
Femoral	910 (21.9)	621 (20.2)	289 (27.1)	

Revascularization method - no. (%)				
PCI	3462 (83.5)	2560 (83.1)	935 (87.6)	**0.001**
CABG	589 (14.2)	437 (14.2)	112 (10.5)	**0.002**
Both	96 (2.3)	51 (1.7)	12 (1.1)	0.22
Medications - Follow-up - no. (%)				
ASA	3393 (81.8)	2579 (83.8)	814 (76.2)	**<0.0001**
P2Y12	2360 (56.9)	1749 (56.8)	611 (57.2)	0.82
ACEi/ARB	2529 (61.0)	1913 (62.1)	61 (57.7)	**0.01**
B-blocker	2678 (64.6)	1998 (64.9)	680 (63.7)	0.47
Calcium Channel Blocker	518 (12.5)	372 (12.1)	146 (13.7)	0.18
Statin	3484 (84.0)	2619 (85.1)	865 (81.0)	**0.002**
DAPT score ≥ 2 - no. (%)	1354 (32.7)	1039 (33.7)	315 (29.5)	**0.01**

BMI - body mass index, PCI - percutaneous coronary intervention, MI - myocardial infarction, CABG - coronary artery bypass graft, PAD - peripheral arterial disease, CVA - cerebrovascular accident, ASA - acetylsalicylic acid, ACEi/ARB - angiotensin-converting enzyme inhibitor/angiotensin-receptor blocker, CAD - coronary artery disease

In those failing to achieve LDL targets, 5% were not on a statin. BMI was recorded in 3,484 patients at the time of the index procedure and at one-year in 2,762 patients. 2,145 patients (77.7%) were overweight/obese, 594 patients (21.5%) were normal weight, and 23 patients (0.8%) were underweight. Weight loss >10% was achieved at follow-up in 175 patients (6.3%) who were overweight/obese and in 31 patients (1.1%) with normal weight **(Figure 10d)**. Females were less likely to achieve target LDL and smoking cessation in follow-up compared to males **(Figure 11)**.

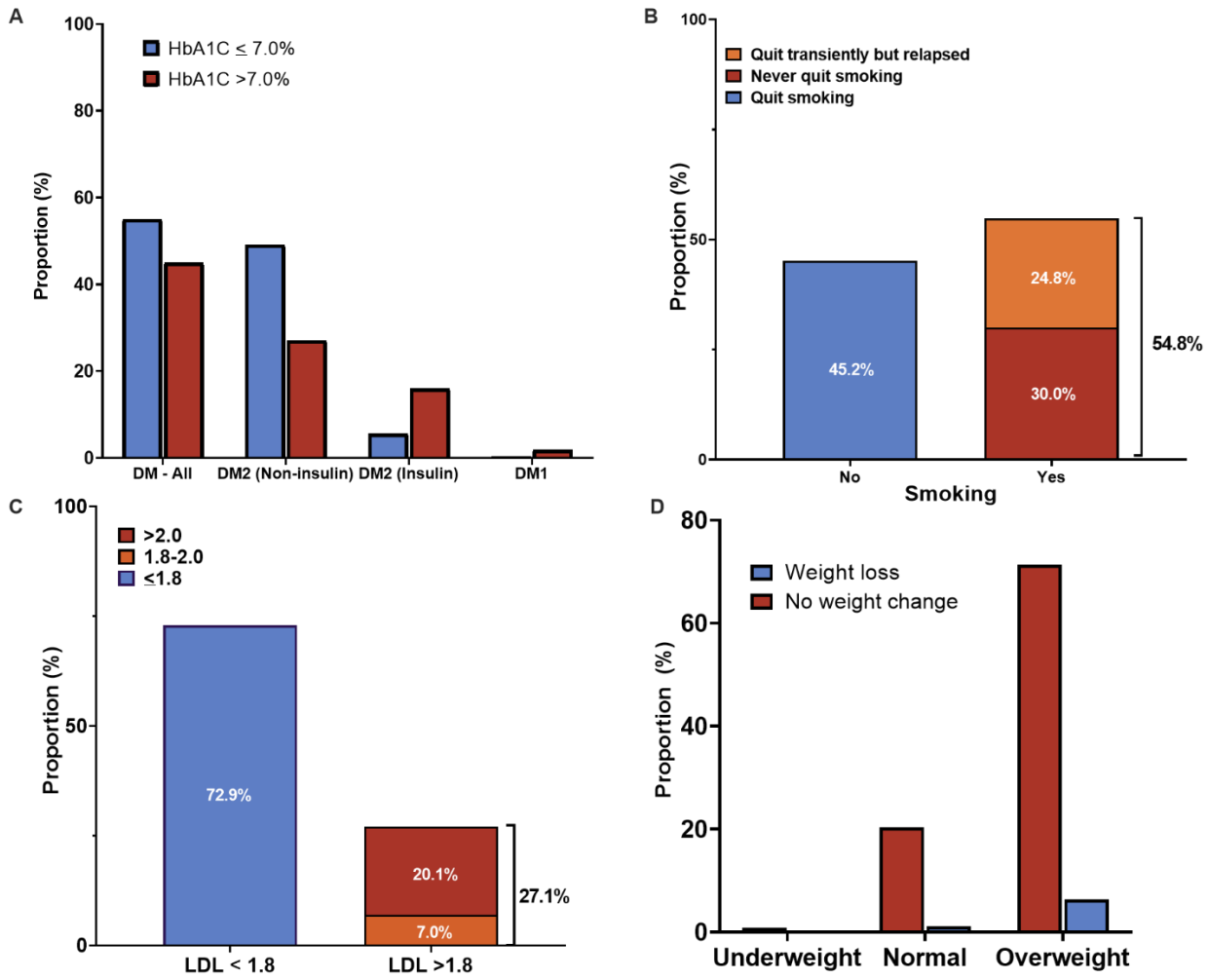

Figure 10. Risk factor management at one year

(A) Glycemic control defined as HbA1c ≤ 7.0% was achieved in 55.0% patients. Of the 335 patients with DM who failed to achieve target HbA1c at 1 year (45.0%), 14(1.9%) had type I diabetes, 202(27.1%) type II diabetes (non-insulin-dependent) patients, and 119 (16%) had type II diabetes (insulin-dependent) patients **(B)** Baseline active smokers were assessed at the time of follow-up for smoking cessation. 45.2% had quit smoking in follow-up, while 54.8% continue to smoke (24.8% having quit but relapsed, 30.0% having never quit). **(C)** Lipid control was defined as LDL ≤ 1.8 mmol/L, 137 patients (7.0%) had an LDL level between 1.8-2.0 mmol/L, and 393 patients (20.1%) had an LDL > 2.0 mmol/L. **(D)** Weight loss >10% was achieved at follow-up in 175 patients (6.3%) who were overweight/obese and in 31 patients (1.1%) with normal weight.

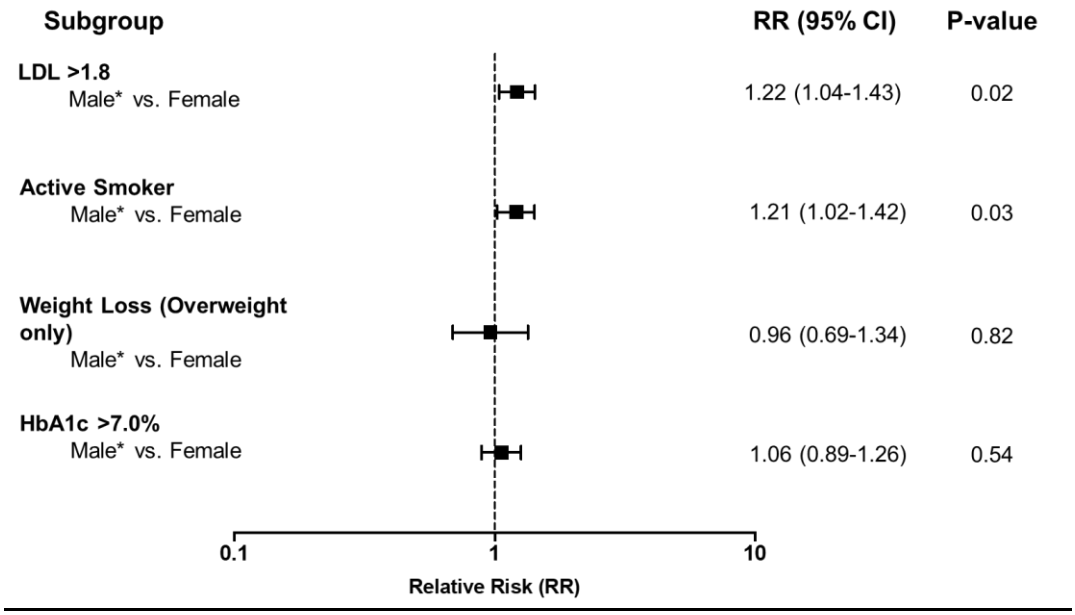

Figure 11. Sex-based differences in optimal risk factor management

Subgroup analysis of risk factor control stratified by sex. Unadjusted relative risk of respective risk factor management with 95% confidence interval are shown above. Each subgroup was dichotomized and the reference group is denoted with an asterisk. P-value less than 0.05 was considered statistically significant. LDL – low density lipoprotein.

Modifiable risk factor burden

47.7% of patients had ≥2 modifiable risk factors identified at one-year follow-up **(Figure 12a)**. Associations between risk factors were noted with overweight patients more likely to have DM (OR 1.56; 95% CI, 1.26-1.94) and to have LDL ≥1.8 mmol/L (OR 1.34; 95% CI, 1.08-1.68), whereas active smokers were more likely to have LDL ≥ 1.8 mmol/L (OR 1.46; 95% CI, 1.21-1.75) at one year **(Figure 12b-e)**. Subgroup analysis of individual risk factors demonstrated that patients with DM had markedly elevated rates of MI-revascularization at one year (HR, 1.84; 95% CI, 1.40 to 2.42; p<0.0001**, Figure 13).**

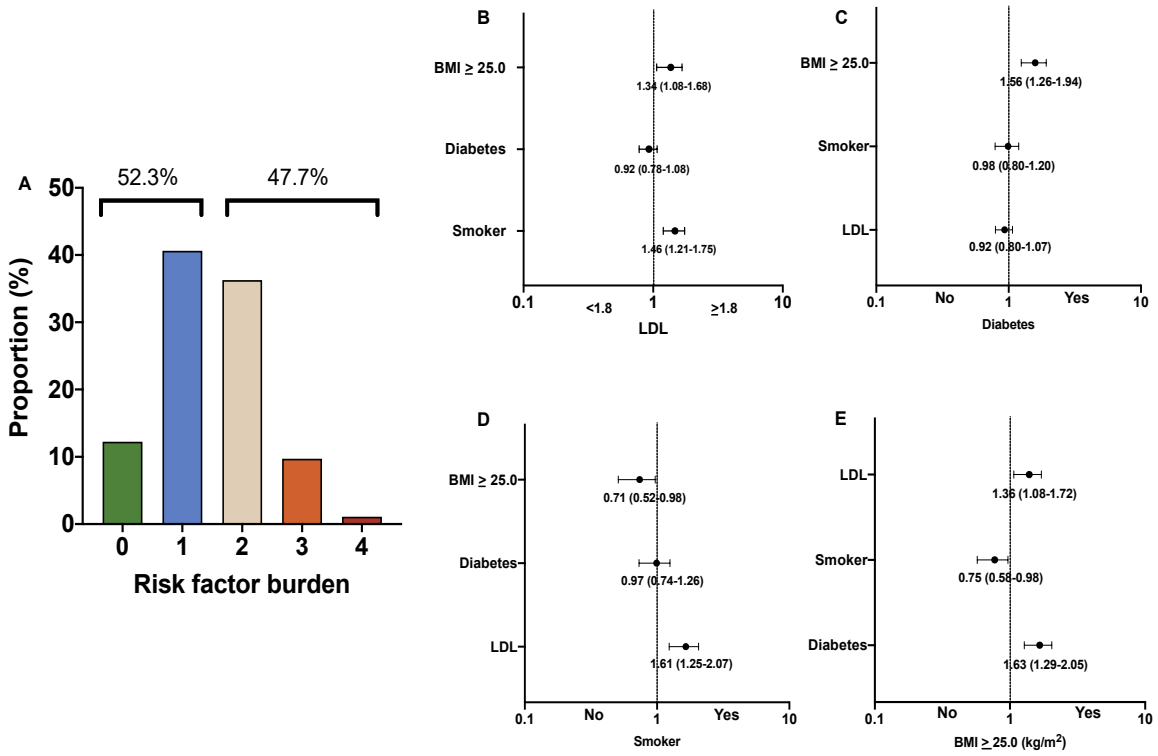

Figure 12. Modifiable risk factor burden

(A) Burden of modifiable risk factors displayed on a patient-level and dichotomized to those patients with ≥2 (47. 7%) or <2 (52.3%) risk factors. (B-E) Interactions between risk factors were noted with overweight patients being more likely to have diabetes (odds ratio, 1.56; 95% CI, 1.26-1.94) while both overweight patients (odds ratio, 1.34; 95% CI, 1.08-1.68) and smokers (odds ratio, 1.46; 95% CI, 1.21-1.75) were more likely to have LDL ≥1.8 mmol/L.

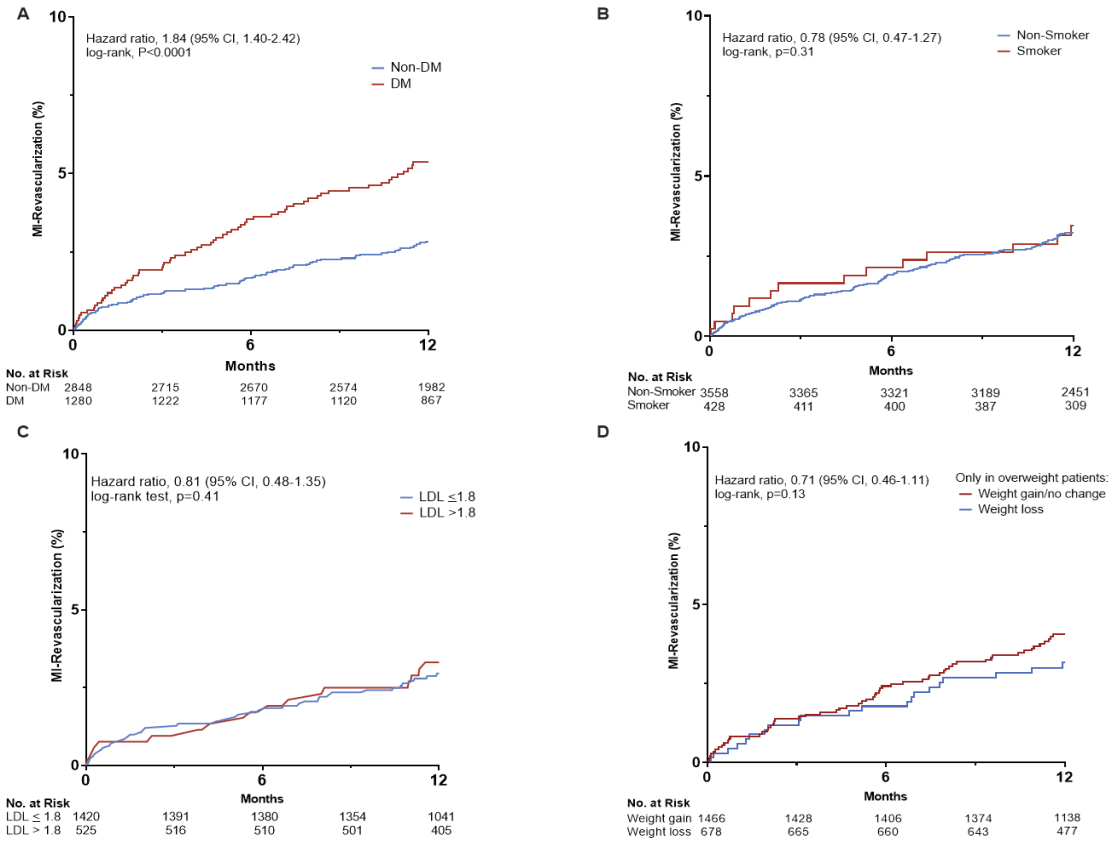

Figure 13. Association of modifiable risk factors with clinical outcomes.

(A) Patients with diabetes had elevated rates of myocardial infarction and unplanned revascularization (MI-revascularization) (hazard ratio, 1.84; 95% CI, 1.40 to 2.42; p<0.0001). **(B)** Active smoking was not associated with increased rates of MI-revascularization (hazard ratio, 0.78; 95% CI, 0.47 to 1.27; p=0.31). **(C)** LDL control ≤1.8 mmol/L was not associated with MI-revascularization (hazard ratio, 0.81; 95% CI, 0.48 to 1.35; p=0.41). **(D)** Weight loss in overweight patients was not associated with MI-revascularization (hazard ratio, 0.71; 95% CI, 0.46 to 1.11; p=0.13). Kaplan-Meier curves were generated and compared by log-rank test and hazard ratios were evaluated using the Cox proportional hazards model. P<0.05 is considered statistically significant.

2.6 Discussion

We established a post-revascularization program to standardize clinical follow-up of patients in the year following revascularization, with the goal of optimizing risk factor management and implementing secondary prevention strategies. Our real-world experience demonstrates several important points. First, significant residual risk exists in this cohort with nearly 1 in 10 patients experiencing MACE in the first 12 months, with females being disproportionately at risk. Second, there is a high prevalence of uncontrolled risk factors, with a quarter not achieving target LDL, half of smokers continuing to smoke, and half of patients with DM not achieving target HbA1c. Finally, risk factor clustering is common, with half of patients having ≥2 factors **(Figure 14)**. Overall, these data highlight that the post-revascularization patient is at high risk for adverse events with a significant number of patients failing to achieve optimal risk factor management during their highest risk period.

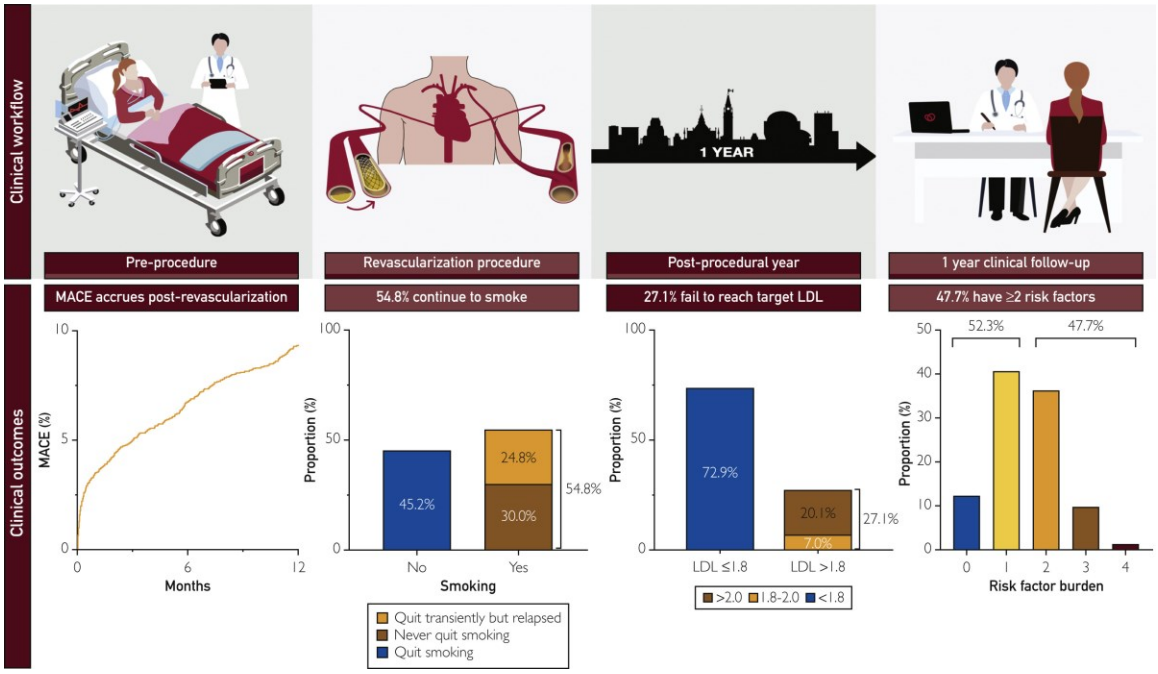

Figure 14. Central Illustration

Clinical workflow of post-revascularization clinic including pre-procedure, revascularization procedures, post-procedural year and one-year clinical follow-up. Summarized clinical outcomes over that time including continual accruing MACE, half of smokers failing to quit and a quarter failing to reach target lipid levels, while half of patients have $\geq$ 2 cardiovascular risk factors.

Established cardiovascular risk factors are known to increase the risk of adverse events, thus motivating targeted interventions.(257) Unfortunately, however, risk factor burden often does not translate into behavioral changes, including among patients at highest cardiac risk.(294) Cardiac rehabilitation with focused risk factor management programs have made considerable strides in this regard.(295) Similarly, dedicated diabetes management programs can enhance glycemic control, translating to improved cardiovascular outcomes.(296,297) Smoking cessation programs can be similarly impactful.(298) Our center previously developed the Ottawa Model for Smoking Cessation, which has yielded cessation rates of up to 44-61% at 6 months and improved

outcomes.(278,279,299) Yet, despite these and other programs being in place at our center, in our study, many patients failed to adequately modify their cardiovascular risk factors. This includes the inability to achieve target HbA1c, weight loss or smoking cessation in follow-up, in part highlighting the difficult nature of modifying patient behaviour.(294) Healthcare providers may also be contributing to suboptimal risk factor control by undertreating certain patients. For instance, lipid control has been well established as a strategy to reduce cardiovascular risk(300,301), with current guidelines suggesting maximal dose statin therapy and treating to an LDL target of 1.8-2.0mmol/L, depending in part on the clinical presentation.(289-291) In our cohort, a quarter of patients failed to achieve guideline-directed targets (7% with LDL 1.8-2.0 and 20.1% with LDL >2.0), including 5% of whom were not on any statin therapy at follow-up. Females were disproportionately affected, being less likely to achieve target LDL and be on statin therapy, in keeping with previous reports.(302) Taken together, these findings highlight potential gaps in cardiovascular risk reduction in routine post-revascularization care.

Accurately predicting the risk of adverse events post-revascularization is important for guiding targeted follow-up and therapies. Patients remain at high risk of adverse events post-revascularization with females being disproportionately afflicted by elevated risk of death and MACE.(303) The precise etiology for these disparities remains unclear with some postulating that despite presenting with less extensive disease (i.e. non-three vessel disease), females may carry a more aggressive CAD phenotype.(303) Without a doubt, the importance of monitoring for sex-specific differences remains and cannot be

understated, similar to other cardiovascular interventions.(304) Despite this, cardiovascular trials continue to report declining rates of female enrollment, (305,306) prompting calls for improved methodological rigor(71) with strategies including standardized checklists(307) to improve sex-specific outcome reporting.

Indeed, the additive impact of cumulative risk factors has been previously discussed.(308) In our study, risk factor clustering was observed. The significance of this phenomenon is clear when one considers that patients with DM are known to have elevated cardiovascular risk(309), but when combined with additional risk factors, their risk of mortality doubles.(310) In our study, we demonstrate that half of patients have two or more risk factors and may influence one another. For instance, overweight individuals were more likely to have DM and were less likely to achieve target LDL. Smokers were similarly less likely to achieve target LDL than non-smokers. Some of these associations may be physiologic in nature whereas others may reflect underlying behavioral tendencies. Regardless, identifying high risk patients, particularly those in whom intervention may be beneficial, could improve post-revascularization care and focus efforts and resources on efforts with greatest likelihood of impact.

Limitations

Our data are subject to selection bias, including survival bias, in that the risk factors of patients who were lost to follow-up or who died prior to their planned follow-up are unknown. However, patients who return for follow-up are likely to be more adherent with medical therapy and have improved risk factor management. Due to selection and

survival bias, our study potentially overestimates the effectiveness of current post-revascularization care strategies of secondary prevention. Our report is limited to the first year post-revascularization, while known to be the highest risk period in this patient population, long term insights are limited. While our established cardiac rehabilitation program is offered to all revascularization patients, a detailed assessment of this was beyond the scope of this study. Therefore, our findings highlight important areas for potential improvement in patient care post-revascularization. Heightened and focused efforts on early and sustained cardiovascular risk reduction in this patient population are warranted.

2.7 Conclusion

Patients who have undergone coronary revascularization are at high risk of MACE and often have sub-optimally managed modifiable risk factors at one-year post-procedure. Targeted efforts to identify this subset of patients and to effectively reduce their risk of future cardiovascular events should be prioritized. Optimal follow-up pathways must be established to maximize the clinical benefits of revascularization.

2.8 Acknowledgements

The authors gratefully acknowledge the efforts of Rebecca Ganz, Rebecca Visser, Caroline Lachance and Cathy Lacey to ensuring this program's success as well as the artistic contributions of Micaela Labinaz to this work.

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2.10 Conflicts of Interest

None applicable

Chapter 3

Evaluation of Plasma Adenosine as a Marker of Cardiovascular Risk: Analytical and Biological Considerations

3.1 Preface

This chapter has been previously published in the *Journal of the American Heart Association*

> **Simard T,** Jung R, Labinaz A, Faraz MA, Ramirez FD, Di Santo P, Perry-Nguyen D, Pitcher I, Motazedian P, Gaudet C, Rochman R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo JJ, Couture E, Promislow S, Beanlands RS, Hibbert B. Evaluation of Plasma Adenosine as a Marker of Cardiovascular Risk: Analytical and Biological Considerations. J Am Heart Assoc. 2019 Aug 6;8(15):e012228. doi: 10.1161/JAHA.119.012228. Epub 2019 Aug 5. PMID: 31379241; PMCID: PMC6761640.
>
> *All authors made substantial contributions to the conception/ design of the work; and/or the acquisition, analysis, or data interpretation; and, (ii) drafted and/or revised the content critically; and, (iii) provided final approval of the published version; and, (iv) agree to be accountable for the work.*

3.2 Abstract

Background:

Adenosine is a ubiquitous regulatory molecule known to modulate signaling in many cells and processes vital to vascular homeostasis. While studies of adenosine receptors have dominated research in the field, quantification of adenosine systemically and locally remains limited owing largely to technical restrictions. Given the potential clinical implications of adenosine biology, there is a need for adequately powered studies examining the role of plasma adenosine in vascular health. We sought to describe the analytical and biological factors that affect quantification of adenosine in humans in a

large, real-world cohort of patients undergoing evaluation for coronary artery disease (CAD).

Methods and results:

Between November 2016 and April 2018, we assessed 1,141 patients undergoing angiography for evaluation of CAD. High-performance liquid chromatography was employed for quantification of plasma adenosine concentration (PAC), yielding an analytical coefficient of variance (CVa) of 3.2%, intra-subject variance (CVi) 35.8% and inter-subject variance (CVg) 56.7%. Traditional cardiovascular risk factors, medications and presentation had no significant impact on adenosine levels. Conversely, increasing age ($p=0.02$) and the presence of obstructive coronary artery disease ($p=0.026$) were associated with lower adenosine levels. Adjusted multivariable analysis supported only age being inversely associated with adenosine levels $p=0.039$).

Conclusions:

Plasma adenosine is not significantly impacted by traditional cardiovascular risk factors; however, advancing age and presence of obstructive CAD may be associated with lower adenosine levels. The degree of intra- and inter-subject variance of adenosine has important implications for biomarker use as a prognosticator of cardiovascular outcomes and as an endpoint in clinical studies.

3.3 Introduction

Adenosine is a purine nucleoside that serves as a crucial intracellular and extracellular regulatory molecule regulating numerous blood and vascular cell types.(1,311) The metabolism of adenosine is regulated by a close balance of production, transport

(primarily via equilibrative nucleoside transporters –ENTs) and degradation (primarily via adenosine deaminase – ADA).(3,4,8) Adenosine circulating in the extracellular space signals primarily via P1 purinergic receptors, G-protein-coupled receptors (GPCRs) with differential responses to adenosine depending on which of the 4-subtypes of adenosine receptors (ADOR) are stimulated – ADOR-A1, ADOR-A2A, ADOR-A2B, and ADOR-A3.(20) Numerous preclinical studies have suggested adenosine regulates vascular homeostasis, with regulatory implications for inflammatory cells, smooth muscle cells, endothelial cells and platelets.(30-32,39,244,312) However, in humans little is known about variance of plasma adenosine concentration (PAC) or factors which influence PAC owing to technical challenges in quantifying levels in large cohorts of patients.

Therapeutically, adenosine's clinical applications have been relatively focused. Intravenous adenosine boluses are predominantly used as a diagnostic and therapeutic agent in the management of tachyarrythmias. Secondarily, adenosine and agents that augment PAC have been used for induction of coronary hyperemia for flow-related non-invasive and invasive assessment of myocardial perfusion.(91) Dipyridamole, acting primarily via ENT inhibition to augment adenosine levels, is more broadly employed for its flow-mediated effects and less commonly as an anti-platelet agent.(113) To minimize off target effects, small molecule agents have been developed to target specific adenosine receptors, such as regadenoson (an ADORA2A specific agonist) for maximizing coronary vasodilation.(98) Nonetheless, despite promising preclinical studies, a translational gap exists whereby the therapeutic application of adenosine modulation has

been hampered by complex receptor biology and a limited understanding of adenosine levels in human disease pathogenesis.

Clinically, the measurement of circulating adenosine has seen limited use - though some studies have either reported prognostic significance in small cohorts or used PAC as an endpoint in clinical trials.(149,313) Quantification of PAC by high-performance liquid chromatography (HPLC) is an established methodology with reported analytical variability (CVa) ranging from 6-7%(314) and up to 10%(315) previously, with more contemporary assays yielding CVs of 1-3%, in keeping with clinical assay standards.(316) With these protocols, some small studies (n=10) have demonstrated reduced local circulating adenosine levels via coronary sampling immediately following balloon angioplasty for coronary artery disease (CAD).(247) Others report elevated PAC in patients (n =71) with chronic congestive heart failure (CHF), proposing it provides protective effects from rising norepinephrine levels.(229) Interestingly, genetic studies in patients with adenosine monophosphate deaminase locus 1 (AMPD1) mutations (putatively augmenting adenosine) demonstrate improved survival in CHF patients.(230,231) AMPD1 carriers have also demonstrated improved cardiovascular survival in those with angiographically documented CAD(243), though this did not hold true for patients post-revascularization.(248) While all of these studies invoke an adenosine-mediated mechanism, definitive links between atherosclerotic risk factors, disease burden and adenosine levels have yet to be established.(311)

Given the prognostic and therapeutic implications of adenosine levels and the lack of robust human data, we set out to systematically evaluate the analytic characteristics of PAC quantification and determine if traditional cardiac risk factors, cardiac therapies, and/or disease burden are associated with PAC.

3.4 Methods

Adenosine sample collection and processing:

Blood samples were collected at the time of angiography via a 6-French plastic arterial access sheath (Terumo Medical, Somerset, New Jersey, USA) placed in the radial artery. Rarely, if this was not possible, then venous samples were collected via peripheral venipuncture. Blood samples (6mL) were collected in Greiner BioOne Vacuette tubes pre-injected with 2mL of ice-cold stop solution. Stop solution was composed of 100uM dipyridamole, 2.5uM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), 1U/mL heparin in 0.9% saline. Tubes were inverted and connected to the access sheath to ensure rapid and direct mixing of blood with stop solution and were maintained on ice prior to and following draws until processing. Samples were centrifuged at 4^0C, 1200g for 10 minutes without brakes to limit platelet activation and the supernatant collected and stored at -80^0C until processing. Hemolyzed samples (which result in markedly elevated PAC levels) were excluded on a biological basis in keeping with prior studies.(314) The data that support the findings of this study are available from the corresponding author upon reasonable request.

Aliquots were then thawed at room temperature and centrifuged at 1000g for 3 minutes and 500uL of sample was diluted in 500uL of 4% phosphoric acid. This 1mL combined solution was then loaded onto a Waters Oasis MCX (Mixed-mode, strong Cation-eXchange) 1mL cartridge and the sample was eluted with vacuum assistance through the column as per protocol. The sample was then washed with 1mL of 2% formic acid followed by 500uL of 100% methanol with vacuum assistance between each wash. The final sample was then eluted using two sequential 125uL elutions with MCX eluting solution (5%NH4OH in 60/40 acetonitrile/methanol) followed by vacuum assistance to ensure all sample was collected from the vial. Samples were then transferred to vials to undergo HPLC analysis. Adenosine standards were prepared using pharmaceutical grade adenosine (Sigma PHR11380-1G) diluted in MCX eluting solution.

Samples were analyzed using HPLC on a Waters Alliance E2695 separating module system with sample quantification by Waters 2489 UV/visible detector at 260nM. The mobile phase was composed of a mixture of Mobile Phase A (10uM ammonium formate pH3 in 50% acetonitrile:50% water, ranging 1-50%) and Mobile Phase B (10µM Ammonium Formate pH3 in 95% Acetonitrile:5% Water, ranging 50-99%) with a sample temperature of 4^0C and column temperature 24^0C. Samples were then processed through a Waters Xbridge BEH amide SP Vanguard Cartridge pre-column and subsequent Waters Xbridge BEH Amide 2.5um, 4.6x150mm column XP. Data processing was completed using Waters Empower 3 Software.

Assay validation for plasma adenosine quantification

Quantification of variance is achieved via relative standard deviation (RSD) and the coefficient of variation (CV). Both RSD and CV are percentages representing the standard deviation (SD) divided by the mean value to standardize the variability for a given result. RSD is an absolute value, while CV is not. We report CV in keeping with prior studies.(317) The CV is assessed at multiple stages of our assay and defined accordingly (i) CVa – CV analytical – the variation of the HPLC assay itself including the processing and analysis of samples (generated via multiple aliquots obtained from a single tube drawn from a single patient), (ii) CVi – CV individual – the intra-subject variation over time generated from serial samples from the same patient collected on different days via $CV_i = (CV_t^2 – CV_a^2)^{1/2}$ and (iii) CVg – the inter-subject variation within the population of subjects studied (generated from different samples collected from different patients at different times).(318,319) The reference change value (RCV) was calculated via $2.77 (CV_a^2 + CV_i^2)^{1/2}$, while the index of individuality (II) was calculated by $(CV_a^2 + CV_i^2)^{1/2} / CV_g$, in keeping with prior reports.(283,318-320) The validation of our HPLC methodology followed good practice guidelines as published previously.(321) Specificity of the assay was maximized by adjusting the gradients and temperatures until adequate separation of the adenosine peak of interest was achieved from the surrounding peaks. The specific identity of the adenosine peak was confirmed by focused degradation of adenosine by ADA followed by quantification to demonstrate loss of the adenosine peak (**Figure 15a**). Repeatability was assessed in both the standards and samples to determine the intra-day assay precision utilizing the same conditions. Standards in eluting solution were injected 10 sequential times from the same vial, while samples prepared

with the MCX system were injected 6 sequential times from the same vial. Both the retention times and peak areas were recorded for each run and a mean, SD and CV reported **(Table 4)** Linearity and range were assessed by creating three individual sample preparations of standards ranging in concentration from 100nM to 15,000nM representing 10%-1500% of the target concentration of adenosine (1000nM) **(Figure 15b, Table 5)** These individual samples were run on the same machine on the same day to generate the appropriate curves from which the retention time and peak areas were recorded across each individual preparation with means, standard deviation (SD) and CV then calculated (**Table 5**). The adenosine standard curve **(Figure 15b)** was then assessed for linearity over a range from 100nM-15,000nM adenosine concentrations in both elution buffer and matrix (adenosine-depleted plasma generated via ADA degradation of endogenous adenosine).

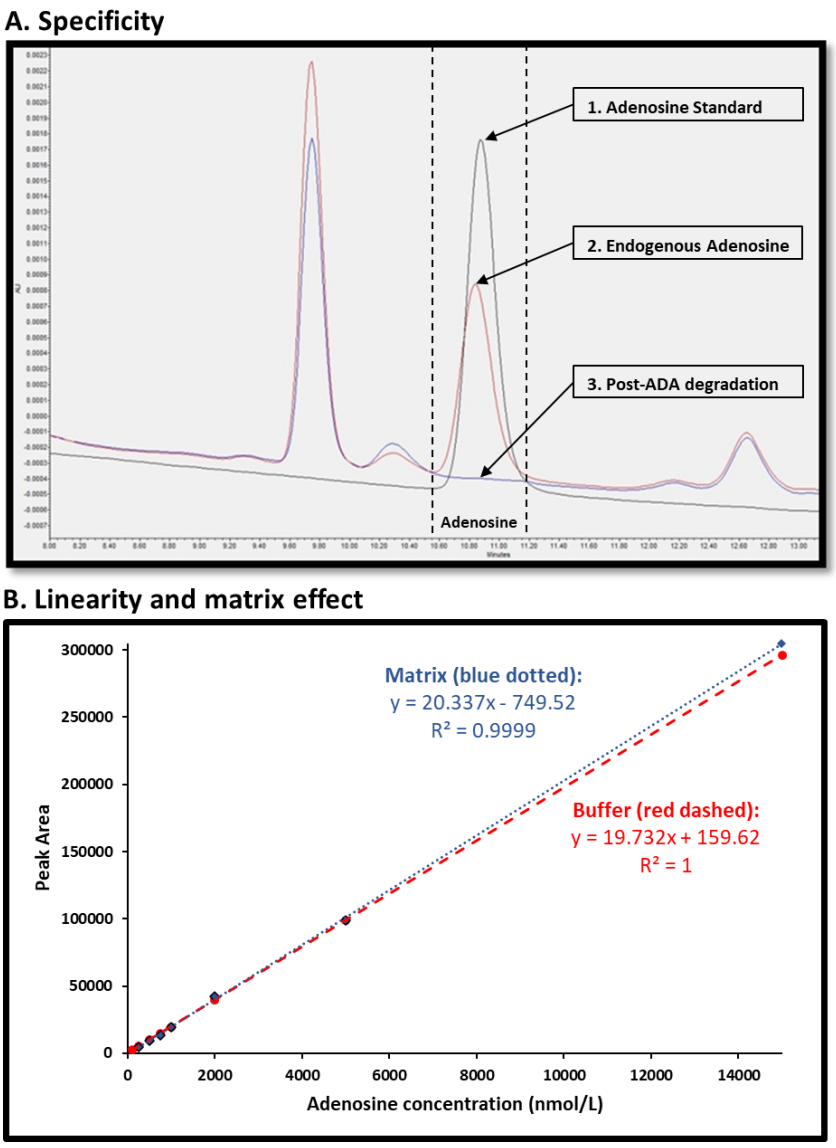

Figure 15. Validation of adenosine HPLC methodology.

(A) Superimposed chromatograms of three separate samples demonstrating a distinct adenosine peak free of interfering peaks. Multiple superimposed peaks including (i) adenosine standard in elution buffer (ii) endogenous adenosine in plasma (matrix) sample and (iii) endogenous plasma sample following degradation of adenosine with adenosine deaminase (ADA) resulting in no detectable adenosine peak confirming specificity. (B) Curve generated by plotting peak areas by adenosine concentration for both standards in buffer (dashed red line) and standards in plasma sample matrix (dotted blue line) demonstrating excellent linearity and minimal matrix effect.

Table 4. Repeatability assessment of adenosine assay

	Injection	Retention Time	Peak Area	Peak Height
Standards	1	10.677	19635	1494
	2	10.665	19584	1460
	3	10.665	19210	1477
	4	10.656	19369	1473
	5	10.651	19194	1483
	6	10.65	18989	1477
	7	10.65	19029	1476
	8	10.643	18751	1469
	9	10.637	18403	1464
	10	10.638	18521	1471
	Mean	10.65	19068.50	1474.40
	SD	0.01	416.88	9.62
	CV (%)	0.12	2.19	0.65
	Injection	**Retention Time**	**Peak Area**	**Peak Height**
Samples	1	10.887	5906	384
	2	10.934	6088	374
	3	10.932	6024	391
	4	10.928	5861	384
	5	10.931	5906	389
	6	10.93	6036	396
	Mean	10.92	5970.17	386.33
	SD	0.02	90.85	7.55
	CV (%)	0.16	1.52	1.96

Generated via 10 sequential injections of 1000nM adenosine standard for standards assessment and 6 sequential injections of a single patient sample for samples assessment. Retention time in minutes. SD – standard deviation, CV – coefficient of variation

Table 5. Linearity and range assessment of standards

HPLC Parameters (N=3/level)		Retention Time (min)			Peak Area		
Adenosine (nM)	**% Target**	**Mean**	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
15000	**1500**	10.63	0.01	0.11	296199.00	2312.07	0.78
5000	**500**	10.65	0.01	0.05	98654.67	480.60	0.49

2000	**200**	10.68	0.01	0.09	39632.33	569.87	1.44
1000	**100**	10.66	0.02	0.16	19773.00	261.86	1.32
750	**75**	10.66	0.02	0.19	14807.67	371.25	2.51
500	**50**	10.68	0.00	0.02	9886.00	334.39	3.38
250	**25**	10.73	0.02	0.22	5397.33	379.43	7.03
100	**10**	10.75	0.01	0.07	2322.33	298.89	12.87

Generated via 3 separate injections of each concentration of adenosine standard ranging from 10-1500% of the target adenosine concentration. HPLC – high performance liquid chromatography, SD – standard deviation, CV – coefficient of variation

Ongoing data validation during the sample collection phase was ensured by repetition of a standardized protocol including blank injection prior to, in the middle of, and following sample injections. Similarly, a blank phosphate-buffered saline (PBS) sample is processed through the MCX column and quantified. Standards are run with each grouping of samples and the curves are monitored for stability including slope, intercept and R^2. The stability of samples during the HPLC analysis period is ensured by performing 3 injections of a given sample at the start, middle and end of each run to ensure consistent results. We also utilize one sample with which we perform (i) ADA degradation (ii) ADA degradation followed by adenosine spiking post MCX column and (iii) adenosine spiking pre and post MCX columns. This process is then repeated in PBS with both an adenosine spike and an adenosine spike followed by ADA degradation. In this way, constant monitoring of the quality and reliability of results generated is ensured over time.(321)

Biological sample and clinical data collection

The University of Ottawa Heart Institute is a high volume, tertiary care center providing coronary revascularization services to over 1.2 million people.(277) From November 2016 to April 2018, 7,252 patients were prospectively enrolled in the CAPITAL (Cardiovascular And Percutaneous ClInical TriALs) revascularization registry which indexes clinical data points on patients undergoing coronary angiography and revascularization. In the CAPITAL revascularization registry, coronary artery disease (CAD) was defined as obstructive stenosis $\geq$ 50% at the time of angiography in keeping with current clinical standards.(322) Acute coronary syndrome (ACS) was composed of troponin-positive presentations including both non-ST-elevation myocardial infarction (NSTEMI) and ST-elevation myocardial infarction (STEMI) cases. Diabetes mellitus (DM) was based on either a hemoglobin A1c (HbA1c) $\geq$6.5% on presentation or a prior DM diagnosis or presence of medical therapy for DM. Tobacco use was dichotomized into smokers (active smoking at the time of sample collection) or non-smokers (not smoking at the time of sample collection). Positive family history was defined as CAD in a first degree relative <55 years of age for males and <65 years for females. Dyslipidemia and hypertension were defined as either a prior diagnosis of either condition or the presence of the appropriate medical therapy for either diagnosis on presentation. This study received approval from the University of Ottawa Heart Institute ethics review board (Protocols #20180562-01H, #20160516-01H and #20170126-01H) and informed consent was completed. Of this cohort of patients undergoing evaluation for coronary artery disease, 1,174 patients had blood samples collected for analysis.

Statistical methods

Data are reported as mean +/- standard deviation (SD), median +/- interquartile range (IQR) or number and percentage (%) where appropriate. Statistical testing was completed using GraphPad Prism 7.04, SigmaStat and SASS v9.4. Biological data was assessed for normality using D'Agostino and Pearson and Shapiro-Wilk normality tests. Following log transformation of the dataset, no statistical outliers were identified. Comparisons of two groups of non-parametric data based on ranks was performed with a Mann-Whitney test. All analyses defined significance as a two-tailed $p<0.05$, unless otherwise specified. Log-transformation of all data was completed prior to regression analysis. Linear regression was performed for age with demonstrated 95% confidence intervals. Univariable linear regression (ULR) was similarly performed for all factors with a pre-determined $p<0.2$ used to identify factors for inclusion in subsequent multivariable linear regression (MLR) analysis with significance defined as $p<0.05$ in keeping with prior studies.(260)

3.5 Results

Analytic evaluation of HPLC assay and PAC as a biomarker

HPLC methodology validation and analytical variability

Robust assay specificity was demonstrated generating a discernible adenosine peak free of interference from surrounding peaks. Focused degradation of adenosine by ADA demonstrated complete abrogation of the adenosine peak (Figure 15a). Repeatability of the HPLC assay itself was assessed by sequential injections from the same preparation of (i) standard (10 sequential injections on the same day of 1000nM adenosine, representing 100% target concentration) and (ii) sample (6 sequential injections on the same day of

serum from a single subject). This approach demonstrated a CV for retention time, peak area and peak height of 0.12%, 2.19% and 0.65% for standards and 0.16%, 1.52% and 1.96% for samples, respectively (**Table 4**). Our assessment of linearity and range included reporting the retention time and peak area for all adenosine values ranging from 100nM to 15,000nM (10%-1500% of target) – producing CV ranging from 0.02-0.22% for retention time, while the CV for peak areas ranged from 0.78% to 7.03% at 250nM **(Table 5)**. The lowest quantified value, 100nM of adenosine, demonstrated a variance of 12.87% identifying a lower limit for reliable quantification by this assay. Plotting the peak areas generated as a function of the adenosine concentrations generates a line of best fit with equation $y=19.732x+159.62$, $R^2=1$ (Figure 15b, dashed line). The impact of matrix (plasma) was assessed with the same range of standard concentrations and compared to the curve generated with standards in elution buffer on the same plot **(Figure 15b,** dotted line), resulting in a trendline of $y=20.337x - 749.52$, $R^2=0.9999$.

Intra and inter-subject variability

The variability of our assay and methodology was assessed at each level of quantification in an unselected cohort of patients undergoing assessment for coronary artery disease `(**Table 6**). First, intra-tube variability was assessed using multiple aliquots from the same blood tube drawn from one subject demonstrating a CV of 3.2% - establishing the analytical CV (CVa) for our assay. Next, intra-subject variability was assessed, first for inter-tube variability using separate draws at the same time point yielding a CV of 23.0%. Intra-subject variation was then assessed within the same day and on separate days. Adenosine levels drawn throughout a single day incrementally increased the CV to

30.1%, while serial collections on the same patient across multiple days (mean 35.8+/-33.1 days) further increased the CV to 35.8% - establishing the CV_i for our assay. Lastly, inter-subject variation (CV_g) was assessed utilizing all adenosine levels in the entire cohort, noting a CV_g of 56.7% **(Table 7).** Overall, this resulted in a RCV of 98.9% and II of 0.63.

Table 6. Intra and inter-subject variation

	No. of subjects	No. of samples	Adenosine (nM)	SD (nM)	CV (%)
Intra-subject					
Intra-tube, same time (CVa)	18	92	1042.0	33.5	**3.2**
Inter-tube, same time	29	95	1240.0	256.1	**23.0**
Different time, same day	17	39	1090.8	349.3	**30.1**
Different time, different day (CVi)	31	64	1216.6	457.3	**35.8**
Inter-subject					
Different time, different day (CVg)	1,141	-	1067.2	605.3	**56.7**

PAC – plasma adenosine concentration, SD – standard deviation, CV – coefficient of variation, CVa – analytical CV, CVi – intra-subject CV, CVg – inter-subject CV

Table 7. Comparison of adenosine variation to established markers

	CVi (%)	CVg (%)
Adenosine	**35.8**	**56.7**
C-reactive protein (CRP)	42.2-52.6	84.4-92.5
N-terminal (NT)-proBNP	30-50	99-130
Insulin	21.1	58.3
Vanillylmandelic acid (24 hour urine)	22.2	47
Cortisol	20.9	45.6
Creatinine kinase (CK)	22.8	40

Calcium (24 hour urine)	27.5	36.6
Haptoglobin	20.4	36.4
Lipase	23	33.1
Thyroid stimulating hormone (TSH)	19.7	27.2
Low-density lipoprotein (LDL)	8.3	25.7

CV – coefficient of variation,CVi – intra-subject CV,CVg–inter-subject CV. Source - *Ricos et al, Macy et al, Clerico et al* Ricos et al [27], Macy et al [28] and Clerico et al [37]

Impact of biologic and therapeutic interventions on PAC

Patient and procedural characteristics

From an initially recruited 1,174 patients, we excluded duplicate samples of the same patients (32 in total) leaving 1,141 patients included in the final analysis. The cohort's baseline demographics are summarized in **Table 8.** The average age was 66.3+/-11.8 years of age (**Figure 16**) with 70.8% being male. The cohort underwent angiography for indications that included ACS (39.5%) and stable CAD (39.9 %). Risk factors included 30.4% diabetics, 18.5% active smokers, 61.3% with dyslipidemia, 16.5% with positive family history and 64.7% with hypertension. Coronary artery disease was known prior to angiography in 38.1% patients, with 24.7% reporting a prior myocardial infarction (MI) and 34.6% having had prior revascularization with either percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). Medical therapy in the cohort included 53.5% on ACEi/ARB, 59.4% on beta blockers, 79.7% on statins, 88.4% on aspirin and 87.7% on P2Y12 inhibitors. A total of 25 recruited patients did not undergo angiography, but had samples collected via venous access, leaving 1,116 patients who underwent angiography for which procedural details were indexed (**Table 9**). After

excluding those with prior revascularization, CAD or MI, 633 cases remained that underwent angiography, of which 431 cases remained that had *de novo* obstructive CAD at the time of sample collection – ranging from 1 vessel (40.1%), 2 vessel (28.5%) to 3 vessel (31.3%) disease. Of the entire cohort, 23.5% underwent percutaneous coronary intervention with placement of one stent (46.2%), two stents (32.4%) or 3 or more stents (21.4%).

Table 8. Baseline demographics

	Number or Mean	Proportion (%) or Standard Deviation (SD)
Number of patients	1,141	-
Age	66.3	11.8
Male	806	70.8
Indication for angiography		
Acute coronary syndrome	451	39.5
ST-elevation myocardial infarction	24	2.1
Non ST-elevation myocardial infarction	292	25.6
Unstable angina	135	11.8
Stable coronary artery disease	455	39.9
Staged percutaneous coronary intervention	124	10.9
Shock	2	0.2
Arrhythmia	19	1.7
Heart failure/left ventricular dysfunction	90	7.9
Past medical history		
Diabetes	347	30.4
Type I	7	2.0
Type II – Diet controlled	13	3.7
Type II – Non-insulin therapy	233	67.1
Type II – Insulin therapy	94	27.1
Smoking	211	18.5
Dyslipidemia	699	61.3
Family History	188	16.5
Hypertension	738	64.7
Prior cerebrovascular accident	80	7.0
Peripheral arterial disease	84	7.4
Atrial fibrillation	119	10.4
Prior coronary artery disease	435	38.1
Prior myocardial infarction	282	24.7
Prior angiogram	462	40.5
Prior percutaneous coronary intervention	317	27.8
Prior coronary artery bypass grafting	78	6.8
Medications		
ACE inhibitor/Angiotensin receptor blocker	610	53.5
Beta blocker	678	59.4
Calcium channel blocker	158	13.8
Statin	909	79.7
Oral anticoagulation	61	5.3
Intravenous unfractionated heparin	132	11.6
Subcutaneous low-molecular weight heparin	123	10.8
Acetylsalicylic acid	1009	88.4
P2Y12	1001	87.7
Clopidogrel	685	68.4
Ticagrelor	315	31.5
Prasugrel	1	0.1

Table 9. Procedural details

	Number or Mean	Proportion (%) or Standard Deviation (SD)
Number of patients undergoing	1,116	-
angiography	1,015	90.9
Access	97	8.7
Radial	4	0.4
Femoral		
Brachial		
Access site medications	585	52.4
Calcium channel blocker	420	37.6
Nitroglycerin		
Procedural medications	966	88.1
Heparin	6678	2378
Mean dose (IU)	53	4.7
Bivalirudin	2	0.2
Glycoprotein IIa/IIb inhibitors	68	6.1
Adenosine	30	2.7
Intravenous	36	3.2
Intracoronary	457	40.9
Nitroglycerin		
Number of cases with *de novo* obstructive	431	38.6
(≥50%) CAD		28.1
Lesion-burden	121	22.5
1 lesion	97	15.8
2 lesions	68	13.7
3 lesions	59	20.4
4 lesions	88	40.1
5+ lesions		28.5
Vessel-burden	173	31.3
1 vessel	123	
2 vessel	135	
3 vessel		
Number of cases with a stent deployed	262	23.5
1 stent	121	46.2
2 stents	85	32.4
3+ stents	56	21.4

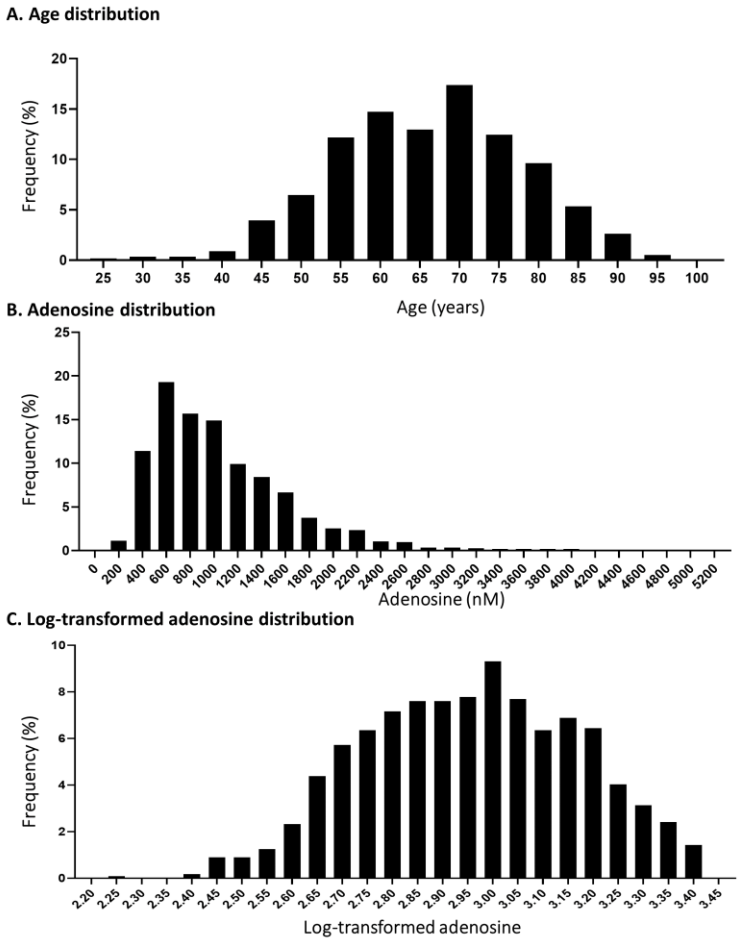

Figure 16. Distribution of adenosine and age.

Histograms depicting the relative frequency in percentage (%) for each designated bin of term. Specifically, (A) demonstrates age distribution throughout the entire cohort (N=1,141) of patients undergoing workup for coronary artery disease, while (B) provides adenosine distribution across the entire cohort. **(C)** Logarithmic transformation of adenosine values across the entire cohort.

Impact of cardiovascular risk factors on PAC

Established cardiovascular risk factors were assessed for impact on circulating adenosine levels **(Table 10)**. Smokers did not show a statistical difference in adenosine levels compared to non-smokers (917 [607-1325] versus 932 [635-1357], p=0.858). As well, there was no statistical difference in adenosine levels between those that did and did not

have a history of dyslipidemia (909 [626-1350] versus 953 [645-1390], p=0.292), hypertension (936 [634-1363] versus 917 [621-1353], p=0.701), or family history of CAD (953 [609-1376] versus 926 [635-1350], p=0.896). Sex did not impact adenosine levels with males (925 [630-1345]) (demonstrating statistically similar levels to females (949 [645-1398], p=0.293). Diabetes as a dichotomized variable did not significantly impact adenosine levels with diabetics and non-diabetics (974 [604-1438] versus 913 [639-1313], p=0.238) Moreover, in the 294 diabetic patients with HbA1c values available, there was no significant relationship between HbA1c and adenosine levels ($r=0.03$, $R^2=0.001$, $p = 0.59$, **Figure 17a**). The impact of age on adenosine levels was also assessed **(Figure 17b)** showing a statistically significant inverse association between age and PAC ($R^2=0.005$, $r=-0.07$, $p=0.02$). Next, we performed additional analysis following division of the cohort into those age $\leq$ 65 (n =533) and those >65 (n =608) years of age, demonstrating reduced adenosine in the >65 cohort (895 [610-1315]) than the $\leq$ 65 cohort (971 [649-1397], p=0.027).

Table 10. Impact of risk factors, medications and coronary artery disease on adenosine

	Present		Absent		p	
Cardiovascular Risk Factors	**n**	**Median [IQR]**	**n**	**Median [IQR]**	*** <0.05**	
Age >65	608	895 [610-1315]	533	971 [649-1397]	0.027	*
Diabetes	347	974 [604-1438]	793	913 [639-1313]	0.238	
Smoking	211	917 [607-1325]	930	932 [635-1357]	0.858	
Dyslipidemia	699	909 [626-1350]	442	953 [645-1390]	0.292	
Family History	188	953 [609-1376]	953	926 [635-1350]	0.896	
Hypertension	738	936 [634-1363]	403	917 [621-1353]	0.701	
Male	806	925 [630-1345]	335	949 [645-1398]	0.293	
Medications	**n**	**Median [IQR]**	**n**	**Median [IQR]**		
Acetylsalicylic acid (ASA)	1023	919 [626-1361]	117	958 [683-1285]	0.650	

Clopidogrel	685	953 [637-1400]	456	904 [609-1289]	0.158	
Ticagrelor	315	875 [595-1254]	826	955 [650-1408]	0.012	*
ACE inhibitor/ARB	610	943 [645-1353]	531	909 [620-1363]	0.419	
Beta blocker	678	958 [644-1356]	463	887 [615-1357]	0.317	
Calcium channel blocker	158	974 [618-1398]	983	919 [634-1346]	0.433	
Statin	909	919 [618-1341]	232	937 [690-1419]	0.141	
Unfractionated heparin	132	985 [619-1397]	1009	925 [634-1350]	0.549	
Low-molecular weight heparin	123	907 [654-1400]	1018	932 [630-1350]	0.568	
Coronary artery disease	**n**	**Median [IQR]**	**n**	**Median [IQR]**		
Coronary artery disease (CAD)	941	909 [618-1325]	200	995 [686-1460]	0.026	*
De novo CAD burden > 1 vessel	548	902 [616-1306]	315	926 [604-1356]	0.676	
Acute coronary syndrome	311	932 [637-1346]	830	928 [630-1363]	0.971	

Median +/- interquartile range (IQR)

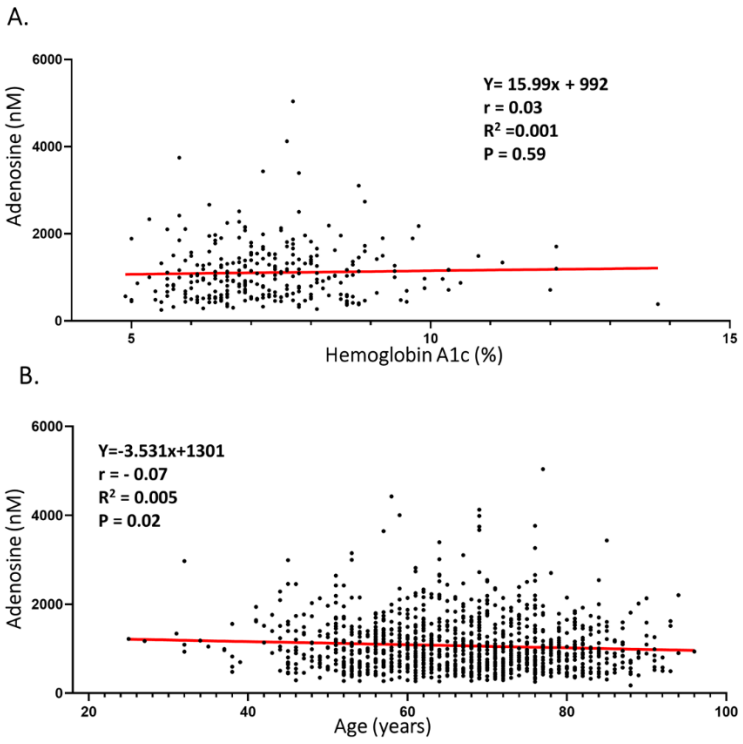

Figure 17. Association of hemoglobin A1c and age with adenosine

(A) Univariable analysis completed for all diabetic patients with a hemoglobin A1c indexed (N =294), demonstrating no significant relationship between HbA1c and adenosine (p=0.59). **(B)** Univariable linear regression of adenosine levels and age (years) in the entire cohort (N= 1,141) demonstrating a negative correlation (p=0.02).

Impact of medical therapy on plasma adenosine levels

Medical therapy for cardiovascular risk reduction was assessed for impact on PAC **(Table 10)**. No difference in adenosine levels was noted comparing patients taking to those not taking ACE inhibitors/ARBs (943 [645-1353] versus 909 [620-1363], p=0.419) beta blockers (958 [644-1356] versus 887 [615-1357], p=0.317) calcium channel blockers (974 [618-1398] versus 919 [634-1346], p=0.433) and statins (919 [618-1341] versus 937 [690-1419], p=0.141). Similarly, anticoagulants utilized preceding angiography did not impact adenosine levels with unfractionated heparin (985 [619-1397] versus 925 [634-1350], p=0.549) or subcutaneous low-molecular weight heparin (LMWH) (907 [654-1400] versus 932 [630-1350], p=0.568). Finally, we evaluated the impact of antiplatelet therapy on PAC as previous data suggesting ticagrelor may impact adenosine metabolism **(Table 10)**.(149,313) No difference in adenosine was seen with acetylsalicylic acid (ASA) (919 [626-1361] versus 958 [683-1285], p=0.65). Similarly, in the class of P2Y12 inhibitors, clopidogrel did not affect adenosine levels (953 [637-1400] versus 904 [609-1289], p=0.158). Interestingly, ticagrelor therapy was associated with a reduction in adenosine levels compared to those not on ticagrelor (875 [595-1254] versus 955 [650-1408], p=0.012).

Impact of coronary artery disease on plasma adenosine levels

Finally, we assessed the impact of CAD presence and burden on plasma adenosine levels **(Table 10)**. In the total cohort, absence of obstructive CAD was associated with higher adenosine levels than patients with obstructive CAD (909 [618-1325] versus 995 [686-1460], p=0.026 (No differences between patients presenting as ACS (NSTEMI/STEMI)

versus non-ACS and were observed (n =311, 932 [637-1346]) versus n =830, 928 [630-1363], p=0.971). Disease burden, as assessed by presence of *de novo* multivessel (>1 vessel) disease, failed to show any association with PAC levels compared to those with single-vessel disease (902 [616-1306] versus 926 [604-1356], p=0.676).

Multivariable linear regression analysis

To assess the association of variables with PAC, we first performed a log-transformation of the adenosine values (**Figure 16b,c)** followed by a ULR to identify potential associated variables **(Table 11)**. Individual variables associated with a $p<0.2$ were identified including total number of vessels (p=0.104), age (p=0.009), hemoglobin A1c (p=0.062), sex (p=0.193), statin (p=0.176), P2Y12 (clopidogrel and ticagrelor) (p=0.195). After multivariable analysis **(Table 12)**, only age (p=0.039) remained inversely associated with PAC.

Table 11. Univariable linear regression

	p	P<0.2
Age	0.009	*
Sex	0.193	*
Coronary artery disease - number of vessels	0.104	*
Acute coronary syndrome	0.950	
ST-elevation myocardial infarction	0.395	
Non-ST-elevation myocardial infarction	0.609	
Diabetes	0.219	
Smoking	0.998	
Dyslipidemia	0.298	
Family History	0.993	
Hypertension	0.553	
Prior coronary artery disease	0.536	
Prior myocardial infarction	0.243	
Prior percutaneous coronary intervention	0.387	
Prior coronary artery bypass grafting	0.734	
ACE inhibitor/Angiotensin receptor blocker	0.483	
Acetylsalicylic acid	0.864	
Beta Blocker	0.422	
Calcium Channel Blocker	0.379	
Statin	0.176	*
Intravenous unfractionated heparin	0.558	
Subcutaneous low molecular weight heparin	0.516	
P2Y12	0.195	*
Hemoglobin A1c	0.062	*
Creatinine	0.816	
Hemoglobin	0.921	

Table 12. Multivariable linear regression

	p	p<0.05
Age	0.039	*
Sex	0.237	
Clopidogrel	0.297	
Ticagrelor	0.537	
Statin	0.504	
Coronary artery disease - number of vessels	0.332	
Hemoglobin A1c	0.088	

3.6 Discussion

Despite abundant preclinical research linking adenosine to vascular disease, the current study is the first to evaluate the relationship of plasma adenosine levels with known cardiovascular risk factors, medical therapy and disease presence in humans. Herein, we report the performance of a high-throughput protocol for rapid HPLC-based adenosine quantification with performance parameters in congruence with good practice guidelines.(321) In the current cohort, our assay produces intra-subject and inter-subject variability consistent with other biomarkers of cardiovascular disease. Notably, in the current dataset traditional cardiovascular risk factors and medical therapies were not associated with significant changes in plasma adenosine levels. In contrast, age and CAD presence were inversely associated with plasma adenosine levels – a finding for which age alone remained statistically significant after multivariable adjustment.

In our study of over 1,100 patients, traditional cardiovascular risk factors including hypertension, diabetes, family history, smoking, dyslipidemia and sex did not associate with adenosine levels, while age was inversely correlated. Age is known to impact other established markers of cardiovascular disease. For example, low-density lipoprotein (LDL) is known to diminish with advancing age at a rate of only 0.8% annually, though this still translates to important clinical implications.(323) Similarly, NT-proBNP is an established marker for diagnosis, monitoring and outcomes in heart failure with biological variance closely mirroring that of adenosine.(324,325) However, it is impacted by age, necessitating age-specific reference intervals and having diminished predictive abilities at more advanced ages.(326,327) Hence, while the annual incremental impact of

advancing age may be small, the cumulative impact of age over time remains an important consideration when establishing adenosine's performance as a diagnostic, prognostic and monitoring clinical test. Smokers have lower adenosine levels in their sputum, with increased adenosine levels and ADORA3 and 1 noted post cessation.(328) However, there has been no definitive link between smoking and circulating adenosine levels in keeping with our data. Similarly, extensive literature links diabetes to adenosine levels – however, these associations typically focus on augmented ADA levels, postulating that this leads to reduced circulating adenosine.(199) Our data does not demonstrate any significant differences in PAC in those with and without diabetes, while not evaluating an impact on receptor activity *nor* in specific vascular beds. Nonetheless, our study provides adequate power across subgroups to evaluate the impact of risk factors in humans and suggests that age may incrementally contribute to a decline in PAC – a finding which confounds smaller observational studies.

Similarly, the use of medications for cardiovascular risk reduction did not demonstrate any significant associations with adenosine levels, with none of ACE inhibitors/ARBs, beta blockers, calcium channel blockers, statins, or heparins demonstrating any significant differences. In contrast, antiplatelet medications have been studied extensively for their putative impact on PAC. Specifically, ticagrelor has garnered significant attention with postulations that observed pleiotropic effects may stem from modulation of adenosine biology. In one study, 60 ACS patients were randomized to ticagrelor or clopidogrel with ticagrelor increasing plasma adenosine levels compared to those receiving clopidogrel, ostensibly via inhibition of red cell uptake.(149) However, a recent

randomized crossover study in 54 ACS patients compared ticagrelor, prasugrel and clopidogrel – failing to demonstrate any significant augmentation in adenosine levels with ticagrelor compared to clopidogrel or prasugrel.(313) In our all-comers cohort with over 300 patients on ticagrelor therapy, reduced PAC was noted in those on ticagrelor compared to those not receiving ticagrelor. However, any non-randomized dataset is innately confounded by the fact that ticagrelor is differentially employed in clinical practice, with ACS patients preferentially receiving ticagrelor given its superior clinical outcomes in ACS patients.(144) Indeed, this was observed in our dataset with 68.8% of patients on ticagrelor presenting as an ACS versus only 27.9% of those not on ticagrelor. The differential use of ticagrelor in our cohort leads to innate differences between the populations which limit further analysis. Hence, our study was not intended to specifically address the impact of P2Y12 agents on adenosine levels,but adds to the growing debate of the impact of ticagrelor on circulating adenosine levels.

Preclinical research has suggested adenosine plays an important role in modulating the pathogenesis of atherosclerosis particularly with modulation of systemic inflammation.(239-241) Indeed, our data suggests an inverse association between obstructive CAD and PAC. However, subgroup analysis failed to show any significant differences in adenosine levels across a spectrum of disease burden (i.e. multivessel disease) or presentation (i.e. acute coronary syndrome). Animal studies have noted increased activity of vascular ADA (resulting in reduced circulating adenosine levels) as a mediator of atherosclerosis – proposing ADA inhibition (augmenting circulating adenosine) as a possible therapeutic approach.(329) Similarly, genetic studies in humans

lend support to the hypothesis that adenosine is a vascular protective molecule.(230,231,243) In humans, patients with CAD and genetic variations that purportedly augment circulating adenosine levels have reduced adverse cardiovascular events.(243) Our data now lends credence to this hypothesis – establishing a potential relationship between the presence and absence of disease. Whether adenosine acts as a prognosticator of events needs to be established in larger cohorts.

In spite of intensive research in the field of adenosine biology, the systematic development and evaluation of adenosine as a potential biomarker has not been previously performed owing to the technical limitations of sampling and existing quantification methods.(319) The currently reported assay yields technical performance that meets and exceeds good practice guidelines.(321) With a CVa 3.2%, CVi 35.8%, CVg 56.7%, RCV 98.9% and II of 0.63 our assay performed in line with many known markers of coronary artery disease – such as C-reactive protein (CRP).(330-332) Indeed, from an assay perspective, a CVa of 3.2% is markedly improved over early assays reporting CVa ranging from 6-7%(314) and up to 10%(315), while falling closely in line with contemporary assays yielding CVa's of 1-3%, keeping with clinical assay standards.(316) The balance of these variances is crucial to assessing the clinical utility of a test. A test with high index of individuality (>1.4) will perform well as a diagnostic test based on population-level reference intervals, while a low index (<0.6) will not as significant changes for a given subject may still fall within a population-based reference range.(319,320) Comparatively, CRP has a CVa 5.2%, CVi 42.2% and CVg 92.5%.(318) Having a large CVg coupled with a relatively smaller CVi means that individuals could

have early disease-related changes without rising above a given reference interval, requiring relatively large changes in value before confidence in its significance is noted (Supplemental Table 3).(317,318) Indeed, the RCV (smallest percentage change not likely due to CVa or CVi at significance of $p \leq 0.05$) is 118% for CRP and index of individuality was 0.46 – a substantial change in value.(318) Comparatively, the RCV for adenosine in our assay is 98.9% with an II of 0.63, translating to similar considerations when determining its clinical utility and optimal interpretation. In fact, the variance of CRP leads to up to 46% of patients alternating between low and high risk categories despite a stable clinical status, translating to a 10-20% probability of making an erroneous risk assignment based on a single CRP value.(333) Despite this, CRP remains an established predictor of cardiovascular outcomes in those with(330) and without CAD(331) and predicts reduction of cardiovascular events in response to medical therapy (332) – supporting its role in current guidelines.(289,334) We demonstrate a similar variance profile to CRP for PAC in humans – meaning the variability in humans will require large sample sizes to adequately detect disease associations *or* to evaluate the impact of therapies on PAC.(335) Thus, clinical tests with this variance profile, such as adenosine, will have little utility in identifying early disease-related changes in the context of a healthy reference interval, favoring serial monitoring for significant changes in individual patients instead (318) – important implications for interpreting previous studies in humans and powering future evaluations of PAC as a marker or endpoint.

Certainly, our study is not without its limitations. The data is observational in nature and subject to all the limitations of this design. However, clinical and procedural data were

prospectively collected in a nested registry design, limiting potential biases a solely retrospective approach may introduce. Second, the relatively large variability does open the possibility of regression dilution bias whereby significant differences may not be seen on account of inherent measurement errors.(336) Hence, despite being substantially larger than any previous human study, we are at risk of not detecting a modest association where one exists. Third, differences in absolute PAC values exist across varying collection methodologies reported. However, the uniform processing procedures and robust analytical variation of this study lends itself to unidirectional variance – whereby any potential errors would exist uniformly throughout the cohort and not impact the ability to detect biological differences present. Finally, our protocol was designed to evaluate PAC assayed by peripheral collection which is the primary method performed in humans. These values may not reflect local tissue levels or levels in specific vascular beds and thus does not preclude organ/tissue specific associations of adenosine. However, as demonstrated by our analysis, adequately powered studies to assess local adenosine levels may be difficult owing to technical factors and variability with robust protocols and statistical procedures forming the basis of any rigorous future analytic studies.

3.7 Conclusion

In humans, plasma adenosine levels are not significantly impacted by traditional cardiovascular risk factors or medical therapy for cardiovascular disease; however, advancing age and the presence of coronary artery disease may be associated with diminishing adenosine levels. Large prospective studies of basal levels and variation of adenosine for prediction of future cardiovascular events are warranted.

3.8 Sources of Funding

Dr. Rob Beanlands is a Career Investigator supported by the Heart and Stroke Foundation of Ontario, a Tier 1 University of Ottawa Chair in Cardiovascular Research and Vered Chair in Cardiology.

3.9 Conflicts of Interest

None relevant

Chapter 4

Performance of Plasma Adenosine as a Biomarker for Predicting Cardiovascular Risk

4.1 Preface

This chapter has been previously published in *Clinical and Translational Science*:

> **Simard T**, Jung RG, Di Santo P, Ramirez FD, Labinaz A, Gaudet C, Motazedian P, Parlow S, Joseph J, Moreland R, Marbach J, Boland P, Promislow S, Russo JJ, Chong AY, So D, Froeschl M, Le May M, Hibbert B. Performance of Plasma Adenosine as a Biomarker for Predicting Cardiovascular Risk. Clin Transl Sci. 2021 Jan;14(1):354-361. doi: 10.1111/cts.12886. Epub 2020 Dec 2. PMID: 33264483; PMCID: PMC7877863.

> *All authors made substantial contributions to the conception/design of the work; and/or the acquisition, analysis, or data interpretation; and, (ii) drafted and/or revised the content critically; and, (iii) provided final approval of the published version; and, (iv) agree to be accountable for the work.*

4.2 Abstract

Adenosine boasts promising pre-clinical and clinical data supporting a vital role in modulating vascular homeostasis. Its widespread use as a diagnostic and therapeutic agent has been limited by its short half life and complex biology, though adenosine-modulators have shown promise in improving vascular healing. Moreover, circulating adenosine has shown promise in predicting cardiovascular events.

We sought to delineate whether circulating plasma adenosine levels predict cardiovascular events in patients undergoing invasive assessment for coronary artery disease (CAD).

Patients undergoing invasive angiography had clinical data prospectively recorded in the CAPITAL revascularization registry and blood samples collected in the CAPITAL Biobank from which adenosine levels were quantified. Tertile-based analysis was used to assess prediction of major adverse cardiovascular events (MACE - composite of death, myocardial infarction, unplanned revascularization, and cerebrovascular accident). Secondary analyses included MACE subgroups, clinical subgroups and adenosine levels.

1815 patients undergoing angiography had blood collected with adenosine quantified in 1323. Of these, 51.0% were revascularized. 7.3% of patients experienced MACE in 12 months of follow-up. Tertile-based analysis failed to demonstrate any stratification of MACE rates (log-rank, p=0.83), when comparing low-to-middle (hazard ratio, HR 1.10; 95% CI, 0.68-1.78, p=0.70) or low-to-high adenosine tertiles (HR 0.95; 95% CI, 0.56-1.57, p=0.84). In adjusted analysis, adenosine similarly failed to predict MACE. Finally, adenosine did not predict outcomes in ACS patients, revascularized patients or those treated medically.

Plasma adenosine levels do not predict subsequent cardiovascular outcomes or aid in patient risk stratification.

4.3 Introduction

Adenosine serves as a crucial regulatory molecule in both intra and extracellular processes.(311) Adenosine homeostasis is closely regulated by an ongoing balance of production, degradation and transport.(3,8) Adenosine signaling is complex with four primary adenosine receptors (1, 2A, 2B, 3) facilitating extracellular signaling. These receptors are found on a multitude of cells with differing responses based upon the cell and receptor subtypes. Accordingly, adenosine signaling has been implicated in vascular

homeostasis with considerable pre-clinical data supporting its role in vasculoprotection.(311)

Therapeutically, adenosine is limited by its relative instability and short half-life. As a hyperemic agent, it has found limited use in coronary flow assessment,(91) though dipyridamole is more commonly used to augment adenosine levels given its improved stability.(113) Dedicated molecules specifically targeting adenosine receptors of interest are also in use to further optimize the intended versus unintended effects of adenosine modulation.(98) Indeed, adjunctive agents which augment adenosine levels have demonstrated promising results. Dipyridamole, augmenting adenosine through equilibrative nucleoside transporter-1 (ENT-1) inhibition, has demonstrated improved vascular patency and reduced restenosis in pre-clinical and clinical studies.(311) Similarly, cilostazol, modestly augments adenosine levels in addition to cAMP, (337) yielding reduced restenosis following coronary stenting.(338) Lastly, ticagrelor, a $P2Y_{12}$ receptor inhibitor, demonstrates improved clinical outcomes aside from its anti-platelet effects proposed to be related to pleiotropic effects via augmentation of adenosine (147) (via ENT-1 inhibition)(148) and possibly cAMP.(150) However, the precise impact of ticagrelor on circulating adenosine levels in clinical trials remains the subject of debate.(149,313) Taken together, pre-clinical and clinical data supports that adenosine augmentation may portend vasculoprotective effects and thereby improve cardiovascular outcomes.

Diagnostically, circulating adenosine levels have not been widely adopted owing in part to the technical challenges with its quantification and notable biological variability.(284) Despite this, small series have suggested a role for adenosine levels in risk stratifying cardiovascular outcomes. Subjects with coronary artery disease (CAD) and genetically reduced adenosine deaminase (AMPD1) activity, theoretically increasing adenosine levels, have demonstrated improved cardiovascular survival.(243) Similarly, we previously reported diminished adenosine levels in those with advanced age and obstructive coronary artery disease (CAD), though no association with established risk factors was noted.(284) However, following revascularization, AMPD1 patients did not demonstrate improved cardiovascular outcomes.(248) This study was limited by its small size, non-conventional revascularization strategies and lack of difference in adenosine levels between the cohorts – supporting the challenges of assessing adenosine as a biomarker.(248) Patients post-revascularization are at elevated risk of recurrent cardiovascular events,(276) and exogenous adenosine has shown promise in improving vascular healing in pre-clinical models.(339) Given the uncertainty of the utility of adenosine as a biomarker, we sought to determine whether circulating adenosine levels are predictive of future cardiovascular events in patients undergoing invasive assessment for CAD.

4.4 Methods

Adenosine sample collection and quantification

Adenosine samples were collected a the time of invasive angiography as previously described.(266) Briefly, blood samples were collected via the plastic arterial access sheath (Terumo Medical, Somerset, New Jersey, USA) or, rarely, if required by

peripheral venipuncture. Samples were collected in pre-filled Greiner BioOne Vacuette tubes containing 2ml of ice-cold stop solution as reported.(266) Hemolyzed samples were excluded.(314) Samples were centrifuged and stored at -80°C to await processing. Samples were then processed via the Waters Oasis MCX (Mixed-mode, strong Cation-eXchange) columns and transferred for HPLC analysis on a Waters Alliance E2695 separating module system with sample quantification by Waters 2489 UV/visible detector at 260nM as previously reported.(266) This methodology underwent thorough development and validation with ongoing quality-of-care metrics to ensure robust adenosine quantification as described.(266)

Population and patient outcomes

The University of Ottawa Heart Institute provides sole revascularization services to over 1.2 million people.(277) The CAPITAL (Cardiovascular And Percutaneous ClInical TriALs) revascularization registry is a web-based registry that prospectively indexes data on patients undergoing coronary angiography. Baseline demographics, cardiovascular risk factors and definitions have been previously described.(266) Patients undergo follow-up assessment in the year following their angiography where clinical outcomes in the year following revascularization are also recorded in the registry. Outcomes were prospectively recorded by individuals blinded to adenosine levels. Myocardial infarction (MI) was recorded according to the universal definition of MI or clinical diagnosis of MI by treating physicians.(288) Repeat unplanned revascularization included patients undergoing repeat coronary intervention not planned following their index procedure and included both target vessel and non-target vessel revascularization. Death included both cardiac and non-cardiac death. Stroke included both ischemic or hemorrhagic events as

diagnosed by treating neurologist or on cross-sectional imaging. The primary outcome of interest was major adverse cardiovascular events (MACE) composed of death, MI, stroke or unplanned revascularization at one-year follow-up. Secondary outcomes of interest included individual MACE components and clinical sub-groups. The collection and use of blood samples in the CAPITAL Biobank was approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB Protocol #: 20160516-01H) and informed consent was obtained from all patients for collection. The outcomes study assessment was approved by OHSN-REB (#20190224-01H) to evaluate clinical outcomes following revascularization.

Statistical analysis

Data are reported as mean +/- standard deviation (SD), median +/- interquartile range (IQR) or number and percentage (%) where appropriate. Plasma adenosine levels were compared using Mann-Whitney U-tests. Plasma adenosine levels were categorized into tertiles (33rd and 67th percentiles) and Kaplan-Meier plots generated to assess event distributions with comparison by log-rank tests. Subsequent Cox proportional-hazard models were then used to determine hazard ratios (HR) and 95% confidence intervals (CI). Multivariable Cox proportional-hazards model was also performed incorporating variables known to impact adenosine levels and post-revascularization MACE including age (years), sex, type 2 diabetes, hypertension, dyslipidemia, coronary artery disease, acute coronary syndrome and revascularization as documented previously and highlighted in univariate analysis (Supplemental Table 1).[282,284] We estimated an incidence rate of 9.5% and 15.5% (effect size of 60%) between the lowest and highest

adenosine tertiles with an α=0.05 and β=0.20 which yielded a sample size of 476 per tertile for a total sample of 1428 subjects. All statistical analyses was completed using SAS v9.4 (SAS Institute, Inc., Cary, NC, USA). Furthermore, all figures were created using GraphPad Prism v8 (GraphPad Software, La Jolla, CA, USA). P<0.05 was considered statistically significant.

4.5 Results

Patient characteristics

During the study period, 1815 patients underwent angiography and had blood collected in the CAPITAL Biobank for further analysis with one-year follow-up completed. Within the collected samples, 1323 had adenosine levels quantified from the time of index angiography and were included in our analysis. Of the 1323 patients included, 675 (51.0%) underwent revascularization by either percutaneous coronary intervention (70.8%, PCI) or coronary artery bypass grafting (28.7%, CABG) or both (0.4%). Medical therapy was pursued in 648 (49.0%) patients with 64.5% having obstructive CAD and 35.5% have no obstructive CAD at the time of angiography **(Figure 18).** Mean age was 66.8 +/- 11.6 years, 29.3% female, risk factors included 65.2% hypertension, 61.1% dyslipidemia, 30.5% diabetes, 18.1% active smokers, and 14.9% family history of CAD. Indications for angiography included 59.7% ACS and 37.7% stable CAD. Patients had a prior history of PCI (27.7%), MI (23.3%), CABG (6.5%), peripheral artery disease (6.7%), and cerebrovascular accident (CVA, 6.6%). Medications at the time of angiography included ASA (90.9%), P2Y12 (89.4%), ACE/ARB (53.5%), beta-blocker (59.2%) and statin (80.9%) **(Table 13)**. Adenosine values were stratified according tertile

(i) 0-33rd (low adenosine, n=436) with a cut-off of 693.2 nM; (ii) 34-67th (middle adenosine, n=451) with a cut-off level of 902.9 nM; and (iii) 68-100th (high adenosine, n=436) with a cut-off level of 1141.9 nM with baseline characteristics stratified by tertiles (**Table 13)**.

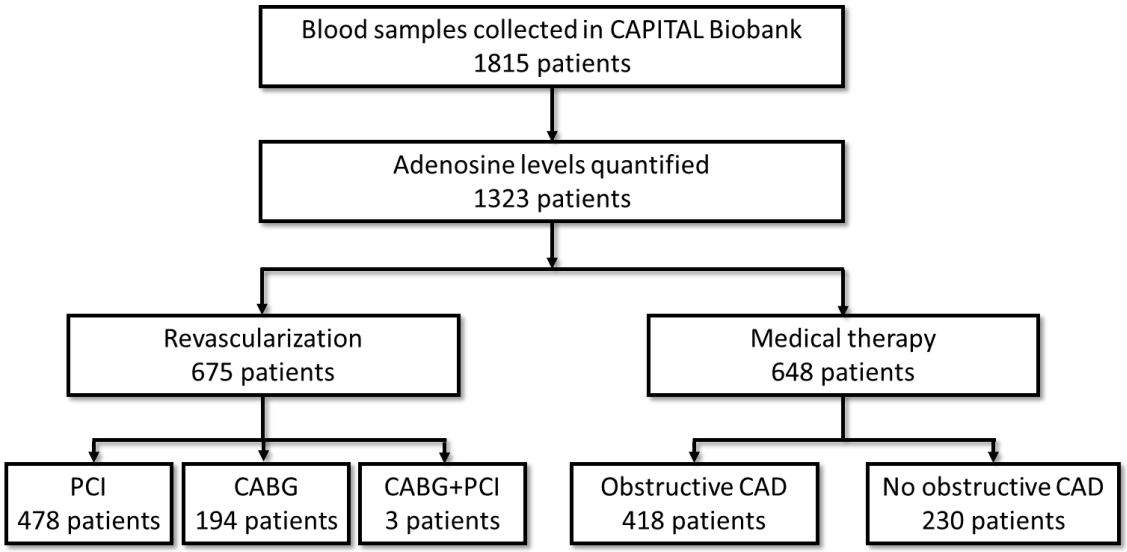

Figure 18. Patient flow diagram.

CAPITAL- Cardiovascular and Percutaneous Intervention Trials, PCI – percutaneous coronary intervention, CABG – coronary artery bypass grafting, CAD – coronary artery disease

Table 13. Baseline characteristics

			Adenosine Tertiles					
	Total Cohort		**Low**		**Middle**		**High**	
Number	1323		436		451		436	
Age - mean +/- SD	66.8	11.6	67.0	11.4	67.3	11.8	66.2	11.6
Sex (female) - no. (%)	387	29.3	112	25.7	144	31.9	131	30.0
Hypertension - no. (%)	863	65.2	287	65.8	281	62.3	295	67.7
Dyslipidemia - no. (%)	808	61.1	266	61.0	277	61.4	265	60.8
Diabetes - no. (%)								
Type I	8	0.6	4	0.9	1	0.2	3	0.7
Type II	396	29.9	132	30.3	116	25.7	148	33.9
Diet	24	1.8	8	1.8	7	1.6	9	2.1
Oral hypoglycemic agents	266	20.1	85	19.5	82	18.2	99	22.7

Insulin	106	8.0	39	8.9	27	6.0	40	9.2
Smoking - no. (%)								
Never	765	57.8	261	59.9	253	56.1	251	57.6
Remote	319	24.1	95	21.8	113	25.1	111	25.5
Active	239	18.1	80	18.3	85	18.8	74	17.0
Family history of CAD - no. (%)	197	14.9	71	16.3	56	12.4	70	16.1
Indication for angiography - no. (%)								
ACS	790	59.7	176	40.4	176	39.0	181	41.5
Staged PCI	150	11.3	54	12.4	52	11.5	44	10.1
Stable CAD	499	37.7	174	39.9	173	38.4	152	34.9
Atrial fibrillation - no. (%)	137	10.4	38	8.7	47	10.4	52	11.9
Previous History - no. (%)								
PCI	366	27.7	124	28.4	119	26.4	123	28.2
MI	308	23.3	107	24.5	92	20.4	109	25.0
CABG	86	6.5	26	6.0	28	6.2	32	7.3
PAD	89	6.7	25	5.7	30	6.7	34	7.8
CVA	86	6.5	24	5.5	23	5.1	39	8.9
Bleed	23	1.7	9	2.1	4	0.9	10	2.3
Heart failure	100	7.6	33	7.6	25	5.5	42	9.6
Medications - no. (%)								
ASA	1203	90.9	404	92.7	404	89.6	395	90.6
P2Y12	1183	89.4	401	92.0	395	87.6	387	88.8
ACEi/ARB	708	53.5	224	51.4	242	53.7	242	55.5
Beta-blocker	783	59.2	255	58.5	273	60.5	255	58.5
Calcium Channel Blocker	185	14.0	62	14.2	62	13.7	61	14.0
Statin	1070	80.9	364	83.5	360	79.8	346	79.4
Revascularized (stent + CABG) - no. (%)	675	51.0	238	54.6	214	47.5	223	51.1

Patient outcomes

Patient outcomes were assessed at one year from their revascularization procedure as previously described.(284) MACE occurred in 7.3% of patients at one-year. When stratifying outcomes by adenosine tertiles we noted no significant differences in MACE rates between the tertiles (log-rank p=0.83). Similarly, no differences in hazard ratios (HR) were noted when comparing the low to middle (HR 1.10; 95% CI, 0.68-1.78,

p=0.70) or low to high adenosine tertiles (HR 0.95; 95% CI, 0.56-1.57, p=0.84, **Figure 19a).**

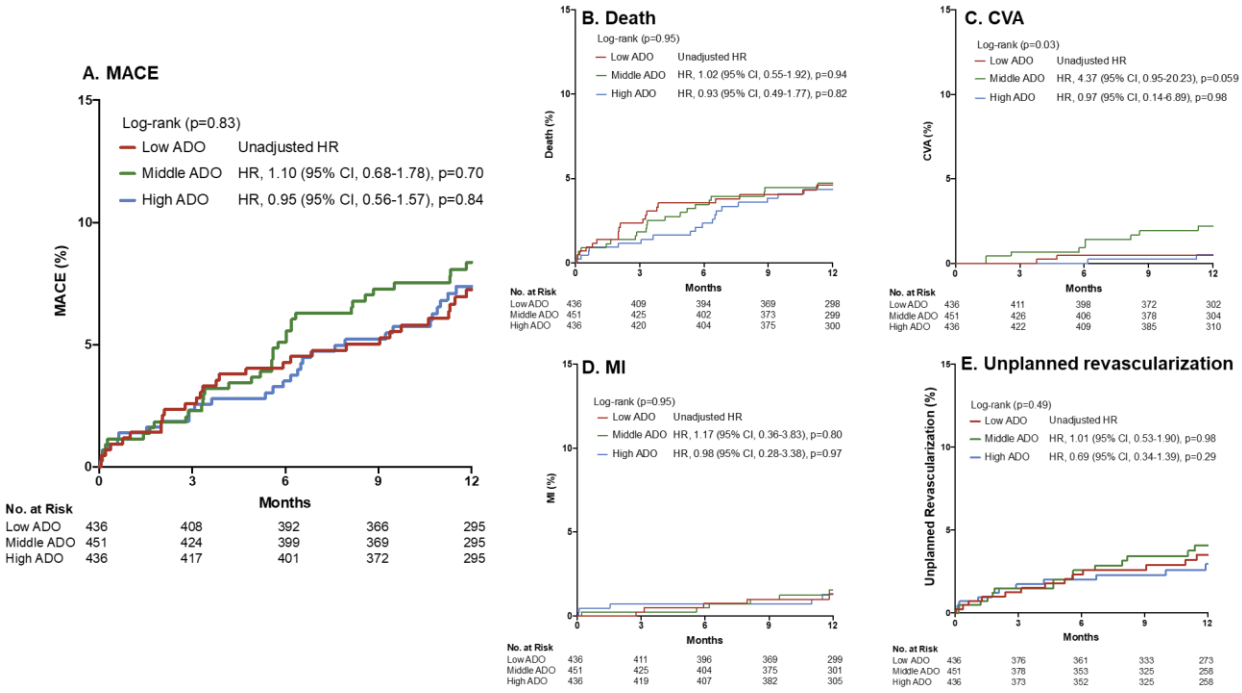

Figure 19. Adenosine and cardiovascular outcomes

(A) Major adverse cardiac events (MACE) including death, cerebrovascular accident (CVA), myocardial infarction (MI) and unplanned revascularization did not demonstrate any difference between middle or high adenosine tertiles. Similar trends were observed when analyzing by subgroups with no differences between tertiles noted for **(B)** Death, **(D)** MI**, (E)** Unplanned revascularization**. (C)** CVA – demonstrated higher incidence of stroke in the middle adenosine tertile. Adenosine levels stratified into tertiles (0-33rd, 34-66th, and 67-100th) for analysis. Kaplan-Meier curves generated and compared via log-rank with subsequent unadjusted hazard ratios (HR) compared by Cox proportional hazards model. P<0.05 was defined as statistically significant.

Secondary outcome analysis of the individual MACE components similarly revealed no changes amongst the subgroups **(Figure 19b-e)**: death (log-rank p=0.95) and low vs middle (HR 1.02, 95% CI 0.55-1.92, p=0.94) or low to high adenosine tertiles (HR 0.93, 95% CI 0.49-1.77, p=0.82); MI (log-rank p=0.95), low versus middle (HR 1.17, 95% CI 0.36-3.83, p=0.80) or low to high adenosine tertiles (HR 0.98, 95% CI 0.28-3.38,

p=0.98); or unplanned revascularization (log-rank p=0.49) and low versus middle (HR 1.01, 95% CI 0.53-1.90, p=0.98) or low versus high adenosine tertiles (HR 0.69, 95% CI 0.34-1.39, p=0.29). When assessing CVA individually there was a noted difference with CVA stratified by adenosine tertiles (log-rank p=0.03), with no differences observed between low versus middle (HR 4.37, 95% CI 0.95-20.23, p=0.059) or low versus high adenosine tertiles (HR 0.97, 95% CI 0.14-6.89, p=0.98) **(Figure 19c)**. Adjusted analyses for age, ACS, DM2, sex, CAD, revascularization, hypertension, and dyslipidemia did not change the observed trends with no difference seen in MACE between low versus middle (HR 1.11; 95% CI, 0.68-1.80, p=0.68) or low versus high adenosine tertiles (HR 1.05; 95% CI, 0.63-1.74, p=0.85) nor in unplanned revascularization between low versus middle (HR 1.06; 95% CI, 0.56-2.01, p=0.86) or low versus high adenosine tertiles (HR 0.70; 95% CI, 0.34-1.42, p=0.32**, Table 14**).

Table 14. Clinical outcomes predicted by plasma adenosine levels

	MACE			**Unplanned revascularization**		
	Event Rates (%)	**Hazard Ratio (95% CI)**	**p**	**Event Rates (%)**	**Hazard Ratio (95% CI)**	**p**
Low adenosine	7.1	-		4.4	-	
Unadjusted analysis (relative to low adenosine)						
Middle Adenosine	7.8	1.10 (0.68-1.78)	0.70	4.2	1.01 (0.53-1.90)	0.98
High Adenosine	6.9	0.95 (0.58-1.57)	0.84	3.0	0.69 (0.34-1.39)	0.29
Adjusted analysis (relative to low adenosine)*						
Middle Adenosine	7.8	1.11 (0.68-1.80)	0.68	4.2	1.06 (0.56-2.01)	0.86
High Adenosine	6.9	1.05 (0.63-1.74)	0.85	3.0	0.70 (0.34-1.42)	0.32

*adjusted for age (years), sex, type 2 diabetes, hypertension, dyslipidemia, coronary artery disease, acute coronary syndrome and revascularization

Sub-group analyses were performed to further assess three cohorts of patients – demonstrating no significant differences between adenosine tertiles in MACE or unplanned revascularization: (i) medical therapy for obstructive CAD **(Figure 20)** – MACE (log-rank p=0.16) and low versus middle (HR 0.68; 95% CI, 0.36-1.26, p=0.22) or low versus high adenosine tertiles (HR 0.54; 95% CI, 0.27-1.06, p=0.07) and unplanned revascularization (log-rank p=0.77) and low versus middle (HR 1.02; 95% CI, 0.41-2.58, p=0.96) or low versus high adenosine tertiles (HR 0.72; 95% CI, 0.26-2.03, p=0.54) (ii) Revascularization cohort **(Figure 21) –** MACE (log-rank p=0.16) and low versus middle (HR 1.89; 95% CI, 0.75-4.74, p=0.17) or low versus high adenosine tertiles (HR 2.34; 95% CI, 0.96-5.68, p=0.06) and unplanned revascularization (log-rank p=0.86) and low versus middle (HR 0.92; 95% CI, 0.32-2.08, p=0.86) or low versus high adenosine tertiles (HR 0.79; 95% CI, 0.31-2.00, p=0.62) and (iii) Acute coronary syndrome cohort **(Figure 22)** – MACE (log-rank p=0.32) and low versus middle (HR 0.57; 95% CI, 0.27-1.21, p=0.14) or low versus high adenosine tertiles (HR 0.74; 95% CI, 0.37-1.50, p=0.41) and unplanned revascularization (log-rank p=0.96) and low versus middle (HR 1.12; 95% CI, 0.45-2.75, p=0.81) or low versus high adenosine tertiles (HR 1.01; 95% CI, 0.40-2.55, p=0.98). Moreover, from a clinical perspective, those who experienced MACE were more likely to be older (72.4+/-11.5 versus 66.1+/-11.4 years, p<0.0001) and have hypertension (73.4% vs 64.1%, p=0.02), dyslipidemia (72.2% versus 59.6%, p=0.002), diabetes (46.2% vs 27.7%, p<0.0001) and a prior history of MI (30.4% vs 22.3%, p=0.02), CABG (10.8% vs 5.9%, p=0.02) and PAD (11.4% vs 6.1%, p=0.01). Patients with MACE were less likely to present as stable CAD (29.1% versus 38.9%,

p=0.02) and less likely to undergo revascularization (39.2% vs 52.6%, p=0.002) **(Table 15)**

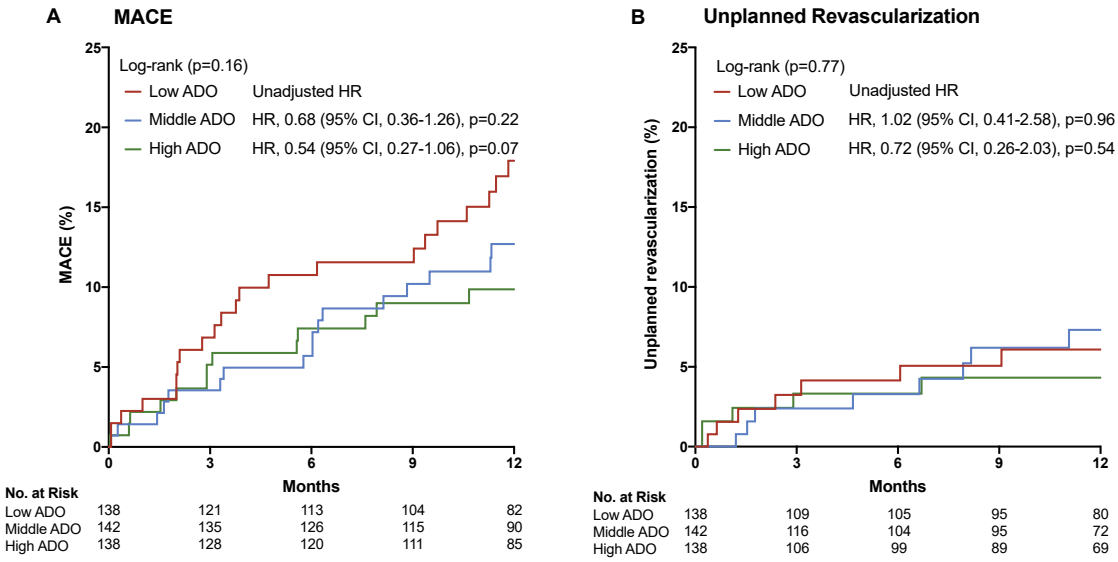

Figure 20. Outcomes stratified by adenosine levels in the medical therapy for obstructive CAD cohort.

(A) Major adverse cardiac events (MACE) including death, cerebrovascular accident (CVA), myocardial infarction (MI) and unplanned revascularization did not demonstrate any difference between middle or high adenosine tertiles nor when assessing unplanned revascularization (B) individually. Adenosine levels stratified into tertiles (33rd, 67th) for analysis. Kaplan-Meier curves generated and compared via log-rank with subsequent hazard ratios (HR) compared using Cox proportional hazards model with $p<0.05$ as significant. ADO, adenosine levels; CAD, coronary artery disease.

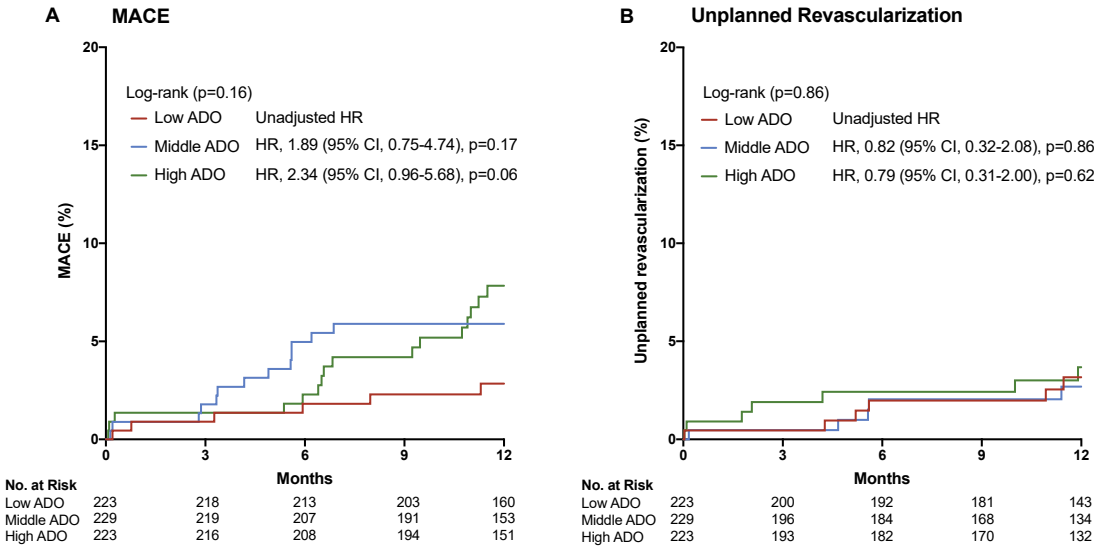

Figure 21. Outcomes stratified by adenosine levels in the revascularization cohort.

(A) Major adverse cardiac events (MACE) including death, cerebrovascular accident (CVA), myocardial infarction (MI) and unplanned revascularization did not demonstrate any difference between middle or high adenosine tertiles nor when assessing unplanned revascularization (B) individually. Adenosine levels stratified into tertiles (33rd, 67th) for analysis. Kaplan-Meier curves generated and compared via log-rank with subsequent hazard ratios (HR) compared using Cox proportional hazards model with $p<0.05$ as significant. ADO, adenosine levels.

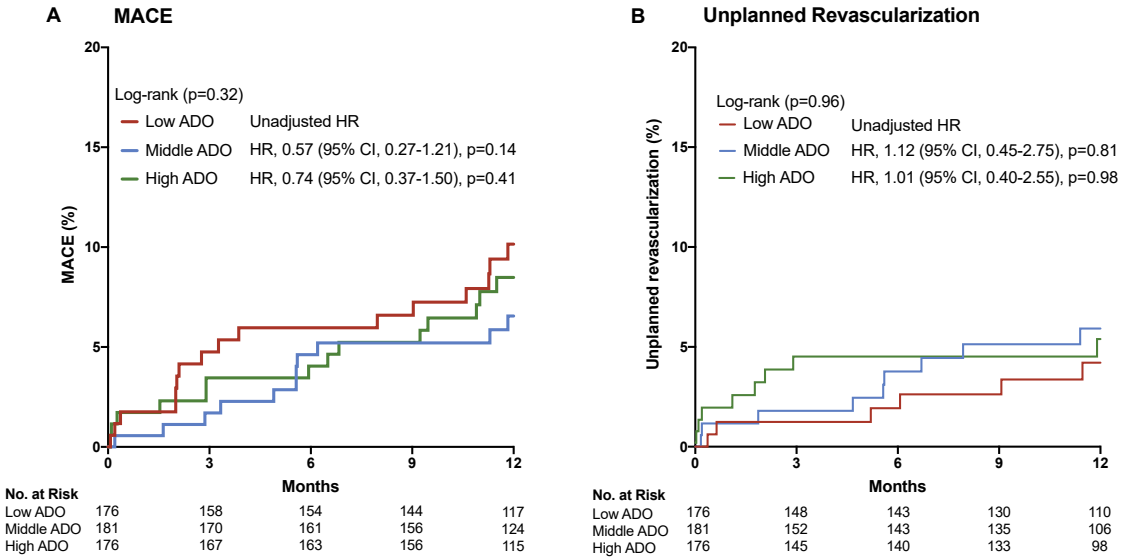

Figure 22. Outcomes stratified by adenosine levels in the acute coronary syndrome cohort

(A) Major adverse cardiac events (MACE) including death, cerebrovascular accident (CVA), myocardial infarction (MI) and unplanned revascularization did not demonstrate any difference

between middle or high adenosine tertiles nor when assessing unplanned revascularization (B) individually. Adenosine levels stratified into tertiles (33rd, 67th) for analysis. Kaplan-Meier curves generated and compared via log-rank with subsequent hazard ratios (HR) compared using Cox proportional hazards model with $p<0.05$ as significant. ADO, adenosine levels

Table 15. Baseline characteristics by MACE

Major adverse cardiovascular events (MACE)	Absent (n=1165)		Present (n=158)		
	N	%	N	%	P value
Age - mean +/- SD	66.1	11.4	72.4	11.5	<0.0001
Sex (female) - no. (%)	333	28.6	54	34.2	0.15
Hypertension - no. (%)	747	64.1	116	73.4	0.02
Dyslipidemia - no (%)	694	59.6	114	72.2	0.002
Diabetes - no. (%)					
Type I	8	0.7	0	0.0	
Type II	323	27.7	73	46.2	<0.0001
Diet	20	1.7	4	2.5	
Oral hypoglycemic agents	218	18.7	48	30.4	
Insulin	85	7.3	21	13.3	
Smoking - no. (%)					0.46
Never	674	57.9	91	57.6	
Remote (quit >1 month ago)	276	23.7	43	27.2	
Active	215	18.5	24	15.2	
Family history of CAD - no. (%)	178	15.3	19	12.0	0.28
Indication for angiography - no. (%)					
ACS	459	39.4	74	46.8	0.07
Staged PCI	138	11.8	12	7.6	0.11
Stable CAD	453	38.9	46	29.1	0.02
Atrial fibrillation - no. (%)	114	9.8	23	14.6	0.06
Previous History - no. (%)					
PCI	317	27.2	49	31.0	0.32
MI	260	22.3	48	30.4	0.02
CABG	69	5.9	17	10.8	0.02
PAD	71	6.1	18	11.4	0.01
CVA	70	6.0	16	10.1	0.049
Bleed	18	1.5	5	3.2	0.14
Heart failure	83	7.1	17	10.8	0.10
Medications - no. (%)					
ASA	1058	90.8	145	91.8	0.69
P2Y12	1043	89.5	140	88.6	0.72
ACEi/ARB	629	54.0	79	50.0	0.35
Beta-blocker	688	59.1	95	60.1	0.80
Calcium Channel Blocker	156	13.4	29	18.4	0.09
Statin	935	80.3	135	85.4	0.12
Revascularized (stent + CABG) - no. (%)	613	52.6	62	39.2	0.002

Finally, we evaluated adenosine values within each outcome cohort with no significant differences noted in any comparison **(Figure 23)**: (i) MACE - absent (n=1227) versus present (n=96) (902.2nM [621.4-1292.3nM] versus 914.2 585.0-1324.5nM], p=0.86) (ii) Death - absent (n=1266) versus present (n=57) (904.1nM [621.4-1285.2 nM] versus 857.0nM [540.8-1418.7nM], p=0.68) (iii) MI - absent (n=1307) versus present (n=16) (902.8nM [619.9-1292.3 nM] versus 838.7nM [562.8-1294.9nM], p=0.77) (iv) CVA - absent (n=1310) versus present (n=13) (902.2nM [619.1-1295.7nM] versus 953.2nM [802.0-1007.3nM], p=0.82) (v) Unplanned revascularization - absent (n=1272) versus present (n=51) (904.3nM [619.9-1297.9 nM] versus 770.7nM [615.4-1144.8],p=0.26).

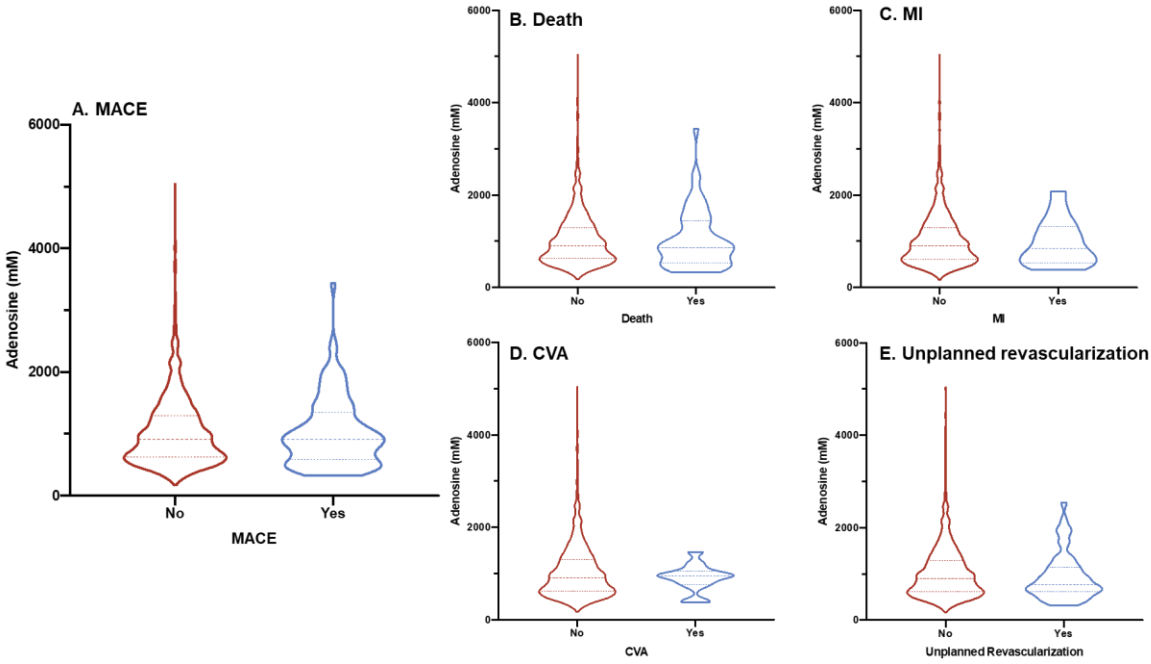

Figure 23. Comparison of adenosine levels across cardiovascular outcome groups.

Circulating adenosine values presented according to each cohort with no significant differences noted between: (A) MACE - absent (n=1165) versus present (n=158) (902.8nM [619.9-1285.2nM] versus 904.0 [616.7-1295.7nM], p=0.99). (B) Death - absent (n=1255) versus present (n=68) (903.3nM [621.4-1283.1 nM] versus 897.8nM [543.9-1458.1nM], p=0.99). (C) MI - absent (n=1293) versus present (n=30) (902.8nM [619.9-1292.3 nM] versus 907.0nM [709.2-1341.0nM], p=0.63). (D) CVA - absent (n=1294) versus present (n=29) (897.6nM [618.3-1292.3nM] versus 970.0nM [837.0-1256.7nM], p=0.25). (E) Unplanned revascularization -

absent (n=1263) versus present (n=60) (905.7nM [619.6-1302.2 nM] versus 789.8nM [616.0-1127.2],p=0.15). Data was presented as Median(IQR) and compared using Mann-Whitney U-test. P<0.05 was defined as statistically significant.

4.6 Discussion

In the current study, we sought to evaluate the ability of plasma adenosine levels to risk stratify patients and predict major adverse cardiovascular events. Contrary to our hypothesis, adenosine levels were not found to predict MACE rates at one year of follow-up, nor did it predict any of the individual components. Multivariable adjustment for factors known to influence outcomes and adenosine levels similarly did not demonstrate a clear benefit of adenosine for risk stratifying patients at the time of invasive angiography. Hence, this study does not support the use of plasma adenosine in risk stratification of cardiac patients for 1 year events.

Prior to our analysis, adenosine's role as a predictor of cardiovascular outcomes was inconclusive. Individuals with genetic mutations (AMPD1) leading to reduced adenosine deaminase (ADA) activity provide a unique perspective on the role of adenosine in cardiovascular outcomes. Elevated adenosine levels have been noted in heart failure (HF) patients previously, supporting a compensatory role as a counter-regulatory molecule in response to rising catecholamine levels endemic to HF patients.[229] Therapeutically, dipyridamole-induced adenosine augmentation was suggested to improve HF severity.(340) Moreover, less-adverse outcomes have been noted in HF patients with AMPD1 mutations; though adenosine levels were not assessed, limiting discernment as to whether circulating or tissue adenosine led to the observed changes.(230,231) Indeed, AMPD1 is most active in the skeletal muscle, suggesting tissue levels may be more

directly affected.(341) Similarly, when 367 patients with obstructive CAD were stratified by AMPD1 status, they demonstrated improved CV outcomes, though adenosine levels were not quantified.(243) While 161 patients undergoing revascularization (PCI and CABG) did not demonstrate differential circulating adenosine levels by AMPD1 status, nor did AMPD1 status predict CV outcomes, suggesting alternative pathways for its observed effects.(248) Indeed, these discrepant results reflect an incomplete understanding of adenosine biology, in that the observed outcome effects may actually be driven by modification of local cardiac/vascular adenosine production with systemic adenosine levels providing a relatively minor role as supported by work in HF patients.(248,342) Similarly, our findings may suggest that local adenosine levels may be more relevant and predictive of vascular healing. While coronary sampling has demonstrated reduced local adenosine levels post PCI(247), the technical barriers of this approach would preclude meaningful adoption as a clinical tool.

While circulating adenosine levels may not predict future events, this does not preclude adenosine as a viable therapeutic target, particularly when considering its tissue-level characteristics. As discussed, numerous agents known to modulate adenosine have shown favorable impacts on vascular healing and cardiovascular outcomes including dipyridamole,(311) cilostazol, (337)-(338) and ticagrelor.(147) While all of these agents have demonstrated favorable impact on clinical outcomes, the mechanism of action may be on local rather than circulating adenosine levels. Dipyridamole is commonly used in IV formulation leading to transient peaks in central adenosine levels(72) lessening with

repeated stimuli(120), while sustained oral administration up to 5 days demonstrated early changes in circulating adenosine, lessening after 48 hours.(121) However, dipyridamole is known to more potently inhibit ENT-1 than ticagrelor, prolonging detectable levels of adenosine following adenosine administration in their presence.(122) While ticagrelor has been shown to augment circulating adenosine in the setting of ACS, other studies have failed to consistently demonstrate this effect.(149,313) Taken together, these findings suggest that while therapeutic modulation of adenosine may represent a viable target, the effect on adenosine may occur briefly at a systemic level, with more sustained augmentation locally. Moreover, adenosine receptor levels have been shown to dynamically respond to changes in adenosine levels, reflecting yet another pathway by which vasculoprotective effects are mediated, while not appreciably impacting systemic adenosine levels.(343) Indeed, using systemic adenosine levels as a target diagnostically or therapeutically is complicated by its innate properties, variations between local and systemic levels and the complex homeostatic mechanisms which regulate tissue levels. Hence, while monitoring adenosine levels may not provide prognostication abilities, modulating adenosine biology still carries significant promise as a therapeutic approach and remains the focus of ongoing investigation.

Our study is not without limitations. First, our population is heterogenous with a variety of indications for invasive angiography and differential revascularization strategies, though assessment of these various sub-groups similarly failed to demonstrate difference in levels. Second, our follow-up period was limited to one year and we cannot rule out the possibility that adenosine may predict longer term outcomes; however, we are unable

to determine this within the current study. Finally, systemic quantification of adenosine levels are subject to biological and analytical variation which may impact levels quantified.(284)

4.7 Conclusion

Systemic adenosine levels are not predictive of cardiovascular events in patients undergoing angiography. While therapeutic targeting of adenosine and associated pathways may yet be beneficial, risk stratification using this molecule is not clinically useful.

4.8 Funding sources

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4.9 Conflicts of Interest

None declared

Chapter 5

Revisiting the Evidence for Dipyridamole in Reducing Restenosis: A Systematic Review and Meta-analysis

5.1 Preface

This chapter has been previously published in the *Journal of Cardiovascular Pharmacology*

> **Simard T,** Motazedian P, Dhaliwal S, Di Santo P, Jung RG, Ramirez FD, Labinaz A, Short S, Parlow S, Joseph J, Rasheed A, Rockley M, Marbach J, Domecq MC, Russo JJ, Chong AY, Beanlands RS, Hibbert B. Revisiting the Evidence for Dipyridamole in Reducing Restenosis: A Systematic Review and Meta-analysis. J Cardiovasc Pharmacol. 2021 Apr 1;77(4):450-457. doi: 10.1097/FJC.0000000000000976. PMID: 33760800.

> *All authors made substantial contributions to the conception/ design of the work; and/or the acquisition, analysis, or data interpretation; and, (ii) drafted and/or revised the content critically; and, (iii) provided final approval of the published version; and, (iv) agree to be accountable for the work.*

5.2 Abstract

Atherosclerosis remains a leading cause of morbidity and mortality, with revascularization remaining a cornerstone of management. Conventional revascularization modalities remain challenged by target vessel re-occlusion – an event driven by mechanical, thrombotic and proliferative processes. Despite considerable advancements, restenosis remains the focus of ongoing research. Adjunctive agents, including dipyridamole, offer a multitude of effects which may improve vascular homeostasis. We sought to quantify the potential therapeutic impact of dipyridamole on vascular occlusion.

We performed a literature search (EMBASE, Medline) examining studies which encompassed three areas: (1) one of the designated medical therapies applied in (2) the setting of a vascular intervention with (3) an outcome including vascular occlusion rates and/or quantification of neointimal proliferation/restenosis. The primary outcome was vascular occlusion rates. Secondary outcome was the degree of restenosis by neointimal quantification. Both human and animal studies were included in this translational analysis.

6,839 articles were screened from which 73 studies were included, encompassing 16,146 vessels followed up for a mean 327.3 days (range 7-3650 days). Pre-clinical studies demonstrate that dipyridamole results in reduced vascular occlusion rates (24.9% versus 48.8%, RR 0.53 [95% CI 0.40-0.70], I^2 = 39%, p<0.00001), owing to diminished neointimal proliferation (SMD -1.13 [95% CI -1.74- -0.53], I^2=91%, p=0.0002). Clinical studies similarly demonstrated reduced occlusion rates with dipyridamole therapy (23.5% versus 31.0%, RR 0.77[95% CI 0.67-0.88], I^2=84%, p<0.0001).

Dipyridamole may improve post-intervention vascular patency and mitigate restenosis. Dedicated studies are warranted to delineate its role as an adjunctive agent following revascularization.

5.3 Introduction

Atherosclerosis involving the coronary, cerebral and peripheral vasculature is a leading cause of morbidity and mortality.(258,259) Revascularization of obstructive stenoses is

typically achieved via either surgical means with endarterectomy or bypass grafting or percutaneous means with angioplasty and stenting. In coronary interventions, balloon angioplasty alone was limited by abrupt vessel closure, arterial recoil and dissection with 15% experiencing acute occlusion. Comparatively, restenosis occurs later and can affect 35-40% of patients.(344) Mechanical support in the form of metallic scaffolds, known as stents, improved luminal diameters and acute complications with reduced repeat revascularizations, though restenosis still persisted in 20% of patients at 6 months.(344) In response, drug-eluting stents (DESs) carry an anti-proliferative agent to mitigate restenosis, though at the cost of increased risk of stent thrombosis due to delayed healing serving as a nidus for thrombus formation.(345) Despite ongoing improvements, contemporary DESs still yield repeat revascularization rates of 3.3% annually.(270) From a surgical perspective, coronary grafting with contemporary coronary-artery bypass grafting (CABG) techniques yield graft failure rates of 5% for arterial and up to 25% for venous conduits at one year.(274) Similarly, bypass grafts for peripheral arterial disease fail at rates of 10-20% per year depending on the location, grafts and approaches utilized.(346) Improving the patency rates of revascularization modalities remains the focus of ongoing efforts.(244,347)

The mechanisms leading to re-occlusion of revascularized vessels are multifactorial and incompletely understood. First, technical factors, including vascular recoil leading to physical occlusion, balloon injury and dissection and anastomotic techniques/graft materials all contribute. Second, platelet and fibrin deposition with subsequent thrombus formation can also lead to acute vessel occlusion (i.e. stent thrombosis) or form the

framework for subsequent restenosis. Last, the growth of hyper-proliferative "scar tissue", known as neointima (NI), in response to vascular injury is also described.(244) The pathophysiology behind NI formation remains elusive, reflecting the complexities of this biological process.(31) Early thrombotic processes give way to inflammatory, smooth muscle and even circulating progenitor cell involvement, reflecting an imbalance in vascular homeostasis.(31) Hence, ongoing efforts to mitigate restenosis and improve vascular patency continue, addressing numerous potential targets.(66,311,348-350)

Adjunctive agents have shown promise in improving vascular healing. Dipyridamole (DP), a phosphodiesterase (PDE) 5/6 inhibitor, prevents the breakdown of both cAMP (leading to inhibition of platelet aggregation and increasing vasodilation) and cGMP (augmenting vasodilation and smooth muscle cell relaxation).(113) Moreover, DP is known to enact further vasculoprotective effects via augmenting extracellular adenosine levels via re-uptake inhibition – leading to vasodilation and inhibiting proliferation and inflammatory effects.(311) To date, DP has primarily been employed for its vasodilatory and anti-platelet effects, though pre-clinical and clinical data strongly suggest an added benefit in mitigating vascular restenosis.(311) Other approaches similarly focus on modulating cAMP levels. Cilostazol, augments cAMP/cGMP via PDE3 inhibition and may modestly augment adenosine levels(337), reducing smooth muscle cell proliferation and NI hyperplasia, mitigating restenosis following coronary stenting.(338) Moreover, preclinical studies with forskolin (activating adenylyl cyclase to augment cAMP)(351) or direct administration of exogenous cAMP(352) have similarly shown promise in mitigating NI proliferation. While DP has yielded way to more potent antiplatelet

regimens, a host of data and alternative mechanisms may support a contemporary role. Accordingly, assessing both preclinical and clinical studies, we sought to quantify the therapeutic benefit of adjunctive agents for preserving vascular patency following revascularization.

5.4 Methods

Systematic Review

A medical librarian (MCD) designed the literature search using key terms and headings. Databased interrogated included Medline (1946-) and Embase (1947-) using search strategies in each as outlined **(**

Figure 24) for English-language articles to a final search date of April 2019**.** Studies of interest encompassed three broad areas of interest; (1) one of the designated medical therapies applied in (2) the setting of a vascular intervention with (3) an outcome reported that included vascular occlusion rates and/or quantification of neointimal proliferation/restenosis as a continuous variable. Details of each include: (1) Medical therapies of interest included adenosine/adenosine-receptor/purine-receptor modulating agents, DP (a.k.a. persantine, Aggrenox) and ticagrelor (a.k.a Brilinta). (2) Interventions of interest included balloon angioplasty, stenting, arterial injury (wire, balloon, anastomosis), bypass grafting (venous, arterial, prosthetic grafts) applied to coronary, carotid or peripheral vascular beds. Both randomized and retrospective studies were included addressing human and animal species in this translational analysis. Exclusions included insufficient data reporting, lack of suitable control group (no therapy/placebo/anti-platelet included), lack of vascular intervention, lack of either

patency or NI outcomes. Protocol is under assessment with PROSPERO. Studies were reviewed for inclusion and data of included studies was extracted by two authors with a third reviewer to verify any disagreements which were resolved by consensus. Quality assessment was performed using previous approaches(271,353) employing the Cochrane risk-of-bias tool for randomized clinical studies **(Figure 25)**.(354)

Database: Ovid MEDLINE(R) ALL <1946 to April 08, 2019>
Search Strategy:

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1 exp Adenosine/ (49642)
2 exp Receptors, Purinergic P1/ (7500)
3 (Adenosine or Dipyridamole or Persantine or Aggrenox or Ticagrelor or Brilinta or Regadenoson or ADORA or (Purinergic adj2 receptor*) or (Purine adj2 receptor*)).tw,kf. (118213)
4 or/1-3 (145151)
5 Neointima/ (1683)
6 coronary stenosis/ or coronary restenosis/ (17404)
7 angioplasty/ or exp angioplasty, balloon/ (58358)
8 Graft Occlusion, Vascular/ (10075)
9 ((Angioplast* adj2 balloon*) or Neointima or (Intimal adj2 thickening) or (Tunica adj2 intima) or ISR or Restenosis or (Lumen adj2 loss) or (Luminal adj2 area) or (Luminal adj2 diameter) or Patency or Occlusion or (stenosis adj2 (coronary or vessel?))).tw,kf. (209402)
10 or/5-9 (257190)
11 4 and 10 (4028)
12 limit 11 to english (3764)
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Database: Embase Classic+Embase <1947 to 2019 April 08>
Search Strategy:

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1 adenosine/ (44188)
2 exp purinergic receptor/ (11689)
3 exp adenosine receptor/ (15389)
4 (Adenosine or Dipyridamole or Persantine or Aggrenox or Ticagrelor or Brilinta or Regadenoson or ADORA or (Purinergic adj2 receptor*) or (Purine adj2 receptor*)).tw. (143511)
5 or/1-4 (164142)
6 neointima/ (4117)
7 "stenosis, occlusion and obstruction"/ (7082)
8 restenosis/ or in-stent restenosis/ (30993)
9 angioplasty/ or percutaneous transluminal angioplasty/ or transluminal coronary angioplasty/ (76095)
10 coronary artery obstruction/ (33333)
11 ((Angioplast* adj2 balloon*) or Neointima or (Intimal adj2 thickening) or (Tunica adj2 intima) or ISR or (Lumen adj2 loss) or (Luminal adj2 area) or (Luminal adj2 diameter) or Patency or Occlusion or (stenosis adj2 (coronary or vessel?))).tw. (278317)
12 or/6-11 (368567)
13 5 and 12 (6471)
14 limit 13 to english (6080)
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Figure 24. Search strategy

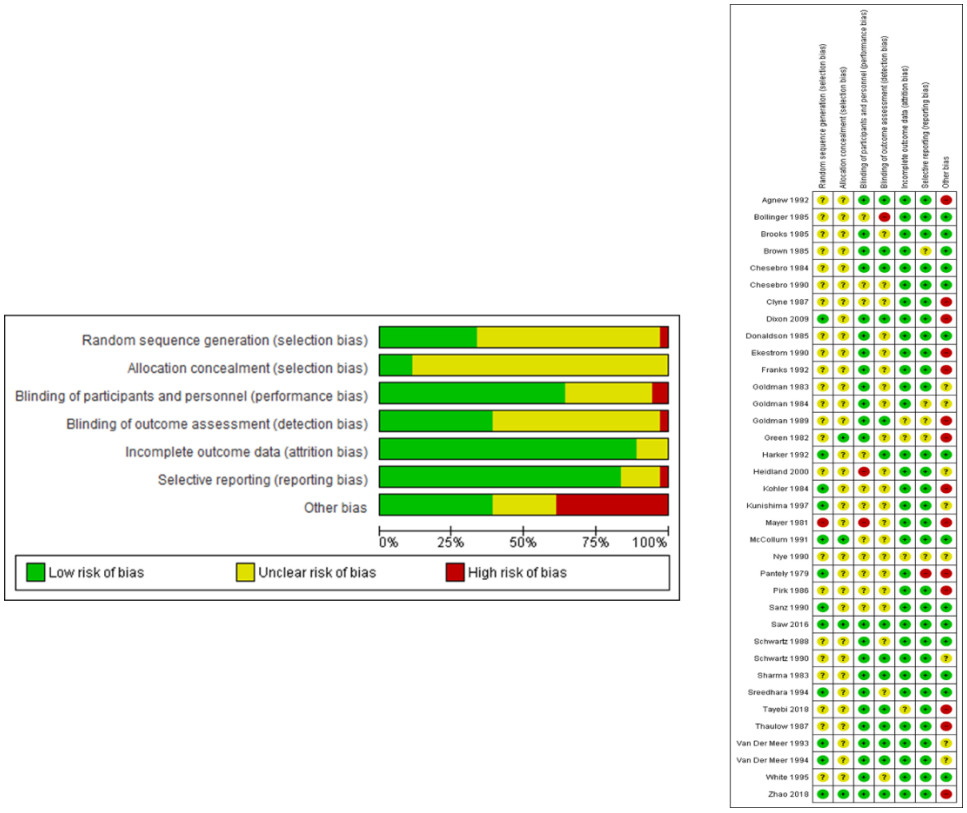

Figure 25. Cochrane risk of bias assessment for randomized clinical studies

Included Studies

Following exclusion of duplicate articles, 6,839 titles and abstracts were screened from which 223 underwent full text assessment, yielding 73 articles for analysis **(Figure 26).** Data was preferentially extracted at a vessel versus subject-level, when available. These 73 articles encompassed 16,146 vessels for analysis (individual study range from 7-2237 vessels, mean 221.2 vessels) followed up for a mean 327.3 days (range 7-3650 days). Vascular beds assessed varied by study including 6 native coronary studies, 20 CABG studies, 9 carotid studies, 4 aorta studies, 32 peripheral studies and 2 multiple site studies. 53 studies (15,107 vessels) reported occlusion rates alone, 14 studies (403 vessels) reported NI size alone, while 6 studies (636 vessels) reported both occlusion rates and NI

size. Funnel plots for publication bias demonstrated moderate symmetry by visual analysis **(Figure 27)**.

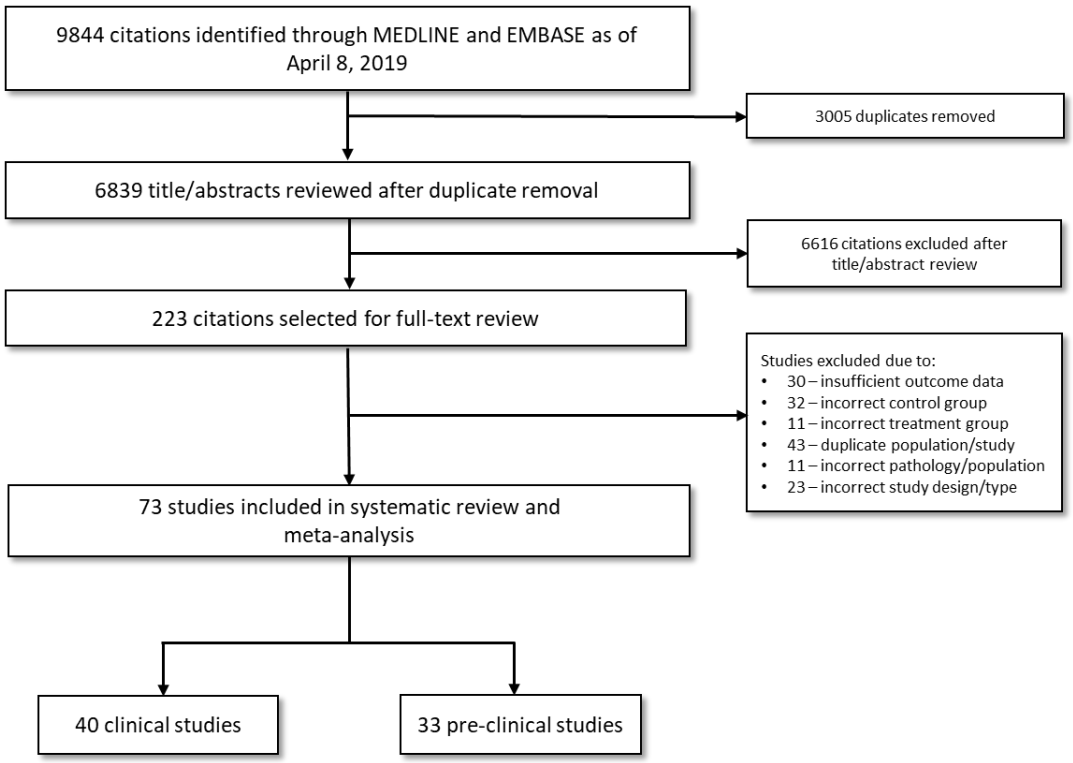

Figure 26. Study flow diagram

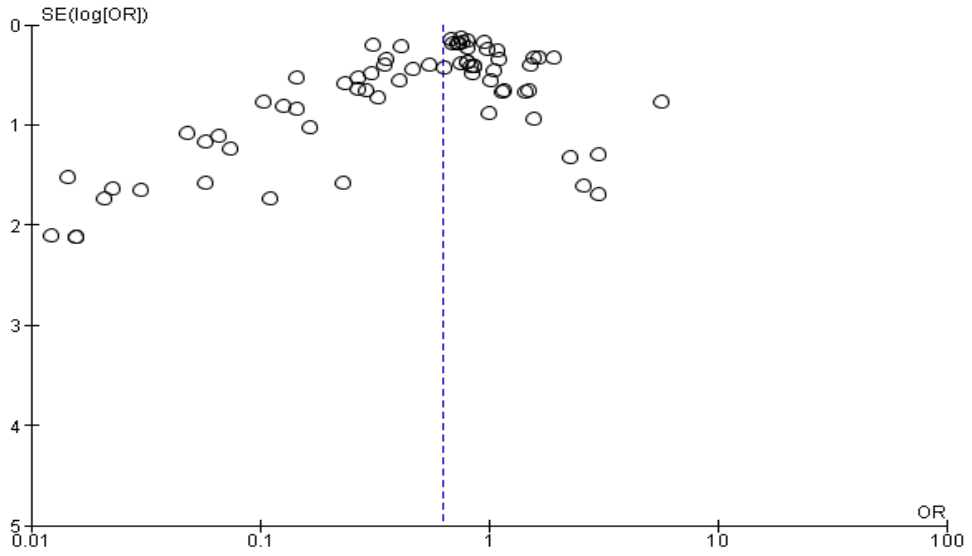

Figure 27. Assessment of publication bias in all studies via visual assessment with funnel plot

The impact of DP on vascular occlusion was assessed in 26 preclinical studies (n=1046). These studies were divided across varying species as follows: 2 goat/sheep studies, 16 dog studies, 7 rat/mice studies, 5 rabbit studies, 2 primate studies and 1 swine study. Average follow-up was 70.7 days (range 7-480 days). In total, 896 vessels were assessed for patency for preclinical studies. Secondarily, we assessed the impact of DP therapy on NI formation in preclinical studies. A total of 20 studies (1039 vessels) reported NI quantifications across varying treatments and subjects with study breakdown as follows: 2 human studies, 8 dog studies, 5 mice/rat studies, 3 rabbit studies, 1 primate study and 1 swine study.

Clinical assessment included a total of 37 studies including 13,339 vessels were assessed with an average of 360.5 vessels/study (range 38-2237 vessels/study). Studies assessed

various vascular beds dichotomized into non-peripheral (coronary – 5 studies, CABG – 16 studies) and peripheral (2 carotid studies, 14 peripheral extremity studies). Quality assessments were performed as described yielding moderate level of quality for randomized clinical studies **(Figure 25)**. Interventions performed included balloon angioplasty (4 studies), autografting (18 studies), prosthetic grafting (6 studies), arterial injury (2 studies), and mixed interventions (7 studies). 36 (90%) of clinical studies were randomized clinical trials. Follow-up was 565.9 days (range 28 – 3650 days). 33 (89.2%) of the studies compared DP therapy to placebo/vehicle controls with the remaining 4 studies (10.8%) comparing to ASA.

Outcomes and analysis

The primary outcome was assessment of vascular patency/occlusion rates. Secondary outcome of interest was NI quantification. Studies which reported absolute NI proliferation as a continuous variable were included to quantify the magnitude of NI reduction achieved with therapies. Varying means of quantification of NI volumes were encountered (1) NI thickness (mm), (2) NI area (mm^2), (3) NI:media ratio and (4) percentage of restenosis (%). If NI size was quantified in multiple locations then the most distal vessel values were employed. These values were analyzed as reported and standardized mean differences (SMD) were employed to unify the magnitude of treatment effect across multiple output formats while utilizing a random-effects model given the variability in populations presented. For errors reported, standard calculations for error were performed, while standard errors and median/interquartile ranges were converted to mean/SD using previously reported methods.(355,356) Analyses was

completed with Review Manager (RevMan) 5.3 (Cochrane Collection, Copenhagen, Denmark).

5.5 Results

Pre-clinical studies

When compared to standard therapy, DP reduced the rate of vascular occlusion by half with 109/437 (24.9%) occlusion events in the treatment arm and 224/459 (48.8%) occlusion events in the control arm (RR 0.53 [95% CI 0.40-0.70], I^2 = 39%, p<0.00001, **Figure 28**). Sensitivity analyses were also performed to assess treatment subgroups (i) No DP vs DP – 5 studies with 194 vessels (RR 0.66[95% CI 0.28-1.54], I^2=59%, p=0.33, **Figure 29**), (ii) No DP versus ASA/DP – 15 studies with 522 vessels (RR 0.46[95% CI 0.33-0.62], I^2=27%, p<0.00001, **Figure 30),** (iii) ASA versus ASA/DP, 5 studies with 190 vessels (RR 0.75[95% CI 0.26-2.17], I^2=44%, p=0.6, **Figure 31**).

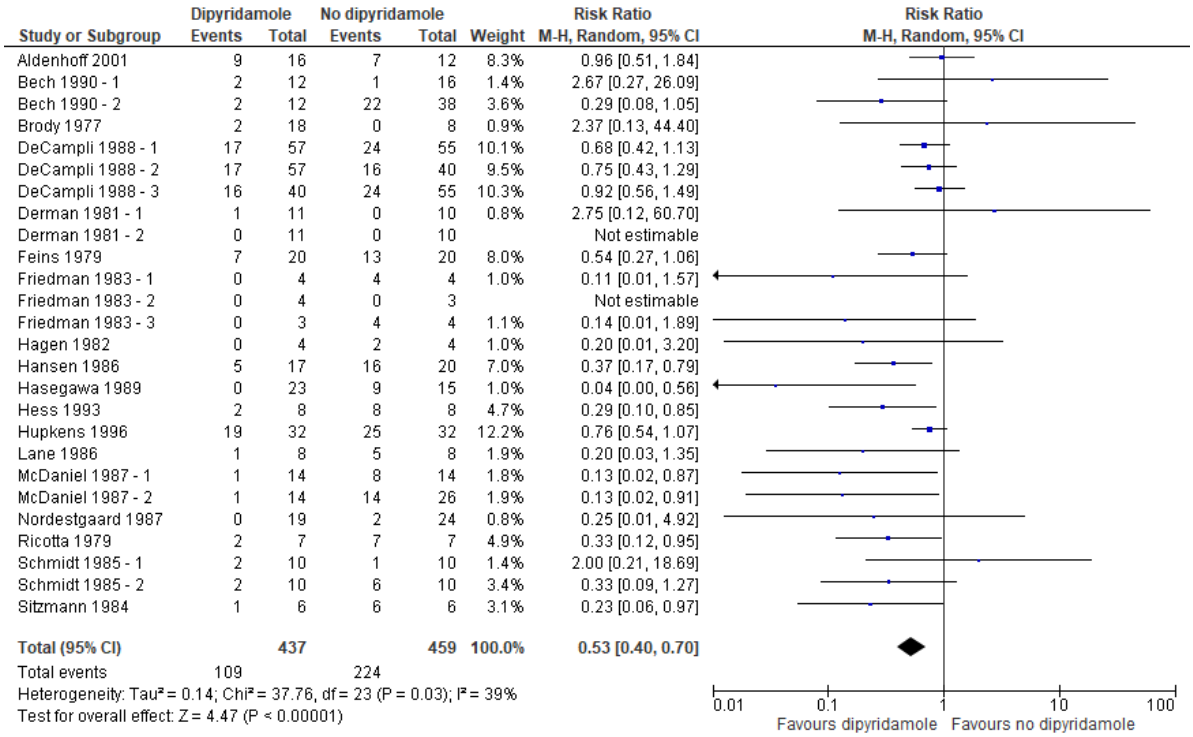

Figure 28. Impact of dipyridamole on vascular occlusion rates in pre-clinical studies

Forest plot depicting all studies assessing no dipyridamole versus dipyridamole for vascular occlusion rates in non-human models.

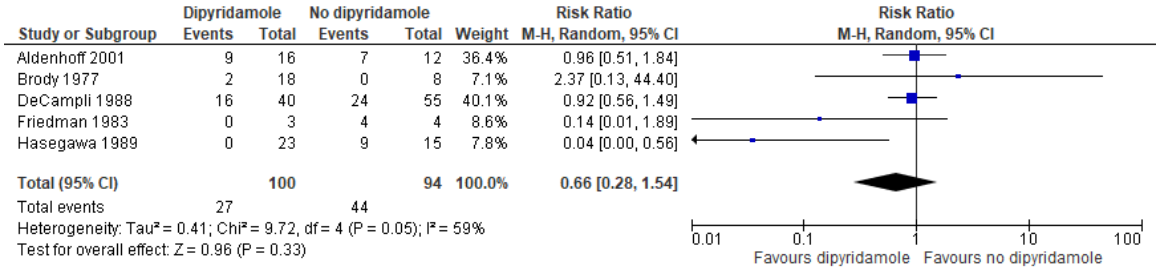

Figure 29. Pre-clinical - No dipyridamole versus dipyridamole monotherapy on vascular occlusion rates.

Forest plot depicting all studies assessing no dipyridamole versus dipyridamole monotherapy for vascular occlusion rates in non-human models.

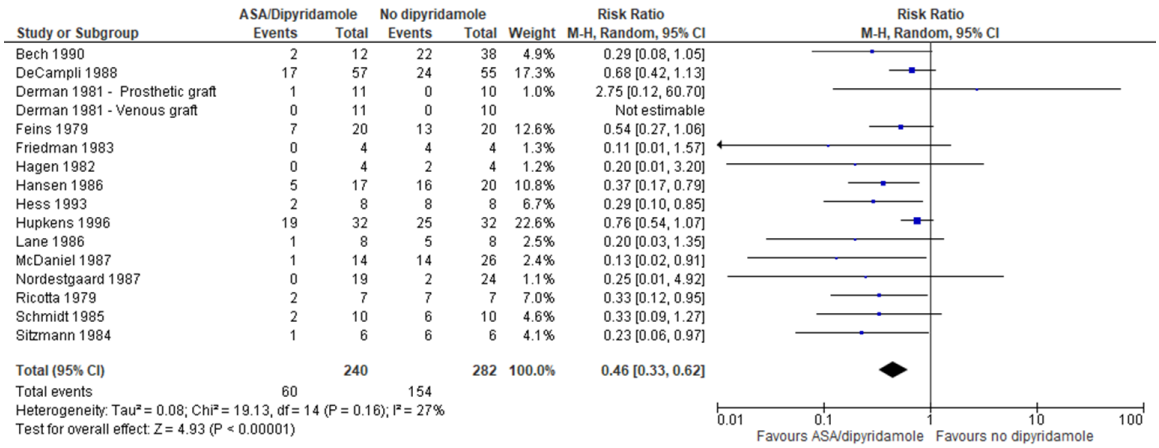

Figure 30. Pre-clinical - No dipyridamole versus ASA/dipyridamole on vascular occlusion rates.

Forest plot depicting all studies assessing no dipyridamole versus ASA/dipyridamole for vascular occlusion rates in non-human models.

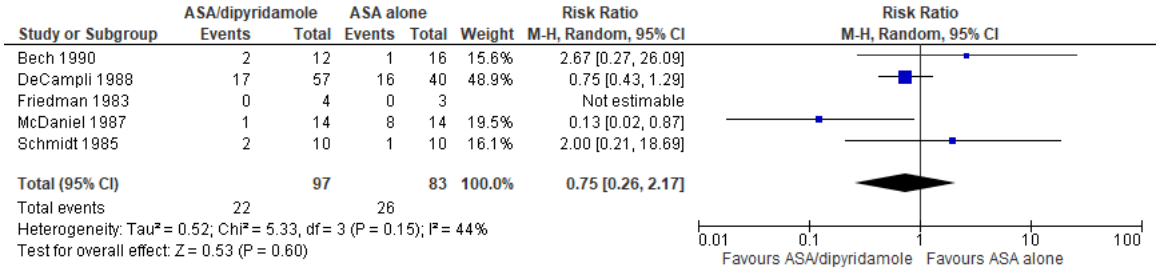

Figure 31. Pre-clinical - ASA monotherapy versus ASA/dipyridamole on vascular occlusion events.

Forest plot depicting all studies assessing ASA monotherapy versus ASA/dipyridamole for vascular occlusion rates in non-human models.

Specifically, NI development was reduced with DP therapy when compared to no DP therapy in 681 vessels assessed (standard mean difference (SMD) -1.13 [95% CI -1.74- -0.53], I^2=91%, p=0.0002, **Figure 32**). In addition to DP's modest anti-platelet effects, DP is also known to augment cAMP and adenosine levels – possibly mediating additional pleiotropic vasculoprotective effects outside of anti-platelet effects which do not clearly impact NI formation. Accordingly, we assessed other adjuvant therapies noted in our

search targeting cAMP modulation (exogenous cAMP analog(352) and cilostazol(357-359)) and adenosine modulation (exogenous adenosine(339), adenosine analogs(33), adenosine receptor 2B agonist(360) and ticagrelor(361)) on neointimal proliferation. Both cAMP modulation (SMD -1.23[95% CI -2.20- -0.27, I^2=70%, p=0.01) and adenosine modulation (SMD -1.14 [95% CI -1.59- -0.69, I^2=5%, p<0.00001) demonstrated diminished NI sizes **(Figure 33).** Indeed, just two studies reported on vascular patency with cAMP modification, noting beneficial effects.(351,358)

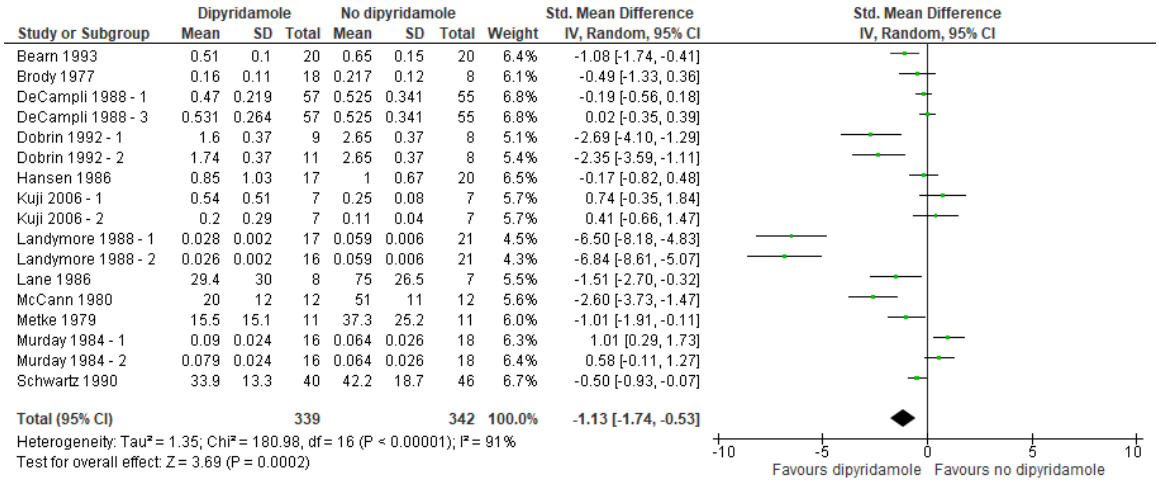

Figure 32. Impact of dipyridamole on neointimal proliferation.

Forest plot depicting all studies assessing no dipyridamole versus dipyridamole and reporting neointimal quantification.

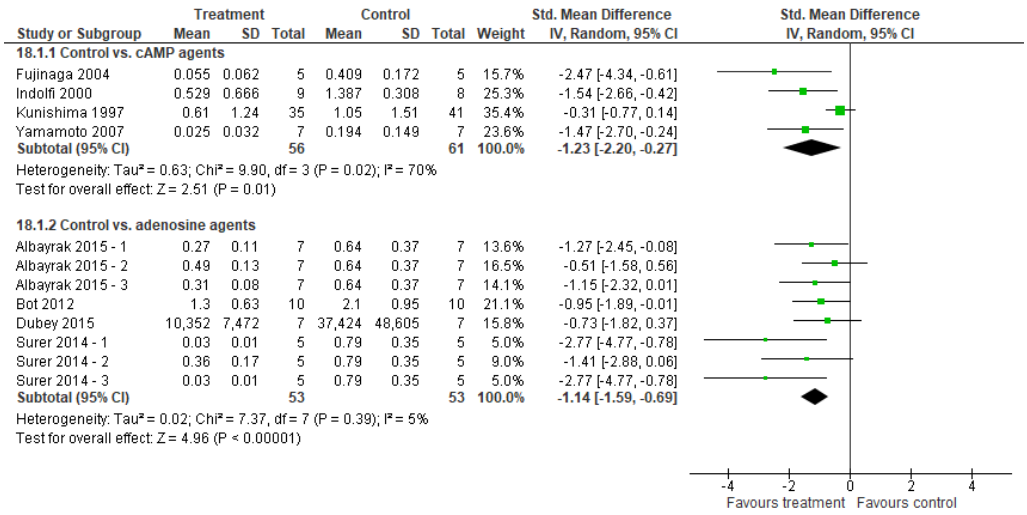

Figure 33. Impact of adjunctive agents on neointimal proliferation.

Forest plot depicting all studies assessing agents which augment cyclic AMP (cAMP) (upper panel) and adenosine levels (lower panel) and reporting neointimal quantification.

Clinical studies

The clinical use of DP in humans was assessed in 14,007 vessels, demonstrating a reduction in vascular occlusion from 2180/7031(31.0%) without DP to 1641/6976 (23.5%) with DP therapy (RR 0.77[95% CI, 0.67-0.88], I^2=84%, p<0.0001) **(Figure 34)**. Sensitivity analysis of individual treatment regimens was also assessed as follows: (i) No DP versus DP (3 studies, 1306 vessels, RR 0.86[95% CI, 0.73-1.00], I^2=0%, p=0.05, **Figure 35**), (ii) No ASA/DP versus ASA/DP (27 studies, 8523 vessels, RR 0.77[95% CI, 0.67-0.88], I^2=74%, p=0.0001, **Figure 36**) and (iii) ASA versus ASA/DP (11 studies, 4181 vessels, RR 0.80[95% CI, 0.44-1.43], I^2=95%, p=0.45, **Figure 37**). Clinical studies were also assessed according to vascular bed (**Figure 38**) – demonstrating preserved treatment effects in both coronary/CABG cohorts (14 studies, 6172 vessels, RR 0.75[95% CI, 0.59-0.94], I^2=84%, p=0.01) and peripheral/carotid cohorts (14 studies, 2351 vessels, RR 0.81[95% CI, 0.69-0.95], I^2=45%, p=0.008). Similarly, sub-group analyses stratified

by mechanisms of revascularization demonstrated persistent benefit of DP therapy in those undergoing CABG with venous conduits or peripheral interventions, but not in those with coronary balloon angioplasties or CABG with arterial grafts **(Figure 39)** Adjunctive therapy was limited in human studies, though also suggestive of reduced rates of vessel occlusion with cilostazol(358) and ticagrelor.(362,363)

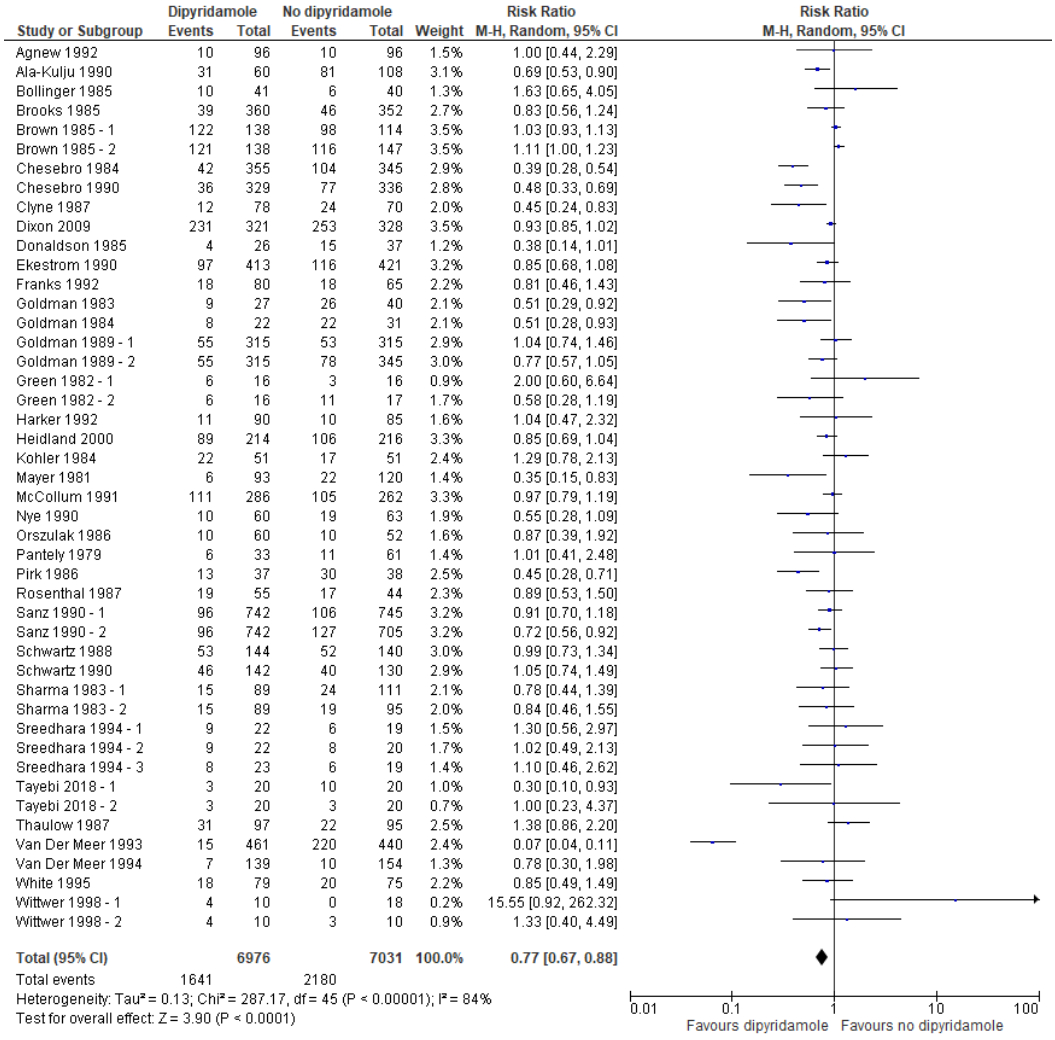

Figure 34. Impact of dipyridamole on vascular occlusion in humans.

Forest plot depicting all studies assessing no dipyridamole versus dipyridamole for vascular occlusion rates in human studies.

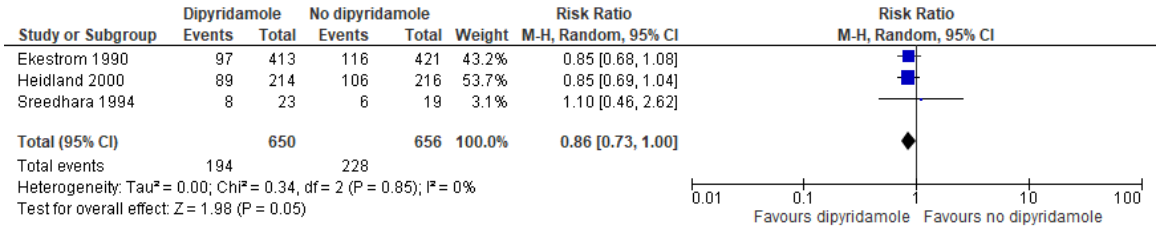

Figure 35. Clinical - No dipyridamole versus dipyridamole monotherapy on vascular occlusion rates.

Forest plot depicting all studies assessing no dipyridamole versus dipyridamole monotherapy for vascular occlusion rates in human studies.

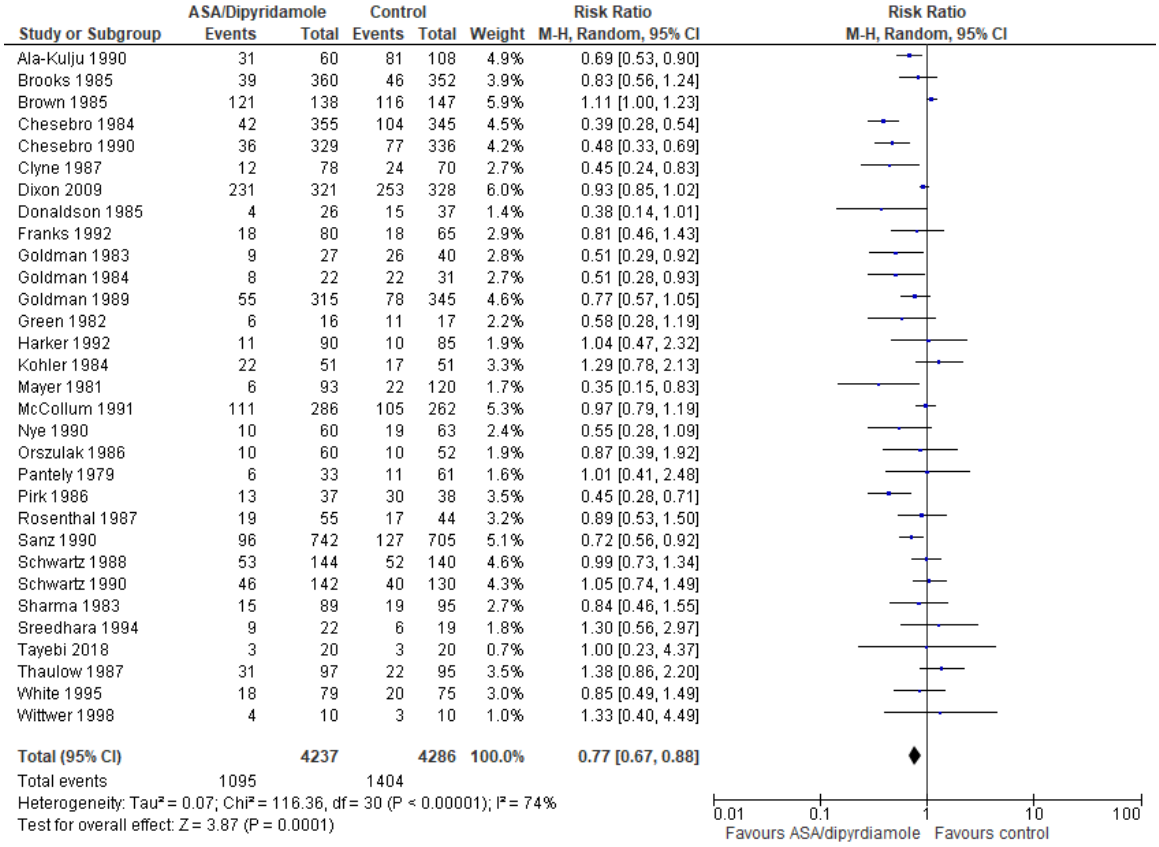

Figure 36. Clinical - No dipyridamole versus ASA/dipyridamole on vascular occlusion rates.

Forest plot depicting all studies assessing no dipyridamole versus ASA/dipyridamole for vascular occlusion in human studies.

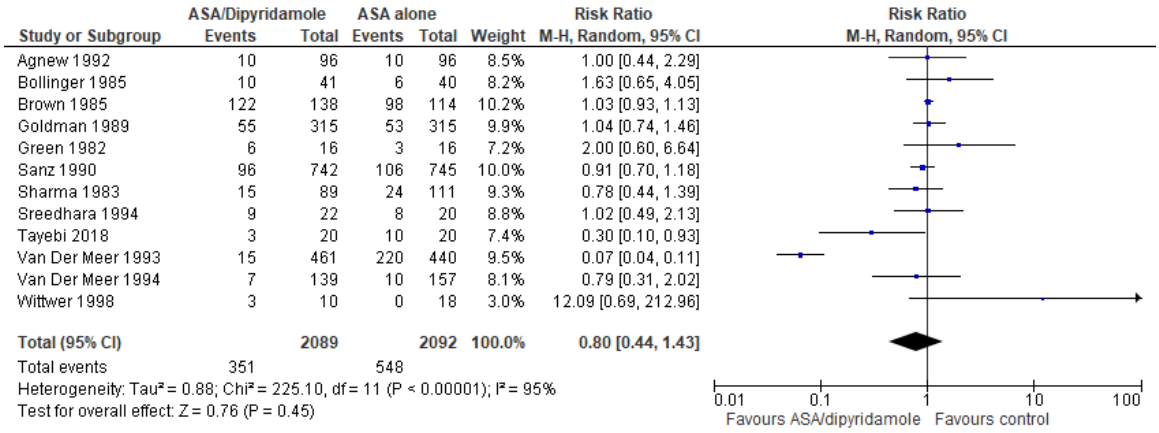

Figure 37. Clinical - ASA versus ASA/dipyridamole on vascular occlusion rates.

Forest plot depicting all studies assessing ASA versus ASA/dipyridamole for vascular occlusion rates in human studies.

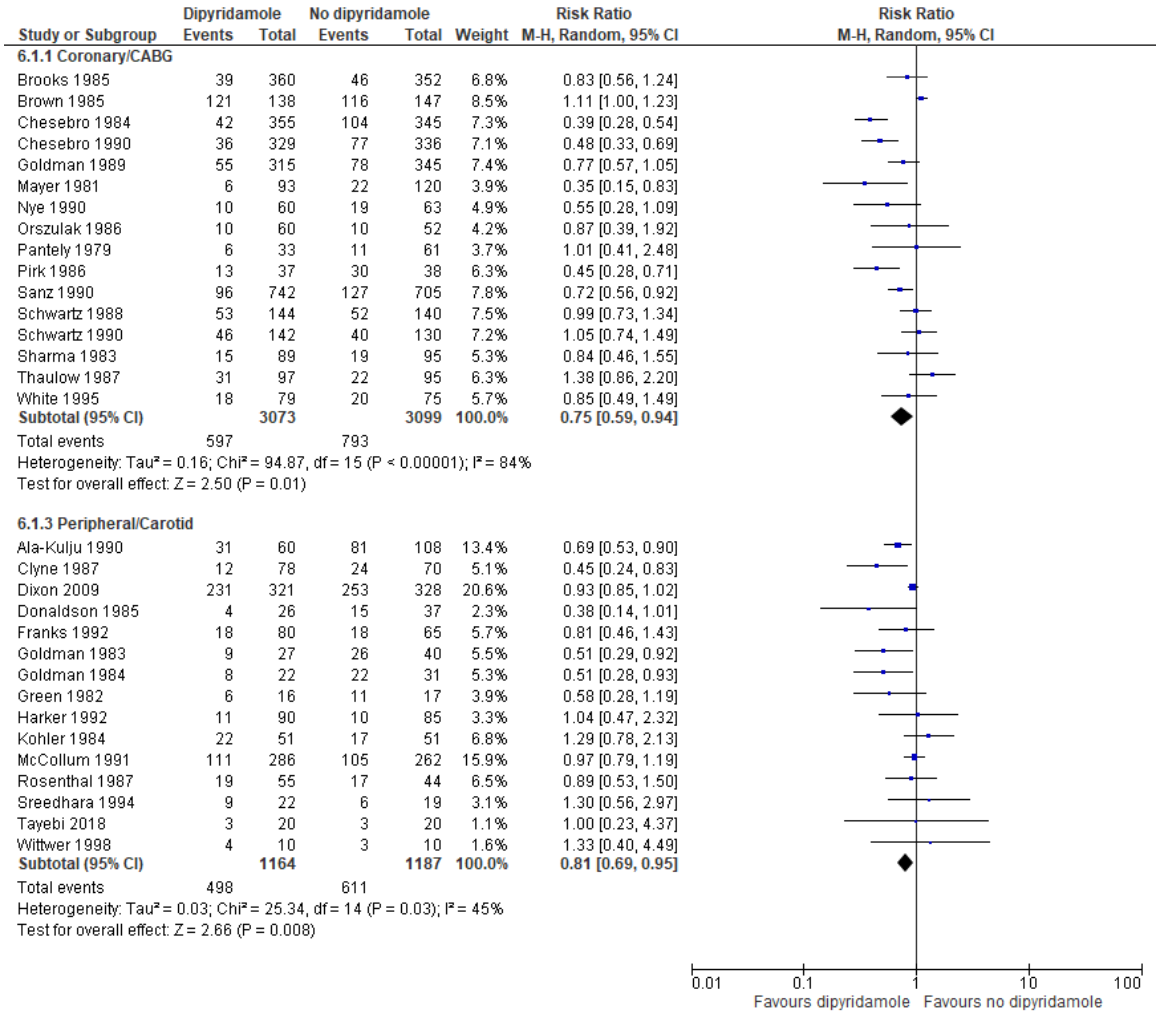

Figure 38. Clinical - Impact of dipyridamole on vascular occlusion by vascular bed.

Forest plot depicting all studies assessing no dipyridamole versus dipyridamole for vascular occlusion rates in human studies dichotomized into coronary/CABG (upper panel) and peripheral/carotid (lower panel).

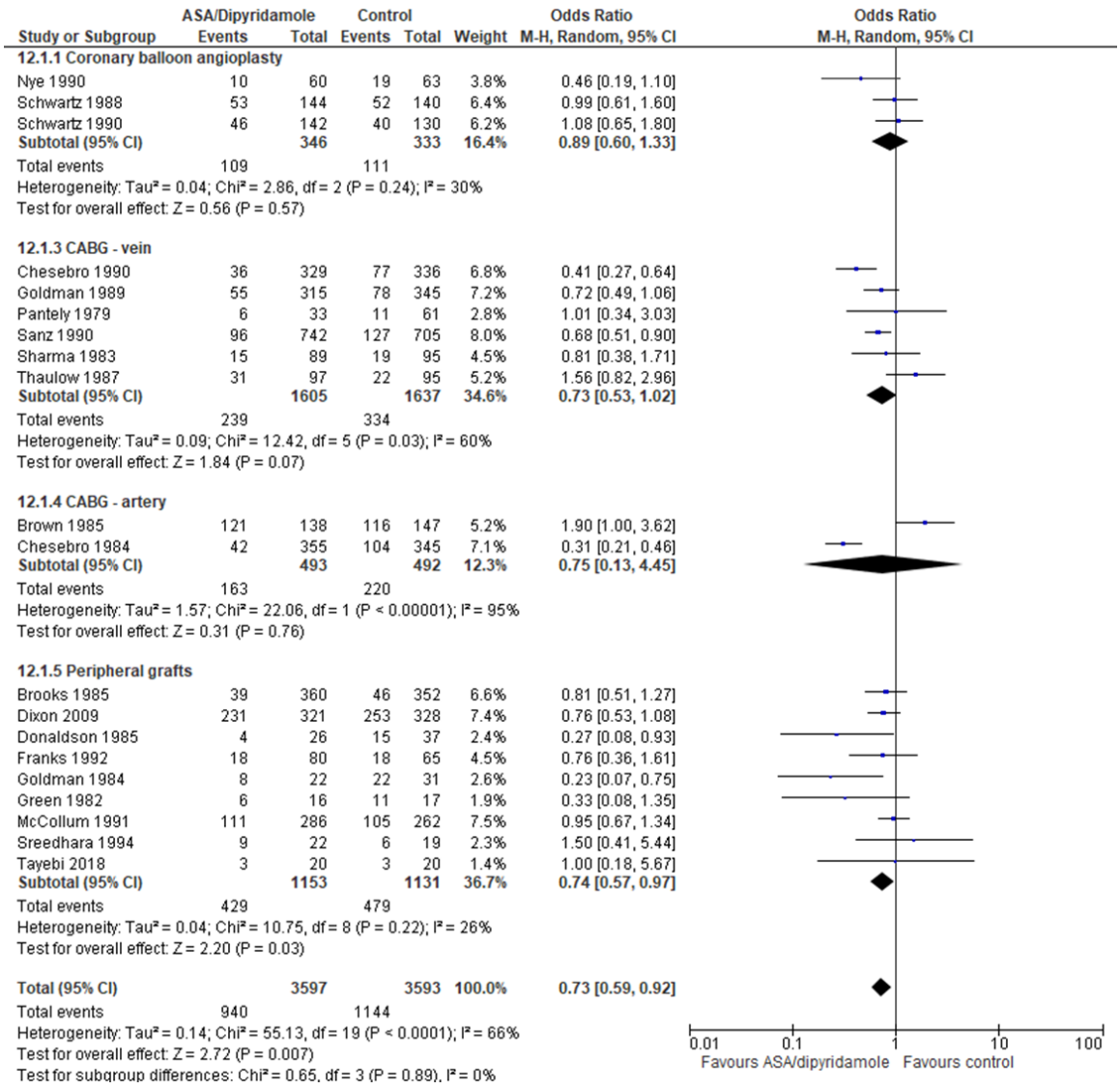

Figure 39. Clinical - Impact of dipyridamole on vascular occlusion by revascularization approach.

Forest plot depicting all studies assessing dipyridamole versus controls for vascular occlusion rates in human studies. Sub-groups divided into coronary balloon angioplasty, CABG – vein, CABG – artery and peripheral grafts.

5.6 Discussion

We sought to evaluate the totality of evidence supporting the role of DP in vascular protection following vascular intervention. In our review of pre-clinical and clinical

evidence, we identified a wide breadth of data that suggests DP may improve vascular patency and reduce NI proliferation. Moreover, multiple downstream mechanisms, namely modulation of cAMP and adenosine, may contribute to the observed effects. While contemporary studies including modern device and medical therapeutic regimens are needed – the available evidence suggests that revisiting dipyridamole therapy for improvement of clinical outcomes following revascularization procedures may be warranted.

In both clinical trials and observational datasets, patients who have undergone revascularization procedures remain at elevated risk for adverse events following their intervention – a risk which persists despite optimal management. This is driven considerably by recurrent ischemic events necessitating repeat revascularization procedures.(276) Accordingly, considerable efforts have been placed on optimizing the technical, pharmacological and risk factor management to improve outcomes. Indeed, the advent of DESs mitigated rates of repeat revascularization, though adverse clinical events remain largely unchanged.(270) Meanwhile cardiac rehabilitation improved smoking cessation, nutrition and physical rehabilitation also improving outcomes.(295,299) Pharmacologically, dual-antiplatelet therapy remains the standard of care to mitigate thrombotic events following revascularization, both percutaneously(364) and surgically.(363) Moreover, secondary prevention strategies continue to evolve with novel anticoagulant(365), lipid lowering (366) and anti-glycemic(367) agents also incrementally improving outcomes. Our review suggests rationale that addition of DP to

current optimal medical therapy should be contemporaneously evaluated for safety and efficacy.

The drivers behind repeat revascularization remain incompletely understood, encompassing both thrombotic and restenotic processes. While DESs(270) and antiplatelets(364) have made considerable strides to improving patency rates, restenosis remains an ongoing challenge, owing to an incomplete biological understanding and the use of non-selective agents.(31,244) Indeed, direct administration of endogenous cAMP reduces NI volume,(352) supporting this metabolites role in improving vascular homeostasis. Cilostazol, via cAMP and (to a lesser extent) adenosine augmentation, has also shown promise in reducing restenosis following revascularization with balloon angioplasty and early stents.(338,358) However, it has yet to achieve broad clinical implementation, with conflicting outcomes in the peripheral arterial disease population.(368) Conversely, while DP was widely used historically for a variety of indications, its role as a neointimal suppressant has been largely unexplored. First, by augmenting cAMP it facilitates vasodilation and anti-platelet effects.(113) Second, DP augments circulating adenosine (more so than cilostazol),(120,121) (369) yielding additional cAMP augmentation while facilitating adenosine-mediated inhibition of inflammation and smooth muscle cell proliferation – key factors in NI propagation.(38) Taken together, DP offers multiple promising mechanisms to facilitate vascular healing following intervention beyond its traditional role as an antiplatelet agent.

The need for adjuvant agents targeting alternative pathways is highlighted by the persistent risk of recurrent ischemic events despite refined technical and anti-platelet regimes. DP has shown promise in incrementally reducing the risk of recurrent episodes in those with cerebral ischemic events, though no clear benefit was seen in those with coronary or peripheral arterial disease.(370) DP has also shown promise in improving CABG(371) and hemodialysis(128) graft patency; though, discrepant results remain.(372,373) Similarly, we suggest that DP improves graft patency in both CABG and peripheral graft settings. For coronary interventions, early studies with balloon angioplasty were not suggestive of benefit – although early vessel failure in the balloon angioplasty era was largely due to elastic recoil.(374) With stents, dual antiplatelet therapy has improved markedly and is mandated to prevent thrombotic complications, potentially affording pleiotropic augmentation of adenosine levels – a source of debate.(149,284,313) Interestingly, DP also augments adenosine levels, more so than ticagrelor, offering an alternative pathway for vasculoprotection.(148) While prior stroke studies did not demonstrate added benefit to DP in addition to dual antiplatelets, these studies did not include stent placement.(375) Indeed, reduced restenosis was noted with DP and dual antiplatelets in the presence of cerebrovascular stenting, supporting its role as an adjunctive NI suppressant.(376) Hence, our review supports re-evaluation of DP as an additive therapy to existing anti-platelet regimens, particularly given DP's favorable cost profile - akin to recent studies repurposing colchicine to improve outcomes in cardiovascular patients.(377,378)

Certainly our study is not without limitations. By design, our population is heterogenous, encompassing a variety of vascular beds and sizes, interventions, species, timepoints and pharmacological regimens – highlighting the breadth of populations DP has been assessed in. However, this also introduces significant heterogeneity into our analysis, a limitation we aimed to mitigate with the sub-group analyses presented within. Moreover, we are unable to report on important differences in baseline comorbidities or sex, with most clinical studies demonstrating a greater proportion of males enrolled, confounding the outcomes reported and limiting its broad applicability. NI quantification analysis is limited to pre-clinical models given the need for histology – although development of intravascular imaging modalities like optical coherence tomography and intravascular ultrasound now offer the ability to serially evaluate NI development in a robust manner in clinical studies. Finally, the broad applicability to conventional clinical practice is limited by the lack of atherosclerosis in most pre-clinical models as well as the lack of contemporary stent and medical regimens - though this should be the focus of ongoing future studies.

5.7 Conclusion

Optimization of arterial healing post-intervention remains the focus of ongoing efforts to improve patient outcomes. DP may improve vascular healing through pleiotropic mechanisms and reduce neointimal proliferation following revascularization. Contemporary clinical studies are warranted.

5.8 Sources of funding

Dr. Rob Beanlands is a Career Investigator supported by the Heart and Stroke Foundation of Ontario, a Tier 1 University of Ottawa Chair in Cardiovascular Research and Vered Chair in Cardiology.

5.9 Conflicts of interest

None relevant

Chapter 6

Contrast-Free Optical Coherence Tomography: Systematic Evaluation of Non-Contrast Media for Intravascular Assessment

6.1 Preface

This chapter has been previously published in the *PLOS ONE*

> **Simard T,** Motazedian P, Majeed K, Sarathy K, Jung RG, Feder J, Ramirez FD, Di Santo P, Marbach J, Dhaliwal S, Short S, Labinaz A, Schultz C, Russo JJ, So D, Chong AY, Le May M, Hibbert B. Contrast-free optical coherence tomography: Systematic evaluation of non-contrast media for intravascular assessment. PLoS One. 2020 Aug 20;15(8):e0237588. doi: 10.1371/journal.pone.0237588. PMID: 32817672; PMCID: PMC7446899.

> *All authors made substantial contributions to the conception/ design of the work; and/or the acquisition, analysis, or data interpretation; and, (ii) drafted and/or revised the content critically; and, (iii) provided final approval of the published version; and, (iv) agree to be accountable for the work.*

6.2 Abstract

Background: Coronary revascularization using imaging guidance is rapidly becoming the standard of care. Intravascular optical coherence tomography uses near-infrared light to obtain high resolution intravascular images. Standard optical coherence tomography imaging technique employs iodinated contrast dye to achieve the required blood clearance during acquisition. We sought to systematically evaluate the technical performance of saline as an alternative to iodinated contrast for intravascular optical coherence tomography assessment.

Methods and Results: We performed bench top optical coherence tomography analysis on nylon tubing with sequential contrast/saline dilutions to empirically derive adjustment coefficients. We then applied these coefficients *in vivo* in an established rabbit abdominal

stenting model with both saline and contrast optical coherence tomography imaging. In this model, we assessed the impact of saline on both quantitative and qualitative vessel assessment. Nylon tubing assessment demonstrated a linear relationship between saline and contrast for both area and diameter. We then derived adjustment coefficients, allowing for accurate calculation of area and diameter when converting saline into both contrast and reference dimensions. *In vivo* studies confirmed reduced area with saline versus contrast [7.43 (5.67-8.36) mm^2 versus 8.2 (6.34-9.39) mm^2, p=0.001] and diameter [3.08 mm versus 3.23 mm, p=0.001]. Following correction, a strong relationship was achieved *in vivo* between saline and contrast in both area and diameter without compromising image quality, artefact, or strut assessment.

Conclusion: Saline generates reduced dimensions compared to contrast during intravascular optical coherence tomography imaging. The relationship across physiologic coronary diameters is linear and can be corrected with high fidelity. Saline does not adversely impact image quality, artefact, or strut assessment.

6.3 Introduction

Coronary angiography and percutaneous coronary intervention (PCI) are predominantly performed based on fluoroscopic and cineangiographic images for determining vessel pathology and sizing. Coronary assessment has greatly improved with the advent of intravascular imaging modalities – specifically intravascular ultrasound (IVUS) and intravascular optical coherence tomography (OCT). OCT uses near-infrared light (wavelength ~1300nm) to generate tomographic images with histological-grade resolution (10-20um). This enhanced resolution enables OCT to provide detailed intravascular assessment for thrombus, plaque morphology, intimal lesions (neointima,

dissection) and stent evaluation (apposition, sizing, coverage).(379) However, OCT requires exclusion of intraluminal blood as it causes significant attenuation of light energy owing to absorption by hemoglobin and scattering by red blood cells.(380) Hence, the use of a flushing solution to clear intraluminal blood is needed for intravascular OCT.

Contemporary FD-OCT (frequency or Fourier-domain OCT) provides pullback speeds up to 75mm/sec, enabling scanning of up to 5-7cm of an epicardial coronary vessels depending on the resolution. The first in-human studies of this technology demonstrated that this improved speed eliminates the need for proximal balloon occlusion as a single, high rate bolus injection was sufficient to exclude blood for imaging. (381) During initial development, saline was employed as a flushing medium with subsequent work demonstrating improved blood exclusion and longer imaging durations (~10 seconds) with viscous contrast media over crystalloids.(381-383) As such, viscous contrast media became the standard imaging medium used in commercial FD-OCT protocols.

Optimizing the imaging medium has garnered significant interest recently as a means of improving image quality while minimizing adverse effects. Indeed, the administration of viscous contrast media is not without risk, namely that of acute kidney injury and contrast-induced nephropathy which, though poorly understood, have been linked to long-term adverse events.(384) The side effect profile is dose-dependent, limiting the number and duration of OCT imaging runs that can be performed. This is particularly relevant in complex cases where a significant contrast load may already have been used for angiography, thus limiting OCT assessment in whom it may be the most beneficial. Accordingly, recent efforts have sought to identify alternative imaging mediums with

improved side effect profiles, in particular colloids and crystalloids.(385,386) Herein, we report the systematic evaluation of saline and saline-diluted contrast as an alternative to iodinated-contrast imaging medium for OCT-based vascular imaging.

6.4 Methods

OCT and imaging media

OCT scans were acquired using a Dragonfly Imaging Catheter (St. Jude Medical, Minnesota, USA) in conjunction with the ILLUMIEN system (St. Jude Medical, Minnesota, UA). Omnipaque 300 mg/mL (GE Healthcare, NJ, USA) was used as contrast media and 0.9% sodium chloride was used as the saline solution with varying dilutions generated via mixtures of these two agents.

Bench top model for dimensional analysis

Nylon 11 D.O.T. tubing (Freelin-Wade, Oregon, USA) with known internal diameters of 2.0/0.079, 3.0/0.118 and 4.3/0.170 mm/inches (+/- 0.08mm/0.003") were used to identify the measured differences between saline and contrast injections. We demonstrate histological and OCT cross-sections of each. **(Figure 40a**). Varying solutions were instilled through the tubing for OCT assessment, including 100% saline, varying dilutions of saline/contrast (25%/75%, 50%/50% and 75%/25%) and 100% contrast. Representative images for a selected subset of 100% saline, 50%/50% saline/contrast and 100% contrast demonstrated in **Figure 40b**. For each tube size and saline/contrast combination, 15 matched images were identified and dimensional analyses were performed to assess the internal area and diameter. This process was performed for both the derivation (n=15) and validation (n=15) groups to generate and validate a correction factor between saline and contrast for area and diameter.

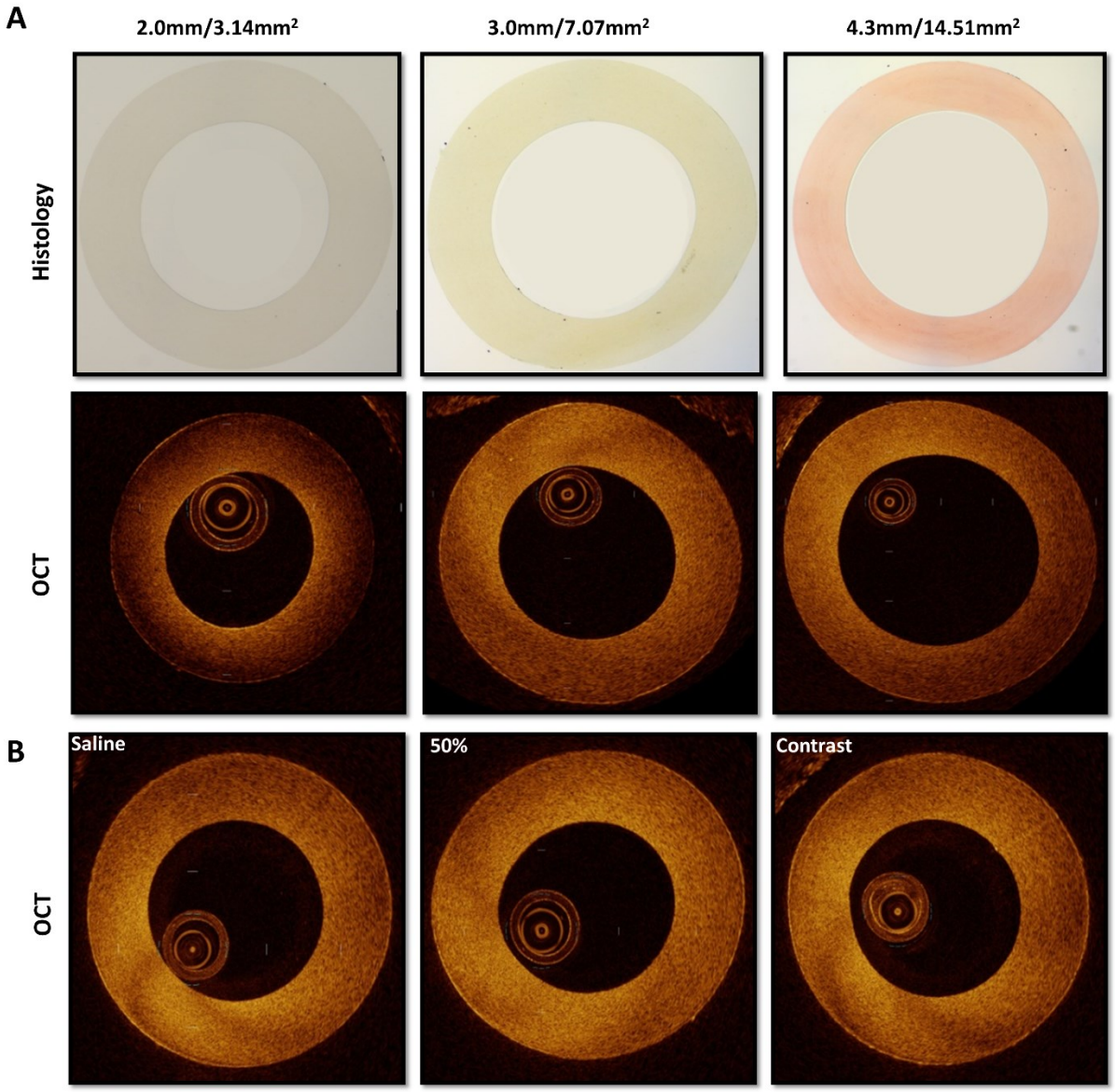

Figure 40. OCT and histological sections of nylon tubing.

(A) Representative images of histological cross-sectional images of nylon tubing across physiologic size range for human coronary artery (2.0mm, 3.0mm, and 4.3mm) with corresponding OCT images of these same nylon tubes with 100% contrast flushing agent below. **(B)** Representative images from a select subset utilizing 100% saline, 50%/50% mixture of saline/contrast, and 100% contrast on identical nylon tubing segment.

Animals and experimental protocol

All animal protocols are in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the guidelines of the Canadian Council on Animal Care. The animal protocols were approved by the University of Ottawa Animal Care Committee. Rabbits are known to have abdominal aortas and iliac vessels similar in sizing to human coronaries (244,387), making them a viable model for studying stent implantation and healing.(388,389) Twenty-one male New Zealand White Rabbits (2kg, NZW, Charles River Laboratories) were maintained in individual confinements with ample food and water supply. Animal health was monitored daily and animals were allowed regular activity periods outside of the cage. Local and systemic analgesics were employed liberally to limit any suffering and distress. Humane endpoints were continuously monitored by the animal care staff and veterinarians. Rabbits were euthanized by lethal doses of pentobarbital sodium. No mortality occurred outside the planned euthanasia or humane endpoints. Rabbits were randomly assigned by online randomization tool to undergo implantation in the abdominal aorta of either a bioresorbable vascular scaffold (BVS) (n=11) or drug-eluting stent (DES) (n=10). A convenience sample was employed based on available animals. Stent implantations were performed during daytime hours in a dedicated operating suite with induction by propofol and subsequent general inhaled anesthesia with vascular access achieved via femoral cut-down approach enabling placement of a 6-French femoral access sheath with subsequent closure by femoral ligation as previously reported (349,390) Intravenous unfractionated heparin (150U/kg) is administered intra-procedurally to prevent thrombosis, while post-stent implantation rabbits were maintained on dual anti-platelet therapy with rectal

acetylsalicylic acid (ASA) 10mg/kg daily and intra-dermal clopidogrel 14mg daily to prevent stent thrombosis. OCT analyses were then performed using saline and contrast as flush solutions at two time-points – (i) immediately following stent implantation and (ii) 6-weeks post-implantation at the time of sacrifice. Three randomly selected unmatched images deemed representative of their respective scans were flagged for qualitative and quantitative evaluation in both the saline and contrast groups.

***In vivo* qualitative analysis**

Identical de-identified images in both the saline and contrast groups underwent subjective grading to compare diagnostic quality and the presence of artifacts between the two groups. Scoring was completed by blinded, independent, and trained evaluators based on five pre-determined criteria in a binary fashion (no= 0, yes=1), in keeping with similar scoring systems.(391) Cumulative quality and artifact scores were assigned to each image ranging from 0 to 4. The following criteria were used to determine diagnostic quality: (1) the presence of a clear border between lumen and luminal wall; (2) the presence of a defined border between the tunica interna and tunica media; (3) the ability to confidently identify whether stent struts were covered or uncovered; and (4) whether the overall image quality was satisfactory for diagnostic interpretation. To assess for difference in the observed artifacts between saline and contrast, four pre-established criteria were employed. Cumulative artifact scores ranging from 0 to 4 were calculated from the binary scores based on the presence or absence of the following: (1) blood swirl or speckle occupying >50% of the lumen, or the presence of thrombus; (2) sew-up artifact; (3) ghost reflections on >1 strut; and (4) saturation artifact.

***In vivo* quantitative analysis**

Dimensional analysis similar to the bench top model was performed to assess both the luminal area and diameter with saline and contrast *in vivo*. Area and diameter were calculated via the automatic area function or manually if the software was unable to detect the luminal wall. The number of stent struts and coverage of struts were manually counted based on the presence of strut and strut reflection shadow. All measurements were completed by independent reviewers blinded to the treatment groups.

Statistical Analysis

Continuous variables are reported as either mean (±SD) or median (IQR). Categorical variables are reported as either frequencies and/or percentages. All plotted relationships were linear with appropriate linear trendline fitting applied and the resulting line of best fit and R^2 displayed for each. The linear equation derived from the line of best fit was then used to derive correction formulas for each contrast concentration with validation performed in a separate cohort. Categorical variables were compared using chi-square tests, whereas continuous variables were compared using either Student t-tests or Mann-Whitney *U* tests, where appropriate.

6.5 Results

Bench top dimensional analysis

We first set out to empirically derive adjustment coefficients for dilutions of contrast on the bench top. Nylon tubing was assessed with varying concentrations of saline/contrast and the generated areas and diameters were plotted as a function of the known reference area and diameters of the tubing, demonstrating a linear relationship across all dilutions **(Figure 41).** As well, a progressively decreasing slope was noted as one reduced contrast content for both dimensions **(Figure 41, Table 16).** The calculated

linear relationships of the measured versus reference dimensions **(Table 16)** were then used to derive a conversion formula for the reported contrast dilution ratios. These conversion formulas allow for the calculation of the corresponding 100% contrast dimension **(Table 17a)** or the reference dimension **(Table 17b)** for any measured dimension at a pre-defined contrast percentage (defined as X in **Table 17).** We then applied these correction formulas to the raw data obtained in a separate validation cohort by plotting the corrected values versus the corresponding 100% contrast and reference values for both area and diameter. With this approach, a 1:1 relation of corrected dimension to contrast or reference dimension would indicate a reliable correction. Indeed, this was observed with validation slopes ranging from 0.98-1.03 with R^2 all greater than 0.97 **(Table 17),** indicating a reliable adjustment for area and diameter across the varying flush solution compositions.

Table 16. Dimensional analysis (a) measured versus reference area (b) measured versus reference diameter

A	Reference (mm^2)	3.14		7.07		14.52		Measured versus Reference	
Measured (mm^2)	Contrast (%)	Mean (mm^2)	SD	Mean (mm^2)	SD	Mean (mm^2)	SD	Relation	R^2
	100	2.80	0.07	6.48	0.21	13.32	0.71	y = 0.9235x – 0.077	1
	75	2.68	0.11	6.24	0.22	13.15	1.01	y = 0.9202x – 0.2303	1
	50	2.67	0.09	5.89	0.31	12.67	0.60	y = 0.8829x – 0.203	0.9993
	25	2.59	0.08	5.71	0.41	12.05	1.05	y = 0.8331x – 0.0844	0.9997
	0	2.59	0.12	5.55	0.29	11.66	0.80	y = 0.7997x + 0.0051	0.9996

B	Reference (mm)	2.00		3.00		4.30		Measured versus Reference	
Measured (mm)	Contrast (%)	Mean (mm)	SD	Mean (mm)	SD	Mean (mm)	SD	Relation	R^2
	100	1.89	0.02	2.87	0.05	4.12	0.11	y = 0.968x – 0.0426	0.9999
	75	1.85	0.04	2.82	0.05	4.09	0.16	y = 0.9742x – 0.1036	1
	50	1.84	0.03	2.73	0.07	4.01	0.10	y = 0.946x – 0.0696	0.9993
	25	1.82	0.03	2.69	0.10	3.91	0.18	y = 0.9112x – 0.0194	0.9997
	0	1.81	0.04	2.66	0.07	3.85	0.13	y = 0.8859x + 0.0254	0.9995

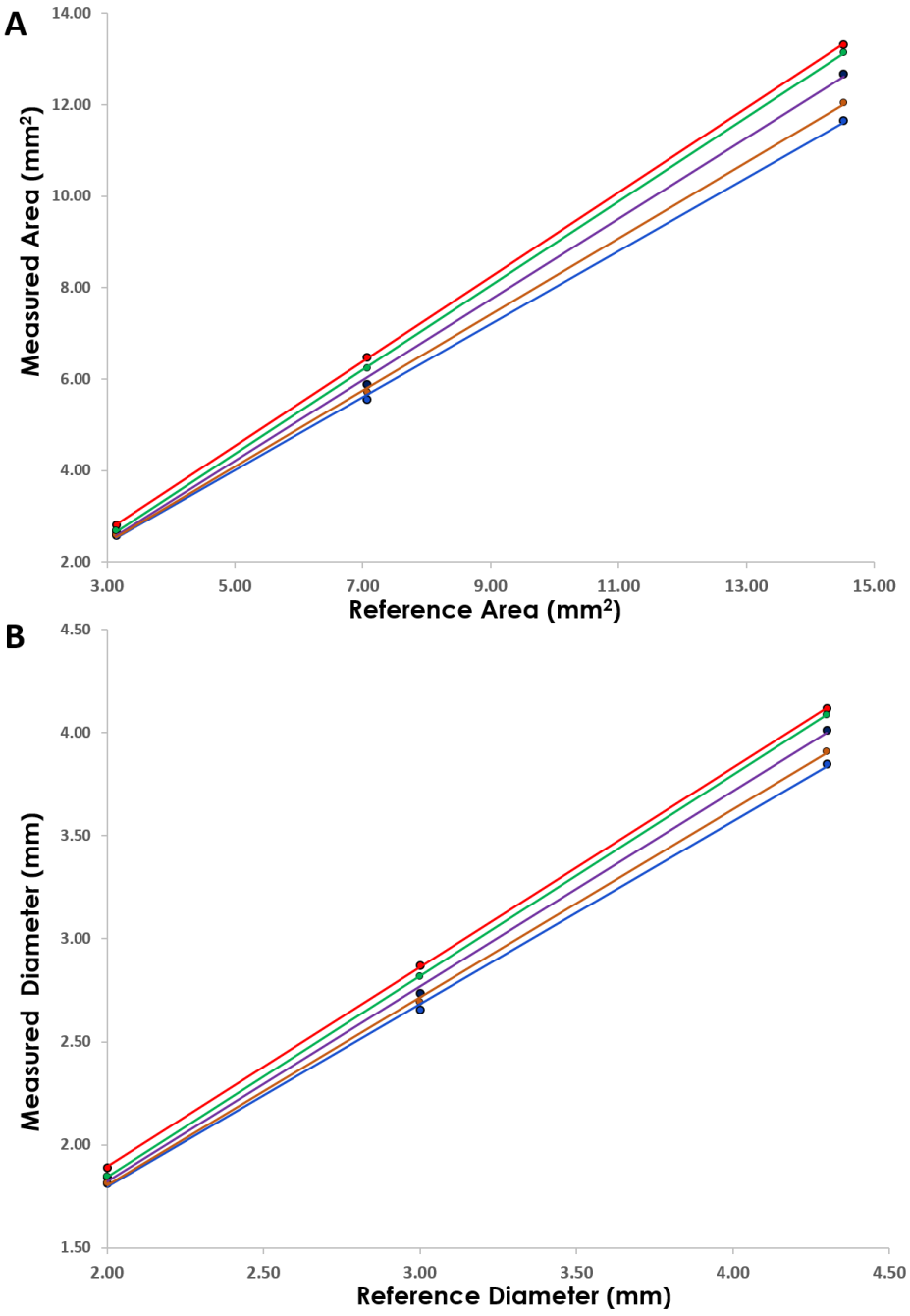

Figure 41. Measured versus reference dimensional analysis.

Dimensional analysis of measured areas (A) and diameters (B) via OCT compared to reference nylon tubing dimensions. Demonstrates linear relationship with differing slopes varying by the different flush solutions employed for each. Refer to Table 1a and 1b for trendline formulas and correlation coefficients. Red – 100% contrast, Green – 75% contrast, Purple – 50% contrast, Orange – 25% contrast, Blue – 0% contrast (pure saline).

Table 17. Conversion factors (a) Adjust measured sizing to contrast sizing (b) Adjust measured sizing to reference sizing

A

Contrast (%)	Derivation (N=45)	Validation (N=45)	
Area	**Conversion formula**	**Slope**	**R^2**
75	Contrast = ((0.9194(X+0.1584))/0.9063)-0.0391	1.0228	0.9952
50	Contrast = ((0.9194(X+0.1149))/0.8706)-0.0391	1.0169	0.9966
25	Contrast = ((0.9194(X+0.0928))/0.831)-0.0391	1.0029	0.992
0	Contrast = ((0.9194(X-0.0501))/0.7926)-0.0391	1.0127	0.996
Diameter	**Conversion formula**	**Slope**	**R^2**
75	Contrast = ((0.9708(X+0.1340))/0.9862)-0.0518	0.9824	0.996
50	Contrast = ((0.9708(X+0.1060))/0.9577)-0.0518	0.9796	0.9961
25	Contrast = ((0.9708(X+0.0255))/0.9147)-0.0518	1.0003	0.995
0	Contrast = ((0.9708(X-0.0057))/0.8928)-0.0518	0.9902	0.996

B

Contrast (%)	Derivation (N=45)	Validation (N=45)	
Area	**Conversion formula**	**Slope**	**R^2**
100	Reference = (X+0.0391)/0.9194	1.0094	0.9905
75	Reference = (X+0.1584)/0.9063	1.0313	0.9834
50	Reference = (X+0.1149)/0.8706	1.0287	0.9913
25	Reference = (X+0.0928)/0.831	1.0055	0.9692
0	Reference = (X-0.0501)/0.7926	1.0184	0.9791
Diameter	**Conversion formula**	**Slope**	**R^2**
100	Reference = (X+0.0518)/0.9708	0.9942	0.9941
75	Reference = (X+0.1340)/0.9862	0.9757	0.9881
50	Reference = (X+0.1060)/0.9577	0.9756	0.9936
25	Reference = (X+0.0255)/0.9147	0.9924	0.9847
0	Reference = (X-0.0057)/0.8928	0.9845	0.9904

In vivo quantitative analysis

Following our bench top assessment, we sought to assess the impact of imaging media in an in vivo model. This model utilizes stent implantation in the abdominal aortas of New Zealand White Rabbits with intravascular OCT assessment capable of generating histological grade imaging with both 100% saline and 100% contrast imaging media **(Figure 42).** Findings in this model mirrored our bench top findings with the saline cohort demonstrating a 9.4% reduction in area [7.43 (5.67-8.36) versus 8.2 (6.34-9.39 mm2), p=0.001] and 5% reduction in diameter [3.08 (2.68-3.26) versus 3.23 (2.84-3.46) mm, p=0.001] in comparison to its contrast counterpart **(Figure 43a,b**).

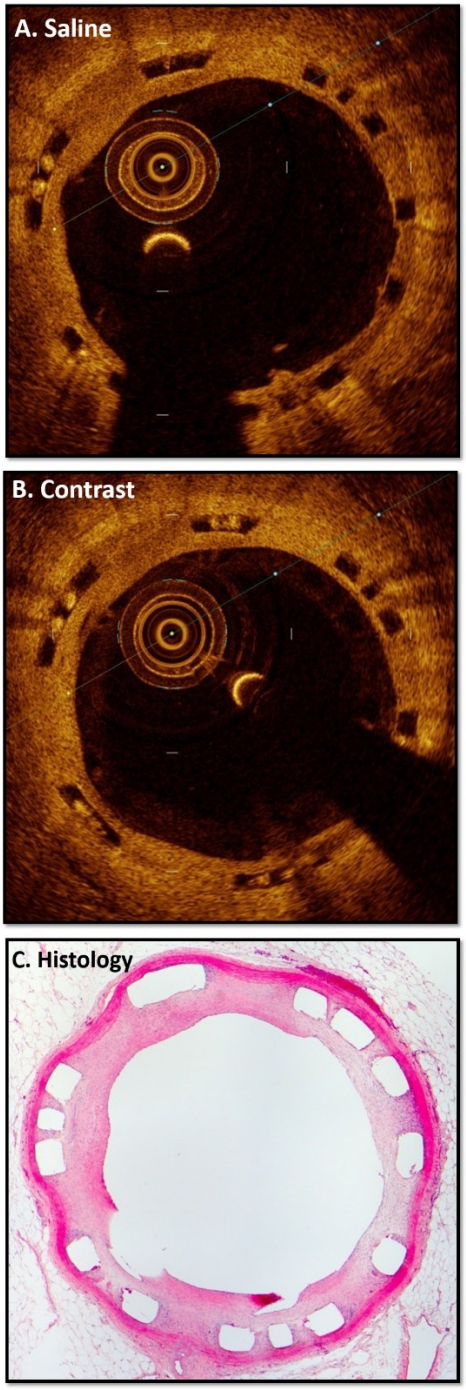

Figure 42. In vivo comparison of OCT versus traditional histology

Images of identical rabbit arterial segment with scaffold in situ and subsequent arterial healing and neointima formation. Specifically note of robust virtual histology with OCT utilizing both 100% saline (A) and 100% contrast (B) flushing agents when compared to traditional histology with haematoxylin and eosin (H&E) staining (C).

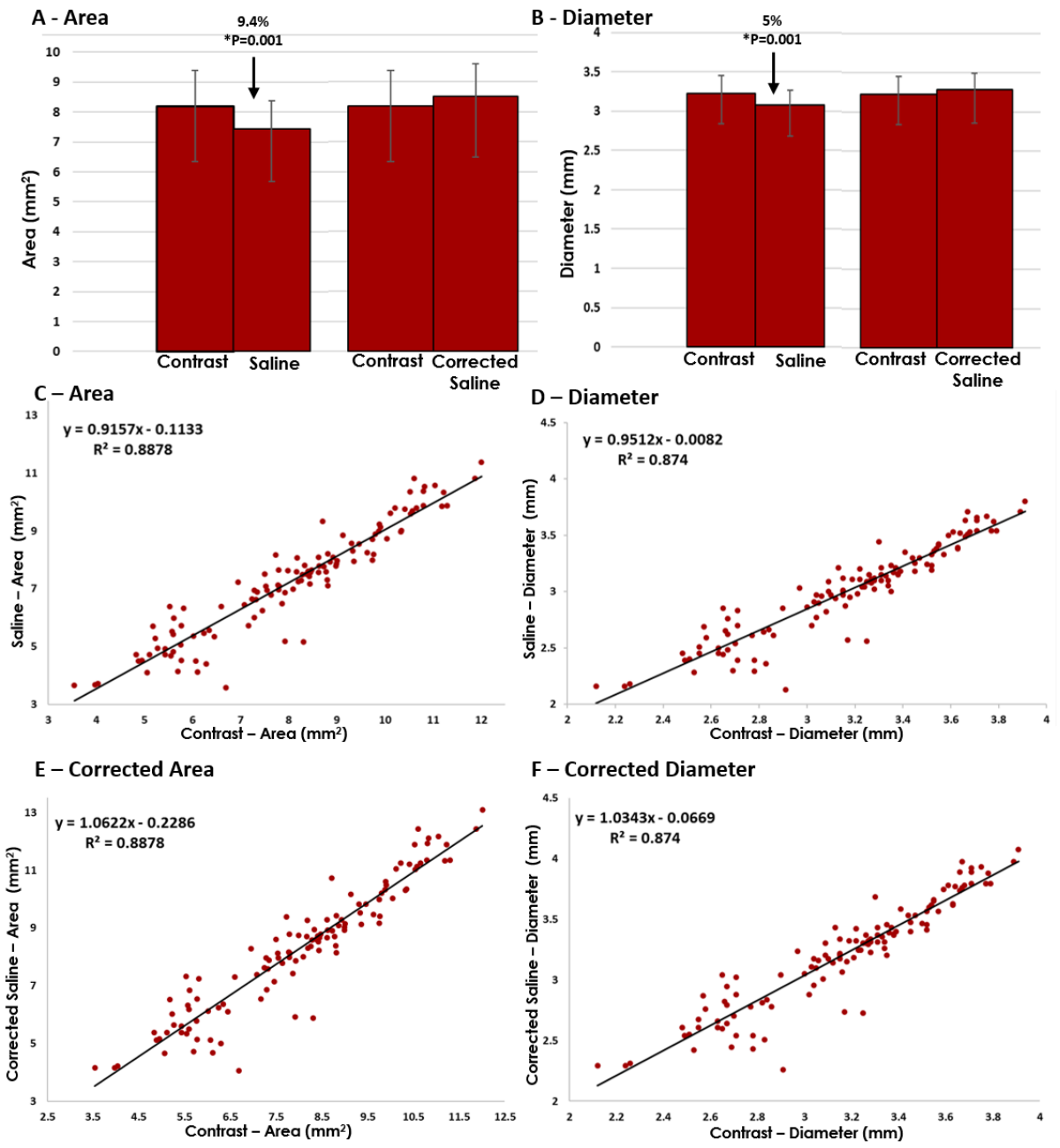

Figure 43. In vivo quantitative analysis.

Dimensional analysis of area (A) and diameter (B) in rabbit model of intravascular OCT. Note of a significant 9.4% reduced area and 5% reduced diameter in the saline versus contrast cohorts. Following application of empirically derived correction factors no further significant difference between either cohorts. Graphical depiction of individual data points for saline versus contrast assessments for both area (C) and diameter (D) demonstrating linear relationship with slopes of 0.9157 and 0.9512 respectively. Following application of empiric correction factors note of augmented slope to 1.0622 and 1.0343 respectively for area I and diameter (F).

Next, we applied the bench top derived and validated dimensional correction formulas **(Table 17a**) to the data generated from our in vivo model. Specifically, we applied the correction formula for 100% saline to 100% contrast [Contrast = ((0.9194(X-0.0501))/0.7926)-0.0391] to the raw data obtained in vivo with 100% saline, generating corrected saline values. This correction eliminated differences between corrected saline and contrast in both area [8.52 (6.48-9.60) vs 8.20 (6.34-9.39) mm2, 9.4% to 3.9% absolute difference, p=0.35, Figure 43a) and diameter [3.29 (2.86-3.49) vs 3.23 (2.84-3.46) mm, 5% to 1.9% absolute difference, p=0.40, Figure 43b). Similarly, when the corrected saline values were plotted as a function of the measured contrast values, they demonstrated a strong 1:1 linear relationship indicating robust correction for both area (slope = 1.06, R2=0.89, **Figure 43e**) and diameter (slope = 1.03, R2=0.87, **Figure 43d**) when compared to uncorrected dimensions with saline versus contrast (**Figure 43c,d**). Last, we used the in vivo images to quantify stent struts and arterial healing post stent implantation. The ability to identify stent struts and describe their appearance (i.e. exposed or covered) is of particular importance to assessing stent deployment and pathology. We did not observe any differences between saline or contrast with regards to the total number of stent struts, exposed struts, or covered struts **(Table 18**). Furthermore, neointima quantification by OCT imaging with saline and contrast similarly demonstrated no differences between groups (32.3+/-6.46 versus 30.3+/-5.56%, p=0.25) (**Table 18).**

Table 18. *In vivo* quantitative stent analysis

	Saline		Contrast		
	Mean	**SD**	**Mean**	**SD**	**p**
Strut Assessment (N)	117		117		
Exposed struts	5.81	6.63	5.44	6.06	0.92
Covered struts	6.29	6.78	6.79	6.74	0.55
Total strut number	12.10	4.42	12.23	3.68	0.9
Neointima assessment (N)	25		25		
Neointima size (%)	32.3	6.46	30.3	5.56	0.25

In vivo qualitative analysis

Based on the 4-point quality scoring system, all *in vivo* images for saline (n=117) and contrast (n=117) were assessed by independent reviewers for each quality component to generate a summative overall quality score (**Table 19).** Ultimately, no significant differences between any of the individual quality components, or in the overall quality scores were observed. Similarly, independent reviewers evaluated both the saline (n=117) and contrast (n=117) cohorts for the presence of common artefacts known to compromise intravascular OCT imaging **(Table 20**; examples are demonstrated in **Figure 44**). Overall, no significant differences in any of the artefacts screened for were demonstrated between either groups. Both saline and contrast yielded image quality on par with histological-grade images, with corresponding histological sections demonstrating striking similarities **(Figure 42).**

Table 19. In vivo qualitative analysis – quality score

	Saline	%/SD	Contrast	%/SD	p
N	117		117		
Luminal border	109	93.1	108	92.3	1.00
Intima/media border	43	36.8	37	31.6	0.49
Stent strut	100	85.4	95	81.2	0.48
Diagnostic quality	99	84.6	93	79.5	0.39
Overall score	3.0	1	2.85	1.06	0.06

Table 20. In vivo qualitative analysis – artefact score

	Saline	%	Contrast	%	p
Number	117		117		
Blood score	23	19.7	12	10.3	0.067
Sew-up	0	0.0	1	0.9	1.000
Ghost-strut	0	0.0	0	0.0	-
Saturation	0	0.0	0	0.0	-

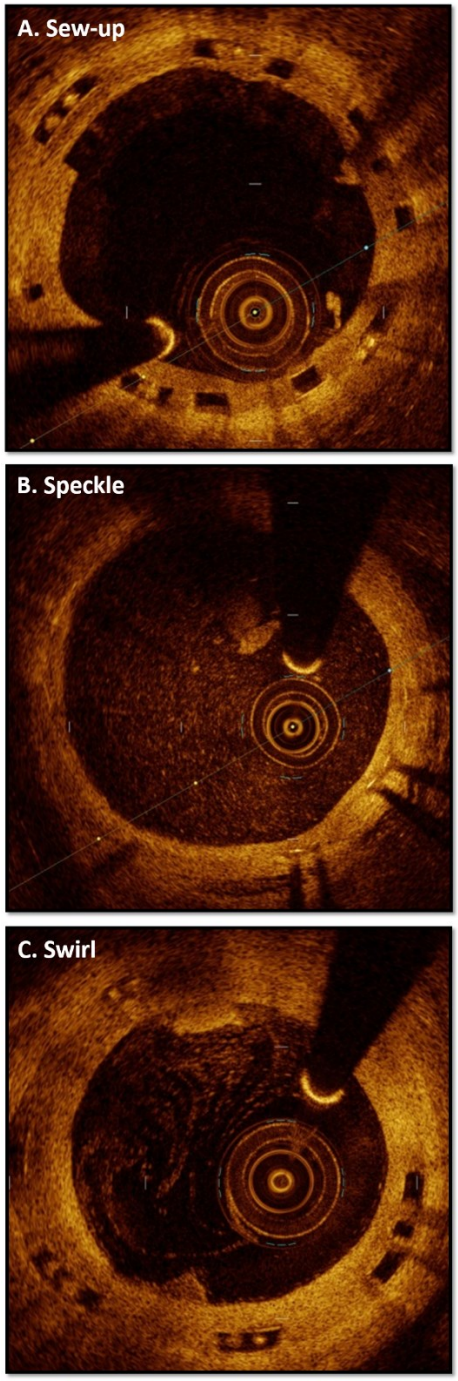

Figure 44. Artefacts with intravascular OCT

Examples of common artefacts with intravascular OCT including sew-up artefact (A) related to motion, as well as speckle (B) and swirl (C) artefacts related to insufficient intravascular blood flushing.

6.6 Discussion

Intravascular OCT provides histological-grade intracoronary assessment, but requires clearance of intraluminal blood via flushing with viscous contrast solution, which carries risks. (392) Preliminary work has investigated the utility of varying flush solutions including dextran and saline with promising results.(393,394) However, a thorough preclinical assessment of the qualitative and quantitative properties has yet to be reported. Herein, we provide empirically-derived and validated correction formulas for dimensional analysis for saline-based imaging. Moreover, we demonstrate the utility of these correction formulas in an *in vivo* setting for dimensional analysis while also demonstrating no significant impact on quantitative and qualitative stent assessments in a preclinical rabbit model.

Dimensional analysis

Our bench top and *in vivo* models enable assessment of the impact of the contrast-content of imaging media on measured intravascular areas and diameters. Both models demonstrate a significant difference with respect to the dimensions reported between saline and contrast in keeping with previous reports.(395) The observed differences in dimensions are likely related to the varying refractive indices between saline (1.33) and contrast (1.44), with most commercial systems employing a refractive index of 1.4 for dimensional calculations.(395) In our bench top model we similarly noted a 10% difference in sizing that, once corrected, yielded no difference between the two groups (4%). Similarly, when assessed in the *in vivo* setting no differences in dimensions remained following correction, with only a 4% difference in area and 2% difference in diameter remaining. This is in keeping with previous work in a swine model that reported

a variance in area of 18% that was reduced to 2.9% with correction based upon the refractive index of the solution.(395) Our empirically-derived correction factors thus enable robust adjustment for a broad range of contrast dilutions across a physiologically-relevant range of luminal diameters.

Qualitative and quantitative assessments in diagnostic quality

In addition to vessel sizing, OCT is commonly performed to clarify unclear anatomy, assess for dissections, and thrombus, and/or plaque morphology. Thus, acquiring a diagnostic image to permit assessment of the vessel architecture is paramount for imaging performance. Accordingly, we assessed qualitative measures in an established rabbit stenting model by performing a standardized assessment of a reviewers' ability to identify key vessel architectural features and artefact presence. (391) In our study, we found no significant differences between saline and contrast in any of the individual quality components, nor in the overall sum quality score suggesting that saline performs comparably to contrast in our system. Similarly, reviewers assessed for the presence of artefacts and did not identify any differences between saline and contrast, though there may be a suggestion of more blood artefact in the saline cohort. This is most likely related to reduced flushing efficacy with saline owing to its >10-fold lower viscosity than iodinated contrast.(395,396) Indeed, the dynamics of flushing has been explored previously with viscosity, flow rate and flush duration identified as the predominant factors impacting blood displacement.(395) We employed a standardized flushing protocol with a rate sufficient to clear blood and to initiate auto-triggering of the OCT run. This rate was then maintained for a fixed duration for each run; therefore, any suggested any variance in image quality would be most likely related to differing

viscosities of the two agents. To overcome the reduced viscosity, one could improve blood clearance by either increasing the flush duration and/or flow rate. (395) Moreover, this effect is likely magnified in the rabbit model as flushing is performed in a retrograde fashion against aortic blood flow as opposed to an antegrade approach in human coronaries.

Study Limitations

Our work is not without limitations. First, our bench top model is based on standardized internal diameters, which, while stringently manufactured and controlled for, still report variances in sizing of ±0.08mm/0.003". However, this variance should minimally impact our adjustment coefficients, given its low magnitude and equal distribution in both groups. Second, the importance of arterial blood clearance is paramount for robust image production. The abdominal aorta of rabbits is an established model for assessment of stent implantation and healing.(388,389) However, while it provides a comparable physiologic system and dimensions to human coronary arteries it is less complex than the human coronary tree. Clinical performance in human coronaries would be needed to corroborate our findings and to optimize and validate saline as a dedicated contrast agent for OCT imaging during diagnostic and interventional coronary procedures. While anecdotal use of saline is reported, varying parameters such as volumes, injection parameters and coronary complexity would need to be standardized for evaluation.

6.7 Conclusion

In summary, saline generates reduced dimensions in a linear fashion, enabling robust correction to contrast values. Our model suggests no significant difference in image quality with saline flushing on account of its reduced viscosity, while any differences could likely be mitigated by varying the flush rate and/or duration. Clinical studies investigating the efficacy of saline as a contrast agent for OCT imaging are warranted.

6.8 Sources of Funding

This work was supported by the UOHIAMO AFP Innovations Funding Competition for Innovative Clinical Projects and CFI (Canadian Foundation for Innovation). The Vered-Beanlands Endowed Fellowship (TS). The Canadian Institutes of Health Research [Vanier Research Graduate Scholarship (RGJ) and Banting Postdoctoral Fellowship (FDR)], the Royal College of Physicians and Surgeons of Canada [Detweiler Travelling Fellowship (FDR)]

6.9 Conflicts of Interest

None declared

Chapter 7

Evaluation of a rabbit model of vascular stent healing: application of optical coherence tomography

7.1 Preface

This manuscript has been published in the *Journal of Cardiovascular Translational Research:*

> Simard T, Jung R, Di Santo P, Sarathy K, Majeed K, Motazedian P, Short S, Dhaliwal S, Labinaz A, Sarma D, Ramirez FD, Froeschl M, Labinaz M, Holmes DR, Alkhouli M, Hibbert B. Evaluation of a Rabbit Model of Vascular Stent Healing: Application of Optical Coherence Tomography. (2023) *J Cardiovasc Transl Res*. doi: 10.1007/s12265-023-10399-1. PMID: 37227686.

All authors made substantial contributions to the conception/ design of the work; and/or the acquisition, analysis, or data interpretation; and, (ii) drafted and/or revised the content critically; and, (iii) provided final approval of the published version; and, (iv) agree to be accountable for the work.

7.2 Abstract

Percutaneous coronary intervention (PCI) is a management strategy for symptomatic obstructive coronary artery disease (CAD). Despite advancements, in-stent restenosis (ISR) still imparts a 1-2% annual rate of repeat revascularization – a focus of ongoing translational research. Optical coherence tomography (OCT) provides high resolution virtual histology of stents. Our study evaluates the use of OCT for virtual histological assessment of stent healing in a rabbit aorta model, enabling complete assessment of intraluminal healing throughout the stent. ISR varies based on intra-stent location, stent length and stent type in a rabbit model – important considerations for translational

experimental design. Atherosclerosis leads to more prominent ISR proliferation independent of stent-related factors. The rabbit stent model mirrors clinical observations, while OCT-based virtual histology demonstrates utility for pre-clinical stent assessment. Pre-clinical models should incorporate clinical and stent factors as feasible to maximize translation to clinical practice.

7.3 Introduction

Percutaneous coronary intervention (PCI) has evolved considerably with conventional stent and drug therapies. (244) Despite this, stent-related adverse events still occur at a rate of 5% in the first year after stenting and beyond the first year this risk remains 2% annually without a notable plateau.(276) Considering the volume of stent implantation, this residual risk is of importance.(397,398) Stent failure arises from multiple etiologies with a major driver being in-stent restenosis (ISR), a challenging pathology with limited therapeutic options. (31,397,399,400) Moreover, in those requiring intervention for ISR, 10-20% will experience recurrent ISR for which interventions become progressively more challenging.(397)

The underlying drivers of ISR remain poorly defined, in part due to the complex pathologies that collectively contribute to its development.(31,244) However, patient-level factors, such as diabetes mellitus (DM), and vessel-level factors, such as stent undersizing and underexpansion, are known to contribute to ISR.(31,400) Intravascular imaging with intravascular ultrasound (IVUS) or optical coherence tomography (OCT) provide incremental intracoronary assessment to optimize PCI with improved stent-

related clinical outcomes. (399,401-403) OCT specifically affords detailed vessel sizing, tissue characterization and pathologic insights which can guide pathology-specific interventional approaches.(397,404,405) Moreover, advancements in stent scaffold designs, coating polymers and pharmacologic targets, both systemic and local, continue to be evaluated to further improve stent-related outcomes.(244,284,285,397,398,406-408)

To facilitate these advancements, translational animal models are required to ensure robust evaluation and reliable translation to clinical trials.(389) Pragmatic models for evaluating coronary stents are limited; however, rabbit abdominal aortas and iliac vessels are similar in size to human coronaries(387) and studies of rabbit models for development of atherosclerosis and diabetes exist (388,389). Moreover, rabbits have been previously employed to evaluate carotid healing using OCT and validated with traditional histology. (409) We sought to evaluate a rabbit abdominal aorta model of stent healing to enable focused assessment of the impact of biologic and stent factors on stent healing, while utilizing OCT to both guide stent implantation and serially evaluate stent healing.

7.4 Methods

Animal design

Animal care protocol #2746 was approved by the University of Ottawa Animal Care Committee and standardized animal care provided by the University of Ottawa Heart Institute Animal Care and Veterinary Services team. New Zealand White (NZW) rabbits were obtained at 2 kilograms in size (Charles River Laboratories, Wilmington,MA) with a total of 39 NZW rabbits utilized. For assessment of atherosclerosis, non-atherosclerotic rabbits (n=12, 6 male, 6 female) received regular chow diet (Hi-Fiber Rabbit Diet, Teklad

Envigo, Madison, WI); while atherosclerotic rabbits (n=11, 5 male, 6 female) received the same chow diet supplemented with andomized erol (Teklad Envigo, Madison, WI) for 6 weeks prior to the stent implantation, in keeping with previously described methods.(410) Assessment of DM was performed with induction in 5 male rabbits via a single administration of Alloxan 100mg/kg (dissolved in sterile NS at 5% w/v) though marginal ear vein intravenous. Over the first 48 hours rabbits develop hypoglycemia which was supported with administration of 10cc glucose (5% w/v) subcutaneously, with ad libitum food and 20% glucose in tap water (weight/volume) available. On days 7-10, rabbits had daily AM testing of glucose via middle ear vein prick to confirm diabetic status with 11-30mmol/L defined as mild-moderate DM, while any rabbits with >30mmol/L levels were sacrificed given high probably of developing diabetic ketoacidosis as described in the literature.(411)

Stent implantation and assessment

Using our previously reported methodologies, all rabbits underwent coronary stent implantation in their abdominal aortas. (349,390,405) Utilization of the abdominal aorta for assessment of stent implantation and diet-induced atherosclerosis is an established approach (84,412) with stent dimensions reflecting those used clinically. (413) Transfemoral access with retrograde aortic assessment affords several technical advantages over alternative locations including (i) faster and safer vascular access with more rapid animal recovery, (ii) ability to achieve larger bore 6Fr vascular access (iii) improved retrograde flushing/OCT imaging quality particularly with low-viscosity saline flushing, (iv) ability for multiple access procedures for sequential imaging in survival

experiments of the same animal and (v) improved stent harvest location for histologic analysis.(84,414) Briefly, rabbits under general anesthetic underwent femoral cutdown to visualize the neurovascular bundle. The common femoral artery was dissected and a 6/5-French Glidesheath Slender (Terumo, New Jersey, USA) was inserted via standard Seldinger technique. An 0.014" workhorse coronary intervention wire was advanced through the access sheath and parked in the descending thoracic aorta. Intravascular OCT (Dragonfly Imaging Catheter, Abbott Lifesciences, Illinois, USA) was then used to determine vessel sizing and guide stent selection. **(Figure 45)** We evaluated Driver bare-metal stents (BMS, Medtronic, Minneapolis, USA), Resolute Integrity Drug Eluting stents (DES, Medtronic, Minneapolis, USA) and Absorb Bioresorbable Vascular Scaffold (BVS, Abbott Lifesciences, Illinois, USA). **(Figure 46A,C,E)** Rabbits underwent implantation of either BMS (n=23, 11 male, 12 female), DES (n=7 male) and BVS (n=9 male) within their respective evaluation groups. Stents were implanted at a 3-3.5mm diameter with average stent lengths of 18.1 ± 6.2mm. The distribution of stent lengths implanted included: 7 (17.9%) ≤14mm, 9 (23.1%) 15mm, 11 (28.2%) 16-19mm, 7 (17.9%) 21-26mm and 5 (12.8%) ≥31mm stent lengths. Post stent implantation subjects were maintained on dual anti-platelet therapy with rectal acetylsalicylic acid (ASA) 10mg/kg daily and intra-dermal clopidogrel 14mg daily to prevent stent thrombosis. The sheath was then removed, and hemostasis achieved via arterial ligation. OCT imaging was then performed at 2 weeks (via contralateral access) and 6 weeks post stent implantation at the time of animal sacrifice to assess stent healing. **(Figure 45)**

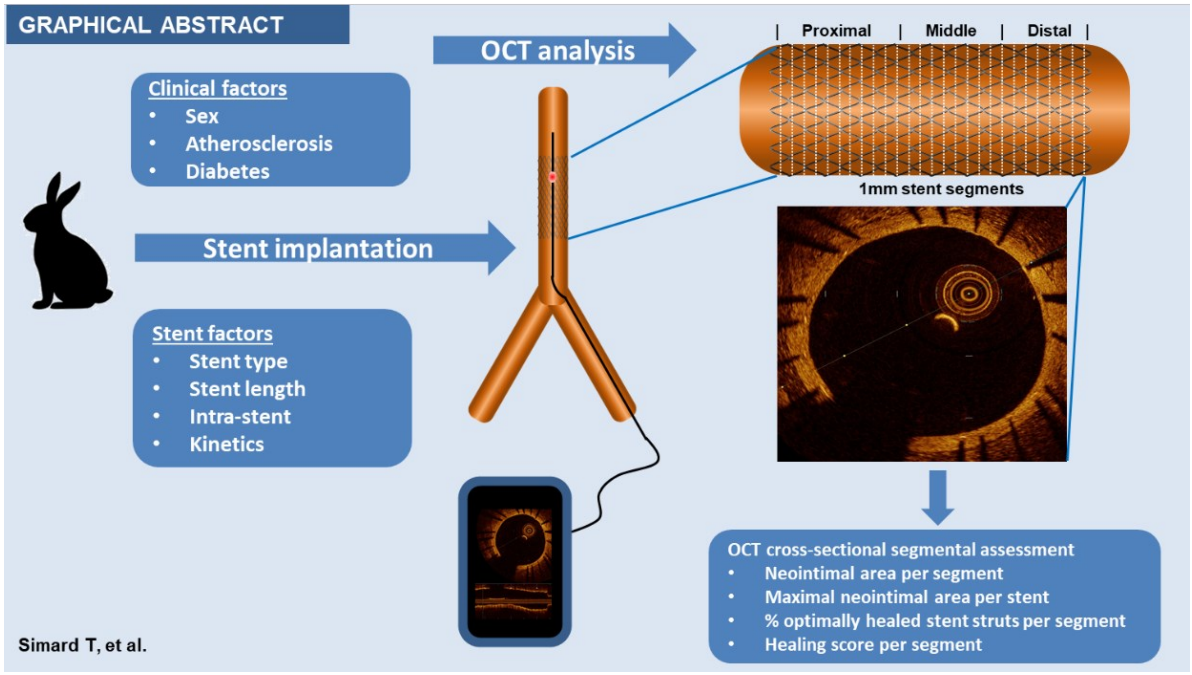

Figure 45. Graphical Abstract - Pre-clinical rabbit stent model with optical coherence tomography (OCT) imaging for assessment of vascular healing.

Stent implantation in abdominal aorta enables OCT quantification of neointimal proliferation and stent strut healing to evaluate the impact of device and disease factors.

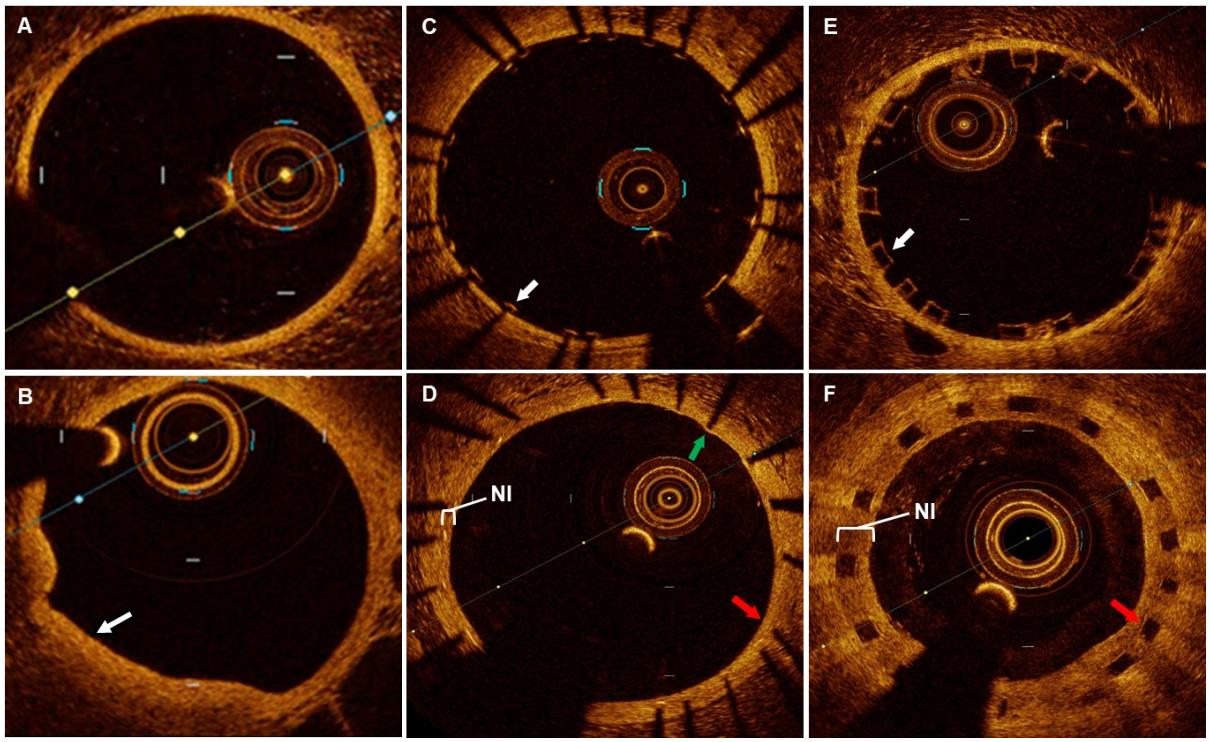

Figure 46. Intravascular optical coherence tomography (OCT)

Representative intravascular optical coherence tomography (OCT) imaged in rabbit abdominal aortas. **(A)** Baseline imaging demonstrating vascular dimensions for stent size selection. **(B)** Baseline imaging of high-cholesterol diet-induced atherosclerosis with visible atherosclerotic plaque (white arrows) presence confirmed prior to stent implantation in this region. **(C)** Immediately following drug-eluting stent (DES) implantation readily demonstrating excellent apposition and expansion and visible low profile individual stent struts (white arrow). **(D)** 6 weeks post DES implantation with relatively thin neointimal (NI) tissue proliferation present (white brackets) with optimally healed stent strut (green arrow) and suboptimal healed stent strut (red arrow) demonstrate**(E)** Immediately following bioresorbable vascular scaffold (BVS) implantation with high profile individual stent struts readily visualized (white arrow). **(F)** 6 weeks following BVS implantation demonstrating suboptimal healing of all stent struts (red arrow delineates single strut) due to prominent diffuse NI proliferation (white bracket delineating) throughout the segment.

OCT analysis of stent healing

Detailed OCT analysis was performed using the ILLUMIEN OCT Data Analysis Station (St. Jude Medical, Minnesota, USA). Atherosclerotic lesions were readily visualized on OCT as described previously **(Figure 46B)**.(415)Independent evaluators were blinded to

each rabbit, stent type and treatment group characteristics completed the OCT analysis similar to prior analyses.(405) Stent analysis was undertaken utilizing a standardized algorithm as adapted from previously published approaches.(416-418) First, the ends of each stent were delineated by the first and last image in which struts were visualized in all four quadrants. Next, we empirically established 1mm sections from proximal to distal stent edge to encompass the entire stented segment, these were then analyzed individually. Stented segments were empirically divided into thirds based on the overall stent length to generate the respective proximal, mid, and distal sections composed of the individual 1mm segments delineated above. **(Figure 45)** Neointimal assessment was performed via measurement of the medial area (MA, mm^2) and luminal area (LA, mm^2) with subsequent neointima (NI) size represented as NI% = [MA– LA] / MA x 100%. NI volume was reported as NI burden (%) and as maximal NI area (%) throughout the entire stent, equivalent to a minimal luminal area (MLA).(416) Strut healing analysis was performed by assessing each individual strut within the same pre-defined segments as the NI analysis. The total number of struts within the segment was recorded as well as the total number of optimally healed struts defined as those that were well apposed with peri-strut rhombus tissue present and either an absent or minimal tissue presence on the luminal aspect of the strut. Struts that were not apposed, did not have NI rhombus tissue present or had markedly prominent tissue present on the luminal aspect of the strut were considered to not be optimally healed. **(Figure 46D,F)**(419) Optimal strut healing (OSH) was then expressed as the proportion of optimally healed struts from the total number of struts in the given segment [OSH% = number of optimally healed struts / total number of struts x 100%]. To incorporate a unified healing score the OSH% and NI% data were

divided to generate a healing score ratio encompassing both the strut healing and NI proliferation aspect of a given segment with a higher ratio representing a more optimally healed segment (i.e. a higher proportion of OSH with low NI volume), while a lower ratio represents a suboptimal healed segment (i.e. a lower proportion of OSH with a higher NI volume).(417,418) **(Figure 45)**

Statistical analysis

Mean or median ± SEM presented, and comparisons performed with unpaired Student's t-test or Mann-Whitney as appropriate. Multiple comparisons with one-way ANOVA. $P<0.05$ considered significant. Significance denoted as ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Analysis performed with GraphPad Prism 9.4.1 (GraphPad Software).

7.5 Results

Cohort

The overall cohort included 39 NZW rabbits with 39 stents implanted , within each stent, 1mm segments were analyzed , yielding a total of 1,076 segments undergoing OCT analysis. Serial OCT analyses were planned at 2 weeks and 6 weeks post stent deployment to assess the kinetics of NI formation in our model, with no significant differences in NI burden between 2 week and 6 week assessments by segmental ($p=0.67$) or maximal NI burden ($p=0.62$) analyses. **(Figure 47)** Given this, sequential OCT segments were pooled for subsequent analyses.

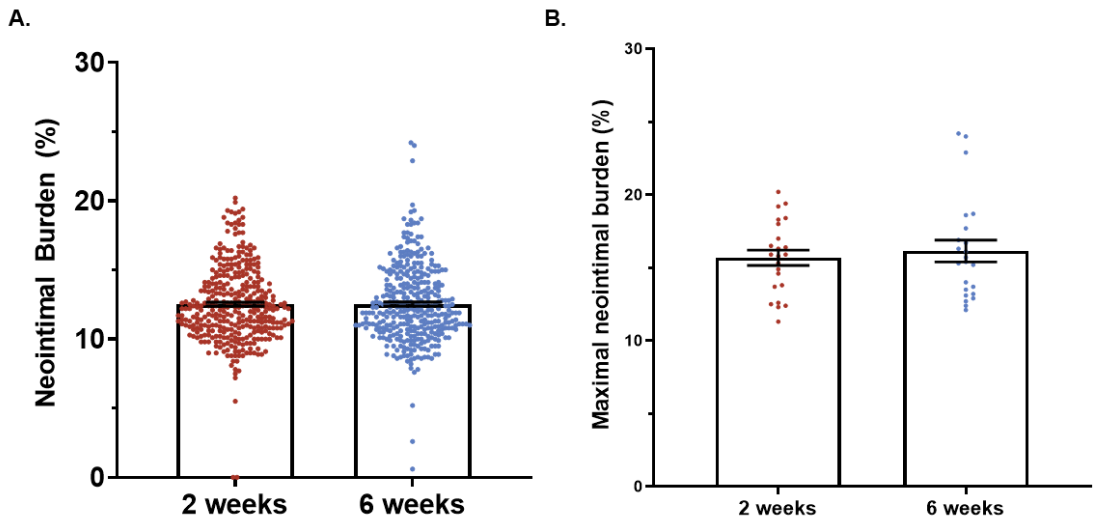

Figure 47. Neointima kinetics

Assessment of 359 1mm stent segments assessed sequentially at 2 weeks and then again at 6 weeks by intravascular optical coherence tomography (OCT). No significant difference in NI burden between 2wks and 6wks when assessed by (A) individual segmental analysis (12.5±0.1% vs 12.5±0.1%, p=0.67) or (B) maximal NI burden per stent (15.7±0.5% vs 16.1±0.7%, p =0.62). Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

Stent-related factors influencing stent healing

Stent factors impacting stent healing were evaluated. First, NI proliferation patterns within the length of the BMSs were assessed, with segments grouped into thirds based on the proximal, middle, or distal portion of the stent. This demonstrated a consistent trend that NI burden was primarily focal, with the NI burden at the stent ends being 8-11% greater than the middle section (p<0.001) by segmental analysis, while a similar trend was observed with the maximal NI noted per section. **(Figure 48 A,B)** Assessment of strut healing demonstrated similar trends with the middle segments demonstrating a greater proportion of optimally healed struts/section (42.8±1.5%) compared to the proximal (26.9±1.7%, p<0.0001) and distal (31.8±1.7%, p<0.0001) sections. **(Figure 48C)** Collectively, the healing score demonstrated a consistent trend with improved healing in the middle section compared to the proximal (p<0.0001) and distal (p=0.009) sections.(**Figure 48D)**

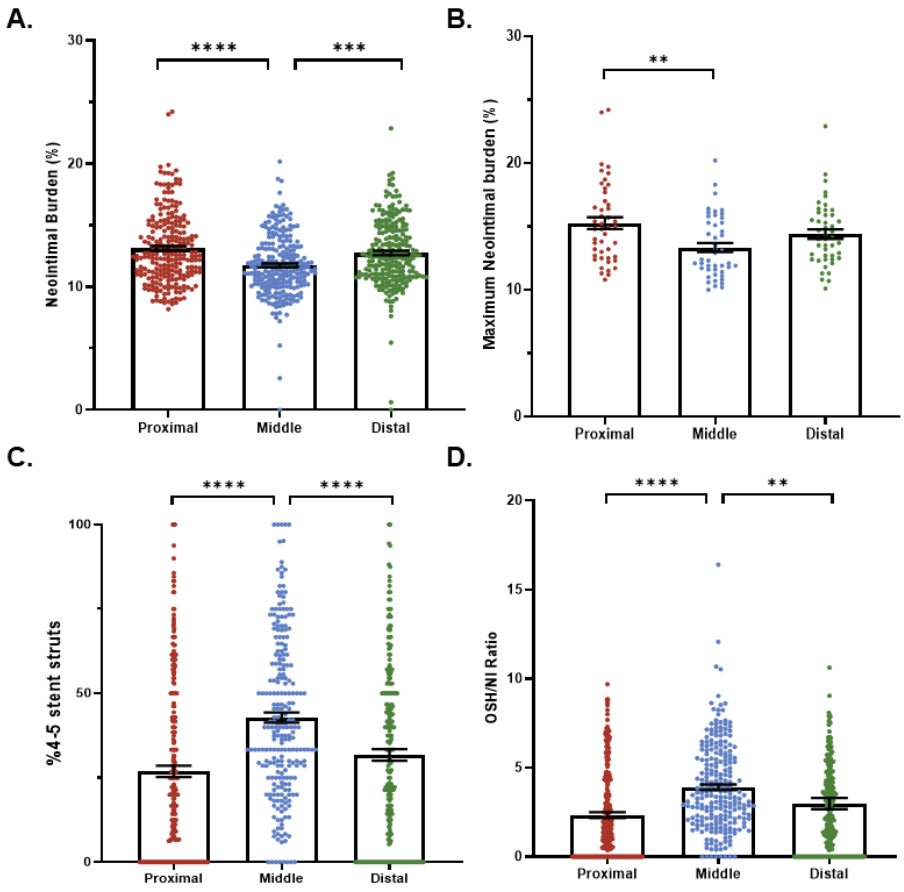

Figure 48. Intra-stent location and healing

Assessment of 718 1mm stent segments grouped into proximal (n=240 segments), middle (n=242 segments) and distal (n=236 segments). (A) Segmental analysis generating mean neointimal (NI) burden throughout each stent section demonstrating that both proximal (13.1±0.2%, p<0.0001) and distal ends (12.7±0.2%, p=0.0002) demonstrating more prominent NI burden than the middle portion (11.7±0.2%). (B) Stent analysis by maximal NI burden per stent demonstrating significant proximal (15.3±0.5%, p=0.003) and non-significant distal (14.4±0.4%,p=0.15) difference compared to the middle portion (13.3±0.4%). (C) Optimal strut healing (OSH) segmental analysis demonstrating reduced proportion of OSH in the proximal (26.9±1.7%, p<0.0001) and distal (31.8±1.7%, p<0.0001) compared to the middle section (42.8±1.5%). (D) Healing score analysis (generated via ratio of % OSH to % NI, higher ratio signifying more optimal healing) demonstrating consistent trend of reduced healing in the proximal (2.3±0.2, p<0.0001) and distal (3.0±0.3, p=0.009) compared to the middle section (3.9±0.2). Mean ± SEM presented, and comparisons performed with one-way ANOVA with significance denoted as ** p<0.01, *** p<0.001, **** p <0.0001.

Next, the impact of stent length on healing patterns was assessed. The BMS cohort (718 one millimeter segments) were divided based upon whether the implanted stent was longer or shorter than the average stent size of the cohort, yielding 398 segments from short stents and 320 segments from long stents. Short stents were noted to have lesser NI burden both by segmental analysis (11.9% relative reduction, p<0.0001) and by maximal NI burden per stent (12.9% relative reduction, p=0.02). **(Figure 49A,B)** Similarly, segmental strut analysis demonstrated a 31.8% relative increase in optimally healed struts in the short stent cohort (p<0.0001). **(Figure 49C)** Collectively, short stents demonstrated improved healing scores compared to long stents (p<0.0001) and the pattern of healing demonstrated more diffuse suboptimal healing in the ends of the stents extending towards the middle, compared to their short stent counterparts. **(Figure 49D,E)**

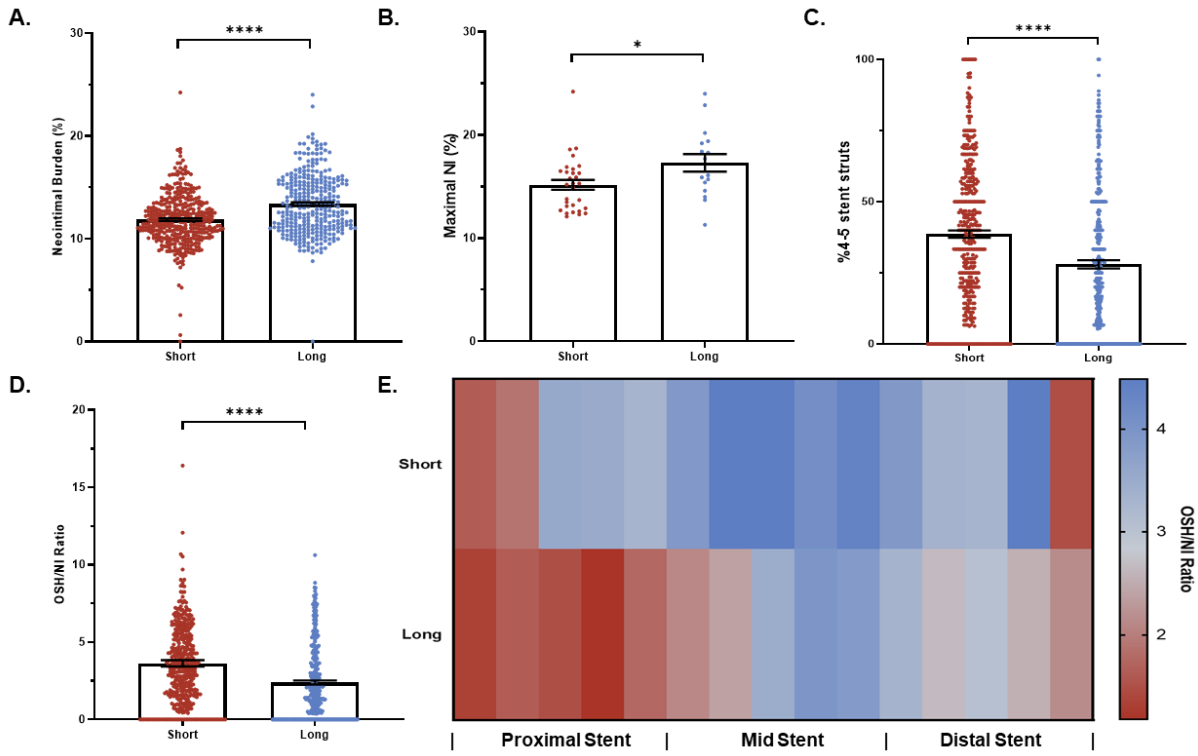

Figure 49. Stent length and healing

Assessment of 718 1mm stent segments were empirically divided into those contained within short stents (398 segments) and long stents (320 segments). Overall NI burden was less with short versus long stents when assessed by both **(A)** individual segment analysis (11.9±0.1% vs 13.4±0.2%, p<0.0001) and **(B)** maximal NI area per stent (15.2±0.5% vs 17.3±3.4%, p=0.02). **(C)** Optimal strut healing (OSH) segmental analysis demonstrating a greater proportion of optimally healed struts in short compared to long stents (38.6± 1.3% vs 28± 1.4%, p<0.0001). **(D)** Healing score analysis (ratio of %OSH to %NI) demonstrated an improved healing profile in short stents (3.6±0.2 vs 2.4±0.1, p<0.0001). **(E)** Heat map subjectively demonstrating more prominent NI in

long stents extending through the end sections compared to shorter stents. Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

Last, the impact of stent type on healing was assessed with comparison of DES and BVS implanted in the same experimental design were made. This demonstrated reduced NI burden with DES compared to BVS when assessed segmentally (**Figure 50A**, 13.5±0.4% vs 31.9±7.1%, p<0.0001) and by maximal NI per stent (**Figure 50B**, 28.8±2.8% vs 44.0±1.6%, p=0.0002). While segmental analysis demonstrated no statistical difference in segmental strut healing **(Figure 50C)** the overall healing score was improved for the DES cohort (**Figure 50D**, p<0.0001) Subjective analysis of the heat map for the overall stent healing score demonstrated marked diffuse suboptimal healing throughout the stent in the BVS cohort, differing substantially from the primarily focal, end-centric adverse healing noted in DESs **(Figure 50E).**

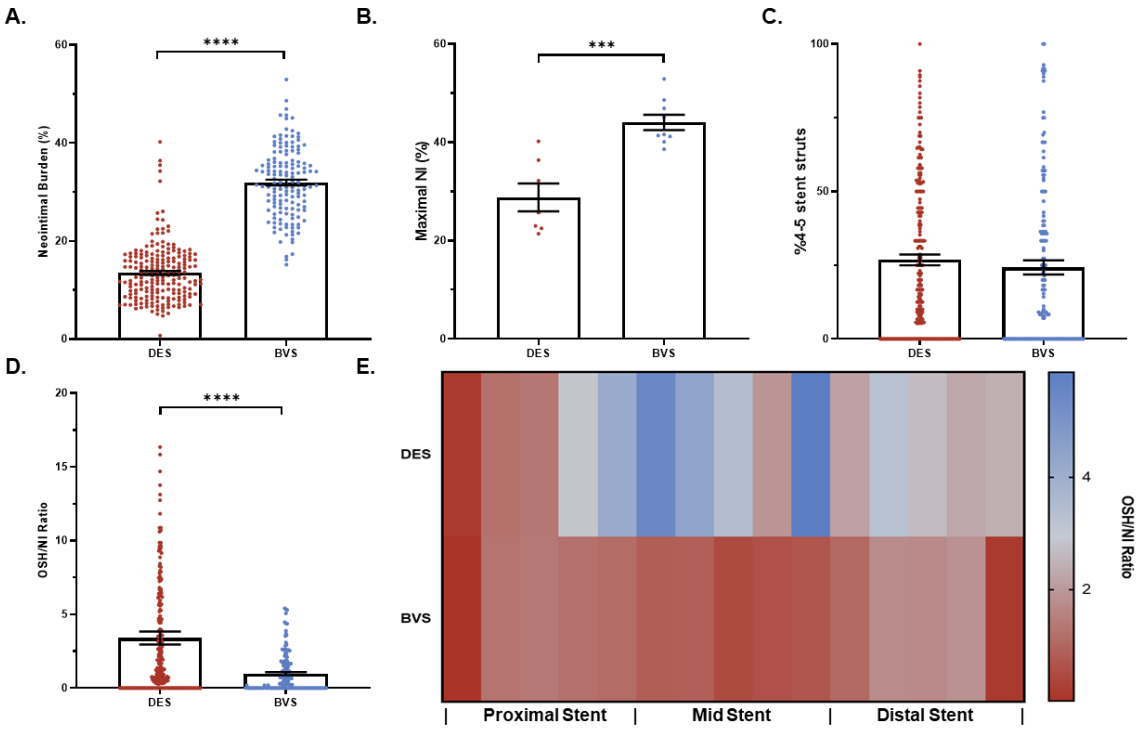

Figure 50. Stent type and healing

Assessment of 358 1mm stent segments including drug-eluting stents (DES, 209 segments) and bioresorbable vascular scaffolds (BVS, 149 segments). Neointima (NI) burden was less with DES compared to BVS when assessed by both **(A)** individual segment analysis (13.5±0.4% vs 31.9±7.1%, p<0.0001) and **(B)** maximal NI area per stent (28.8±2.8% vs 44.0±1.6%, p=0.0002). **(C)** Optimal strut healing (OSH) segmental

analysis with no difference in proportion of optimally healed stent struts between DES and BVS (26.8± 1.8% vs 24.3±2.4%, p=0.4). **(D)** Healing score analysis (ratio of %OSH to %NI) demonstrated an improved healing profile in DES compared to BVS stents (3.4±0.4 vs 1.0±0.1, p<0.0001). **(E)** Heat map subjectively demonstrating markedly different healing pattern with diffuse suboptimal healing pattern throughout the BVS stents compared to DES. Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

Impact of risk factors on stent healing

Assessment of the impact of clinical risk factors including sex, atherosclerosis and diabetes on stent healing were subsequently assessed in our rabbit stent model. First, the impact of sex was assessed via comparison of females (356 stent segments) and males (362 stent segments) with a slight trend towards increased NI volume in males by segmental analysis (**Figure 51A,** 12.3±0.1% vs 12.8±0.2%, p=0.02) but without differences in maximal NI volume **(Figure 51B,** p=0.43), optimal strut healing (**Figure 51C**, p=0.52) or healing scores (**Figure 51D**, p=0.67) with subjective healing patterns remaining similar between both sexes **(Figure 51E).** In the atherosclerosis cohort, successful induction of atherosclerosis with high cholesterol diet was documented on OCT with diffuse atherosclerotic plaques throughout the abdominal aortas with macrophage-rich plaques noted on immunohistochemistry. **(Figure 46B, Figure 53).** Atherosclerosis relatively increased the NI burden by 15.9% (p<0.0001) as assessed by segmental NI volume **(Figure 52A)** and 16.9% (p=0.0002) by maximal NI thickness per stent **(Figure 52B).** Similarly, those with atherosclerosis had a 16.9% reduction in proportion of optimally healed stent struts **(Figure 52C)** and collective healing scores **(Figure 52D)** with subjective healing patterns demonstrating more marked distal adverse healing profiles compared to the non-atherosclerotic counterparts **(Figure 52E).** Additional analyses demonstrated a consistent augmentation of NI burden regardless of stent section, stent length or kinetics – suggesting the consistent impact of atherosclerosis on NI formation **(Figure 54).** Last, assessment of diabetes on stent healing in a subgroup of rabbits demonstrated no overt differences in NI volume, stent strut healing or healing scores **(Figure 55A-D).** Healing pattern analysis demonstrated a more diffuse adverse healing profile in the stent ends of diabetic rabbits **(Figure 55E).**

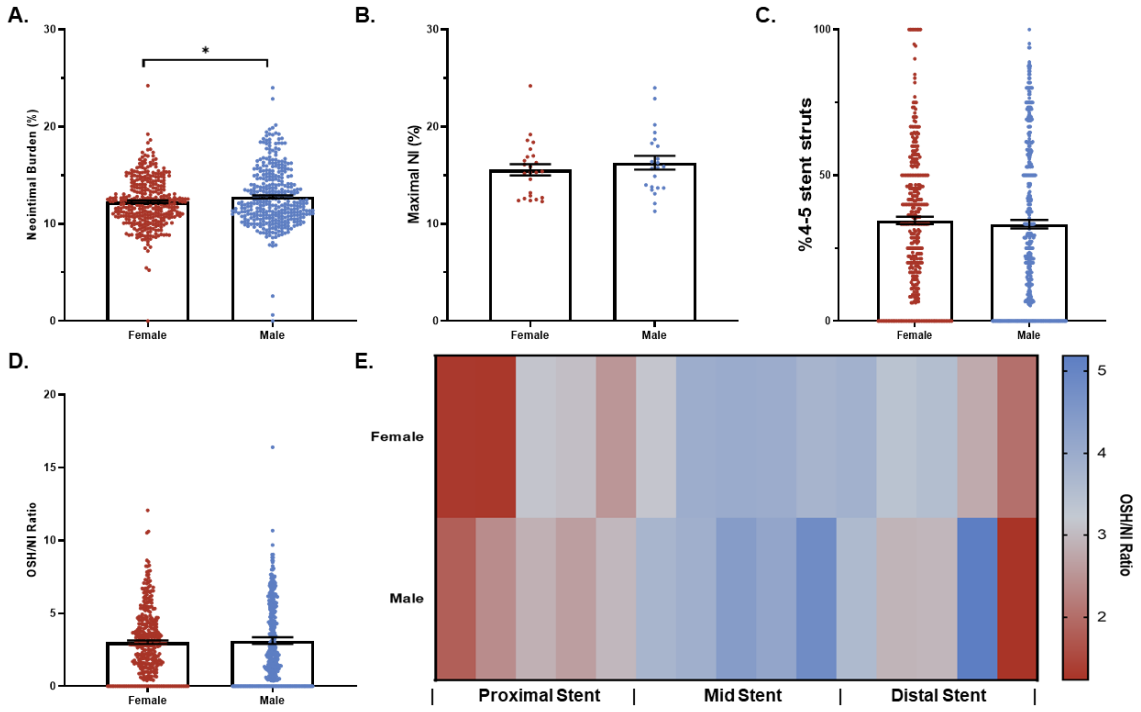

Figure 51. Sex and stent healing

Assessment of 718 1mm stent segments implanted in females (356 segments) or males (362 segments). Neointima (NI) burden differed with **(A)** individual segment analysis revealing statistically less NI burden in females vs males (12.3±0.1% vs 12.8±0.2%, p=0.02) with **(B)** maximal NI area per stent demonstrating no difference (15.6±0.6% vs 16.3±0.7%, p=0.43). **(C)** Optimal strut healing (OSH) segmental analysis with no difference in optimally healed stent struts in females compared to males (34.5± 1.3% vs 33.2±1.5%, p=0.52). **(D)** Healing score analysis (ratio of %OSH to %NI) demonstrated no difference in healing profile in females vs males (3.0±0.1 vs 3.1±0.2, p=0.67). **(E)** Heat map demonstrating subjectively similar focal healing patterns favoring the stent ends. Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

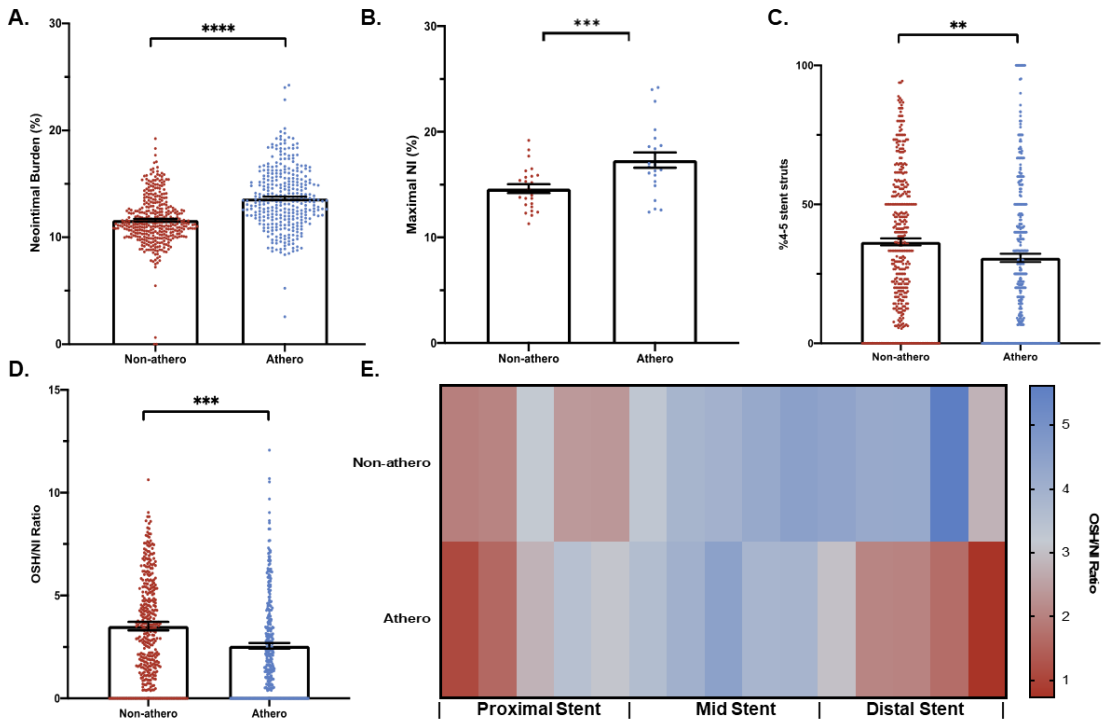

Figure 52. Atherosclerosis and stent healing

Assessment of 718 1mm stent segments implanted in the absence (390 segments) or presence (328 segments) of diet-induced atherosclerosis. Neointima (NI) burden was less in the absence of atherosclerosis when assessed by both **(A)** individual segment analysis (11.6±0.1% vs 13.6±0.2%, p<0.0001) and **(B)** maximal NI area per stent (14.6±0.4% vs 17.3±0.7%, p=0.0002). **(C)** Optimal strut healing (OSH) segmental analysis with greater proportion of optimally healed stent struts in those without atherosclerosis (36.5± 1.3% vs 30.8±1.5%, p=0.0002). **(D)** Healing score analysis (ratio of %OSH to %NI) demonstrated an improved healing profile in non-atherosclerotic subjects compared to those with atherosclerosis (3.5±0.2 vs 2.6±0.1, p=0.003). **(E)** Heat map subjectively demonstrating more prominent suboptimal healing extending further into stent in the setting of atherosclerosis. Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

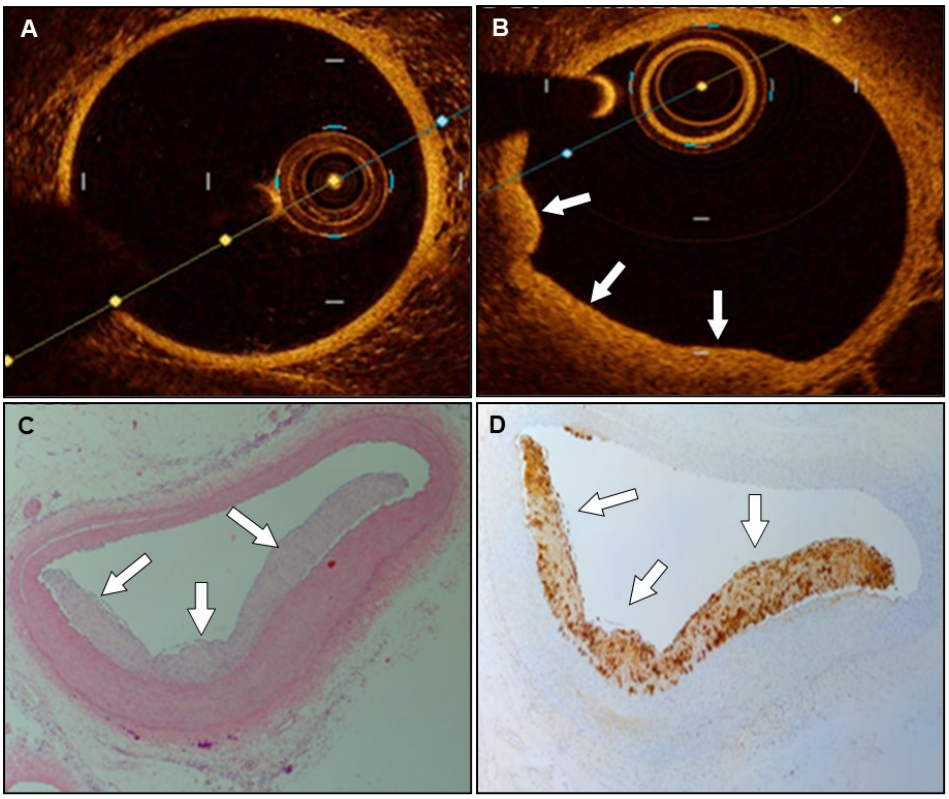

Figure 53. Atherosclerosis on optical coherence tomography and immunohistochemistry

(A) Baseline normal vessel on pre-stent implantation optical coherence tomography (OCT) assessment for comparison. **(B)** Atherosclerotic lesion readily visualized on OCT assessment with bulky lesion protruding into the lumen and signal attenuation noted beyond the lesion. Immunohistochemistry assessment of arterial cross-section with **(C)** hematoxylin and eosin staining demonstrating atherosclerotic plaque presence (white arrows) and **(D)** RAM-11 staining of cross section demonstrating atherosclerotic plaque (white arrows) with dense macrophage content (brown).

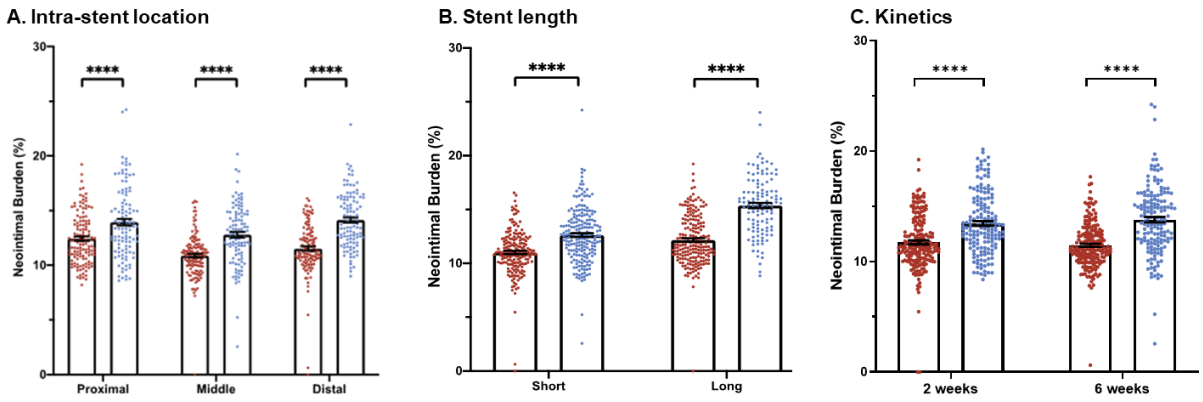

Figure 54. Atherosclerosis and neointima burden

Assessment of 718 1mm stent segments implanted in the absence (390 segments, red) or presence (328 segments, blue) of diet-induced atherosclerosis. Neointima (NI) burden was less without atherosclerosis compared to with atherosclerosis across all comparisons as follows: **(A)** Intra-stent segments – proximal (12.4±0.2% vs 13.9±0.3%, p<0.0001), middle (10.9±0.2% vs 12.8±0.3%, p<0.0001) and distal (11.5±0.2% vs 14.1±0.2%, p<0.0001); **(B)** Stent sizes - short stents (11.0±0.2% vs 12.6±0.2%,p<0.0001) and long stents (12.2±0.2% vs 15.4±0.2%, p<0.0001)**(C)** Kinetic assessment with 2 week (11.7±0.2% vs 13.5±0.2%, p<0.0001) and 6 week NI burden (11.5±0.2% vs 13.8±0.2%, p<0.0001) demonstrating similar trends comparing non-atherosclerosis to atherosclerosis. Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

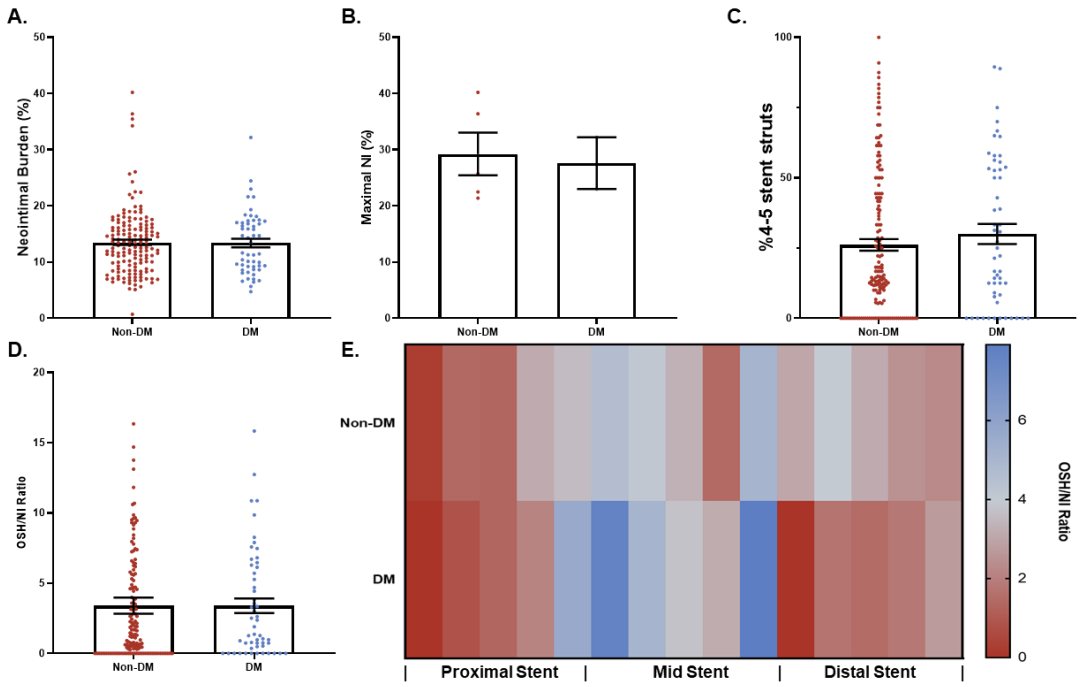

Figure 55. Diabetes and stent healing

Assessment of 209 1mm stent segments implanted in diabetic (54 segments) or non-diabetic subjects (155 segments). Neointima (NI) burden did not differ between the presence or absence of diabetes by either **(A)** individual segment analysis (13.5±0.5% vs 13.4±0.8%, p=0.9) or **(B)** maximal NI area per stent (29.2±3.8% vs 27.6±4.6%, p=0.82). **(C)** Optimal strut healing (OSH) segmental analysis with no difference in optimally healed stent struts (26.1± 2.1% vs 30.0±3.6%, p=0.34). **(D)** Healing score analysis (ratio of %OSH to %NI) demonstrated no difference in healing profile in non-diabetics vs diabetics (3.4±0.6 vs 3.4±0.5, p=1.0). **(E)** Heat map demonstrating subjectively similar focal healing patterns favoring the stent ends with more extensive adverse healing in the diabetic cohort. Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

7.6 Discussion

Validated translational models for evaluation of vascular healing following stent implantation are critical for evaluation of novel stent and systemic therapies. We evaluated a translational model for assessment of coronary stent healing utilizing stent implantation in rabbit abdominal aortas with evaluation by virtual histology with intravascular OCT. Our model mirrors clinical observations including the impact of stent length, stent type/strut thickness and differential intra-stent healing patterns observed.

Evaluable clinical factors include atherosclerosis, associated with augmented NI burden, while the impact of sex and diabetes, though readily evaluable with this model, remain to be discerned. This model facilitates refined preclinical experimental design, ensuring maximal resource optimization while maintaining clinical translatability.

Stent factors

We evaluated the impact of stent related factors on the healing patterns relative to clinical observations. IVUS studies of human ISR patterns include type I (focal, <10mm), type II (ISR >10mm within stent), III (ISR >10mm outside the stent) and pattern IV (complete occlusion of the stent).(400) Clinically, most patients demonstrate focal ISR with predictors of TLR including diabetes, recurrent ISR and higher ISR class.(399,400) Our rabbit model is similar with a primarily focal ISR pattern and diminished healing profile favoring the stent ends while sparing the middle. Stent size is also of importance, with NI thickness known to progress independent of vessel size; hence, disproportionately affecting smaller diameter vessels, while longer stents similarly demonstrate augmented ISR rates.(413) We demonstrate more marked NI proliferation and diminished strut healing with longer stents coupled with a preserved focal healing pattern, in keeping with clinical observations.(413) Considering the consistent aortic size at 3-3.5mm of our rabbit model, we did not assess the impact of varying stent diameters, though this size range is in keeping with >70% of the stent diameters implanted clinically, ensuring the broad applicability of the sizes presented to human coronary diameters. (413) Moreover, our model would accommodate for smaller diameters via either iliac or carotid stent implantation if desired. Lastly, we assessed the impact of stent type and strut profile by comparing BVS stents to their DES counterparts, demonstrating more marked and diffuse NI proliferation coupled with diminished healing when compared to their DES counterparts, matching that seen clinically.(399,400) A likely driver of the marked NI proliferation is the greater strut thickness of BVS stents (150um), with strut thickness being an independent predictor of lumen loss.(420,421) While assessment of late lumen loss is challenging in a preclinical model, device thrombosis and TLR were primary drivers of the BVS's low demand and subsequent withdrawal from the market,

highlighting the challenges with direct translation of animal models to clinical practice.(422) Collectively, our translational model demonstrates consistent stent-related observations to humans with respect to ISR. This supports the model's translatability to clinical practice, while also highlighting the key stent factors to be considered in experimental design.

Clinical factors

Our preclinical model enables assessment of some clinical factors. Sex remains an important factor dictating discrepant outcomes across numerous clinical interventions and outcomes, with females often under-represented in cardiovascular trials.(71,398,423-426) Sex-related stent outcomes remain a subject of debate, without a clear clinical signal towards differential ISR rates in either sex following coronary stent implantation.(427) Our model demonstrates a small difference suggesting greater NI burden in males though overall similar NI burdens, strut healing and patterns were observed, mirroring clinical observations. While our model is not adequate to exclude sex-based impact on stent healing, it suggests any potential impact may not be of significant magnitude in the rabbit model. However, it would remain prudent to maintain equal sex distribution in pre-clinical and clinical studies evaluating stent healing to ensure no differential effects with novel therapies. In humans, stents are generally implanted and heal in the presence of atherosclerosis.(244) Clinical studies support the presence of early ISR with homogeneous NI hyperplasia followed by late ISR which is typically attributed primarily by neoatherosclerosis.(404,428) In rabbit atherosclerosis models, stent implantation is a known nidus for acute phase response and systemic inflammation leading to non-target plaque progression (429). Our model demonstrates the adverse impact of atherosclerosis on stent healing with a 16-17% relative increase in NI volumes and reduction in optimal strut healing. Hence, while atherosclerosis is a heterogenous pathology that is difficult to replicate, atherosclerosis should be included in the evaluation of novel stent therapeutics, given the impact this pathology has on stent healing and implications for therapeutic efficacy. Lastly, diabetes is an established independent predictor of NI hyperplasia leading to more diffuse and advanced stages of ISR, (400) with studies suggesting more

prominent type Ib ISR (ISR primarily focused to the stent edges).(430) Our model of diabetes induction is successful, while also demonstrating similar stent healing patterns observed clinically, with more prominent and diffuse ISR extending from the stent edges, supporting the models validity. While we did not show statistical difference in NI burden, this likely reflects the small sample size in this more complex experimental model, warranting further dedicated evaluation in future studies.

Translational study design

Our study has several implications for the translational research on stent healing. First, we describe pre-clinical approaches to assess the technical, stent-specific, and clinical risk factors impacting stent healing – important considerations for future pre-clinical studies. Second, considering the differential intra-stent healing patterns observed, traditional evaluation of stent healing by manual histologic analysis of a pre-determined portion(s) of a stent is insufficient to fully evaluate the totality of stent healing. Hence, virtual histology with OCT should be a standard approach, with a hybrid model including traditional histology focused for specific biological questions of interest to maximize resource utilization. Third, NI assessment by OCT can be reported by either segmental analysis or minimal luminal area (MLA). Both provide consistent measures of NI burden, with MLA being the simplest metric, while also demonstrating clinical utility both for assessment of obstructive coronary stenoses (431) and post-stent optimization.(401) Going forward, OCT is well suited to artificial intelligence (AI) evaluation to incorporate the vast datapoints generated and enable granular intravascular assessment both structurally (432) and functionally (433). Intravascular imaging further facilitates patient-specific treatment algorithms (404) and post-stent optimization indices(434), improving clinical outcomes. Fourth, optimal timing of preclinical post-stent assessment remains unknown. Our model demonstrates similar stent healing profiles at 2 and 6 weeks, an important consideration for experimental design, though the impact on pre-clinical study design and translatability to humans remains to be discerned. (404,413) Regardless, the presented model readily facilitates kinetics assessments via serial imaging in the same

animals, mitigating animal requirements and allowing for efficient pre-clinical assessment to further evaluate this important aspect of stent healing.

Limitations

Our study is not without limitations as our model facilitates efficient assessment of variables impacting stent healing, though pre-clinical findings require validation in clinical trials. Given the consistent size of rabbit abdominal aortas, stent implantation ranged at sizes of 3.0-3.5mm stents precluding assessment of varying stent diameters, a known driver of restenosis. However, this could be accomplished via varying rabbit sizes or implantation in the carotids or iliofemoral vessels. Similarly, our model does not assess mechanical factors, also known to drive stent-related outcomes. However, assessment of stent under-expansion or mal-apposition could be achieved with intentional under-sizing and under-deployment based upon pre-stent OCT assessment. Assessment of clinical parameters including sex, atherosclerosis and diabetes are inherently limited in a translational model, and while they may share similarities they never replicate the complexity of the corresponding human processes. However, our model shows promising parallels in stent healing to clinical observations and warrants further detailed evaluation to further refine and improve translatability of the model. Experimental design did not allow for dedicated randomization or sample size calculations for each endpoint presented, which may impact the results observed. Non-target lesion events also contribute clinical outcomes(429), while this was not a focus of the current study, the model could readily accommodate such assessment, amongst other variables.

7.7 Conclusion

Optical coherence tomography assessment of vascular healing in a rabbit aorta stent model demonstrates similar characteristics to human coronary healing with regards to device and disease specific factors. This model may serve as a valuable tool for pre-clinical assessment of novel therapeutics and device development.

7.8 Sources of Funding

This work was supported by the UOHIAMO AFP Innovations Funding Competition for Innovative Clinical Projects and CFI (Canadian Foundation for Innovation). The Vered-Beanlands Endowed Fellowship (TS). The Canadian Institutes of Health Research [Vanier Research Graduate Scholarship (RGJ) and Banting Postdoctoral Fellowship (FDR)], the Royal College of Physicians and Surgeons of Canada [Detweiler Travelling Fellowship (FDR)]

7.9 Conflicts of Interest

None declared

Chapter 8

Dipyridamole and vascular healing following stent implantation

8.1 Preface

This chapter is currently under review for publication.

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8.2 Abstract

Introduction

Patients undergoing coronary stent implantation still incur a 2% annual rate of adverse events, largely driven by in-stent restenosis (ISR) due to neointimal (NI) tissue proliferation, a process in which smooth muscle cell (SMC) biology is felt to play a central role. Dipyridamole (DP) is an approved therapeutic agent with data supporting improved vascular patency rates. Pre-clinical data supports that DP may enact its vasculoprotective effects via adenosine receptor A2B (ADOR-A2B). We sought to evaluate the efficacy of DP to mitigate ISR in a pre-clinical rabbit stent model.

Methods & Results

24 New Zealand White Rabbits were divided into two cohorts – non-atherosclerosis and diet-induced atherosclerosis (n=12/cohort, 6 male and 6 female). Following stent implantation, rabbits were randomized 1:1 to control or oral dipyridamole therapy for 6 weeks followed by optical coherence tomography (OCT) and histology assessment of NI

burden and stent strut healing. Compared to control, DP demonstrated a 16.6% relative reduction in NI volume (14.7±0.8% vs. 12.5±0.4%, p=0.03) and a 36.2% relative increase in optimally healed stent struts (37.8±2.8% vs 54.6±2.5%, p<0.0001). Atherosclerosis demonstrated attenuated effect with no difference in NI burden (15.2±1.0% vs. 16.9±0.8%, p=0.22) and only a 14.2% relative increase in strut healing (68.3±4.1% vs 78.7±2.5%, p=0.02). DP treated rabbits demonstrated a 44.6% (p=0.045) relative reduction in NI SMC content. *In vitro* assessment of DP and coronary artery SMCs demonstrated dose-dependent reduction in SMC migration and proliferation. Selective small molecule antagonism of ADOR-A2B abrogated the effects of DP on SMC proliferation. DP modulated SMC phenotypic switching with ADOR-A2B siRNA knockdown supporting its role in the observed effects.

Conclusion

Dipyridamole reduces NI proliferation and improves stent healing in a preclinical model of stent implantation with conventional antiplatelets. Atherosclerosis attenuates the observed effect. Clinical trials of DP as an adjunctive agent may be warranted to evaluate for clinical efficacy in stent outcomes.

8.3 Introduction

Percutaneous coronary intervention (PCI) with stent implantation (244) is still challenged by a 2% annual rate of stent-related adverse events which persist in follow-up.(276,397,398) In-stent restenosis (ISR) due to hyperproliferative neointimal (NI) tissue proliferation, remains a leading cause of stent related adverse events. Despite this, the pathophysiology behind NI proliferation remains incompletely understood, with pathologic smooth muscle cell (SMC) migration and proliferation felt to contribute to

some extent.(31,244,397) ISR remains the focus of ongoing investigation to improve our understanding of the underlying biology and develop novel therapeutics to improve stent-related outcomes.

Adenosine (ADO) carries numerous regulatory roles both intracellularly and extracellularly, impacting several cell populations involved in vascular homeostasis, including SMCs. (3,8,311) Adenosine signals primarily via 4 receptors with preclinical work suggesting a potential role for adenosine receptor (ADOR)-A2B in the regulation of the vascular effects observed, particularly NI proliferation.(32) Indeed, *in vitro* and *in vivo* arterial injury models support adenosine mitigating NI formation via ADOR-A2B-mediated inhibition of SMC proliferation.(33,38,435) Despite this promising preclinical work, ADO has yet to establish itself as a viable therapeutic approach for ISR, owing in part to its innate limitations. (91) However, focused ADOR small molecule agents have demonstrated improved utility and are used broadly clinically.(98) Hence, evaluation of alternative agents which modulate adenosine signaling, but with improved clinical pragmatism holds promise for potential therapies.

Dipyridamole (DP) is an established, cost-effective, FDA-approved therapy with a broad range of clinical indications.(113) DP improves vascular outcomes in several settings and mitigates restenosis rates following revascularization in both preclinical and clinical studies.(408) DP functions primarily as a phosphodiesterase (PDE) 5/6 inhibitor, mitigating cAMP/cGMP breakdown, a mechanism which previously demonstrated clinical benefit for ISR reduction.(338) However, DP is also known to augment

circulating ADO levels primarily via ENT-1 re-uptake inhibition.(311,408) Preclinical studies suggest that DP may enact its vasculo-protective effects via ADOR-A2B-mediated inhibition of SMC proliferation, though this is based upon *in vitro and* preclinical models with limited data in the context of stent implantation and conventional antiplatelet therapy.(38)

Given the hypothesized impact of adenosine biology on vascular remodeling after stenting, we sought to evaluate the efficacy of DP to mitigate ISR in a conventional preclinical rabbit stent model.

8.4 Methods

Preclinical rabbit model of stent healing

We utilized our established translational model of stent evaluation in New Zealand White Rabbits in which coronary stents were implanted in the abdominal aortas of equal numbers of male and female rabbits via femoral access.(349,390,405) Evaluation of stent implantation in atherosclerotic lesions was performed via addition of 1% cholesterol to the regular diet (Hi Fiber Rabbit Diet, Teklad Envigo, Madison, WI) for 6 weeks prior to stent implantation as reported previously with plaques visualized on OCT. (410) Following stent implantation all rabbits were maintained on dual antiplatelet therapy with ASA (PR 35mg/2ml PR gel q2d) and clopidogrel (SQ (ear) 14mg day prior, then 3.5mg daily thereafter). Rabbits (n=12) were then randomized to either control (n=6) or dipyridamole (n=6) therapy for 6 weeks followed by evaluation by both virtual histology with OCT and traditional histology **(Figure 56)**. In the atherosclerosis cohort, 12 separate

rabbits underwent stent implantation following induction of atherosclerotic lesions visualized on OCT(415), followed by randomization to either control (n=6) or dipyridamole (n=6) therapy. Dipyridamole was administered daily orally (2.5mg/kg/day, range 5-7.5mg/day). Animal studies were approved by the University of Ottawa Animal Care Committee with care provided by the Animal Care and Veterinary Services team. At 6 weeks, virtual histology was performed with intravascular optical coherence tomography (OCT) (Dragonfly Imaging Catheter, Abbott Medical, Minnesota, USA) calibrated per clinical standards, utilizing methodologies previously reported.(416-418) Detailed OCT analysis was performed by evaluators blinded to the rabbit sex and treatment group. NI was quantified by measurement of the medial area (MA, mm^2) and luminal area (LA, mm^2) combined to report NI quantification as NI% = [MA– LA] / MA x 100%.(405) On the vast majority of assessments, the intimal:medial border was readily visualized by OCT and directly measured. However, when this was not well visualized in some areas of a segment, then the outer stent borders were instead measured, and when these were not well visualized then the known stent strut thickness measured from the luminal aspect of the stent strut was used to approximate the location of the intimal:medial border. **(Figure 57)**. %NI was quantified in 1mm segments throughout the stent with the maximal NI area (%) throughout the stented segment, or minimal luminal area (MLA) ultimately analyzed.(416) The primary endpoint of maximal NI burden per stent was reported as a relative difference on OCT-based quantification to optimize reproducibility. Optimal strut healing (OSH) was quantified as the proportion (%) of OSH struts per 1mm cross-sectional segment divided by the total number of stent struts present per segment. OSH was defined as prior as the presence of either rhomboid NI

tissue on the strut sides with or without minimal luminal strut tissue coverage, while struts that were mal-apposed, uncovered, or had prominent luminal tissue present were not considered optimally healed.(419) **(Figure 57)** Plasma adenosine levels were quantified as reported previously.(284,285)

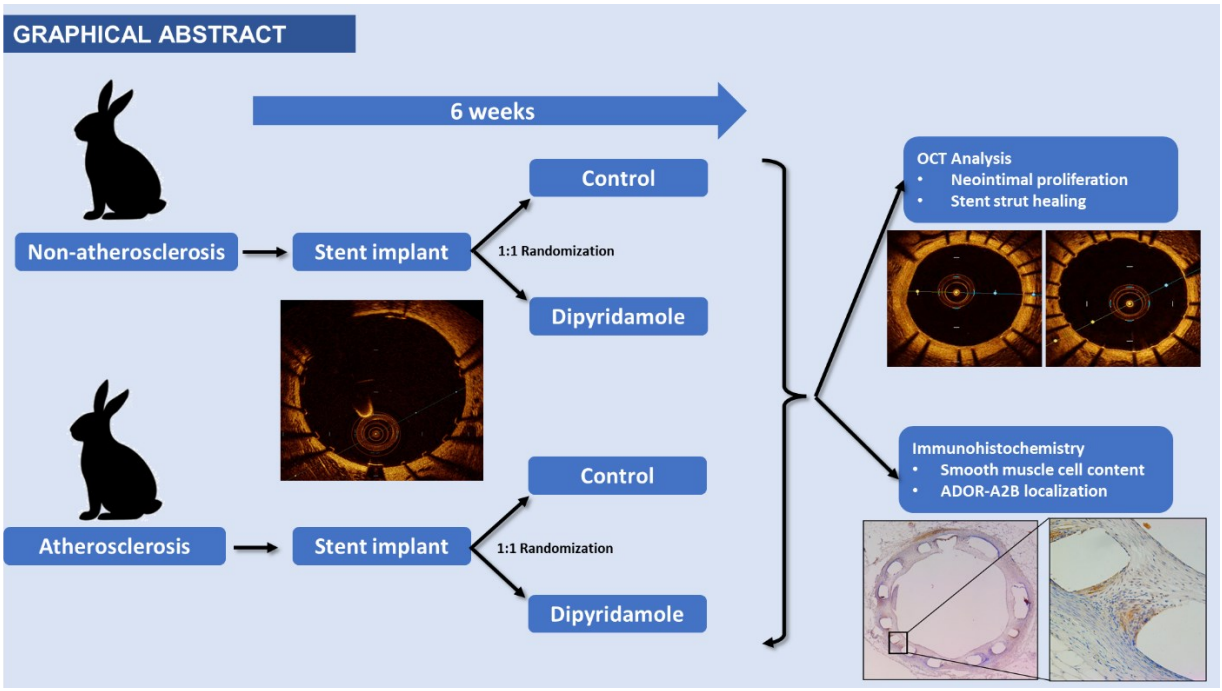

Figure 56. Graphical abstract

Non-atherosclerotic and atherosclerotic rabbits were randomized to control or dipyridamole for 6 weeks followed by assessment by intravascular OCT and histology.

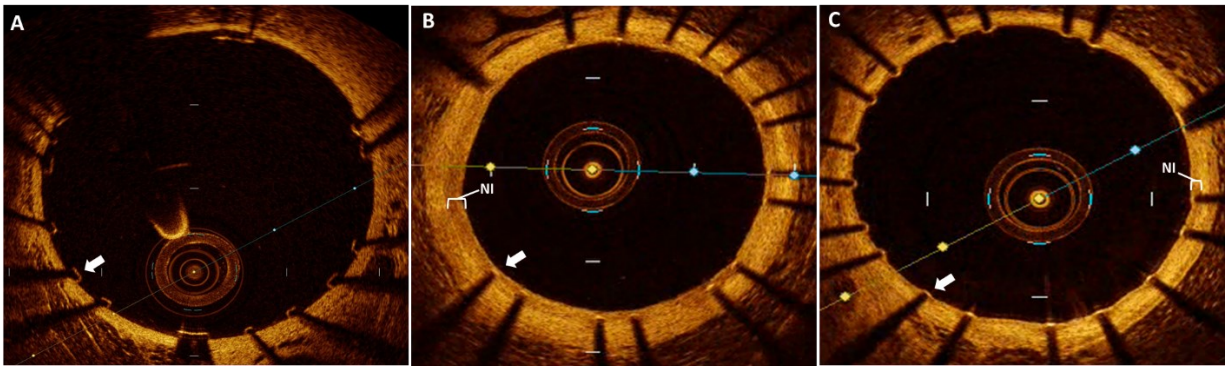

Figure 57. Intravascular optical coherence tomography (OCT) assessment of stent healing

Representative OCT images of rabbit aorta following human coronary stent implantation. **(A)** immediately following stent implantation demonstrating well apposed and completely uncovered stent struts without neointimal (NI) tissue. Followed by 6 weeks post stent implantation with **(B)** control group demonstrating a prominent neointimal (NI, white bracket) burden with all stent struts sub optimally healed due to prominent NI burden overlaying each strut (white arrow), while **(C)** dipyridamole treated subjects demonstrate a minimal NI burden (white bracket) just covering the stent struts with all individual struts optimally healed (white arrow).

Immunohistochemistry

At 6 weeks post stent implantation, rabbit aortas were harvested, the stent struts removed and paraffin embedded. They were then deparaffinized, serially washed, incubated in 3% H2O2, washed, blocked for 10min with 10% goat serum, followed by the respective primary antibody diluted in 1:50 blocking solution and incubated at 4C overnight. Primary antibodies included: SMC (monoclonal, actin antibody, mouse anti-rabbit, MA5-11869, Invitrogen, Carlsbad, CA) and ADORA-2B (polyclonal, goat anti-Human, Novus Biological, Oakville, ON, Canada). Following washing, Vectastain Avidin-Biotin Complex (ABC) kit (Vector Laboratories, PK-6100) was utilized per manufacturer's instruction s (Vector Laboratories, Newark, CA, USA). Biotinylated secondary antibody was then incubated for 10 minutes at room temperature and washed. Detection was then performed with DAB (3,3'-Diaminobenzidine) solution (Sigma, D5905) and following preparation, 3% H2O2 was added to the solution. DAB solution was then added to the tissue, incubated and washed. Counterstaining with hematoxylin was then performed for 1min, washed, incubated in PBS for 2 minutes and then dehydrated. Slides were then mounted with permount solution for examination. Automated quantification of actin antibody signal intensity within the NI tissue was performed and pixels quantified to compare SMC content in control and DP treated cohorts.

In vitro SMC assessment

(i) Cell culture and treatments.

In vitro approaches were used to assess the impact of DP on SMC biology as well as focused ADOR-A2B small molecule agents. Treatments included control (dimethyl sulfoxide, DMSO) and escalating doses of DP (10uM to 200uM). ADOR-A2B specific small molecule agents included A2B agonist (BAY60-6583, 10uM, Cat #4472, Tocris, Bio-Techne, Minneapolis, MN, USA) and A2B selective antagonist (GS-6201, 1uM, Cat #4727, Tocris, Bio-Techne, Minneapolis, MN, USA). These treatments were then utilized in varying combinations to delineate the effects of DP and ADOR-A2B-specific factors on SMC biology utilizing our previously described methods.(406) Briefly, human coronary artery smooth muscle cells (SMCs, C0175C, ThermoScientific) were maintained at baseline in Medium 231 (M231500, ThermoScientific) with SMC growth supplement (S00725, ThermoScientific) with specific assays as outlined below:

(ii) Migration analysis. SMCs were plated onto 96-well plates and incubated until confluence was achieved, labelled with CellMask Orange (C10045, ThermoScientific) and washed. Uniform scratch was then performed with a P200 tip, washed and the appropriate treatments were then added in Medium 231 with serial imaging every 3 hours for 24 hours with Cytation 5 (BioTek, Winooski, Vermont, USA) and wound closure quantified on the Cytation Gen5 software as percentage confluence.

(iii) Proliferation and apoptosis analysis. Cultured SMCs were treated with 5uM CellTrace Violet (C34557, ThermoScientific) and seeded onto 96-well plates ($5.0x10^3$ SMC/mL) for 24 hours. Following washing, they were then treated with DMSO, DP and ADOR-A2B agonists and antagonists for 48 hours at 37°C and 5.0% CO2. Proliferation and apoptosis were then quantified using a MACSQuant Analyzer 10 (software version 2.8.1618.16380, Miltenyi Biotec Inc, Auburn, CA) assessing the CellTrace Violet quantified proliferation (reported as proliferation index ratio to control) and apoptosis via Annexin V-FITC kit (130-092-052, Milteny Biotec) per the instructions for use and reported as % apoptotic cells/total cells present.

(iv) SMC phenotypic switching - real-time gene expression analysis. Cultured SMCs on 6-well plates in Medium 231 were treated with DMSO, DP and A2B agonists/antagonists. Total RNA was then extracted with TRIzol LS (10296010, ThermoScientific) and reverse transcription performed with SuperScriptTM IV VILO Master Mix (11766050, ThermoScientific). The cDNA generated was then diluted 1:10 for real-time PCR analysis with SYBR Select Master Mix for CFX (4472942, ThermoScientific) utilizing primers for KLF4, KLF5, SMC actin (ACTA2) and GAPDH to assess SMC phenotypic switching as previously described.(406,436)

(v) ADOR-A2B Knockdown. ADOR-A2B knockdown was performed in SMCs in keeping with previously reported approaches in SMCs.(33,437) siRNA analysis was performed utilizing siRNA specific for ADOR-A2B (Catalog # 4390824, ThermoFisher) and silencer selective negative control siRNA (Cat# 4390843, Ambion). These were transfected into SMCs using lipofectamine RNAiMAX reagent (Invitrogen) with

successful knockdown of ADOR-A2B confirmed with Western blot analysis for ADOR-A2B 2B (primary antibody, goat anti-Human, Novus Biological, Oakville, ON, Canada) and GAPDH loading control. This approach was replicated with siRNA against ADOR-A2B and negative siRNA control administration followed by treatment with DP 20uM and A2B agonist and subsequent quantification of SMC phenotypic switching as outlined above by RT-PCR analysis of KLF4, KLF5 and ACTA2 gene expression.

Statistics

We *a priori* calculate the sample size for rabbit size based on our prior data suggesting a control % NI proliferation of 15±1.5%, we calculated a relative treatment effect of 15% reduction translating to a treatment group NI volume of 12.75% with an alpha of 0.05 and power 0.9 (beta 0.1) which translated to n=6 per group. Mean ± SEM with comparisons by unpaired Student's t-test and Mann-Whitney where appropriate. Analysis performed with Graphpad Prism 9.4.1 (GraphPhad Software). $P<0.05$ considered significant with significance denoted as *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

8.5 Results

Dipyridamole and in-stent restenosis

Utilizing our established preclinical rabbit stent model we performed OCT-guided stent implantation **(Figure 57A)** followed by intravascular OCT at 6 weeks to assess for stent healing, specifically differences in NI volume and the proportion of optimally healed stent struts between control **(Figure 57B)** and DP treated cohorts **(Figure 57C).** In the initial cohort, 12 rabbits underwent stent implantation (1 stent per rabbit) followed by randomization to control (N=6) or dipyridamole (n=6). Dipyridamole treated rabbits

demonstrating a 16.6% relative reduction in maximal %NI/stent (14.7±0.8% vs. 12.5±0.4%, p=0.03) and 36.2% relative increase in the proportion of optimally healed stent struts (37.8±2.8% vs 54.6±2.5%, p<0.0001). **(Figure 58A,B)** Next, we assessed the efficacy of DP following stent implantation in the setting of atherosclerosis with 12 rabbits undergoing stent implantation (1 stent per rabbit) followed by randomization (n=6/group) to control or dipyridamole. One rabbit (control group) died prior to the 6 week end point and was unavailable to analyze. In the setting of atherosclerosis, we noted andomfference in NI burden between control and dipyridamole (15.2±1.0% vs. 16.9±0.8%, p=0.22) and a 14.2% relative increase in strut healing with dipyridamole (68.3±4.1% vs 78.7±2.5%, p=0.02). **(Figure 58C,D)** There was no significant difference in circulating adenosine levels between control and dipyridamole treated rabbits across the entire cohort at baseline, 2,4 and 6 weeks post stent implantation. **(Figure 59)** No overt differences in bleeding diathesis were noted between the cohorts, though hemoglobin levels were not empirically quantified. Immunohistochemistry in the non-atherosclerosis cohort was performed, demonstrating a 44.6% (p=0.045) relative reduction in SMC content in the NI of rabbits treated with dipyridamole over controls with peri-strut localization of ADOR-A2B noted **(Figure 60)**

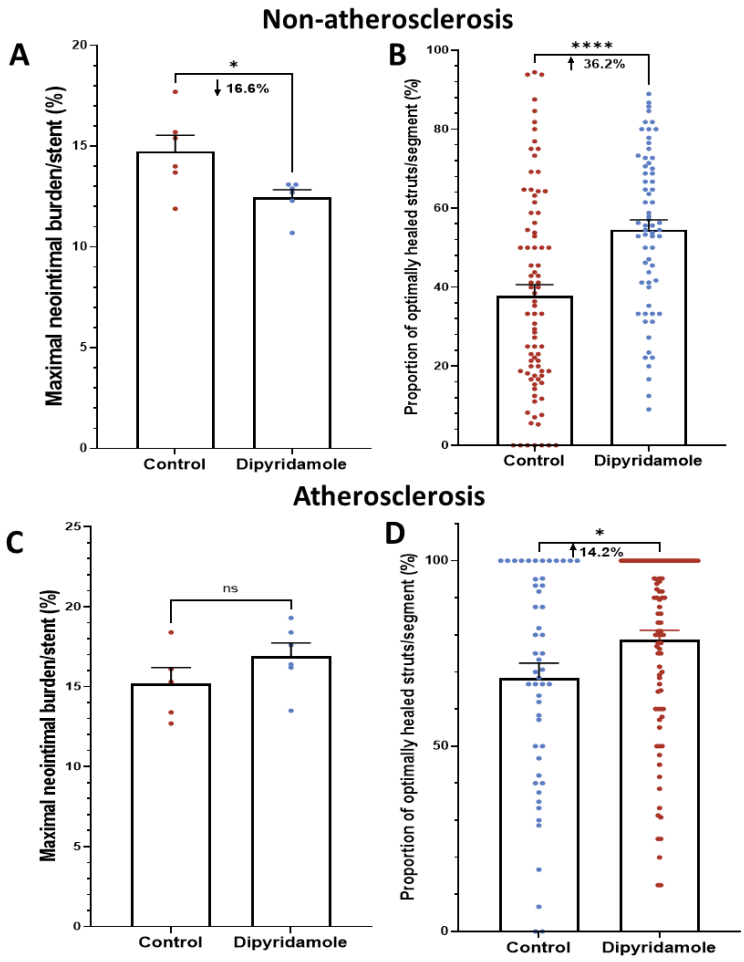

Figure 58. Dipyridamole and stent healing

Non-atherosclerosis cohort. Assessment of 12 stents (169 1mm segments) randomized to control (n=6, 94 segments) or dipyridamole (n=6, 75 segments). (A) Reduced neointimal burden (%) with dipyridamole assessed by maximal NI volume per stent (16.6% relative reduction, 14.7±0.8% vs. 12.5±0.4%, p=0.03). (B) Improved proportion of optimally healed stent struts per segment with dipyridamole (36.2% relative increase, 37.8±2.8% vs 54.6±2.5%, p<0.0001). Atherosclerosis cohort. Diet-induced atherosclerosis via administration of 6 weeks of high-cholesterol diet prior to stent implantation. Assessment of 11 stents (143 1mm segments) randomized to control (n=5, 51 segments) or dipyridamole (n=6, 92 segments). (B) No difference in maximal NI volume per stent with dipyridamole (15.2±1.0% vs. 16.9±0.8%, p=0.22). (D) Improved proportion of optimally healed stent struts per segment with dipyridamole (14.2% relative increase, 68.3±4.1% vs 78.7±2.5%, p=0.02). Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

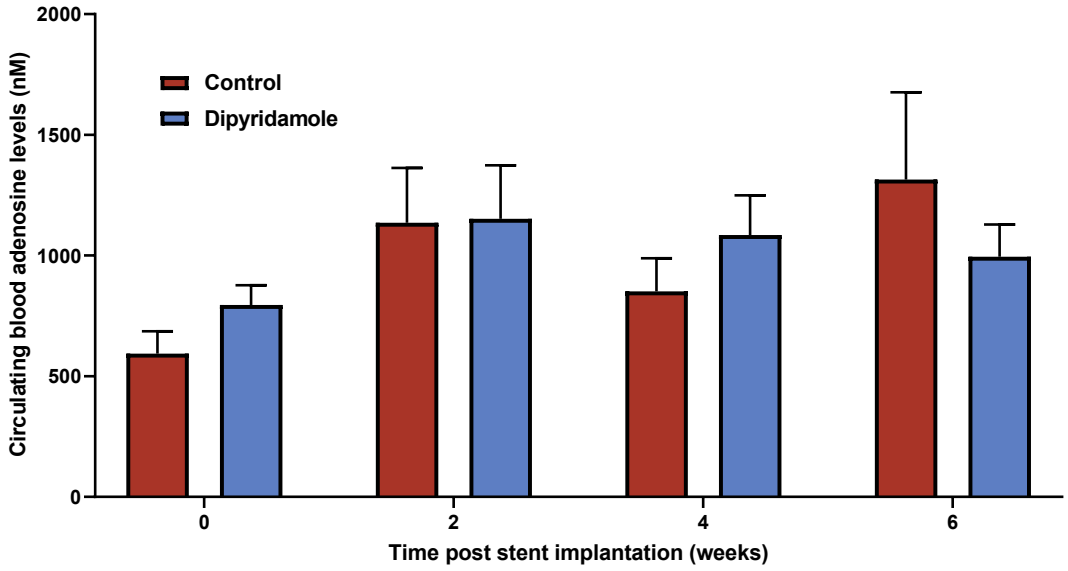

Figure 59. Dipyridamole and circulating adenosine levels.

Blood samples collected via either ear vein draw (4 and 6 weeks) or via vascular access sheath (0 and 2 weeks) with adenosine levels quantified via high performance liquid chromatography. No overt differences noted in circulating adenosine levels between control and dipyridamole treated subjects at 0 weeks (n=7, 593.4±93nM vs. n=8, 795.1±81.8nM, p=0.13); 2 weeks (n=16, 1135.7±226.8nM vs. n=14, 1151.9±222.1nM, p=0.96); 4 weeks (n=10, 851.2±137.7nM vs. n=15, 1084.5±164.7nM, p=0.32); 6 weeks (n=13, 1314.5±361.8 vs. n=15, 994.8±133.3nM, p=0.39). Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

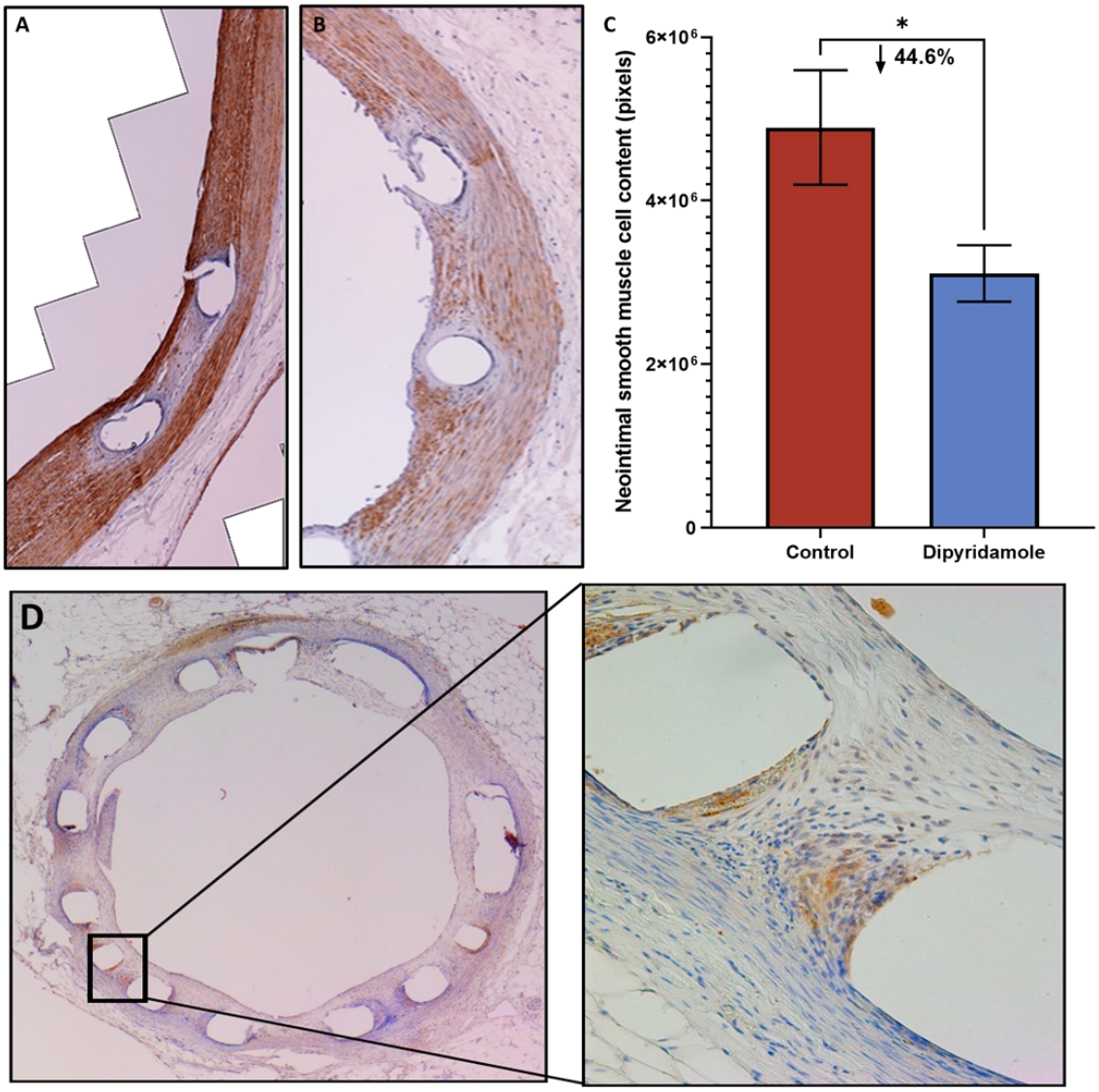

Figure 60.Immunohistochemistry of neointimal tissue

Assessment of neointimal (NI) tissue smooth muscle cell (SMC) content via immunohistochemistry for alpha-SMC actin 6 weeks post stent deployment with either control (n=6) or dipyridamole (n=6) therapy. Representative immunohistochemistry images of a **(A)** control vessel demonstrating prominent NI tissue with dense SMC content (brown staining) versus **(B)** dipyridamole treated vessel with less prominent NI tissue and SMC content (brown staining). **(C)** Quantification of SMC content by automated detection of signal intensity by pixels with note of 44.6% relative reduction in SMC content with dipyridamole (control 4895600±699514 pixels vs dipyridamole 3109225±345330 pixels, p=0.045). **(D)** Cross-section of rabbit aorta post stent processing with immunohistochemistry for adenosine receptor A2B demonstrating presence of this receptor primarily localized to the peri-strut locations (brown). Mean ± SEM with comparisons by unpaired Student's t-test. Significance denoted as *p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.

Dipyridamole and the role of ADOR-A2B in SMC migration and proliferation

In vitro assessment of human coronary SMCs was employed to further evaluate the impact of DP and ADOR-A2B mediated effects. SMC migration was assessed utilizing a scratch assay on confluent monolayer of SMCs **(Figure 61A)** with escalating doses of DP demonstrating a progressive reduction in SMC migration. **(Figure 61B)** At 24 hours, 20uM DP led to a 44.4% relative reduction in wound closure compared to DMSO control (40.7±4.7% vs 25.9±2.3%, p=0.04) while 200uM DP dosing was suggestive of SMC cell death. Next, more granular assessment of proliferation and apoptosis was performed utilizing flow cytometry with escalating DP dosing **(Figure 61C)**. Similarly, this demonstrated a dose-dependent reduction in SMC proliferation with a concomitant augmentation in SMC apoptosis. Assessment of these curves noted that dipyridamole 20uM dosing led to proliferation inhibition without a concomitant rise in SMC apoptosis, supporting its dosing in subsequent assessments. **(Figure 61D)** *In vitro* assessment of SMCs for proliferation indices demonstrated a 26.7% relative reduction in SMC proliferation with DP 20uM over DMSO control, while addition of DP and A2B agonist (10uM BAY60-6583) led to a 54.0% relative reduction in SMC proliferation over control. Administration of DP and an A2B antagonist (1uM GS-6201) abrogated the effect of DP monotherapy with no relative reduction in proliferation noted. **(Figure 62A)** Collectively, this supports that the inhibition of SMC proliferation may, at least in part, be mediated by ADOR-A2B.

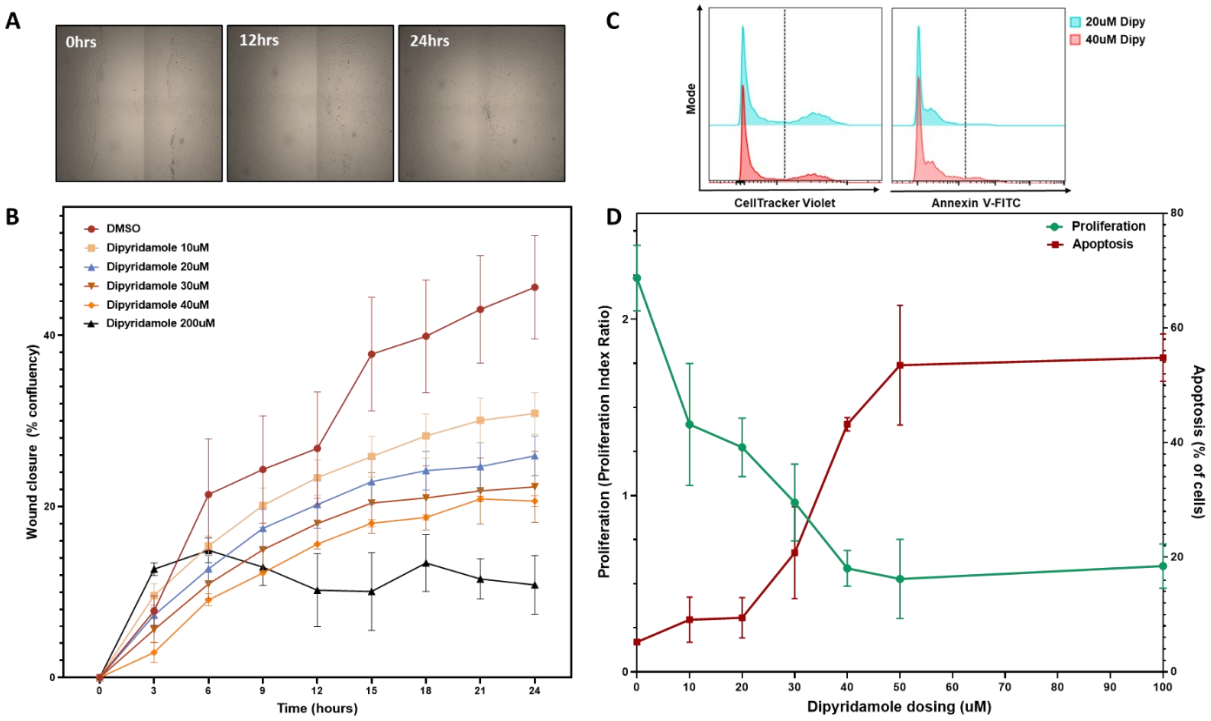

Figure 61. Dipyridamole dose-dependently inhibits coronary SMC migration and proliferation

In vitro experiments utilizing human coronary smooth muscle cell (SMC) with treatments as outlined. **(A)** Wound closure assessed by scratch assay on confluent monolayer of SMCs assessed over 24 hours with sequential imaging.**(B)** Percentage confluency presented for DMSO control (n=9) and sequential dosing of dipyridamole (n=3/dose) at 10,20,30,40 and 200uM dosing with diminishing wound closure rates noted with escalating dipyridamole dose compared to DMSO control. At 24 hours, wound confluence with DMSO was 40.7±4.7% compared to 25.9±2.3% with 20uM dipyridamole (44.4% relative reduction, p=0.04). Mean ± SEM with comparisons by Mann-Whitney test. **(C)** Flow cytometry gating for CellTracker Violet (proliferation) and Annexin-V FITC (apoptosis) with representative histograms for 20uM and 40uM demonstrating reduced proliferation and increased apoptosis**. (D)** Dose escalation studies with DMSO control (n=3, denoted 0uM) and sequential escalation of dipyridamole dosing with 10uM, 20uM, 30uM, 40uM, 50uM and 100uM dosing (n=3/dose) demonstrating progressive dose-dependent reduction in proliferation indices (green) with concomitant rise in proportion of apoptotic cells (red). Crossover between 30-40uM dipyridamole dose and maximal inhibition of proliferation without significant rise in apoptosis noted at 20uM dipyridamole dosing. Mean ± SEM demonstrated.

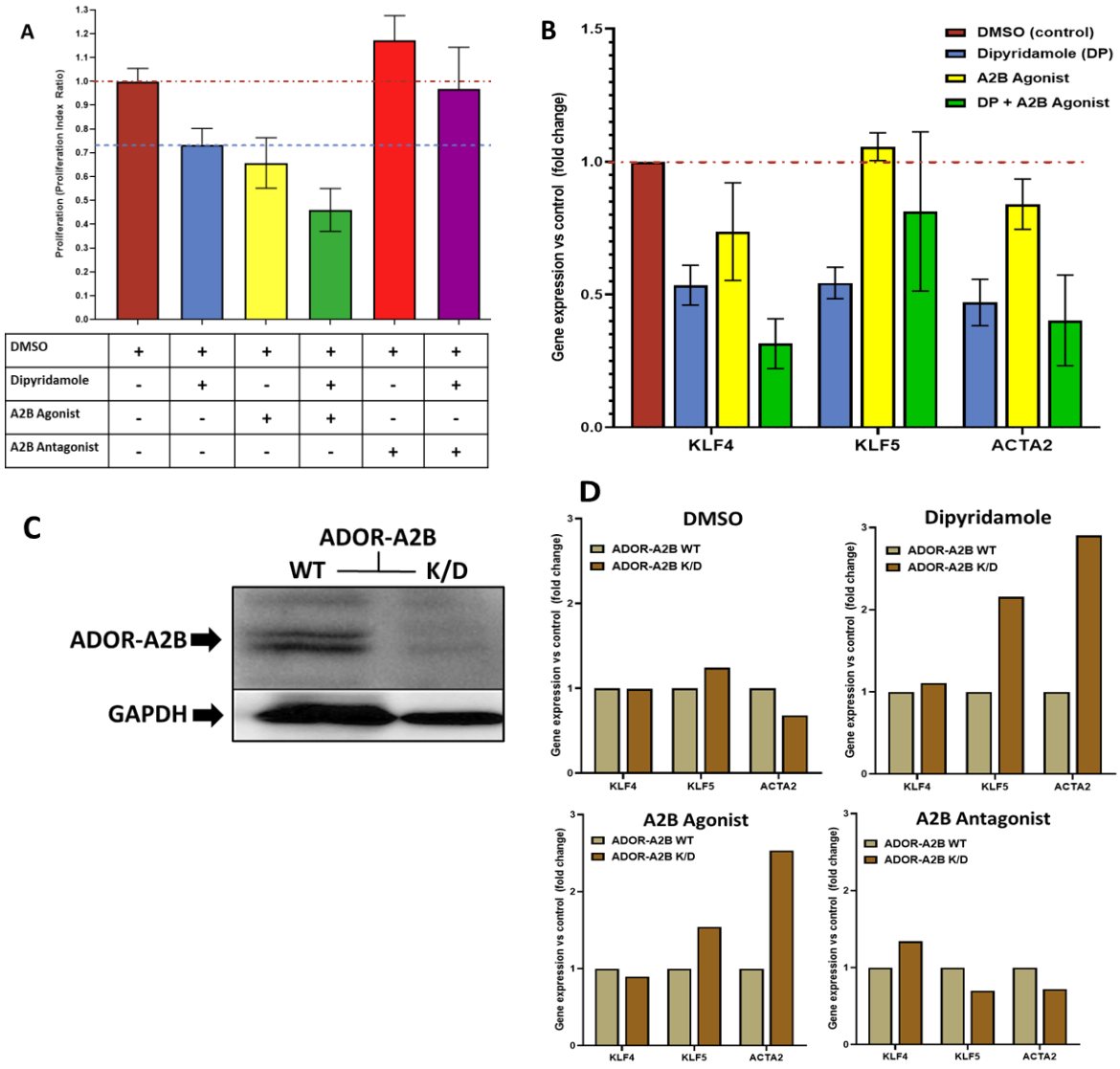

Figure 62. Dipyridamole, ADOR-A2B and the effects on SMC proliferation and phenotypic modulation

(A) Flow cytometric assessment of cell proliferation via proliferation index utilized to assess treatment impact on coronary smooth muscle cell proliferation *in vitro* following 48 hours of therapy. All treatments indexed to DMSO control (n=22) with red dashed line at unity level designating no difference from control. Dipyridamole (DP) 20uM (blue, n=20) with 26.7% relative reduction in proliferation index to 0.73±0.07 and blue dashed line designating level of proliferation inhibition achieved with dipyridamole for comparison. A2B agonist (yellow, n=10, BAY60-6583, 10uM) with 34.3% relative reduction in proliferation to 0.66±0.11. Combination of DP 20uM and A2B agonist (green, n=10, BAY60-6583, 10uM) with 54.0% relative reduction in proliferation to 0.66±0.11. A2B antagonist (red, n=10) with 17.2% increase in proliferation to 1.17±0.10. Combination of DP and A2B antagonist (n=10, purple, GS-6201 1uM) with abrogation of previously noted proliferation inhibition with DP to 3.3% relative reduction in proliferation (proliferation index 0.97±0.18). SMC phenotypic modulation assessment via RT-PCR evaluation of SMC differentiation markers KLF4, KLF5 and ACTA2 **(B, D). (B)** DMSO (n=6), dipyridamole (DP, n=6), A2B agonist (BAY60-6583, n=3), DP+A2B agonist (n=4)

assessed with reported fold changes in gene expression relative to DMSO control presented. **(C)** Western blot for ADOR-A2B with GAPDH loading control demonstrating successful knockdown of ADOR-A2B with administration of specific siRNA (K/D) and continued presence of ADOR-A2B with siRNA negative control (WT). **(D)** Assessment of therapies in the context of ADOR-A2B WT and siRNA K/D with RT-PCR for KLF4, KLF5 and ACTA2 with DMSO (n=4) and dipyridamole (n=3), A2B agonist (BAY60-6583, 10uM) and A2B antagonist (GS-6201 1uM, n=4). Mean ± SEM presented.

Dipyridamole and the role of ADOR-A2B in SMC phenotypic switching

In vitro assessment of SMCs was further utilized to assess modulation of SMC phenotypic switching. Administration of therapies including DMSO control, DP (20uM), A2B agonist (BAY60-6583 10uM), DP+A2B agonist with differential modulation of gene expression for KLF4, KLF5 and ACTA2 – genes known to characterize differential states of SMC phenotypic switching **(Figure 62B)**. Selective siRNA for ADOR-A2B were transfected into SMCs with confirmed gene knockdown of ADOR-A2B by Western blot assessment **(Figure 62C)**. ADOR-A2B siRNA knockdown was then performed in SMCs prior to treatment with DMSO, DP 20uM, A2B agonist (BAY60-6583, 10uM) and A2B antagonist (GS-6201 1uM) with assessment of gene expression relative to siRNA negative controls demonstrating differential modulation of KLF4, KLF5 and ACTA2 with ADOR-A2B knockdown **(Figure 62D).**

8.6 Discussion

In-stent restenosis stemming from NI tissue proliferation continues to cause adverse events following percutaneous treatment of obstructive CAD. DP is an established therapeutic agent for improving vascular outcomes with purported adenosine-mediated effects. In a translational stent model with conventional DAPT, we demonstrate that dipyridamole mitigates NI tissue formation and improves stent strut healing, with

diminished intimal reduction in the setting of atherosclerosis. DP mediates these effects, in part, from reduced SMC content driven by diminished SMC proliferation and migration while altering SMC phenotypic switching. Selective modulation of ADOR-A2B and siRNA knockdown supports an ADOR-A2B mediated component to these observed effects. Thus, therapeutic efficacy derived through either reduction in NI or optimized stent healing may be achievable by either repurposing DP and/or development of ADOR-A2B specific agonists.

Contextualizing our preclinical results within the known clinical data is of importance. Despite advancements and conventional DESs, target lesion failure (TLF) continue to occur at rates of 5.0% in the first year, followed by an annual 2% annual rate up to 5 years which does not plateau – a focus of ongoing study.(276) Clinically, DP has had mixed results in reducing restenosis with early PTCA RCTs – which don't use a vascular scaffold like a stent – demonstrating no difference in ISR rates. (374) Subsequently, RCTs assessing hemodialysis graft patency rates have shown improved graft patency rates from 23% to 28% at 1 year with DP.(128) Meta-analyses of all-comer human revascularization studies assessing DP demonstrated a reduction in vascular occlusion rates from 31% without to 23.5% with DP (RR 0.77, 95% CI 0.67-0.88), with a consistent effect across medical regimens and vascular beds.(408) However, clinical data assessing DP with conventional stent placement and DAPT remains very limited.(376) Hence, the reported 16.6% NI burden relative reduction and improved strut healing in the presence of stent placement is promising, though the abrogated effect on NI formation observed in the presence of atherosclerosis may suggest attenuated benefit in the context

of established disease. Nonetheless, given the approved status of DP, rapid translation to clinical evaluation of DP as an adjunct to DAPT following conventional DES implantation could readily be performed.

Dipyridamole's effect on restenosis and vascular SMCs is well described in preclinical models. Meta-analyses of preclinical models assessing DP therapy demonstrated a 47% relative risk reduction in vascular occlusion post intervention (RR 0.53, 95% CI 0.4-0.7, $p<0.00001$) in addition to a 13% relative reduction in mean difference of NI burden (standard mean difference -1.13, 95% CI -1.74- - 0.53, $p=0.0002$).(408) Faxon *et al* assessed the utility of ASA and DP in rabbit iliac angioplasty model with diet-induced atherosclerotic lesions utilizing histologic assessment at 4 weeks demonstrating an improvement in luminal diameter with oral ASA+DP therapy compared to control (1.3±0.6mm vs 0.7±0.6mm, $p<0.05$).(126) This effect was felt to be, in part, related to early platelet accumulation with notable thrombosis present in the control group.(126,438) Singh *et al* subsequently employed a rabbit model of femoral and carotid balloon injury utilizing local DP delivery (to abrogate concerns of binding to serum proteins systemically), demonstrating a 63% inhibition of SMC proliferation with a 20% reduction in NI thickness ($p<0.05$).(127) Dubey *et al* provided mechanistic insights into these observed DP effects with *in vitro* human aortic SMCs demonstrating inhibition of SMC proliferation and diminished collagen synthesis/extracellular matrix deposition, a plausible means by which DP mitigates NI proliferation.(38) However, these studies did not employ conventional antiplatelet regimens nor did they assess stent implantation – important limitations in

translating to current day interventional practice. Hence, our study sought to replicate contemporary medical and device therapy finding reduced NI burden, improved strut healing and reduced NI SMC content in the presence of stent placement. Moreover, the dose-dependent inhibition of SMC migration and proliferation observed *in vitro* were robust, with apoptosis noted with escalating doses, but without concern of a cytotoxic/apoptotic effects for the 20uM dose of DP selected for our *in vitro* experiments. Considering the lessons learned from excessive cellular inhibition with first generation DESs, namely incomplete healing and thrombosis, the appropriate balance of cellular inhibition without toxicity is critical for any stent related therapeutic agent, with dosing varying on whether it is employed systemically or locally.(276)

The mechanisms underpinning the purported benefits of DP in mitigating NI proliferation and SMC modulation remain complex, but substantial data supports a connection to adenosine and ADOR-A2B signaling. Preclinical murine wire injury models in ADOR-A2B knockout mice demonstrated augmented NI formation in mice lacking ADOR-A2B.(32) Use of 2-chloroadenosine (stable adenosine analog) has been shown to mitigate SMC proliferation, migration and collagen synthesis via activation of ADOR-A2B receptors.(33,38) Dubey *et al* documented mechanistic insights centered around cyclin D based on *in vitro* human coronary SMCs with siRNA induced ADOR-A2B knockdown. Briefly, ADOR-A2B signaling leads to activation of adenylyl cyclase leading to augmentation of cAMP and PKA; PKA subsequently inhibits proliferation by blocking several pathways (ERK1/2, Akt and Skp2) that converge at Cyclin D with the collective result being diminished G1 cyclin expression and activity and cell cycle

progression as a result.(33) This mechanism is also maintained in circulating progenitor SMCs, another documented source of NI proliferation.(31,435) The role of adenosine and circulating progenitors is also supported by rescue of the observed effects by wild type bone marrow transplant in ADOR-A2B knockout mice.(32) Hence, ADO is intricately linked to vascular homeostasis and this mechanism demonstrates multiple potentials for DP modulation. DP enacts many effects via PDE 5/6 inhibition which leads to cAMP/cGMP breakdown, hence DP will augment cAMP levels which would stimulate PKA. Indeed, breast malignancy studies have suggested DP induced inhibition of cyclin D1 expression as a mechanism behind diminished malignant cell proliferation.(439) Moreover, DP is known to inhibit adenosine re-uptake, thereby augmenting circulating ADO levels from SMC culture *in vitro*.(38,311) Additionally, Dubey *et al* suggested that the DP augmentation of ADO levels may mitigate SMC proliferation and collagen synthesis via activation of ADOR-A2B receptors.(38) Lastly, altered SMC collagen and extracellular matrix generation suggests another mechanistic insight – phenotypic modulation. Vascular SMCs maintain plasticity allowing substantial variations in phenotype varying from differentiated quiescent forms to de-differentiated forms in response to injury. Numerous signaling pathways modulate this process including SM-alpha-actin (ACTA2) and Kruppel-like factor 4 and 5 (KLF4,5). In response to injury, the de-differentiated "synthetic" phenotype prevails being characterized by a loss of contractile markers and augmentation of proliferation, migration and protein synthesis – if this process persists beyond the initial injury repair, pathologic NI tissue formation ensues.(440)

Collectively, our results align with the mechanisms previously reported. We demonstrate that DP has beneficial effects in mitigating SMC content, migration and proliferation without augmenting apoptosis. These beneficial effects are likely mediated via ADOR-A2B-related mechanisms with abrogation of the DP effect noted with selective A2B inhibition. Moreover, DP, selective A2B modulation and A2B siRNA knockdown all result in modulation of SMC phenotypic switching factors – supporting the role of DP and ADOR-A2B in this process. This aligns with prior studies noting DP's impact on diminishing collage synthesis,(38) while also being able to reverse the inhibitory effects of DP via administration of PDGF, supporting a potential role of DP and SMC phenotypic switching.(127) While we did not note a clear relation between DP administration and elevated circulating adenosine levels, this is not unexpected considering the complex processes involved in circulating adenosine levels.(284,285) Moreover, unchanged systemic adenosine levels do not necessarily preclude tissue-level alterations in adenosine biology which may enact the observed effects.Going forward, clinical evaluation of DP is warranted coupled with ongoing preclinical evaluation of A2B specific agents for mitigating restenosis.

Limitations

Our study is not without limitations. Our stent model simulates ideal stent implantation technical parameters with non-drug eluting stents, enabling refined examination of the biology involved. Similarly, the atherosclerosis model follows an established approach of diet-induced atherosclerotic lesions, though the plaques developed do not reflect the range of pathologies encountered in human lesions.

However, future clinical studies should explore conventional DES implanted in the context of atherosclerosis with complex anatomy where stent and technical stent factors may also contribute to the ISR observed. Nonetheless, these results support further studies in a clinical setting to validate these preclinical findings. Dose selection for DP and empiric selection of 6 week timepoint may not reflect the optimal timepoint for NI tissue assessment with differential kinetics contributing to the results observed. This could be improved with dedicated experiments. The A2B agonists and antagonists employed are highly selective for their specified receptors, though we cannot exclude off target effects on other ADOR's. While the ADOR-A2B siRNA K/D is reassuring as to ADOR-A2B mediated effects, a gene knockout model would be beneficial to fully explore the ADOR-A2B mediated effects.

8.7 Conclusion

Dipyridamole mitigates neointimal formation and improves stent strut healing in a preclinical model of stent implantation with conventional antiplatelet therapy. This therapeutic effect is abrogated in the presence of atherosclerosis. Clinical studies of DP in addition to conventional therapy post stent implantation may be warranted to evaluate for clinical efficacy.

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8.9 Conflicts of Interest

None noted.

Chapter 9

General Discussion

9.1 Summary of Findings

We explored the role of adenosine biology as both a biomarker and therapeutic target for mitigating NI proliferation, a pathology which leads to a 2% annual risk of adverse events clinically.(244,311) Our first objective was to explore ADO role as a biomarker. We established a post-revascularization clinic linked with a web-based clinical registry and blood biobank of all-comers undergoing invasive coronary angiography, documenting a significant residual risk of adverse events up to 11% at 1 year.(398) Next, we sought to evaluate whether circulating ADO levels were predictive of adverse events. This required establishing the biological and analytical variance of circulating adenosine, while adjusted analysis demonstrated that advancing age was inversely associated with circulating ADO levels.(284) We then evaluated whether circulating ADO levels at the time of angiography were predictive of adverse cardiac events at one year, demonstrating no clear association between ADO levels and clinical events at one year.(285)

We then assessed the therapeutic potential of modulation of ADO biology. Considering the innate challenges of utilizing ADO as a therapeutic agent, we utilized dipyridamole (DP). First, DP was re-evaluated in both pre-clinical and clinical studies, demonstrating a consistent beneficial effect with respect to improved clinical outcomes and reduced vascular events. **(Chapter 5)**(408) We then established a rabbit stent model to facilitate assessment of DP as a therapeutic agent. This evaluation included documenting saline as a viable imaging medium for OCT analysis, with adjustable dimensional reductions noted

and no significant reduction to image integrity. **(Chapter 6)**(405) We then established the clinical translatability of our rabbit stent model utilizing OCT assessment. Our model reports variability of NI proliferation depending on intra-stent location, stent length and stent type, mirroring clinical observations, while being able to assess the impact of sex, atherosclerosis and diabetes on stent healing. **(Chapter 7)** Utilizing this model, we performed a randomized pre-clinical assessment of DP therapy, demonstrating reduction in ISR and improved stent healing with DP compared to control. The therapeutic effect was abrogated in the presence of atherosclerosis. **(Chapter 8)** Mechanistically, the observed benefits of DP were thought to be related to modulation of ADO biology, specifically ADOR-A2B. *In vivo,* DP therapy demonstrated reduced rabbit NI smooth muscle cell (SMC) content. While *in vitro* DP evaluation demonstrated dose-dependent inhibition of SMC proliferation and migration with modulation of SMC phenotypic switching. Similarly, i*n vitro* selective modulation of ADOR-A2B and ADOR-A2B knockdown support an ADOR-A2B-mediated component to the observed DP effects. **(Chapter 8)**

9.2 Circulating Adenosine as a Biomarker

Adenosine biology has several links to vasculo-protective cells and effects, supporting its role as a marker of vascular events.(311) Clinically, quantification of circulating ADO has seen limited use owing to its innate instability and challenging methodology to achieve reliable quantification. Despite this, data supports detectable alterations in circulating ADO levels following balloon angioplasty(247) as well as in response to differing therapeutics with potential connections to clinical outcomes.(149,313)

Moreover, studies in patients with adenosine monophosphate deaminase locus 1 (AMPD1) mutations (leading to augmented circulating ADO levels) have demonstrated improved cardiovascular outcomes in those with CAD(243), albeit with mixed results following revascularization.(248)

We demonstrate several important findings related to circulating ADO levels in humans. First, established cardiovascular risk factors (hypertension, diabetes, family history, smoking and dyslipidemia were not associated with circulating ADO levels. However, age was inversely associated with circulating ADO levels – an observation that remained after adjustment for covariates. **(Chapter 3)** This observation is akin to NT-proBNP, an established heart failure marker, which demonstrates similar assay parameters to adenosine (324,325) NT-proBNP is similarly impacted by age, requiring age-specific reference intervals and reducing prognostic ability with advancing age.(326,327) Hence, the importance of age in interpretation and utilization of ADO levels cannot be underestimated. Next, we do not demonstrate a clear association between cardiovascular medications and ADO levels. Specifically, ticagrelor had been thought to augment circulating ADO levels, with postulation this may lead to the improved clinical outcomes observed with this agent.(149) However, subsequent randomized studies did not demonstrate a clear effect of ticagrelor on circulating ADO.(313) We similarly did not demonstrate overt differences in circulating ADO levels by antiplatelet utilized. However, our study is innately limited by the differential use of ticagrelor in acute coronary syndrome patients, leading to important limitations in interpreting circulating ADO levels in the context of ticagrelor.(144) Nonetheless, the impact of antiplatelet

agents on circulating ADO levels remains a subject of debate. Lastly, our unadjusted analysis supports a potential relationship between the presence and absence of CAD, though following adjusted analysis this effect no longer persisted. This is in line with prior work suggesting genetic origins of augmented adenosine are associated with reduced cardiovascular events.(243) Hence, we undertook additional study to discern the added prognostic benefits of ADO.

The role of ADO as a biomarker includes several important considerations and comparisons to existing markers. The use of high-performance liquid chromatography (HPLC) for quantifying circulating ADO is an established methodology with contemporary assays reporting analytical variability (CVa) of 1-3%, in keeping with clinical assay standards.(316) Indeed, our reported CVa 3.2%, CVi 35.8% and CVg 56.7% align well with contemporary clinical testing. For comparison, C-reactive protein (CRP), reports variances of CVa 5.2%, CVi 42.2% and CVg 92.5%.(318) This profile has important implications, with individual CRP levels generating a 10-20% risk of erroneous risk assignment.(333) Despite this, CRP remains an established predictor of CV events (330,331) and response to therapy.(332) Our analysis of 1,323 patients undergoing invasive angiography did not support circulating ADO as predictive of one year cardiovascular events in those undergoing invasive angiography. This observation held in both unadjusted and adjusted analysis as well as in subgroups including ACS patients, revascularized patients or those treated medically.(285) Hence, we refute the hypothesis that ADO levels predict one year events. **(Chapter 4)**

While we did not demonstrate a prognostic role for circulating ADO to risk stratify at one year, this does not preclude the importance of ADO biology in vascular homeostasis. First, our outcomes were limited to one year and considering the non-plateauing 2% annual rate of adverse events reported, we cannot exclude that ADO may risk stratify with additional event time accrued. (276,397,398) Next, the population analyzed reflects real-world patients undergoing invasive angiography with heterogenous pathologies and management strategies; hence, the potential benefit in a specific sub-group is not excluded. Lastly, we cannot exclude that tissue-level changes in ADO biology may not carry importance, while not being detected with our peripheral quantification. While tissue-level ADO levels may not provide a practically useful clinical test, the modulation of tissue-level ADO levels or ADO receptor expression may still reflect an important therapeutic target, irrespective of circulating ADO levels.(343) Hence, exploration of therapeutic targets modulating ADO biology remains a viable target.

While ADO itself has seen limited clinical use owing to its innate properties, small molecule agents targeting ADO receptors have been utilized as drug targets.(91) DP is an established clinical therapeutic agent with vasculo-protective features that is known to impact ADO biology.(113) Moreover, oral administration of DP has been demonstrated to impact circulating ADO levels.(121) Hence, to better characterize the potential therapeutic impact of DP, we evaluated pre-clinical and clinical studies evaluating the role of DP in improving vascular patency rates and NI proliferation. Pre-clinically our work supports a reduction in vascular occlusion rates with diminished NI proliferation. **(Chapter 5)** Similarly, assessment of clinical studies evaluating DP therapy supports

reduced occlusion rates with DP therapy **(Chapter 5)**.(408) Hence, further assessment of DP as an adjunctive therapy to improve vascular healing post stent implantation are warranted. Moreover, understanding the ADO-dependent mechanisms by which DP enacts the observed effects may enable further refinement of therapeutic strategies.

9.3 Rabbit stent model for evaluation of stent healing

To evaluate the potential efficacy of DP therapy and ADO biology, we refined a rabbit model of stent implantation to evaluate. Rabbits are known to have vessels similar in size to human coronaries(387) with reported models for development of atherosclerosis and diabetes (388,389). OCT has demonstrated validity to histology when employed in rabbit carotid stents (409) Hence, we evaluated a rabbit abdominal aorta model of stent healing to determine the translatability of this model to clinical observations, while assessing the role of intravascular OCT to guide stent implantation and evaluate stent healing.

First, we assessed the impact of differing flush solutions as imaging mediums for acquisition of OCT imaging.(405) Using a benchtop tubing model we compared saline, contrast and varying dilutions therein to assess the impact of differing imaging mediums and generate adjustment coefficients to adjust for the differences observed. We report reduced dimensions with saline compared to contrast, with a linear relationship noted enabling calculation of adjustment coefficients. We then validated these adjustment coefficients *in vivo* utilizing our rabbit pre-clinical model. *In vivo,* we noted similar reduced dimensions with saline with robust correction achieved with the benchtop

coefficients. Moreover, we did not observe any adverse effects in image quality, artefact or strut assessment with saline as a flush solution. Hence, saline is a viable flushing medium for pre-clinical assessment.**(Chapter 6)**

Next, we assessed the translatability of our rabbit stent model to clinical observations of stent healing **(Chapter 7)**. Clinically, most patients demonstrate focal ISR, (399,400) an observation mirrored in our rabbit model with primarily focal ISR patterns observed, favoring the stent ends primarily. Clinical studies describe greater ISR rates in longer stents.(413) Similarly, our model demonstrates more prominent NI formation and diminished stent strut healing with longer stents. While our model did not assess varying stent diameters, another factor known to predict ISR rates, the 3-3.5mm size of the rabbit aorta does reflect >70% of stents implanted clinically and so the translatability of the results should be maintained.(413) Stent strut thickness is yet another established clinical predictor of NI proliferation and vascular patency.(420,421) Utilizing BVS stents with greater strut thickness than metallic stents we demonstrated augmented and diffuse NI proliferation with diminished strut healing. Next, we evaluated the impact of sex on stent healing, without a clear signal of significant difference in healing between either sex observed. While not excluding a potential sex impact, this observation does mirror that observed clinically, where sex does not appear to be a primary driver of NI related events, though sex should continue to be adjusted for in ongoing evaluations in case of differential therapeutic effects.(427)

Next, stents are typically implanted clinically in the presence of atherosclerosis and so pre-clinical evaluation should incorporate this into any study. **(Chapter 7)** We note a 17% relative increase in NI burden in the presence of atherosclerosis with diminished stent strut healing. Lastly, diabetes is an established predictor of NI proliferation and adverse events following stent implantation with clinical studies demonstrating prominent NI patterns favoring the stent edges.(430) Our model demonstrates similar healing patterns of ISR noted, comparable to those seen clinically, though without significant difference in overall NI volumes, likely reflecting the low sample size . Overall, the reported rabbit stent model utilizing intravascular OCT demonstrates excellent translatability to clinical observations and highlights several important considerations for pre-clinical study design. Incorporation of these observations promises to improve experimental design and resource utilization, while maintaining maximal clinical translation of the results obtained.

9.4 Dipyridamole mitigates ISR: a potential role for ADOR-A2B

Assessment of DP as a novel therapeutic agent to facilitate stent healing was further explored. Our work suggests the consistent benefit in improved vascular patency rates in both pre-clinical and clinical studies.(408) While DP is thought to act primarily through phosphodiesterase (PDE) 5/6 inhibition, thereby augmenting cAMP/cGMP; DP also augments circulating ADO levels via ENT-1 re-uptake inhibition.(311,408) Moreover, pre-clinical studies support the role of DP mediating these vasculo-protection via modulation of ADORA-A2B and subsequent inhibition of SMC proliferation.(38) However, work to date has been limited to models not examining stent implantation, nor

in the context of contemporary antiplatelet regimens – important limitations to clinical translation. Hence, we sought to evaluate the therapeutic effect of DP to mitigate ISR formation in a pre-clinical stent model utilizing conventional antiplatelets. Furthermore, we performed *in vitro* evaluation of SMCs to elucidate the ADOR-A2B related mechanisms for the effects observed of DP. **(Chapter 8)**

Our randomized pre-clinical study assessing DP versus control therapy demonstrated therapeutic benefit, with a 16.6% relative reduction in NI burden and improved stent strut healing in those treated with DP. While surrogates of clinical endpoints are not feasible in pre-clinical evaluation, the translation to clinical studies is extrapolated from surrogates such as NI or OSH. Early clinical studies assessing balloon angioplasty without stent placement did not demonstrate an effect of DP therapy,(374) though subsequent trials demonstrate improved dialysis graft patency with DP.(128) However, data assessing DP in the context of stents and antiplatelets remains limited, though the collective data assessing DP supports a vasculo-protective role.(408) Interestingly, replication of this experiment in the presence of diet-induced atherosclerosis abrogated the observed therapeutic benefit. **(Chapter 8)** We previously reported that atherosclerosis led to a 16-17% increase in NI burden and diminished stent strut healing,**(Chapter 7)** ostensibly augmenting the potential for quantification of therapeutic benefit. Despite this, we observed attenuated therapeutic effect of DP, with non-significant reductions in NI burden and only modest improvement in stent strut healing observed.**(Chapter 8)** Considering stents are implanted clinically in the presence of atherosclerosis, this is an important observation. Indeed, the benefits observed may not translate to the clinical

pathology and inflammatory milieu in which it is utilized. This warrants further evaluation with dedicated clinical studies.

The mechanisms underpinning the observed therapeutic effects of DP are key to further therapeutic refinement. The important role that SMCs play in NI proliferation and DP therapeutic effect are well documented. Prior rabbit arterial injury models with local DP administration demonstrated 63% inhibition of SMC proliferation with 20% reduction in NI burden.(127) This was further supported by *in vitro* studies noting DP-induced inhibition of SMC proliferation and collagen synthesis/extracellular matrix deposition.(38) We similarly report dose-dependent reductions in SMC migration and proliferation without concerning apoptosis at the 20uM DP doses employed. **(Chapter 8)** Additionally, pre-clinical studies support the role of ADOR-A2B with gene knockout murine studies demonstrating augmented NI burden.(32) Similarly, ADO leads to ADOR-A2B-mediated inhibition of SMC proliferation, migration and collagen synthesis.(33,38) The cellular mechanisms focus on cyclin D with augmented cAMP, PKA stimulation and reduction in G1 cyclin expression as the final common pathway.(33) The purported ADOR-A2B mediated effects of DP follows. First, DP augments cAMP levels which would similarly stimulate PKA, with oncology studies supporting DP inhibition of cyclin D1 as an anti-neoplastic effect.(439) Moreover, DP augments circulating ADO *in vitro* with resultant reductions in SMC proliferation and collagen synthesis via ADOR-A2B receptors.(38) Lastly, vascular SMCs maintain plasticity with varying phenotypes arising in response to differential stimuli, with vascular injury promoting a de-differentiated synthetic phenotype which can ultimately

translate to pathologic NI proliferation.(440) DP is known to reduce collagen synthesis,(38) while the DP effects on SMCs are reversible with administration of PDGF, supporting a potential role of DP and SMC phenotypic switching.(127)

Our results align with these mechanisms.**(Chapter 8)** DP mitigates NI tissue SMC content while demonstrating *in vitro* reductions in SMC migration and proliferation without augmenting apoptosis. Selective modulation of ADOR-A2B in the presence of DP supports an ADOR-A2B-mediated effect on SMC proliferation. Lastly, ADOR-A2B modulation via small molecule agents and siRNA-gene knockdown suggests that DP may impact SMC phenotype switching via ADOR-A2B. Collectively, our *in vitro* work supports that DP impacts SMC biology via ADOR-A2B mediated effects - a finding which may have implications for therapeutic development and targeting.

9.5 Conclusions and Future Directions

Our study has several implications for evaluation of adenosine as a marker and mediator of vascular homeostasis. First, while circulating ADO levels did not show prognostic benefit for CV events at one year, this does not definitively exclude the predictive potential. It is possible that the predictors of CV events at 1 year are driven primarily by alternative factors, with ADO-related effects taking longer than 1 year to accrue and detect. Hence, further evaluation at delayed time points could still bear clinical relevance for risk prognostication and the absence of risk stratification at this stage would definitively exclude any meaningful role for ADO as a risk stratifying biomarker. Should circulating ADO not bore value as a clinical prognostic tool, it does not exclude the

potential of tissue-level changes in ADO being prognostic. However, considering the clinical impracticality required in obtaining such samples, the yield of exploring this further is likely limited. Alternatively, evaluating the prognostic benefit of ADO receptor levels could also be considered as well, though the practicality of this approach may face similar challenges.

Next, evaluation of DP and ADO biology are promising therapeutic targets for improving vascular healing post stent implantation. Our pre-clinical randomized study supports the additive benefit of DP to DAPT with stent implantation, albeit with diminished benefit in the setting of atherosclerosis. The next step to definitively assess the net clinical benefit would be a randomized clinical trial. Considering that DP is already an approved and cost-effective clinical agent, this could be readily initiated with relative pragmatism. Such a study could randomize DP versus non-drug control following stent implantation with a primary endpoint of NI volume by OCT analysis, a clinically relevant, yet pragmatic, endpoint. This would enable evaluation of the biological hypothesis clinically, while not needing to power to clinical endpoints, thereby mitigating sample size. Lastly, selective modulation of ADOR-A2B should be further explored in pre-clinical and, ultimately, clinical studies to discern the role receptor-specific modulation has in vascular healing. Multiple selective ADOR-A2B modulators are available and should be evaluated *in vitro* followed by *in vivo* randomized pre-clinical studies. In this way, high yield small molecules with respect to reductions in NI burden can be identified, enabling rapid translation to clinical trials for definitive assessment of efficacy and safety in a clinical

setting. Collectively, DP and modulation of ADOR-A2B biology show promise as targets for novel therapeutic approaches to improve outcomes following stent implantation.

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Chapter 10

Appendix I: Productivity during PhD enrollment

10.1 Preface

During PhD enrollment, inclusive of the studies noted above, I have authored/co-authored a total of 105 publications (including 30 as first/co-first/corresponding author) and 36 conference abstracts. See selected list below and link to online academic profile/electronic abstracts here:

- Google Scholar Profile
- PubMed Profile

10.2 Peer-reviewed publications

1. **Simard T**, Jung R, Di Santo P, Sarathy K, Majeed K, Motazedian P, Short S, Dhaliwal S, Labinaz A, Sarma D, Ramirez FD, Froeschl M, Labinaz M, Holmes DR, Alkhouli M, Hibbert B. Evaluation of a Rabbit Model of Vascular Stent Healing: Application of Optical Coherence Tomography. **(2023)** *J Cardiovasc Transl Res*. doi: 10.1007/s12265-023-10399-1. PMID: 37227686.

2. Di Santo P, Abdel-Razek O, Jung R, Parlow S, Poulin A, Bernick J, Morgan B, Robinson L, Feagan H, Wade J, Goh CY, Singh K, Froeschl M, Labinaz M, Fergusson DA, Coyle D, Kyeremanteng K, Abunassar J, Wells GA, **Simard T,** Hibbert B. Rationale and Design of the Rivaroxaban Post-Transradial Access for the Prevention of Radial Artery Occlusion Trial (CAPITAL-RAPTOR). **(2023)** *BMJ Open.* doi: 10.1136/bmjopen-2022-070720. PMID: 37173116; PMCID: PMC10186464.

3. Abdel-Razek O, Marbach JA, **Simard T,** Almufleh A, Di Santo P, Jung R, Parlow S, Mathew R, Hibbert B, Froeschl M. Formalized Research Training Increases Fellowship and Early Career Academic Productivity: A Five-Year Follow-up. **(2023)** *Can J Cardiol.* doi: 10.1016/j.cjca.2023.04.027. Epub ahead of print. PMID: 37149217.

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10.3 Conference Abstracts

1. Alarouri, HS, Samimi S, Ponce AC, **Simard T**, Killu A, Alkhouli M. Assessment of the hemodynamic effects of left atrial appendage occlusion with invasive left atrial pressure measurements. **(2023)** *ACC 2023.*

2. Motazedian P, Marbach J, Prosperi-Porta G, Parlow S, Di Santo P, Jung R, Abdel-Razek O, Bradford W, Tsang M, Hyon M, Pacifici S, Mohanty S, Huggins GS, **Simard T**, Hibbert B. Point-of-Care Ultrasonography: Artificial Intelligence in the Assessment of Left Ventricular Function **(2022).** *Poster presentation. AHA 2022.*

3. Jung R, **Simard T,** Gillmore T, Rizk M, Di Santo P, Joseph J, Stotts C, Motazedian P, Prosperi-Porta G, Abdel-Razek O, Parlow S, Lepage-Ratte M, Shorr R, Ramirez FD, Alkhouli M, Rodes-Cabau J, Holmes Jr DR, Hibbert B. Incidence and Timing of Device-Related Thrombus in Percutaneous Implantable Cardiac Devices: A Systematic Review and Meta-Analysis **(2022)** *Oral presentation, TCT 2022.*

4. Abdel-Razek O, Jung R, Di Santo P, Gillmore T, Stotts C, Soriano J, Verreault-Julien L, Goh CY, Parlow S, Sypkes C, Ramirez FD, Chan V, Toeg H, **Simard T,** Froeschl M, Labinaz M, Hibbert B. Impact of Preexisting and New Atrial Fibrillation on Major Adverse Cardiac Events After Coronary Revascularization **(2022)** *Oral presentation, TCT 2022.*

5. Chavez Ponce A, El Shaer A, Sulaiman S, Harris A, Hohmann S, **Simard T,** Holmes Jr D, Goldsweig A, Alkhouli M. Contemporary Trends in Same-Day vs Deferred Discharge After Left Atrial Appendage Occlusion in the United States (2016-2021). **(2022)** *Oral poster presentation, TCT 2022.*

6. Ramirez FD, Jung R, Di Santo P, **Simard T**, Parlow S, Weng W, Abdel-Razek O, Malhotra N, Cheung M, Hutson J, Marbach J, Thibert M, Fernando S, Nery P, Nair

G, Russo J, Hibbert B. Arrhythmic events in patients with cardiogenic shock on inotropic support: results of the DOREMI randomized trial. **(2022)** Heart Rhythm Society, San Francisco, CA.

7. Alkhouli M, Du C, Killu A, **Simard T,** Noseworthy P, Friedman P, Curtis J, Freeman J, Holmes DR. Residual leaks post left atrial appendage occlusion. **(2022)** American College of Cardiology, Washington DC, United States. *Late Breaking Clinical Trial - Oral Presentation.*

8. Yao X, Ngufor C, Van Houten H, Abraham N, Graff-Radford J, Alkhouli M, **Simard T**, Killu A, Rabinstein A, Friedman P, Holmes DR, Noseworthy P. Machine learning identified subset of AF patients who benefit from left atrial appendage occlusion versus NOAC. **(2022)** American College of Cardiology, Washington DC, United States. *Moderated Poster*

9. Hatoum H, Gadhave R, Lilly SM, Alkhouli M, **Simard T.** Impact of hypertensive conditions and arterial load on transcatheter aortic valve performance and coronary perfusion**. (2021).** American College of Cardiology Scientific Sessions. Atlanta, Georgia, United States. *Online. Poster Presentation.*

10. Mathew R, Di Santo P, Marbach J, Hutson J, **Simard T,** Jung R, Merdad A, Almufleh A, Wen W, Abdel-Razek O, Fernando S, Kyeremanteng K, Bernick J, Wells G, Froeschl M, Labinaz M, Le May M, Russo J, Hibbert B. Capital Do-Re-Mi: A randomized trial of dobutamine compared to milrinone in cardiogenic shock. **(2020)** American Heart Association: Scientific Sessions Virtual Meeting, Chicago, United States. *Oral presentation*.

11. Jung RG, **Simard T,** Di Santo P, Duchez AC, Labinaz A, Parlow S, Yang J, Dhaliwal S, Ramirez FD, Xiao F, Lordkipanidze M, Burger D, Hibbert B. Discovery of plasminogen activator inhibitor-1 platelet -derived extracellular vesicles to predict major adverse cardiac events. (**2020**). American Heart Association: Scientific Sessions Virtual Meeting, Chicago, United States. *Moderated Poster.*

12. Jung RG, **Simard T,** Kovach C, Flint K, Don C, Di Santo P, Adamo M, Branca L, Valentini F, Benito-Gonzalez T, Vazquez FF, Estevez-Loureiro R, Berardini A, Conti N, Rapezzi C, Biagini E, Parlow S, Shorr R, Levi A, Manovel A, Cardenal-Piris R, Diaz JF, Shuvy M, Haberman D, Alessandra S, Marini C, Bargagna M, Schiavi D, Paolo D, Buzzatti N, Markovic S, Chan V, Hynes M, Mesana T, Labinaz M, Markovic S, Pappalardo F, Taramasso M, Hibbert B. Transcatheter mitral-valve repair in cardiogenic shock and severe mitral regurgitation: a patient-level multi-center analysis. (**2020**). Transcatheter Cardiovascular Therapeutics (TCT) Connect, United States. Oral abstract.

13. Q Barry, A Fu, R Boudreau, D Perry-Nguyen, U Tran, **T Simard,** M Le May, M Labinaz, A Dick, C Glover, M Froeschl, B Hibbert, A Chong, D So. Outcomes of appropriate versus inappropriate de-esclation of P2Y12 inhibitor therapy post

percutaneous coronary intervention: a retrospective cohort study. **(2019)** Canadian Cardiovascular Congress, Montreal, Quebec, Canada. *Poster Presentation.*

14. **Simard T**, Labinaz M, Zahr F, Nazer B, Gray W, Hermiller J, Chaudhry S-P, Guimaraes L, Philippon F, Rodés-Cabau J, Sorajja P, Hibbert B. Levoatrial to coronary sinus shunting as a novel strategy for symptomatic heart failure: first-in-human experience. **(2019)** Transcatheter cardiovascular therapeutics, San Francisco, USA. *Keynote Oral Presentation.*

15. Jung R, Parlow S, **Simard T**, Chen C, Ghataura H, Kishore A, Perera A, Moreland R, Hughes I, Tavella R, Hibbert B, Beltrame J, Singh K. Clinical Features, Sex Differences, and Outcomes of Myocardial Infarction With Nonobstructive Coronary Arteries (MINOCA): A Multicenter, International, Registry Analysis. **(2019)** Transcatheter cardiovascular therapeutics, San Francisco, USA. *Moderated Poster Presentation.*

16. Boudreau R, Fu A, Barry Q, Perry-Nguyen D, Tran U, **Simard T,** Le May M, Labinaz M, Dick A, Glover C, Froeschl M, Hibbert B, Chong AY, So D. Comparing treatment recommendations for the DAPT and PRECISE-DAPT scores after percutaneous coronary intervention. **(2019)** European Society of Cardiology Congress, Paris, France. *Poster presentation.*

17. Boudreau R, Fu A, Barry Q, Perry-Nguyen D, Tran U, **Simard T,** Le May M, Labinaz M, Dick A, Glover C, Froeschl M, Hibbert B, Chong AY, So D. Comparing outcomes following 1 year of dual antiplatelet therapy in patients with high and low PRECISE-DAPT scores. **(2019)** American College of Cardiology, New Orleans, Louisiana, USA. *Poster presentation.*

18. Aleksova N, So D, **Simard T,** Davies R, Stadnick E, Mielniczuk L, Chih S. Antiplatelet therapy and platelet reactivity after heart transplantation. **(2018)** Canadian Cardiovascular Congress, Toronto, Ontario, Canada. *Oral presentation.*

19. Fu A, Barry Q, Boudreau R, Perry-Nguyen D, Tran U, **Simard T**, Le May M, Labinaz M, Dick A, Glover C, Froeschl M, Hibbert B, Chong A, So D. Incidence and rationale for P2Y12 inhibitor switching in patients within 1 year of percutaneous coronary intervention. **(2018)** Canadian Cardiovascular Congress, Toronto, Ontario, Canada. *Moderated poster presentation.*

20. Barry Q, Fu A, Boudreau R, Perry-Nguyen D, Tran U, **Simard T**, Le May M, Labinaz M, Dick A, Glover C, Froeschl M, Hibbert B, Chong A, So D. Appropriate verses inappropriate de-escalation of P2Y12 inhibitor therapy post percutaneous coronary intervention: a retrospective cohort study. **(2018)** Canadian Cardiovascular Congress, Toronto, Ontario, Canada. *Poster presentation.*

21. Boudreau R, Fu A, Barry Q, Perry-Nguyen D, Tran U, **Simard T**, Le May M, Labinaz M, Dick A, Glover C, Froeschl M, Hibbert B, Chong A, So D. Do the DAPT

and PRECISE-DAPT scores provide concordant recommendations for duration of P2Y12 inhibitor treatment after percutaneous coronary intervention. **(2018)** Canadian Cardiovascular Congress, Toronto, Ontario, Canada. *Moderated poster presentation.*

22. **Simard T,** Di Santo P, Ramirez FD, Jung R, Labinaz A, Pitcher I, Motazedian P, Rochman R, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, So D, Chong AY, Le May M, Hibbert B. Achieving target LDL following percutaneous coronary revascularization – contemporary results in a nationalized health care system. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster presentation.*

23. Di Santo P, Labinaz A, Rochman R, Jung R, **Simard T,** Ramirez FD, Pitcher I, Motazedian P, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, Le May M, Labinaz M, Glover C, Hibbert B. Sex differences in symptom pattern of chest pain and resolution following coronary revascularization. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster presentation.*

24. Jung R, **Simard T,** Labinaz A, Ramirez FD, Di Santo P, Pitcher I, Motazedian P, Rochman R, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, Froeschl M, Dick A, Hibbert B. Impact of general cardiology follow-up on optimal cardiovascular risk factor management post-revascularization: outcomes in a nationalized healthcare system. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster presentation.*

25. **Simard T,** Jung R, Di Santo P, Ramirez FD, Labinaz A, Pitcher I, Motazedian P, Rochman R, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, Dick A, Froeschl M, Le May M, Hibbert B. The impact of weight loss on cardiovascular outcomes following percutaneous coronary revascularization – a contemporary, real world experience. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster.*

26. Di Santo P, Labinaz A, Rochman R, Jung R, **Simard T,** Ramirez FD, Pitcher I, Motazedian P, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, Le May M, Labinaz M, Glover C, Hibbert B. Sex differences in symptom pattern of chest pain and resolution following coronary revascularization. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster presentation.*

27. Sarathy K, Jung R, Labinaz A, Rochman R, Pitcher I, Motazedian P, Ramirez FD, Di Santo P, Marbach J, Boland P, Hibbert B, **Simard T,** Alghofaili S, Russo J, Couture E, Glover C, Labinaz M. Patterns of proton pump inhibitor use in patients post coronary revascularization: impact of bleeding risk on prescription patterns. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster presentation.*

28. Sarathy K, Jung R, Labinaz A, Rochman R, Pitcher I, Moreland R, Motazedian P, Ramirez FD, Di Santo P, Marbach J, Boland P, **Simard T,** Alghofaili S, Russo J, Couture E, Labinaz M, Glover C, Hibbert B. Physician predictors of mortality outcomes in the cardiac cath lab: comparisons to a validated risk score. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster presentation.*

29. Russo J, Aleksova N, Pitcher I, Faraz M, Couture E, Parlow S, Visintini S, **Simard T,** Boland P, Di Santo P, Marbach J, Ramirez FD, So D, Labinaz M, Le May M, Hibbert B. Left ventricular decompression during extracorporeal membrane oxygenation for cardiogenic shock: a systematic review and meta-analysis. **(2018)** Transcatheter Cardiovascular Therapeutics (TCT). San Diego, California, USA. *Moderated poster presentation.*

30. Fu A, Perry-Nguyen D, Tran U, **Simard T**, Le May M, Labinaz M, Dick A, Glover C, Froeschl M, Hibbert B, Chong AY, So D. Outcomes of switching P2Y12 inhibitors in patients who underwent percutaneous coronary intervention: a retrospective cohort study. **(2018)** American College of Cardiology, Orlando, Florida, USA. *Poster Presentation.*

31. Russo J, James T, Di Santo P, Hibbert B, Ramirez D, **Simard T,** Wells G, Le May M. Optimal mean arterial pressure in comatose survivors of out-of-hospital cardiac arrest: an analysis of area below and between blood pressure thresholds. **(2017)** American Heart Association. Anaheim, California, USA. *Oral Presentation.*

32. **Simard T,** Motazedian P, Ramirez FD, Jung R, Feder J, Di Santo P, Russo JJ, Pourdjabbar A, Le May M, So D, Chong AY, Hibbert B. Pre-clinical comparison of saline and contrast for intravascular imaging using optical coherence tomography **(2017)** The Canadian Cardiovascular Congress, Vancouver, British Columbia, Canada. *Moderated poster.*

33. Ramirez FD, Chen Y, Di Santo P, **Simard T**, Motazedian P, Birnie D, Hibbert B. The cumulative burden of potentially modifiable cardiac risk factors is modestly associated with a perceived need to improve physical health: a population-based study of Canadian adults. (**2016**). American Heart Association, New Orleans, LA, USA. *Poster.*

34. **Simard T (co-first author, presenting author)**, Hibbert B, Ramirez FD, Di Santo P, Johnson C, Turek M, Labinaz M, Chow B, Le May M. Impact of routine handheld focused cardiac ultrasonography on the diagnosis and management of hospitalized cardiac patients: the CAPITAL FoCUS registry. (**2016**). AHA, New Orleans, LA, USA. *Oral presentation.*

35. Di Santo P, Harnett D, **Simard T**, Ramirez FD, Pourdjabbar A, Yousef A, Moreland R, Bernick J, Wells G, Dick A, Le May M, Labinaz M, So D, Chong AY, Hibbert B. Photoplethysmography using a smartphone application for assessment of ulnar

patency prior to transradial angiography: a randomized clinical trial. **(2016).** American Heart Association, New Orleans, LA, USA. *Poster*.

36. MacDonald, Z.D., Yousef, A., **Simard, T.**, Russo, J.J., Feder, J., Froeschl, M.V., Dick, A., Glover, C., Burwash, I.G., Hibbert, B., Labinaz, M. **(2016)** Transcatheter aortic valve implantation (TAVI) in native aortic valve regurgitation: a systematic review. The Canadian Cardiovascular Congress, Oral Presentation. Montreal, Quebec, Canada. *Oral presentation.*

Chapter 11

Appendix II: Curriculum Vitae (Publications/Abstracts Focused)

Publications:

1. **Simard T**, Jung R, Di Santo P, Sarathy K, Majeed K, Motazedian P, Short S, Dhaliwal S, Labinaz A, Sarma D, Ramirez FD, Froeschl M, Labinaz M, Holmes DR, Alkhouli M, Hibbert B. Evaluation of a Rabbit Model of Vascular Stent Healing: Application of Optical Coherence Tomography. **(2023)** *J Cardiovasc Transl Res*. doi: 10.1007/s12265-023-10399-1. PMID: 37227686.

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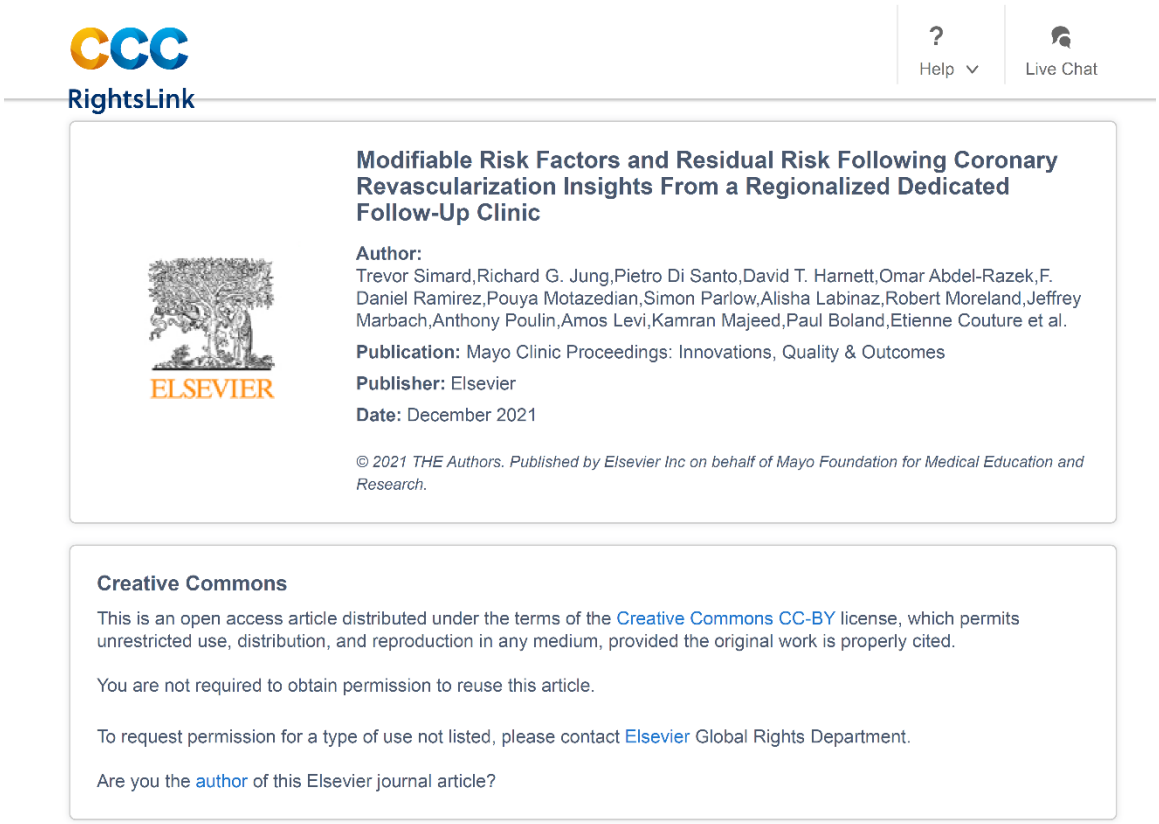

Chapter 3

Evaluation of Plasma Adenosine as a Marker of Cardiovascular Risk: Analytical and Biological Considerations

Trevor Simard, Richard Jung, Alisha Labinaz, Mohammad Ali Faraz, F. Daniel Ramirez, Pietro Di Santo, Dylan Perry-Nguyen, Ian Pitcher, Pouya Motazedian, Chantal Gaudet, Rebecca Rochman, Jeffrey Marbach, Paul Boland, Kiran Sarathy, Saleh Alghofaili, Juan J. Russo, Etienne Couture, Steven Promislow, Rob S. Beanlands and Benjamin Hibbert

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Revisiting the Evidence for Dipyridamole in Reducing Restenosis: A Systematic Review and Meta-analysis

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Chapter 6

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Chapter 7

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