Molecular Mechanisms of Sex-Differences in Vascular Reactivity of Human Internal Mammary Artery

A Thesis by Publication Submitted to The University of Adelaide as the requirement for the degree of Doctor of Philosophy

Victor Lamin

Faculty of Health Sciences, Adelaide Medical School, Discipline of Medicine,

The University of Adelaide

South Australia

March 2017

TABLE OF CONTENT

AUTHOR'S DECLERATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and through web search engines, unless permission has been granted by the University to restrict access for a period of time.

……..

Victor Lamin
March 2017

THESIS DEDICATION

I dedicate this thesis to the Almighty God, Creator of the Heavens and the Earth, the giver of wisdom and understanding, above all, the source of my inspiration, knowledge and strength during my candidature.

It is by his grace, love and kindness that I was able to complete this thesis by trusting in him with all my heart, acknowledged, and yielded to his direction.

ACKNOWLEDGEMENT

This thesis represents not only my research work at the laboratory, it is a completion of a milestone of work started about a decade ago by The Translational Vascular Function Research Collaborative Group, Department of Cardiology, The Queen Elizabeth Hospital and I would like to take this opportunity to thank the following people that contributed immensely to the realization of this thesis.

First, My sincere thanks and appreciation goes to my primary supervisor Professor John F. Beltrame for the support he gave me during my Ph.D. research, his patience, and immense knowledge impacted in me, particularly so when exploring new ideas and communicating it to the scientific audience. His critical analysis contributed enormously to the writing of this thesis. In addition, I acknowledge the great contribution of Dr. David P. Wilson to this work by serving as a mentor and second supervisor. I further acknowledge his helpful suggestions, scientific advice and knowledge gained during many insightful discussions that serves as my primary resource for getting my science questions answered.

Second, I am also so thankful to Dr. Amenah Jaghoori for her contribution to this work and my colleagues for their input in to this thesis through their productive critics especially at the lab meetings that provided new ideas to the work.

Third, I am indebted to some people who also contributed to the success of this work, namely Irene Stafford and Tamila Heresztyn for their support in mass spectrometry assay development, Dr. John Licari in the EPR assay development and the surgical team of Royal Adelaide Hospital, D'Arcy Sutherland Cardiothoracic Surgical Unit, for their support during samples collection.

Forth, I acknowledge The University of Adelaide, International Postgraduate Research Scholarship and the Australian Postgraduate Award for the funding that paid for my time at

the University of Adelaide through the Basil Hetzel Institute of Translational Health Research, The Queen Elizabeth Hospital. I am grateful for their support.

Finally, My thanks and appreciation goes to my beloved family, Amayoh, Rejoice and Divine Lamin, my parents Jestina and Gabriel Lamin, Mariam and Umaru Kamara for their support and the love they share with me during my research.

I love you all

LIST OF THESIS FIGURES

LIST OF THESIS TABLES

LIST OF THESIS ABBREVIATIONS

5-HT: 5-hydroxytryptamine

6-keto PGF1α: Prostaglandin I_2 metabolite

AA: Arachidonic acid

ACE-inhibitor: Angiotensin Converting Enzyme Inhibitor

ACS: Acute Coronary Syndrome

BH4: Tetrahydrobiopterin

BK_{Ca}: Large conductance Ca^{2+}-activated K^+ channels

BMI: Body Mass Index

Ca^{2+}: Cytosolic Ca2+ concentration

CABG: Coronary Artery Bypass Graft

CaM: Calmodulin

cGMP: Cyclic guanosine monophosphate

COX: Cyclooxygenase

CPI-17: C kinase-activated phosphatase inhibitor

DAG: Diacylglycerol

EDHF: Endothelial-derived hyperpolarising factor

eNOS: Endothelial nitric oxide synthase

ET-1: Endothelin-1

EPR: Electron paramagnetic resonance

GPCR G protein-coupled receptor

IgG: Immunoglobulin G

IMA: Internal Mammary Artery

IP3: Inositol trisphosphate

IP3R: Inositol trisphosphate receptor

IK_{Ca}: Intermediate conductance Ca^{2+}-activated K^+ channels

KPSS: High K^+ solution

L-NAME: Nω-nitro-L-arginine methyl ester hydrochloride

LC-MS/MS: Liquid chromatography tandem-mass spectrometry

MI: Myocardial Infarction

MLCK: Myosin light chain kinase

MLCP: Myosin light chain phosphatase

Mn^{2+}: Manganese

NO: Nitric oxide

NOS: Nitric oxide synthase

PE: Phenylephrine

PG: Prostaglandin

PGF2α: Prostaglandin F2α

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PMCA: Plasma membrane Ca2+ ATPase

ROCC: Receptor-operated Ca2+ channel

ROK: Rho-associated kinase

RyR: Ryanodine receptor

SEM: Standard error of mean

Ser: Serine

SERCA: Sarco-endoplasmic reticular Ca2+ ATPase

SK_{Ca}: Small conductance Ca^{2+}-activated K^+ channels

SOCC: Store-operated Ca2+ channel

SR: Sarcoplasmic reticulum

SSRI: Selective Serotonin Reuptake Inhibitor

SV: Saphenous vein

TBS-T: Tris-buffer saline – Tween 20

TCA: Trichloroacetic acid

Thr: Threonine

U46619: Stable analogue of thromboxane A2

VGCC: Voltage-gated Ca2+ channel

VSMC: Vascular smooth muscle cell

THESIS ABSTRACT

Background: Clinical and epidemiological evidences have revealed that females have increased post-operative mortality and worsen outcome following coronary artery bypass grafting (CABG) compared to males. The potential reason for the sex-disparity is multifactorial and may include a combination of clinical (for example, smaller vessel size), psychosocial (for example, depression) and biological factors (for example, increase platelet activity and postmenopausal status) in female compared to male. Additional biological factor could include vascular reactivity of the conduit vessels that has not been previously assessed. Previous work in our group has revealed female internal mammary artery (IMA) graft conduit hypersensitivity to phenylephrine (PE) and serotonin (5-HT) but not endothelin-1 (ET-1) and thromboxane A_2 stable mimetics U46619 when subjected to ex-vivo organ bath experiment.

Presently, the mechanisms responsible for the female IMA hypersensitivity to PE and 5HT are unknown. The present study evaluates potential mechanisms responsible for IMA hypersensitivity to PE and 5-HT specifically assessing the role of: (1) the endothelium, (2) nitric oxide (NO) pathway, (3) prostaglandin (PG) pathway, and/or (4) specific vascular receptors.

Methods: Human IMA segments were obtained from 160 males (with mean age 67.3 ± 5.9 years) and 99 females (with mean age 67.8± 6.0 years) undergoing CABG and subjected to the following experimental conditions. (1) Contractile responses of isolated human IMA in the presence or absence of an intact endothelium were evaluated in an organ bath preparation coupled with challenge by cumulative doses of PE (0.01 μM -300 μM), 5-HT (0.001-300 μM), ET-1 (0.01-300 μM) and U46619 (0.001-300 μM). (2) The role of nitric oxide was determined by assessing the influence of the nitric oxide synthase (NOS) inhibitor, N-w-

Nitro-L-arginine Methyl Ester (L-NAME, 300 μM) on PE-and 5-HT mediated vasoconstriction. Electron paramagnetic resonance spectrometry (EPR) coupled with the NO spin trap iron (II) dithiocarbamate complexes containing diethyldithiocarbamate; Fe $(DETC)_2$ was used to quantify NO release. (3) The role of prostaglandins in mediating PE and 5-HT-vasoconstrictor responses was assessed using the cyclooxygenase inhibitor indomethacin (10 μM). Liquid chromatography/tandem mass spectrometry (LC/MS/MS) was used to evaluate prostanoids metabolite including prostaglandin E_2 (PGE_2), PGD_2, $PGF_2\alpha$, 6-keto PGF1α, and TXB_2 in vessel in IMA segments in response to A23187 (10 μM) (4) Phos-tag™ western blot was used to quantify abundance and phosphorylation status of α_1-adrenergic and 5-HT_{2A+2B} receptors in IMA segments. Data are expressed as mean ± SEM. Statistical analysis of unpaired data has been made using Student t-test. Differences are considered significant at $p < 0.05$.

Results: Compared with males, female IMA were hypersensitive to 5-HT and PE but not U46619 and ET-1. The female IMA hypersensitivity was (1) abolished by endothelial denudation, (2) unaffected by NOS inhibition, with no difference in EPR-assessed NO levels, (3) abolished by COX inhibition, with a trend in reduced 6-keto PGF1α but not $PGF_2\alpha$ levels in females, and (4) unrelated to the abundance and phosphorylation of α_1-adrenergic and 5-HT_{2A+2B} receptors.

Conclusions: This data indicate that female IMA hypersensitivity is agonist specific; attributable to an endothelium-dependent COX pathway involving possible impaired 6-keto PGF1α productions in female. Thus, post-operative inotrope use or serotonin release from platelet following CABG in females will produce more IMA vasoconstriction than male, which may result in significant anterior wall ischaemia and contribute to their poorer outcomes.

PREFACE

This thesis investigates the mechanisms of sex-differences in vasoconstrictor response of human internal mammary artery graft conduit to phenylephrine and serotonin in an organ bath setup. The thesis is structured into five chapters as listed below:

1. An introductory chapter (Chapter 1) that provide background information relevant to the study, followed by the aims and objectives of this thesis.

2. A method chapter (Chapter 2) that provide detailed experimental protocol (from human samples collection to assays development). A portion of this chapter has already been published before the completion of this thesis.

3. Four experimental studies presented as two individual chapters (Chapter 3 and Chapter 4) that are distinct bodies of work that address the experimental aims of this thesis. Chapter 3 and 4 are ready to be submitted for review before completion of this thesis.

4. And a concluding chapter (Chapter 5) that presents a discussion and conclusion section summarizing the key findings of the three experimental studies in this thesis and provides information regarding the possible practical and/or clinical application of these findings.

The thesis was prepared in a thesis by publication style and each experimental chapter is formatted to conform to the style of the journal to which it was published or ready to be submitted. Author contribution statements are at the beginning of each chapter.

1. CHAPTER 1: INTRODUCTION

Background information relevant to the study, aims, and objectives of this thesis

---

Cardiovascular diseases (CVDs) continue to dominate the Westernised health profile as the major cause of death, accounting for more than 17 million deaths globally each year (32% of all deaths in female and 27% in males), and this figure is expected to grow to 23.6 million by 2030.[1, 2] The main contributor of this mortality is coronary artery disease (CAD) that is responsible for about 49% of all CVDs death.[3]

In Australia, CAD is a major public health concern with 25% mortality occurring within the first hour of myocardial infarction (MI) and mortality increases to 40% with in a year.[4]

The pathophysiology of CAD is characterized by an inadequate supply of oxygen-rich blood to the myocardium as a result of narrowing or blocking of a coronary artery leading to myocardial ischaemia.[5] The treatment of CAD has evolved significantly due in part to improvements over the past decades in both medical therapy and invasive revascularization techniques (such as percutaneous coronary intervention (PCI) and coronary artery bypass graft surgery (CABG). The first line of treatment of patients with chronic stable angina is medical therapy, directed towards decreasing myocardial oxygen demand through either influencing vascular function and/or influencing lipid profile.[6] If medical therapy is unsuccessful in alleviating angina, myocardial revascularization with either percutaneous coronary intervention (PCI), with or without placement of a stent, or CABG may be appropriate.[6]

Several contemporary trials comparing PCI and CABG have reported no difference with regards mortality and MI. [6-8] However, long term outcome suggested reduction in MIs with CABG, [9] making CABG the treatment of choice for severe multiple vessels disease. [10, 11]

1.1. THE EVOLUTION OF CORONARY ARTERY BYPASS GRAFT (CABG) SURGERY

CABG is an invasive (open heart) surgical procedure in which autologous graft conduit is used to bypass atherosclerotic narrowing of the coronary artery, thereby improving blood supply to an ischemic territory. The procedure has considerably evolved since it was first introduced. This section will provide a summary of some historical recapitulation that gave birth to 'modern' CABG procedure.

1.1.1. CABG History

Although Francois Franck performed the first surgical treatment (sympathectomy) for the alleviation of angina pectoris in 1899, modern CABG began following the introduction of coronary angiography by Mason Sones.[12] Invasive selective coronary angiography provided the 'diagnostic roadmap' to enable Goetz and colleagues to perform the first non-sutured internal mammary artery (IMA) anastomosis using tantalum ring as a connector device in 1960.[6, 12] This procedure serves as the foundation for the contribution of Vasilii Kolesov, who first performed sutured IMA anastomosis to the left anterior descending artery (LAD), followed by saphenous vein aorta-coronary bypass led by Michael DeBakey.[12] Though the primary aim of the surgical intervention by then was to significantly decreased the symptoms of angina and prevent myocardial infarction (MI) was met in some patients, there was no consistency in the surgical procedure and surgical outcome until a team led by Rene´ Favaloro in 1968, documented the systematically implantation of IMA on 191 patients using sutured technique, exhibiting surgical consistency.[12] The prevalence of modern CABG has increased regularly across the world and safety improved. It is

now considered a well-established treatment modality for angina pectoris and prolonging the lives of patient with multiple vessel disease.

1.1.2. CABG Procedure

The anastomosis of the IMA conduit to the LAD is considered the gold standard procedure of modern CABG. The IMA is used in combination with other conduits in multiple grafting procedures. Presently, CABG can be successfully performed with or without cardiopulmonary bypass machine (CPB or heart lung machine), called the on-pump CABG- the traditional way, or the off-pump CABG-the newer procedure or can be achieved minimal invasively.[13]

1.1.2.1. On-pump CABG (ONCAB)

ONCAB is the conventional procedure involving sternotomy as a means to access the heart, and the use of cold potassium cardioplegia solution to stabilize the heart during the anastomosis of the conduit vessel.[14] The CPB machine is used to support perfusion for the body during surgery.[14] CPB use is associated with increased post-operative risks including the development of whole body inflammation,[15] cognitive decline,[16] stroke,[16] myocardium damage[17] and bleeding,[18] which has a greater possibility in influencing postoperative outcome.

1.1.2.2. Off-pump CABG (OPCAB)

OPCAB is the modern procedure carried out on a beating heart, using a specialize equipment to position the heart during anastomosis of the conduit without the use of CPB machine. This alternative technique was introduced as a means of decreasing the risk associated with CPB use in CABG. However, studies of the Randomized On/Off Bypass (ROOBY) trial and the Five-Year Outcomes after Off-Pump or On-Pump

Coronary-Artery Bypass Grafting reveals no significant difference at 30 days, 1 and 5 year in the rate of the composite outcome of death, stroke, and myocardial infarction respectively.[13, 19]

1.1.2.3. Minimal Invasive CABG (MICAB)

MICAB is a novel procedure performed on a beating heart on a patient in closed anterior thorax position using minimal invasive technique.[20] This procedure has additional benefits such as reduced operation and recovery time, decreased need for blood transfusion, less pain and less time under anaesthesia, decrease length of hospital stay.[20] However, the total number of bypass conduit is reduced, making it useful to selected group of patients.

1.2. SEX-DIFFERENCES IN CABG OUTCOME

The words sex and gender in medical literature are commonly used interchangeably. However, sex refers to biological, physiological and anatomical differences, whiles gender refers to behaviours, roles, expectations, and activities in society. In this thesis, sex will be used to distinguish male and female in relation to their biological, physiological and anatomical differences.

1.2.1. Sex-differences in in-hospital mortality post CABG

Sex-differences in in-hospital mortality following CABG have been extensively studied, categorized, and documented. In the last two decades, the available postoperative data reveals increased in-hospital mortality for adjusted and unadjusted data in female compared to male.[21-23] Stratification of in-hospital mortality according to age reveals a considerably higher mortality among young females aged 50 years and below with a 3-fold increase in hospital mortality (3.4% for females versus 1.1%

for males), and females 50 to 59 years of age with a 2.4 fold increase in mortality (2.6% for females versus 1.1% for males) compared to their males counterpart (Figure 1).[21] Furthermore, the recent data by Swaminathan *et al.*, (2016) with over 2.5 million participants further revealed an increase in the overall unadjusted in-hospital mortality in female (3.2% for females versus 1.8% for males).[24]

This sex-disparity have led to the inclusion of female sex as postoperative risk factor for adverse outcome post CABG in the Euro-SCORE algorithm, a validated model for CABG. Thus suggesting the existence of sex-specific risk factors predisposing female to worsen post-operative CABG outcome.

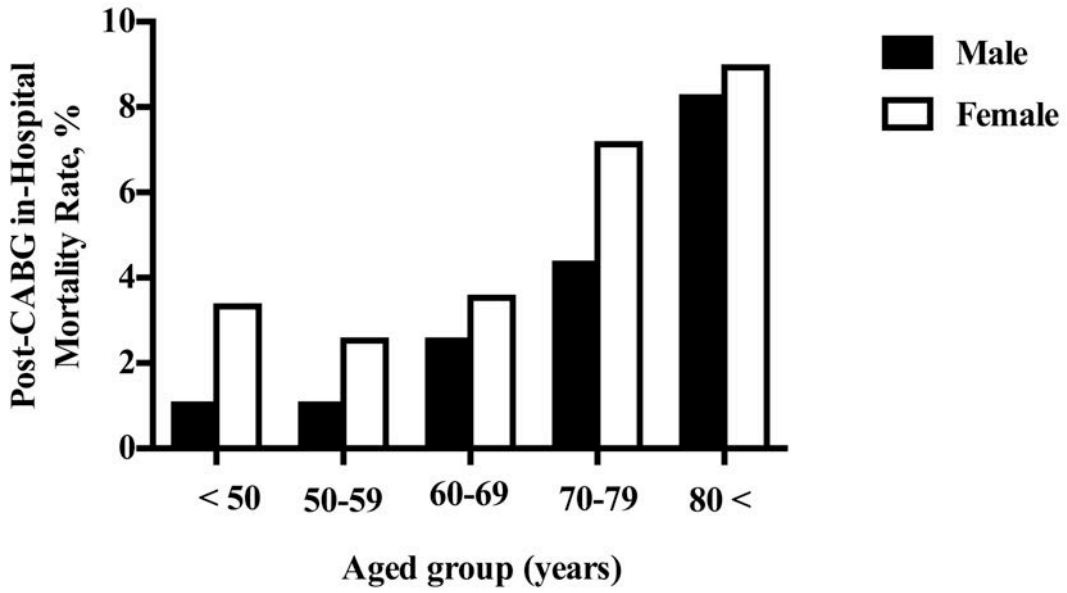


Figure 1: Stratification of post-operative in-hospital mortality based on aged: Female possess increase mortality in all age groups with a considerable higher mortality in young females aged 50 years and below. Modified from Vaccarino *et al.*, 2002.[21]

1.2.2. Sex-differences in post-operative recovery

Considering the nature of modern CABG procedure, in most cases involving sternotomy and the use of CPB, difference in recovery has been reported. Sex-differences in this recovery pattern have been an area of interest in the past 2 decades. Female CABG patients have higher incidence of deep sternal wound infections than male CABG patients.[25] Females have been recorded with worsen recovery pattern with increase rate of hospital readmission up to 1-year compared with males,[26] a problem likely due in part to their higher rate of comorbidity on the initial surgical admission. This sex-disparity in recovery was associated to worsening functional and mental health status in females compared to males,[27] with increase scores for depression and anxiety, and sleep disturbance compared to their male counterpart.[28] Thus raised the question whether females show the same quality benefit of CABG procedure compared to males.

1.3. POSSIBLE CAUSES TO THE INCREASE IN HOSPITAL MORTALITY OF FEMALE POST CABG

The potential reason for the differential outcome of female post CABG is multifactorial and may include a combination of clinical, psychosocial and biological factors.

1.3.1. Clinical factors

The next section of this thesis will focus on clinical factors as a contributor to this differential outcome of female including (1) Clinical presentation (2) Disease management (3) Pre-operative (4) Intra-operative and (5) Post-operative risk factors.

1.3.1.1. Clinical presentation

Clinical presentation and disease management is significant in examining clinical factors as contributor to the worsen outcomes of female post CABG. It is well documented that the diagnosis of coronary artery disease (CAD) in premenopausal female is less reliable than their male counterparts.[29-31] This is probably due to the fact that premenopausal females are more likely to report atypical angina [29-31] compared to their aged counterpart males are associated with ST-elevation myocardial infarction (MI).[32, 33] Furthermore, females are less likely to identify their initial symptoms as expression of disease development, and they will rather associate the onset of disease in the form of physical signs or symptoms to gastrointestinal distress, stress, or anxiety and ignore the immediate seeking of medical attention.[34] This has resulted in the misperception that occurs at two levels, the patient, and the clinician, since both believe that CAD is 'rare' in young female, with a greater possibility of misdiagnosing of symptoms that represent myocardial ischaemia as early in premenopausal female compare to postmenopausal female or their aged counterpart male.[35, 36] This patient/clinician misperception can possibly influence the likelihood to be referred for interventional procedure and later present with advance stage of CAD and could further influence post-operative outcome in female.[33, 37]

1.3.1.2. Disease management

Managing angina and other symptoms is important in females because of their misperception in symptoms and increase in other comorbidities during presentation compared to males.[22] Female with acute MI have been shown to receive less cardio protective medications such as renin–angiotensin–aldosterone system (RAAS) inhibitor,[38, 39] anti-platelet,[40] lipid lowering drugs[41] and beta-blockers[42] and

receive less mechanical revascularization procedure (PCI and CABG) that can influence their survival outcome in MI.

1.3.1.3. Pre-operative risk factors

These well-defined risk factors are associated with coronary artery disease development that can further influence CABG outcome.[42-46] Pre-operative risk factors can be considered as either modifiable or non-modifiable, or female-specific factors.

1.3.1.3.1. Modifiable clinical risk factors

The INTERHEART study has well documented the risk factors of cardiovascular disease that are considered modifiable.[47] For the purpose of this thesis, risk factors including hypertension, diabetes, obesity, smoking and tobacco use and physical inactivity will be discussed.

1.3.1.3.1.1. Hypertension

Although blood pressure targets and thus the threshold definition for hypertension are currently in a state of flux, hypertension is generally defined as a systolic blood pressure (SBP) of 140 mm Hg or above or diastolic blood pressure (DBP) of 90 mm Hg and above.[48] Approximately 95% of hypertensive patients have 'essential' or primary hypertension, where the cause is unknown. However 5% have an identifiable cause such as primary hyperaldosteronism or phaemochromocytoma.[49] Pathophysiological findings of blood pressure management reveals that for every 20 mmHg SBP elevation or 10 mmHg DBP elevation, this will directly corresponds to a double risk for adverse cardiac event[50] manifested as mostly damaging the function of critical organs of the body.[51, 52] Many individual with undetected hypertension

might have a devastating effect on their cardiovascular health as a result of late presentation.[51]

Sex difference in the prevalence of hypertension revealed that young males have an increase SBP and DBP compared to aged matched females.[53] At age 50-64 years, the prevalence of hypertension is similar between male and females until after 64 where the prevalence of female becomes higher because of decline in oestrogen levels in menopause.[54, 55]

Hypertensive patients are associated with about 40% increase risk of adverse outcome compared to normotensive patients,[48] although the reasons are unclear. Hypertension can be modified by either, lifestyle measures and/or drug therapy. Randomized controlled trial of anti-hypertensive treatment have demonstrated reduced mortality but not cardiovascular events.[56]

1.3.1.3.1.2. Diabetes Mellitus

According to the American Diabetes Association, the clinical definition of diabetes is fasting blood glucose of ≥ 7.0 mmol/l.[57] Using the American Diabetes Association diagnostic parameter, there are about 135 million (4.0%) of people reported with diabetes globally in the last decade and this is said to increase to 300 million (5.4%) by 2025.[58] Sex-differences in the prevalence of diabetes revealed more males living with diabetes than females, with the number of affected females slightly increased post menopause,[59, 60] suggesting the protective effect of oestrogen to diabetes development.

The pathophysiology of diabetes on cardiovascular health is devastating. Diabetic female have 3- to 6-fold increased risk of MI compared to diabetic male with 2- to 4-fold increase mortality.[61] This reveals that female with diabetes have worsen

outcome post MI compared to male with diabetes.

The influence of diabetes in CABG outcome has revealed conflicting results. For example, some studies showed increase postoperative mortality in diabetic female compared with diabetic male,[62, 63] with higher incidence of bypass graft dysfunction. [64, 65] In contrast, others reported no sex-difference in 12 months morbidity outcomes,[66] including the use of IMA conduit as the conduit vessel of choice between diabetic and non-diabetic patients,[67] suggesting that IMA possess some protective mechanism against diabetes related graft dysfunction.

Medical intervention in diabetic patients with established atherosclerotic condition can extend overall survival; improves quality of life; decreases the need for intervention procedures and reduces the incidence of cardiac event.[68]

1.3.1.3.1.3. Hyperlipidaemia

Hyperlipidaemia refers to increased levels of circulating lipids such as cholesterol and triglycerides in the body, which increased risk of developing MI. Hyperlipidaemia is associated with 4.4 million deaths each year.[69] The clinical definition of hyperlipidaemia is total cholesterol >5.5mmol/L and/or triglyceride > 2mmol/L. [70] In a primary prevention strategies, a total cholesterol above 6.8mmol/l warrants treatment.[71] Postmenopausal females have dyslipidaemia with a total cholesterol levels rise by 10 % compared to age counterpart, with no rise in high-density lipoprotein (HDL) cholesterol pattern.[72, 73]

Total cholesterol is a strong predictor of cardiac event among young individuals, whereas hypertriglyceridemia is a stronger predictor among old individuals.[74] In addition, low/decrease HDL level is a stronger predictor of adverse cardiac event in female compared to male.[74] Hyperlipidaemia is a risk factor for cardiovascular

events because of atherosclerosis development in the graft conduit, but there was no association with postoperative in-hospital mortality in male and female.[75]

1.3.1.3.1.4. Obesity

Obesity is considered an abnormal or excessive fat accumulation in the body, classified as an individual having a Body Mass Index (BMI) of ≥30 that may impair health in an individual. The prevalence of BMI greater than and equal to 25 in the adult population increased from 29·8% to 38·0% in female and 28·8% to 36·9% in male in the past two decades.[76] The pathophysiology of abnormal or excessive fat accumulation reveals that for every 5-unit of elevated BMI, there is a corresponding 34% increase CVD mortality in male and by 29% CVD mortality in female,[77] suggesting a devastating effect of obesity in male compared to female.

The waist-hip ratio is another predictive marker of obesity proposed to be better than BMI alone.[78] In male, the waist-hip ratio predicts CVD incidence only in normal weight individual with BMI ≤ 25, but not for overweight or obese male. In female, waist-hip ratio is a predictor of adverse CVD events irrespective of BMI.[79]

Cardiac performance maybe impaired following induction of anaesthesia and tracheal intubation in the obese patient during surgery.[80] Furthermore, there is a strong association between obesity and elevated postoperative risk of wound infections compared to normal weight patients post CABG,[70] that carries a mortality of about 25%.[70]

1.3.1.3.1.5. Smoking and Tobacco use

Smoking is one of the most important factors that double the risk of MI.[81-83] Smoking is also associated with inhibition of wound healing mechanism,[84] thrombosis[85] and endothelial dysfunction in smokers compared to non-smokers.[86] The influence of smoking in male and female reveals 6-fold increase risk of MI in female, with only 3-fold increase risk of MI in male smoker compared with non-smoker, suggesting that smoking is more detrimental in female than male.[87]

Presently there is no association between smoking and postoperative in-hospital mortality post CABG. However, smokers going through CABG receive increase doses of muscle relaxants and other anesthetics in CABG compared to nonsmoker.[88, 89] with increases risk of significant lung complications. Therefore, it is advisable to quit smoking before surgery for better surgical outcome.[90]

1.3.1.3.1.6. Physical in activity/sedentary life style

Physical activity and good health outcome has been well documented, with a decrease risk of developing CVD compared to physical inactivity or sedentary individual**.** Generally, male are more active than female.[91] Mild exercise activity of a minimum of 150 minutes or 75 minutes of active exercise per week is generally recommended for both male and female to experience healthy outcome.[92, 93] Exercise activity can improve other risk factors such as hypertension, decrease sugar level, improve lipid profile,[94] it lowers the prevalence of obesity, type 2-diabetes, enhance functional endothelium and improve coronary circulation.[95]

Physical inactivity in CABG carries an elevated risk of postoperative complications and an increase hospital stay.[96, 97]

1.3.1.3.2. Non-modifiable clinical risk factors

These are risk factors associated with CVD development and worsen outcome that we have little or no control over. For the purpose of this thesis, the following will be discussed (1) age (2) family history of cardiovascular event and (3) female related risk factors.

1.3.1.3.2.1. Age

The prevalence of MI has increased in premenopausal female, while declining in aged matched male.[98] Furthermore, the incidence of stroke occurs at an average age of 71 and 75 years in male and female respectively,[99] this is reversed in the oldest group aged 85-94 years, with stroke incidence higher in female than in males.[99] Furthermore, the influence of age on postoperative outcome in CABG revealed that increase in age is associated with increase in hospital mortality in both male and female.[21] This demonstrates the ability of young patients to withstand the surgical trauma compared to their older counterpart. In addition, although female going through are older than male, they still show worsen outcome compared to their aged matched counterpart.[21]

1.3.1.3.2.2. Family history of coronary artery disease

Strong association exist between family's history of cardiovascular events and disease progression because of shared genetic and biochemical components that predispose the individual to disease development in later life. Therefore, if a male first-degree relative such as father/brother or a female first-degree relative such as mother/sister) suffered MI before the age of 55 and 65 respectively, there is a greater chances of CVD development compared to individual with no shared genetic and

biochemical components.[100] Furthermore, if both parents have suffered from MI before the age of 55, your risk rise to 50% for developing CAD independent of other risk factors.[100, 101] Sex-difference in the association of family history as a predictor of CVD outcome is still debatable. Some reported a significantly predictive mortality for male aged 40 years and above and this prediction further prevail even after CVD risk factors adjustment.[101] In contrast, others reported family history as a strong predictor for female.[102] However, the association of family history of CVD and CABG outcome has been less investigated, as this might be beneficial to understand family history as a predictor for postoperative outcome in CABG.

1.3.1.3.3. Female related clinical risk factors

Female related risk factors are general factors that can affect CVD health in female which include (1) post-menopausal status and (2) polycystic ovarian syndrome.

1.3.1.3.3.1. Postmenopausal status

Menopause is the permanent cessation of menstrual periods in the life of a female for at least 12 months without any obvious pathological cause.[103] The loss of oestrogen is associated with an acceleration of atherosclerotic disease in female, [104] and further impacts CVD risk factors and vascular endothelial dysfunction.[104] Most female experience menopause at an average age around 51 years, however, about 1% experience menopause at the age of 40 years and below, a medical condition refers to as premature menopause or premature ovarian insufficiency (POI). Female with premature or early menopause have 80% increased risk for CVD[103] with further two-year decrease in expectancy compared to female with normal menopause.[104]

Oestrogen/hormonal replacement therapy is proven beneficial to relieve symptoms of menopause until their median age.[105] However, its clinical use has declined as a result of large-scale prospective clinical trials that revealed its risks exceeded it benefits with greater risk of adverse outcome.[106, 107] Pre-menopausal female (<50 years) going through CABG has a worsen outcome compared to post-menopausal and their male counterpart.[21] Further, oestrogen/hormonal replacement therapy administration before CABG does not influence post-surgical outcome.[108]

1.3.1.3.3.2. Polycystic ovarian syndrome (PCOS)

PCOS is an endocrine disorder characterized by ovarian dysfunction, affecting about 8 to 10% of the female population that serve as a risk factors for female.[109] According to the Rotterdam consensus clinical definition of PCOS, the criteria require the presence of two of the following: oligo/anovulation, hyperandrogenism or polycystic ovaries on ultrasound.[110] PCOS can affect risk factors for CVD such as obesity,[109] impaired glucose tolerance[111] and hypertension.[110, 112] Presently there is no association of PCOS's to postoperative outcome. However, there is an association of PCOS and adverse cardiovascular event rate in postmenopausal female.[113]

1.3.1.4. Intra-operative risk factors

These are risk factors that are considered iatrogenic in clinical settings. Iatrogenic complications of cardiothoracic surgery are potentially devastating and are associated with a significant patient morbidity and mortality.[114] Some iatrogenic complications in CABG includes graft damage during surgery (as a result of graft harvest or graft anostosis), iatrogenic aortocoronary arteriovenous fistula (resulting from placement of an arterial graft to a cardiac vein) and clips retention.[115]

Currently, there is limited information with regards sex-differences in iatrogenic complications in CABG. This is important as it could provide information with regards iatrogenic complication in CABG as a contributing factor in the worsen outcome of female in CABG.

1.3.1.5. Post-operative risk factors

Postoperative care of CABG patient is challenging and conditions such as preoperative as well as intraoperative risk factors should be considered in postoperative care to enhance recovery outcome. Female with more complex clinical preoperative risk factors are more likely to be readmitted with unstable angina and congestive heart failure post CABG compared to male with the same perioperative risk score.[116] With regards mobilization and pain management from 12 hours to 72 hours post-surgery, male are associated with increase mobilization and pain medication as compared with female with decrease mobilization and increase sedative medication use.[117] Furthermore, female sex is an independent risk factor for ICU stay ≥3 days compared to the mean ICU stay of 1.9±1.5 days in males independently of age, off-pump CABG, stroke and renal failure.[118]

1.3.2. Psychosocial factors

In addition to the clinical risk factors, psychosocial factors including (1) socioeconomic status (2) Depression and (3) Anxiety and Stress, can contributor to the sex-difference in CVD outcome.

1.3.2.1. Socioeconomic status

Socioeconomic status (SES), such as occupation, income and education is strongly related to health outcomes.[119] This result into lack of autonomy of an individual,

decrease resources to participate in leisure-time physical activities and the acquisition of beliefs and knowledge and enables people to have a sense of control over their health, and makes them more likely to pass on healthy habits to their children.[120] Female posses lower SES compared to male.[119] Lower SES is associated to 2-fold elevated risk of CVD mortality in female between the aged of 30-59 years compared to aged match male.[121, 122] Furthermore, lower-income patients hospitalized for MI are mostly female relatively limited access to cardiovascular procedures.[123]

1.3.2.2. Depression

The effect of depression on CVD outcome has been studied intensely. An estimated two-thirds of MI patients are associated with benign forms of depression[124] which result to a 3-fold increase in mortality even after CVD risk adjustment,[125, 126] suggesting that depression is a powerful predictor of survival post MI. The global prevalence of depression is higher in females with a 1.7-fold greater incidence and serves as an independent risk factor for adverse events in females such as cardiovascular death and all-cause mortality compared to males.[127-129] Furthermore, case-controlled studies suggested treating depression yielded a 42% reduction in death or recurrent myocardial infarction when compared with depressed patients not treated with an antidepressant. [127, 130] However, a large randomised controlled trial demonstrated that antidepressant did not improve cardiovascular events.[131]

The prevalence of depression among CABG patients is 15% to 20% in male and female,[132] compared to 5% to 9% in females and 2% to 3% in males in a healthy population.[133] Preoperative depression is associated with elevated 2-fold to 3-fold postoperative mortality post CABG independent of other risk factors. [134-136]

1.3.2.3. Anxiety and stress

Similar to the increased prevalence of depression in females, the prevalence of an anxiety and stress is higher in females than in males.[137-142] This can manifests as an activator of sympathetic and parasympathetic nervous systems and cardiovascular excitation that can exacerbate CAD symptoms.[143] Anxiety is more prevalence in patients with an unknown surgery date because of fear for dying before the procedure.[144] In the Post-CABG Trial, the presence of anxiety symptoms was significantly associated with a higher incidence rate of death or myocardial infarction after a median follow-up time of 4.3 years following CABG.[143]

1.3.3. Biological factors

Biological variations that can influence CABG outcome in female includes (1) Coronary artery sizes, (2) Microvascular dysfunction (3) Atherosclerosis and (4) Bleeding thrombosis and coagulation

1.3.3.1. Coronary artery sizes

The diameter of the coronary arteries in females is less than that of males, with about 10 to 15% smaller even after adjusting for different body size.[145, 146] This difference in vessel sizes is associated with difficulty in graft anastomosis in female,[146, 147] and further decrease graft patency,[146, 147] that may eventually leads to an increase graft failure in female compared to male, and further influence post operative outcome.

1.3.3.2. Coronary micro-vascular dysfunction

In an invasive coronary angiography for the evaluation for ischaemia, about 50% of females compared to only 17% of males are found to have normal or insignificant obstructive CAD (defined to be >50% luminal stenosis in a coronary artery).[148-150]

This phenomenon has prompted the recent hypothesis of an alternative, 'female specific pattern of ischemic heart disease known as coronary microvascular dysfunction or microvascular angina.[151] Female with coronary microvascular dysfunction not only continued to experience ongoing symptoms but also suffered poorer outcomes with 3.5-fold higher risk of major adverse events, including death, myocardial infarction, stroke, and heart failure compared to female without coronary microvascular dysfunction.[152]

1.3.3.3. Atherosclerosis

Atherosclerosis is an inflammatory condition characterized by the influx of immune cells into the media of the vessel wall of an artery. The severity of atherosclerosis formation in female has been found to increase following menopause, resulting in more female "living" with atherosclerosis than male with different plaque morphology between sexes.[153-155] Culprit lesions causing acute coronary syndrome (ACS) or sudden cardiac death in female harbour less extensive atherosclerosis and evidence of rupture.[155-159] However, female have reported with a higher prevalence of plaque erosion compared to males [155-159] that was made up of primarily thrombus formation.[93, 155]

1.3.3.4. Platelet activity

Platelets are key mediators that function in vascular homeostasis as well as the development of atherosclerosis and other cardiovascular disease conditions. However, controlling their function by irreversible inhibiting their activities can leads to elevated risk of bleeding in CABG.[160, 161] Female have increase total platelet count compared to male.[162, 163] In addition, younger female and male have increase platelet count than their older counterpart.[162, 163] Furthermore, female have increase

platelet reactivity compared to male [163-165] both with and without antiplatelet therapy.[164-166] This could potentially leads to increase in production of platelet derived vasoconstriction in circulation in female compared to male, predisposing their vessel to spasm.

1.3.3.5. Bleeding, thrombosis and coagulation

Severe bleeding as a result of surgical operation is a serious complication post CABG that can influence morbidity and mortality post CABG.[167, 168] Bleeding related re-operation is estimated to be 2.3% to 8% patients post CABG, [167-170] which is often multifactorial and influenced both by surgical factors and an impaired haemostasis.[171, 172] The administration of coagulation mediators during cardiopulmonary bypass has also been associated to coagulopathy post CABG.[171, 172] In addition, females are more likely to suffer from higher rates of bleeding compared to males.[173, 174] Furthermore, female sex is a predictive factor for blood transfusion post CABG compared to male, [175-177] and this is associated with increased morbidity and mortality.[178, 179]

1.4. THE VASCULAR SYSTEM

The vascular system is a closed loop system of blood vessels that play an integral role in the movement of blood throughout the circulatory system. It consists of an extensive network of arteries, arterioles, capillaries, venules and veins through which cellular function such as growth and development, absorption of essential nutrients, and removal of cellular and metabolic waste products take place.[180]

Oxygenated blood returning from the lungs is ejected from the left ventricle of the heart into a large network of arteries. As a result of the pressure generated by the

heart in an artery, large arteries are more elastic in nature to withstand the pressure; while small arteries are less compliant.[181] The continuous branching of arteries results to blood flow within the smallest blood vessels of the arterial tree called arterioles.[181] The resistance of arterioles is significantly higher than arteries, and they regulate their diameter to control the amount of blood flow to the respective organs, making them crucial in determining the mean arterial pressure. The bifurcations of arterioles form capillaries that connect the arterial vascular tree to the venous system where exchange of nutrients and gases between cells and blood take place via diffusion (Figure 2). The capillaries also regulate the microcirculation by altering the tone of the smooth muscle in the arteriolar wall.[180] There are different distinguished forms of capillaries including continuous, fenestrated, and discontinuous.[180] The return of deoxygenated blood to the heart is via the venous system, and it begins with its movement into venule formed from capillary beds that vary widely in size and character but function to return blood to the heart. Venule merge to form larger veins that eventually return blood to heart (Figure 2).

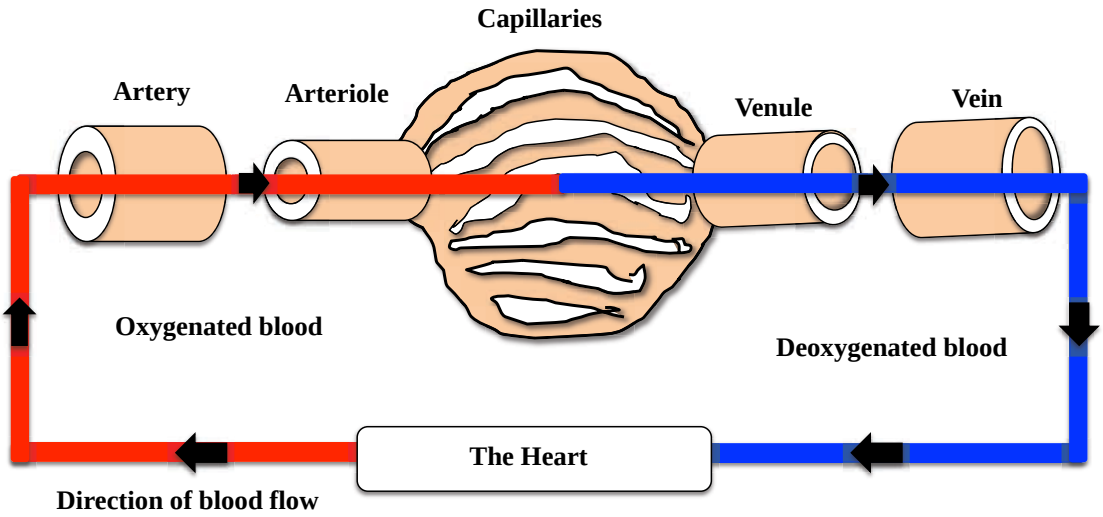


Figure 2: Schematic diagram summarizing the sequence of blood flow through the heart via the arteries, arterioles, capillaries, venules, veins, then back to the heart.

1.4.1. Anatomy of blood vessel

Three anatomical features are distinct to all blood vessels such as tunica intima, tunica media, and tunica externa has been revealed.[181] However, these features are different in their structure and morphology in vein and an artery giving each vessel it unique characteristic features that is well adapted for specific function (Figure 3).

The tunica intima composed of thin lining of specialized epithelium called the endothelial layer. Below the endothelium, exist a fibro-elastic connective tissue layer and an organized layer of internal elastic lamina that provides flexibility and stability for endothelial cells and perivascular cells.[182]

The tunica media is the middle layer of the vessel that contains predominantly smooth muscle cells and elastin fibres and collagen. This region is considered denser and more organized in arteries than it is in veins as a result their physiological function. Furthermore, this layer also contains an external elastic lamina that provides structural support.[181]

The tunica externa is a sheath of connective tissues that blend with the surrounding connective tissue outside the vessel holding it in relative position. Lymphatic and nerve plexi are observed in the adventitia in addition to vaso vasorum that provides larger arteries and veins with adequate nutrients and oxygen.[181]

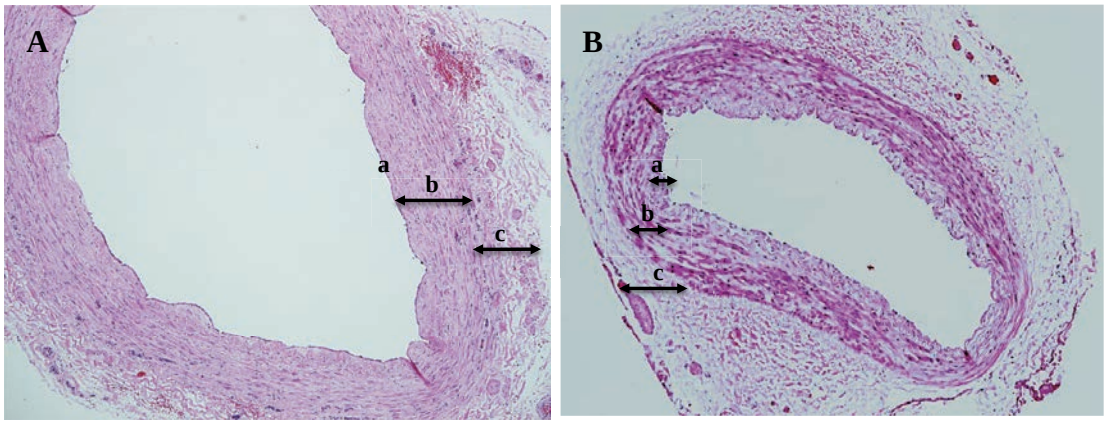


Figure 3: Histological examination by H&E summarises the structural differences between an artery and vein: A, saphenous vein and B, internal mammary artery. a=tunica intima containing endothelial layer, b=tunica media composing of smooth muscle cell, elastic lamina and collagen, and c= tunica externa composing of the adventitia and perivascular adipose tissue and nerve endings

1.4.2. The coronary circulation

The coronary circulation refers to a specialized system of blood vessels dedicated to delivers oxygenated blood to the myocardium. The circulation is from the coronary artery through the microcirculation and provides drainage systems to remove deoxygenated blood through coronary veins.

1.4.2.1. Coronary arteries

There are 2 major coronary arteries that branch off from the root of the aorta behind the right and left cusps of the aortic valve.[183] They provide the entire blood supply to the myocardium as follows:

1. The right coronary artery branches into the right marginal artery and the posterior descending artery-that supplies blood to the right atrium, right ventricle and the bottom portion of both ventricles and back of the septum

(Figure 4A).[183]

2. The left main coronary artery (also called the left main trunk): The left main branches into the circumflex artery-that supplies blood to the left atrium, side and back of the left ventricle and the left anterior descending artery (LAD)-that supplies the front and bottom of the left ventricle and the front of the septum (Figure 4B).[183]

1.4.2.2. Coronary microcirculation

The coronary microcirculation consists of terminal arterioles, precapillary sinuses, capillaries, and venules that function together to delivered nutrients and exchange of gases in the myocardium. During diastole, the precapillary sinuses serve as a blood reservoir and eject the blood during diastole to sustain myocyte perfusion.[183] Furthermore, there are arteriosinusoidal, arterioluminal, and thebesian vessels that communicate between the vessels of the myocardium and the cardiac chambers**.** The arteriosinusoidal vessels penetrate the chamber walls and divide into irregular, endothelium-lined sinuses. The arteriosinusoidal vessels open directly into the atria and ventricles and the thebesian vessels are small veins that connect capillary beds directly with the cardiac chambers.[183]

1.4.2.3. Coronary veins

The major venous vessels of the human heart are: coronary sinus, the anterior interventricular veins, left marginal veins, posterior veins of the left ventricle, and the posterior interventricular veins. They collect and return blood to the right atrium via the coronary sinus.

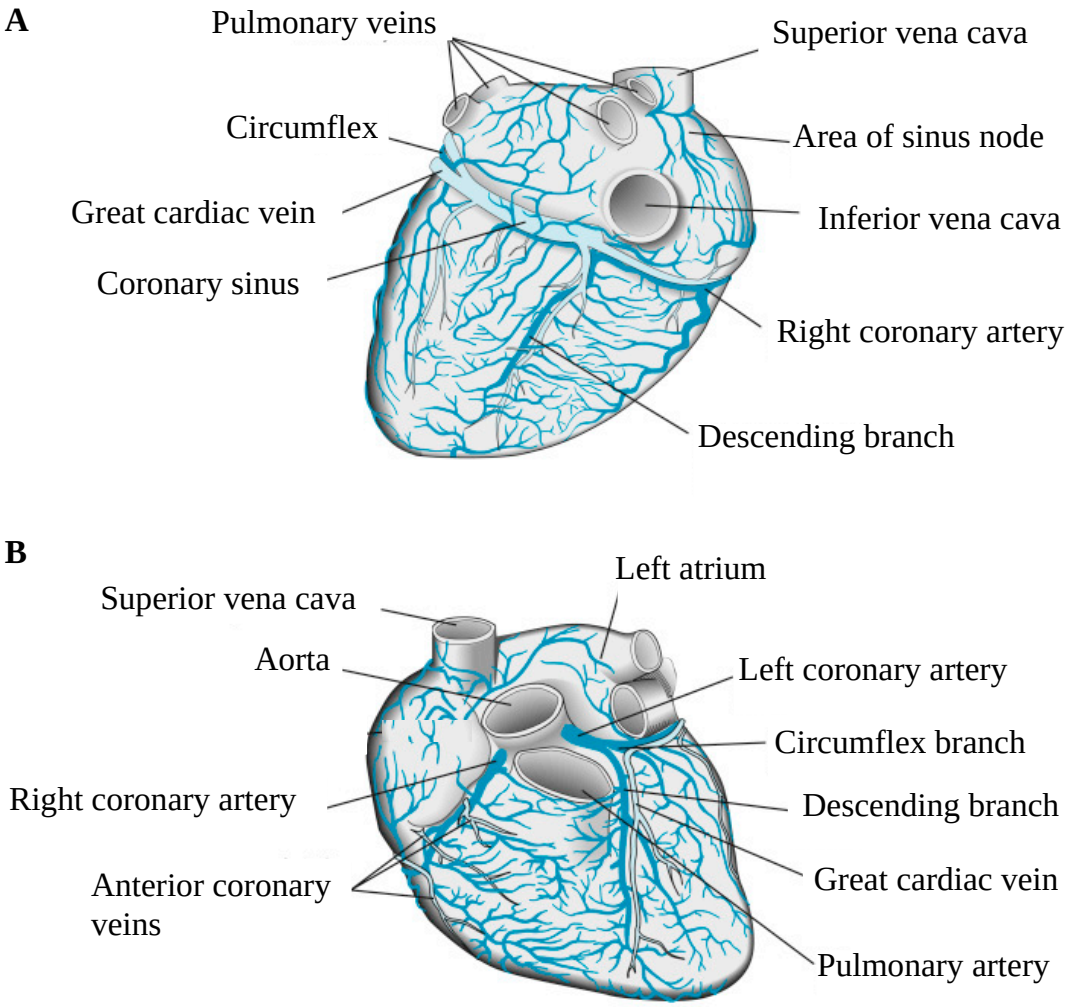


Figure 4: Schematic diagram illustrating the location and distribution of the principal coronary vessels: (A) Anterior view and (B) posterior view of the heart. Modified from Pappano *et al.*, 2013[183]

1.4.3. CABG graft conduits

The patency of CABG graft is essential for the long-term survival of the recipient patient post CABG. Various conduits have been explored from different sites of the body in the quest for better survival outcome. For the purpose of this thesis, the following autologous graft conduit will be discusses: (1) the great saphenous vein (SV), (2) the internal mammary artery (IMA), (3) gastroepiploic artery (GEA), (4)

inferior epigastric artery and (5) radial artery (RA).

1.4.3.1. Saphenous vein

Saphenous vein graft is currently the only vein graft and the most frequently chosen conduit because of it availability to perform multiple grafts in CABG; relative to its large diameter that makes it is technically easy to use. The 5 years patency of SVG is 65% to 80%, at 7 to 10 years, is about 50% to 60% and at 10-15 years is 50%. [184] However, the mismatch in diameter between the right coronary artery and the SV is known to impact on the patency rate of the SV. [185] Sex-differences in SVG patency revealed lower 2-year vein graft patency in female compared to male (female (76.4%) and male (82.1%), [186] no sex-differences in survival rates between 5 to 15 years [186] was observed post CABG. The SV can be used in different configurations with different anastomosis (e.g., sequential, composite, or Y-graft). [187]

1.4.3.2. Internal mammary artery

The IMA graft conduit is a widely used conduit because of its excellent patency when anastomose to the LAD.[184] The methods of harvesting the IMA have been proposed to influence it patency and postoperative outcome. Some surgeons are in support of harvesting the conduit vessel in a skeletonized fashion giving advantages of reducing trauma and further influence postoperative complications. [188] In contrast, others prefer it to be harvested as a pedicle grafts and left attached to its origin before grafting. [189, 190] The introduction of a second IMA conduit during CABG is linked to an improved overall survival, with decrease need for repeat surgical procedure compared to single IMA graft. [191] In addition, it also reverses the sex-differences in in-hospital mortality and the further long-term differences.[192-195] The anastomosis of the IMA conduit to the LAD is considered the gold standard procedure of modern

CABG. Sex-differences in IMA use revealed that female have less frequent use of IMA compared to male. [24]

1.4.3.3. Radial artery

The RA is part of the brachial artery that descends along the lateral side of the forearm to the wrist, with a luminal diameter of 2–3 mm that qualifies it as a perfect match for the coronary artery compared to SV with a larger lumen. [196] The RA is mostly used as an alternative arterial graft in combination with IMA for reason since additional arterial grafts may improve result relative to overall survival outcomes of CABG surgery.[197-199] The patency of RA is reported to be between 70.7% and 98.3%, [200] with 95% at 1 year, and 88% at 4 years.[201-204] Sex- differences in late survival post CABG reveal no difference RA use in male and female in combination with IMA.[205]

1.4.3.4. Inferior epigastric artery

The IEA arises from the external iliac at a point cranial to the inguinal ligament and harvested from the inside of the lower abdominal wall.[206] About two decades ago, the IEA was thought to be the best replacement for the SV graft. [206] However, it use was discontinued due to doubts regarding it behaviour, complex harvesting and technical problems associated anastomosis the conduit and uncertain patency rate. [207] Currently there is limited information regarding the use of this conduit in male and female in association to influencing postoperative outcome.

1.4.3.5. Gastroepiploic artery

The GEA is suitable conduit for CABG in which it current use is not common. It is general considered as a free and redo graft in patients with IMA.[208] The patency of

GEA to the right coronary artery at 15-year is 71.3% and survival rates of 88.6%. [209, 210] However, it is technically difficult to harvest which further decrease it use in modern CABG procedure. There is currently limited information available regarding sex-differences in the use of GEA in CABG.

1.5. MECHANISM OF VASCULAR SIGNALING

For the purpose of this thesis, the mechanism of vascular signalling will focus on G protein-coupled receptors (GPRCs) signalling with regards the endothelium, smooth muscle and their regulatory mechanisms.

1.5.1. G protein-coupled receptors (GPRCs) signalling

GPCRs is a family of cell surface receptors that are capable of transducing a wide range of signals across the plasma membrane into varieties of information that are capable of influencing diverse biological functions.[211] Structurally they contain a 7 trans-membrane (7TM) domain with an extracellular amino terminus that is highly specific to a particular signal and an intracellular carboxyl terminus that interact with a G-protein.[212, 213] The G-protein contain three subunits: Gα, Gβ and Gγ,[211] of which the Gα and the Gγ subunits are attached to the plasma membrane by a covalent lipid anchors (Figure 5A). [212, 213]

Activation of GPCRs by ligand binding on the extracellular 7TM domains, results in a conformational change of the receptor leading to motif functioning as a guanine nucleotide exchange factor (GEF) that promotes the release of GDP bound to the Gα subunit, for a GTP, resulting in dissociation of the G-protein complex into Gα and Gβγ subunits (Figure 5B).[211, 214] The activated Gα bound to GTP diffuses along the membrane surface to activate target proteins and enzymes such as Ca^{2+}, K^{+} channels, adenylyl cyclase, phospholipase C (PLC), phospholipase D (PLD) and protein

kinases to generate various second messengers that influences diverse biological functions. Furthermore, the Gβγ complex is also able to diffuse along the inner membrane to activate the muscarinic K^+ channel and positively regulate adenylyl cyclase, phospholipase C-β (PLC-β), phospholipase A2 (PLA2), phosphoinositide 3-kinase (PI3-kinase), and β-adrenergic receptor kinase (Figure 5C).[131, 211, 215, 216]

There are four main classes of heterotrimeric G proteins, described based on the Gα component and their coupling specificity: (i) $G_{\alpha q/11}$, (ii) $G_{\alpha s}$, (iii) $G_{\alpha i}$ and (iv) $G_{\alpha 12/13}$[217]

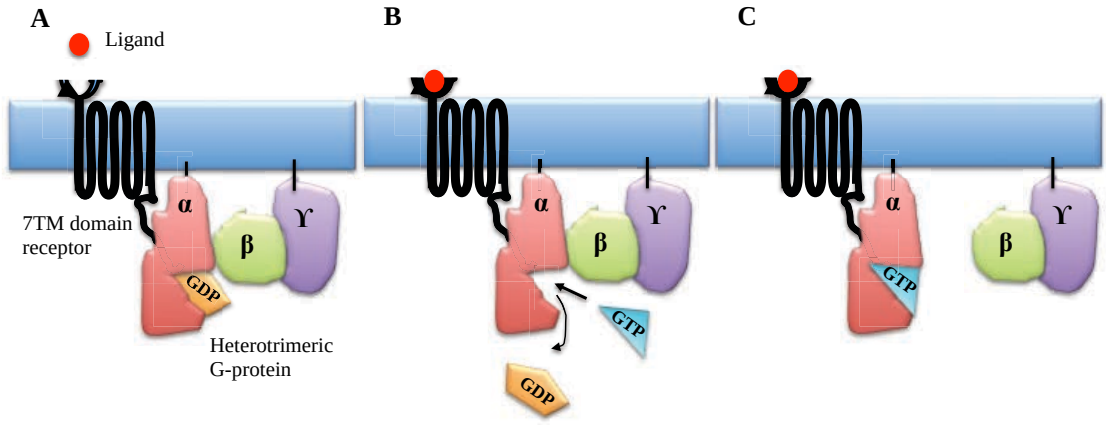


Figure 5: Schematic diagram illustrating activation and structural change of GPCRs. GPCRs are 7TM receptor containing three different subunits: G_α, G_β and G_γ subunits. The G_α and the G_γ subunits are attached to the plasma membrane by lipid anchors. (A and B) Activation of GPCR by ligand binding to the extracellular receptor results in a conformational change of the G-protein that promotes the release of GDP bound to the Gα subunit, for a GTP. (C) The activated GPRC results in a conformational change leading to the dissociation of the G-protein complex into G_α and $G_{\beta\gamma}$ subunits, and the G_α bound to GTP activate target proteins and enzymes to generate second messengers that influences diverse biological functions. In addition the $G_{\beta\gamma}$ complex is also able to diffuse along the inner membrane surface and affect protein activity.

1.5.1.1. $G_{\alpha q/11}$-coupled receptors

$G_{\alpha q/11}$ is a family of receptors that consists of four members, including $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, and $G_{\alpha 15/16}$.[218-220] A wide variety of hormones, neurotransmitters, and locally acting vasoactive substances use this signal transduction pathway to mediate their biological effects.[218] Activation of these receptors leads to activation of β-isoforms of phospholipase C, resulting in the hydrolysis of phosphoinositol 4,5-bisphosphate (PIP2) to release inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG).[221, 222] These second messengers serve to propagate and amplify the signals leading to the activation of SR Ca^{2+} and voltage-gated L-type Ca^{2+} and PKC dependent activation of DG/ CPI-17 resulting in smooth muscle contraction, [218, 221-223] balanced with endothelial activation and the release of endothelial-dependent vasodilator substances including nitric oxide (NO) prostanoids and endothelium derived hyperpolarizing factor. [218, 221-223]

Sex-differences in total abundance of $G\alpha_{q/11}$ have been reported in mice, with females expressing higher levels than males.[224] Although the phosphorylated state between sex was not revealed, one will suggests that females are having increase $G_{\alpha q/11}$ activity compared to males. $G_{\alpha q/11}$ is also playing a crucial role in survival, mice lacking $G_{\alpha q/11}$ family die at day 10.5 of embryonic development with aberrant vasoconstriction response to angiotensin II or phenylephrine agonist that functional affect blood pressure,[225, 226] where as mice lacking a specific $G\alpha_{q/11}$ family member $G\alpha_{11}$ did not show any phenotypic abnormalities,[226] which might be as a result of the high functional redundancy between the family.[227]

1.5.1.2. Gα$_s$-coupled receptors

Gα$_s$-coupled receptors play a central role in promoting vascular relaxation.[211] The majority of studies with regards G$_{\alpha s}$-coupled receptor signalling focuses on β_2-adrenergic receptor signalling because of it prevalence.[211] G$_{\alpha s}$-coupled receptor activation stimulates adenylyl cyclase (AC) release, which catalyses the formation of cAMP. There are three main effectors of cAMP: cAMP-dependent protein kinase (protein kinase A or PKA), the guanine-nucleotide-exchange factor (GEF) EPAC and cyclic-nucleotide-gated ion channels.[225] However, PKA activity is responsible for much of the cellular actions elicited G$_{\alpha s}$-coupled receptor. PKA has been shown to regulate other signalling pathways that leads to the inactivation of phospholipase C (PLC) β_2, decreases the activities of Raf and Rho and modulates ion channel permeability.[228-231] Research has found no sex-differences in total abundance of the Gαs protein in mice, [224] suggesting no sex-difference in activity state.

1.5.1.3. Gα$_i$-coupled receptors

The majority of known GPCRs are preferentially couple to members of the G$_{\alpha i}$-family.[232] G$_{\alpha i}$-coupled receptor activates Rho through Rho guanine nucleotide exchange factors (Rho GEFs),[233, 234] and RAS by various pathways, resulting in the RAF–MAP kinase kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) stimulation, that is associated with inhibition of G$_{\alpha s}$-stimulated AC activity and thus reduced cAMP generation as illustrated in figure 6.[235]

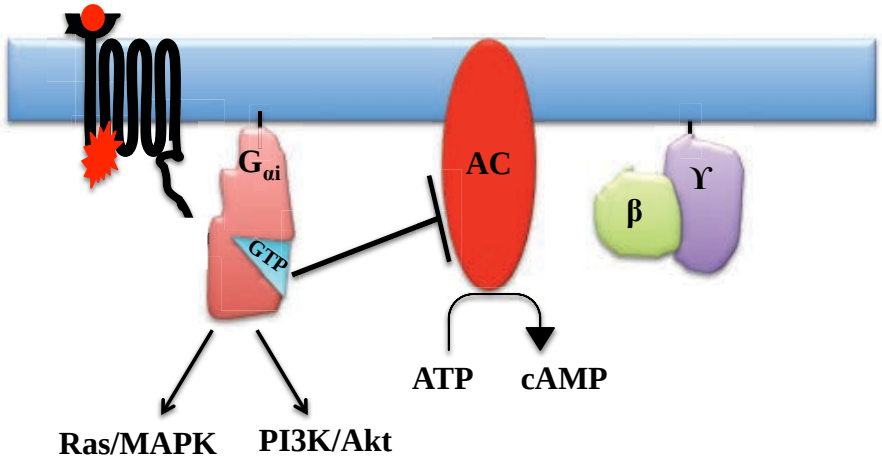


Figure 6: Schematic illustration depicting inhibition of adenylyl cyclase (AC) by $G_{\alpha i}$. In addition $G_{\alpha i}$ also leads to the activation of Ras/MAPK and PI3K/Akt pathway

1.5.1.4. $G\alpha_{12/13}$-coupled receptors

The $G\alpha_{12/13}$-coupled receptor family is composed of 2 members $G_{\alpha 12}$ and $G_{\alpha 13}$ that posses slow rates of nucleotide exchange and GTP hydrolysis.[211, 212] $G_{\alpha 12/13}$-coupled receptor family specifically targets the Rho GEFs that function both as a GAP and an effector for $G_{\alpha 12/13}$.[212, 236] Activation of $G\alpha_{12/13}$-coupled receptor leads to translocation of Rho GEF from the cytosol to the plasma membrane to interact with Rho A that activates Rho kinases (ROCK1/2) and downstream pathways.[237-241] ROCK causes the phosphorylation of the regulatory myosin-binding subunit of myosin phosphatase, which results in the inhibition of the enzyme favouring the myosin light chain phosphorylation and smooth muscle contraction. [213, 214, 217, 219, 220, 242, 243]

Smooth-muscle-specific deficiency of $G\alpha_{12/13}$-coupled receptors mediated signalling does not lead to alteration of the basal vascular tone under normotensive conditions.[244]

1.5.2. Regulation of GPCR activity

GPCR-mediated signal transduction is strictly regulated by phosphorylation of the receptor by two currently recognized classes of serine/threonine protein kinases: (1) Second messenger-dependent kinases: In this process, the elevation of second messengers (PKA/PKC) is able to phosphorylate both agonist occupied and unoccupied GPCRs. This phosphorylation causes desensitization of the GPCR by uncoupling it from G protein or in the case of the agonist unoccupied receptor by preventing GPCR coupling to G protein.[245] This generalized cellular hypo-responsiveness phosphorylation is referred to as heterologous desensitization (Figure 7A). Whether or not heterologous desensitization of GPCR leads to arrestin binding or internalization of the receptor depends upon the particular GPCR subtype in question.[245]

(2) G protein-coupled receptor kinases (GRKs): In this process, the agonist-occupied GPCR is subsequently phosphorylated by GRK. This increases its affinity for β-arrestin, acting as a scaffold to bring together other intracellular signalling proteins that binds to the receptor. This reduces signalling by preventing the association with the G-protein, initiating receptor-specific, or homologous desensitization.[246, 247] In addition, they can either target the receptor for endocytosis, leading to receptor down regulation (1) or they can activate beta-arrestin-dependent signalling pathways that are independent of G-protein signalling (2) (Figure 7B).[245, 248]

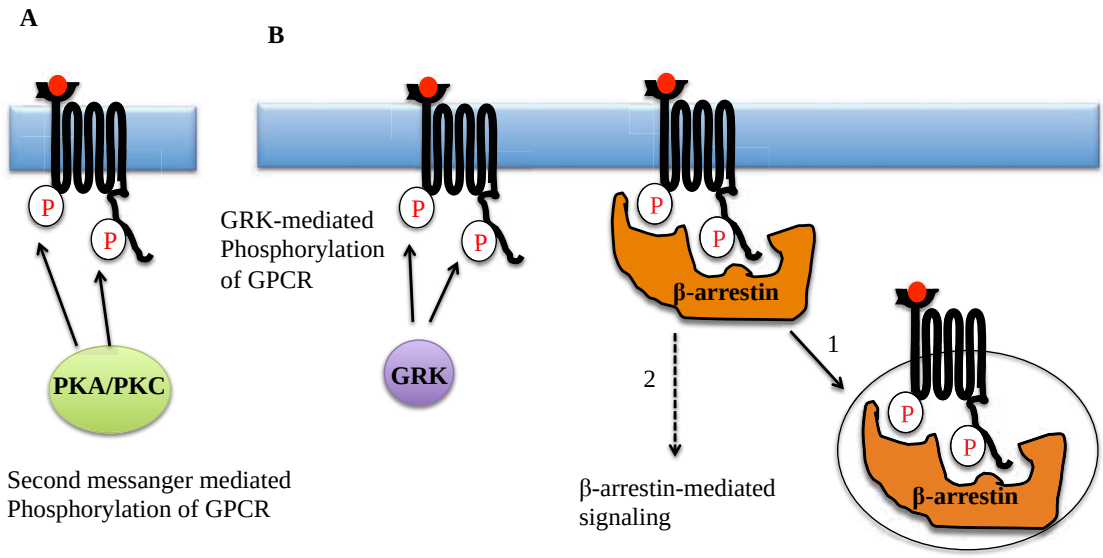


Figure 7: G protein-coupled receptor (GPCR) desensitization mechanism (**A**) Second messenger dependent heterologous desensitization. The agonist binds to the GPCR leading increased levels of second messenger that can further phosphorylate both agonist occupied and unoccupied GPCRs. (B) Classical model of receptor-specific, or homologous desensitization of GPCR by GRKs and arrestins. The agonist-occupied GPCR is subsequently phosphorylated by GRK, and arrestin binds to the phosphorylated GPCR, leading to receptor desensitization and further endocytosis (1) or arrestin-mediated cell signalling (2)

1.5.3. Vascular endothelial cell signalling

The vascular endothelium is a monolayer layer of specialized cells that line the entire lumen of blood vessels.[249, 250] These cells function by sensing and/or response to mechanical (shear stress and increased blood flow) or chemical (hormonal) stimuli producing vasoactive autocoids that act in either paracrine or autocrine manner in maintaining vascular haemostasis, influence smooth muscle function, inhibit platelet adhesion and aggregation as well as influencing blood fluidity. [251, 252] The endothelium derived vasoactive autacoids include both vasodilatory mediators such

as nitric oxide, prostanoid (both vasodilator and vasoconstrictor prostanoids), endothelial derived hyperpolarizing factor and endothelial derived constrictor factor (endothelin-1).

1.5.3.1. Nitric Oxide (NO)

Nitric oxide (NO) is a soluble gas that function as a potent biological mediator with diverse physiologic and pathophysiologic roles. [219,] NO is produced from L-arginine by the family of three nitric oxide synthase (NOS) enzymes identified in different tissues including: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) [253], utilizing the same substrates and cofactors. eNOS is predominantly responsible for production of endothelium dependent vasodilatory NO. NO produced from nNOS functions primarily as a neurotransmitter, where as NO produce from iNOS function in immune modulation.[254, 255]

In an inactive endothelium, eNOS is bound to caveolae coated protein-caveolin-1, serving as an inhibitor of eNOS activity limiting the production of tonic NO.[256, 257] The recruitment of Ca^{2+}-calmodulin complex to eNOS, leads to displacement of caveolin-1 from the enzyme and enzyme activation.[258, 259] This triggered nicotinamide-adenine-dinucleotide phosphate (NADPH)-mediated electron transfer within the eNOS domains that enables L- arginine to be convert into NO and L-citrulline in the presence of molecular oxygen and cofactors such as Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6*R*-) 5,6,7,8-tetrahydro-l-biopterin (BH4). [253, 260] eNOS can also be activated by stimuli that do not produce sustained increases in intracellular $Ca^{2+,}$ but still induce a long-lasting release of NO through phosphorylation of several serine (Ser1177) or decrease NO activity by inhibiting it function through phosphorylation of threonine (Thr495) residues. [261, 262]

In addition, hormones such as oestrogen and insulin has been shown to phosphorylate eNOS mainly via Akt, whiles shear stress elicits phosphorylation mainly by activating protein kinase A (PKA) leading to NO generation (Figure 8). [263] [264]

1.5.3.2. The Prostanoids

Prostanoids (Prostaglandins (PGs) and thromboxane (TXA_2) are lipid biomolecules that plays a crucial role in the regulation of vascular function and platelet reactivity. Prostanoids are synthesize by the hydrolysis of membrane-bounds phospholipids by phospholipase A_2 leading to the production of arachidonic acid, that is further metabolized by the enzymes cyclooxygenase 1 and 2 (COX-1 and COX-2), generating prostaglandin H_2 (PGH_2), which is then catalysed by specific prostanoids synthases to produce various prostanoids, including prostaglandin PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A2 (TXA_2) 91.[265]

Following production, prostanoids are transported rapidly out of the cell by their respective prostaglandin transporter (PGT) to activate a specific G-protein-coupled receptors such as prostacyclin (IP) receptor, $PGF_{2\alpha}$ (FP) receptor, and TXA_2 (TP) receptor, PGE_2 (EP) receptors and PGD_2 (DP) receptor to affect their function (Figure 8) [266]

1.5.3.2.1. Prostaglandin D_2 (PGD_2)

PGD_2 is an important prostaglandin that is abundantly produced that exerts its biological function via 2 distinct receptors: the prostaglandin D (DP) receptor and the chemo-attractant receptor-homologous molecule expressed on Th2 cells (CRTH2).[267, 268] Both receptors are members of the $G\alpha_s$-coupled receptors.[269] Although the effects of PGD_2 on vascular function are not well known, activation of

DP receptor has been shown to increases blood flow and vascular permeability,[270] induce endothelium NO-dependent relaxation[271] and further mediates endothelium-independent contractions through activation of TP receptors.[272, 273]

1.5.3.2.2. Prostaglandin E_2 (PGE_2)

PGE_2 is the most abundant prostaglandin in the human body. They are involved in multiple physiological effects including vascular tone.[274] There are four subtypes of PGE_2 receptors designated as EP_1, EP_2, EP_3, and EP_4 and multiple splicing isoforms of the EP_3 subtype.[275] EP_2 and EP_4 are coupled to the $G\alpha_s$ receptor, EP_1 is coupled to $G_{\alpha q/11}$ and EP_3 is coupled to $G\alpha_i$ of the GPCRs respectively.[276]

1.5.3.2.3. Prostaglandin F2α (PGF2a)

$PGF_{2\alpha}$ has diverse physiological actions ranging from being a potent luteolytic agent for normal parturition,[277] to a potent vasoconstrictor of smooth muscle vasculature (C,D,E). $PGF_{2\alpha}$ exerts it function through prostaglandin F (FP) receptor that is coupled to the $G_{\alpha q/11}$ family of GPCRs.[278] Interestingly, the vasoconstrictor properties of this prostaglandin have been shown to be inhibited by selective thromboxane receptor (TP-) antagonists, suggesting a mechanism of action via the TP-receptor.[279] Additionally, FP receptor on endothelial cells have been shown to induce endothelium- NO-dependent relaxations through DP, EP2 and IP, indicating a cross stimulation of $PGF_{2\alpha}$ agonist.[280, 281]

1.5.3.2.4. Prostacyclin (PGI_2)

Prostacyclin (PGI_2) is a potent vasodilator and inhibitor platelet aggregation. [282] The half-life of PGI_2 is very short and they rapidly converted into 6-keto-PGF1α. [283] PGI_2 elicits its biological effects through prostaglandin I (IP) receptors. The IP receptor has reveals differences in coupling to G-proteins, depending upon the

cellular context and species sequence variations. Over-expressed IP receptors in an in-vitro set up leads to $G\alpha_s$ and $G\alpha_{q/11}$ activation.[284-286] Furthermore, persistent activation leads to decrease cAMP, suggesting potentially $G_{\alpha i}$ coupling in these expression systems.[285] In another studies involving smooth muscle,[287] and platelets [288] reveals that IP coupling to $G\alpha_s$ but not to $G\alpha_{q/11}$, and the mouse IP receptor has been shown to couple to $G\alpha_i$.[286] Thus, G-protein coupling to an IP receptor can be cell and species dependent.

The basal function of prostacyclin can be up regulated by cytokines, growth factors, sex hormones and age via activation of PKA and ERK-1/2.[289] In addition, PGI_2 can cross talk with it endothelium counterpart, nitric oxide in the control of vascular relaxation upon endothelial activation. It is important to note that in the presence of functional endothelium with greater NO producing capacity, blocking PGI_2 production has no effect on vasodilatation.[290] However, when NO is blocked, the residual dilation is due to increased PGI_2 synthesis, [291] suggesting that PGI_2 plays a compensatory role in dilation of the vessel when NO is reduced.

In human, defect in prostacyclin receptor signaling, as observed in patients with a dysfunctional IP receptor mutation or impaired PGI_2 production has been reported in several cardiovascular disorders including pulmonary hypertension, [292, 293] peripheral vascular disease [294] and ischemic heart disease.[295] Synthetic PGI_2 is a logical choice for the treatment of these diseases. Presently there are different analogues of PGI_2 formulations available with different route of administration including intravenous use (Epoprostenol), subcutaneous injection (**T**reprostinil**)**, and inhalation (Iloprost). These analogues have shown to improve hemodynamic parameters, symptoms,

increase exercise capacity and improves survival in patients with pulmonary hypertension [296] and peripheral atherosclerotic arterial disease. [297]

1.5.3.2.5. Thromboxane A_2 (TXA_2)

TXA_2 is widely implicated in the pathogenesis of a variety of cardiovascular diseases including ischemic heart disease, atherosclerosis, hypertension, preeclampsia, and stroke, owing to its acute and chronic effects in promoting platelet aggregation, vasoconstriction, and proliferation.[298-301] There are two isoforms of TP receptor in humans TP_α and TP_β, differ only in their C-terminal portions with TP_α shorter than the TP_β isoform with identical ligand binding site. TP receptors are coupled to $G_{\alpha q/11}$ and the $G_{\alpha 12/13}$ families.[302]

In an experimental mice model with TP receptors deletion, the phenotype reveals normal blood pressure due to the short half-life of endogenous thromboxane,[303] in addition to decreases vascular proliferation and decrease platelet activity. This suggests that TXA_2 appears to have little influence on baseline regulation of blood pressure. However, TP receptor antagonists attenuate the development of hypertension and TP receptor–deficient mice were further shown to be resistant to the development of hypertension.[301] Furthermore, the stable analogue of TXA_2 (U-46619) has been revealed to induces contraction via activation of the Ca^{2+}/calmodulin/MLCK pathway and maintain it Ca^{2+} sensitization mechanism via RhoA/ROK pathway independent of PKC dependent activation of CPI-17.[304] These data suggest therefore that normal levels of TxA_2 may not contribute to pathological vasoconstriction, but that during hypertension elevated TxA_2 does play a role

1.5.3.3. Cyclooxygenase enzymes (COX)

Cyclooxygenase (COX) is an enzyme that catalyses the initial step in the conversion

of arachidonic acid to prostaglandins (PGs). They and are expressed in various cell types including the vasculature, platelets, neurons, inflammatory cells, gastric mucosa and the heart.[305, 306] There are two isoforms of COX; COX-1 and COX-2 with remarkably similar structures.[306]

COX-1 is thought to serve "housekeeping" functions as a constitutive enzyme but also contributes to overall prostaglandin production. In the platelets, COX-1 is the main isoform and is responsible for production of TXA_2, while COX-2 is highly inducible by diverse stimuli including cytokine and growth factor.[305, 306] However, in an hypertensive rat aorta, the endothelial dependent production of PGI_2 metabolite 6-keto-prostaglandin F1α (6-keto-PGF1α) was almost completely blocked by inhibiting COX-1, but only partly reduced by inhibition of COX-2, arguing for the major role of COX-1 in PGI_2 synthesis in hypertensive rat.[273] In addition, mice with isoform-specific COX deficiency (COX-1 knockout or COX-2 knockout), 6-keto-PGF1α productions was abolished by COX-1 knockout but unaltered by COX-2 knockout.[307] Thus demonstrate an explicit role for COX-1 in endothelial PGI_2 synthesis, suggesting that in some arteries COX-1 can be the predominant functional form.

1.5.3.4. COX-inhibition

COX inhibitors are associated with a number of side effects including gastrointestinal toxicity, and renal and hepatic insufficiency the rate of glomeruleral filtration is reduced. Such critical adverse reactions have been shown to highly dependent on COX-1 inhibition.[308]

Aspirin, is the most commonly used non-selective COX inhibitor that irreversibly acetylate the amino acid at serine 529, which inhibits arachidonic acid from binding

to the enzyme's catalytic site.[309] Although aspirin is considered non-selective for both COX-1 and 2, research has indicated that it inhibits COX-1 more than COX-2.[309] The biological effect of aspirin can persist for a maximum of 10 days resulting in long-term aspirin therapy for cumulative effects.[310, 311] Other group of non-steroidal anti-inflammatory drugs (NASIDs) including ibuprofen, naproxen, ketoprofen, fenbufen, tiaprofenic acid, fenprofen have been shown to be non-specific COX inhibitor.[312, 313]

Selective COX-2 inhibitors whose therapeutic effects are as strong as conventional NSAIDs but with fewer side effects such as Celecoxib and Rofecoxib (Vioxx) have been developed since they have less adverse effects.[312, 313] However, within five years after its release to the public, Vioxx was withdrawn from the market owing to the adverse cardiovascular effects of the drug.[314] In addition, highly selective COX-2 inhibitory drugs coxibs (i.e. Etoricoxib, Valdecoxib, Parecoxib and Lumiracoxib) with increased biochemical COX-2 selectivity over that of Rofecoxib and Celecoxib have been developed.[312, 313] However, as selective COX-2 inhibitors do not inhibit TXA_2, incidence of bleeding is reduced. It has been hypothesized that these drugs cannot be used instead of aspirin.

1.5.3.5. Endothelin-1 (ET)

There are structurally three isoforms of ET that have been described, i.e., ET-1, ET-2, ET-3 and a vasoactive intestinal constrictor form.[315] Each isoforms is playing a specific physiological role in different tissue types.[315, 316]Amongst the three ET isopeptides, the 21-amino acid peptide ET-1 is regarded as the most prominent isoform in the cardiovascular system, accounting for the majority of the biological effects of ETs.[315, 317] Endothelial cells are the main producers of ET-1,[315] whose

synthesis depends on various stimuli including thrombin, angiotensin II, cytokines, hypoxia and shear stress.[318] ET-1 is synthesized as an inactive precursor molecule, preproET-1, which is then cleaved into "big" ET-1, and further catalysed to yield the bioactive ET-1 peptide.[318] Regulation of ET-1 production as well as its release is stimulated by inflammatory cells such as interleukins and TNF-α and decreased by NO and PGI_2 activity.[319, 320] There are 2 subtypes of ET-1 receptor, ET_A and ET_B; both are coupled to $G_{\alpha q/11}$ and the $G_{\alpha 12/13}$ families of GPCRs.[321] In the VSMCs, both ET_A and ET_B receptors are highly expressed, where as ET_B receptors are also expressed in the endothelial cells mediating relaxation.[322, 323]

1.5.3.6. Endothelium-Derived Hyperpolarizing Factor (EDHF)

EDHF is the 3rd endothelial mediated smooth muscle relaxation independent of NO and PGI_2 known to hyperpolarized smooth muscle. Potential EDHF differ by vascular bed, with similar function by increasing K^+ mediated depolarization of smooth muscle.[324] Physiological as well as chemical mediators that can activate the endothelium to release NO has been shown at the same time induces EDHF mediated smooth muscle relaxation.[324-326] However, this process was blocked upon endothelium removal, [324-326] but unaffected by NOS or COX inhibition. Despite the fact that reactive oxygen species (ROS) appear to function as EDHF, their physiologic role is still debatable because in large vessel, ROS inhibition did not inhibit EDHF mediated relaxations.[327, 328] It is suggested that EDHF is particularly important in the small resistant vessels, thereby influencing flow distribution and pressure.[329]

The epoxyeicosatrienoic acids (EETs), arachidonic-acid-derived products of cytochrome P450 have been revealed to play a role as EDHF in large arteries.[330] Furthermore, cytochrome P450 inhibitors completely blocked the EDHF-mediated

response in bovine,[331] porcine artery,[332, 333] Canine[334] and human coronary arteries.[335] This suggests that activation of a cytochrome P450 epoxygenase is a prerequisite for the generation of EDHF-mediated relaxation in large artery (Figure 8).

1.5.4. Endothelial dysfunction

Endothelial dysfunction is a pathological condition, characterized by a shift in the normal function of the endothelium toward reduced vasodilation, a proinflammatory and prothrombic states.[336] Endothelial dysfunction is associated as the initiating factor of conditions, such as hypertension, coronary artery disease, peripheral vascular disease, diabetes, and the progression of atherosclerotic lesions that potentially leads to clinical target organ damage. [337] Endothelial dysfunction occurs earlier in male than female [338] and is partly associated to aging, that adversely affect the availability of NO through decreased L-arginine and the cofactors required for NO synthesis which results in eNOS uncoupling that replaces NO synthesis for superoxide anion, [339-341], that further increases in the expression of pro-inflammatory mediators. [342]In addition, the inhibitory phosphorylation of Thr495 eNOS, increased oxidative stress and elevated levels of asymmetrical dimethylarginine (ADMA), an endogenous competitive inhibitor of eNOS, also leads to reduced NO production.[343-347]

Presently, various techniques have been developed to assess functional endothelial status both in preclinical and clinical settings. [348] In preclinical settings, endothelial function can be evaluated using classical myography technique involving measuring isometric tension with a force transducer of an isolated blood vessel in response to endothelial-dependent vasodilators to confirm functional endothelial integrity. In clinical settings, endothelial function can be evaluated using invasive and non-

invasive techniques by measuring the vasodilatory effect of the endothelium.[348] Invasively, endothelial function of the coronary microvasculature and epicardial arteries can be assessed through intracoronary agonist infusion combined with quantitative angiography.[349] This process initiate vasodilation in normal coronary arteries in the presence of intact endothelium and hence in the presence of endothelial dysfunction or in the absence of the endothelium, acetylcholine leads to vasoconstriction as a result of its direct effect on the underlying smooth muscle cells.[349] In a non-invasive method in the periphery, endothelial dysfunction can measured by flow mediated dilation (FMD) and peripheral artery tonometry in response to vasoactive agents or hyperaemia.[349, 350]

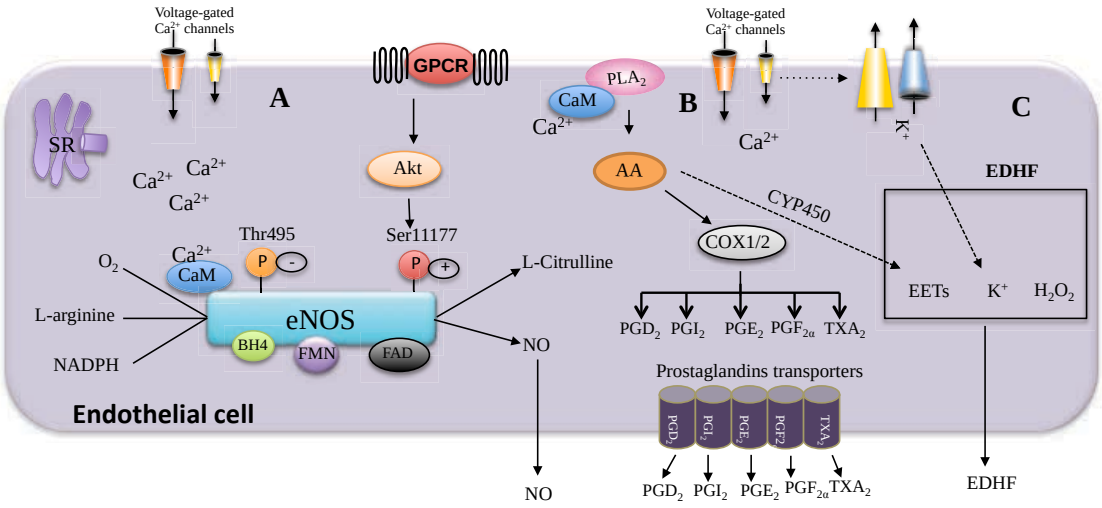


Figure 8: Schematic diagram illustrating signal transduction in vascular endothelial cell involved in nitric oxide, prostaglandin and endothelial hyperpolarizing factor production. (A) eNOS catalyses the conversion of L-arginine to NO and L-citrulline in the presence of oxygen. In addition, eNOS activity can also be increased through direct phosphorylation of serine residue (Ser1177) via the Akt. (B) The prostanoids are synthesized from membrane phospholipids by phospholipase A_2. AA is subjected to bisoxygenase and peroxidase activities of the cyclooxygenases (or prostaglandin

G/H synthases) to synthesize the individual classes of prostanoids that are transported by their respective transporters. (C) Depending on the species and vascular bed, the nature of the latter has been narrowed to include epoxyeicosatrienoic acids, K^+ ions and H_2O_2. The subsequent activation of a cytochrome P450 epoxygenase results in the generation of EETs that leads to relaxation in large vessel. AA: Arachidonic acid; NOS: Nitric oxide synthase; COX: Cyclooxygenase; CYP 450: Cytochrome P450.

1.5.5. Vascular smooth muscle cell (VSMC) signalling

VSMC are the main constitutive stromal cells of the vascular wall that function as the work-engine of the vessel. By contraction and relaxation, they alter the luminal diameter, which enables blood vessels to maintain an appropriate blood pressure.

1.5.5.1. Mechanism of Ca^{2+}-dependent smooth muscle contraction

Contraction of vascular smooth muscle is dependent on increase cytosolic Ca^{2+} that function as second messenger in activating contractile apparatus. Contractile agonist binds to a G protein coupled ligand gated channel receptor leading to phospholipase C activation. Activated phospholipase C cleaves membrane lipid phosphoinositide 4, 5- bisphosphate (PIP2), resulting in the generation of IP3 and DAG.[351] IP3 stimulates intracellular Ca^{2+} release from the sarcoplasmic reticulum and DAG causes protein kinase C (PKC) activation.[218] Additionally, different Ca^{2+} entry channels, such as voltage-operated (VOC), receptor-operated (ROC), and store-operated (SOC) Ca^{2+} channels, as well as Ca^{2+}-permeable non-selective cation channels (NSCC), are involved in the elevation of intracellular Ca^{2+} concentration.[352] [353-355] The increased in cytosolic free Ca^{2+} bind to calmodulin and induce a structural change in calmodulin, the Ca^{2+}-calmodulin complex activates myosin light chain kinase (MLCK) that further phosphorylates myosin light chain (MLC20) on serine 19,[354]

enabling cross-bridge formation between the myosin heads and the actin filaments and in the presence of ATP cross bridge cycling and contraction ensues (Figure 9).[356, 357]

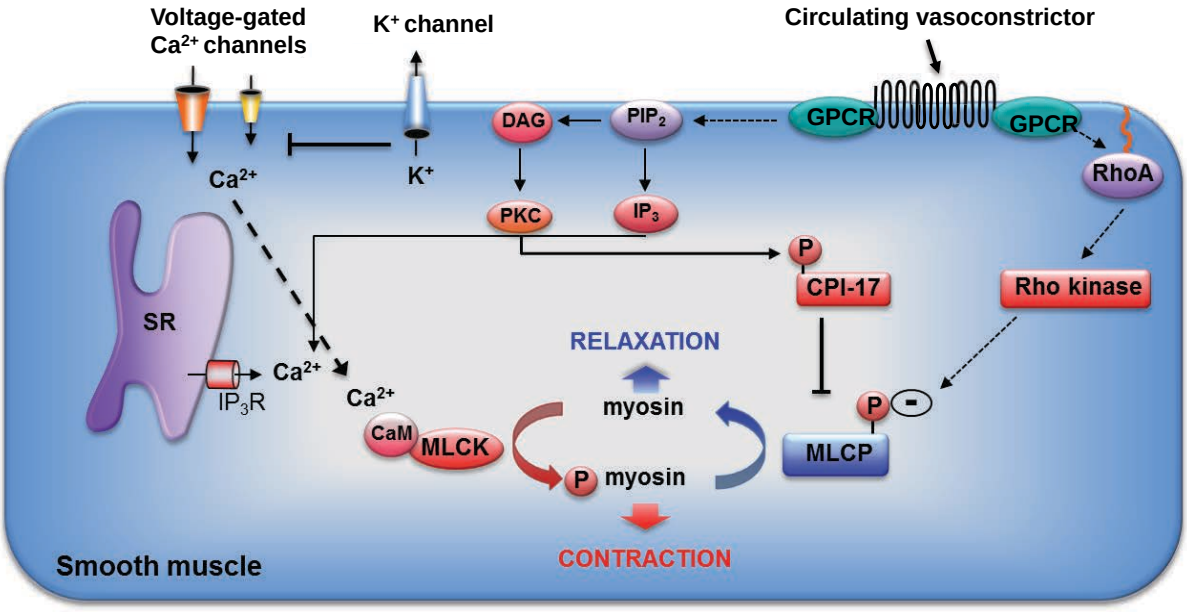


Figure 9: Schematic diagram illustrating Ca^{2+} dependent vascular smooth muscle contraction: Activation of $G_{\alpha q/11}$ coupled receptors leads to an increase cytosolic Ca^{2+} that activate myosin light chain kinase (MLCK), which in turn phosphorylates myosin to initiate the myosin-actin cross bridge cycling and VSMC contraction. This action is opposed by myosin light chain phosphatase (MLCP) dephosphorylating myosin. The function of MLCP is directly inhibited by protein kinase C (PKC) mediated CPI-17, and/or by the inhibitory phosphorylation via RhoA/Rho kinase (ROK) through activation of $G\alpha_{12/13}$ pathway. In addition, cytosolic Ca^{2+} are lowered by extrusion via plasma membrane Ca^{2+} ATPase (PMCA) and/or SR uptake through sarco- endoplasmic reticulum Ca^{2+} ATPase (SERCA) favouring relaxation. Modified from Wilson *et al.*, (2011).

1.5.5.2. Ca^{2+} sensitization

Ca^{2+} sensitization in smooth muscle is the production of an increase in contractile force for a given concentration of free intracellular Ca^{2+}.[358, 359] The process of Ca^{2+} sensitization is agonist specific. For example, contractile agonist-induced force is often higher than depolarization (high K^+)-induced force at similar, or even lower cytosolic Ca^{2+}.[360-362] The mechanism of Ca^{2+} sensitization involves inhibition of myosin phosphatase by RhoA/Rho kinase[363] and/or PKC-dependent phosphorylation of CPI-17 [351] supporting vascular contraction.

1.5.5.3. Ca^{2+} dynamics in smooth muscle

Ca^{2+} is an important trigger for vascular contraction. Therefore, it is important to understand the mechanism of Ca^{2+} regulation in vascular smooth muscle. For the purpose of this thesis, the regulation of increase and decrease cytosolic Ca^{2+} levels in smooth muscle will be discussed.

1.5.5.3.1. Mechanism of increase cytosolic Ca^{2+} in smooth muscle

The mechanisms responsible for increase cytosolic Ca^{2+} in smooth muscle can be grouped into two categories; (1) Ca^{2+} release from intracellular stores such as sarcoplasmic reticulum (SR) and (2) Ca^{2+} influx from extracellular space across the plasma membrane.

Ca^{2+} release from intracellular stores such as sarcoplasmic reticulum (SR): The SR is the largest internal pooled of Ca^{2+} in smooth muscle cell equipped with two major Ca^{2+} inducing channels; the IP3 and ryanodine receptors channels. The IP3 receptor channel is the major Ca^{2+} release channel that is activated by the IP3 second messenger generated from phospholipase C activation as discussed above. The other

Ca^{2+} release channel is the ryanodine receptor channel whose function depends on Ca^{2+} and ATP for their activation and inhibited by Mg^{2+} [364]

Ca^{2+} influx from extracellular space across the plasma membrane: Ca^{2+} channels on the plasma membrane are the major routes through which large amount of Ca^{2+} enters smooth muscle from extracellular space. This process is mediated by: (1) voltage-dependent Ca^{2+} channel and, (2) receptor- dependent Ca^{2+} channel.

Ca^{2+} influx via voltage-dependent Ca^{2+} channels (VDCC) is considered the major routes through which large amount of Ca^{2+} enters smooth muscle from extracellular space. The function of VDCC is dependent on membrane potential on the cell surface.[365] Depolarization of the membrane potential opens VDCC and Ca^{2+} enters the cell across the plasma membrane and they are closed by membrane hyperpolarization.[352]

Presently, several subtypes of VDCC have been identified with the two most clinically relevant are the L (High-voltage activated Ca^{2+} channels/long lasting activation), and provide the greatest Ca^{2+} entry in most agonist mediated vasoconstriction can be blocked by calcium channel blocker, [366-368] and the T (low-voltage-activated Ca^{2+} channels/transient activation).[352]

The high-voltage- L-type VDCC requires large signal conductance amplitude for their activation and they further inactivate slowly [369, 370]. Their activation is achieved by both Ca^{2+} and strong depolarization signal and thus considered to be bi-exponential.[369, 370] Low-voltage activated T-type VDCC requires small amplitude of signal for their activation, and they further inactivate rapidly, and thus consider mono-exponential.[369, 370]

Ca^{2+} influx via receptor operated Ca^{2+} channels (ROCC): ROCC are surface membrane receptors that are activated by PLC coupled GPCRs resulting in a sustained Ca^{2+} influx from the extracellular space across the membrane into the cytosol upon their activation.[352]

1.5.5.3.2. Mechanism of decrease cytosolic Ca^{2+} in smooth muscle

There are several mechanisms employed to reduce cytosolic free Ca^{2+} in smooth muscle cell. For the purpose of this thesis, the following will be discussed: (1) the plasma membrane Ca^{2+}- ATPase (PMCA), (2) the SR Ca^{2+}-ATPase (SERCA), and (3) through activation of Ca^{2+} activated K^+ channels.

Plasma membrane Ca^{2+} ATPase (PMCA): The PMCA is a Ca^{2+} pump with high Ca^{2+} affinity and low transport capacity which couples ATP hydrolysis to the efflux of Ca^{2+} from the cytosol across the plasma membrane. [371-373] They are activated by calmodulin and protein kinases and are almost inactive at very low Ca^{2+} concentrations.[374]

Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA): The SERCA is the only active Ca^{2+} transporter in the SR that regulates Ca^{2+} reuptake in the SR ATP. It function by transporting one Ca^{2+} into the lumen of the SR while extruding one molecule of K^+ and H^+ to the cytosol. [261, 372, 375] The function of SERCA is modulated by the endogenous molecules phospholamban (PLB).[376]

Ca^{2+}activated K^+ channels (K_{Ca}): K_{Ca} are a group of K^+ gated channels, activated in response to the influx Ca^{2+}via voltage-VDCC resulting to an increase efflux of K^+ from the cytosol to the extracellular space resulting in membrane hyperpolarization that subsequent deactivate VDCC. [377] They are classified into three subtypes including (1) large conductance Ca^{2+} activated K^+ channels (BK_{Ca}), (2) intermediate

conductance Ca^{2+} activated K^+ channels (IK_{Ca}) and (3) small conductance Ca^{2+} activated K^+ channels (SK_{Ca}).[378]

BK_{Ca} can be phosphorylated by PKA and PKG resulting in their activation and increasing its open probability.[379, 380] In addition, protein kinase C (PKC) phosphorylation is known to inhibit the BK_{Ca} in vascular smooth muscle. [379, 380] IK_{Ca} channels on the other hands are found on both smooth muscle and endothelial cells [381]. On the endothelium, IK_{Ca} alone are sufficient to mediate hyperpolarization and consequent relaxation in the presence of inhibitors of the NO pathway through increase endothelium Ca^{2+} influx resulting in NO production. [382] The SK_{Ca} channels are known to contain the Ca^{2+} calmodulin binding domain and respond increase in cytosolic Ca^{2+}. [377, 381]

1.5.5.4. Mechanism of smooth muscle relaxation

Relaxation of smooth muscle cell is dependent on two mechanisms: inhibition vascular smooth muscle Ca^{2+} entry and activation of the phosphatase as illustrated in figure 6. The inhibition vascular smooth muscle Ca^{2+} entry is achieved through increase in formation of cyclic guanosine monophosphate (cGMP), [357] that activates protein kinases, Ca^{2+} Mg^{2+}-ATPase pumps that extrudes cytosolic Ca^{2+}, Na+/Ca2+ exchangers and further inhibits ion channels that prevents the Ca^{2+} influx as discussed above.[354] The decrease in intracellular Ca^{2+} concentration leads to an inactivation of Ca^{2+}-calmodulin complex that no longer able to activate myosin light chain kinase (MLCK). [383]

Furthermore, Ca^{2+} depletion also increases the activity of myosin light chain phosphatase (MLCP) leading to de-phosphorylation of myosin light chain and RhoA promoting the phosphorylated state of MLC and the dissociation of the actin-myosin

cross-bridge and smooth muscle relaxation ensues. [356]

1.5.6. Circulating vasoconstrictors

Many circulating molecules can influence vascular tone and regulate blood flow. For the purpose of this thesis, only adrenergic system, and serotonin will be discussed.

1.5.6.1. The adrenergic system

The adrenergic system is the major regulator of vascular function through activation of it two major classes of receptors: alpha (α) and beta (β) adrenergic receptors. Various subtypes of these receptors have been identified that mediate the functional effects of catecholamine like epinephrine and norepinephrine Table 2[384]. The α_1 adrenergic receptor is coupled to $G_{\alpha q/11}$ and $G_{\alpha 12/13}$ whereas the α_2 is coupled to $G_{\alpha i}$.[385, 386] Both β_1 and β_2 adrenergic receptor subtypes can couple to $G_{\alpha s}$,[387] β_2 receptors are also able to couple to $G_{\alpha i}$.[387] Furthermore, β_3 is coupled to $G_{\alpha i}$ as well as to $G_{\alpha s}$.[388]

Phenylephrine (PE) is a synthetic α_1 adrenergic receptor agonist that is widely used in clinical settings. Researched have demonstrated that nanomolar concentrations of PE, which are devoid of any contractile effect can induce slight endothelium-dependent vasorelaxation.[389]

1.5.6.2. Serotonin (5-HT)

Serotonin or 5-hydroxytrptamine (5HT) is a well-known monoamine neurotransmitter with vascular and platelets effects.[390] The majority of 5-HT is generated by enterochromaffin cells in the intestinal mucosa, which is further incorporate and stored by circulating platelets.[391] Thus, most of the peripheral 5-HT is platelet associated and is released from activated platelets, increasing plasma 5-HT levels that

leads to vascular contraction, proliferation, migration or release of vasoactive mediators.[392]

Furthermore, there is recent evidence of local 5-HT synthesis (similar to local vascular Angiotensin-II synthesis) from peripheral arteries that possibly contributes to local and perhaps the circulating 5-HT.[393, 394] Increased levels of 5-HT have been implicated in several pathological conditions including pulmonary hypertension.[395, 396] 5-HT signalled through it serotonergic receptors that are grouped in seven classes (known as 5-HT_{1-7}) and various subtypes. [397] However, for the purpose of this thesis, only 5-HT_2 receptors will be discussed as the major receptor mediating serotonin-induced in vascular smooth muscle that has been implicated in coronary spasm[398-400] and the dominant receptor in platelets that promote the aggregation of platelets, resulting in release of more 5-HT.[398, 399] These receptors are coupled to the $G_{\alpha q/11}$ and $G_{\alpha 12/13}$ family of the G-protein (Table 1).[401, 402]

Table 1: Summary of contractile agonists, signalling receptors and their related second-messenger.

Contractile Agonist	Receptor subtype	Vascular location	GPCR subtype	Second Messenger
Endothelin-1	ET_A	VSMC	$G\alpha_{q/11}/G\alpha_{12/13}$	PKC/CPI-17, IP3, RhoA/ROK
	ET_B	VSMC/EC	$G\alpha_{q/11}$	PKC/CPI-17, IP3,
Thromboxane A2	TPα	VSMC	$G\alpha_{q/11}/G\alpha_{12/13}$	IP3, RhoA/ROK
	TPβ	VSMC	$G\alpha_{q/11}/G\alpha_{12/13}$	IP3, RhoA/ROK
Phenylephrine	α_1-AR	VSMC/EC	$G\alpha_{q/11}/G\alpha_{12/13}$	PKC/CPI-17, IP3, RhoA/ROK
Serotonin (5-HT)	5-HT_{2A}	VSMC/EC	$G\alpha_{q/11}/G\alpha_{12/13}$	PKC/CPI-17, IP3, RhoA/ROK
	5-HT_{2B}	VSMC/EC	$G\alpha_{q/11}/G\alpha_{12/13}$	PKC/CPI-17, IP3, RhoA/ROK
	5-HT_{2C}	VSMC/EC	$G\alpha_{q/11}/G\alpha_{12/13}$	PKC/CPI-17, IP3, RhoA/ROK

1.5.7. Ex-vivo vascular reactivity Studies

Vascular reactivity studies of isolated vascular segment has been used as a tool to study the physiological and pharmacological properties of isolated vessel in an ex-vivo set up.[403] Three common methods have been utilised in vascular reactivity studies which include, organ bath (ring or strip-mounted), wire (ring-mounted) myograph, and pressure myograph (pressurized vessel-mounted).[403]

1.5.7.1. Sex-differences in isolated vessel preparation

Sex differences in vascular reactivity of isolated vessel preparation to circulating catecholamines have been investigated in both humans and animal model of research. The result of each research is summarized in table 2 below.

Table 2: Summery of studies involving sex-differences in vascular reactivity of isolated vascular segments in both humans and animal models of research. NE: Noradrenalin, 5-HT: Serotonin, Ach: Acetylcholine, NE: Norepinephrine, KCL: High potassium solution, U46619: Thromboxane agonist.

Species	Vascular bed	Contractile/relaxation agonist	Result	Reference
Human	IMA	NA, Levosimendan	Male IMA vasodilate more to levosimendan than female in the presence of NA	Aka et al., 2007[404]
Human	IMA	NE, 5-HT, KCl	Male and female posses equal strength of contraction to KCL and NE, but female IMAs had greater strength of contraction to serotonin	Dignan et. al., 1992[405]
Human	SV	ET-1	Male have increase contraction to ET-1-than female	Ergul et al. 1998[406]
Streptozotocin (STZ)-induced type 1 diabetic mice	Aorta	ACh, NE	Diabetic Females have increase contraction to NE than diabetic male. In addition, ACh relaxations were impaired only in the diabetic female	Takenouchi et el., 2010[407]
Wistar rats	Aorta	ET-1, NE, 5-HT, Ach	Female are hypersensitive to NE and 5-HT, but not ET	Tostes et al, 2000[408]
Mice	Aorta	KCl U46619 and 5-HT	Male have increase contraction to 5-HT but not KCl and U46619	Nuno et al., 2007[409]

1.5.8. Previous research in our group

Previous work in our group have revealed female IMA hypersensitivity to phenylephrine and serotonin but not endothelin-1 and thromboxane A_2 stable mimetics U46619 when subjected to ex-vivo organ bath experiment (Figure 10).[410]

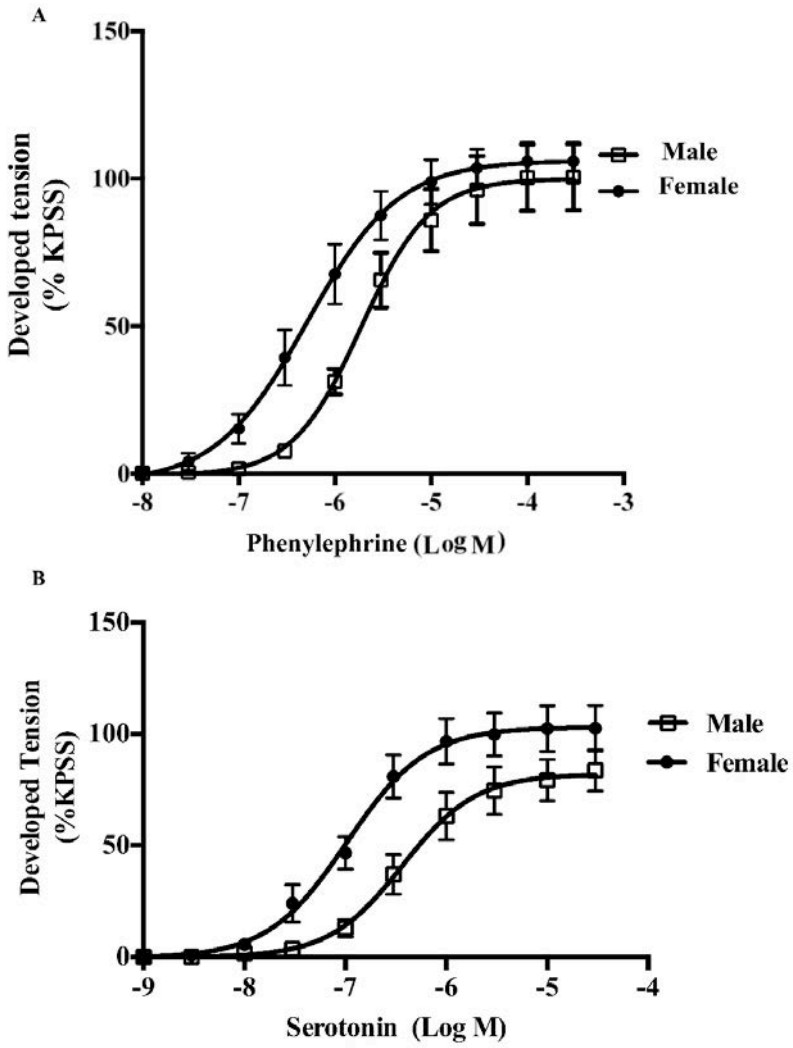


Figure 10. Sex-difference in vascular reactivity to phenylephrine and serotonin: (A) Phenylephrine: EC_{50} (Male=1.14±0.23 μM and female 0.52 ±0.15 μM, n=13-15 and $p<0.05$) and (B) Serotonin: EC_{50} (Male= 0.82 ± 0.32 μM and female 0.14 ±0.03 μM, n=7-10 and $p<0.05$). Adapted from Jaghoori's thesis 2014[410]

Furthermore, the sex-difference in vascular reactivity to PE was abolished when the production of the endothelial derived relaxing factors such as Nitric Oxide (NO) and prostaglandin (PGs) were blocked with Nitro-L-arginine methyl ester (L-NAME) and Indomethacin (INDO) respectively (Figure 11).

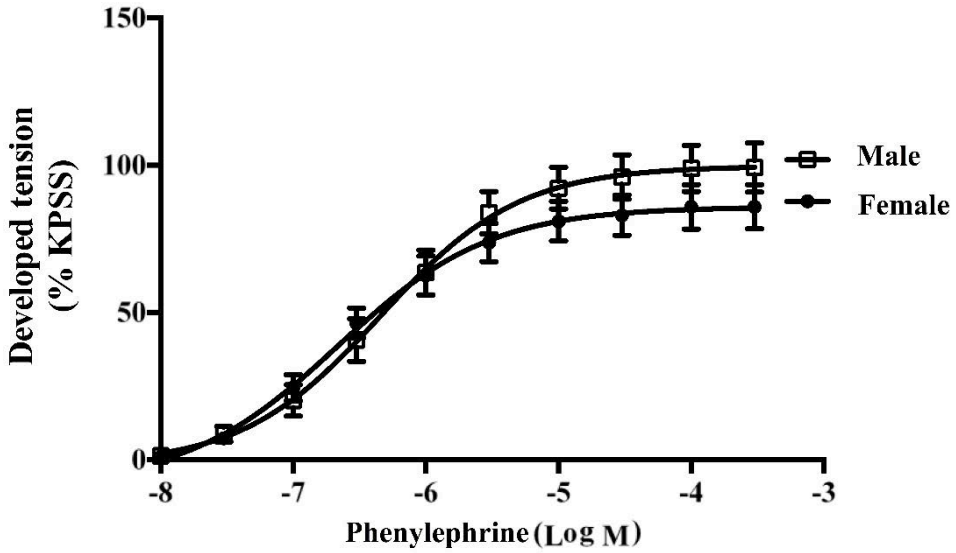


Figure 11. Sex-difference in vascular reactivity to PE in IMA was abolished when NO and PGs were attenuated by L-NAME and INDO. EC_{50} (Male = 1.25 ± 0.25 μM and female 0.51 ± 0.14 μM, n=10-15 and $p>0.05$). Adapted from Jaghoori's thesis 2014[410]

This suggests that the sex difference in reactivity of the vascular conduit used in grafting to constrictors to PE is endothelium dependent, and it might be a contributing factor to differential outcome following CABG.

Presently, *t*he molecular mechanism responsible for this endothelial dependent sex-difference in vascular response to PE and 5HT is unknown.

1.5.9. Thesis aims and objectives

This thesis expands on the above previous work, focusing on evaluating the mechanisms responsible for the sex-differences in female IMA hypersensitivity to phenylephrine and serotonin as illustrated in figure 12.

Thus, the aims of this thesis are outlined below.

A. To determine the role of endothelial integrity in mediating female IMA hypersensitivity to phenylephrine and serotonin, with the specific objectives including:
    1. To evaluate sex-difference in vasoconstrictor response of functional IMA segments with an intact/denuded endothelium to phenylephrine.
    2. To evaluate sex-difference in vasoconstrictor response of functional IMA segments with endothelium intact/denuded to serotonin.

B. To determine the role of nitric oxide synthase and nitric oxide in mediating female IMA hypersensitivity to serotonin, with the specific objectives including:
    1. To evaluate sex-difference in vasoconstrictor response of functional IMA segments with an intact endothelium in the presence of nitric oxide inhibition.
    2. To evaluate sex-differences in the capacity of functional IMA to generate NO in an ex-vivo preparation.

C. To determine the role of cyclooxygenase and vascular prostanoids in mediating female IMA hypersensitivity to serotonin, with the specific objectives including:
    1. To evaluate sex-difference in vasoconstrictor response of functional IMA segments with intact endothelium in the presence of cyclooxygenase inhibition.

2. To evaluate sex-differences in the capacity of functional IMA to express vascular prostanoids in an ex-vivo preparation, utilising measurement of prostanoids metabolites.

D. The role of α_1-adrenergic and 5-$HT_{2A+\ 2B}$ receptors activity state in mediating female IMA sensitivity to 5-HT and PE

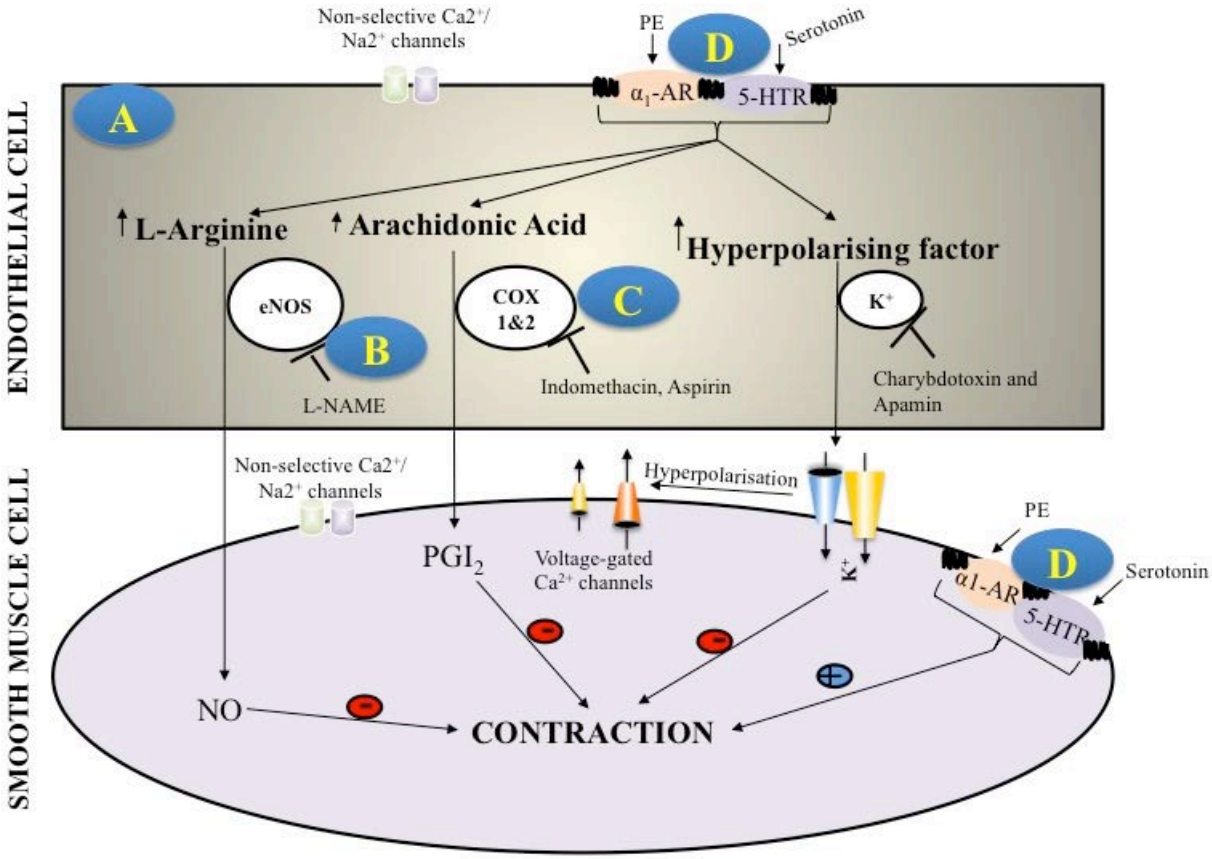


Figure 12: Schematic diagram illustrating the present aims of the thesis: (A) Vasculum endothelium, (B) NO pathway, (C) Prostanoids pathway and (D) phenylephrine and serotonin signalling receptors. ⊥ = Inhibition, - = opposes contraction and, + = support contraction.

2. CHAPTER 2

Thesis Methods

This chapter provides detailed descriptions of the methods employed in this thesis. All experiments were based upon isolated human internal mammary artery vessels. The methods used include (1) Clinical Procedures – in obtaining the human IMA segments, (2) Vascular Reactivity Studies – using an organ bath preparation, (3) Nitric Oxide Quantification – by electron paramagnetic resonance, (4) Prostaglandin Metabolite Quantification – via liquid chromatography/mass spectrometry, (5) Vascular Receptor Quantification – utilising Western blot methodology, and (6) Data Analysis Methods. Also included in this chapter (Section 2.7) is a published manuscript that details the development of endothelial denudation techniques for the IMA preparation.

2.1. PARTICIPATING MEMBERS

Victor Lamin BSc (Hons), MSc (PhD Candidate),[1,2] Amenah Jaghoori BSc (Hons), PhD(Colleague),[1,2] Rachel Jacobczak BSc (Hons) (Research Associate),[1] Irene Stafford BSc (Research Associate),[2] Tamila Heresztyn BSc, MSc (Research Associate), [2] Michael Worthington MBBS, FRCS (Cardiothoracic Surgeon),[3] James Edwards MBBS, FRCS (Cardiothoracic Surgeon),[3] Fabiano Viana MBBS, FRCS (Cardiothoracic Surgeon),[3] Robert Stuklis MBBS, FRCS (Cardiothoracic Surgeon),[3] David P. Wilson BSc, MSc, PhD (Secondary Supervisor),[1,2] John F. Beltrame BSc, BMBS, PhD, FRCP (Primary Supervisor)[1,2]

2.2. AFFILIATIONS

1. Discipline of Medicine, Adelaide Medical School, University of Adelaide, South Australia
2. Cardiology Research Unit, Basil Hetzel Institute for Translational Health Research, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville SA, 5011, South Australia
3. D'Arcy Sutherland Cardiothoracic Surgical Unit, Royal Adelaide Hospital North Terrace , SA 5000 , South Australia

2.3. CLINICAL PROCEDURES

2.3.1. Patient Recruitment

The study was approved by the Royal Adelaide Hospital Human Ethics Committee on 28th April 2009, with approval number RAH PROTOCOL NO: 090411, Title: The effect of selective/non-selective endothelin blockage on statin inhibition of endothelin vasoconstriction responses.

Patients scheduled for elective CABG between 2013 and 2016 were consented pre-operatively for the use of their discarded left IMA tissue.

2.3.1.1. Inclusion Criteria

All patients undergoing elective CABG surgical procedures at the Royal Adelaide Hospital were approached either a day before the surgery (7 pm) or in the morning before surgical preparation (6 am) to be recruited for participation in the study. Only patients consented for the use of their discarded vessel were recruited.

2.3.1.2. Exclusion Criteria

Although the study was not a patient interventional study, 12% of the patient declined consent to out study. This was due to either language barrier (with no interpreter available) or as a result, the patient stressed about the surgical procedure especially those seen as day-of-surgery admission patients (DOSA).

Furthermore, patients were excluded if their IMA have been treated with papaverine hydrochloride to induce dilation, preventing arterial spasm following the graft anastomosis, which might influence vascular response. The patient recruitment process is summarized below in the flow diagram in figure 13.

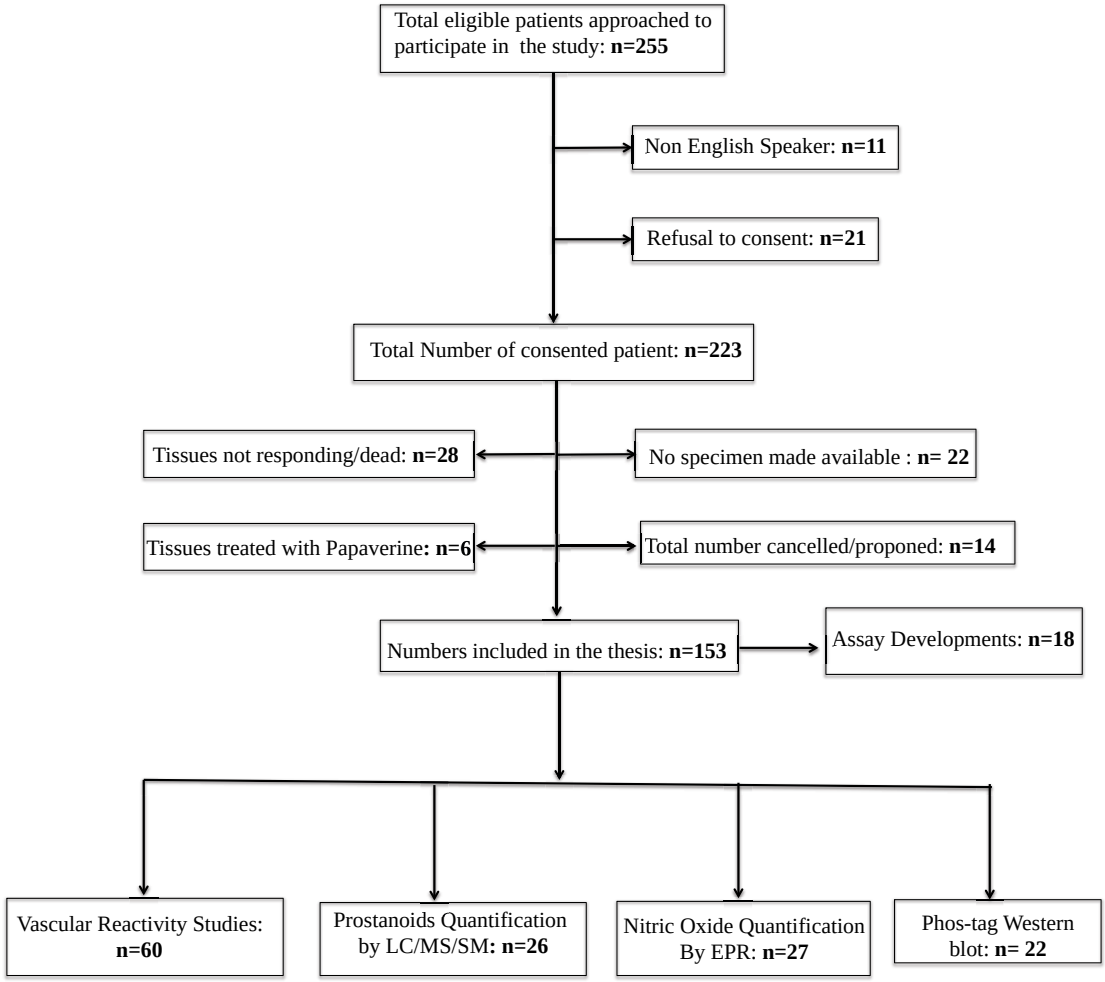


Figure 13: Flow diagram presenting the process of patient recruitment and sample

size in each study group.

2.3.2. Surgical Procedure

On the day of surgery, the patient's clinical history and medications were recorded for each patient including: (a) baseline demographics, specifically age, body mass index (BMI) and blood pressure, (b) pre- existing conditions and co-morbidities, including hypertension, hypercholesterolemia, diabetes and tobacco use and (c) pre-operative mediations administered 24 hours prior to surgery. The clinical profile of the recruited patients for the individual research studies, are listed in the respective baseline characteristics tables in chapters 3 and 4.

The four participating surgeons followed the standard coronary artery bypass graft procedure. Anaesthesia was undertaken using standard cardiac protocols including: fentanyl pre-medication, thiopentone induction, and isofluorane maintenance anaesthesia and muscle relaxation with rocuronium. Surgery was undertaken via a median sternotomy approach with the left IMA harvested as a pedicle graft and the excess vessel length trimmed. The remnant IMA (generally discarded) was available for the experimental studies. Patients were grouped based on biological sex.

2.4. VASCULAR REACTIVITY STUDIES

In this thesis, the potential mechanisms responsible for the observed sex-differences in vascular response to serotonin and phenylephrine of the human IMA graft conduit segments was evaluated in relation to the (i) endothelium, (ii) nitric oxide (NO) pathway, (iii) prostaglandin (PG) pathway, and (iv) vascular receptors.

2.4.1. Study drugs and chemicals

Serotonin, U46619 (stable analogue of thromboxane A_2), A23187 (calcium ionophore), indomethacin, N-w-Nitro-L-arginine Methyl Ester (L-NAME), $FeSO_4$, diethyldithiocarbamate (DETC), Phenylephrine (PE) and sodium nitroprusside (SNP) were all obtained from Sigma Chemical Company, St. Louis, MO. Endothelin-1 was purchased from Auspep, Australia. Other chemicals utilised and their source included: SDS, 1× Complete™ protease inhibitor cocktail from Roche, Mannheim, Germany; di-isopropylfluorophosphate from Sigma-Aldrich, Caste Hill, Australia; and Phos-tag™ Acrylamide from Wako Laboratory Chemicals, Japan. Prostanoids (5PGmix- containing: PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ and TXB_2), deuterated prostanoids ($PGF_{2\alpha}$ -d4) and DEA nonate were obtained from Cayman Chemical Co. (Ann Arbor, MI). All organic solvents for the mass spectrometry analysis were of liquid chromatography mass spectroscopy (LCMS) grade.

Serotonin was prepared by dissolving the compound in 0.1M HCl and further diluted in double distilled water to the required concentration. PE, ET-1 and L-NAME were prepared in double distilled water, with U46619, indomethacin and A23187 prepared in 100% ethanol. The Krebs-bicarbonate solution contained (in mmol/L): NaCl 118, KH_2PO_4 1.18, KCl 3.89, $NaHCO_3$ 25, $MgCl_2$ 1.05, $CaCl_2$ 2.34, EDTA 0.01 and glucose 5.56, while the high K^+ (KPSS) solution replaced NaCl in normal Krebs with an equimolar amount of KCl (yielding a final KCl [121.89] mmol/L) both solutions were aerated with carbogen gas (95% O_2 and 5% CO_2 to maintain pH 7.4. All drugs were diluted to the required final concentration in physiological Krebs-bicarbonate solution previously aerated with carbogen gas (95% O_2 and 5% CO_2). The concentration of each drug was expressed as final concentration in μM.

2.4.2. Vascular Segment Preparation and contractile agonist dose response

IMA segments obtained during CABG were immediately placed in ice-cold Krebs-bicarbonate solution. Each artery segment was dissected free from perivascular fat and external loose tissues and cut into 3mm wide rings.

Vascular reactivity was assessed using an organ bath preparation with the isolated IMA rings mounted on stainless steel hooks and the artery tension measured with a force transducer (from AD Instruments – Australia). The organ bath preparation utilised a 15ml water-jacketed organ bath containing carbogen-bubbled Krebs solution. Isometric tension was recorded using Lab chart 6 software (from AD Instruments – Australia) with the system being calibrated daily. The experimental protocol is summarized in figure 12. Arteries were equilibrated at a resting tension of 19.6 mN for 60 minutes at 37°C before undergoing KPSS- dependent contraction three times to allowed us to assess several physical factors, including tissue handling during the surgical process, dissection and mounting in the laboratory, could influence vascular integrity.

Upon reaching a plateau, the high K^+ buffer was washed-out 3 times and the segments allowed equilibrating for 30 minutes. This step was repeated three times and arteries were subjected to cumulative doses of either phenylephrine (PE, 0.01 - 300µM), or serotonin (5HT, 0.001 - 300µM). Following the maximum agonist-dependent response, arteries–were placed in normal Krebs for 60 minutes and subsequently contracted to an EC_{70} dose of the agonist (a concentration that produced 70% maximum constriction to that agonist). As previously described the calcium ionophore (A23187) was used at a concentration of 2 µM, to activate endothelial Ca^{2+} entry and verify endothelium-dependent vasodilatation as illustrated in figure 14.[411] All arteries that produced a force less than 19.6 mN when

submaximally contracted with KPSS solution are considered damage during isolation and excluded from the study.

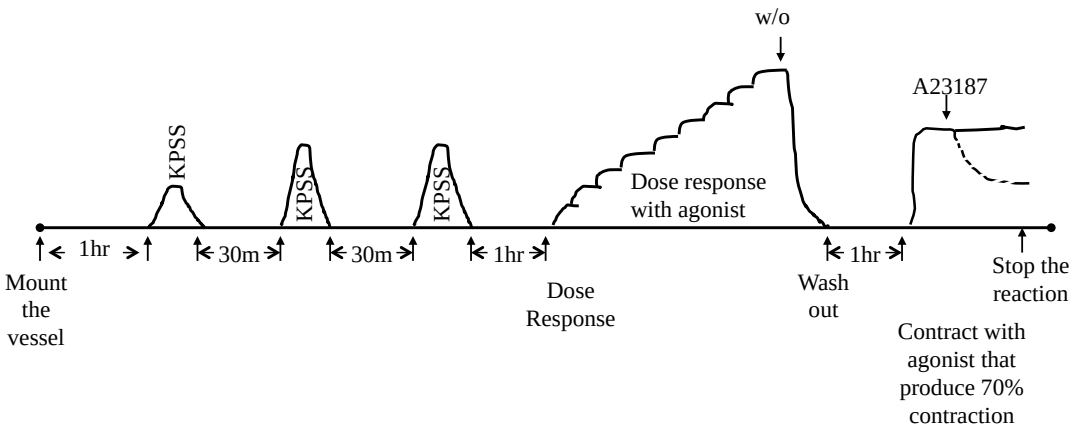


Figure 14: Experimental protocol for vascular reactivity study. KPSS=High K^+ solution, W/O= wash out, A23187: Ca^{2+} ionophore, hr.: hour, m=minute

2.4.3. Mechanistic Studies

Vasoconstrictor agonists producing a sex-difference in vascular responses underwent investigation to elucidate the underlying vascular mechanism including the role of the endothelium, NO pathway and PG pathway.

(i). The Endothelium. The role of the endothelium in mediating sex-differences in vascular response to phenylephrine and serotonin was evaluated by endothelium denudation. An endothelium denudation technique was established by mechanical luminal shear abrasion for the human IMA. Please find attached the manuscript for the endothelial denudation method. [412] (Please find manuscript below).

(ii) Nitric Oxide (NO) Pathway. The role of NO on sex-differences in vascular responses to phenylephrine and serotonin stimulation was investigated in in chapter 3 and 4 by NOS inhibition using L-NAME (300 μM). Following the 3rd KPSS stimulation and the 30-minute equilibration, the vessels were incubated with nitric

oxide synthase blocker L- NAME (300µM) for 60-minutes followed by cumulative dose response with either phenylephrine or serotonin as described above.

(iii) Prostaglandin (PG) Pathway . The role of the PG pathway on sex-differences in vascular responses to phenylephrine and serotonin stimulation was investigated in in chapter 3 and 4 by cyclooxygenase inhibition (10 µM, indomethacin)**.**

Following the 3rd KPSS stimulation and the 30-minute equilibration, the vessels were incubated with the non-selective cyclooxygenase inhibitor (10 µM, indomethacin) for 60-minutes followed by cumulative dose response with either phenylephrine or serotonin as described above.

2.5. NITRIC OXIDE QUANTIFICATION

Electron paramagnetic resonance (EPR) - spin trapped based quantification of stimulated nitric oxide release in endothelium-intact IMA segments. Specifically, NO was spin-trapped from IMA segments in response to the addition of SNP, pravastatin or the calcium ionophore A23187 (10µM). The spin trap included Fe (II) dithiocarbamate complexes containing diethyldithiocarbamate (Fe $(DETC)_2$) prepared by dissolving 0.45mg/ml $FeSO_4$ or 0.72 mg/ml DETC in 0.9% NaCl bi-distilled water (EPR-grade) and aerated with nitrogen gas > 20 min on ice. Three IMA segments (each 3mm in length) were pooled placed in Krebs/HEPES buffer prior to the addition of stimulus or inhibitors and incubated with the spin trap (equal volume of FeSO4 and DETC) complex for 120 min at 37 ° C. Signals were measured using ESR-Spectrometer e-scan equipped with Temperature & Gas Controller, and Shear Stress Controller- Noxygen Science Transfer & Diagnostics GmbH Bruker Bio Spin Corp. A triplet EPR spectrum of $(DETC)_2$-Fe (II)-NO with a (N) = 12.8 gauss and g = 2.04 was observed, a characteristic signal for NO in the above spectrum.[413] The (DETC)(2)-Fe (II)-NO signal in the IMA segment was markedly suppressed by

endothelium denudation and pre-administration of the nitric oxide synthase (NOS) inhibitor, (L-NAME) in rat aorta, confirming that the NO detected from the artery was produced enzymatically by NOS. Calibration solutions of DEA nonoate were prepared at final concentrations 2 µM, 4 µM, 8 µM and 16 µM and the signal were measured as NO concentrations/double integral.

2.6. PROSTAGLANDIN METABOLITE QUANTIFICATION

Sex- differences in prostaglandin metabolite levels of 6-keto $PGF1_{1\alpha}$, TXB_2, $PGF_{2\alpha}$, PGD_2 and PGE_2 were quantified in the IMA segment by liquid chromatography/electrospray ionization tandem mass spectrometry. This was undertaken in Chapter 4. During assay development, we realize that the minimum amount of tissue prostaglandin detectable by our LC/MSMS is 200mg and stimulating human IMA with serotonin and phenylephrine liberate prostaglandins metabolites below our detection limit. A23187 was therefore used to augment PG production

Following activation of calcium entry using the calcium ionophore A23187 (2µM) (n=10-12), as previously described by Furugen *et al.*, (2011).[414] PGs were isolated from 96-350mg IMA segments ground under LN_2 and solubilize in 2 ml PG homogenizing buffer containing (1X PBS, 1 mM EDTA, 10 μM indomethacin, pH 7.4) in a pre-coated tube with 2mg butylated hydroxytoluene (BHT). Samples were further sonicated for 30 seconds and followed by the addition of 200 $ngml^{-1}$ of internal standard $PGF_{2\alpha}$ -d4. Following the addition of 1ml acetone and a 2 minute vortex samples were allowed to stand on ice for 20 minutes and centrifuged at 3000 × g for 15 min at 4 ◦C. The supernatant was transferred into fresh tube and the acetone layer evaporated nitrogen gas. PH was adjusted to 3 before solid-phase extraction.

HLB column was used in solid-phase extraction conditioned with 3ml methanol and 2% formic acid. Samples were washed with 3ml solution containing 5% methanol, 2% formic acid, followed by 3ml 25% methanol and 3ml hexane before eluting with 2 ml LCMS grade methanol and dried under nitrogen gas. Samples were re-constituted in 100µl 25% acetonitrile. Calibration curves of for the prostaglandins were linear in the range from 0.2 to 50 $ngml^{-1}$ ($r2 > 0.99$). Calibration curves were constructed by plotting peak area ratio (exogenous standard to internal standard) versus the nominal concentration and were fitted using least-squares regression.

2.7. VASCULAR RECEPTOR QUANTIFICATION

To evaluate vascular receptor density as a mechanism underlying observed sex-differences in vascular responses, isolated blood vessels were stored and receptors quantified using Western blot. This was undertaken in Chapter 3 and 4. Vascular receptors were quantified in relation to both total abundance and phosphorylation status. Mn^{2+} Phos-tag™ phosphorylation-dependent mobility shift analysis was used to quantify total and phosphorylated protein expression of $serotonin_{2A}$ and $serotonin_{2B}$ receptor subtypes.

Proteins were extracted from each 3 mm IMA rings using 2x Laemmli sample buffer (containing 65.8mM Tris-HCl, 26.3% (w/v) glycerol, 2.1% SDS and 0.01% bromophenol blue), followed by SDS PAGE, coomassie stained, and the entire lane of protein for each sample quantified as an index of extractable vascular proteins and adjusted the concentration to be equal for all arteries. Following normalization of protein levels, samples were subject to a Phos-tag™ SDS-PAGE containing 7.5% polyacrylamide gel with 25 µM Phos-tag™ and 50 µM $MnCl_2$ at 150 V for 90 min, as described previously by Tomida *et al.*, (2008). [415] Proteins were transferred, using

the BioRad Transblot® Turbo system™, onto 0.22 µm PVDF at 25 V and 1.3 amps for 7 min. Non-specific binding sites were blocked with 3% non-fat dry milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% (*v*/*v*) Tween-20) for 60 minutes, followed by incubation with TBS-T containing a 1:1000 dilution of either a mouse-derived affinity-purified anti-serotonin$_{2A}$ or rabbit-derived serotonin$_{2B}$ antibody (Santa Cruz Biotechnology) for 60 minutes. PVDF membranes were washed four times in TBS-T and incubated for 60 minutes in TBS-T containing a1: 10000 dilution of anti-mouse (for the 2A receptor) or anti rabbit (for the 2B receptor) IgGs coupled with Dylight™ 800 fluorochromes (Pierce Thermo Scientific, Rockford, IL, USA)

2.8. DATA ANALYSIS

Sample size calculation for the vascular reactivity study was performed based on previously published studies utilising human isolated blood vessels using PE concentration-response curve in a predominantly male patient cohort to be used as the standard representative for all agonists in both sexes.[410] Therefore, to identify a 20% difference in the effective concentration that elicited 50% of the maximal response (EC_{50} expressed as log EC_{50}) values of 6.2 ± 0.529[416] between males and female with 90% power, a sample size of 4 patients was required in each group. Sample size calculation was performed on STATA version 11.

All data are expressed as mean ± SEM. In all experiments, n refers to the number of patient from which IMA were taken. Contractile responses were expressed in milli-Newton (mN) normalized with the corresponding KPSS response for that artery, to correct for any small differences in vessel length and diameter. Relaxation responses were expressed as the percentage reduction in the agonist-induced contraction by the calcium ionophore A23187 (2 µM). Maximal responses (E_{max}) and log EC_{50} obtained

from concentration–response curves by non-linear curve fitting using Graph pad Prism 6 (San Diego, CA). Statistical analysis has been made using multiple regression and student t-test. Statistically significant differences are considered at $p < 0.05$.

Statement of Authorship

TITLE of Paper	Mechanisms responsible for serotonin vascular reactivity sex-differences in the internal mammary artery
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted w ork w ritten in manuscr pt style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Victor Lamin
Contribution to the Paper	Victor Lamin contributed to the following experiments: (1) Sample collection, (2) Contractile response to serotonin with endothelium (n=10-12), without endothelium (n=10-11), (3) the mechanism of serotonin response, L-NAME (n=7-10) and indomethacin (n=7-10), (4) Nitric oxide quantification using EPR (n=8), (5) quantification of prostanoids using LC-MS/MS (n=10-12), (6) Phos-tag™ western blot analysis and (7) writing the manuscript
Overall percentage (%)	90.1%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 17/10/2016

Co-Author Contributions

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mr Michael Worthington		
Contribution to the Paper	Mr. Michael Worthington provided isolated vascular segments and reviewing the manuscript		
Signature		Date	4.11.16

Name of Co-Author	Mr Fabiano Viana		
Contribution to the Paper	Mr. Fabiano Viana provided isolated vascular segments and reviewing the manuscript		
Signature		Date	9.11.16

Name of Co-Author	Mr James Edwards		
Contribution to the Paper	Mr. James Edwards provided isolated vascular segments and reviewing the manuscript		
Signature		Date	3.11.16

Name of Co-Author	Mr Robert Stuklis		
Contribution to the Paper	Mr. Robert Stuklis provided isolated vascular segments and reviewing the manuscript		
Signature		Date	3.11.16

Name of Co-Author	Dr. David P. Wilson		
Contribution to the Paper	David Wilson participated in experimental design, data analysis and critical revisions of the manuscript.		
Signature		Date	28/11/2016

Name of Co-Author	Professor John F. Beltrame		
Contribution to the Paper	Professor John F. Beltrame participated in experimental design, data analysis and critical revisions of the manuscript.		
Signature		Date	17/10/16

Please cut and paste additional co-author panels here as required.

2.9. ABSTRACT

BACKGROUND: Endothelial denudation is an important approach to evaluate the role of the endothelium in vascular reactivity studies. Although approaches to remove the endothelium are well established in animal models, these methods have proved difficult to effectively translate to remnants of human internal mammary artery (IMA) obtained during coronary bypass. This study sought to identify the optimal technique for endothelial denudation of IMA while preserving vascular contractile responses.

METHODS: IMA segments were subject to endothelial denudation using one of the following techniques: (1) surface abrasion, rubbing with a stainless steel wire, (2) vasoconstriction abrasion or (3) shear abrasion via infusion of an effervescent solution. Following intervention, IMA segments were evaluated by: (1) histochemistry to quantify structural damage and endothelial cell abundance and (2) functional endothelium-dependent vasodilator response using vascular myography in an organ bath preparation.

RESULTS: Vasoconstriction abrasion removed endothelial cells and caused disruption of the internal elastic lamina, these vessels failed to respond to the vasoconstrictor phenylephrine (PE) or the endothelium-dependent vasodilator A23187. Surface abrasion alone was incomplete in removing endothelial cells, vessel vasodilated partially when challenged with A23187 in the presence of PE. Shear abrasion removed endothelial cells most effectively, as these pre-constricted vessels did not relax to A23187 but demonstrated increased sensitivity to PE.

CONCLUSIONS: In this controlled comparative study assessing both structural and functional endpoints of endothelial denudation techniques, we have demonstrated that shear abrasion by infusion of an effervescent solution is the optimal technique to remove the endothelium and preserve vascular function in human IMA.

KEY WORDS: Internal mammary artery, endothelium denudation and vascular reactivity.

2.10. INTRODUCTION

Advances in research in the last 3 decades have led to a revolution in our understanding of the complexity of the vascular endothelium. Previously perceived as an inert cellular lining, it is now appreciated that it is a complex endocrine organ regulating vascular function via the release of vasoactive autocoids as well as influencing platelet activity [249, 417]. Vascular endothelial denudation leads to loss of endothelial function and a subsequent increase vascular sensitivity to vasoactive autocoids [418, 419]. This has been a fundamental method to assess the role of the endothelium of an isolated vessel preparation in vascular reactivity studies. Previous vascular reactivity studies have denuded the vascular endothelium of isolated vessel via mechanical and chemical approaches. Chemical endothelial denudation through treatment with detergent [420, 421] is considered unsafe and has been found to compromise the viability of the smooth muscle cell [422].

Mechanical endothelial denudation has been undertaken in both animal and human isolated vessel via (1) the gentle rubbing of the luminal surface of the vessel with wooden stick [423], syringe needle [424], stainless steel wire [425, 426] forcept [427] blunt seeker [428] and balloon catheter [429, 430] (2) intense vascular constriction upon a intravascular rod [430] and (3) the passing of air bubbles through the lumen of the vessel [431]. Similar studies utilizing the human internal mammary artery (IMA) have been problematic and met with variable success in achieving consistent endothelial denudation, since the vessel is not amenable to the above established denudation techniques.

The objective of this study is to determine the most effective endothelial denudation technique for IMA in isolated vessel preparations, as assessed by vascular reactivity studies.

2.11. METHODS

Patients scheduled for elective coronary artery bypass grafting (CABG), were consented pre-operatively for the use of their discarded IMA vessel.

The study was approved by the Royal Adelaide Hospital Human Ethics Committee on 28th April 2009, with approval number RAH PROTOCOL NO: 090411, Title: The effect of selective/non-selective endothelin blockage on statin inhibition of endothelin vasoconstriction responses. Standard cardiac surgical procedures were undertaken utilizing a combination of opioid (fentanyl), inhalation (isoflurane) and muscle relaxant (rocuronium) in anaesthesia, followed by a primary median sternotomy approach.

The left IMA was harvested as a pedicle graft with the excess vessel length trimmed. The remnant IMA segment from the region before the bifurcation was available for the experimental studies before the treatment with papaverine.

2.11.1. Vessel Isolation

The IMA segments were immediately placed into ice-cold Krebs-bicarbonate solution containing in mmol/L: NaCl 118, KH_2PO_4 1.18, KCl 3.89, $NaHCO_3$ 25, $MgCl_2$ 1.05, $CaCl_2$ 2.34, EDTA 0.01 and glucose 5.56 at pH 7.4, previously aerated with carbogen gas (95% O_2 and 5% CO_2). Each vascular segment was dissected free of connective tissue and cut into 3mm wide segments.

2.11.1.1. Vessel Isolation and Denudation

The IMA segments were assigned to either a control group (no endothelial denudation) or one of the following experimental luminal mechanical endothelial denudation approaches

2.11.1.1.1. Surface abrasion method

This traditional approach involves mechanical removal of the endothelium by gentle rubbing of the intimal surface with stainless-steel wire as described previously by Wackenfors [425] and Aleksandar [426].

2.11.1.1.2. Constrictor abrasion method

Constrictor abrasion method. In this technique, a stainless steel rod is inserted through the lumen of the vascular segment, ensuring that the rod occupies ≥ 90% of the vessel lumen. The vessel (with the rod in-situ), is then immersed in high potassium (KPSS) solution containing in mmol/L: KH_2PO_4 1.18, KCl 16, $NaHCO_3$ 25, $MgCl_2$ 1.05, $CaCl_2$ 2.34, EDTA 0.01 and glucose 5.56 at pH 7.4) previously aerated with Carbogen gas (95% O_2 and 5% CO_2) to induce vascular constriction upon the stainless steel rod. The rod was then carefully moved back and forth three times within the vessel lumen thereby removing the endothelium. This is a hybrid approach to similar methods that utilise an inflated balloon catheter rather than the stainless steel rod as described previously by Lu [430].

2.11.1.1.3. Shear abrasion method

Shear abrasion method. This technique involves high velocity intraluminal infusion of an effervescent solution consisting of air and physiological Krebs solution through a catheter, moving the catheter tip within the lumen for 90 seconds as described previously by Guyton [431] with some modifications.

2.11.2. Assessment of Endothelial Integrity

Control and experimental isolated IMA preparations were fixed in 10% buffered neutral formalin, embedded in paraffin before being sectioned transversely (5 μm

sections), and mounted on glass slides. Sections were immuno-stained for CD 34 and Hematoxylin and eosin (H&E) for histological examination. The total number of CD 34 positive cells per view was estimated as the average number of cells in 20 views at different location. Images were captured using a Nikon Eclipse 90i microscope and recorded on a Nikon cool snap HQ camera.

2.11.2.1. Structural Assessment

Control and experimental isolated IMA preparations were fixed in 10% buffered neutral formalin, embedded in paraffin before being sectioned transversely (5 μm sections), and mounted on glass slides. Sections were immuno-stained for CD 34 and Haematoxylin and eosin (H&E) for histological examination. The total number of CD 34 positive cells per view was estimated as the average number of cells in 20 views at different location. Images were captured using a Nikon Eclipse 90i microscope and recorded on a Nikon cool snap HQ camera.

2.11.2.2. Functional Assessment

Vascular reactivity was assessed using vascular myography in an organ bath preparation with the isolated IMA segments mounted on stainless steel hooks and vessel tension measured with a force transducer. The organ bath preparation involved a 15 ml jacketed organ bath containing Krebs solution bubbled with Carbogen. Isometric tension was recorded using Lab chat 6 software with the system being calibrated each day before the start of any experiment. The vessel segments were allowed to stabilize at a resting tension of 19.6 mN for 1 hour at 37 °C before the experiments commenced. The vessels were submaximally contracted with KPSS solution three times, to confirm a functional vessel and optimize the vascular contractile apparatus function. Following a further 30-minute equilibration period, a cumulative dose response curve to the phenylephrine agonist (PE, 0.01 μM - 300 μM)

was generated and the dose producing a half maximal response determined. After the last response, vessel was allowed to equilibrate for 1 hour and pre-contracted to a dose of the agonist that produces 70% constriction. Upon reaching a plateau constrictor response, the endothelium-dependent vasodilator A23187 (a calcium ionophore, 2 μM), was added to confirm endothelial integrity

2.11.3. Data analysis

Vascular constriction was expressed as change in weight per mili-Newton (mN) normalized with the KPSS response and relaxation responses as the percentage reduction in the agonist-induced constriction. Dose-response curve to PE constriction was obtained. Data are expressed as mean ± SEM. Statistical analysis of paired data has been made using student t-test was used to compare two and analysis of variance (ANOVA) more than two groups. Differences are considered significant at $p < 0.05$.

2.12. RESULTS

IMA segments were obtained from 15 patients (60% male) with mean age: 68.0 ± 13.25) undergoing CABG. Patient characteristics are summarized in Table 3. A total of 48 segments were studied with similar luminal diameters between the study groups. A total of 12 experimental conditions were done per intervention.

2.12.1. Structural Assessment

Histological examination of the control group revealed a continuous lining of endothelial cells, separated from the smooth muscle layer by an intact continuous internal elastic lamina (Figure 15). An intact internal elastic lamina was also evident in all the segments subjected to the mechanical shear abrasion method, suggesting intact sub-intimal layers despite denudation of the endothelial lining (Figure 15).

IMA segments subjected to the mechanical surface abrasion method demonstrated variable amounts of distortion in the elastic lamina, which occasionally extended into the smooth muscle layer (Figure 15). In the mechanical constrictor abrasion method, there was substantial disruption in the elastic lamina, which extended into the smooth muscle layer. Endothelial denudation by shear abrasion demonstrated very few endothelial cells remaining within the luminal lining and minimal damages to the vascular smooth muscle cell layer. This was confirmed by quantification of the average number of CD 34 positive cells per view (intact IMA= 13.15 ± 6.3, surface abrasion IMA = 3.7 ± 2.8 and shear abrasion IMA = 1.6 ± 1.4, Figure 16). This suggest that endothelial denudation by the surface abrasion method was not as effective as the shear abrasion method.

2.12.2. Functional Assessment

IMA denudation using surface and shear abrasion method have equivalent contraction responses to the potassium depolarizing solution, and constriction abrasion has a reduced response compare to the intact IMA (Intact IMA: 27.3 ± 17.8 mN, surface abrasion: 20.9 ± 16.7 mN, constriction abrasion: 7.5 ± 1.15 mN and shear abrasion: 23.7 ± 12.2 mN, (Figure 17).

Vasoconstrictor responses to incremental PE concentrations produced concentration-dependent contractions in intact and denuded IMA segments. There was a significant difference in endothelium dependent vascular relaxation to A23187 between control IMA (84 ± 2.0 %), IMA subjected to surface abrasion (29.43 ± 1.5 %) and shear abrasion (0.04 ± 0.3 %).

There was no difference in the EC_{50} of the concentration-response curve of the surface abrasion (compared to intact vessel), indicating equal sensitivity to PE. However, there was a difference in the EC_{50} of the concentration-response curve of the mechanical shear abrasion denudation (compare to intact IMA), indicating that they are more sensitive to PE than the intact IMA (EC_{50}: intact vessel= 2.41 ± 0.34 μM, surface abrasion=2.07 ± 1.33 μM, and shear abrasion: =1.29 ± 0.34 μM, Figure 18).

2.13. DISCUSSION

This study is the first comprehensive systematic evaluation of endothelial denudation techniques for IMA. Using both structural and functional assessments, the study demonstrates that shear abrasion technique is the optimal method for denuding the endothelium from isolated IMA segments obtained from patients undergoing elective CABG. Although there was no differences between intact IMA, surface and shear abrasion in response to the standard potassium depolarizing stimulus, there was a trend of reduced responses in the surface abrasion suggesting that part of it smooth muscle cell apparatus was damaged. Constriction abrasion (compare to intact IMA) had the least potassium contractile response (generated a force < 9.8 mN) indicating that there was substantial smooth muscle cell damaged during the denudation procedure. They were considered unresponsive tissue without further challenge with PE or A23187.

The mechanical shear abrasion method is similar in effect to the bubble passage techniques for arterial injury used previously by Guyton et al. [(431)]. As expected from an endothelium-denuded vessel, IMA segments subjected to this denudation process demonstrated denuded endothelial cells on histological examination, and functional studies showed a hyper constrictor response to vasopressor (due to loss of endogenous endothelial vasodilatory factors) and failure to respond to the endothelium-dependent vasodilator. In contrast, IMA subjected to surface abrasion technique leads to a success rate of about 50% of IMA showing insufficient disruption of endothelial layer and subsequent endothelium-dependent vasodilator responses to A23187. Constrictor abrasion method on the other hand produced severe damage to the smooth muscle cell so that constrictor responses to vasopressor could not be evoked.

Other endothelial denudation technique that utilises surface abrasion method such as wooden stick [423], and stainless steel wire [430, 432], and constriction abrasion [433], has been successfully applied to denude the vascular endothelium of animal vessel. Wang successfully used a sand paper polished cat whisker in surface abrasion method to denude the endothelium of foetal pulmonary resistance artery [434]. He obtained total endothelial removal as depicted by vascular hypersensitivity after endothelial removal. Lu used a specifically designed catheter in constriction abrasion method to successfully denude the artery of Wistar rat [430]. In addition, similar methods were also reported to successfully denude animal vessel in vascular reactivity studies such include: gentle rubbing of the luminal surface of the vessel with wooden stick [423], syringe needle [424], stainless steel wire [425, 426], forcept [427], blunt seeker and balloon catheter [429, 432]and intense vascular constriction upon a intravascular rod [430]. However, utilising these methods in similar studies involving human IMA have been problematic, since the vessel is not amenable to regular denudation techniques.

The extent of endothelium denudation is important for mechanistic vascular studies involving smooth muscle cell. However, some approaches have been ineffective in abolishing the endothelium-dependent response in human IMA. For example, Lu, applied mechanical surface abrasion techniques to denude IMA and subjected them to vasoactive intestinal peptide (VIP) to evoke endothelium dependent relaxation and observed 20% relaxation to the VIP. This is consistent in our studies involving surface abrasion demonstrating that there is residual endothelial vasodilatory function in response to VIP [435]. Similar result was also observed by Pagan using urotensin II as a vasodilator after IMA denudation and observed a residual 20% endothelium-dependent relaxation response [436]. Thus although these studies reported IMA denudation by surface abrasion, the residual endothelium-dependent relaxation

responses infer that an endothelium-dependent mechanism has not been completely excluded.

The human IMA is an outstanding conduit of choice for CABG. Unlike saphenous vein graft, which has a median patency of 7 years, more than 90% of IMA grafts are patent at 15 years [433]. This in part is attributable to its endothelial function. Moreover, despite the CABG having established atherosclerotic disease and significant CV risk factors, all which contribute to endothelial dysfunction, all the IMA segments demonstrated intact endothelial responses with little or no atherosclerotic plaque formation prior to the interventions.

The challenge of denuding the IMA is due to its unique structural and morphological arrangement compared to other vessel [437]. Structural and morphological analysis has revealed that the media of IMA is organized into two distinct layers: the internal muscular layer and the external layer with spirally oriented elastic lamellae which houses the smooth muscle cells in between [438]. The endothelial layer shows fewer fenestrations and lower intercellular junction permeability [437] which contributes to it resilient nature to atherosclerotic plaque formation, and renders it endothelium with greater attachment to it luminal surface and makes it very difficult to successfully and consistently denude it endothelium by gentle rubbing of the inter-luminal surface with stainless steel wire and the vascular constriction upon an intravascular rod techniques. However, our procedure based on shear abrasion technique has been shown to be safe and effective to remove the endothelium of isolated IMA vessel and provides significant benefit over the surface and constriction abrasion methods used in this research. It also has an additional theoretical advantage of keeping the vascular segment under a more physiologic condition during the denudation process and

requires less skill to be successful.

2.14. CONCLUSION

In this systematic, comprehensive, controlled comparative study assessing both structural and functional endpoints of endothelial denudation techniques, we have demonstrated that shear abrasion method is the optimal technique for the IMA segments endothelial denudation. This should be employed in future isolated IMA in vascular reactivity studies that require endothelial denudation as well as in other resilience vessel.

2.15. LIST OF FIGURES AND TABLE

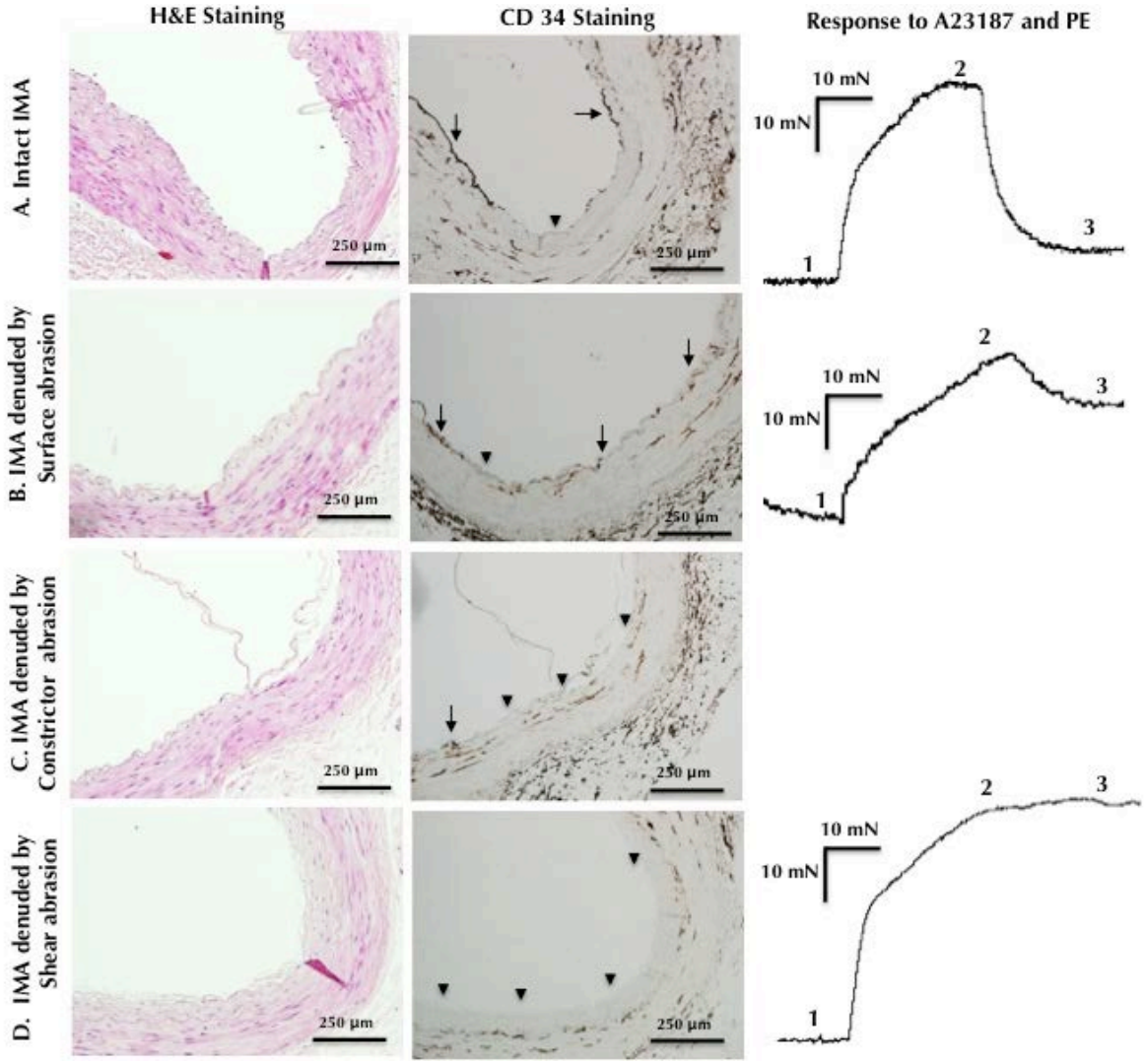


Figure 15: Structural & Functional Findings from Endothelial Denudation of Human IMA Segments. (A) Control segments with intact endothelial lining (H&E and CD34 staining; black arrow indicates endothelial cell lining and arrowhead indicates denuded area) that are pre-constricted with PE (1) and exposed to an endothelium-dependent vasodilator (2) demonstrate a vasodilator response (3). (B) Surface abrasion produces endothelium removal, but there were still viable endothelial cells produces endothelium-dependent vasodilator response (C) Constriction abrasion produces distortion of the internal elastic lamina and vessel were unable to respond to PE or A23187 and (B) Shear abrasion produces significant removal of endothelial

cells and hyper constriction to PE with a loss of the endothelium-dependent vasodilator response.

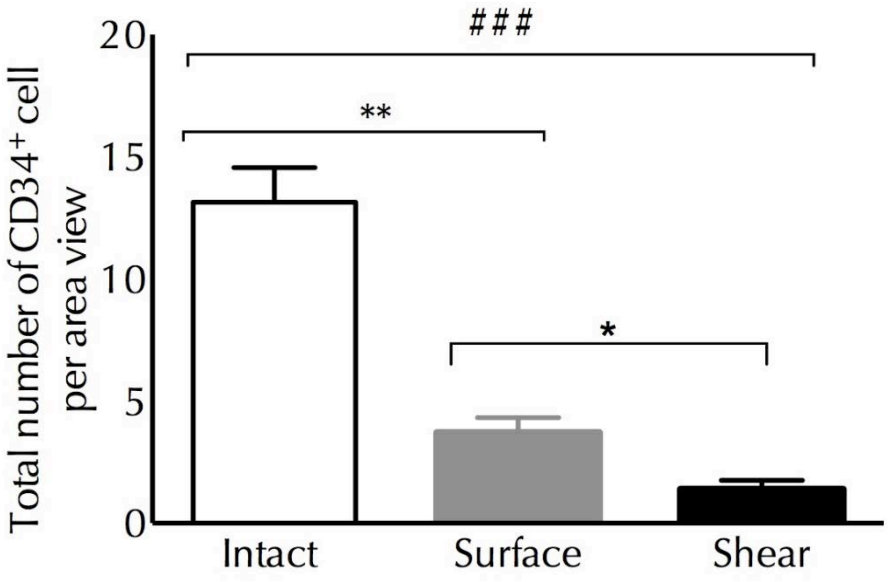


Figure 16: Endothelial Cell Loss with Denudation. Shear abrasion leads to decrease endothelial cell number compared to intact IMA (intact IMA-white bar: 13.15±6.3 n=12, surface abrasion-grey bar: 3.7±2.8 n=6 and shear abrasion-back bar: 1.6± 1.4 n=12). Results are presented as mean ± standard error of the mean. *, * and #p > 0.05.

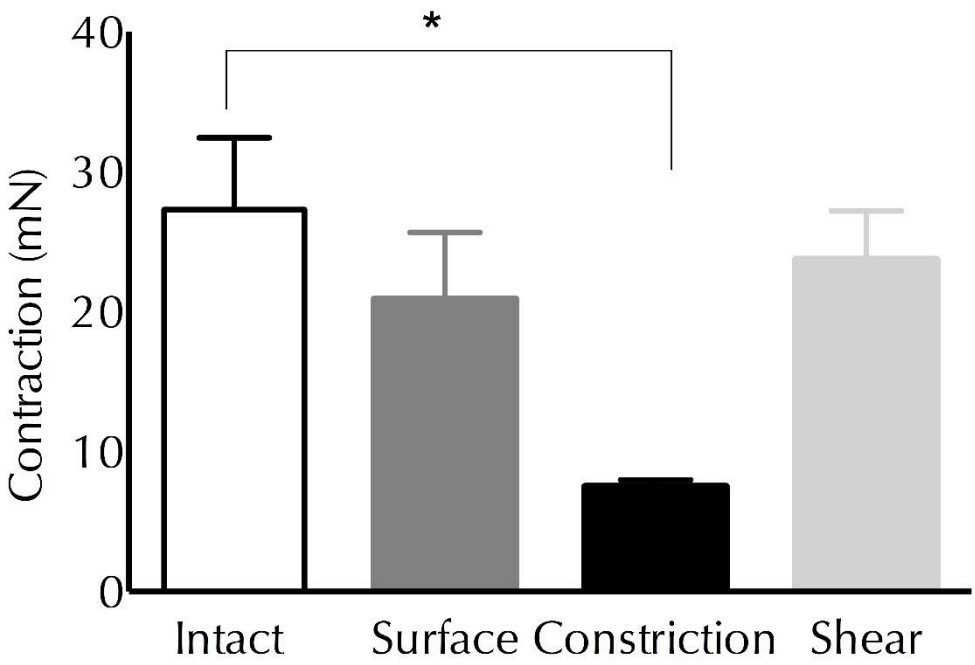


Figure 17: Contractile Responses to potassium ion following Mechanical Denudation**.** Shear abrasion has equal strength of contraction to potassium ion as compared to Intact IMA (Intact IMA-white bar: 27.3 ± 17.8 mN, n=12), surface abrasion-dark grey bar: 20.9 ± 16.7 mN, n=12, shear abrasion-black bar: 23.7 ± 12.2 mN, n=12) and constriction abrasion-grey bar: 7.5 ± 1.15 mN, n=6). Results are presented as mean ± standard error of the mean, $p > 0.05$.

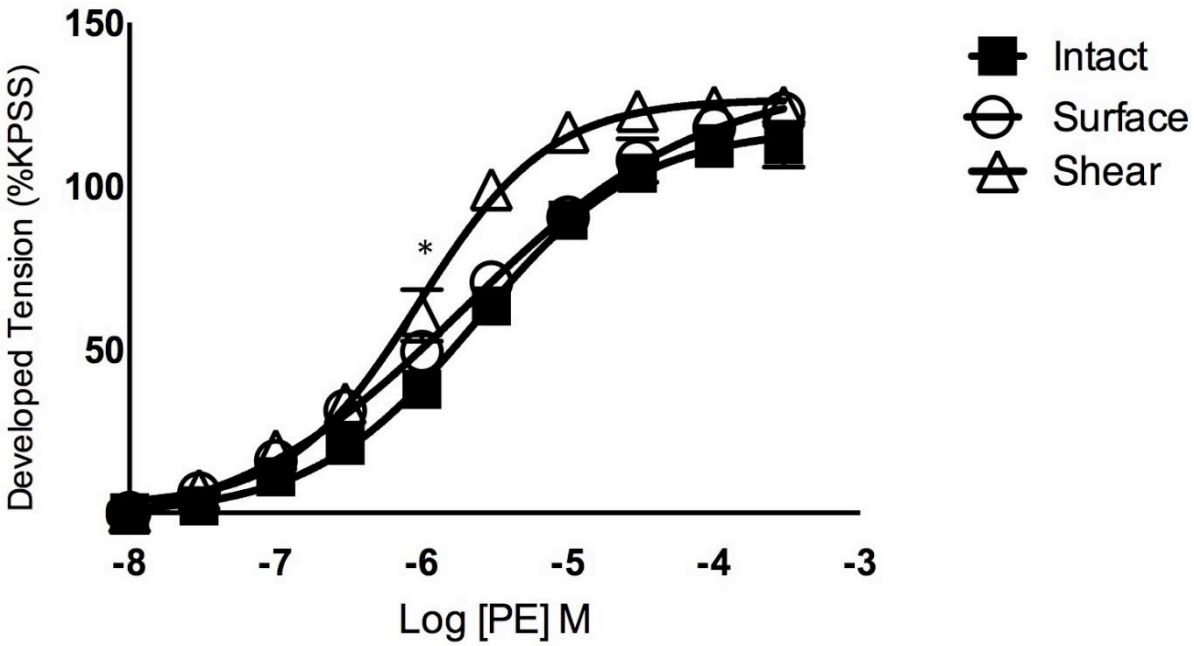


Figure 18: Vascular Hypersensitivity to PE Following Endothelium Denudation. Mechanical shear abrasion increase vascular sensitivity to PE compared to the intact IMA (Intact IMA EC_{50}: 2.41 ± 0.34, n=12 close square, surface abrasion EC_{50}: 2.07 ± 0.13, n=6 close circle and shear abrasion EC_{50}: 1.29 ± 0.34, n=12 open square and) respectively. *p > 0.05

Table 3: Patient characteristics and pre-operative medications for included patients.

TOTAL PATIENT	**15**
Male	60%
Mean age	68.0 ± 13.25
Vascular Risk Factors	
Current smoker	2 (13%)
Ex-smoker	3 (20%)
Diabetic	6 (40%)
Hypertensive	11 (73%)
Hypercholesterolemia	8 (53%)
BMI	26.7 ± 4.50
Drug therapy	
Anti-platelet	9 (60%)
Statin	13 (86%)
Beta-Blocker	11 (73%)
ACE-Inhibitor	6 (40%)
Calcium Channel Blocker	4 (26%)
Nitrate	2 (13%)
Diuretic	5 (33%)
SSRI	2 (13%)
Angiotensin Receptor Blocker	4 (26%)

3. CHAPTER 3

Sex Differences in Vascular Reactivity of Coronary Artery Bypass Graft Conduits to Phenylephrine and Endothelin-1

Statement of Authorship

Title of Paper	Sex differences in vascular reactivity of coronary artery bypass graft conduits.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Dr. Amenah Jaghoori
Contribution to the Paper	Dr. Amenah Jaghoori contributed to the following experiments: (1) Sample collection, (2) Contractile response to PE with endothelium (n=25-32), Contractile response to ET-1 with endothelium (n=11-15), (3) the mechanism of PE response, L-NAME (n=11-16) and Indomethacin (n=9-13) and (4) writing the manuscript
Overall percentage (%)	45.1%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 1/11/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Victor Lamin		
Contribution to the Paper	Victor Lamin contributed to the following: (1) Sample collection, (2) Contractile response to PE with endothelium (n=10-12), Contractile response to PE without endothelium (n=10-11), (3) Phos-tag™ western blot analysis (n=5-6) (4) participated in writing the manuscript.		
Signature		Date	17/10/2016

Name of Co-Author	Rachel Jacobczak		
Contribution to the Paper	Rachel Jacobczak was involved in sample collection, experimental set up and vascular reactivity studies		
Signature		Date	17/10/2016

Name of Co-Author	Mr Michael Worthington		
Contribution to the Paper	Mr. Michael Worthington provided isolated vascular segments and reviewing the manuscript		
Signature		Date	4/11/16

Name of Co-Author	Mr Fabiano Viana		
Contribution to the Paper	Mr. Fabiano Viana provided isolated vascular segments and reviewing the manuscript		
Signature		Date	9/11/16

Name of Co-Author	Mr James Edwards

Contribution to the Paper	Mr. James Edwards provided isolated vascular segments and reviewing the manuscript		
Signature		Date	

Name of Co-Author	Mr Robert Stuklis		
Contribution to the Paper	Mr. Robert Stuklis provided isolated vascular segments and reviewing the manuscript		
Signature		Date	3.11.16

Name of Co-Author	Dr. David P. Wilson		
Contribution to the Paper	Dr. David Wilson participated in experimental design, data analysis and critical revisions of the manuscript.		
Signature		Date	28/11/2016

Name of Co-Author	Professor John F. Beltrame		
Contribution to the Paper	Professor John F. Beltrame participated in experimental design, data analysis and critical revisions of the manuscript.		
Signature		Date	17/10/16

Please cut and paste additional co-author panels here as required.

3.1. ABSTRACT

Rationale: Females have increase in-hospital mortality and poorer outcomes following coronary artery bypass grafting (CABG). Biological differences in the reactivity of the graft conduits to circulating catecholamine may contribute to this sex-difference.

Objective: This study examined sex differences in the vasoconstrictor responses of internal mammary artery (IMA) and saphenous vein (SV) conduits to phenylephrine (PE) and endothelin-1 (ET-1).

Methods and results: Functional IMA and SV were obtained from 78 male and 50 female patients undergoing CABG (67.7±11 and 69±10 years, respectively) and subjected to the following experimental conditions. (1) Concentration response curves for PE and ET-1 were generated in an intact IMA and SV and endothelium denuded IMA segments, (2) in the presence of the nitric oxide synthase inhibitor (L-NAME) or the cyclooxygenase inhibitor (Indomethacin) in an endothelium intact IMA and (3) the activity state (abundance and phosphorylation) of the α_1-adrenergic receptor was investigated using Phos-tag™ western blot analysis.

(1) Compared to male, female IMA and SV were hypersensitive to PE but not ET-1 ($p<0.05$). The female IMA hypersensitivity response to PE was abolished following endothelial denudation, (2) persisted in the presence of L-NAME, but was abolished in the presence of indomethacin and (3) there was no sex-differences in the abundance and phosphorylation of the α_1-adrenergic receptor in IMA.

Conclusion: Female IMA and SV graft conduits are hypersensitive to α_1-adrenergic stimuli. The female IMA hypersensitive to PE is mediated by endothelial cyclooxygenase pathway. Therefore, post-operative inotrope use may produce

excessive IMA and SV graft constriction in females that might eventually contribute to their poorer CABG outcomes.

3.2. INTRODUCTION

Following coronary artery bypass grafting (CABG), female experience poorer outcomes compared to their male counterpart [439]. In particular, females have increased postoperative mortality and morbidity risks, as well as an increased 30-day mortality and readmission rates following CABG.[21, 45, 440, 441] The mechanisms responsible for these sex-differences are likely to be multifactorial including clinical, psychosocial and biological factors. Clinical factors include being older age, more risk factors especially hypertension [442-444] and a delay in presentation/diagnosis of coronary artery disease. Psychosocial factors may include a higher prevalence of depression [445, 446] and poorer socioeconomic status.[447, 448] Biological factors could include increased platelet aggregation,[166, 449] smaller vessel sizes and post-menopausal loss of oestrogen's cardio-protective effects.

An additional biological mechanism that has not received significant attention is sex differences in vascular reactivity. Important endogenous vasoconstrictors include the catecholamines and endothelin-1.

The α_1-adrenergic agonist (Phenylephrine, PE) can stimulates the α_1-adrenergic receptors, mimicking the effects of sympathetic adrenergic nerve activation in blood vessels acting as potent endogenous vasoconstrictors. ET-1 on the other hands is one of the most potent endogenous vasoconstrictors produced by the endothelium. ET-1 is associated to development of atherosclerosis through vascular stimulation and VSMC proliferation [450] migration and contraction[451]. Both α_1-adrenergic and ET-1 receptors have been found to increase in human internal mammary artery (IMA) segment, implicating their role in CABG graft spasm. [452-454]

Thus, the objective of this study was to determine if there are sex differences in vascular reactivity to PE and ET-1 in isolated IMA and SV segments in an ex-vivo organ bath preparation.

3.3. METHODS

3.3.1. Patient subjects

The study was approved by the Royal Adelaide Hospital Human Ethics Committee on 28th April, 2009, with approval number RAH PROTOCOL NO: 090411, Title: The effect of selective/non-selective endothelin blockage on statin inhibition of endothelin vasoconstriction responses, for the use of the use of patient discarded IMA. Patient scheduled for elective CABG were consented pre-operatively with their medications were recorded.

Standard cardiac anaesthesia included a fentanyl pre-medication, thiopentone induction, isoflurane maintenance anaesthesia and muscle relaxation with rocuronium was achieved, followed by surgical procedure by median sternotomy approach with the left IMA harvested as a pedicle graft and the excess vessel length trimmed. The remnant IMA (generally discarded) was available for the experimental studies. Patients were grouped based on biological sex.

3.3.2. Study drugs and chemicals

Phenylephrine, calcium ionophore A23187, indomethacin, N-w-Nitro-L-arginine Methyl Ester (L-NAME), all from Sigma Chemical Company, St. Louis, MO, SDS, Endothelin-1 was purchased from Auspep, Australia, 1× Complete™ protease inhibitor cocktail (Roche, Mannheim, Germany), di-isopropylfluorophosphate (Sigma-Aldrich, Caste Hill, Australia), Phos-tag™ Acrylamide from Wako Laboratory Chemicals, Japan, 30% acrylamide/0.8% N, N'-methylenebisacrylamide solution, Tween-20, and 0.22 μm PVDF membrane were from BioRad Laboratories, Australia

PE, ET-1 and L-NAME were prepared by dissolving the compound in double distilled water to the required concentration; indomethacin and A23187 were prepared in 100% ethanol. Krebs-bicarbonate solution contained in mmol/L: NaCl 118, KH_2PO_4 1.18, KCl 3.89, $NaHCO_3$ 25, $MgCl_2$ 1.05, $CaCl_2$ 2.34, EDTA 0.01 and glucose 5.56, while the high K^+ (KPSS) solution replaced NaCl in normal Krebs with an equimolar amount of KCl (yielding a final KCl [121.89] mmol/L) both solutions were aerated with carbogen gas (95% O_2 and 5% CO_2 to maintain pH 7.4. All drugs were diluted to the required concentration in physiological Krebs-bicarbonate solution previously aerated with carbogen gas (95% O_2 and 5% CO_2). The concentration of each drug was expressed as final concentration in μM.

3.3.3. Functional Isolated Vessel Studies

Segments of IMA obtained during surgery were placed in ice-cold Krebs solution and transported to the basic vascular laboratory. The vessels were dissected to remove peri-adventitial fat and cut into vascular rings (2-3mm wide) then suspended on wires and mounted in a water-jacketed organ bath. The vessels were bathed in Krebs solution with a resting tension of 19.6mN was used for the IMA segments. [455]

Following a 60-minute equilibration period, the contractile responses of the vessels were assessed in response to a depolarizing potassium (112mmol/L) physiological salt solution (KPSS). The KPSS response was consecutively repeated 3 times, vessels showing no/poor response were considered to have been damaged by handling and were discarded. The final contractile response to KPSS was used as the reference benchmark to normalize agonist mediated contractile responses.

Following a 30-minute equilibration, cumulative concentration-response curves of the IMA segments were established to the following agonists: Phenylephrine (PE, 0.01 -

300µM) and Endothelin-1 (ET-1, 0.01 - 300nM). Endothelial integrity was assessed at the end of the concentration-response curves utilizing a bolus dose of the Ca^{2+} ionophore A23187 (2 µM) in PE pre-constricted vessels.

3.3.4. Endothelium dependent mechanism

As described previously, endothelial denudation was undertaken using shear abrasion via infusion of an effervescent solution through the vessel lumen [412] Functional denudation was confirmed by abolition of the Ca^{2+} ionophore A23187 (2 µM) vasodilatory response. Mechanistic studies elucidating the role of endothelial-derived factors were performed for observed sex-differences in the above vasomotor reactivity experiments. Vascular segments were incubated for 30 minutes with (1) L-nitroarginine methyl ester (L-NAME; nitric oxide synthase blocker) or (2) indomethacin (non-specific cyclooxygenase inhibitor). Following these pre-treatment incubations, concentration-response curves to PE were assessed in the presence of L-NAME or Indomethacin.

3.3.5. Receptor Quantification

Relevant vascular receptors were quantified both in relation to abundance and phosphorylation status. Mn^{2+} Phos-tag™ phosphorylation-dependent mobility shifts analysis was used to quantify total and phosphorylated protein expression of α_1-adrenergic receptor. The principle of this procedure involves tagging a phosphorylated protein with Mn2+ Phos-tag™ thereby slowing it movement on the SDS PAGE differentiating phosphorylated and un-phosphorylated protein on the same gel as previously described by Tomida *et al.*, (2008).[415]

Proteins were extracted from each 3 mm rings using a modified Laemmli sample buffer, electrophoresed on an SDS PAGE, coomassie stained, and the entire lane of protein for each sample quantified as an index of extractable vascular proteins and adjusted the concentration to be equal for all arteries. Samples were electrophoresed on a Phos-tag™ gels containing 7.5% polyacrylamide gel with 25 µM Phos-tag™ and 50 µM $MnCl_2$ at 150 V for 90 min. Proteins were transferred, using the BioRad Transblot® Turbo system™, onto 0.22 µm PVDF at 25 V and 1.3 amps for 7 min. Non-specific binding sites were blocked with 3% non-fat dry milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% (*v*/*v*) Tween-20) for 60 min, followed by incubation with TBS-T containing rabbit-derived affinity-purified anti-α_1 antibody (1:1000). PVDF membranes were washed three times in TBS-T and incubated with a 1:1000 dilution (in TBS-T). Anti-Mouse and Anti rabbit IgGs coupled with Dylight™ 800 fluorochromes (Pierce Thermo Scientific, Rockford, IL, USA): anti-mouse IgG (for 1:10,000), for 60 min before another three washes with TBS-T.

3.3.6. Data Analysis

The concentration response curves to all the agonists were expressed as the percentage of the final KPSS contraction. Sigmoid curves of best fit were constructed by performing nonlinear regression analyses using Graph Pad Prism, Version 6 (Graph Pad Software Inc, La Jolla, USA). The EC_{50} (agonist concentration eliciting half the maximal response) and E_{max} (maximum response) were derived for each curve. Student's unpaired *t*-tests were used to identify statistically significant sex-differences for both the vascular responses and western blot experiments. Data is presented as mean ± SEM with $p < 0.05$ considered statistically significant. For western blot experiments data is presented as mean integrated intensity ± SEM and

unpaired student's *t*-test was used to identify statistical differences ($p<0.05$) between male and female patient samples.

3.4. RESULTS

IMA and SV samples were obtained from 78 males and 50 females undergoing CABG. The clinical characteristics of these patients are summarized in Table 4. There were no significant differences between sexes in demographics, clinical risk factors or pre-operative medications (Table 4). All female patients were post-menopausal and none were on hormone replacement therapy.

3.4.1. Vascular Functional Studies

Endothelium-dependent vasodilator responses were initially intact for all vessels.

3.4.2. Vasoconstrictor responses

Female IMA and SV exhibit an increased sensitivity to PE with no difference in maximal contraction. The concentration-responses curves and EC_{50}/E_{max} values are detailed in Table 5 and Figure 19 respectively.

In contrast, there was no sex-difference in IMA and SV response to ET-1 (Table 5 and Figure 20)

With no sex difference observed in response to endothelin-1, no further studies were conducted using this vasoconstrictor and with SV. However, mechanistic studies exploring the role of the endothelium and α-adrenergic receptor, in the female hypersensitivity response to phenylephrine, were undertaken in IMA and described below.

3.4.2.1. Role of endothelium in the sex difference in IMA vasoconstrictor responses to phenylephrine

In the absence of the endothelium, the sex-difference observed with PE in endothelium-intact IMA was abolished, suggesting an endothelium-dependent mechanism (Figure 21A). As expected, denudation (compared to intact vessel)

increases sensitivity to PE (Figure 21 B&C) due to the loss of endogenous endothelial vasodilatory substances. Consistent with the mechanical denudation finding, combined inhibition of the endothelial nitric oxide and prostaglandin pathway also revealed abolition of the observed sex difference to PE.

Furthermore, pre-treatment with L-NAME alone did not influence the sex-difference in PE responses in relation to vessel sensitivity (Table 6, figure 22A and B), whereas indomethacin alone abolished the female IMA hypersensitivity (Table 6, Figure 22C and D). Furthermore, individual analysis within sex in the presence or absence of indomethacin revealed a significant shift in the male but not the female PE concentration response (Figure 23).

Multiple regression analysis reveals no association between sex, EC_{50} and E_{max}, risk factors, age and medication for the intact IMA. However, age, hypertension, statin, beta-blocker, calcium channel blocker and SSRI added statistically significantly to the prediction of the denuded IMA treated with PE, $p < .05$. (Table 7)

3.4.3. IMA Receptor Expression

Total protein expression of α_1- receptors revealed no relative sex-differences in (abundance: male= 16. 6 $X10^4 \pm 7.7X10^3$ and female= 17. 1 X104 $\pm$ $8.6X10^3$, n=5-6) and (% phosphorylation: male= 16.22 ± 4.9, and female= 17.26 ± 3.7, n=5-6, Figure 24)

3.5. DISCUSSION

This study demonstrates a sex difference in the response to α_1-adrenergic receptor activation in human IMA and SV segments. The mechanism of the female IMA hypersensitivity to α_1-adrenergic receptor activation is endothelium-dependent and particularly related to endothelial prostanoids. Further analysis reveals that it is the absence of an endothelial vasodilatory PG that account for the difference. In contrast, there was no difference in male and female IMA and SV to Endothelin-1.

Although both Endothelin-1 and α_1-adrenergic stimulation can activate PLA2, resulting in the production and release of prostanoids including PGI_2 in various cell types.[456-460] However, there was no sex-difference in IMA vascular contraction to ET-1. Previous work by Seo *et al.*, 1994 revealed that although smooth muscle oh human IMA expresses both ET_A and ET_B receptors and the endothelium express ET_B receptors, the endothelial ET_B receptor in human IMA is not functionally linked to Nitric oxide and prostanoids production.[461] This suggests that dose response with ET-1 agonist will leads to decrease in endothelial PGI_2 production in a comparable manner to the pharmacological blocking with indomethacin in male IMA and potentially explain the no-sex-difference observed with ET-1 agonist. However, this pathway needs further investigation to confirm the effect of ET-1 on vascular PGI_2 production in human IMA.

The sex-differences in the α_1-adrenergic stimulation are on a background of homogeneous responses to KPPS in all vessels (indicating uniform activity of voltage gated Ca^{2+} channels between males and females). This selective hyper-reactivity to α_1-adrenergic agonists is particularly pertinent in the clinical setting of CABG and may contribute to the poorer outcomes experienced by women in the post-operative period.

Post-menopausal females have increased adrenergic influences compared with males, as evidenced by (i) increased sympathetic activity assessed by clinical measures of autonomic function including heart rate variability analysis [462], and muscle sympathetic nerve activity [463], (ii) increased plasma noradrenaline levels [464]. This may contribute to the increased systemic vascular resistance [464] and thus the higher prevalence of hypertension and stroke observed in females. [465]

In the current isolated vessel study, we have for the first time conclusively demonstrated that post-menopausal vessels are hypersensitive to a conventional α_1-adrenergic agonist, independent of extravascular neural and humoral adrenergic influences. Thus, when the increased vascular sensitivity to alpha adrenergic stimuli is combined with the in-vivo increased systemic adrenergic activity, it would be expected that female show an exaggerated vasoconstrictor response to adrenergic stimuli, which may contribute to their increased morbidity and mortality following CABG surgery.

The mechanism/s responsible for the female IMA hypersensitivity to α_1-adrenergic stimulation is not related to an increased number of α_1-receptors or their phosphorylation dependent activation state on the blood vessels, as demonstrated by our receptor studies. Furthermore, endothelial nitric oxide synthase (eNOS) inhibition by L-NAME did not attenuate the relative female-male hypersensitivity to the α_1-adrenergic agonist, indicating nitric oxide did not contribute to this sex-difference. In contrast, inhibition of prostaglandin synthesis with the cyclooxygenase inhibitor indomethacin did attenuate the sex-difference, implicating a key role in the vascular prostanoids pathway.

Sex-differences in the effects of vascular prostanoids have received limited attention. Indomethacin inhibits the vasodilator (prostacyclin, PGI_2) and vasoconstrictor

prostanoids (thromboxane A_2, TxA_2; prostaglandin F_{2a}, PGF_{2a}). However, additional analysis reveals that it is the absence of an endothelial vasodilatory PG in female that account for the difference. Furthermore, in the presence of indomethacin, that blocked the male vasodilatory PG, results in the shifting of the male dose response curve making them to behave like female.

Isolated rat vessel studies have also reported sex-differences. Aortic ring studies from male normoglycaemic rats have showed an increased constrictor response to PE compared with females [(466)]. In contrast, female diabetic rat vessels were hyper-reactive to PE compared to male diabetic vessels [(466)]. These hyper-reactivity responses to PE were attenuated by indomethacin [(466)].

3.6. STUDY LIMITATIONS

These studies were conducted in an isolated human IMA model, which enabled us to focus on interpretation of vasomotor responses to individual agonists, independent of platelets, circulating hormones and nerve activation. However, utilizing an isolated vessel sourced from the distal IMA with the adventitia removed, devoid the vessel of its usual compensatory autonomic influences that may play a role in vasomotor responses of the *in-situ* IMA (especially the proximal segments that are not mobilized during surgery for IMA *in-situ* grafting). Furthermore, the luminal blood milieu that would traverse an intact IMA graft has been excluded in this model and thus limits interpretation to *in-vivo* responses since: (a) the impact of synergistic vasoconstrictors on the IMA response has not been assessed, and (b) compensatory local vasodilatory mechanisms may have been excluded. The western blot analysis represent total receptor density in the IMA segments, which include the inactive intracellular and the functional plasma membrane receptors. A better approach would have been to specifically quantify the plasma membrane receptors only as they are responsible for agonist mediated stimulation of the cells. Despite these limitations, this isolated vessel model has provided an opportunity to identify sex-differences in human vasoconstrictor responses that is inherent to the blood vessel.

3.7. CLINICAL IMPLICATIONS

Combinations of clinical, psychosocial and biological factors are likely to be responsible for the poor outcomes experienced by females undergoing CABG. This study identifies an additional biological factor inherent to the CABG conduits that may contribute to the poorer post-CABG outcomes experienced by women. The female hypersensitivity to α_1-adrenergic stimuli is particularly concerning in the post-operative period, since during this period there is a hyper-adrenergic state both from (a) endogenous catecholamine release, [467, 468] and (b) exogenous exposure, with the frequent administration of inotropes. Thus, the exaggerated IMA vasoconstriction to these α_1-adrenergic agonists may result in vasoconstriction of the IMA graft and thus myocardial ischaemia (frequently involving the anterior wall, where the IMA is often grafted) thereby compromising left ventricular function. Furthermore, when high dose inotropes are used in an already compromised post-operative patient, this phenomenon will be further aggravated in female patients and could lead to their demise. Thus, these pathophysiological findings raise concern about the judicious use of α_1- agonists (inotropes) in the post-operative period, especially in females. Further clinical studies are required to elucidate the role of this biologic mechanism contributing to an adverse clinical outcome.

3.8. CONCLUSION

This study has identified a biological sex differences in the vascular reactivity of the IMA conduit used in CABG that could result in exaggerated IMA vasoconstriction in females to α-adrenergic receptor agonist. Thus, the use of alpha-agonists in the post-operative period in women should be cautiously undertaken.

3.9. LIST OF FIGURES AND TABLES

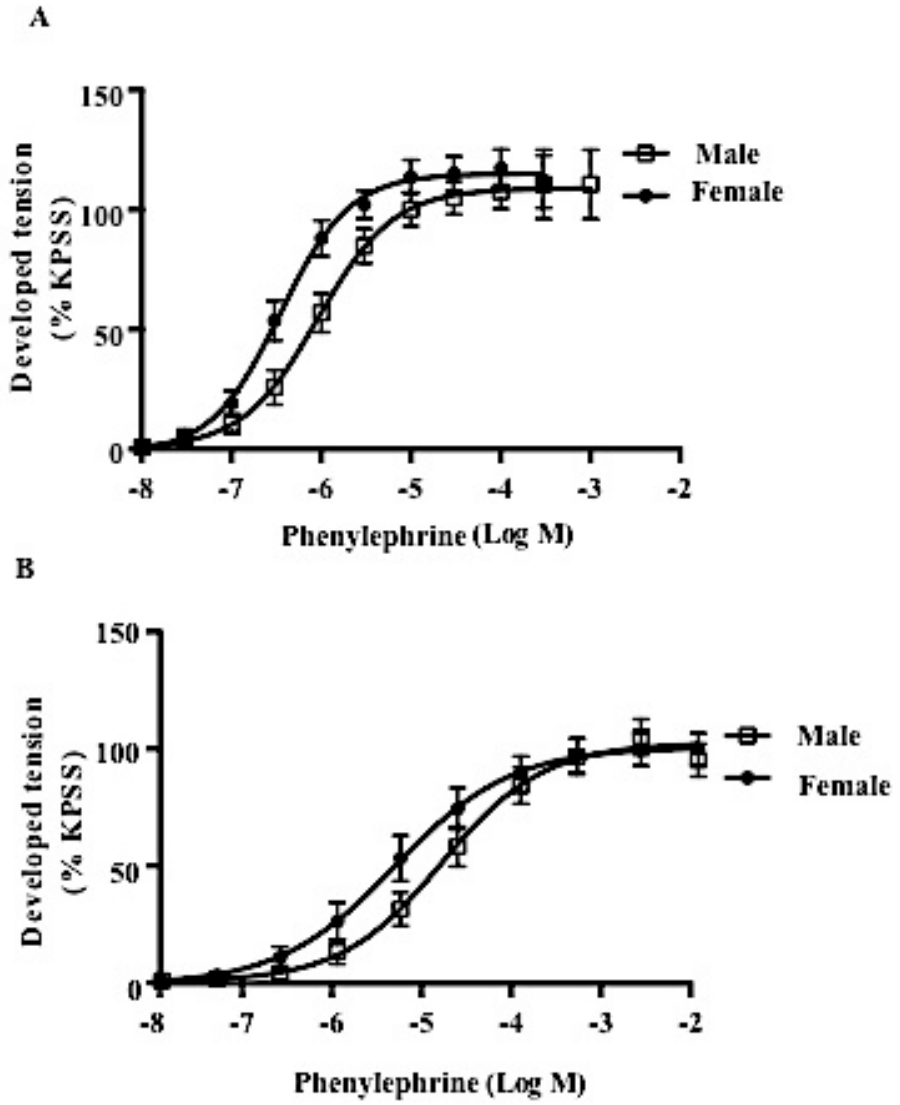


Figure 19: Female IMA and SV are hypersensitivity to PE than aged match male. (A) Cumulative dose response curves to PE (n=35-43) in isolated rings of human IMA (Log EC_{50}: male = -5.83±0.06 and female = -6.22 ± 0.07), maximal contraction (E_{max}: male= 105.9±6.81 and female=112.2 ± 9.44). (B) Cumulative dose response curves to PE (n=10-19) in isolated rings of human SV (Log EC_{50}: male = -5.63±0.11 and female = -6.01±0.14) maximal contraction (E_{max}: male= 107.4 ± 8.09 and female=99.89 ± 6.80). Values are expressed as mean ± SEM and are normalized to the maximal contraction to KPSS.

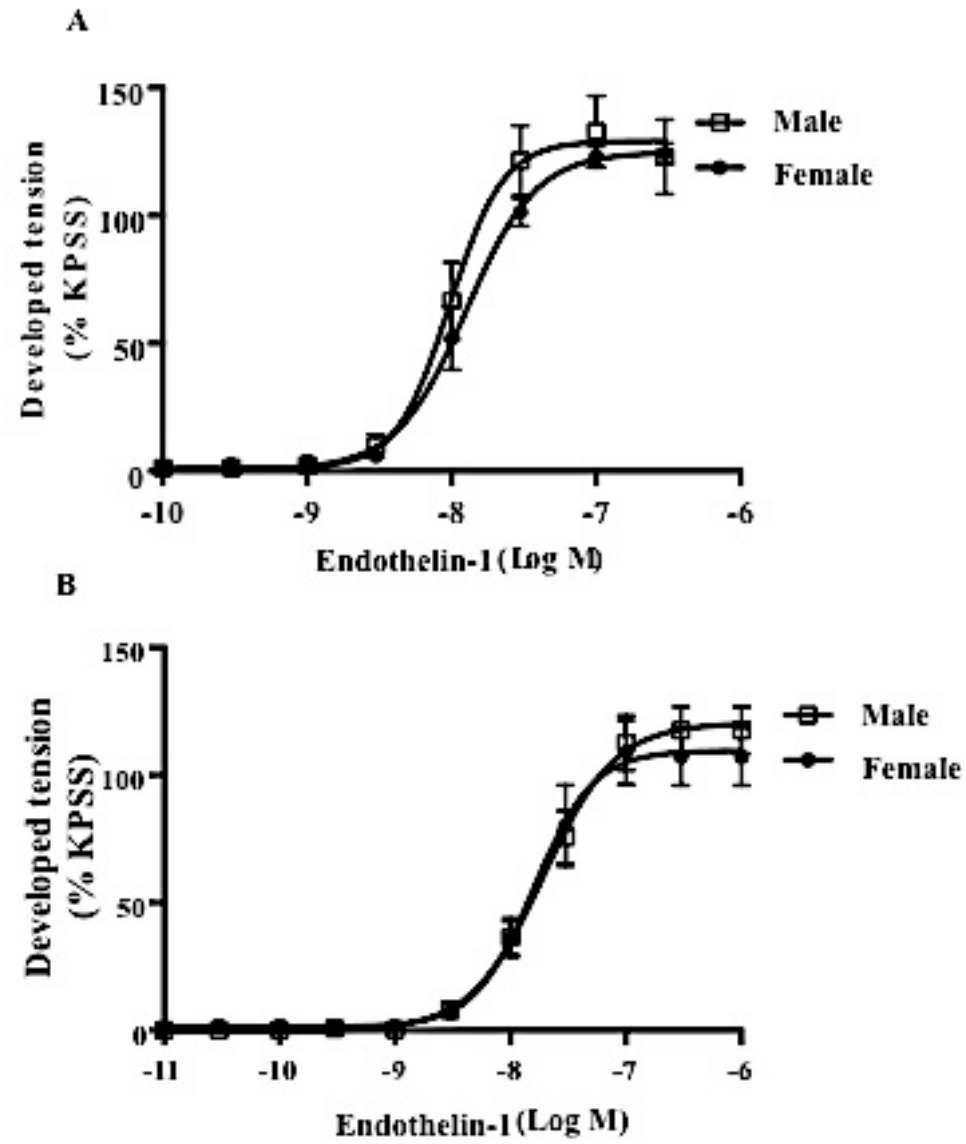


Figure 20: Male and Female IMA and SV have equal response to ET-1. (A) Cumulative dose response curves to ET-1 in isolated rings of human IMA (Log EC_{50}: male = -7.94±0.06 and female = -7.89±0.08) and maximum response (E_{max}: male= 133.4±14.04 and female=124.4±4.14 n=11-14). (B) Cumulative dose response curves to ET-1 in isolated rings of human SV (Log EC_{50}: male = -7.52±0.14 and female = -7.61±0.13) and maximum response (E_{max}: male= 144.4±12.16 and female=129.2±14.71, n=7-10). Values are expressed as mean ± SEM and are normalized to the maximal contraction to KPSS.

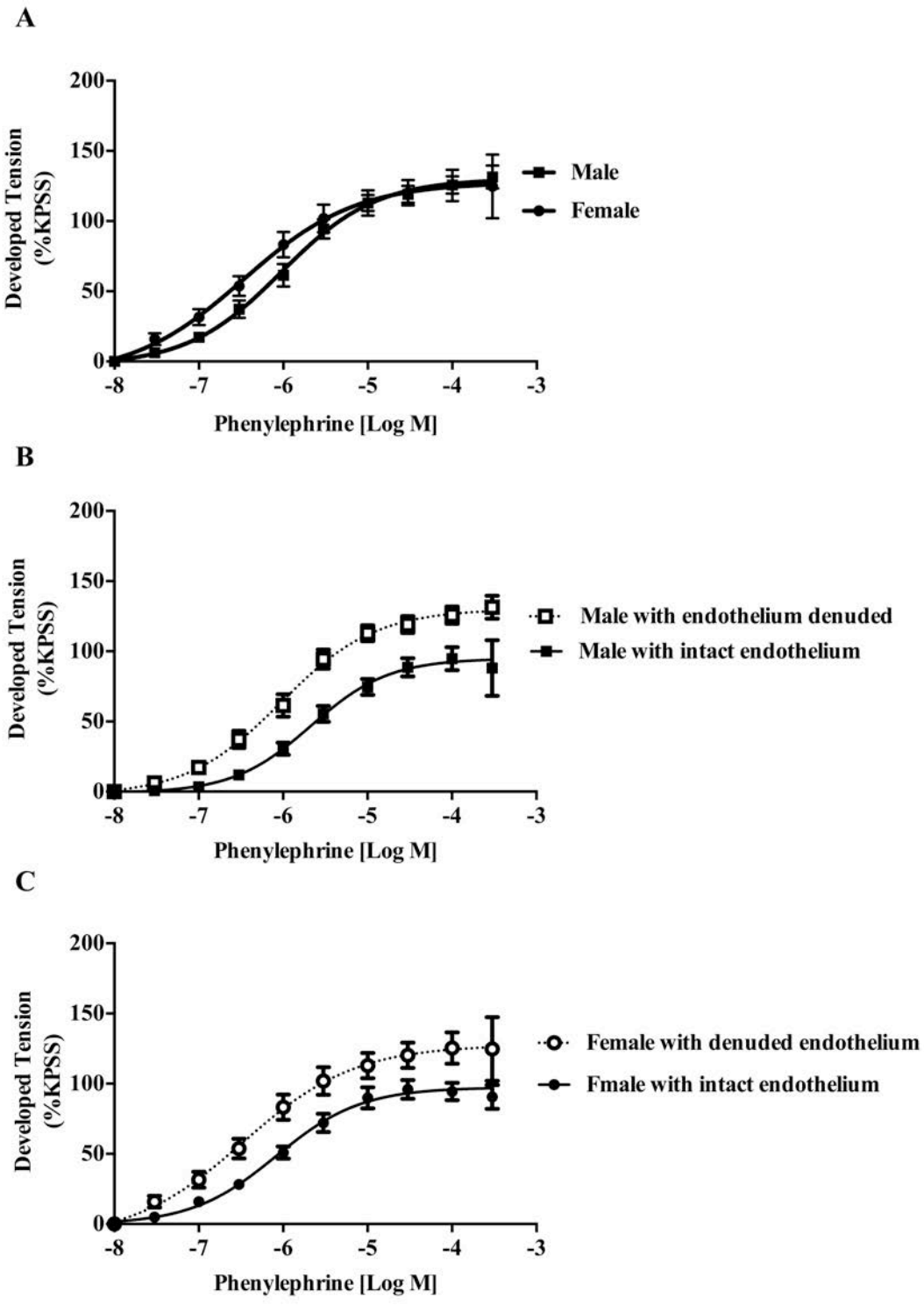


Figure 21: Female IMA hypersensitivity to PE is endothelium dependent. (A) Cumulative dose response curves to PE (n=10-12) in isolated rings of endothelium denuded human IMA. Endothelium removal abolished female IMA hypersensitivity to PE (Log EC_{50}: male= -6.08±0.12 and female= -6.65±0.27), with no change in

maximum response (E_{max}: male= 124.1 ±6.63 and female =127.4±10.10). Further analysis demonstrated that denudation (compared to intact vessel) increase sensitivity to PE (B&C). Values are expressed as mean ± SEM and are normalized to the maximal contraction to KPSS.

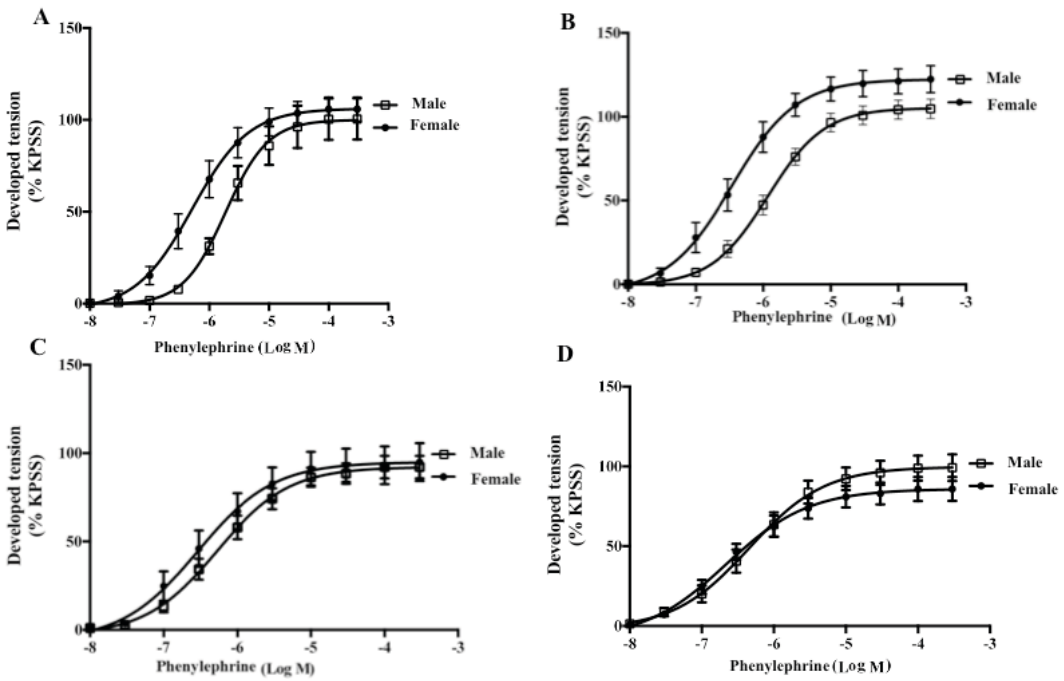


Figure 22: Female IMA hypersensitivity to PE is endothelium nitric oxide synthase (NOS) independent but dependent on cyclooxygenase (COX). Cumulative dose response curves to PE in isolated rings of human IMA. (A) Control IMA (n=11-16). Female IMA are hypersensitivity than aged male with no sex-difference in maximal contractile response (B) IMA in the presence of the NOS inhibitor (L-NAME, n=10-15). NOS inhibition did not influence female IMA hypersensitivity or maximal contractile response (C) IMA in the presence of the non-selective COX inhibitor (Indomethacin, n=9-13). COX inhibition abolished female IMA hypersensitivity with no difference in maximum. (D) A combination of COX and NOS inhibition abolished female IMA hypersensitivity with no difference in maximum contraction. Values are expressed as mean ± SEM and are normalized to the maximal contraction to KPSS.

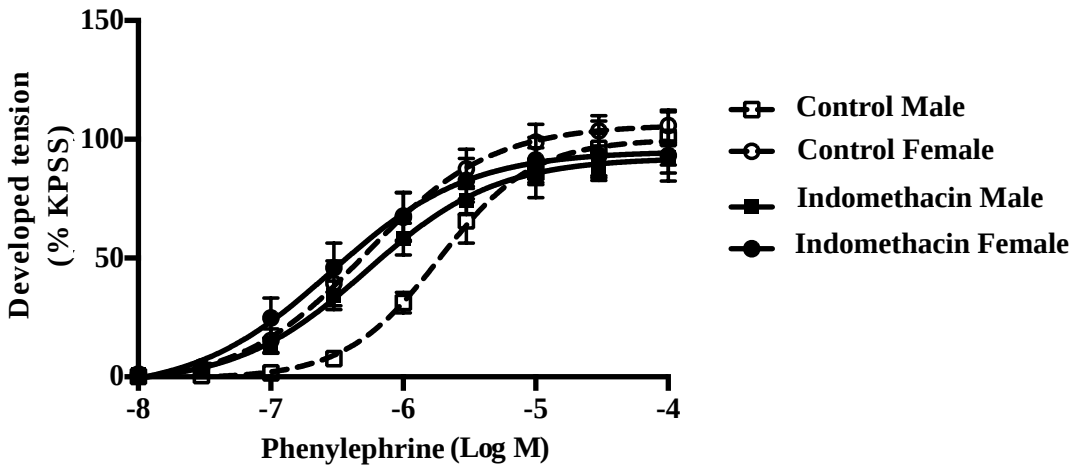


Figure 23: Individual vascular response with in sex with and without Indomethacin reveals a significant shift in the male but not the female PE concentration response.

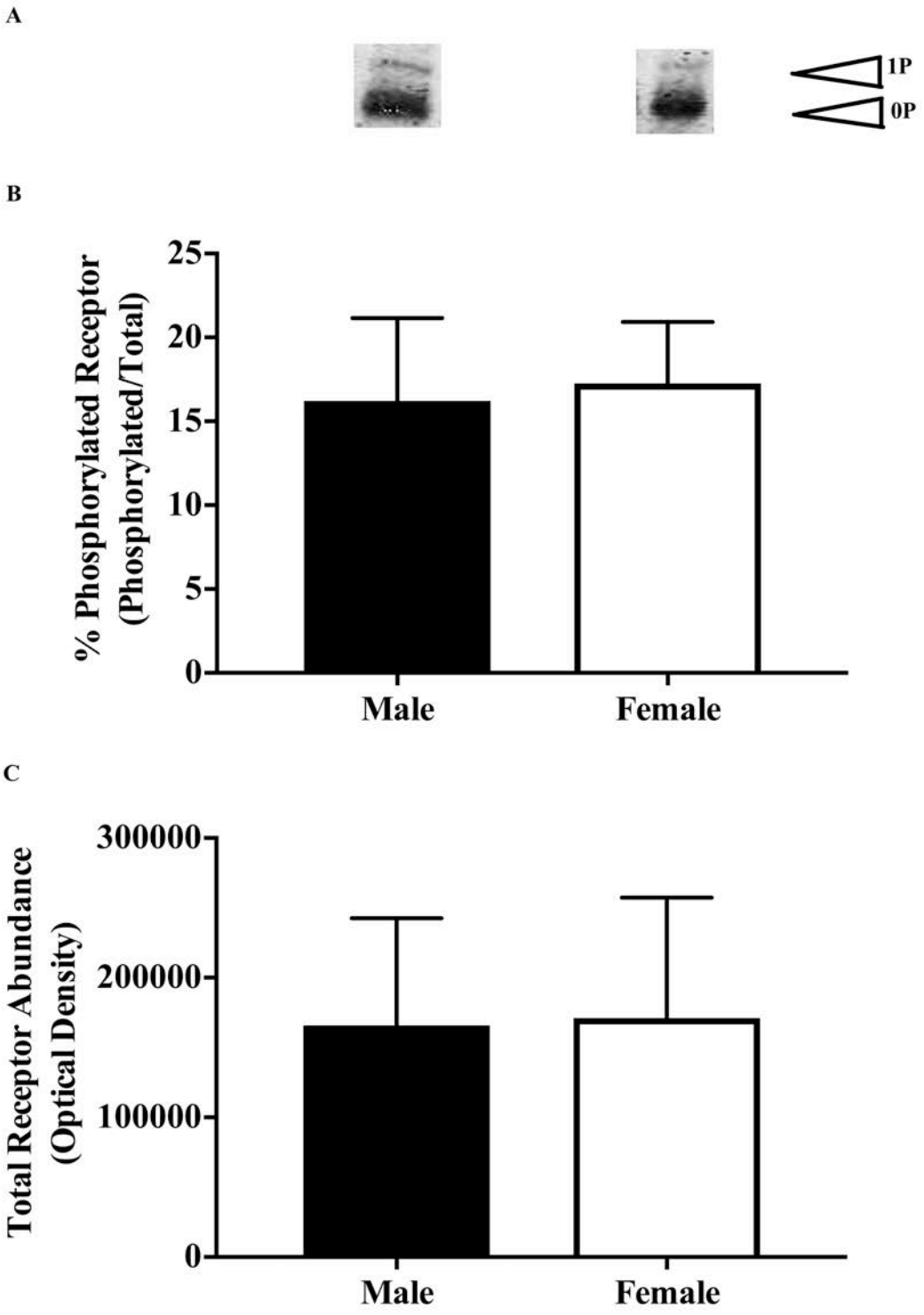


Figure 24: Female IMA hypersensitivity is independent of α_1-Adrenergic receptor activity. (A) Illustrate representative western blot of α_1-adrenergic receptor in the phosphorylated and un-phosphorylated state. (B) Illustrate cumulative data of the rate

of phosphorylated to total α_1-adrenergic receptor. (C) Illustrate total α_1-adrenergic receptor abundance in Male and Female IMA (n=5 females & 6 males).

Table 4. Clinical Characteristics and maintenance medications of Study Patients-1

	MALE (N=78)	**FEMALE (N=50)**
Age (mean years ± STDV)	67.7 ± 10.5	69 ± 10.1
BMI (mean ± STDV)	28.9 ± 5.3	30.4 ± 8.4
Hypertension (%)	49 (62.8)	33 (66.0)
Hypercholesterolemia	43 (55.1)	31 (62.0)
Diabetes Mellitus (%)	31 (39.7)	22 (44.0)
Current Smoker (%)	12 (15.4)	11 (22.0)
Anti-Platelets (%)	47 (60.3)	27 (54.0)
Statins (%)	64 (82.1)	36 (72.0)
Angiotensin Converting Enzyme inhibitor (%)	41 (52.6)	20 (40.0)
Angiotensin receptor Blocker (%)	14 (18.0)	17 (34.0)
Calcium Channel Blocker (%)	33 (42.3)	17 (34.0)
Beta-Blocker (%)	37 (47.4)	23 (46.0)
Long acting Nitrates (%)	33 (42.3)	19 (38.0)

Table 5: EC_{50} and corresponding E_{max} responses to phenylephrine (PE) and endothelin-1 (ET-1) in endothelial intact and denuded (E-) internal mammary artery and saphenous vein segments of male and female patient.

	No. of Subjects		EC_{50} (Log M)		E_{max} %	
	Male	Female	Male	Female	Male	Female
IMA						
PE	43	35	-5.83±0.06	-6.22±0.07**	105.9±6.81	112.2±9.44
Et-1	14	11	-7.94±0.06	-7.89±0.08	133.4±14.04	124.4±4.14
PE (E-)	12	10	-6.08±0.12	-6.65±0.27	124.1 ±6.63	127.4±10.10
SV						
PE	19	10	-5.63±0.11	-6.01±0.14*	107.4±8.09	99.89±6.80
Et-1	10	7	-7.52±0.14	-7.61±0.13	144.4±12.16	129.2±14.71

Values are mean ± SEM. EC_{50}, concentration of agonist producing 50% of the maximal contractile response; E_{max}, maximal contractile response presented as the % of KPSS (potassium physiological salt solution) mediated vasoconstriction. Mean values for PE EC_{50} are significantly different between sexes (*$p<0.05$, **$p<0.005$ for male vs. female).

Table 6: EC_{50} and corresponding E_{max} responses to phenylephrine (PE) in endothelial intact internal mammary artery segments of male and female patient pre-treated with L-NAME and indomethacin.

	No. of Subjects		PE EC_{50} (Log M)		PE E_{max} %	
	Male	Female	Male	Female	Male	Female
PE (µM)	15	11	-5.70±0.10	-6.24±0.14**	101.1±10.11	111.2±8.03
PE (L-NAME + Indomethacin)	15	10	-6.39±0.12	-6.66±0.16	100.4±7.30	87.44±7.48
PE (L-NAME)	16	11	-6.01±0.09	-6.47±0.11**	104.6±5.39	121.8±7.96
PE (Indomethacin)	13	9	-6.23±0.12	-6.48±0.14	92.54±5.93	100.2±10.93

Values are mean±SEM. (**p<0.005 for male vs. female). Data derived from figure 21.

Table 7: Multiple regression analysis using EC_{50} and E_{max} as dependent variables, sex as independent variable of IMA treated with phenylephrine

Sex + Confounder	With endothelium (p-value)		Without endothelium (p-value)	
	EC_{50}	E_{max}	EC_{50}	E_{max}
Age	0.327	0.362	0.027*	0.647
Hypertension	0.583	0.578	0.016*	0.611
Diabetes Mellitus	0.244	0.391	0.097	0.909
Hypercholesterolemia	0.159	0.320	0.060	0.760
Current Smoker	0.359	0.486	0.051	0.863
Ex-smoker	0.363	0.391	0.070	0.769
Aspirin	0.361	0.412	0.021*	0.975
Statin	0.359	0.461	0.043*	0.887
Beta-blocker	0.279	0.351	0.043*	0.814
Calcium channel blocker	0.159	0.328	0.058	0.751
ACE Inhibitor	0.282	0.343	0.076	0.788
Angiotensin Receptor Blocker	0.336	0.678	0.066	0.948
GTN	0.317	0.373	0.066	0.733
SSRI	0.338	0.374	0.045*	0.836
Body Mass Index	0.368	0.320	0.055	0.858

*$p<0.005$. Data derived from multiple regression, n: male=12 and female=10 for each parameter.

4. CHAPTER 4

Sex-differences in vascular reactivity of the internal mammary artery especially involving serotoninergic mechanisms

---

Statement of Authorship

TITLE of Paper	Mechanisms responsible for serotonin vascular reactivity sex-differences in the internal mammary artery
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Victor Lamin
Contribution to the Paper	Victor Lamin contributed to the following experiments: (1) Sample collection, (2) Contractile response to serotonin with endothelium (n=10-12), without endothelium (n=10-11), (3) the mechanism of serotonin response, L-NAME (n=7-10) and indomethacin (n=7-10), (4) Nitric oxide quantification using EPR (n=8), (5) quantification of prostanoids using LC-MS/MS (n=10-12), (6) Phos-tag™ western blot analysis and (7) writing the manuscript
Overall percentage (%)	90.1%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 17/10/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr. Amenah Jaghoori		
Contribution to the Paper	Dr. Amenah Jaghoori contributed the following experiments: (1) Contractile response to serotonin with endothelium (n=10-11), (2) thromboxane A2 (U46619, n=10-11)		
Signature		Date	1/11/16

Name of Co-Author	Rachel Jakobczak		
Contribution to the Paper	Rachel Jakobczak was involved in sample collection, experimental set up and vascular reactivity studies		
Signature		Date	17/10/2016

Name of Co-Author	Irene Stafford		
Contribution to the Paper	Irene Stafford was involved in mass spectrometry assay development and vascular reactivity experiments.		
Signature		Date	17.10.16

Name of Co-Author	Tamila Heresztyn		
Contribution to the Paper	Tamila Heresztyn was involved in mass spectrometry assay development		
Signature		Date	17.10.16

Name of Co-Author	Mr Michael Worthington		
Contribution to the Paper	Mr. Michael Worthington provided isolated vascular segments and reviewing the manuscript		
Signature		Date	4/11/16

Name of Co-Author	Mr Fabiano Viana		
Contribution to the Paper	Mr. Fabiano Viana provided isolated vascular segments and reviewing the manuscript		
Signature		Date	9/11/16

Name of Co-Author	Mr James Edwards		
Contribution to the Paper	Mr. James Edwards provided isolated vascular segments and reviewing the manuscript		
Signature		Date	9/11/16

Name of Co-Author	Mr Robert Stuklis		
Contribution to the Paper	Mr. Robert Stuklis provided isolated vascular segments and reviewing the manuscript		
Signature		Date	9/11/16

Name of Co-Author	Dr. David P. Wilson		
Contribution to the Paper	Dr. David Wilson participated in experimental design, data analysis and critical revisions of the manuscript.		
Signature		Date	28/11/16

Name of Co-Author	Professor John F. Beltrame		
Contribution to the Paper	Professor John F. Beltrame participated in experimental design, data analysis and critical revisions of the manuscript.		
Signature		Date	17/10/16

Please cut and paste additional co-author panels here as required.

4.1. ABSTRACT

Rationale: The increased adverse cardiac events in females undergoing coronary artery bypass grafting (CABG) is multifactorial and may include clinical, psychosocial and biological factors. A potential contributing biological factor could include vascular hyper-reactivity of the internal mammary artery (IMA) to endogenous vasoconstrictors in females resulting in a predilection to myocardial ischaemia.

Objective: This study evaluated sex-differences in serotonin and thromboxane A_2 mediated vasoconstrictor responses in human isolated IMA, with the mechanistic role of (1) the endothelium, (2) nitric oxide (NO), (3) prostaglandins (PG) and (4) receptor activity state investigated for any observed sex-difference.

Methods and results: Viable isolated human IMA segments were obtained from 116 patients (aged 67±2 years; 38% female) undergoing CABG. Cumulative concentration-response curves for the serotonin and thromboxane A_2 mimetic, U46619 were determined and revealed an increased sensitivity to serotonin but not U46619 in female IMA segments compared with males. These sex-differences to serotonin were further assessed by: (1) endothelial denudation, (2) endothelial nitric oxide synthase (NOS) inhibition of vascular responses and NO quantification using electron paramagnetic resonance (EPR), (3) cyclooxygenase (COX) inhibition of vascular responses and PG metabolite quantification using mass spectrometry, and (4) quantification of receptor abundance and phosphorylation status. The female hyper-reactivity to serotonin was (1) abolished by endothelial denudation, (2) unaffected by NOS inhibition, with no difference in EPR-assessed NO levels, (3) abolished by COX inhibition (4) unrelated to the abundance and phosphorylation of serotonin$_{2A}$ and serotonin$_{2B}$ receptors.

Conclusions: These data indicate that female IMA are hyper-reactive to serotonin but not U46619, with the former attributable to an endothelium-dependent COX pathway.

4.2. INTRODUCTION

Compared with males, females have poorer outcomes following coronary artery bypass grafting (CABG), including increased in-hospital mortality and hospital readmissions [21, 23, 469].

Although the mechanisms responsible for these sex-differences are likely to be multifactorial they may include clinical (for example, smaller vessel size),[47, 470] psychosocial (for example, depression),[445] and biological factors (for example, increase platelet activity and postmenopausal status)[449] in females compared to males. Biological factors may involve endothelial injury with the consequential platelet activation and aggregation, which are more evident in postmenopausal females. [166, 449]

Importantly, activated platelets liberate serotonin and thromboxane A_2, both of which are potent vasoactive compounds implicated in a number of clinical situations, such as angina, acute coronary syndrome, and vasospasm.[390, 471, 472] Serotonin and thromboxane A_2 function through their cell surface receptors (5-HT_{2A+2B} and TPα+β receptors) coupled to both $G_{q/11}$ and $G_{12/13}$ families of GPCR, which in turn activate sarcoplasmic reticulum calcium (Ca^{2+}) release, voltage-gated L-type Ca^{2+} entry, and increased Ca^{2+} sensitivity via Rho A-mediated activation of Rho kinases and PKC dependent activation of CPI-17 for serotonin but not thromboxane A_2 signalling [351] [302, 402] [363]. Therefore, vascular response to serotonin and thromboxane A_2 involves a balance of direct contractile effects mainly due to activation of their respective cell surface receptors on smooth muscle, balanced with endothelial activation and the release of endothelial-dependent vasodilator substances including nitric oxide (NO) and prostanoids.[473] [474] [475, 476]

The female sex hormone, oestrogen has been reported to regulate vascular physiology and function by modulating ion fluxes on smooth muscle cells and regulate endothelial-dependent vasodilator production and activity. [477, 478] As a result of the positive influence of oestrogen on the vasculature, menopause is therefore accompanied by increased systemic vascular sensitivity, increased platelet activity in female. [166, 449, 464][166, 449, 464] Thus it is hypothesized that postmenopausal females have increased vascular sensitivity to both serotonin and Thromboxane A_2 receptor activation than aged matched males. This differential response could be a potential contributing factor to the worsen outcome of post-menopausal female going through CABG.

The present study evaluates sex-differences in vascular response of human IMA graft conduit segments to serotonin and the thromboxane A_2. Potential mechanisms responsible for any observed sex-differences may involve the (1) endothelium, (2) nitric oxide (NO) pathway, (3) prostaglandin (PG) pathway, and/or (4) quantification of the specific vascular receptors.

4.3. METHODS

4.3.1. Patient Recruitment

The study was approved by the Royal Adelaide Hospital Human Ethics Committee on 28th April, 2009, with approval number RAH PROTOCOL NO: 090411, Title: The effect of selective/non-selective endothelin blockage on statin inhibition of endothelin vasoconstriction responses.

Patients scheduled for elective CABG consented pre-operatively for the use of their discarded IMA tissue. The patient's clinical history and medications were recorded. Anaesthesia was undertaken using standard cardiac protocols including fentanyl pre-medication, thiopentone induction, isofluorane maintenance anaesthesia and muscle relaxation with rocuronium. Surgery was undertaken via a median sternotomy approach with the left IMA harvested as a pedicle graft and the excess vessel length trimmed. The remnant IMA (generally discarded) was available for the experimental studies. Patients were grouped based on biological sex.

4.3.2. Study drugs and chemicals

Serotonin, U46619 (stable analogue of thromboxane A_2), A23187 (calcium ionophore), indomethacin, N-w-Nitro-L-arginine Methyl Ester (L-NAME), $FeSO_4$, diethyldithiocarbamate (DETC) and sodium nitroprusside (SNP) were all obtained from Sigma Chemical Company, St. Louis, MO. Other chemicals utilised and their source included: SDS, 1× Complete™ protease inhibitor cocktail from Roche, Mannheim, Germany; di-isopropylfluorophosphate from Sigma-Aldrich, Caste Hill, Australia; and Phos-tag™ Acrylamide from Wako Laboratory Chemicals, Japan. Prostanoids (5PGmix- containing: PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ and TXB_2), deuterated prostanoids ($PGF_{2\alpha}$ -d4) and DEA nonoate were obtained from Cayman

Chemical Co. (Ann Arbor, MI). All organic solvents for the mass spectrometry analysis were of liquid chromatography mass spectroscopy (LCMS) grade.

Serotonin was prepared by dissolving the compound in 0.1M HCl and further diluted in double distilled water to the required concentration. L-NAME was prepared in double distilled water, with U46619, indomethacin and A23187 prepared in 100% ethanol. The Krebs-bicarbonate solution contained (in mmol/L): NaCl 118, KH_2PO_4 1.18, KCl 3.89, $NaHCO_3$ 25, $MgCl_2$ 1.05, $CaCl_2$ 2.34, EDTA 0.01 and glucose 5.56, while the high K^+ (KPSS) solution replaced NaCl in normal Krebs with an equimolar amount of KCl (yielding a final KCl [121.89] mmol/L) both solutions were aerated with carbogen gas (95% O_2 and 5% CO_2 to maintain pH 7.4. All drugs were diluted to the required final concentration in physiological Krebs-bicarbonate solution previously aerated with carbogen gas (95% O_2 and 5% CO_2). The concentration of each drug was expressed as final concentration in μM.

4.3.3. Vascular Segment Preparation

IMA segments obtained during CABG were immediately placed in ice-cold Krebs-bicarbonate solution. Each artery segment was dissected free from perivascular fat and external loose tissues and cut into 3mm wide rings. Vascular reactivity was assessed using an organ bath preparation with the isolated IMA rings mounted on stainless steel hooks and the artery tension measured with a force transducer (from AD Instruments – Australia). The organ bath preparation utilised a 15ml water-jacketed organ bath containing carbogen-bubbled Krebs solution. Isometric tension was recorded using Lab chart 6 software (from AD Instruments – Australia) with the system being calibrated daily. Arteries were equilibrated at a resting tension of 19.6 mN for 60 minutes at 37°C before undergoing KPSS- dependent contraction three times. Upon reaching a plateau, the high K^+ buffer was washed-out 3 times and the

segments allowed to equilibrate for 30 minutes. This step was repeated three times and arteries were subjected to cumulative doses of either serotonin (0.001 μM - 300 μM) or thromboxane A_2 receptor agonist (0.0001 μM – 300 μM). Following the maximum agonist-dependent response, arteries were placed in normal Krebs for 60 minutes and subsequently contracted to an EC_{70} dose of the agonist (a concentration that produced 70% maximum constriction to that agonist). As previously described, the calcium ionophore (A23187) was used at a concentration of 2 μM, to activate endothelial Ca^{2+} entry and verify endothelium-dependent vasodilatation.[411] All arteries treated with papaverine hydrochloride during surgery or that produced a force less than 19.6 mN when submaximally contracted with KPSS solution were considered to be damaged during isolation and excluded from the study.

4.3.4. Mechanistic Studies.

Vasoconstrictor agonists producing a sex-difference in vascular responses underwent investigation to elucidate the underlying vascular mechanism including the role of the endothelium, NO pathway, PG pathway and vascular receptors.

4.3.4.1. The Endothelium.

Endothelial denudation was performed by mechanical luminal shear abrasion as previously described. [412] Briefly, infusion of an effervescent solution consisting of air and physiological Krebs buffer was injected through the lumen of the artery, moving the tip of the catheter up and down for 90 seconds in a petri-dish to ensure complete removal of the endothelial layer.

4.3.4.2. Nitric Oxide (NO) Pathway.

The role of NO on sex-differences in vascular contractile responses was assessed by (a) NOS inhibition, following the 3rd KPSS stimulation and the 30-minute

equilibration, the vessels were incubated with nitric oxide synthase blocker L- NAME (300μM) for 60-minutes followed by cumulative dose response with serotonin and (b) electron paramagnetic resonance (EPR) - spin trapped based quantification of stimulated NO release in endothelium-intact IMA segments. Specifically, NO was spin-trapped from IMA segments in response to the addition of SNP and the calcium ionophore A23187 (10μM) (n=8). The spin trap included Fe (II) dithiocarbamate complexes containing diethyldithiocarbamate (Fe $(DETC)_2$) prepared by dissolving 0.45mg/ml $FeSO_4$ or 0.72 mg/ml DETC in 0.9% NaCl bi-distilled water (EPR-grade) and aerated with nitrogen gas > 20 min on ice. Three IMA segments (each 3mm in length) were pooled placed in Krebs/HEPES buffer prior to the addition of stimulus or inhibitors and incubated with the spin trap (equal volume of FeSO4 and DETC) complex for 120 min at 37 ° C. Signals were measured using ESR-Spectrometer e-scan equipped with Temperature & Gas Controller, and Shear Stress Controller-Noxygen Science Transfer & Diagnostics GmbH Bruker Bio Spin Corp. A triplet EPR spectrum of $(DETC)_2$-Fe (II)-NO with a (N) = 12.8 gauss and g = 2.04 was observed, a characteristic signal for NO in the above spectrum.[413] The (DETC)(2)-Fe (II)-NO signal in the IMA segment was markedly suppressed by endothelium denudation and pre-administration of the nitric oxide synthase (NOS) inhibitor, (L-NAME) in rat aorta, confirming that the NO detected from the artery was produced enzymatically by NOS. Calibration solutions of DEA nonoate were prepared at final concentrations 2 μM, 4 μM, 8 μM and 16 μM and the signal were measured as NO concentrations/double integral.

4.3.4.3. Prostaglandin (PG) Pathway

The role of the PG pathway on sex-differences in vascular responses was assessed by (a) Cyclooxygenase inhibition, following the 3rd KPSS stimulation and the 30-minute

equilibration, the vessels were incubated with cyclooxygenase inhibitor indomethacin (10μM) for 60-minutes followed by cumulative dose response with serotonin and (b) quantification of stimulated prostaglandin release in endothelium-intact IMA segments.

Prostaglandin metabolite levels of 6-keto $PGF1_{1\alpha}$, TXB_2, $PGF_{2\alpha}$, PGD_2 and PGE_2 were quantified in the IMA segment by liquid chromatography/electrospray ionization tandem mass spectrometry, following activation of calcium entry using the calcium ionophore A23187 (2μM) (n=10-12), as previously described by Furugen et al, (2011).[414] PGs were isolated from 96-350mg IMA segments ground under LN_2 and solubilize in 2 ml PG homogenizing buffer containing (1X PBS, 1 mM EDTA, 10 μM indomethacin, pH 7.4) in a pre-coated tube with 2mg butylated hydroxytoluene (BHT). Samples were further sonicated for 30 seconds and followed by the addition of 200 $ngml^{-1}$ of internal standard $PGF_{2\alpha}$ -d4. Following the addition of 1ml acetone and a 2 minute vortex samples were allowed to stand on ice for 20 minutes and centrifuged at 3000 × g for 15 min at 4 ◦C. The supernatant was transferred into fresh tube and the acetone layer evaporated nitrogen gas. pH was adjusted to 3 prior to solid-phase extraction. HLB column was used in solid-phase extraction conditioned with 3ml methanol and 2% formic acid. Samples were washed with 3ml solution containing 5% methanol, 2% formic acid, followed by 3ml 25% methanol and 3ml hexane before eluting with 2 ml LCMS grade methanol and dried under nitrogen gas. Samples were re-constituted in 100μl 25% acetonitrile. Calibration curves of for the prostaglandins were linear in the range from 0.2 to 50 $ngml^{-1}$ ($r2 > 0.99$). Calibration curves were constructed by plotting peak area ratio (exogenous standard to internal standard) versus the nominal concentration and were fitted using least-squares regression.

4.3.4.4. Vascular Receptors.

Vascular receptors were quantified in relation to both total abundance and phosphorylation status. Mn^{2+} Phos-tag™ phosphorylation-dependent mobility shift analysis was used to quantify total and phosphorylated protein expression of serotonin$_{2A}$ and serotonin$_{2B}$ receptor subtypes.

Proteins were extracted from each 3 mm IMA rings using 2x Laemmli sample buffer (containing 65.8mM Tris-HCl, 26.3% (w/v) glycerol, 2.1% SDS and 0.01% bromophenol blue), followed by SDS PAGE, coomassie stained, and the entire lane of protein for each sample quantified as an index of extractable vascular proteins and adjusted the concentration to be equal for all arteries. Following normalization of protein levels, samples were subject to a Phos-tag™ SDS-PAGE containing 7.5% polyacrylamide gel with 25 µM Phos-tag™ and 50 µM $MnCl_2$ at 150 V for 90 min, as described previously by Tomida et al, (2008). [(415)] Proteins were transferred, using the BioRad Transblot® Turbo system™, onto 0.22 µm PVDF at 25 V and 1.3 amps for 7 min. Non-specific binding sites were blocked with 3% non-fat dry milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% (*v*/*v*) Tween-20) for 60 minutes, followed by incubation with TBS-T containing a 1:1000 dilution of either a mouse-derived affinity-purified anti-serotonin$_{2A}$ or rabbit-derived serotonin$_{2B}$ antibody (Santa Cruz Biotechnology) for 60 minutes. PVDF membranes were washed four times in TBS-T and incubated for 60 minutes in TBS-T containing a1: 10000 dilution of anti-mouse (for the 2A receptor) or anti-rabbit (for the 2B receptor) IgG coupled with Dylight™ 800 fluorochromes (Pierce Thermo Scientific, Rockford, IL, USA)

4.3.5. Data analysis

The data are expressed as mean ± SEM. In all experiments, n refers to the number of patient from which IMA were taken. Concentration-response curves to serotonin and U46619 dependent-contraction were obtained. Contractile responses were expressed in milli-Newton (mN) normalized with the corresponding KPSS response for that artery, to correct for any small differences in vessel length and diameter. Relaxation responses were expressed as the percentage reduction in the agonist-induced contraction by the calcium ionophore A23187 (2 µM). Maximal responses (E_{max}) and the effective concentration that elicited 50% of the maximal response (EC_{50} expressed as log EC_{50}) obtained from concentration–response curves by non-linear curve fitting using Graph pad Prism 6 (San Diego, CA). EC_{50} values were expressed as µM. Statistical analysis has been made using student t-test. Differences are considered significant at $p < 0.05$.

4.4. RESULTS

A total of 116 patients (38% female) with a mean age of 67±2 years were recruited with viable IMA segments. The clinical characteristics of these patients, including their cardiovascular risk factors and regular maintenance medications prior surgery, are recorded in Table-7. There were no differences between males and females in these clinical characteristics, nor was there a difference in the maximum contraction to high K^+ solutions for both intact and endothelium-denuded IMA (intact: male= 23.32 ± 1.92 mN and female= 25.31± 2.54 mN, n=41-52, denuded: male= 21.22 ± 2.69 mN and female= 22.69± 1.93 mN, n=21-27, p=0.35 Figure 25). Of importance, all females recruited to this study were post-menopausal.

4.4.1. Vascular Reactivity to Serotonin & U46619

In the endothelium intact human IMA segments, females exhibit increased sensitivity (Log EC_{50}: male = -6.29 ± 0.07 and female = -6.83 ± 0.09, p =0.001, n=20-21), with no sex-difference in maximal contraction (E_{max}: male= 88.27 ± 6.14 and female=97.80 ± 6.18, p=0.281, n=20-21 Figure 26A) to serotonin.

In contrast, there was no sex-difference in IMA responses, both in vascular sensitivity (Log EC_{50}: male = -8.20 ± 0.08 and female = -8.48 ±0.11, p =0.52, n=9-10) and maximum contraction (E_{max}: male= 179.6 ± 17.67 and female=155.2± 8.80, p=0.218, n=10-11 figure 26B) to thromboxane A_2.

Accordingly, further experiments were undertaken to evaluate the mechanisms responsible for the sex-differences in serotonin responses but no further investigation of the thromboxane A_2 mimetics response were performed.

4.4.2. Mechanisms for Sex-difference in Serotonin Vascular Reactivity

4.4.2.1. The role of endothelium in sex-dependent vascular reactivity to serotonin.

Following endothelial denudation, the sex-difference in vascular sensitivity observed with serotonin in endothelium-intact vessels was abolished (Log EC_{50}: male= -7.04 ± 0.13 and female= -7.02 ± 0.11, p =0.92, n=10-11), with no change in maximal response (E_{max}: male= 125.3 ± 9.03 and female =124.9 ± 19, p =0.98, n=10-11 Figure 27A). Hence the sex-difference in serotonin responses is endothelium-dependent. As expected, denudation produced an increased sensitivity to serotonin in both males and females relative to the intact preparation. (Figure 27B&C).

Furthermore, multiple regression analysis reveals no association between sex, $EC_{50,}$ E_{max}, risk factors medication for both the intact and denuded IMA. (Table 9)

4.4.2.2. The role of NO in endothelial sex-dependent vascular reactivity to serotonin

Serotonin concentration response curves (0.001 μM - 300 μM, n=7-10) were generated in the presence of L-NAME in an endothelium intact human IMA. The NOS inhibition did not affect the sex-difference in IMA sensitivity to serotonin (Log EC_{50}: male= - 6.60 ± 0.14 and female= -7.52 ± 0.24, p = 0.003. n=7-10), or maximal contractile response (E_{max}: male= 130.7 ± 13.38 and female=126.4 ± 12.20, p=0.82, n=7-10 figure 28A). Furthermore, direct measurement of NO release via EPR, showed no sex-difference in response to SNP (total moles of NO generated: male=0.26 ± 0.06 μM and female= 0.22 ± 0.05 μM, p = 0.66 n=8 figure 28B). These NOS inhibition and EPR measured NO release experiments provide supporting

evidence that the endothelial NO pathway does not contribute to the endothelium-dependent sex-difference in serotonin responses.

4.4.2.3. The role of PG in endothelial sex-dependent vascular reactivity to serotonin

The effect of indomethacin on serotonin concentration-response curves (0.001 μM - 300 μM, n=6-9) was assessed on endothelium-intact human IMA segments. Indomethacin abolished the female IMA hypersensitivity (Log EC_{50}: male= - 6.80 ± 0.18 and female= - 6.79 ± 0.48, p=0.97), with increase female maximum contractile response (E_{max}: male= 133 ± 9.0 and female= 144.9 ± 10.37, p = 0.01, figure 29A) to serotonin. Furthermore, individual analysis within sex in the presence or absence of the COX inhibitor indomethacin revealed a significant shift in the male but not the female serotonin concentration response (Figure 29B).

Direct PG metabolite measurement by mass spectrometry revealed no difference in the $PGF_2\alpha$ metabolite level (male= 0.015 ± 0.004 $ngmg^{-1}$ and female= 0.011 ± 0.004 $ngmg^{-1}$, p=0.56 n=10-12 figure 30A), with a comparable trend towards significance (due to insufficient samples) of reduced 6-keto $PGF_1\alpha$ in female IMA (male= 0.035 ± 0.001 $ngmg^{-1}$ and female= 0.017 ± 0.004 $ngmg^{-1}$, p = 0.08 n=10-12 figure 30B), these data suggest that female IMA's produce less prostacyclin compared to males but comparable amounts of the $PGF_2\alpha$ vasoconstrictor.

4.4.2.4. Quantification of total serotonin$_{2A\ +2B}$ abundance and phosphorylation status

Phos-tag™ coupled with western blot analysis revealed equal phosphorylation status and abundance of both serotonin$_{2A}$ receptor (phosphorylation: male= 39.90 ± 12.35 and female= 33.26 ± 6.39, p=0.66, and abundance: male= 2.3×10^4 ± 7.4×10^3 and

female= $2.2\times10^4 \pm 4.5\times10^3$, p=0.99, n=5-6 Figure 31A, C and E) and serotonin$_{2B}$ receptor (phosphorylation: male= 22.93 ± 3.3 and female= 29.32 ± 3.96,p=0.24 and abundance: male= $2.2\times10^6 \pm 4.1\times10^5$ and female= $2.1\times10^6 \pm 5.5\times 10^5$, p=0.87, n=5-6 Figure 31B, D and F) receptor isoform.

4.5. DISCUSSION

We report several novel findings in this study. First, female IMA are hypersensitive to serotonin but not U46619, suggesting that sex-dependent female IMA hypersensitivity is agonist specific. Second, the mechanism responsible for this sex-difference in vascular reactivity to serotonin involves endothelium-dependent factors that are independent of vascular NOS but dependent upon the vascular cyclooxygenase pathway, and unrelated to vascular serotonin receptor abundance or phosphorylation. This endothelium-COX mediated female IMA hypersensitivity to serotonin could be an important biological mechanism precipitating peri-operative graft spasm in females resulting in major adverse cardiac events post CABG.

It is widely recognize that both 5-HT and U46619 can stimulate the synthesis and release of the endogenous PGI_2.[479-483] However, there was no sex-difference in vascular reactivity to U46619. The possible mechanism responsible for the agonist specific response of IMA from postmenopausal females resulting in the no-sex difference in U46619 may be as a result of the cross regulation between U46619 and the PGI_2 release by the endothelium.

Although we do recognize the limitation in cell culture experiment, however, previous research involving bovine aortic endothelial cells initial exposure to U46619 at low concentration leads to the induction of PGI_2 that causes a feedback inhibition of further PGI_2 synthesis.[484] Thus, this it suggests that cumulative dose response with U46619 will leads to a significant decrease in PGI_2 production in this *in-vitro* setup through feedback inhibition mechanism similar to the effect of indomethacin blocking of the male IMA PGI_2 production, making them to behaves live female, masking the COX-mediated sex-difference in vascular reactivity to the agonist. However, this pathway needs further investigation in the context of human IMA.

Few studies have examined sex differences in responses to serotonin and/or thromboxane in an isolated vessel preparation. Nuno *et al.*, (2007) [409] and Lamping *et al.*, (2001) [485] previously revealed male hypersensitivity to serotonin but not thromboxane A_2 in mice. However, no sex-difference serotonin responses were observed in studies utilising porcine vessels.[486, 487] This suggests species-specific, sex-differences in vascular responses to serotonin. In human IMA, a previous study revealed increased female maximum contraction to serotonin, but no female IMA hypersensitivity was observed.[405] The lack of female IMA sensitivity may be due to experimental preparation influencing the findings, since the present study excluded samples that had papaverine administered by the surgeons that could potentially blunt the vasoconstrictor responses, whereas it is unclear if this has been undertaken in the previous study. To the best of our knowledge, no studies have further dissected the mechanism responsible for the differential response to serotonin in human IMA.

It is well known that oestrogen can promote vascular tone by potentiating the actions of endothelium nitric oxide and prostacyclin.[488] We did not assess pre-menopausal vs menopausal differences in vascular reactivity as it was beyond the scope of this thesis. Our data at suggests no fundamental difference in nitric oxide production between male and female at an average aged of 66 years. This is not surprising as the human IMA is an outstanding conduit of choice for CABG, Unlike saphenous vein graft, which has a median patency of 7 years, more than 90% of IMA grafts are patent at 15 years and this in part is attributable to its endothelial function. [433]

The observed sex-difference in vascular response to serotonin is COX-dependent. Within sex analysis, in the presence or absence of the COX inhibitor-indomethacin, revealed a significant shift in the male but not the female serotonin concentration response in the absence of the endothelium (Figure 29B). This suggests that the

abolition of the serotonin sex-difference is due to the males becoming more sensitive to serotonin (i.e. becoming more like females) in the presence of indomethacin. Hence indomethacin may block a male endothelium-dependent vasodilatory prostanoids that accounts for the sex-difference in serotonin responses.

Our cumulative data quantifying vascular prostacyclin metabolite level in the IMA of male and female, reveals a p-value =0.08. However, it is important to note that although we often conveniently use $p \leq 0.05$ to report a difference, it is well recognize that this is an arbitrary figure that carries no information about the magnitude of an effect, which is captured by the effect size and confidence interval. Therefore, the possible increase PGI_2 in males compared with their aged matched females is a clinically relevant finding, suggesting an increased role of prostacyclin in the maintenance of vascular tone in males [(489)]; perhaps as a result of the impaired bioavailability of NO which is evident in coronary artery disease patients.

Prostacyclin is a potent vasodilator that can also limit the activation and aggregation of platelets, prevent atherogenesis and thrombogenesis and prevent the local release of platelet-derived vasoactive mediators [(490)]. Sex-differences in the production and effects of vascular prostacyclin with regards vasomotor regulation have been less investigated. Steinleitner *et al.*, 1989 reveals decrease arterial production of prostacyclin in postmenopausal female compared to their premenopausal counterpart [(491)], suggesting the positive role of oestrogen in mediating vascular tone in premenopausal female.[(488)] Batres *et al.*, 1986 reported decrease prostacyclin in female in vein umbilical cord compared to male [(492)], which implies that the ability to produce prostacyclin in different vascular beds in male and female not only manifested in old age but also during childhood.

In an eNOS knockout model, arterial ACh- dependent relaxation was unaffected by indomethacin in female but greatly reduced in male [493, 494], with increase plasma level of prostacyclin compared to female [493], suggesting that COX activity, to an extent, compensates for lack of NO only in male compared to female [493]. This might be responsible for the increase vascular sensitivity in postmenopausal female compared to their aged match male.

In addition, impaired prostacyclin production has been reported in disorder such as pulmonary hypertension. [292, 293] As a result of the existing sex difference in prostacyclin production, there is the existence of sex difference in incidence and prevalence of this impaired prostacyclin related condition, with female sex associated with increased incidence of pulmonary hypertension [495] compared to their male counterpart. Therefore, the possible decrease in prostacyclin production in the endothelium of female IMA might predispose the IMA graft conduit to post-operative spasm, and might eventually contribute to their worsen outcome following CABG.

4.6. STUDY LIMITATIONS

The above studies were undertaken in an *ex-vivo* preparation that allowed the investigation of vasomotor responses in isolated vessels to individual agonists. This may differ to the *in-vivo* setting, where platelets, circulating hormones and homeostatic mechanisms may influence the vascular response. Furthermore, vascular prostanoids metabolite such as prostaglandin E2 (PGE_2), prostaglandin D2 (PGD_2), and thromboxane B2 (TXB_2) were below the detection limit of our LC-MS-MS, as this would have been important to evaluate the ratio between constrictor and dilatory prostanoids in the IMA of males and females. Furthermore, the relative role of oestrogen was not explored since only post-menopausal women could be recruited in this study.

4.7. CONCLUSION

This study identified biological sex differences in vascular reactivity of the IMA to serotonin but not U46619, with the former attributable to an endothelium-dependent COX pathway involving possible impaired PGI_2 productions in females. This sex difference may predispose women to developing IMA graft spasm when exposed to platelet-derived serotonin and potentially contribute to the increased post-CABG adverse cardiac events experienced by women.

4.8. LIST OF FIGURES AND TABLE

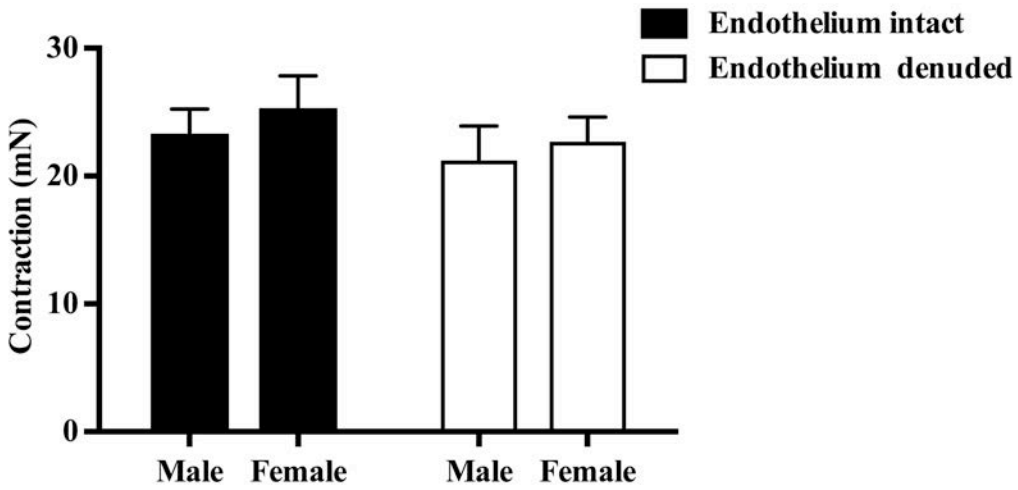


Figure 25: High K^+ -mediated vasoconstriction reveals no sex-difference with regards intact and denuded IMA (n=21-54, p=0.35).

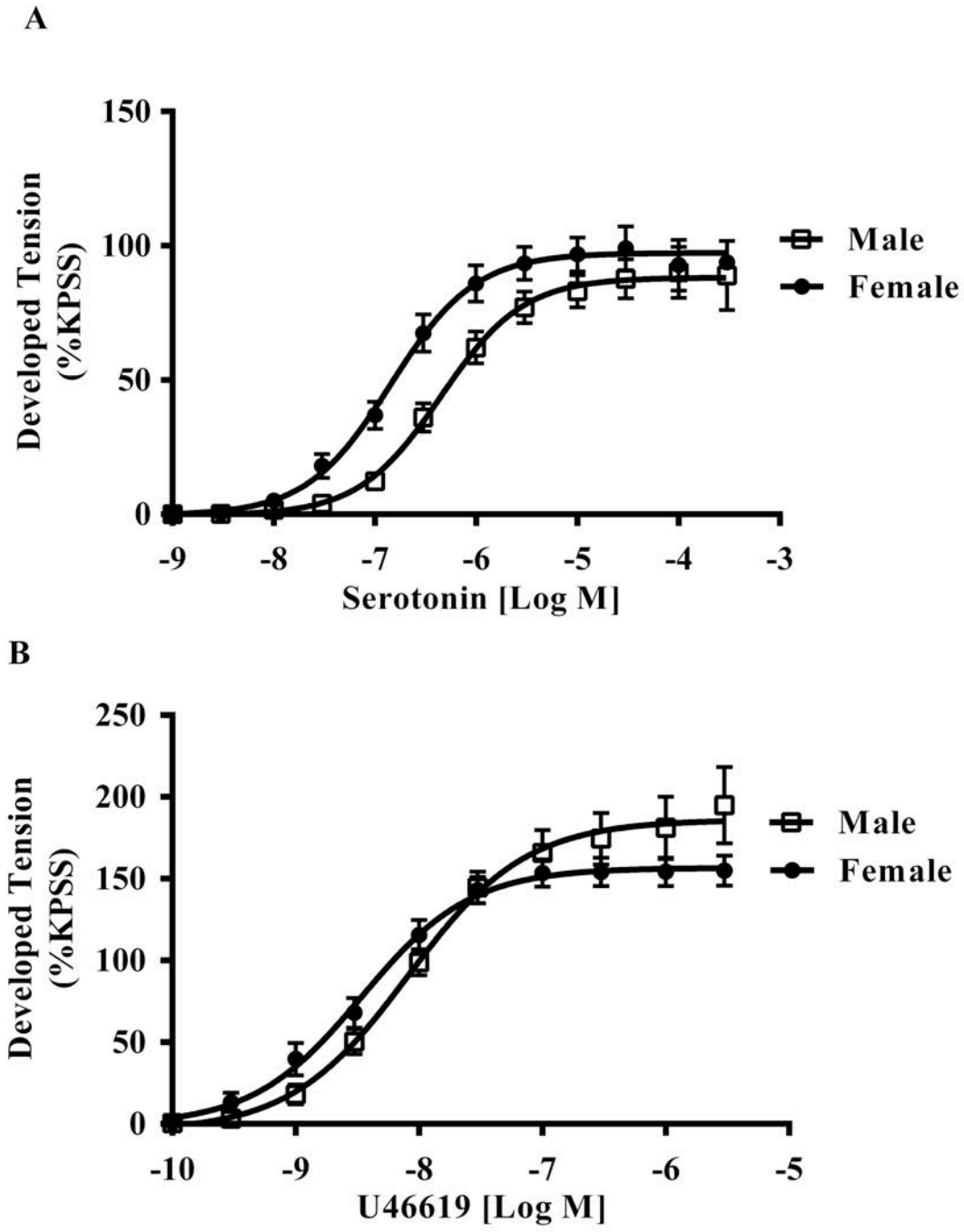


Figure 26: Female IMA are hypersensitive to serotonin but not U46619. (A) Cumulative dose response curves to serotonin (n=21 females & 22 males) in isolated rings of human IMA. Females exhibit increase sensitivity to serotonin than aged matched males (p=0.01), with no sex-difference in maximal contraction (p=0.281, see text for details). (B) Cumulative dose response curves to U46619 (n=9-10) in isolated

rings of human IMA. No sex-differences were observed for both sensitivity ($p = 0.52$) and maximum response ($p = 0.218$) to U46619.

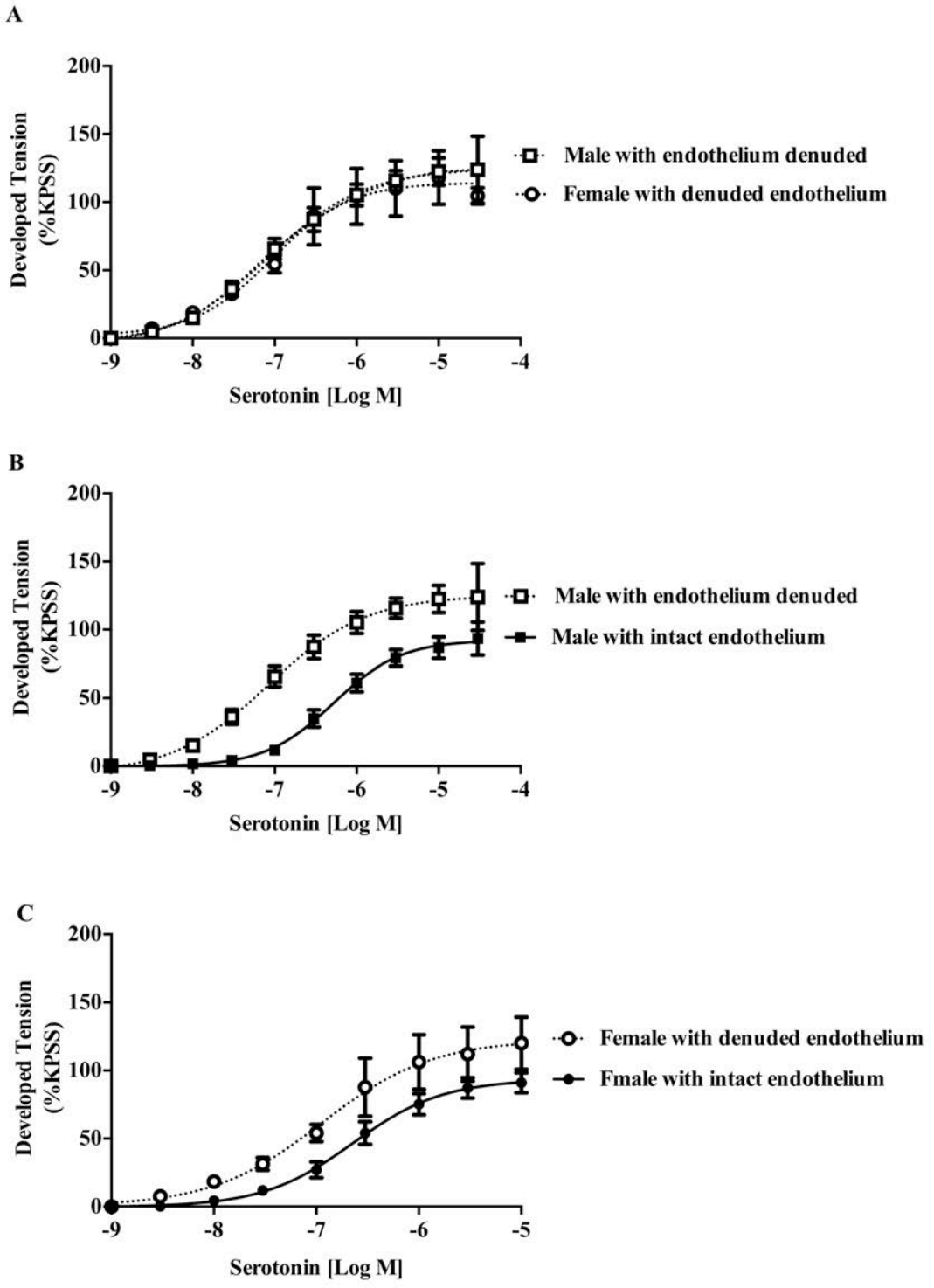


Figure 27: Female IMA hypersensitivity to serotonin is endothelium dependent. Cumulative dose response curves to serotonin (n= 10 females & 11 males) in isolated rings of endothelium denuded human IMA. Endothelium removal abolished female IMA hypersensitivity (p =0.92), with no change in maximum response to serotonin (p

=0.98). Further analysis demonstrated that denudation (compared to intact vessel) increase sensitivity to serotonin (B&C).

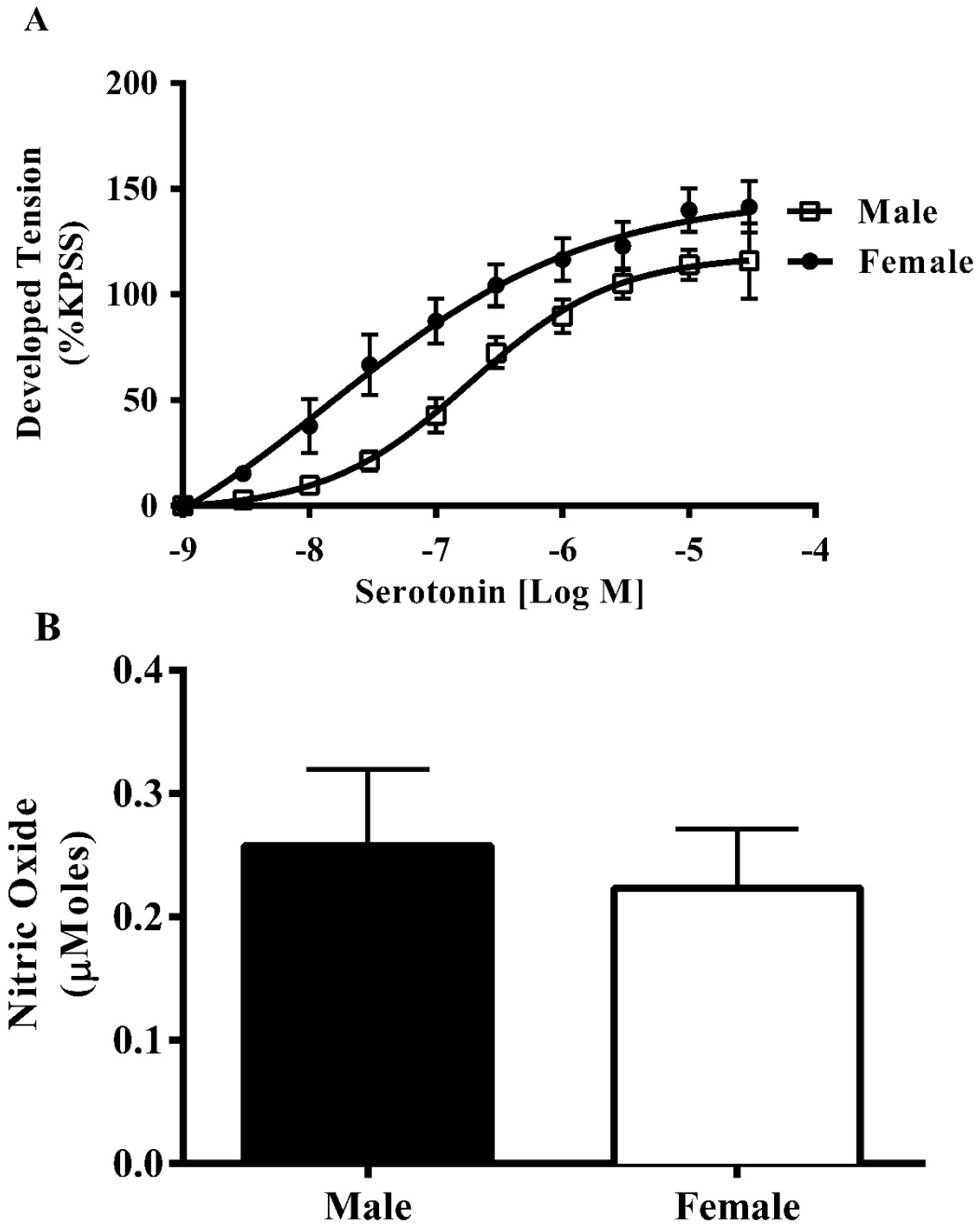


Figure 28: Female IMA hypersensitivity to serotonin is NOS independent. (A) Cumulative dose response curves to serotonin in isolated rings of human IMA (n= 7 females & 10 males) in the presence of the NOS inhibitor (L-NAME). Compared with controls (Figure 1A), NOS inhibition did not influence female IMA hypersensitivity ($p = 0.003$), or maximal contractile response to serotonin ($p = 0.82$).

(B) Nitric Oxide quantification in isolated rings of human IMA in the presence of SNP reveals no sex-difference in NO production in an ex-vivo setup (n= 8 females & 8 males, $p = 0.66$).

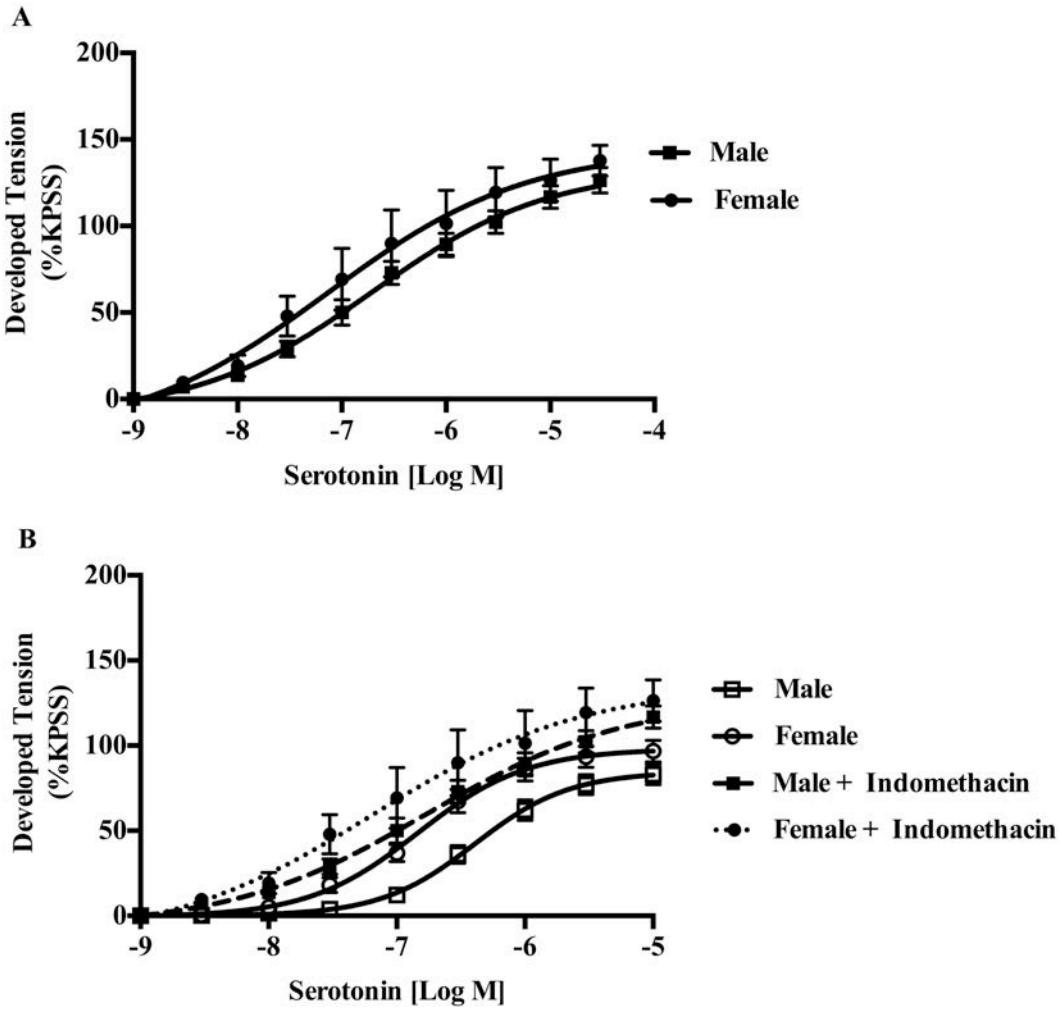


Figure 29: Female IMA hypersensitivity to serotonin is COX dependent with a significant shift in the male curve in the presence of indomethacin**.** (A) Cumulative dose response curves to serotonin (n= 6 females & 9 males) in isolated rings of human IMA in the presence of the non-selective COX inhibitor (Indomethacin). COX inhibition abolished female IMA hypersensitivity (p=0.97), with increase female maximum response (p=0.01) to serotonin. (B) Evaluation of the male and female responses with/without Indomethacin reveals a significant shift in the male but not the female serotonin concentration response (data derived from Figure 2A, Figure 3 and Figure 5A, n=6=22).

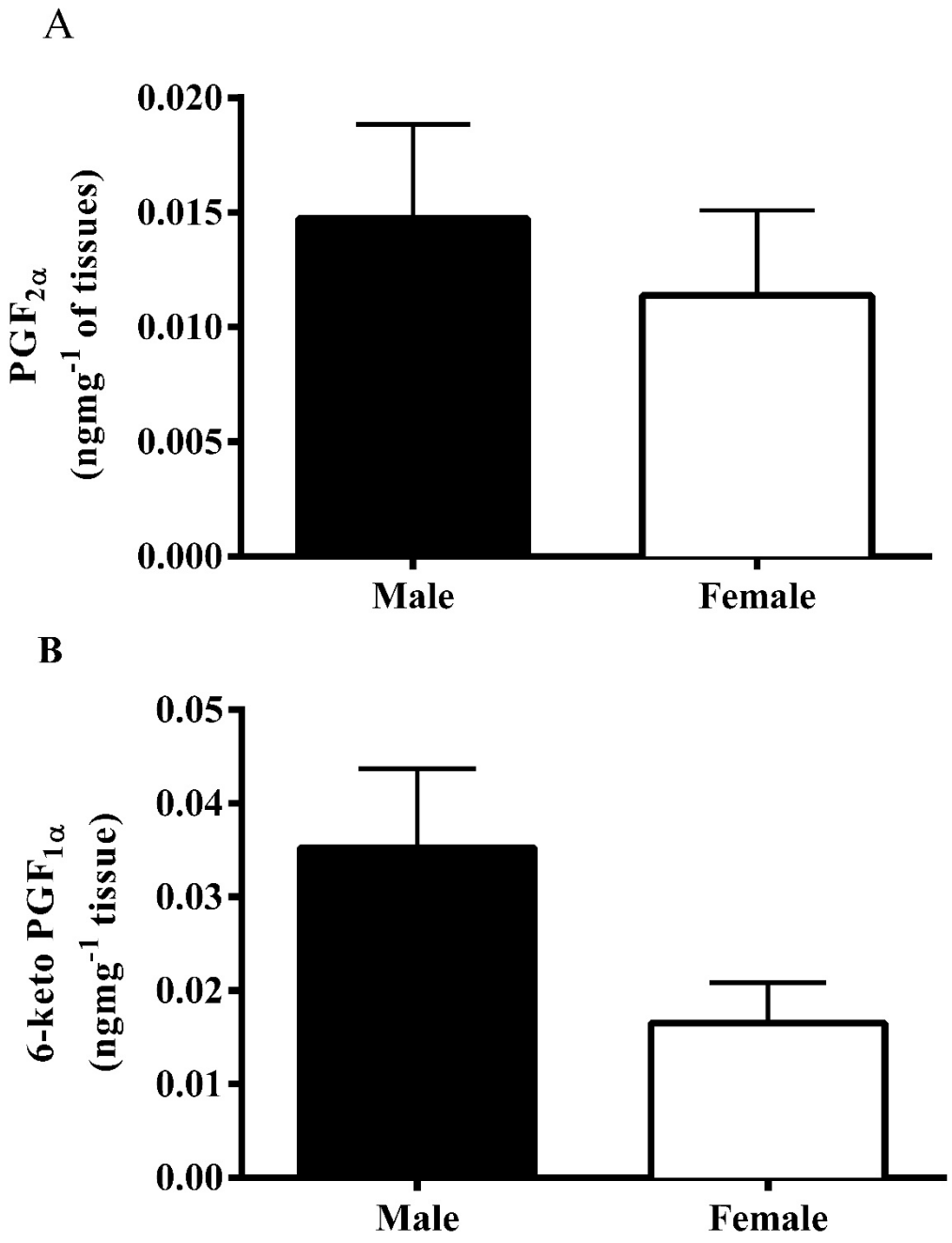


Figure 30: Male and female IMA expresses comparable amount of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$. (A) Quantification of the $PGF_2\alpha$ vasoconstrictor metabolite (p=0.56) and (B) the 6-keto $PGF_1\alpha$ vasodilatory metabolite (p=0.08, n= 10 females & 12 males).

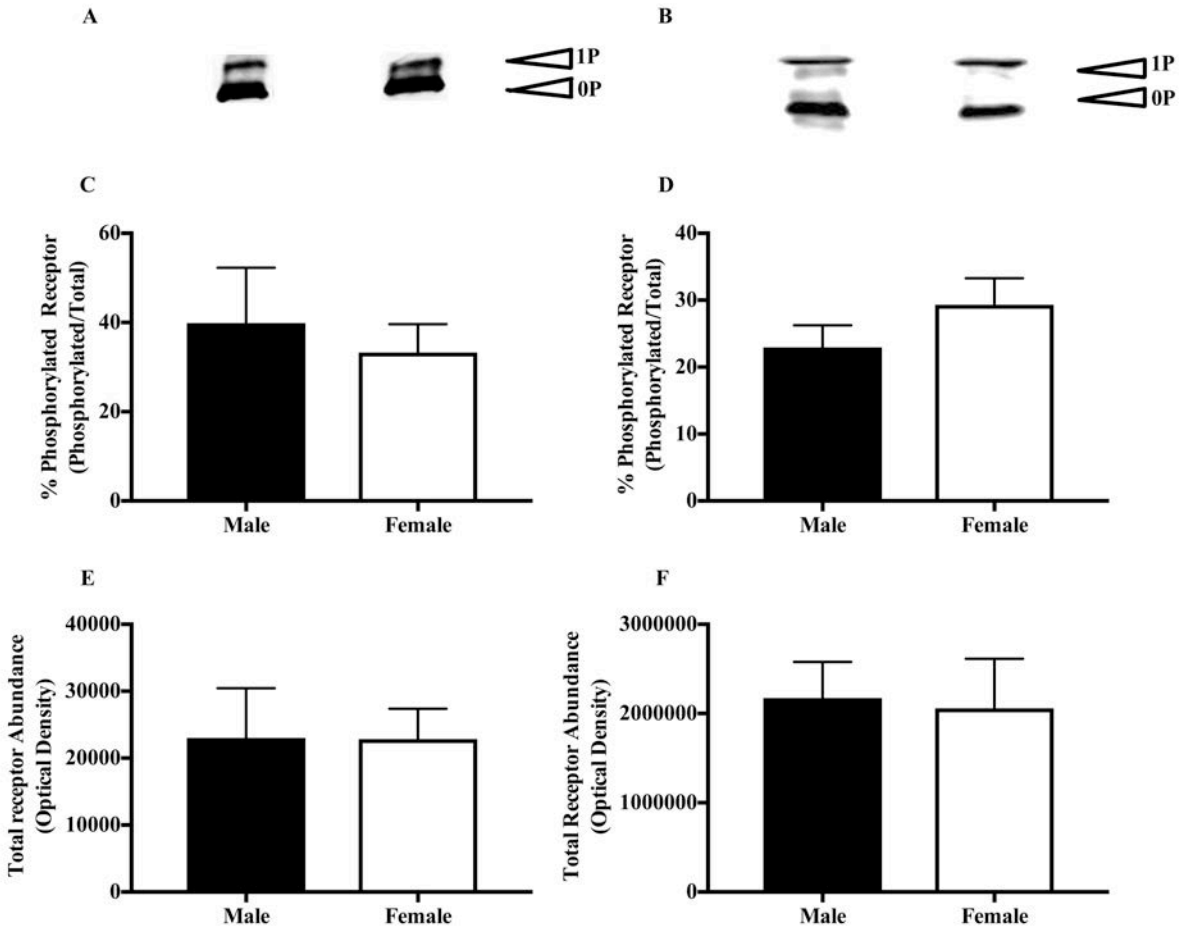


Figure 31: Female IMA hypersensitivity is independent of serotonin$_{2A}$ and serotonin$_{2B}$ receptor activity**.** A & B illustrate representative western blot of serotonin$_{2A}$ and serotonin$_{2B}$ receptors respectively, in the phosphorylated and un-phosphorylated state (n=5 females & 6 males). C & D illustrate cumulative data of the rate of phosphorylated to total serotonin$_{2A}$ and serotonin$_{2B}$ receptors respectively (n=5 females & 6 males). E & F illustrate total serotonin$_{2A}$ and serotonin$_{2B}$ receptors abundance respectively in Male and Female IMA (n=5 females & 6 males).

Table 8. Clinical characteristics and maintenance medications of study patients-2

	CHARACTERISTICS	MALE (n=72)	FEMALE (n=44)	P-value
1	**Age range**			
	Mean age	66.8 ± 1.2	66.6 ± 1.8	0.93
2	**Vascular Risk Factors**			
	Current smoker	19 (26%)	13 (29%)	0.83
	Ex-smoker	16 (22%)	12 (27%)	0.65
	Diabetic	45 (62%)	25 (57%)	0.56
	Hypertension	48 (67%)	34 (77%)	0.29
	Hypercholesterolemia	47 (65%)	30 (68%)	0.84
3	**Maintenance Medications**			
	Anti-platelet	62 (86%)	35 (80%)	0.44
	Statin	68 (94%)	40 (91%)	0.48
	Beta-Blocker	58 (80%)	34 (77%)	0.81
	ACE-Inhibitor	40 (56%)	22 (50%)	0.57
	Calcium Channel Blocker	44 (61%)	28 (63%)	0.85
	Long-acting Nitrate	45 (63%)	29 (66%)	0.84
	Diuretic	26 (36%)	19 (43%)	0.55
	SSRI	12 (17%)	6 (14%)	0.79
	Angiotensin Receptor Blocker	21 (29%)	14 (32%)	0.84

Table 9: Multiple regression analysis using EC_{50} and E_{max} as dependent variables, sex as independent variable of IMA treated with serotonin.

Sex + Confounder	With endothelium (p-value)		Without endothelium (p-value)	
	EC_{50}	E_{max}	EC_{50}	E_{max}
Age	0.119	0.761	0.975	0.033*
Hypertension	0.162	0.921	0.963	0.189
Diabetes Mellitus	0.185	0.337	0.896	0.219
Hypercholesterolemia	0.103	0.742	0.843	0.147
Current Smoker	0.143	0.608	0.909	0.090
Ex-smoker	0.129	0.683	0.915	0.276
Aspirin	0.210	0.637	0.965	0.147
Statin	0.183	0.430	0.961	0.056
Beta-blocker	0.113	0.665	0.984	0.143
Calcium channel blocker	0.135	0.648	0.988	0.152
ACE Inhibitor	0.105	0.628	0.957	0.154
Angiotensin Receptor Blocker	0.129	0.639	0.843	0.185
GTN	0.129	0.683	0.731	0.271
SSRI	0.129	0.686	0.967	0.077
Body Mass Index	0.056	0.533	0.883	0.186

Data derived from multiple regression, n: male=12 and female=10 for each parameter.

5. CHAPTER 5

Thesis Conclusions and Implications

Coronary artery bypass grafting has well documented benefits in selected patients [10, 11] but carries an increase post-operative risk of death, especially amongst female compared to their male counterpart.

The IMA is used as a graft conduit of first choice because of its proven clinical efficacy of 95% patency at 15 years.[184]

We investigated sex-difference in human IMA reactivity to PE, ET-1, 5-HT as a potential contributing biological factor to the excess postoperative in-hospital mortality of female following CABG. We only found sex-differences in PE & 5HT, for which this thesis investigated the mechanisms involved

Chapter 1 details the relevant background information including the clinical background, vascular physiology concepts, and sex-differences.

Chapter 2 details the methodologies employed in this thesis and further explored an optimal endothelial denudation for IMA, as conventional endothelial denudation methods are often ineffective.

Chapter 3 examined sex differences in the reactivity of the IMA to phenylephrine and explored potential mechanisms.

Chapter 4 investigated the mechanism of sex differences in IMA reactivity to serotonin and explored potential mechanisms.

5.1. Thesis major findings

The key findings from this comprehensive assessment includes the following:

1. Consistent with the documented clinical benefits of IMA, isolated vessel studies show robust endothelial integrity that is not amiable to regular endothelium denudation methods, even in patients with coronary atherosclerosis (i.e. endothelial dysfunction), their IMA have intact endothelium.
2. In an ex-vivo preparation utilizing isolated IMA segments from patients with coronary artery disease undergoing CABG, agonist-specific sex-differences in vascular reactivity was observed. Specifically, females exhibit a hypersensitivity to phenylephrine and serotonin compared to males but there was no difference in responses to endothelin-1 and the stable analogue of thromboxane A_2 (U46619).
3. In relation to female IMA hypersensitivity to phenylephrine (α_1 agonist),

The response is endothelium-dependent since the sex-difference is abolished by endothelial denudation.

The endothelium-dependent sex-difference is not mediated via the nitric oxide pathway since there was no difference between males and females in NO generation and the sex-difference was not abolished by NOS inhibition.

Previous studies in our laboratory have demonstrated that the endothelium-dependent sex-difference is mediated via a prostanoid pathway since indomethacin abolished the phenomenon.

There is no sex-difference in IMA α_1-receptor activity (total abundance and phosphorylation).

4. Concerning female IMA hypersensitivity to serotonin;

   (a) The sex-difference is abolished by endothelium denudation, confirming it is endothelium-dependent.

   (b) The endothelium-dependent response is not mediated via the NO pathway since there was no difference between males and females in NO generation and the sex-difference was not abolished by NOS inhibition.

   (c) The prostanoid pathway plays a key role in the sex-difference since indomethacin inhibited the phenomenon and assessment of prostaglandin metabolite levels reveals a decrease trend of vascular PGI_2 production (as indexed by 6-keto PGF-1α production-the stable metabolite of PGI_2)

   (d) Close evaluation of the data reveals that males became hyperactive (similar to females) with indomethacin suggesting that males produce an endothelium-dependent vasodilating prostanoid, that is not found in females and responsible for the relative female hypersensitivity to serotonin

   (e) There was no sex difference in serotonin 5-HT_{2A} and 5-HT_{2B} receptor activity (total abundance and phosphorylation)

5.2. The mechanism of the agonist specific COX-dependent female IMA hypersensitivity

The sex-difference in IMA reactivity is agonist specific, with females exhibiting a hypersensitivity to both PE and 5-HT but not ET-1 and U46619 agonists compared to their males counterpart. Furthermore, the sex-difference in PE and 5-HT was abolished by endothelial denudation and indomethacin, implicating the involvement of COX pathway, with decreases vascular PGI_2 production (as indexed by 6-keto PGF-1α production-the stable metabolite of PGI_2) as illustrated in figure 32A and 32B).

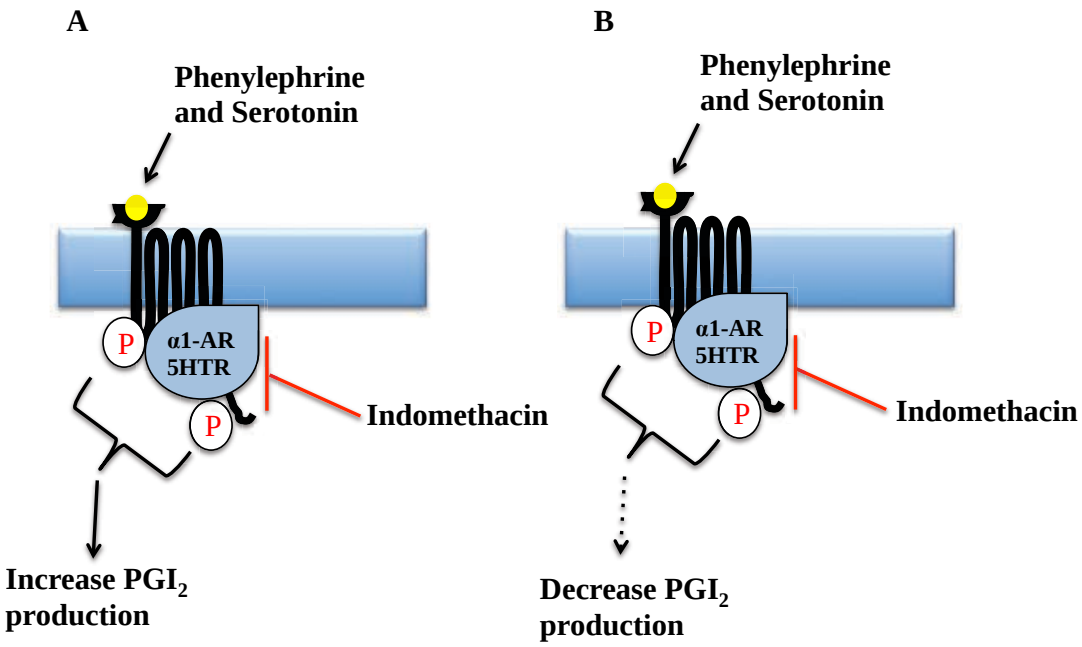


Figure 32: Schematic diagram illustrating the mechanism of endothelial PGI_2 mediated sex-difference in vascular reactivity to PE and 5-HT in human IMA. (A) Illustrating male IMA exposed to PE and 5-HT leading to increase PGI_2 production thereby regulating vascular contraction. (B) Illustrating female IMA exposed to PE and 5-HT leading to decrease PGI_2 production thus leading to hypersensitivity. Broken arrow illustrates decrease PGI_2 production.

Sex-differences in PGI_2 production have also been previously reported. Steinleitner *et al.,* 1989 reveals decrease arterial production of PGI2 in postmenopausal female compared to their premenopausal counterpart [491], suggesting the positive role of oestrogen in mediating vascular tone in premenopausal female.[488] Batres *et al.,* 1986 reported decrease prostacyclin in female in vein umbilical cord compared to male [492], which implies that the ability to produce PGI_2 in different vascular beds in male and female not only manifested in old age but also during childhood.

The mechanism responsible for the decrease in PGI_2 in female IMA is not known. However, previous animal research has revealed increased COX-2 protein expression in male compared to female and a comparable COX-1.[496] Thus it worth suggesting that the sex-difference in PGI_2 is as a result of differential COX- expression in female that might predispose their graft to post operative spasm and possibly contribute to the differential outcome of female post CABG. However, this needs to be investigated in the context of human IMA.

It is interesting to note that although it is widely known that PE, 5-HT, ET-1 and U46619 can activated COX to stimulate the synthesis and release of the endogenous PGI_2 in various cell types,[459, 460, 481-483] there was no sex-difference in IMA response to ET-1 and U46619. This could be related to the possible defect in PGI_2 production in the endothelium of IMA in response to ET-1 and U46619 in male and female due to the fact that the endothelial ET_B receptor on the human IMA has been shown to not functionally linked to PGI_2 production[461] thus leading decrease/no PGI_2 production (Figure 33A), whereas U46619 is known to functionally regulate further PGI_2 production by feedback inhibitory mechanism upon initial stimulation (Figure 33B). [484] Thus, this differential response of ET-1 and U46619 in mediating endothelial PGI_2 production, might potentially mimic the effect of indomethacin blocking of the

male PGI_2 production, abolishing the underline sex-difference in vascular reactivity to ET and U46619.

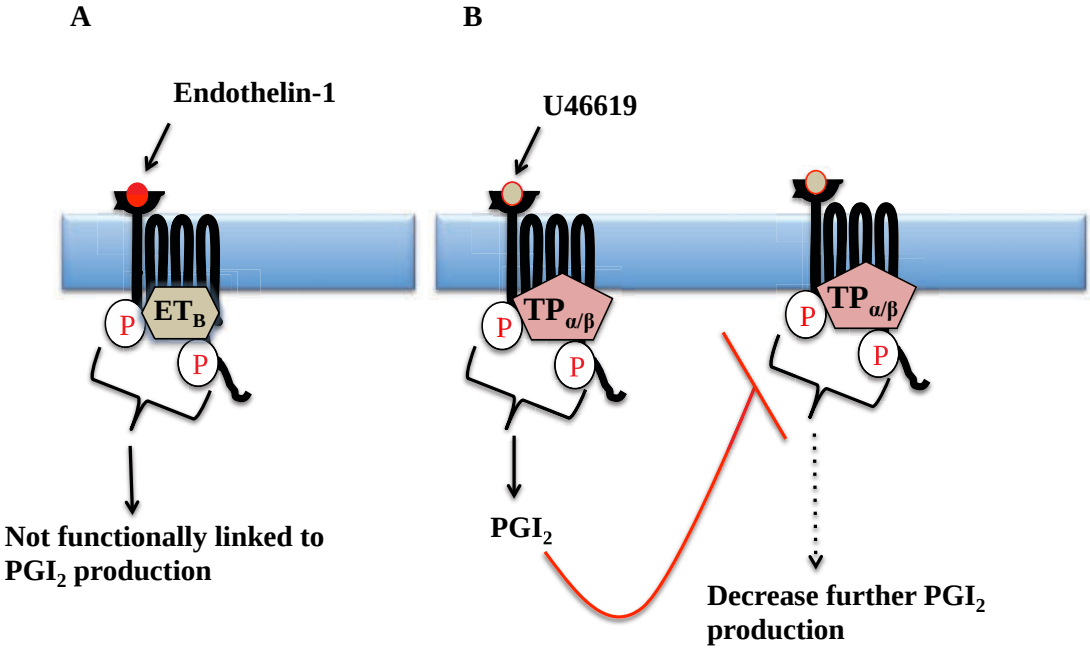


Figure 33: Schematic diagram illustrating the mechanism responsible for the decrease in PGI_2 production that might possibly mediating the no-sex differences in vascular reactivity. (A) Illustrate that in the endothelium of human IMA, the endothelin-1 receptor (ET_B) is not functionally linked to endothelial PGI_2 production. (B) Illustrate that although U46619 can induce endothelium PGI_2 production, the generated PGI_2 leads to further endothelial PGI_2 production through feedback inhibition mechanism

PGI_2 is a potent platelets inhibitor and vasodilatory prostanoids that function particularly in larger arteries, signaling through PGI_2/IP receptors with subsequent activation of the pathway involving a G_s-receptor stimulating the cAMP/PKA pathway, [497] resulting in intercellular Ca^{2+} extrusion and reuptake, activation of K^+ channels [498, 499] and thus vascular relaxation. Furthermore, PGI_2 has also been shown to cross talk with its endothelium counterpart, nitric oxide, in the control of vascular relaxation. During endothelium dysfunction as a result of impaired NO availability, a compensatory increase in the role of PGI_2 have been reported to overcome the increase in vascular resistance. [500-502] Therefore, the identification of a deficiency in IMA endothelial PGI_2 production provides a target for supplementation with synthetic PGI_2 analogues in the acute care of females undergoing CABG.

Finally, It is important to note that 80% of our subjects were on Aspirin, with a maintenance dose of 100 mg at the time of surgery (table-2 and 5). Although aspirin is a well-recognized as an irreversible inhibitor of cyclooxygenase [503] thereby inhibiting the generation of thromboxane with little effect on prostacyclin production, [504, 505] suggesting that the female hypersensitivity response to phenylephrine and serotonin and the decrease trend of vascular PGI_2 production as indexed by 6-keto PGF-1α production-the stable metabolite of PGI_2 is independent on the influence of aspirin.

5.3. CLINICAL IMPLICATIONS

The findings of this thesis show female IMA hypersensitivity to phenylephrine and serotonin as an additional biological contributor factor to the worsen outcome of female post CABG. Thus it implies that postoperative use of inotropes such as phenylephrine in patients with low cardiac output syndrome following CABG could potentially compromise graft blood flow in females more so than males, which may contribute to the significantly poorer short-term outcomes in females following CABG.

In addition, serotonin release as a result of platelet activation/aggregation or high plasma serotonin concentrations may induce IMA spasm in females due to a possible decrease prostacyclin production thereby producing myocardial ischaemia and contribute to their poorer CABG outcomes.

Therefore, the development of sex-specific guidelines for clinically used medications that have vasoactive properties can prove beneficial for female patients post CABG.

5.4. FUTURE DIRECTIONS

The work undertaken in this thesis has shown the presence of endothelial dependent COX-mediated female IMA hyperactivity to phenylephrine and serotonin compared to their aged matched male counterpart. The findings of this thesis have important clinical implications as discussed above. However, further research is required for future translation of this work into clinical practice.

Thus, further studies are required in the following areas:

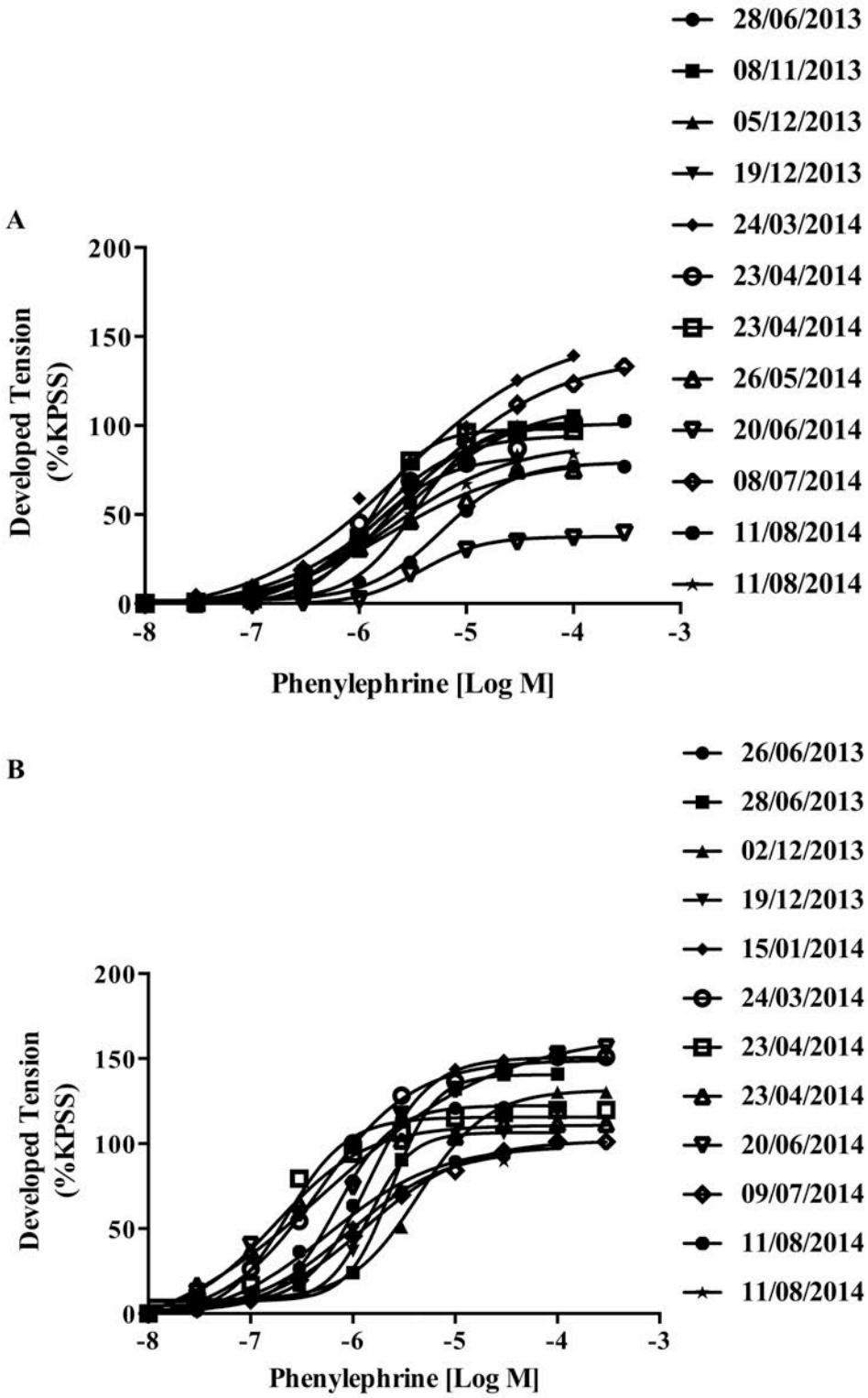
First, females may be more susceptible to post-operative α_1-agonist induced myocardial ischaemia and therefore could have a higher post-operative mortality compared with males, when exposed to these agents.

Second, since reduced PGI_2 production in females may contribute to the increased sensitivity to alpha-adrenergic and serotonergic (platelet-derived) stimuli, PGI_2 replacement therapy may be of benefit for haemodynamically compromised female post CABG patients. A clinical trial is required to confirm this basic laboratory observation.

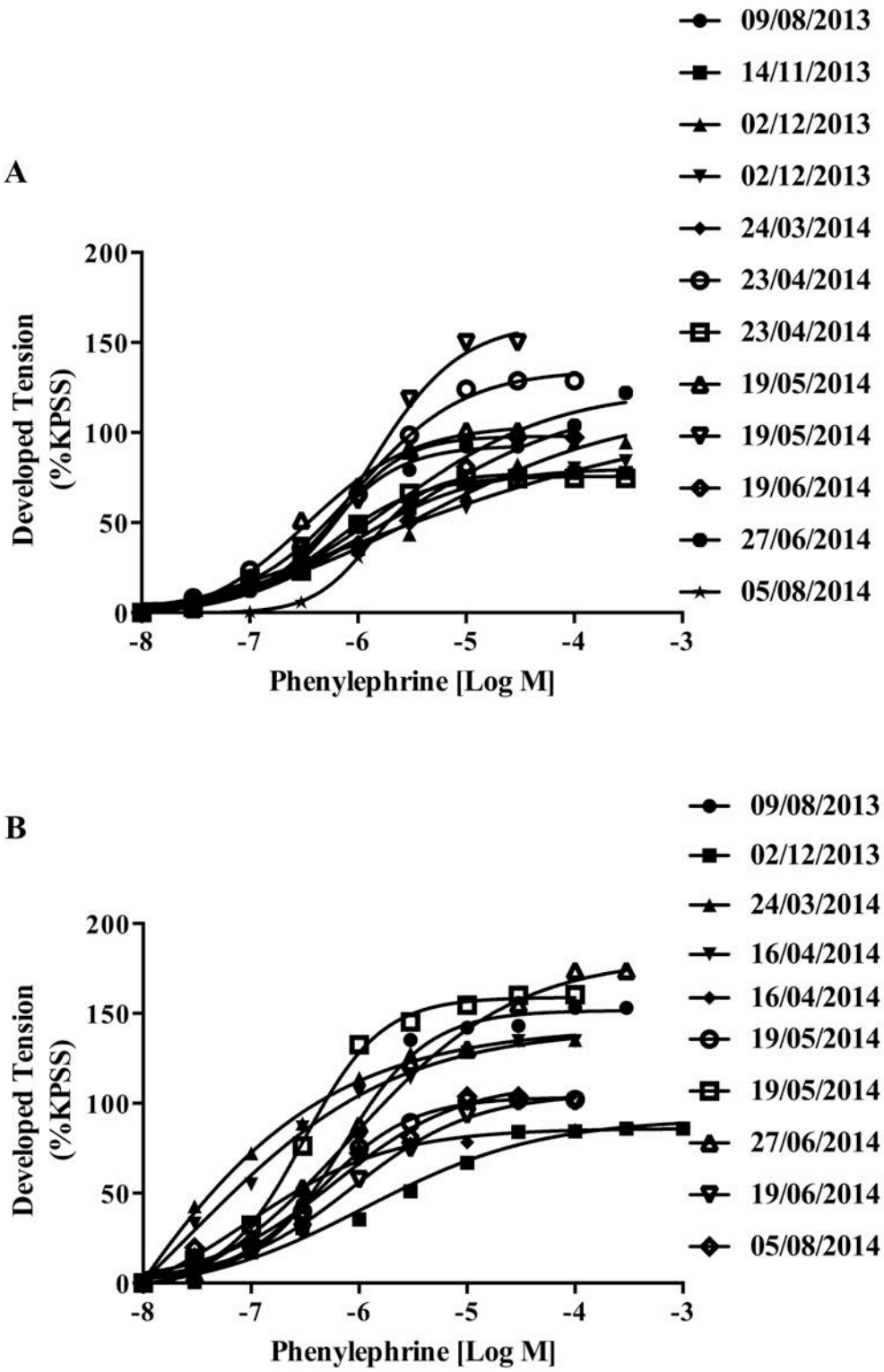
We hoped that the work in this thesis will serves as a stepping-stone and provide background for these future studies.

APPENDICES

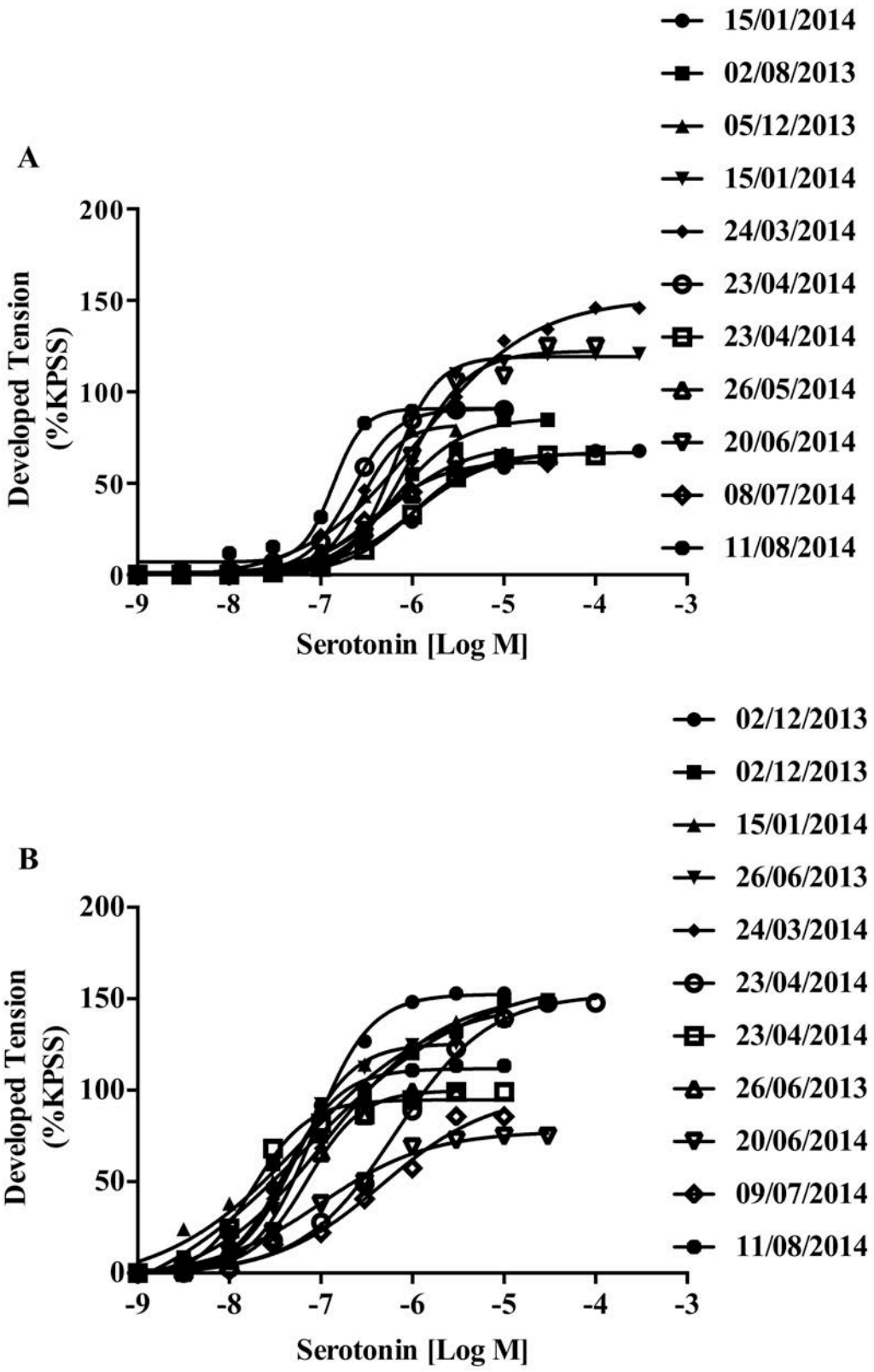
1. VASCULAR REACTIVITY



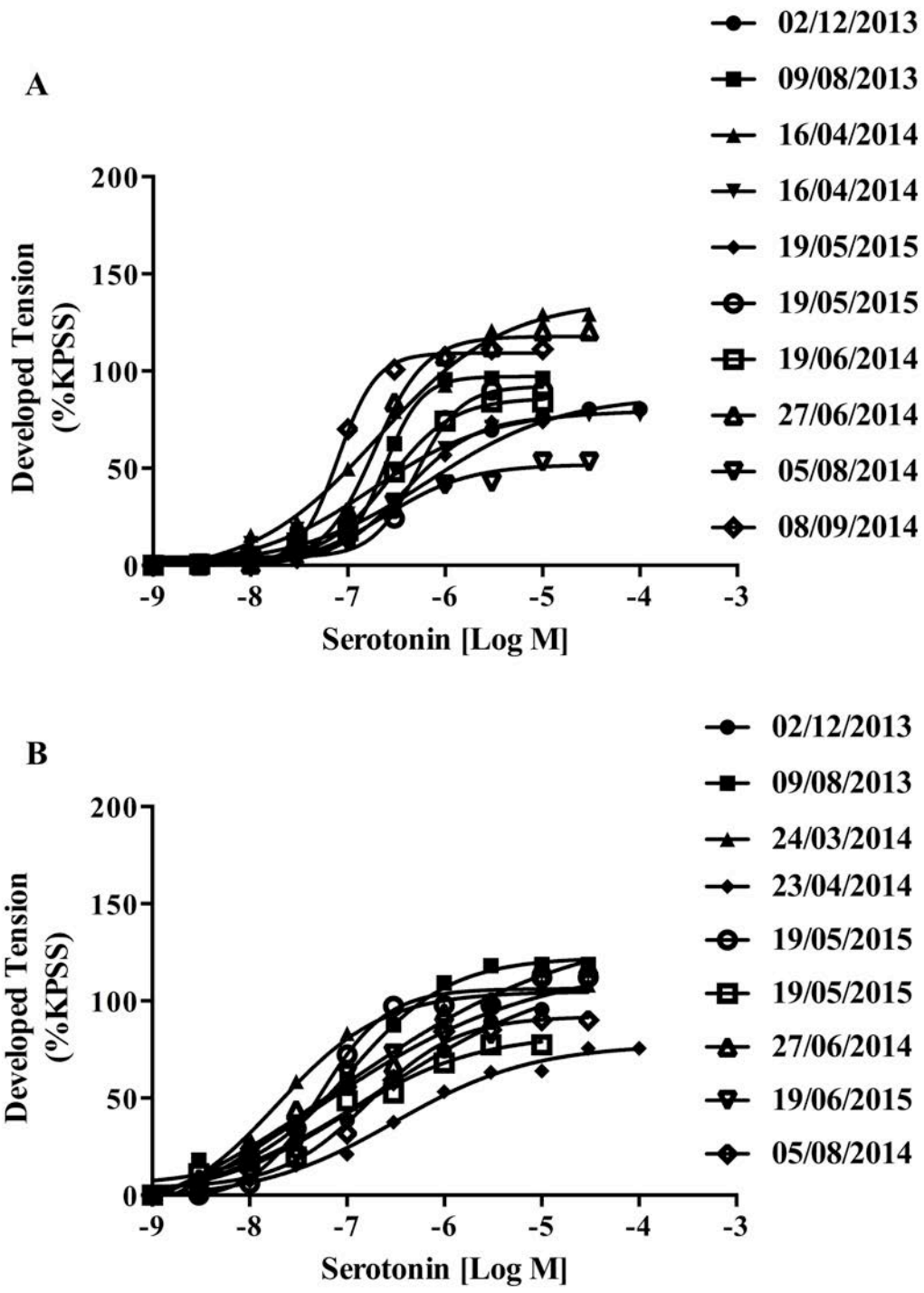
APPENDIX 1: Individual plotting of Male IMA treated with phenylephrine (A) with endothelium intact and (B) with endothelium denuded



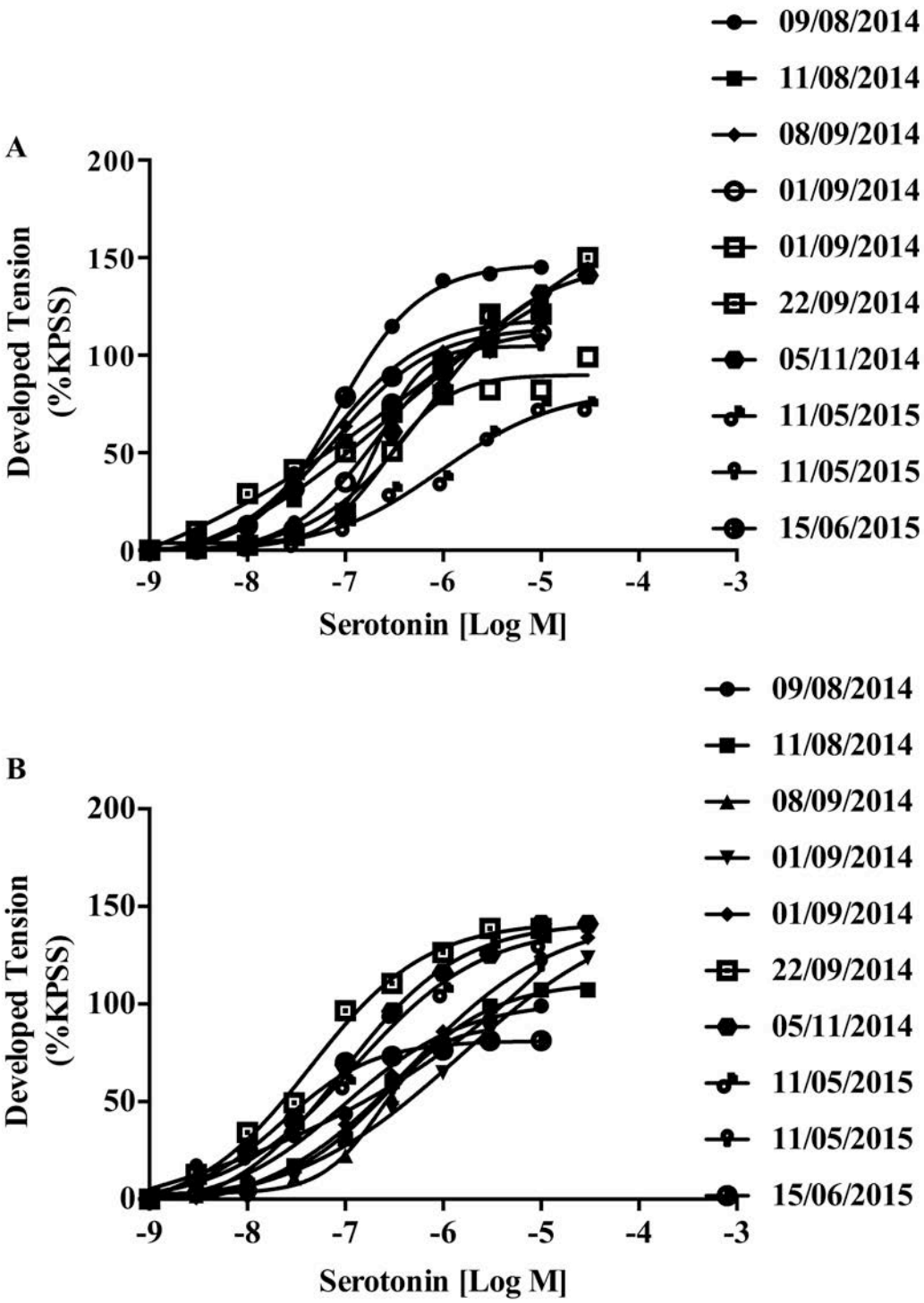
APPENDIX 2: Individual plotting of Female IMA treated with phenylephrine (A) with endothelium intact and (B) with endothelium denuded



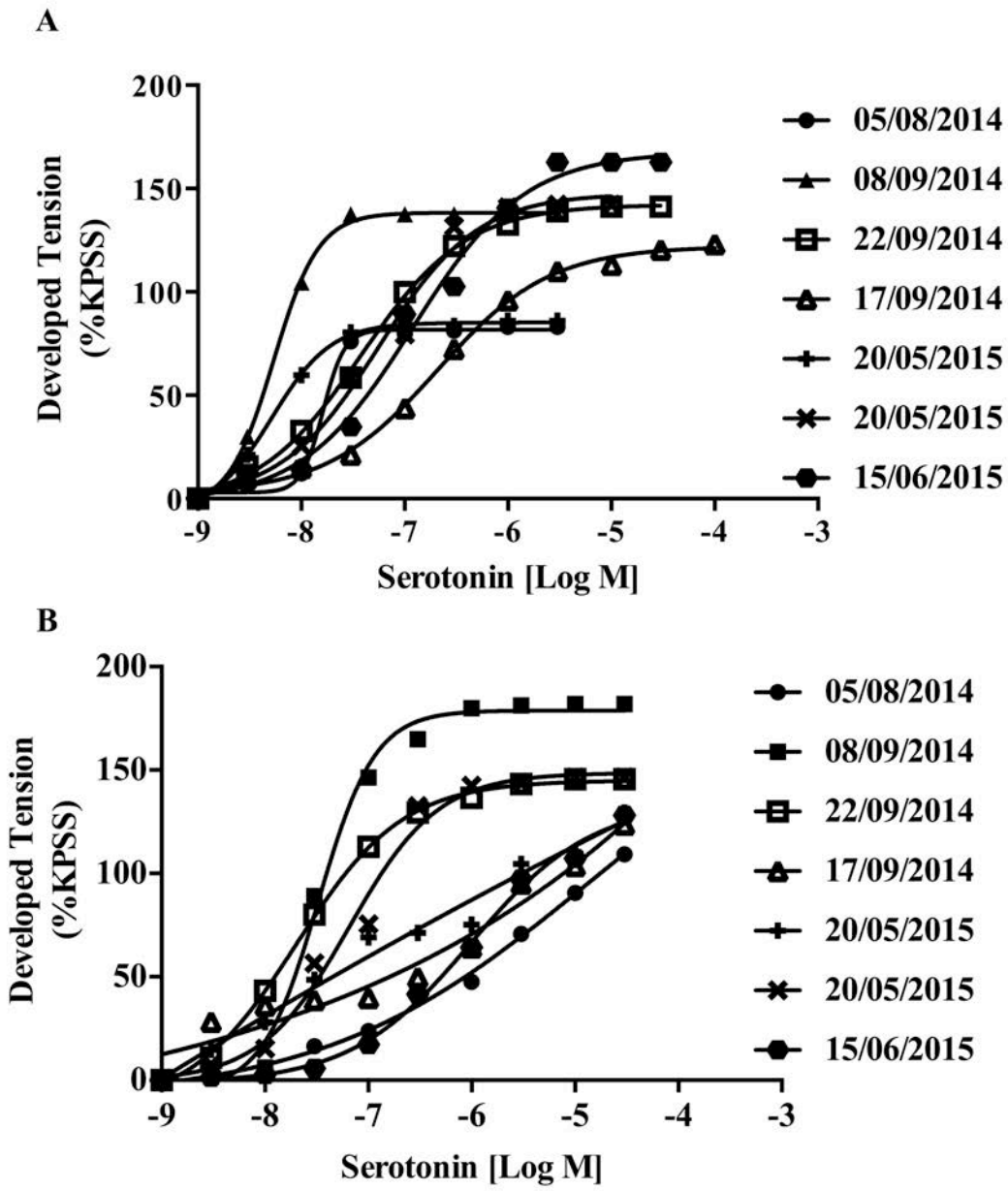
APPENDIX 3: Individual plotting of Male IMA treated with serotonin (A) with endothelium intact and (B) with endothelium denuded



APPENDIX4: Individual plotting of female IMA treated with serotonin (A) with endothelium intact and (B) with endothelium denuded

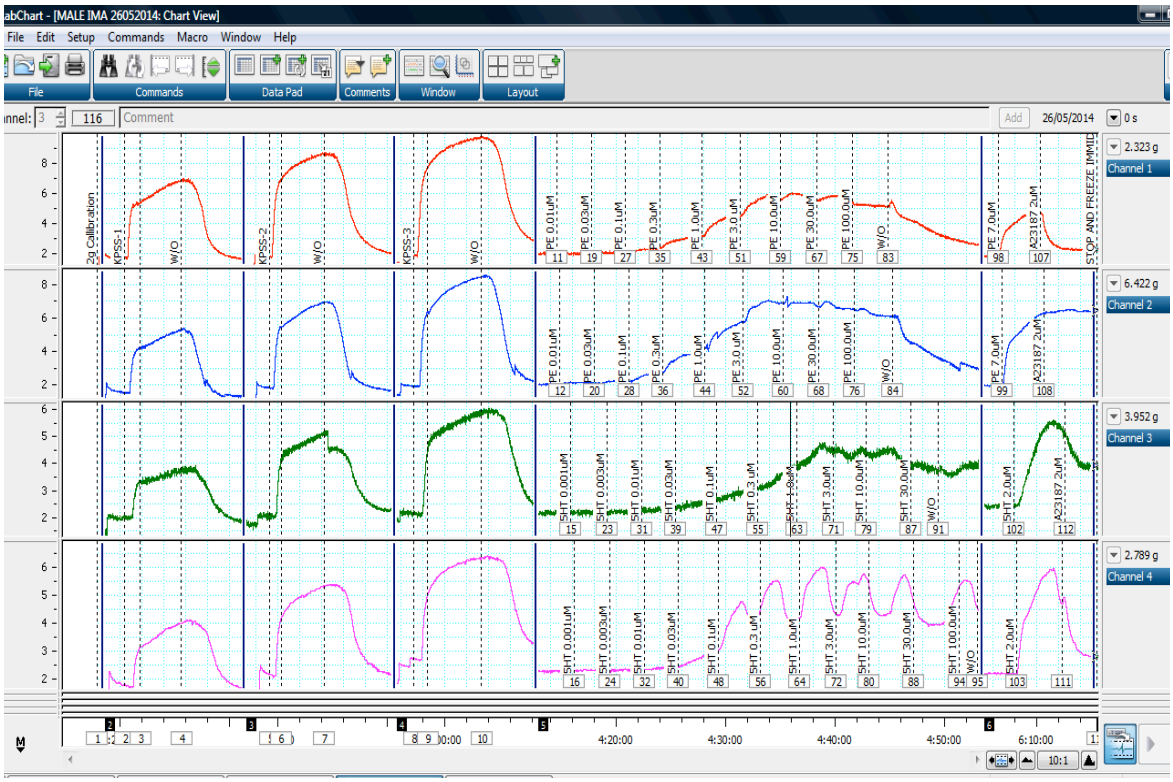


APPENDIX 5: Individual plotting of mechanistic study of Male IMA treated with serotonin (A) Using L-NAME to blockade nitric oxide synthase (B) Using Indomethacin to block cyclooxygenase

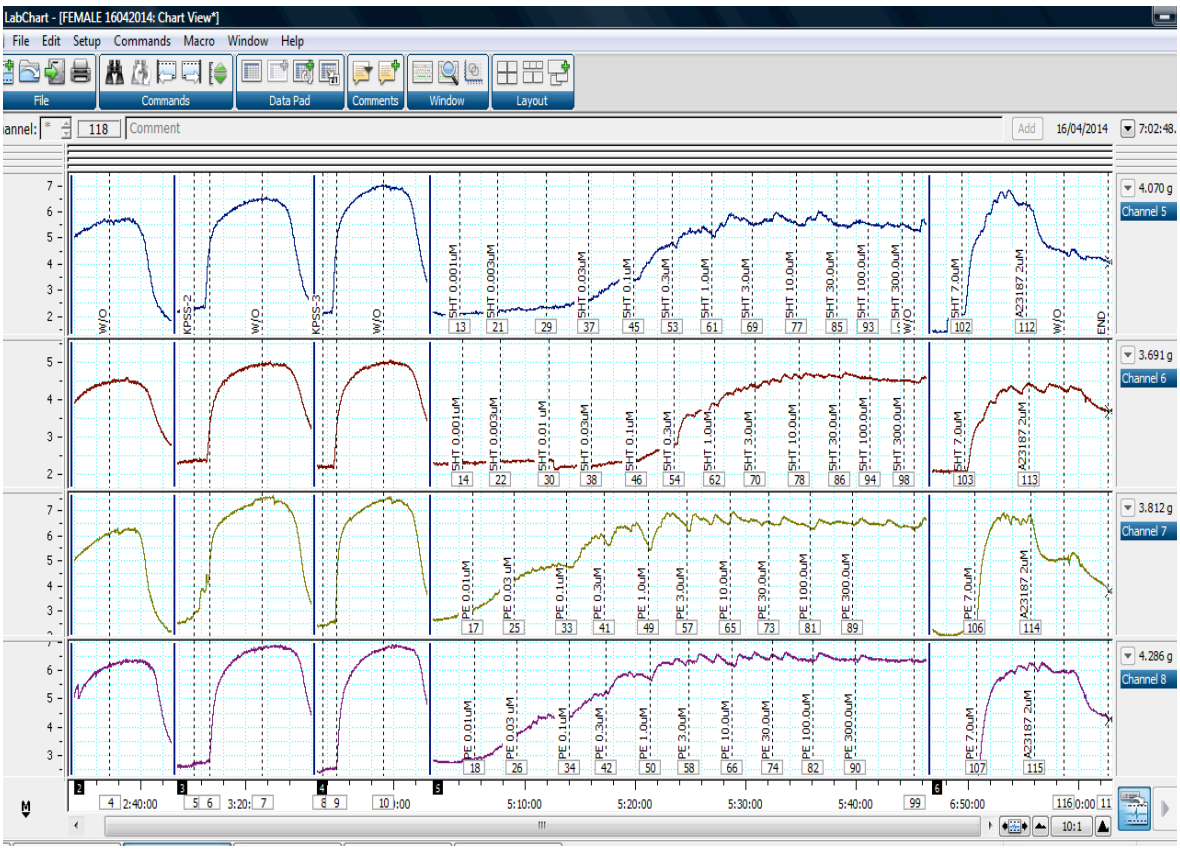


APPENDIX 6: Individual plotting of mechanistic study of female IMA treated with serotonin (A) Using L-NAME to blockade nitric oxide synthase (B) Using Indomethacin to block cyclooxygenase

A

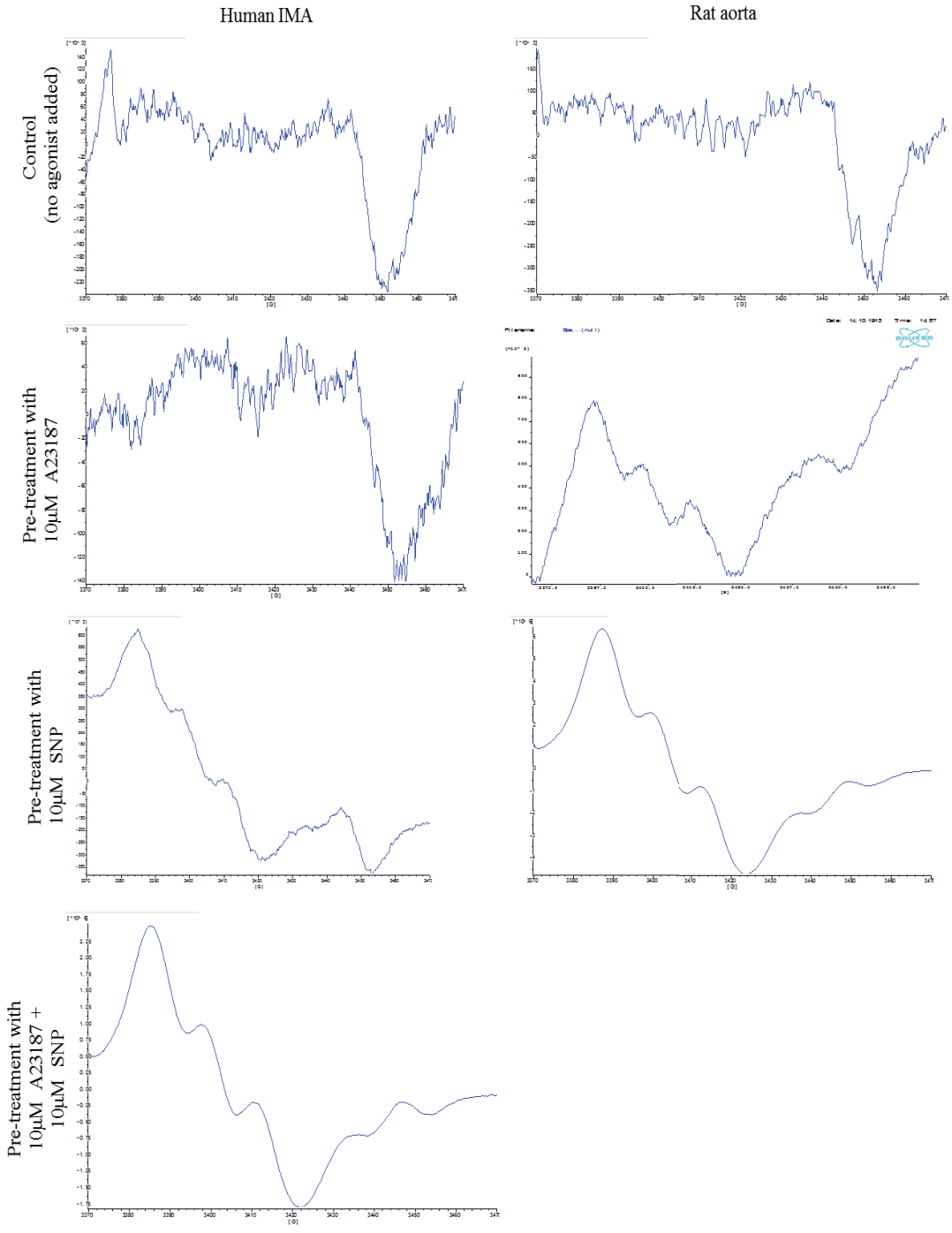


B



Appendix 7: Lab Chart diagram sample showing male (A) and female (B) endothelium intact and denuded IMA in response to PE and serotonin

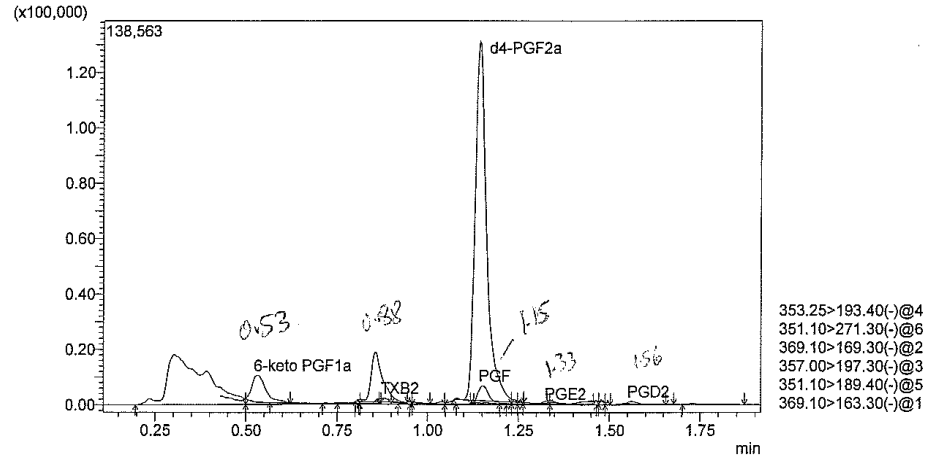
2. NITRIC OXIDE QUANTIFICATION



Appendix 8: Nitric oxide quantification assay development by EPR in human and rat aorta

3. PROSTANOID METABOLITE QUANTIFICATION

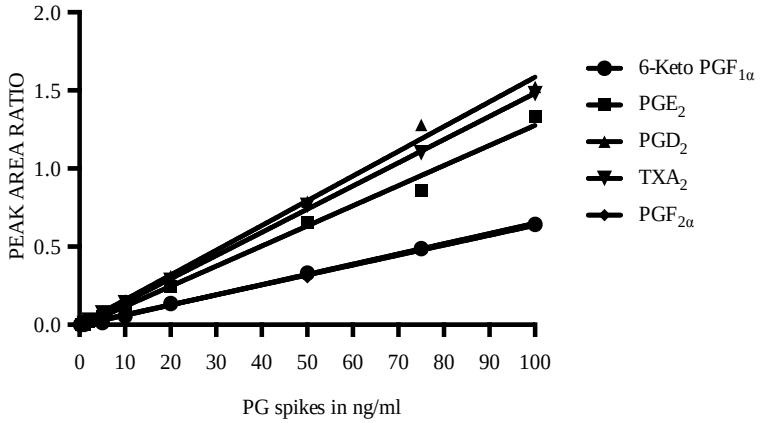
A



MS Quantitative Table

Name	Ret. Time	m/z	Area	Conc.	Unit
PGF	1.152	353.25>193.40	15271	0.000	ppm
PGD2	1.560	351.10>271.30	2588	0.000	ppm
TXB2	0.881	369.10>169.30	6537	0.000	ppm
d4-PGF2a	1.144	357.00>197.30	283099	0.000	ppm
PGE2	1.333	351.10>189.40	2069	0.000	ppm
6-keto PGF1a	0.533	369.10>163.30	35346	0.000	ppm
			344910		

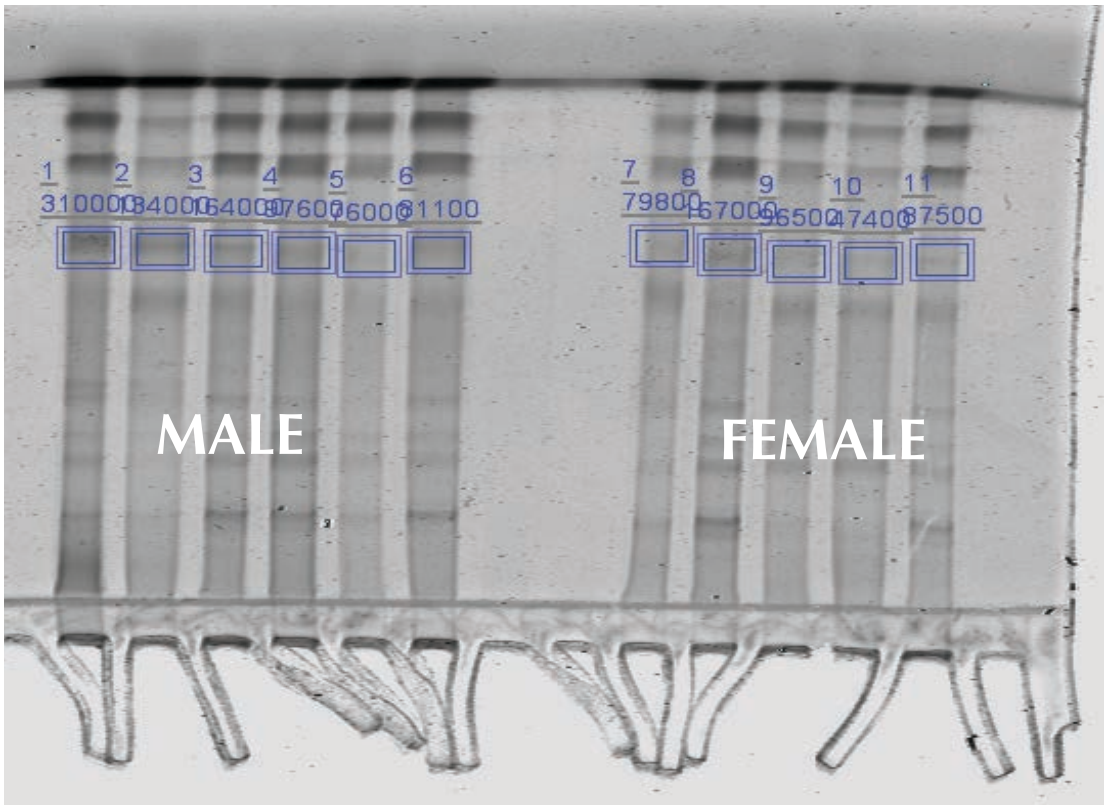
B



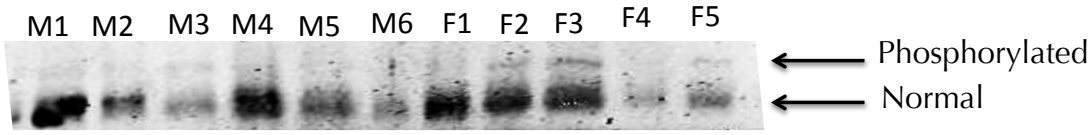
	6-KETO PGF1a	PGE2	PGD2	TXA2	PGF_{2a}
Best-fit values					
Slope	0.006543 ± 8.214e-005	0.01288 ± 0.0004174	0.01584 ± 0.0004006	0.01485 ± 0.0001242	0.006362 ± 6.584e-005
Y-intercept when X=0.0	-0.004738 ± 0.003966	-0.01130 ± 0.01900	0.001525 ± 0.01824	-0.002968 ± 0.005996	0.0005237 ± 0.002844
X-intercept when Y=0.0	0.7242	0.8773	-0.09629	0.1999	-0.08231
1/slope	152.8	77.64	63.14	67.34	157.2
95% Confidence Intervals					
Slope	0.006342 to 0.006744	0.01189 to 0.01387	0.01489 to 0.01679	0.01455 to 0.01515	0.006211 to 0.006514
Y-intercept when X=0.0	-0.01444 to 0.004966	-0.05624 to 0.03364	-0.04161 to 0.04466	-0.01764 to 0.01170	-0.006034 to 0.007082
X-intercept when Y=0.0	-0.7753 to 2.163	-2.751 to 4.170	-2.936 to 2.533	-0.7993 to 1.172	-1.130 to 0.9349
Goodness of Fit					
R square	0.9991	0.9927	0.9955	0.9996	0.9991
Sy.x	0.008264	0.04366	0.04190	0.01242	0.007122
Is slope significantly non-zero?					
F	6345	952.2	1563	14304	9338
DFn, DFd	1.000, 6.000	1.000, 7.000	1.000, 7.000	1.000, 6.000	1.000, 8.000
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant	Significant	Significant	Significant
Data					
Number of X values	8	9	9	8	10
Maximum number of Y replicates	1	1	1	1	1
Total number of values	8	9	9	8	10
Number of missing values	2	1	1	2	0
Equation	Y = 0.006543*X - 0.004738	Y = 0.01288*X - 0.01130	Y = 0.01584*X + 0.001525	Y = 0.01485*X - 0.002968	Y = 0.006362*X + 0.0005237

Appendix 9: Prostanoids assay development using LCMS. (A) Chromatogram illustrating prostanoids analysis of human IMA (B) Calibration curve of prostanoids in human tissue matrix

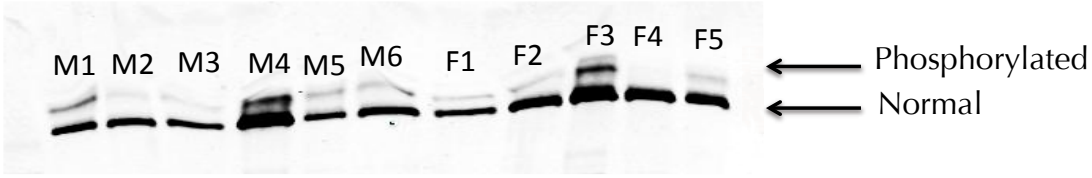
4: PHOS-TAG WESTERN BLOT ANALYSIS



APPENDIX 10: Consistency of protein content staining for male and female IMA using coomassie blue-stained actin



APPENDIX 11: Western blot image of α_1-receptor in the phosphorylated and un-phosphorylated state (M1-6=males and F1-5=Females)



APPENDIX 12: Western blot image of serotonin$_{2A}$ receptor in the phosphorylated and un-phosphorylated state (M1-6=males and F1-5=Females)

APPENDIX 13: Western blot image of serotonin$_{2B}$ receptor in the phosphorylated and un-phosphorylated state (M1-6=males and F1-5=Females)

REFERENCES

1. Wong ND. Epidemiological studies of CHD and the evolution of preventive cardiology. Nature reviews Cardiology. 2014;11(5):276-89.

2. The global burden of disease: 2004 update. http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/ index.html. 2008. Date accessed: 10/11/12.: World Health Organisation (WHO); 2004.

3. Women and heart disease: Cardiovascular profile of women in australia. Australian Institute of Health and Welfare (AIHW); 2011.

4. The shifting burden of cardiovascular disease in Australia. http://s3.amazonaws.com/zanran_storage/ww3.heartfoundation.org.au/ContentPages/106280007.pdf: Access Economics Pty Limited; 2005.

5. S M. Epidemic of coronary heart disease and its treatment in australia. http://www.aihw.gov.au/publication-detail/?id=6442467371. 2002. Date accessed: 20/10/12: Australian Institute of Health and Welfare; 2002.

6. Hueb W, Lopes NH, Gersh BJ, Soares P, Machado LA, Jatene FB, et al. Five-year follow-up of the Medicine, Angioplasty, or Surgery Study (MASS II): a randomized controlled clinical trial of 3 therapeutic strategies for multivessel coronary artery disease. Circulation. 2007;115(9):1082-9.

7. So SI. Coronary artery bypass surgery versus percutaneous coronary intervention with stent implantation in patients with multivessel coronary artery disease (the Stent or Surgery trial): a randomised controlled trial. Lancet. 2002;360(9338):965-70.

8. Daemen J, Boersma E, Flather M, Booth J, Stables R, Rodriguez A, et al. Long-term safety and efficacy of percutaneous coronary intervention with stenting

and coronary artery bypass surgery for multivessel coronary artery disease: a meta-analysis with 5-year patient-level data from the ARTS, ERACI-II, MASS-II, and SoS trials. Circulation. 2008;118(11):1146-54.

9. Kappetein AP, Feldman TE, Mack MJ, Morice MC, Holmes DR, Stahle E, et al. Comparison of coronary bypass surgery with drug-eluting stenting for the treatment of left main and/or three-vessel disease: 3-year follow-up of the SYNTAX trial. European heart journal. 2011;32(17):2125-34.

10. Soran O, Manchanda A, Schueler S. Percutaneous coronary intervention versus coronary artery bypass surgery in multivessel disease: a current perspective. Interactive cardiovascular and thoracic surgery. 2009;8(6):666-71.

11. Rathore SS, Epstein AJ, Volpp KG, Krumholz HM. Hospital coronary artery bypass graft surgery volume and patient mortality, 1998-2000. Annals of surgery. 2004;239(1):110-7.

12. Head SJ, Kieser TM, Falk V, Huysmans HA, Kappetein AP. Coronary artery bypass grafting: Part 1--the evolution over the first 50 years. European heart journal. 2013;34(37):2862-72.

13. Lamy A, Devereaux PJ, Prabhakaran D, Taggart DP, Hu S, Straka Z, et al. Five-Year Outcomes after Off-Pump or On-Pump Coronary-Artery Bypass Grafting. N Engl J Med. 2016.

14. Valtonen M, Vahasilta T, Kaila-Keinanen T, Kuttila K. New mini-extracorporeal circulation system (ECC.O) is a safe technique in coronary surgery. Scandinavian cardiovascular journal : SCJ. 2007;41(5):345-50.

15. Steinberg JB, Kapelanski DP, Olson JD, Weiler JM. Cytokine and complement levels in patients undergoing cardiopulmonary bypass. The Journal of thoracic and cardiovascular surgery. 1993;106(6):1008-16.

16. Selnes OA. Etiology of cognitive change after CABG surgery: more than just the pump? Nature clinical practice Cardiovascular medicine. 2008;5(6):314-5.

17. Fremes SE, Weisel RD, Mickle DA, Ivanov J, Madonik MM, Seawright SJ, et al. Myocardial metabolism and ventricular function following cold potassium cardioplegia. The Journal of thoracic and cardiovascular surgery. 1985;89(4):531-46.

18. Shekar PS. Cardiology patient page. On-pump and off-pump coronary artery bypass grafting. Circulation. 2006;113(4):e51-2.

19. Shroyer AL, Grover FL, Hattler B, Collins JF, McDonald GO, Kozora E, et al. On-pump versus off-pump coronary-artery bypass surgery. N Engl J Med. 2009;361(19):1827-37.

20. McGinn JT, Jr., Usman S, Lapierre H, Pothula VR, Mesana TG, Ruel M. Minimally invasive coronary artery bypass grafting: dual-center experience in 450 consecutive patients. Circulation. 2009;120(11 Suppl):S78-84.

21. Vaccarino V, Abramson JL, Veledar E, Weintraub WS. Sex differences in hospital mortality after coronary artery bypass surgery: evidence for a higher mortality in younger women. Circulation. 2002;105(10):1176-81.

22. Saxena A, Dinh D, Smith JA, Shardey G, Reid CM, Newcomb AE. Sex differences in outcomes following isolated coronary artery bypass graft surgery in Australian patients: analysis of the Australasian Society of Cardiac and Thoracic Surgeons cardiac surgery database. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery. 2012;41(4):755-62.

23. O'Connor GT, Morton JR, Diehl MJ, Olmstead EM, Coffin LH, Levy DG, et al. Differences between men and women in hospital mortality associated with

coronary artery bypass graft surgery. The Northern New England Cardiovascular Disease Study Group. Circulation. 1993;88(5 Pt 1):2104-10.

24. Swaminathan RV, Feldman DN, Pashun RA, Patil RK, Shah T, Geleris JD, et al. Gender Differences in In-Hospital Outcomes After Coronary Artery Bypass Grafting. The American journal of cardiology. 2016;118(3):362-8.

25. Shroyer AL, Coombs LP, Peterson ED, Eiken MC, DeLong ER, Chen A, et al. The Society of Thoracic Surgeons: 30-day operative mortality and morbidity risk models. The Annals of thoracic surgery. 2003;75(6):1856-64; discussion 64-5.

26. Vaccarino V, Lin ZQ, Kasl SV, Mattera JA, Roumanis SA, Abramson JL, et al. Gender differences in recovery after coronary artery bypass surgery. Journal of the American College of Cardiology. 2003;41(2):307-14.

27. Vaccarino V, Lin ZQ, Kasl SV, Mattera JA, Roumanis SA, Abramson JL, et al. Sex differences in health status after coronary artery bypass surgery. Circulation. 2003;108(21):2642-7.

28. Schulz P, Zimmerman L, Barnason S, Nieveen J. Gender differences in recovery after coronary artery bypass graft surgery. Progress in cardiovascular nursing. 2005;20(2):58-64.

29. Shaw LJ, Bugiardini R, Merz CN. Women and ischemic heart disease: evolving knowledge. Journal of the American College of Cardiology. 2009;54(17):1561-75.

30. Kothawade K, Bairey Merz CN. Microvascular coronary dysfunction in women: pathophysiology, diagnosis, and management. Current problems in cardiology. 2011;36(8):291-318.

31. Hemingway H, Langenberg C, Damant J, Frost C, Pyorala K, Barrett-Connor E. Prevalence of angina in women versus men: a systematic review and meta-analysis of international variations across 31 countries. Circulation. 2008;117(12):1526-36.

32. Jacobs AK. Coronary intervention in 2009: are women no different than men? Circulation Cardiovascular interventions. 2009;2(1):69-78.

33. Dey S, Flather MD, Devlin G, Brieger D, Gurfinkel EP, Steg PG, et al. Sex-related differences in the presentation, treatment and outcomes among patients with acute coronary syndromes: the Global Registry of Acute Coronary Events. Heart. 2009;95(1):20-6.

34. Douglas PS, Ginsburg GS. The evaluation of chest pain in women. N Engl J Med. 1996;334(20):1311-5.

35. Pepine CJ, Balaban RS, Bonow RO, Diamond GA, Johnson BD, Johnson PA, et al. Women's Ischemic Syndrome Evaluation: current status and future research directions: report of the National Heart, Lung and Blood Institute workshop: October 2-4, 2002: Section 1: diagnosis of stable ischaemia and ischemic heart disease. Circulation. 2004;109(6):e44-6.

36. Mieres JH, Shaw LJ, Arai A, Budoff MJ, Flamm SD, Hundley WG, et al. Role of noninvasive testing in the clinical evaluation of women with suspected coronary artery disease: Consensus statement from the Cardiac Imaging Committee, Council on Clinical Cardiology, and the Cardiovascular Imaging and Intervention Committee, Council on Cardiovascular Radiology and Intervention, American Heart Association. Circulation. 2005;111(5):682-96.

37. Stangl V, Witzel V, Baumann G, Stangl K. Current diagnostic concepts to detect coronary artery disease in women. European heart journal. 2008;29(6):707-17.

38. Dagenais GR, Pogue J, Fox K, Simoons ML, Yusuf S. Angiotensin-converting-enzyme inhibitors in stable vascular disease without left ventricular systolic dysfunction or heart failure: a combined analysis of three trials. Lancet. 2006;368(9535):581-8.

39. Indications for ACE inhibitors in the early treatment of acute myocardial infarction: systematic overview of individual data from 100,000 patients in randomized trials. ACE Inhibitor Myocardial Infarction Collaborative Group. Circulation. 1998;97(22):2202-12.

40. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK, et al. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med. 2001;345(7):494-502.

41. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet. 2005;366(9493):1267-78.

42. Freemantle N, Cleland J, Young P, Mason J, Harrison J. beta Blockade after myocardial infarction: systematic review and meta regression analysis. Bmj. 1999;318(7200):1730-7.

43. Hannan EL, Bernard HR, Kilburn HC, Jr., O'Donnell JF. Gender differences in mortality rates for coronary artery bypass surgery. American heart journal. 1992;123(4 Pt 1):866-72.

44. Hammar N, Sandberg E, Larsen FF, Ivert T. Comparison of early and late mortality in men and women after isolated coronary artery bypass graft surgery in Stockholm, Sweden, 1980 to 1989. Journal of the American College of Cardiology. 1997;29(3):659-64.

45. Edwards FH, Carey JS, Grover FL, Bero JW, Hartz RS. Impact of gender on coronary bypass operative mortality. The Annals of thoracic surgery. 1998;66(1):125-31.

46. Aldea GS, Gaudiani JM, Shapira OM, Jacobs AK, Weinberg J, Cupples AL, et al. Effect of gender on postoperative outcomes and hospital stays after coronary artery bypass grafting. The Annals of thoracic surgery. 1999;67(4):1097-103.

47. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet. 2004;364(9438):937-52.

48. Sandberg K, Ji H. Sex differences in primary hypertension. Biology of sex differences. 2012;3(1):7.

49. Thomas RM, Ruel E, Shantavasinkul PC, Corsino L. Endocrine hypertension: An overview on the current etiopathogenesis and management options. World journal of hypertension. 2015;5(2):14-27.

50. Jones DW, Hall JE. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure and evidence from new hypertension trials. Hypertension. 2004;43(1):1-3.

51. Zampaglione B, Pascale C, Marchisio M, Cavallo-Perin P. Hypertensive urgencies and emergencies. Prevalence and clinical presentation. Hypertension. 1996;27(1):144-7.

52. Waeber B, de la Sierra A, Ruilope LM. Target organ damage: how to detect it and how to treat it? Journal of hypertension Supplement : official journal of the International Society of Hypertension. 2009;27(3):S13-8.

53. Boynton RE, Todd RL. Blood pressure readings of 75,258 university students. Archives of internal medicine. 1947;80(4):454-62.

54. Dubey RK, Oparil S, Imthurn B, Jackson EK. Sex hormones and hypertension. Cardiovascular research. 2002;53(3):688-708.

55. Barton M, Meyer MR. Postmenopausal hypertension: mechanisms and therapy. Hypertension. 2009;54(1):11-8.

56. Tinetti ME, Han L, McAvay GJ, Lee DS, Peduzzi P, Dodson JA, et al. Anti-hypertensive medications and cardiovascular events in older adults with multiple chronic conditions. PloS one. 2014;9(3):e90733.

57. AMERICAN DIABETES ASSOCIATION, Last Reviewed. Last Edited: June 9, 2015: AMERICAN DIABETES ASSOCIATION September 22, 2014. .

58. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes care. 1998;21(9):1414-31.

59. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes care. 2004;27(5):1047-53.

60. Gale EA, Gillespie KM. Diabetes and gender. Diabetologia. 2001;44(1):3-15.

61. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Executive summary: heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation. 2012;125(1):188-97.

62. Leavitt BJ, Sheppard L, Maloney C, Clough RA, Braxton JH, Charlesworth DC, et al. Effect of diabetes and associated conditions on long-term survival after coronary artery bypass graft surgery. Circulation. 2004;110(11 Suppl 1):II41-4.

63. Leavitt BJ. The effects of diabetes mellitus on coronary artery bypass graft surgery. Current diabetes reports. 2007;7(1):20-4.

64. Mohammadi S, Dagenais F, Mathieu P, Kingma JG, Doyle D, Lopez S, et al. Long-term impact of diabetes and its comorbidities in patients undergoing isolated primary coronary artery bypass graft surgery. Circulation. 2007;116(11 Suppl):I220-5.

65. Gitman MR, Okrainec K, Nguyen H, Duerr R, Del Core M, Fourchy D, et al. Impact of diabetes on 12-month outcomes following coronary artery bypass graft surgery: results from the ROSETTA-CABG Registry. The Canadian journal of cardiology. 2005;21(13):1169-74.

66. Woods SE, Smith JM, Sohail S, Sarah A, Engle A. The influence of type 2 diabetes mellitus in patients undergoing coronary artery bypass graft surgery: an 8-year prospective cohort study. Chest. 2004;126(6):1789-95.

67. Yamamoto T, Hosoda Y, Takazawa K, Hayashi I, Miyagawa H, Sasaguri S. Is diabetes mellitus a major risk factor in coronary artery bypass grafting? The influence of internal thoracic artery grafting on late survival in diabetic patients. The Japanese journal of thoracic and cardiovascular surgery : official publication of the Japanese Association for Thoracic Surgery = Nihon Kyobu Geka Gakkai zasshi. 2000;48(6):344-52.

68. Smith SC, Jr., Blair SN, Criqui MH, Fletcher GF, Fuster V, Gersh BJ, et al. Preventing heart attack and death in patients with coronary disease. Circulation. 1995;92(1):2-4.

69. The World Health Report 2002: Reducing risks, promoting healthy life. Accessed on 04/12/2011.Geneva; 2002. : World Health Organization (WHO). ; 2012.

70. Ardeshiri M, Faritous Z, Ojaghi Haghighi Z, Hosseini S, Baghaei R. Effect of obesity on mortality and morbidity after coronary artery bypass grafting surgery in Iranian patients. Anesthesiology and pain medicine. 2014;4(2):e18884.

71. National Cholesterol Education Program Expert Panel on Detection E, Treatment of High Blood Cholesterol in A. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation. 2002;106(25):3143-421.

72. Matthews KA, Meilahn E, Kuller LH, Kelsey SF, Caggiula AW, Wing RR. Menopause and risk factors for coronary heart disease. N Engl J Med. 1989;321(10):641-6.

73. Abbey M, Owen A, Suzakawa M, Roach P, Nestel PJ. Effects of menopause and hormone replacement therapy on plasma lipids, lipoproteins and LDL-receptor activity. Maturitas. 1999;33(3):259-69.

74. Johansson S, Wilhelmsen L, Lappas G, Rosengren A. High lipid levels and coronary disease in women in Goteborg--outcome and secular trends: a prospective 19 year follow-up in the BEDA*study. European heart journal. 2003;24(8):704-16.

75. Brackbill ML, Sytsma C. Secondary prevention of hyperlipidemia after coronary artery bypass graft: from acute care to primary care. American journal of critical care : an official publication, American Association of Critical-Care Nurses. 2004;13(5):411-5.

76. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2014;384(9945):766-81.

77. Stamler J, Wentworth D, Neaton JD. Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and

graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). Jama. 1986;256(20):2823-8.

78. Lerner DJ, Kannel WB. Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. American heart journal. 1986;111(2):383-90.

79. Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. Circulation. 1983;67(5):968-77.

80. Dudina A, Cooney MT, Bacquer DD, Backer GD, Ducimetiere P, Jousilahti P, et al. Relationships between body mass index, cardiovascular mortality, and risk factors: a report from the SCORE investigators. European journal of cardiovascular prevention and rehabilitation : official journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology. 2011;18(5):731-42.

81. Wilhelmsson C, Vedin JA, Elmfeldt D, Tibblin G, Wilhelmsen L. Smoking and myocardial infarction. Lancet. 1975;1(7904):415-20.

82. Wald NJ, Hackshaw AK. Cigarette smoking: an epidemiological overview. British medical bulletin. 1996;52(1):3-11.

83. Rosenberg L, Kaufman DW, Helmrich SP, Miller DR, Stolley PD, Shapiro S. Myocardial infarction and cigarette smoking in women younger than 50 years of age. Jama. 1985;253(20):2965-9.

84. Theadom A, Cropley M. Effects of preoperative smoking cessation on the incidence and risk of intraoperative and postoperative complications in adult smokers: a systematic review. Tobacco control. 2006;15(5):352-8.

85. Cole CW, Hill GB, Farzad E, Bouchard A, Moher D, Rody K, et al. Cigarette smoking and peripheral arterial occlusive disease. Surgery. 1993;114(4):753-6; discussion 6-7.

86. Vanhoutte PM, Shimokawa H, Tang EH, Feletou M. Endothelial dysfunction and vascular disease. Acta physiologica. 2009;196(2):193-222.

87. Grundtvig M, Hagen TP, German M, Reikvam A. Sex-based differences in premature first myocardial infarction caused by smoking: twice as many years lost by women as by men. European journal of cardiovascular prevention and rehabilitation : official journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology. 2009;16(2):174-9.

88. Teiria H, Rautoma P, Yli-Hankala A. Effect of smoking on dose requirements for vecuronium. British journal of anaesthesia. 1996;76(1):154-5.

89. Nigrovic V, Wierda JM. Post-succinylcholine muscle pain and smoking. Canadian journal of anaesthesia = Journal canadien d'anesthesie. 1994;41(5 Pt 1):453-4.

90. van Domburg RT, Meeter K, van Berkel DF, Veldkamp RF, van Herwerden LA, Bogers AJ. Smoking cessation reduces mortality after coronary artery bypass surgery: a 20-year follow-up study. Journal of the American College of Cardiology. 2000;36(3):878-83.

91. Azevedo MR, Araujo CL, Reichert FF, Siqueira FV, da Silva MC, Hallal PC. Gender differences in leisure-time physical activity. International journal of public health. 2007;52(1):8-15.

92. Gulsvik AK, Thelle DS, Samuelsen SO, Myrstad M, Mowe M, Wyller TB. Ageing, physical activity and mortality--a 42-year follow-up study. International journal of epidemiology. 2012;41(2):521-30.

93. Burke AP, Farb A, Malcom GT, Liang YH, Smialek J, Virmani R. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. N Engl J Med. 1997;336(18):1276-82.

94. Oparil S. Pathophysiology of sudden coronary death in women. Implications for prevention. Circulation. 1998;97(21):2103-5.

95. Perk J, De Backer G, Gohlke H, Graham I, Reiner Z, Verschuren WM, et al. European guidelines on cardiovascular disease prevention in clinical practice (version 2012) : the fifth joint task force of the European society of cardiology and other societies on cardiovascular disease prevention in clinical practice (constituted by representatives of nine societies and by invited experts). International journal of behavioral medicine. 2012;19(4):403-88.

96. Noyez L, Biemans I, Verkroost M, van Swieten H. Is a sedentary lifestyle an independent predictor for hospital and early mortality after elective cardiac surgery? Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation. 2013;21(10):439-45.

97. Nery RM, Barbisan JN, Mahmud MI. Influence of the practice physical activity in the coronary artery bypass graft surgery results. Revista brasileira de cirurgia cardiovascular : orgao oficial da Sociedade Brasileira de Cirurgia Cardiovascular. 2007;22(3):297-302.

98. Towfighi A, Zheng L, Ovbiagele B. Sex-specific trends in midlife coronary heart disease risk and prevalence. Arch Intern Med. 2009;169(19):1762-6.

99. Leening MJ, Ferket BS, Steyerberg EW, Kavousi M, Deckers JW, Nieboer D, et al. Sex differences in lifetime risk and first manifestation of cardiovascular disease: prospective population based cohort study. Bmj. 2014;349:g5992.

100. Sesso HD, Lee IM, Gaziano JM, Rexrode KM, Glynn RJ, Buring JE. Maternal and paternal history of myocardial infarction and risk of cardiovascular disease in men and women. Circulation. 2001;104(4):393-8.

101. Sholtz RI, Rosenman RH, Brand RJ. The relationship of reported parental history to the incidence of coronary heart disease in the Western Collaborative Group Study. American journal of epidemiology. 1975;102(4):350-6.

102. Barrett-Connor E, Khaw K. Family history of heart attack as an independent predictor of death due to cardiovascular disease. Circulation. 1984;69(6):1065-9.

103. Ossewaarde ME, Bots ML, Verbeek AL, Peeters PH, van der Graaf Y, Grobbee DE, et al. Age at menopause, cause-specific mortality and total life expectancy. Epidemiology. 2005;16(4):556-62.

104. Maas AH, Appelman YE. Gender differences in coronary heart disease. Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation. 2010;18(12):598-602.

105. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. Jama. 1998;280(7):605-13.

106. Vickers MR, MacLennan AH, Lawton B, Ford D, Martin J, Meredith SK, et al. Main morbidities recorded in the women's international study of long duration oestrogen after menopause (WISDOM): a randomised controlled trial of hormone replacement therapy in postmenopausal women. Bmj. 2007;335(7613):239.

107. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama. 2002;288(3):321-33.

108. Nussmeier NA, Mora-Mangano C, Fontes M, Schwann NM, Mangano DT, Investigators of the Ischaemia Education F, et al. Hormone replacement therapy is safe in women undergoing coronary artery bypass grafting. Texas Heart Institute journal. 2005;32(4):507-14.

109. Lim SS, Davies MJ, Norman RJ, Moran LJ. Overweight, obesity and central obesity in women with polycystic ovary syndrome: a systematic review and meta-analysis. Human reproduction update. 2012;18(6):618-37.

110. Rotterdam EA-SPCWG. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertility and sterility. 2004;81(1):19-25.

111. Moran LJ, Misso ML, Wild RA, Norman RJ. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. Human reproduction update. 2010;16(4):347-63.

112. Castelo-Branco C, Steinvarcel F, Osorio A, Ros C, Balasch J. Atherogenic metabolic profile in PCOS patients: role of obesity and hyperandrogenism. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2010;26(10):736-42.

113. Shaw LJ, Bairey Merz CN, Azziz R, Stanczyk FZ, Sopko G, Braunstein GD, et al. Postmenopausal women with a history of irregular menses and elevated androgen measurements at high risk for worsening cardiovascular event-free survival: results from the National Institutes of Health--National Heart, Lung, and Blood

Institute sponsored Women's Ischaemia Syndrome Evaluation. The Journal of clinical endocrinology and metabolism. 2008;93(4):1276-84.

114. Christensen JD, Seaman DM, Washington L. Imaging of complications of thoracic and cardiovascular surgery. Radiologic clinics of North America. 2014;52(5):929-59.

115. Gardner JD, Maddox WR, Calkins JB, Jr. Iatrogenic Aortocoronary Arteriovenous Fistula following Coronary Artery Bypass Surgery: A Case Report and Complete Review of the Literature. Case reports in cardiology. 2012;2012:652086.

116. Guru V, Fremes SE, Austin PC, Blackstone EH, Tu JV. Gender differences in outcomes after hospital discharge from coronary artery bypass grafting. Circulation. 2006;113(4):507-16.

117. Calderone KL. The Influence of Gender on the Frequency of Pain and Sedative Medication Administered to Postoperative-Patients. Sex Roles. 1990;23(11-12):713-25.

118. Cacciatore F, Belluomo Anello C, Ferrara N, Mazzella F, Manzi M, De Angelis U, et al. Determinants of prolonged intensive care unit stay after cardiac surgery in the elderly. Aging clinical and experimental research. 2012;24(6):627-34.

119. Carli LL. Gender and Social Influence. Journal of Social Issues. 2001;57(4):725-41.

120. Wardle J, Waller J, Jarvis MJ. Sex differences in the association of socioeconomic status with obesity. American journal of public health. 2002;92(8):1299-304.

121. Naqvi TZ, Naqvi SS, Merz CN. Gender differences in the link between depression and cardiovascular disease. Psychosomatic medicine. 2005;67 Suppl 1:S15-8.

122. Mallik S, Krumholz HM, Lin ZQ, Kasl SV, Mattera JA, Roumains SA, et al. Patients with depressive symptoms have lower health status benefits after coronary artery bypass surgery. Circulation. 2005;111(3):271-7.

123. Philbin EF, McCullough PA, DiSalvo TG, Dec GW, Jenkins PL, Weaver WD. Socioeconomic status is an important determinant of the use of invasive procedures after acute myocardial infarction in New York State. Circulation. 2000;102(19):107-15.

124. Watkins LL, Koch GG, Sherwood A, Blumenthal JA, Davidson JR, O'Connor C, et al. Association of anxiety and depression with all-cause mortality in individuals with coronary heart disease. Journal of the American Heart Association. 2013;2(2):e000068.

125. Frasure-Smith N, Lesperance F, Talajic M. Depression and 18-month prognosis after myocardial infarction. Circulation. 1995;91(4):999-1005.

126. Cay EL, Vetter N, Philip AE, Dugard P. Psychological status during recovery from an acute heart attack. Journal of psychosomatic research. 1972;16(6):425-35.

127. Taylor CB, Youngblood ME, Catellier D, Veith RC, Carney RM, Burg MM, et al. Effects of antidepressant medication on morbidity and mortality in depressed patients after myocardial infarction. Archives of general psychiatry. 2005;62(7):792-8.

128. Ford DE, Erlinger TP. Depression and C-reactive protein in US adults: data from the Third National Health and Nutrition Examination Survey. Arch Intern Med. 2004;164(9):1010-4.

129. Cyranowski JM, Frank E, Young E, Shear MK. Adolescent onset of the gender difference in lifetime rates of major depression: a theoretical model. Archives of general psychiatry. 2000;57(1):21-7.

130. Coulter SA, Campos K. Identify and treat depression for reduced cardiac risk and improved outcomes. Texas Heart Institute journal. 2012;39(2):231-4.

131. Joynt KE, O'Connor CM. Lessons from SADHART, ENRICHD, and other trials. Psychosomatic medicine. 2005;67 Suppl 1:S63-6.

132. Gehi A, Haas D, Pipkin S, Whooley MA. Depression and medication adherence in outpatients with coronary heart disease: findings from the Heart and Soul Study. Arch Intern Med. 2005;165(21):2508-13.

133. Diagnostic and statistical manual of mental disorders. American Psychiatric Association; 2000.

134. Tully PJ, Baker RA, Turnbull D, Winefield H. The role of depression and anxiety symptoms in hospital readmissions after cardiac surgery. Journal of behavioral medicine. 2008;31(4):281-90.

135. Tully PJ, Baker RA, Knight JL. Anxiety and depression as risk factors for mortality after coronary artery bypass surgery. Journal of psychosomatic research. 2008;64(3):285-90.

136. Blumenthal JA, Lett HS, Babyak MA, White W, Smith PK, Mark DB, et al. Depression as a risk factor for mortality after coronary artery bypass surgery. Lancet. 2003;362(9384):604-9.

137. Wittchen HU, Essau CA, von Zerssen D, Krieg JC, Zaudig M. Lifetime and six-month prevalence of mental disorders in the Munich Follow-Up Study. European archives of psychiatry and clinical neuroscience. 1992;241(4):247-58.

138. McLean CP, Anderson ER. Brave men and timid women? A review of the gender differences in fear and anxiety. Clinical psychology review. 2009;29(6):496-505.

139. Leach LS, Christensen H, Mackinnon AJ, Windsor TD, Butterworth P. Gender differences in depression and anxiety across the adult lifespan: the role of psychosocial mediators. Social psychiatry and psychiatric epidemiology. 2008;43(12):983-98.

140. Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S, et al. Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Archives of general psychiatry. 1994;51(1):8-19.

141. Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry. 2005;62(6):617-27.

142. Chaplin TM, Hong K, Bergquist K, Sinha R. Gender differences in response to emotional stress: an assessment across subjective, behavioral, and physiological domains and relations to alcohol craving. Alcoholism, clinical and experimental research. 2008;32(7):1242-50.

143. Zsuzsanna Cserép ASkaBM. Short and Long Term Effects of Psychosocial Factors on the Outcome of Coronary Artery Bypass Surgery2013.

144. Koivula M, Tarkka MT, Tarkka M, Laippala P, Paunonen-Ilmonen M. Fear and anxiety in patients at different time-points in the coronary artery bypass process. International journal of nursing studies. 2002;39(8):811-22.

145. Sheifer SE, Canos MR, Weinfurt KP, Arora UK, Mendelsohn FO, Gersh BJ, et al. Sex differences in coronary artery size assessed by intravascular ultrasound. American heart journal. 2000;139(4):649-53.

146. O'Connor NJ, Morton JR, Birkmeyer JD, Olmstead EM, O'Connor GT. Effect of coronary artery diameter in patients undergoing coronary bypass surgery. Northern New England Cardiovascular Disease Study Group. Circulation. 1996;93(4):652-5.

147. Fisher LD, Kennedy JW, Davis KB, Maynard C, Fritz JK, Kaiser G, et al. Association of sex, physical size, and operative mortality after coronary artery bypass in the Coronary Artery Surgery Study (CASS). The Journal of thoracic and cardiovascular surgery. 1982;84(3):334-41.

148. Humphries KH, Pu A, Gao M, Carere RG, Pilote L. Angina with "normal" coronary arteries: sex differences in outcomes. American heart journal. 2008;155(2):375-81.

149. Daly C, Clemens F, Lopez Sendon JL, Tavazzi L, Boersma E, Danchin N, et al. Gender differences in the management and clinical outcome of stable angina. Circulation. 2006;113(4):490-8.

150. Bugiardini R, Bairey Merz CN. Angina with "normal" coronary arteries: a changing philosophy. Jama. 2005;293(4):477-84.

151. Reis SE, Holubkov R, Lee JS, Sharaf B, Reichek N, Rogers WJ, et al. Coronary flow velocity response to adenosine characterizes coronary microvascular function in women with chest pain and no obstructive coronary disease. Results from the pilot phase of the Women's Ischaemia Syndrome Evaluation (WISE) study. Journal of the American College of Cardiology. 1999;33(6):1469-75.

152. Sedlak TL, Lee M, Izadnegahdar M, Merz CN, Gao M, Humphries KH. Sex differences in clinical outcomes in patients with stable angina and no obstructive coronary artery disease. American heart journal. 2013;166(1):38-44.

153. Villablanca AC, Jayachandran M, Banka C. Atherosclerosis and sex hormones: current concepts. Clinical science. 2010;119(12):493-513.

154. Regitz-Zagrosek V. Therapeutic implications of the gender-specific aspects of cardiovascular disease. Nature reviews Drug discovery. 2006;5(5):425-38.

155. Farb A, Burke AP, Tang AL, Liang TY, Mannan P, Smialek J, et al. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. Circulation. 1996;93(7):1354-63.

156. Virmani R, Burke AP, Kolodgie FD, Farb A. Pathology of the thin-cap fibroatheroma: a type of vulnerable plaque. Journal of interventional cardiology. 2003;16(3):267-72.

157. Mautner SL, Lin F, Mautner GC, Roberts WC. Comparison in women versus men of composition of atherosclerotic plaques in native coronary arteries and in saphenous veins used as aortocoronary conduits. Journal of the American College of Cardiology. 1993;21(6):1312-8.

158. Kramer MC, Rittersma SZ, de Winter RJ, Ladich ER, Fowler DR, Liang YH, et al. Relationship of thrombus healing to underlying plaque morphology in sudden coronary death. Journal of the American College of Cardiology. 2010;55(2):122-32.

159. Burke AP, Farb A, Malcom GT, Liang Y, Smialek J, Virmani R. Effect of risk factors on the mechanism of acute thrombosis and sudden coronary death in women. Circulation. 1998;97(21):2110-6.

160. Nijjer SS, Watson G, Athanasiou T, Malik IS. Safety of clopidogrel being continued until the time of coronary artery bypass grafting in patients with acute coronary syndrome: a meta-analysis of 34 studies. European heart journal. 2011;32(23):2970-88.

161. Mahla E, Metzler H, Tantry US, Gurbel PA. Controversies in oral antiplatelet therapy in patients undergoing aortocoronary bypass surgery. The Annals of thoracic surgery. 2010;90(3):1040-51.

162. Stevens RF, Alexander MK. A sex difference in the platelet count. British journal of haematology. 1977;37(2):295-300.

163. Segal JB, Moliterno AR. Platelet counts differ by sex, ethnicity, and age in the United States. Annals of epidemiology. 2006;16(2):123-30.

164. Zwierzina WD, Kunz F, Kogelnig R, Herold M. Sex-related differences in platelet aggregation in native whole blood. Thrombosis research. 1987;48(2):161-71.

165. Haque SF, Matsubayashi H, Izumi S, Sugi T, Arai T, Kondo A, et al. Sex difference in platelet aggregation detected by new aggregometry using light scattering. Endocrine journal. 2001;48(1):33-41.

166. Bobbert P, Stellbaum C, Steffens D, Schutte C, Bobbert T, Schultheiss HP, et al. Postmenopausal women have an increased maximal platelet reactivity compared to men despite dual antiplatelet therapy. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis. 2012;23(8):723-8.

167. Sellman M, Intonti MA, Ivert T. Reoperations for bleeding after coronary artery bypass procedures during 25 years. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery. 1997;11(3):521-7.

168. Dacey LJ, Munoz JJ, Baribeau YR, Johnson ER, Lahey SJ, Leavitt BJ, et al. Reexploration for hemorrhage following coronary artery bypass grafting: incidence and risk factors. Northern New England Cardiovascular Disease Study Group. Archives of surgery. 1998;133(4):442-7.

169. Unsworth-White MJ, Herriot A, Valencia O, Poloniecki J, Smith EE, Murday AJ, et al. Resternotomy for bleeding after cardiac operation: a marker for increased morbidity and mortality. The Annals of thoracic surgery. 1995;59(3):664-7.

170. Moulton MJ, Creswell LL, Mackey ME, Cox JL, Rosenbloom M. Reexploration for bleeding is a risk factor for adverse outcomes after cardiac operations. The Journal of thoracic and cardiovascular surgery. 1996;111(5):1037-46.

171. Paparella D, Brister SJ, Buchanan MR. Coagulation disorders of cardiopulmonary bypass: a review. Intensive care medicine. 2004;30(10):1873-81.

172. Hartmann M, Sucker C, Boehm O, Koch A, Loer S, Zacharowski K. Effects of cardiac surgery on hemostasis. Transfusion medicine reviews. 2006;20(3):230-41.

173. Hochman JS, Tamis JE, Thompson TD, Weaver WD, White HD, Van de Werf F, et al. Sex, clinical presentation, and outcome in patients with acute coronary syndromes. Global Use of Strategies to Open Occluded Coronary Arteries in Acute Coronary Syndromes IIb Investigators. N Engl J Med. 1999;341(4):226-32.

174. Anand SS, Xie CC, Mehta S, Franzosi MG, Joyner C, Chrolavicius S, et al. Differences in the management and prognosis of women and men who suffer from acute coronary syndromes. Journal of the American College of Cardiology. 2005;46(10):1845-51.

175. van Straten AH, Kats S, Bekker MW, Verstappen F, ter Woorst JF, van Zundert AJ, et al. Risk factors for red blood cell transfusion after coronary artery bypass graft surgery. Journal of cardiothoracic and vascular anaesthesia. 2010;24(3):413-7.

176. Shevde K, Pagala M, Kashikar A, Tyagaraj C, Shahbaz N, Iqbal M, et al. Gender is an essential determinant of blood transfusion in patients undergoing coronary artery bypass graft procedure. Journal of clinical anaesthesia. 2000;12(2):109-16.

177. Karkouti K, Cohen MM, McCluskey SA, Sher GD. A multivariable model for predicting the need for blood transfusion in patients undergoing first-time elective coronary bypass graft surgery. Transfusion. 2001;41(10):1193-203.

178. Murphy GJ, Reeves BC, Rogers CA, Rizvi SI, Culliford L, Angelini GD. Increased mortality, postoperative morbidity, and cost after red blood cell transfusion in patients having cardiac surgery. Circulation. 2007;116(22):2544-52.

179. Engoren MC, Habib RH, Zacharias A, Schwann TA, Riordan CJ, Durham SJ. Effect of blood transfusion on long-term survival after cardiac operation. The Annals of thoracic surgery. 2002;74(4):1180-6.

180. Pugsley MK, Tabrizchi R. The vascular system. An overview of structure and function. Journal of pharmacological and toxicological methods. 2000;44(2):333-40.

181. Borysenko M. BT. Functional Histology. Little, Brown and Company, Boston 1984:195-208.

182. Sims DE. The pericyte--a review. Tissue Cell. 1986;18(2):153-74.

183. Pappano AJ. Coronary Circulation. Cardiovascular Physiology Tenth ed2013. p. 223-36.

184. Sur S, Sugimoto JT, Agrawal DK. Coronary artery bypass graft: why is the saphenous vein prone to intimal hyperplasia? Canadian journal of physiology and pharmacology. 2014;92(7):531-45.

185. Yamane Y, Uchida N, Okubo S, Morimoto H, Mukai S. Impact of the size mismatch between saphenous vein graft and coronary artery on graft patency. General thoracic and cardiovascular surgery. 2016.

186. Loop FD, Golding LR, MacMillan JP, Cosgrove DM, Lytle BW, Sheldon WC. Coronary artery surgery in women compared with men: analyses of risks and

long-term results. Journal of the American College of Cardiology. 1983;1(2 Pt 1):383-90.

187. Hulusi M, Basaran M, Ugurlucan M, Kocailik A, Basaran EK. Coronary artery bypass grafting with Y-saphenous vein grafts. Angiology. 2009;60(6):668-75.

188. Cameron A, Davis KB, Green G, Schaff HV. Coronary bypass surgery with internal-thoracic-artery grafts--effects on survival over a 15-year period. N Engl J Med. 1996;334(4):216-9.

189. Lytle BW, Loop FD, Cosgrove DM, Ratliff NB, Easley K, Taylor PC. Long-term (5 to 12 years) serial studies of internal mammary artery and saphenous vein coronary bypass grafts. The Journal of thoracic and cardiovascular surgery. 1985;89(2):248-58.

190. Gardner TJ. Searching for the second-best coronary artery bypass graft: is it the radial artery? Circulation. 2007;115(6):678-80.

191. Buxton BF, Komeda M, Fuller JA, Gordon I. Bilateral internal thoracic artery grafting may improve outcome of coronary artery surgery. Risk-adjusted survival. Circulation. 1998;98(19 Suppl):II1-6.

192. Popovic B, Voillot D, Maureira P, Vanhuyse F, Agrinier N, Aliot E, et al. Bilateral internal mammary artery bypass grafting: long-term clinical benefits in a series of 1000 patients. Heart. 2013;99(12):854-9.

193. Mohammadi S, Dagenais F, Doyle D, Mathieu P, Baillot R, Charbonneau E, et al. Age cut-off for the loss of benefit from bilateral internal thoracic artery grafting. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery. 2008;33(6):977-82.

194. Kieser TM, Lewin AM, Graham MM, Martin BJ, Galbraith PD, Rabi DM, et al. Outcomes associated with bilateral internal thoracic artery grafting: the

importance of age. The Annals of thoracic surgery. 2011;92(4):1269-75; discussion 75-6.

195. Elmistekawy EM, Gawad N, Bourke M, Mesana T, Boodhwani M, Rubens FD. Is bilateral internal thoracic artery use safe in the elderly? Journal of cardiac surgery. 2012;27(1):1-5.

196. Cohen G, Tamariz MG, Sever JY, Liaghati N, Guru V, Christakis GT, et al. The radial artery versus the saphenous vein graft in contemporary CABG: a case-matched study. The Annals of thoracic surgery. 2001;71(1):180-5; discussion 5-6.

197. Zacharias A, Schwann TA, Riordan CJ, Durham SJ, Shah AS, Habib RH. Late results of conventional versus all-arterial revascularization based on internal thoracic and radial artery grafting. The Annals of thoracic surgery. 2009;87(1):19-26 e2.

198. Zacharias A, Schwann TA, Riordan CJ, Durham SJ, Shah AS, Engoren M, et al. Late outcomes after radial artery versus saphenous vein grafting during reoperative coronary artery bypass surgery. The Journal of thoracic and cardiovascular surgery. 2010;139(6):1511-8 e4.

199. Schwann TA, Zacharias A, Riordan CJ, Durham SJ, Shah AS, Habib RH. Sequential radial artery grafts for multivessel coronary artery bypass graft surgery: 10-year survival and angiography results. The Annals of thoracic surgery. 2009;88(1):31-9.

200. Collins P, Webb CM, Chong CF, Moat NE, Radial Artery Versus Saphenous Vein Patency Trial I. Radial artery versus saphenous vein patency randomized trial: five-year angiographic follow-up. Circulation. 2008;117(22):2859-64.

201. Tatoulis J, Royse AG, Buxton BF, Fuller JA, Skillington PD, Goldblatt JC, et al. The radial artery in coronary surgery: a 5-year experience--clinical and

angiographic results. The Annals of thoracic surgery. 2002;73(1):143-7; discussion 7-8.

202. Possati G, Gaudino M, Prati F, Alessandrini F, Trani C, Glieca F, et al. Long-term results of the radial artery used for myocardial revascularization. Circulation. 2003;108(11):1350-4.

203. Iaco AL, Teodori G, Di Giammarco G, Di Mauro M, Storto L, Mazzei V, et al. Radial artery for myocardial revascularization: long-term clinical and angiographic results. The Annals of thoracic surgery. 2001;72(2):464-8; discussion 8-9.

204. Acar C, Ramsheyi A, Pagny JY, Jebara V, Barrier P, Fabiani JN, et al. The radial artery for coronary artery bypass grafting: clinical and angiographic results at five years. The Journal of thoracic and cardiovascular surgery. 1998;116(6):981-9.

205. Schwann TA, Engoren M, Bonnell M, Clancy C, Habib RH. Comparison of late coronary artery bypass graft survival effects of radial artery versus saphenous vein grafting in male and female patients. The Annals of thoracic surgery. 2012;94(5):1485-91.

206. Buche M, Schroeder E, Gurne O, Chenu P, Paquay JL, Marchandise B, et al. Coronary artery bypass grafting with the inferior epigastric artery. Midterm clinical and angiographic results. The Journal of thoracic and cardiovascular surgery. 1995;109(3):553-9; discussion 9-60.

207. Setina M, Pirk J. [The inferior epigastric artery. Short-term and one-year patency]. Rozhledy v chirurgii : mesicnik Ceskoslovenske chirurgicke spolecnosti. 1997;76(3):129-32.

208. Grandjean JG, Mariani MA, Ebels T. Coronary reoperation via small laparotomy using right gastroepiploic artery without CPB. The Annals of thoracic surgery. 1996;61(6):1853-5.

209. Malvindi PG, Jacob S, Kallikourdis A, Vitale N. What is the patency of the gastroepiploic artery when used for coronary artery bypass grafting? Interactive cardiovascular and thoracic surgery. 2007;6(3):397-402.

210. Kim KB, Cho KR, Choi JS, Lee HJ. Right gastroepiploic artery for revascularization of the right coronary territory in off-pump total arterial revascularization: strategies to improve patency. The Annals of thoracic surgery. 2006;81(6):2135-41.

211. Billington CK, Penn RB. Signaling and regulation of G protein-coupled receptors in airway smooth muscle. Respir Res. 2003;4:2.

212. Penn RB, Pronin AN, Benovic JL. Regulation of G protein-coupled receptor kinases. Trends Cardiovasc Med. 2000;10(2):81-9.

213. Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, et al. Insights into G protein structure, function, and regulation. Endocr Rev. 2003;24(6):765-81.

214. Wettschureck N, Offermanns S. Mammalian G proteins and their cell type specific functions. Physiological reviews. 2005;85(4):1159-204.

215. Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE. The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature. 1987;325(6102):321-6.

216. Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shekter LR, et al. Molecular basis for interactions of G protein betagamma subunits with effectors. Science. 1998;280(5367):1271-4.

217. Guo Y, Li M, Lu M, Wen Z, Huang Z. Predicting G-protein coupled receptors-G-protein coupling specificity based on autocross-covariance transform. Proteins. 2006;65(1):55-60.

218. Zhang L, Shi G. Gq-Coupled Receptors in Autoimmunity. J Immunol Res. 2016;2016:3969023.

219. Berridge MJ. Neuronal calcium signaling. Neuron. 1998;21(1):13-26.

220. Augustine GJ, Santamaria F, Tanaka K. Local calcium signaling in neurons. Neuron. 2003;40(2):331-46.

221. Neves SR, Ram PT, Iyengar R. G protein pathways. Science. 2002;296(5573):1636-9.

222. Berridge MJ. Inositol trisphosphate and calcium signalling. Nature. 1993;361(6410):315-25.

223. Wettschureck N, Moers A, Offermanns S. Mouse models to study G-protein-mediated signaling. Pharmacol Ther. 2004;101(1):75-89.

224. Morton TL, Ansari MA, Jacobson JD. Gender differences and hormonal modulation of G proteins Galpha(q/11) expression in lymphoid organs. Neuroendocrinology. 2003;78(3):147-53.

225. Szekeres M, Nadasy GL, Turu G, Soltesz-Katona E, Benyo Z, Offermanns S, et al. Endocannabinoid-mediated modulation of Gq/11 protein-coupled receptor signaling-induced vasoconstriction and hypertension. Molecular and cellular endocrinology. 2015;403:46-56.

226. Offermanns S, Zhao LP, Gohla A, Sarosi I, Simon MI, Wilkie TM. Embryonic cardiomyocyte hypoplasia and craniofacial defects in G alpha q/G alpha 11-mutant mice. The EMBO journal. 1998;17(15):4304-12.

227. Strathmann M, Simon MI. G protein diversity: a distinct class of alpha subunits is present in vertebrates and invertebrates. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(23):9113-7.

228. Sassone-Corsi P. The cyclic AMP pathway. Cold Spring Harbor perspectives in biology. 2012;4(12).

229. Hallsworth MP, Twort CH, Lee TH, Hirst SJ. beta(2)-adrenoceptor agonists inhibit release of eosinophil-activating cytokines from human airway smooth muscle cells. British journal of pharmacology. 2001;132(3):729-41.

230. Hall IP. Second messengers, ion channels and pharmacology of airway smooth muscle. Eur Respir J. 2000;15(6):1120-7.

231. Ammit AJ, Hoffman RK, Amrani Y, Lazaar AL, Hay DW, Torphy TJ, et al. Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth-muscle cells. Modulation by cyclic adenosine monophosphate. Am J Respir Cell Mol Biol. 2000;23(6):794-802.

232. Joshi S, Abebe W, Agrawal DK. Identification of guanine nucleotide binding regulatory proteins in bovine tracheal smooth muscle. Mol Cell Biochem. 1996;154(2):179-84.

233. Taussig R, Zimmermann G. Type-specific regulation of mammalian adenylyl cyclases by G protein pathways. Adv Second Messenger Phosphoprotein Res. 1998;32:81-98.

234. Hirshman CA, Emala CW. Actin reorganization in airway smooth muscle cells involves Gq and Gi-2 activation of Rho. The American journal of physiology. 1999;277(3 Pt 1):L653-61.

235. Barnes PJ. Pharmacology of airway smooth muscle. American journal of respiratory and critical care medicine. 1998;158(5 Pt 3):S123-32.

236. Sah VP, Seasholtz TM, Sagi SA, Brown JH. The role of Rho in G protein-coupled receptor signal transduction. Annual review of pharmacology and toxicology. 2000;40:459-89.

237. Treisman R, Alberts AS, Sahai E. Regulation of SRF activity by Rho family GTPases. Cold Spring Harb Symp Quant Biol. 1998;63:643-51.

238. Siehler S. Regulation of RhoGEF proteins by G12/13-coupled receptors. British journal of pharmacology. 2009;158(1):41-9.

239. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol. 2003;4(6):446-56.

240. Narumiya S, Ishizaki T, Watanabe N. Rho effectors and reorganization of actin cytoskeleton. FEBS Lett. 1997;410(1):68-72.

241. Meyer BH, Freuler F, Guerini D, Siehler S. Reversible translocation of p115-RhoGEF by G(12/13)-coupled receptors. J Cell Biochem. 2008;104(5):1660-70.

242. Chiba Y, Takada Y, Miyamoto S, MitsuiSaito M, Karaki H, Misawa M. Augmented acetylcholine-induced, Rho-mediated Ca2+ sensitization of bronchial smooth muscle contraction in antigen-induced airway hyperresponsive rats. British journal of pharmacology. 1999;127(3):597-600.

243. Chiba Y, Misawa M. Increased expression of G12 and G13 proteins in bronchial smooth muscle of airway hyperresponsive rats. Inflamm Res. 2001;50(6):333-6.

244. Wirth A, Benyo Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. Nat Med. 2008;14(1):64-8.

245. Kelly E, Bailey CP, Henderson G. Agonist-selective mechanisms of GPCR desensitization. British journal of pharmacology. 2008;153 Suppl 1:S379-88.

246. Krupnick JG, Benovic JL. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annual review of pharmacology and toxicology. 1998;38:289-319.

247. Freedman NJ, Lefkowitz RJ. Desensitization of G protein-coupled receptors. Recent Prog Horm Res. 1996;51:319-51; discussion 52-3.

248. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem. 1998;67:653-92.

249. Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G, et al. The vascular endothelium and human diseases. International journal of biological sciences. 2013;9(10):1057-69.

250. Durand MJ, Gutterman DD. Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. Microcirculation. 2013;20(3):239-47.

251. Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. American journal of physiology Renal physiology. 2001;280(2):F193-206.

252. Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circulation research. 1996;79(5):984-91.

253. Vanhoutte PM. Endothelium and control of vascular function. State of the Art lecture. Hypertension. 1989;13(6 Pt 2):658-67.

254. Zhang YH, Jin CZ, Jang JH, Wang Y. Molecular mechanisms of neuronal nitric oxide synthase in cardiac function and pathophysiology. The Journal of physiology. 2014;592(15):3189-200.

255. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, et al. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science. 1992;256(5054):225-8.

256. Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, et al. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. The Journal of biological chemistry. 2001;276(41):38121-38.

257. Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, et al. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. Science. 2001;293(5539):2449-52.

258. Chidlow JH, Jr., Sessa WC. Caveolae, caveolins, and cavins: complex control of cellular signalling and inflammation. Cardiovascular research. 2010;86(2):219-25.

259. Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, Sessa WC. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. The Journal of biological chemistry. 2000;275(29):22268-72.

260. Pollock JS WF, Mitchell JA & Förstermann U Characterisation of EDRF/NO synthase as a FAD/FMN containing flavoprotein. Endothelium. 1993;1:147-52.

261. Grover AK, Khan I. Calcium pump isoforms: diversity, selectivity and plasticity. Review article. Cell calcium. 1992;13(1):9-17.

262. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. European heart journal. 2012;33(7):829-37, 37a-37d.

263. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. The American journal of physiology. 1986;250(6 Pt 2):H1145-9.

264. Cannon RO, 3rd. Role of nitric oxide in cardiovascular disease: focus on the endothelium. Clinical chemistry. 1998;44(8 Pt 2):1809-19.

265. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiological reviews. 1999;79(4):1193-226.

266. FRESNO M D-MM, CUESTA N, CACHEIRO-LLAGUNO C AND ÍÑIGUEZ MA. Prostanoids actions in cardiovascular physiopathology. An R Acad Nac Farm. 2008;74(4).

267. Kostenis E, Ulven T. Emerging roles of DP and CRTH2 in allergic inflammation. Trends Mol Med. 2006;12(4):148-58.

268. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med. 2001;193(2):255-61.

269. Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. The Journal of biological chemistry. 1995;270(32):18910-6.

270. Smyth EM, Grosser T, Wang M, Yu Y, FitzGerald GA. Prostanoids in health and disease. J Lipid Res. 2009;50 Suppl:S423-8.

271. Abran D, Varma DR, Chemtob S. Regulation of prostanoid vasomotor effects and receptors in choroidal vessels of newborn pigs. The American journal of physiology. 1997;272(3 Pt 2):R995-1001.

272. Kobayashi K, Tsubosaka Y, Hori M, Narumiya S, Ozaki H, Murata T. Prostaglandin D2-DP signaling promotes endothelial barrier function via the cAMP/PKA/Tiam1/Rac1 pathway. Arteriosclerosis, thrombosis, and vascular biology. 2013;33(3):565-71.

273. Gluais P, Lonchampt M, Morrow JD, Vanhoutte PM, Feletou M. Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: the Janus face of prostacyclin. British journal of pharmacology. 2005;146(6):834-45.

274. Legler DF, Bruckner M, Uetz-von Allmen E, Krause P. Prostaglandin E2 at new glance: novel insights in functional diversity offer therapeutic chances. Int J Biochem Cell Biol. 2010;42(2):198-201.

275. Sugimoto Y, Narumiya S. Prostaglandin E receptors. The Journal of biological chemistry. 2007;282(16):11613-7.

276. Liang X, Lin L, Woodling NS, Wang Q, Anacker C, Pan T, et al. Signaling via the prostaglandin E(2) receptor EP4 exerts neuronal and vascular protection in a mouse model of cerebral ischaemia. The Journal of clinical investigation. 2011;121(11):4362-71.

277. Horton EW, Poyser NL. Uterine luteolytic hormone: a physiological role for prostaglandin F2alpha. Physiological reviews. 1976;56(4):595-651.

278. Abramovitz M, Boie Y, Nguyen T, Rushmore TH, Bayne MA, Metters KM, et al. Cloning and expression of a cDNA for the human prostanoid FP receptor. The Journal of biological chemistry. 1994;269(4):2632-6.

279. Morrow JD, Minton TA, Roberts LJ, 2nd. The F2-isoprostane, 8-epi-prostaglandin F2 alpha, a potent agonist of the vascular thromboxane/endoperoxide receptor, is a platelet thromboxane/endoperoxide receptor antagonist. Prostaglandins. 1992;44(2):155-63.

280. Yu Y, Lucitt MB, Stubbe J, Cheng Y, Friis UG, Hansen PB, et al. Prostaglandin F2alpha elevates blood pressure and promotes atherosclerosis. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(19):7985-90.

281. Chen J, Champa-Rodriguez ML, Woodward DF. Identification of a prostanoid FP receptor population producing endothelium-dependent vasorelaxation in the rabbit jugular vein. British journal of pharmacology. 1995;116(7):3035-41.

282. Vane J, Corin RE. Prostacyclin: a vascular mediator. European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery. 2003;26(6):571-8.

283. Moncada S, Vane JR. The role of prostacyclin in vascular tissue. Federation proceedings. 1979;38(1):66-71.

284. Smyth EM, Nestor PV, FitzGerald GA. Agonist-dependent phosphorylation of an epitope-tagged human prostacyclin receptor. The Journal of biological chemistry. 1996;271(52):33698-704.

285. Smyth EM, Li WH, FitzGerald GA. Phosphorylation of the prostacyclin receptor during homologous desensitization. A critical role for protein kinase c. The Journal of biological chemistry. 1998;273(36):23258-66.

286. Miggin SM, Kinsella BT. Investigation of the mechanisms of G protein: effector coupling by the human and mouse prostacyclin receptors. Identification of critical species-dependent differences. The Journal of biological chemistry. 2002;277(30):27053-64.

287. Parfenova H, Zuckerman S, Leffler CW. Inhibitory effect of indomethacin on prostacyclin receptor-mediated cerebral vascular responses. The American journal of physiology. 1995;268(5 Pt 2):H1884-90.

288. Nishimura T, Yamamoto T, Komuro Y, Hara Y. Antiplatelet functions of a stable prostacyclin analog, SM-10906 are exerted by its inhibitory effect on inositol 1,4,5-trisphosphate production and cytosolic Ca++ increase in rat platelets stimulated by thrombin. Thrombosis research. 1995;79(3):307-17.

289. Kasza Z, Fetalvero KM, Ding M, Wagner RJ, Acs K, Guzman AK, et al. Novel signaling pathways promote a paracrine wave of prostacyclin-induced vascular smooth muscle differentiation. J Mol Cell Cardiol. 2009;46(5):682-94.

290. Verma S, Raj SR, Shewchuk L, Mather KJ, Anderson TJ. Cyclooxygenase-2 blockade does not impair endothelial vasodilator function in healthy volunteers: randomized evaluation of rofecoxib versus naproxen on endothelium-dependent vasodilatation. Circulation. 2001;104(24):2879-82.

291. Beverelli F, Bea ML, Puybasset L, Giudicelli JF, Berdeaux A. Chronic inhibition of NO synthase enhances the production of prostacyclin in coronary arteries through upregulation of the cyclooxygenase type 1 isoform. Fundam Clin Pharmacol. 1997;11(3):252-9.

292. Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, et al. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. American journal of respiratory and critical care medicine. 1999;159(6):1925-32.

293. Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, et al. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. N Engl J Med. 1992;327(2):70-5.

294. Mak OT. Prostacyclin production in vascular endothelium of patients with Blackfoot disease. Advances in experimental medicine and biology. 1988;242:119-25.

295. NERI SERNERI G MG, POGGESI L, GALANTI G, MORETTINI A, SCARTI L. REDUCED PROSTACYCLIN IN ISCHEMIC HEART DISEASE. The American journal of cardiology. 1982;49.

296. McLaughlin VV, Genthner DE, Panella MM, Rich S. Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. N Engl J Med. 1998;338(5):273-7.

297. Poredos P. Possibilities for clinical use of prostacyclin in vascular disease. Pflugers Archiv : European journal of physiology. 2000;440(5 Suppl):R137-8.

298. Neri Serneri GG, Gensini GF, Abbate R, Mugnaini C, Favilla S, Brunelli C, et al. Increased fibrinopeptide A formation and thromboxane A2 production in patients with ischemic heart disease: relationships to coronary pathoanatomy, risk factors, and clinical manifestations. American heart journal. 1981;101(2):185-94.

299. Namba T, Sugimoto Y, Hirata M, Hayashi Y, Honda A, Watabe A, et al. Mouse thromboxane A2 receptor: cDNA cloning, expression and northern blot analysis. Biochemical and biophysical research communications. 1992;184(3):1197-203.

300. Mehta JL, Lawson D, Mehta P, Saldeen T. Increased prostacyclin and thromboxane A2 biosynthesis in atherosclerosis. Proceedings of the National Academy of Sciences of the United States of America. 1988;85(12):4511-5.

301. Francois H, Athirakul K, Mao L, Rockman H, Coffman TM. Role for thromboxane receptors in angiotensin-II-induced hypertension. Hypertension. 2004;43(2):364-9.

302. Hirata T, Ushikubi F, Kakizuka A, Okuma M, Narumiya S. Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. The Journal of clinical investigation. 1996;97(4):949-56.

303. Thomas DW, Mannon RB, Mannon PJ, Latour A, Oliver JA, Hoffman M, et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. The Journal of clinical investigation. 1998;102(11):1994-2001.

304. Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP. Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca2+ entry and Ca2+ sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. The Biochemical journal. 2005;389(Pt 3):763-74.

305. Smith WL, Song I. The enzymology of prostaglandin endoperoxide H synthases-1 and -2. Prostaglandins Other Lipid Mediat. 2002;68-69:115-28.

306. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem. 2000;69:145-82.

307. Liu B, Luo W, Zhang Y, Li H, Zhu N, Huang D, et al. Involvement of cyclo-oxygenase-1-mediated prostacyclin synthesis in the vasoconstrictor activity evoked by ACh in mouse arteries. Exp Physiol. 2012;97(2):277-89.

308. Meagher EA. Balancing gastroprotection and cardioprotection with selective cyclo-oxygenase-2 inhibitors: clinical implications. Drug Saf. 2003;26(13):913-24.

309. Frolich JC. A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzymes. Trends in pharmacological sciences. 1997;18(1):30-4.

310. Patrono C, Ciabattoni G, Patrignani P, Pugliese F, Filabozzi P, Catella F, et al. Clinical pharmacology of platelet cyclooxygenase inhibition. Circulation. 1985;72(6):1177-84.

311. Burch JW, Stanford N, Majerus PW. Inhibition of platelet prostaglandin synthetase by oral aspirin. The Journal of clinical investigation. 1978;61(2):314-9.

312. Vane JR, Botting RM. Mechanism of action of anti-inflammatory drugs. Scand J Rheumatol Suppl. 1996;102:9-21.

313. Lee J KS. Inflammation and non-steroidal anti-inflammatory drugs. In: Smith CM RA, Saunders, editor. Textbook of Pharmacology. New York1992. p. 401-36.

314. Orlando BJ, Malkowski MG. Crystal structure of rofecoxib bound to human cyclooxygenase-2. Acta Crystallogr F Struct Biol Commun. 2016;72(Pt 10):772-6.

315. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(8):2863-7.

316. Saida K, Mitsui Y, Ishida N. A novel peptide, vasoactive intestinal contractor, of a new (endothelin) peptide family. Molecular cloning, expression, and biological activity. The Journal of biological chemistry. 1989;264(25):14613-6.

317. Kedzierski RM, Yanagisawa M. Endothelin system: the double-edged sword in health and disease. Annual review of pharmacology and toxicology. 2001;41:851-76.

318. Davenport AP, Maguire JJ. Endothelin. Handbook of experimental pharmacology. 2006(176 Pt 1):295-329.

319. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988;332(6163):411-5.

320. Alonso D, Radomski MW. The nitric oxide-endothelin-1 connection. Heart Fail Rev. 2003;8(1):107-15.

321. Gohla A, Schultz G, Offermanns S. Role for G(12)/G(13) in agonist-induced vascular smooth muscle cell contraction. Circulation research. 2000;87(3):221-7.

322. Liu S, Premont RT, Kontos CD, Huang J, Rockey DC. Endothelin-1 activates endothelial cell nitric-oxide synthase via heterotrimeric G-protein betagamma subunit signaling to protein jinase B/Akt. The Journal of biological chemistry. 2003;278(50):49929-35.

323. Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K, et al. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. The Journal of clinical investigation. 1993;91(4):1367-73.

324. Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine coronary smooth muscle. British journal of pharmacology. 1988;93(3):515-24.

325. Cohen RA, Vanhoutte PM. Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. Circulation. 1995;92(11):3337-49.

326. Bolton TB, Clapp LH. Endothelial-dependent relaxant actions of carbachol and substance P in arterial smooth muscle. British journal of pharmacology. 1986;87(4):713-23.

327. Hamilton CA, Williams R, Pathi V, Berg G, McArthur K, McPhaden AR, et al. Pharmacological characterisation of endothelium-dependent relaxation in human radial artery: comparison with internal thoracic artery. Cardiovascular research. 1999;42(1):214-23.

328. Hamilton CA, McPhaden AR, Berg G, Pathi V, Dominiczak AF. Is hydrogen peroxide an EDHF in human radial arteries? American journal of physiology Heart and circulatory physiology. 2001;280(6):H2451-5.

329. Garland CJ, Hiley CR, Dora KA. EDHF: spreading the influence of the endothelium. British journal of pharmacology. 2011;164(3):839-52.

330. Fleming I, Fisslthaler B, Michaelis UR, Kiss L, Popp R, Busse R. The coronary endothelium-derived hyperpolarizing factor (EDHF) stimulates multiple

signalling pathways and proliferation in vascular cells. Pflugers Archiv : European journal of physiology. 2001;442(4):511-8.

331. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circulation research. 1996;78(3):415-23.

332. Popp R, Bauersachs J, Hecker M, Fleming I, Busse R. A transferable, beta-naphthoflavone-inducible, hyperpolarizing factor is synthesized by native and cultured porcine coronary endothelial cells. The Journal of physiology. 1996;497 (Pt 3):699-709.

333. Hecker M, Bara AT, Bauersachs J, Busse R. Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. The Journal of physiology. 1994;481 (Pt 2):407-14.

334. Widmann MD, Weintraub NL, Fudge JL, Brooks LA, Dellsperger KC. Cytochrome P-450 pathway in acetylcholine-induced canine coronary microvascular vasodilation in vivo. The American journal of physiology. 1998;274(1 Pt 2):H283-9.

335. Quilley J, McGiff JC. Is EDHF an epoxyeicosatrienoic acid? Trends in pharmacological sciences. 2000;21(4):121-4.

336. Endemann DH, Schiffrin EL. Endothelial dysfunction. Journal of the American Society of Nephrology : JASN. 2004;15(8):1983-92.

337. Versari D, Daghini E, Virdis A, Ghiadoni L, Taddei S. Endothelial dysfunction as a target for prevention of cardiovascular disease. Diabetes care. 2009;32 Suppl 2:S314-21.

338. Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE. Aging is associated with endothelial dysfunction in healthy men

years before the age-related decline in women. Journal of the American College of Cardiology. 1994;24(2):471-6.

339. Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. The Biochemical journal. 2002;362(Pt 3):733-9.

340. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(16):9220-5.

341. Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, et al. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. Arteriosclerosis, thrombosis, and vascular biology. 2001;21(4):496-502.

342. Brandes RP, Fleming I, Busse R. Endothelial aging. Cardiovascular research. 2005;66(2):286-94.

343. Yoon HJ, Cho SW, Ahn BW, Yang SY. Alterations in the activity and expression of endothelial NO synthase in aged human endothelial cells. Mechanisms of ageing and development. 2010;131(2):119-23.

344. Smith AR, Visioli F, Frei B, Hagen TM. Age-related changes in endothelial nitric oxide synthase phosphorylation and nitric oxide dependent vasodilation: evidence for a novel mechanism involving sphingomyelinase and ceramide-activated phosphatase 2A. Aging cell. 2006;5(5):391-400.

345. Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. Biochemical and biophysical research communications. 1999;263(3):681-4.

346. Ignarro LJ. Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. Circulation research. 1989;65(1):1-21.

347. Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, et al. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation. 1998;98(18):1842-7.

348. Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, Ganz P, et al. The assessment of endothelial function: from research into clinical practice. Circulation. 2012;126(6):753-67.

349. Tousoulis D, Antoniades C, Stefanadis C. Evaluating endothelial function in humans: a guide to invasive and non-invasive techniques. Heart. 2005;91(4):553-8.

350. Seals DR, Jablonski KL, Donato AJ. Aging and vascular endothelial function in humans. Clinical science. 2011;120(9):357-75.

351. Webb RC. Smooth muscle contraction and relaxation. Advances in physiology education. 2003;27(1-4):201-6.

352. Hughes AD. Calcium channels in vascular smooth muscle cells. Journal of vascular research. 1995;32(6):353-70.

353. MacMillan D, Chalmers S, Muir TC, McCarron JG. IP3-mediated Ca2+ increases do not involve the ryanodine receptor, but ryanodine receptor antagonists reduce IP3-mediated Ca2+ increases in guinea-pig colonic smooth muscle cells. The Journal of physiology. 2005;569(Pt 2):533-44.

354. Hilgers RH, Webb RC. Molecular aspects of arterial smooth muscle contraction: focus on Rho. Experimental biology and medicine. 2005;230(11):829-35.

355. Akata T. Cellular and molecular mechanisms regulating vascular tone. Part 1: basic mechanisms controlling cytosolic Ca2+ concentration and the Ca2+-dependent regulation of vascular tone. Journal of anaesthesia. 2007;21(2):220-31.

356. Woodrum DA, Brophy CM. The paradox of smooth muscle physiology. Molecular and cellular endocrinology. 2001;177(1-2):135-43.

357. Wamhoff BR, Bowles DK, Owens GK. Excitation-transcription coupling in arterial smooth muscle. Circulation research. 2006;98(7):868-78.

358. Sato K, Ozaki H, Karaki H. Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2. The Journal of pharmacology and experimental therapeutics. 1988;246(1):294-300.

359. Himpens B, Kitazawa T, Somlyo AP. Agonist-dependent modulation of Ca2+ sensitivity in rabbit pulmonary artery smooth muscle. Pflugers Archiv : European journal of physiology. 1990;417(1):21-8.

360. Kitazawa T, Kobayashi S, Horiuti K, Somlyo AV, Somlyo AP. Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca2+. The Journal of biological chemistry. 1989;264(10):5339-42.

361. Himpens B, Casteels R. Different effects of depolarization and muscarinic stimulation on the Ca2+/force relationship during the contraction-relaxation cycle in the guinea pig ileum. Pflugers Archiv : European journal of physiology. 1990;416(1-2):28-35.

362. Bradley AB, Morgan KG. Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. The Journal of physiology. 1987;385:437-48.

363. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. Nature. 1994;372(6503):231-6.

364. Marks AR. Calcium channels expressed in vascular smooth muscle. Circulation. 1992;86(6 Suppl):III61-7.

365. Yu FH, Catterall WA. The VGL-chanome: a protein superfamily specialized for electrical signaling and ionic homeostasis. Science's STKE : signal transduction knowledge environment. 2004;2004(253):re15.

366. Inoue Y, Oike M, Nakao K, Kitamura K, Kuriyama H. Endothelin augments unitary calcium channel currents on the smooth muscle cell membrane of guinea-pig portal vein. The Journal of physiology. 1990;423:171-91.

367. Do KH, Kim MS, Kim JH, Rhim BY, Lee WS, Kim CD, et al. Angiotensin II-induced aortic ring constriction is mediated by phosphatidylinositol 3-kinase/L-type calcium channel signaling pathway. Exp Mol Med. 2009;41(8):569-76.

368. Ball CJ, Wilson DP, Turner SP, Saint DA, Beltrame JF. Heterogeneity of L- and T-channels in the vasculature: rationale for the efficacy of combined L- and T-blockade. Hypertension. 2009;53(4):654-60.

369. McDonald TF, Pelzer S, Trautwein W, Pelzer DJ. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. Physiological reviews. 1994;74(2):365-507.

370. Bertolino M, Llinas RR. The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. Annual review of pharmacology and toxicology. 1992;32:399-421.

371. Verma AK, Filoteo AG, Stanford DR, Wieben ED, Penniston JT, Strehler EE, et al. Complete primary structure of a human plasma membrane Ca2+ pump. The Journal of biological chemistry. 1988;263(28):14152-9.

372. Marin J, Encabo A, Briones A, Garcia-Cohen EC, Alonso MJ. Mechanisms involved in the cellular calcium homeostasis in vascular smooth muscle: calcium pumps. Life sciences. 1999;64(5):279-303.

373. Brini M, Carafoli E. The plasma membrane Ca(2)+ ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. Cold Spring Harbor perspectives in biology. 2011;3(2).

374. Di Leva F, Domi T, Fedrizzi L, Lim D, Carafoli E. The plasma membrane Ca2+ ATPase of animal cells: structure, function and regulation. Archives of biochemistry and biophysics. 2008;476(1):65-74.

375. Sumida M, Okuda H, Hamada M. Ca2+,Mg2+-ATPase of microsomal membranes from bovine aortic smooth muscle. Identification and characterization of an acid-stable phosphorylated intermediate of the Ca2+,Mg2+-ATPase. Journal of biochemistry. 1984;96(5):1365-74.

376. Periasamy M, Kalyanasundaram A. SERCA pump isoforms: their role in calcium transport and disease. Muscle & nerve. 2007;35(4):430-42.

377. Fakler B, Adelman JP. Control of K(Ca) channels by calcium nano/microdomains. Neuron. 2008;59(6):873-81.

378. McManus OB. Calcium-activated potassium channels: regulation by calcium. Journal of bioenergetics and biomembranes. 1991;23(4):537-60.

379. Minami K, Fukuzawa K, Nakaya Y. Protein kinase C inhibits the Ca(2+)-activated K+ channel of cultured porcine coronary artery smooth muscle cells. Biochemical and biophysical research communications. 1993;190(1):263-9.

380. Kume H, Takai A, Tokuno H, Tomita T. Regulation of Ca2+-dependent K+-channel activity in tracheal myocytes by phosphorylation. Nature. 1989;341(6238):152-4.

381. Mondin L, Balghi H, Constantin B, Cognard C, Sebille S. Negative modulation of inositol 1,4,5-trisphosphate type 1 receptor expression prevents dystrophin-deficient muscle cells death. American journal of physiology Cell physiology. 2009;297(5):C1133-45.

382. McNeish AJ, Sandow SL, Neylon CB, Chen MX, Dora KA, Garland CJ. Evidence for involvement of both IKCa and SKCa channels in hyperpolarizing responses of the rat middle cerebral artery. Stroke. 2006;37(5):1277-82.

383. Jackson WF. Ion channels and vascular tone. Hypertension. 2000;35(1 Pt 2):173-8.

384. Giovannitti JA, Jr., Thoms SM, Crawford JJ. Alpha-2 adrenergic receptor agonists: a review of current clinical applications. Anaesthesia progress. 2015;62(1):31-9.

385. Lembo G, Iaccarino G, Vecchione C, Barbato E, Morisco C, Monti F, et al. Insulin enhances endothelial alpha2-adrenergic vasorelaxation by a pertussis toxin mechanism. Hypertension. 1997;30(5):1128-34.

386. Freeman JE, Kuo WY, Drenger B, Barnett TN, Levine MA, Flavahan NA. Analysis of lysophophatidylcholine-induced endothelial dysfunction. Journal of cardiovascular pharmacology. 1996;28(3):345-52.

387. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature. 2002;415(6868):206-12.

388. Soeder KJ, Snedden SK, Cao W, Della Rocca GJ, Daniel KW, Luttrell LM, et al. The beta3-adrenergic receptor activates mitogen-activated protein kinase in

adipocytes through a Gi-dependent mechanism. The Journal of biological chemistry. 1999;274(17):12017-22.

389. Filippi S, Parenti A, Donnini S, Granger HJ, Fazzini A, Ledda F. alpha(1D)-adrenoceptors cause endothelium-dependent vasodilatation in the rat mesenteric vascular bed. The Journal of pharmacology and experimental therapeutics. 2001;296(3):869-75.

390. Mohammad-Zadeh LF, Moses L, Gwaltney-Brant SM. Serotonin: a review. Journal of veterinary pharmacology and therapeutics. 2008;31(3):187-99.

391. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. Annu Rev Med. 2009;60:355-66.

392. Hirafuji M. Recent advances in 5-hydroxytryptamine (5-HT) receptor research: how many pathophysiological roles does 5-HT play via its multiple receptor subtypes? Foreword. Biological & pharmaceutical bulletin. 2013;36(9):1391.

393. Ni W, Thompson JM, Northcott CA, Lookingland K, Watts SW. The serotonin transporter is present and functional in peripheral arterial smooth muscle. Journal of cardiovascular pharmacology. 2004;43(6):770-81.

394. Ni W, Geddes TJ, Priestley JR, Szasz T, Kuhn DM, Watts SW. The existence of a local 5-hydroxytryptaminergic system in peripheral arteries. British journal of pharmacology. 2008;154(3):663-74.

395. Kereveur A, Callebert J, Humbert M, Herve P, Simonneau G, Launay JM, et al. High plasma serotonin levels in primary pulmonary hypertension. Effect of long-term epoprostenol (prostacyclin) therapy. Arteriosclerosis, thrombosis, and vascular biology. 2000;20(10):2233-9.

396. Biondi ML, Marasini B, Bianchi E, Agostoni A. Plasma free and intraplatelet serotonin in patients with Raynaud's phenomenon. Int J Cardiol. 1988;19(3):335-9.

397. Filip M, Bader M. Overview on 5-HT receptors and their role in physiology and pathology of the central nervous system. Pharmacological reports : PR. 2009;61(5):761-77.

398. Fleming I, Busse R. Endothelium-derived epoxyeicosatrienoic acids and vascular function. Hypertension. 2006;47(4):629-33.

399. Bath PM, Hassall DG, Gladwin AM, Palmer RM, Martin JF. Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. Arteriosclerosis and thrombosis : a journal of vascular biology. 1991;11(2):254-60.

400. Kaumann AJ, Levy FO. 5-hydroxytryptamine receptors in the human cardiovascular system. Pharmacol Ther. 2006;111(3):674-706.

401. Nichols DE, Nichols CD. Serotonin receptors. Chem Rev. 2008;108(5):1614-41.

402. Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, et al. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacological reviews. 1994;46(2):157-203.

403. Ko EA, Song MY, Donthamsetty R, Makino A, Yuan JX. Tension Measurement in Isolated Rat and Mouse Pulmonary Artery. Drug discovery today Disease models. 2010;7(3-4):123-30.

404. Akar F, Manavbasi Y, Parlar AI, Ulus AT, Katircioglu SF. The gender differences in the relaxation to levosimendan of human internal mammary artery. Cardiovascular drugs and therapy. 2007;21(5):331-8.

405. Dignan RJ, Yeh T, Jr., Dyke CM, Lutz HA, 3rd, Wechsler AS. The influence of age and sex on human internal mammary artery size and reactivity. The Annals of thoracic surgery. 1992;53(5):792-7.

406. Ergul A, Shoemaker K, Puett D, Tackett RL. Gender differences in the expression of endothelin receptors in human saphenous veins in vitro. The Journal of pharmacology and experimental therapeutics. 1998;285(2):511-7.

407. Takenouchi Y, Kobayashi T, Taguchi K, Matsumoto T, Kamata K. Gender differences in vascular reactivity of aortas from streptozotocin-induced diabetic mice. Biological & pharmaceutical bulletin. 2010;33(10):1692-7.

408. Tostes RC, David FL, Carvalho MH, Nigro D, Scivoletto R, Fortes ZB. Gender differences in vascular reactivity to endothelin-1 in deoxycorticosterone-salt hypertensive rats. Journal of cardiovascular pharmacology. 2000;36(5 Suppl 1):S99-101.

409. Nuno DW, Korovkina VP, England SK, Lamping KG. RhoA activation contributes to sex differences in vascular contractions. Arteriosclerosis, thrombosis, and vascular biology. 2007;27(9):1934-40.

410. Jaghoori A. Sex-Dependent Differences in Vasomotor Responses of Old Male and Female Humans: University of Adelaide; 2014.

411. Huraux C, Makita T, Kurz S, Yamaguchi K, Szlam F, Tarpey MM, et al. Superoxide production, risk factors, and endothelium-dependent relaxations in human internal mammary arteries. Circulation. 1999;99(1):53-9.

412. Lamin V, Worthington M. Endothelial Denudation of Isolated Human Internal Mammary Artery Segments. Cardiovascular Pharmacology: Open Access. 2015;5(3).

413. Jackson SK, Thomas MP, Smith S, Madhani M, Rogers SC, James PE. In vivo EPR spectroscopy: biomedical and potential diagnostic applications. Faraday Discuss. 2004;126:103-17; discussion 69-83.

414. Furugen A, Yamaguchi H, Tanaka N, Ito H, Miyamori K, Fujikawa A, et al. Quantification of intracellular and extracellular prostanoids stimulated by A23187 by

liquid chromatography/electrospray ionization tandem mass spectrometry. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2011;879(30):3378-85.

415. Tomida J KH, Kinoshita E and Takata M. Detection of phosphorylation on large proteins by western blotting using Phos-tag containing gel. 2008.

416. Jarajapu YP, Coats P, McGrath JC, MacDonald A, Hillier C. Increased alpha(1)- and alpha(2)-adrenoceptor-mediated contractile responses of human skeletal muscle resistance arteries in chronic limb ischaemia. Cardiovascular research. 2001;49(1):218-25.

417. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980;288(5789):373-6.

418. Urabe M, Kawasaki H, Takasaki K. Effect of endothelium removal on the vasoconstrictor response to neuronally released 5-hydroxytryptamine and noradrenaline in the rat isolated mesenteric and femoral arteries. British journal of pharmacology. 1991;102(1):85-90.

419. Dora KA, Hinton JM, Walker SD, Garland CJ. An indirect influence of phenylephrine on the release of endothelium-derived vasodilators in rat small mesenteric artery. British journal of pharmacology. 2000;129(2):381-7.

420. Tesfamariam B, Halpern W, Osol G. Effects of perfusion and endothelium on the reactivity of isolated resistance arteries. Blood vessels. 1985;22(6):301-5.

421. Gustafsson H, Mulvany MJ, Nilsson H. Rhythmic contractions of isolated small arteries from rat: influence of the endothelium. Acta physiologica Scandinavica. 1993;148(2):153-63.

422. Osol G, Cipolla M, Knutson S. A new method for mechanically denuding the endothelium of small (50-150 microns) arteries with a human hair. Blood vessels. 1989;26(5):320-4.

423. Borna C, Wang L, Gudbjartsson T, Karlsson L, Jern S, Malmsjo M, et al. Contractions in human coronary bypass vessels stimulated by extracellular nucleotides. The Annals of thoracic surgery. 2003;76(1):50-7.

424. Tanaka N, Nakamura E, Ohkura M, Kuwabara M, Yamashita A, Onitsuka T, et al. Both 5-hydroxytryptamine 5-HT2A and 5-HT1B receptors are involved in the vasoconstrictor response to 5-HT in the human isolated internal thoracic artery. Clinical and experimental pharmacology & physiology. 2008;35(7):836-40.

425. Wackenfors A, Ingemansson R, Malmsjo M. Endothelin receptors in endothelium-denuded human coronary artery bypass grafts and coronary arteries. The Annals of thoracic surgery. 2003;75(3):874-81.

426. Jovanovic A, Grbovic L, Tulic I. Predominant role for nitric oxide in the relaxation induced by acetylcholine in human uterine artery. Human reproduction. 1994;9(3):387-93.

427. Allen SP, Chester AH, Dashwood MR, Tadjkarimi S, Piper PJ, Yacoub MH. Preferential vasoconstriction to cysteinyl leukotrienes in the human saphenous vein compared with the internal mammary artery. Implications for graft performance. Circulation. 1994;90(1):515-24.

428. Wiley KE, Davenport AP. Comparison of vasodilators in human internal mammary artery: ghrelin is a potent physiological antagonist of endothelin-1. British journal of pharmacology. 2002;136(8):1146-52.

429. Poppa V, Miyashiro JK, Corson MA, Berk BC. Endothelial NO synthase is increased in regenerating endothelium after denuding injury of the rat aorta. Arteriosclerosis, thrombosis, and vascular biology. 1998;18(8):1312-21.

430. Lu X, Guo X, Linares C, Kassab GS. A new method to denude the endothelium without damage to media: structural, functional, and biomechanical validation. American journal of physiology Heart and circulatory physiology. 2004;286(5):H1889-94.

431. Guyton JR, Dao DT, Lindsay KL. Endothelial denudation and myointimal thickening in the rat carotid artery induced by the passage of bubbles. Experimental and molecular pathology. 1984;40(3):340-8.

432. Lamping KG, Marcus ML, Dole WP. Removal of the endothelium potentiates canine large coronary artery constrictor responses to 5-hydroxytryptamine in vivo. Circulation research. 1985;57(1):46-54.

433. Buxton BF, Hayward PA, Newcomb AE, Moten S, Seevanayagam S, Gordon I. Choice of conduits for coronary artery bypass grafting: craft or science? European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery. 2009;35(4):658-70.

434. Wang Y, Mercer-Connolly A, Lines L, Toyoda O, Coceani F. Endothelium-denuded pulmonary resistance arteries from the fetal lamb: preparation and response to vasoactive agents. Journal of pharmacological and toxicological methods. 1994;32(2):85-91.

435. Luu TN, Chester AH, O'Neil GS, Tadjkarimi S, Pepper JR, Yacoub MH. Different responses of the human gastroepiploic and internal mammary arteries to vasoactive peptides. The American journal of physiology. 1993;264(2 Pt 2):H583-7.

436. Pagan RM, Martinez AC, Hernandez M, Martinez MP, Garcia-Sacristan A, Correa C, et al. Endothelial and neural factors functionally involved in the modulation of noradrenergic vasoconstriction in healthy pig internal mammary artery. Biochemical pharmacology. 2012;83(7):882-92.

437. Otsuka F, Yahagi K, Sakakura K, Virmani R. Why is the mammary artery so special and what protects it from atherosclerosis? Annals of cardiothoracic surgery. 2013;2(4):519-26.

438. Borovic ML, Borovic S, Peric M, Vukovic P, Marinkovic J, Todorovic V, et al. The internal thoracic artery as a transitional type of artery: a morphological and morphometric study. Histology and histopathology. 2010;25(5):561-76.

439. Kim C, Redberg RF, Pavlic T, Eagle KA. A systematic review of gender differences in mortality after coronary artery bypass graft surgery and percutaneous coronary interventions. Clinical cardiology. 2007;30(10):491-5.

440. Bukkapatnam RN, Yeo KK, Li Z, Amsterdam EA. Operative mortality in women and men undergoing coronary artery bypass grafting (from the California Coronary Artery Bypass Grafting Outcomes Reporting Program). The American journal of cardiology. 2010;105(3):339-42.

441. Saxena A, Dinh D, Smith JA, Shardey G, Reid CM, Newcomb AE. Sex differences in outcomes following isolated coronary artery bypass graft surgery in Australian patients: analysis of the Australasian Society of Cardiac and Thoracic Surgeons cardiac surgery database. European Journal of Cardio-Thoracic Surgery. 2011.

442. Fiebach NH, Viscoli CM, Horwitz RI. Differences between women and men in survival after myocardial infarction: Biology or methodology? JAMA. 1990;263(8):1092-6.

443. Kudenchuk PJ, Maynard C, Martin JS, Wirkus M, Weaver WD, The MPI. Comparison of presentation, treatment, and outcome of acute myocardial infarction in men versus women (the Myocardial Infarction Triage and Intervention Registry). The American Journal of Cardiology. 1996;78(1):9-14.

444. Yusuf S, Hawken S, √îunpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. The Lancet. 2004;364(9438):937-52.

445. Frasure-Smith N, Lesperance F, Juneau M, Talajic M, Bourassa MG. Gender, depression, and one-year prognosis after myocardial infarction. Psychosomatic medicine. 1999;61(1):26-37.

446. Naqvi TZ, Naqvi SSA, Merz CNB. Gender Differences in the Link Between Depression and Cardiovascular Disease. Psychosomatic Medicine. 2005;67(Supplement 1):S15-S8.

447. Vogels EA, Lagro-Janssen AL, van Weel C. Sex differences in cardiovascular disease: are women with low socioeconomic status at high risk? The British journal of general practice : the journal of the Royal College of General Practitioners. 1999;49(449):963-6.

448. Lacey EA, Walters SJ. Continuing inequality: gender and social class influences on self perceived health after a heart attack. Journal of epidemiology and community health. 2003;57(8):622-7.

449. Roshan TM, Normah J, Rehman A, Naing L. Effect of menopause on platelet activation markers determined by flow cytometry. American journal of hematology. 2005;80(4):257-61.

450. Yazawa H, Iida-Kubota E, Honma Y, Honda K. Characterization of the endothelin receptor in primary cultures of human aortic smooth muscle cells. Japanese journal of pharmacology. 1993;63(3):313-8.

451. Davenport AP, O'Reilly G, Kuc RE. Endothelin ETA and ETB mRNA and receptors expressed by smooth muscle in the human vasculature: majority of the ETA sub-type. British journal of pharmacology. 1995;114(6):1110-6.

452. Sharma AC, Fogelson BG, Nawas SI, Vigneswaran WT, Sam AD, 2nd, Alden KJ, et al. Elevated coronary endothelin-1 but not nitric oxide in diabetics during CABG. The Annals of thoracic surgery. 1999;67(6):1659-63.

453. He GW, Yang CQ. Inhibition of vasoconstriction by phosphodiesterase III inhibitor milrinone in human conduit arteries used as coronary bypass grafts. Journal of cardiovascular pharmacology. 1996;28(2):208-14.

454. He GW, Shaw J, Hughes CF, Yang CQ, Thomson DS, McCaughan B, et al. Predominant alpha 1-adrenoceptor-mediated contraction in the human internal mammary artery. Journal of cardiovascular pharmacology. 1993;21(2):256-63.

455. Sage PR, de la Lande IS, Stafford I, Bennett CL, Phillipov G, Stubberfield J, et al. Nitroglycerin tolerance in human vessels: evidence for impaired nitroglycerin bioconversion. Circulation. 2000;102(23):2810-5.

456. Resink TJ, Scott-Burden T, Buhler FR. Activation of phospholipase A2 by endothelin in cultured vascular smooth muscle cells. Biochemical and biophysical research communications. 1989;158(1):279-86.

457. Ihara M, Noguchi K, Saeki T, Fukuroda T, Tsuchida S, Kimura S, et al. Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. Life sciences. 1992;50(4):247-55.

458. de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, et al. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. Proceedings of the National Academy of Sciences of the United States of America. 1988;85(24):9797-800.

459. Golub MS, Berger ME, Powell M. Adrenergic stimulation of prostacyclin production in the rat tail artery. I. Response to agonists and antagonists. Prostaglandins, leukotrienes, and medicine. 1985;20(3):299-311.

460. Boeynaems JM, Demolle D, Galand N. Adrenergic stimulation of vascular prostacyclin: role of alpha 1-receptors in smooth muscle cells. Eur J Pharmacol. 1987;144(2):193-200.

461. Seo B, Oemar BS, Siebenmann R, von Segesser L, Luscher TF. Both ETA and ETB receptors mediate contraction to endothelin-1 in human blood vessels. Circulation. 1994;89(3):1203-8.

462. Gautam S, Shankar N, Tandon OP, Goel N. Comparison of cardiac autonomic functions among postmenopausal women with and without hormone replacement therapy, and premenopausal women. Indian journal of physiology and pharmacology. 2011;55(4):297-303.

463. Narkiewicz K, Phillips BG, Kato M, Hering D, Bieniaszewski L, Somers VK. Gender-Selective Interaction Between Aging, Blood Pressure, and Sympathetic Nerve Activity. Hypertension. 2005;45(4):522-5.

464. Sherwood A, Park SB, Hughes JW, Blumenthal JA, Hinderliter A, Trivedi R, et al. Cardiovascular hemodynamics during stress in premenopausal versus postmenopausal women. Menopause. 2010;17(2):403-9.

465. Writing Group M, Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, et al. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation. 2010;121(7):e46-e215.

466. Aloysius UI, Achike FI, Mustafa MR. Mechanisms underlining gender differences in Phenylephrine contraction of normoglycaemic and short-term Streptozotocin-induced diabetic WKY rat aorta. Vascular pharmacology. 2012;57(2-4):81-90.

467. Hall RI, MacLaren C, Smith MS, McIntyre AJ, Allen CT, Murphy JT, et al. Light versus heavy sedation after cardiac surgery: myocardial ischaemia and the stress response. Maritime Heart Centre and Dalhousie University. Anaesthesia & Analgesia. 1997;85(5):971-8.

468. Philbin DM, Rosow CE, Schneider RC, Koski G, D'Ambra MN. Fentanyl and sufentanil anaesthesia revisited: how much is enough? Anesthesiology. 1990;73(1):5-11.

469. Jaglal SB, Tu JV, Naylor CD. Higher in-hospital mortality in female patients following coronary artery bypass surgery: a population-based study. Provincial Adult Cardiac Care Network of Ontario. Clinical and investigative medicine Medecine clinique et experimentale. 1995;18(2):99-107.

470. Fiebach NH, Viscoli CM, Horwitz RI. Differences between women and men in survival after myocardial infarction. Biology or methodology? Jama. 1990;263(8):1092-6.

471. Robich MP, Araujo EG, Feng J, Osipov RM, Clements RT, Bianchi C, et al. Altered coronary microvascular serotonin receptor expression after coronary artery bypass grafting with cardiopulmonary bypass. The Journal of thoracic and cardiovascular surgery. 2010;139(4):1033-40.

472. Metais C, Bianchi C, Li J, Li J, Simons M, Sellke FW. Serotonin-induced human coronary microvascular contraction during acute myocardial ischaemia is blocked by COX-2 inhibition. Basic research in cardiology. 2001;96(1):59-67.

473. Van Nueten JM, Janssens WJ, Vanhoutte PM. Serotonin and vascular reactivity. Pharmacological research communications. 1985;17(7):585-608.

474. Gelosa P, Ballerio R, Banfi C, Nobili E, Gianella A, Pignieri A, et al. Terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, increases survival in stroke-prone rats by preventing systemic inflammation and endothelial dysfunction: comparison with aspirin and rosuvastatin. The Journal of pharmacology and experimental therapeutics. 2010;334(1):199-205.

475. Shirahase H, Fujiwara M, Usui H, Kurahashi K. A possible role of thromboxane A2 in endothelium in maintaining resting tone and producing contractile response to acetylcholine and arachidonic acid in canine cerebral arteries. Blood vessels. 1987;24(3):117-9.

476. Gude NM, Boura AL, King RG, Brennecke SP, Jamal OS, Smith R, et al. Evidence for inhibition by endothelium-derived relaxing factor of thromboxane A2 receptor-mediated vasoconstriction in the fetal vessels of the human perfused placenta. Placenta. 1992;13(6):597-605.

477. Tostes RC, Nigro D, Fortes ZB, Carvalho MH. Effects of estrogen on the vascular system. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas. 2003;36(9):1143-58.

478. Guo X, Razandi M, Pedram A, Kassab G, Levin ER. Estrogen induces vascular wall dilation: mediation through kinase signaling to nitric oxide and estrogen receptors alpha and beta. The Journal of biological chemistry. 2005;280(20):19704-10.

479. Jeremy JY, Mikhailidis DP, Dandona P. The effect of nifedipine, nimodipine and nisoldipine on agonist- and trauma-stimulated vascular prostacyclin synthesis in vitro. Naunyn Schmiedebergs Arch Pharmacol. 1986;332(1):70-3.

480. Jeremy JY, Mikhailidis DP, Dandona P. Thromboxane A2 analogue (U-46619) stimulates vascular PGI2 synthesis. Eur J Pharmacol. 1985;107(2):259-62.

481. el-Kashef HA, Hofman WF, Ehrhart IC. Prostacyclin production with serotonin, increased flow, or elevated venous pressure in dog lung. J Appl Physiol (1985). 1990;69(4):1283-9.

482. Demolle D, Van Coevorden A, Boeynaems JM. Stimulation of aortic smooth muscle prostacyclin by serotonin: role of distinct receptors in contractile and synthetic states. Circulation research. 1989;64(4):806-13.

483. Coughlin SR, Moskowitz MA, Antoniades HN, Levine L. Serotonin receptor-mediated stimulation of bovine smooth muscle cell prostacyclin synthesis and its modulation by platelet-derived growth factor. Proceedings of the National Academy of Sciences of the United States of America. 1981;78(11):7134-8.

484. Nicholson NS, Smith SL, Fuller GC. Effect of the stable endoperoxide analog U-46619 on prostacyclin production and cyclic AMP levels in bovine endothelial cells. Thrombosis research. 1984;35(2):183-92.

485. Lamping KG, Faraci FM. Role of sex differences and effects of endothelial NO synthase deficiency in responses of carotid arteries to serotonin. Arteriosclerosis, thrombosis, and vascular biology. 2001;21(4):523-8.

486. Miller VM, Lewis DA, Barber DA. Gender differences and endothelium- and platelet-derived factors in the coronary circulation. Clinical and experimental pharmacology & physiology. 1999;26(2):132-6.

487. Cox DA, Cohen ML. Influence of gender on vasomotor effects of oxidized low-density lipoprotein in porcine coronary arteries. The American journal of physiology. 1997;272(6 Pt 2):H2577-83.

488. Ospina JA, Krause DN, Duckles SP. 17beta-estradiol increases rat cerebrovascular prostacyclin synthesis by elevating cyclooxygenase-1 and prostacyclin synthase. Stroke. 2002;33(2):600-5.

489. Bunting S, Moncada S, Vane JR. The prostacyclin--thromboxane A2 balance: pathophysiological and therapeutic implications. British medical bulletin. 1983;39(3):271-6.

490. Miller SB. Prostaglandins in health and disease: an overview. Seminars in arthritis and rheumatism. 2006;36(1):37-49.

491. Steinleitner A, Stanczyk FZ, Levin JH, d'Ablaing G, 3rd, Vijod MA, Shahbazian VL, et al. Decreased in vitro production of 6-keto-prostaglandin F1 alpha by uterine arteries from postmenopausal women. American journal of obstetrics and gynecology. 1989;161(6 Pt 1):1677-81.

492. Batres RO, Dupont J. Gender differences in prostacyclin and prostaglandin E2 synthesis by human endothelial cells. Prostaglandins, leukotrienes, and medicine. 1986;22(2):159-71.

493. Scotland RS, Madhani M, Chauhan S, Moncada S, Andresen J, Nilsson H, et al. Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. Circulation. 2005;111(6):796-803.

494. Davis CM, Siler DA, Alkayed NJ. Endothelium-derived hyperpolarizing factor in the brain: influence of sex, vessel size and disease state. Women's health. 2011;7(3):293-303.

495. Mair KM, Johansen AK, Wright AF, Wallace E, MacLean MR. Pulmonary arterial hypertension: basis of sex differences in incidence and treatment response. British journal of pharmacology. 2014;171(3):567-79.

496. Sullivan JC, Sasser JM, Pollock DM, Pollock JS. Sexual dimorphism in renal production of prostanoids in spontaneously hypertensive rats. Hypertension. 2005;45(3):406-11.

497. Majed BH, Khalil RA. Molecular mechanisms regulating the vascular prostacyclin pathways and their adaptation during pregnancy and in the newborn. Pharmacological reviews. 2012;64(3):540-82.

498. Tanaka Y, Yamaki F, Koike K, Toro L. New insights into the intracellular mechanisms by which PGI2 analogues elicit vascular relaxation: cyclic AMP-independent, Gs-protein mediated-activation of MaxiK channel. Current medicinal chemistry Cardiovascular and hematological agents. 2004;2(3):257-65.

499. Dong H, Waldron GJ, Cole WC, Triggle CR. Roles of calcium-activated and voltage-gated delayed rectifier potassium channels in endothelium-dependent vasorelaxation of the rabbit middle cerebral artery. British journal of pharmacology. 1998;123(5):821-32.

500. Zellers TM, Wu YQ, McCormick J, Vanhoutte PM. Prostacyclin-induced relaxations of small porcine pulmonary arteries are enhanced by the basal release of endothelium-derived nitric oxide through an effect on cyclic GMP-inhibited-cyclic AMP phosphodiesterase. Acta pharmacologica Sinica. 2000;21(2):131-8.

501. Puybasset L, Bea ML, Ghaleh B, Giudicelli JF, Berdeaux A. Coronary and systemic hemodynamic effects of sustained inhibition of nitric oxide synthesis in conscious dogs. Evidence for cross talk between nitric oxide and cyclooxygenase in coronary vessels. Circulation research. 1996;79(2):343-57.

502. Henrion D, Dechaux E, Dowell FJ, Maclour J, Samuel JL, Levy BI, et al. Alteration of flow-induced dilatation in mesenteric resistance arteries of L-NAME treated rats and its partial association with induction of cyclo-oxygenase-2. British journal of pharmacology. 1997;121(1):83-90.

503. Schror K. Aspirin and platelets: the antiplatelet action of aspirin and its role in thrombosis treatment and prophylaxis. Seminars in thrombosis and hemostasis. 1997;23(4):349-56.

504. Hartanto MD, Arieselia Z, Setiabudy R, Setiawati A, Baziad A. Urinary 11-dehydro-thromboxane B(2) and 2,3-dinor-6-keto-prostaglandin-F(1)alpha in healthy post-menopausal and pre-menopausal women receiving aspirin 100 mg. Journal of thrombosis and thrombolysis. 2012;34(1):79-84.

505. Awtry EH, Loscalzo J. Aspirin. Circulation. 2000;101(10):1206-18.