

The role of the Toll-like receptors in systemic inflammatory response to cardiac surgery

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Abstract

Background: Cardiac surgery with cardiopulmonary bypass (CPB) can lead to a spectrum of post-operative complications as a result of activation of systemic inflammatory responses. Cellular injury can lead to the release of damage-associated molecular patterns (DAMPs) such as mitochondria DNA (mtDNA), which act as a ligand activating leukocytes and endothelial cells via innate immunity receptors such as Toll-like receptor 9 (TLR9). However, the contributions of DAMPs to inflammatory responses to CPB are unknown.

Aim: This study is to identify the DAMPs and associated receptors that drive systemic inflammatory responses to surgery, which may lead to the identification of novel anti-inflammatory interventions such as TLR antagonists with subsequent translation of our findings to the clinical field. Additionally, Post-operative atrial fibrillation (POAF) is frequent complication in cardiac surgery, which may contribute to the inflammatory response. We aimed to identify a possible predictor for POAF, which may lead to its prevention or early management.

Methods: Sixty-six patients undergoing CABG were recruited, 44 with on-pump and 22 with off-pump CABG (OPCAB) to compare the effect of CPB. To identify the effect of ischemic heart disease (IHD) on the mtDNA release. We recruited a separate group of 22 patients undergoing aortic valve replacement (AVR) with normal coronary angiogram. Blood samples were taken at different time-points to CPB. Quantitative PCR was generated to quantify mtDNA concentration (Chapter 3). Pro-inflammatory biomarkers such as interleukines, interferons, MAP kinases, NF- κ B and other biomarkers were assessed by PCR array (Chapter 5). Both mtDNA and the proinflammatory biomarker were preoperatively compared to assess of the development

of POAF (Chapter 6). Additionally, we used different animal models experiments, to establish the effect of surgery and CPB on TLR9 signalling and to test the effect of blocking TLR9. Specifically, we performed sternotomy in mouse and rat models respectively and performed sternotomy with CPB in the pig model (Chapter 4).

Results: mtDNA was significantly higher in patients with IHD than those without ($p<0.01$). CABG with CPB led to a significant elevation in serum mtDNA levels compared to OPCAB ($p<0.001$ at peak of CPB time). The potential downstream activation of TLR9 also showed significant elevation in all cytokines and chemokines utilizing the TLR9 signalling pathway (p varies from <0.001 - <0.05) but not those using other signalling pathway ($p>0.05$). Blocking the TLR9 in mice has significantly reduced the production of proinflammatory cytokine (IL-6). INF- α and mtDNA were the only independent predictors for POAF development.

Conclusion: Elevation in circulating mtDNA level is related to the extent of the IHD. CPB can influence the release of circulating mtDNA and proinflammatory cytokines production via signalling the TLR9, which may contribute to the initiation of a sterile systemic inflammatory response. The mtDNA and INF- α were independent predictors for development of POAF, which are in agreement with the inflammatory theory that relates the inflammation to the POAF development.

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List of abbreviations

ACT	Active clotting time
AP-1	Activator Protein 1
ATP	Adenosine tri-phosphate
AVR	Aortic valve replacement
bDNA	Bacterial DNA
BMI	Body mass index
BMS	Bare metal stent
CABG	Coronary artery bypass graft
CCS	Canadian cardiovascular society
cDNA	Complementary DNA
CL	Cardiolopin
CLR	C-type lectin receptor
CPB	Cardio pulmonary bypass
CpG	Cytosine-phosphate-guanine
CRP	C-reactive protein
CVB3	Coxsackie virus B3
CXCL9	Chemokine (C-X-C motif) ligand 9
Cyto C	Cytochrome oxidase C
DAMPs	Damage associated molecular patterns
DES	Drug eluting stent
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EF	Ejection fraction
ES	Euroscore
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
HMGB1	High mobility group box-1
HUVEC	Human umbilical cord vein endothelial cells
IHD	Ischemic heart disease
IL	Interleukin
INF-α	Interferon alpha
INF-β	Interferon beta
IR	Ischemic-reperfusion
IRAK	IL-1 receptor-associated kinases
IRF	Interferon regulatory factor
LAD	Left anterior descending
LDF	Leukocyte depleting filters
LIMA	Left internal mammary artery
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MALP	Mycoplasma macrophage-activating lipopeptides
MAPK	Mitogen-Activated Protein Kinases
MELAS	Mitochondrial encephalopathy with lactic acidosis and stroke-like episode
MMP	Matrix metalloproteinase
MOF	Multi organ failure
mtDNA	Mitochondrial DNA
MyD88	Myeloid differentiation factor 88

NADPH	Nicotinamide adenine dinucleotide phosphate
nDNA	Nuclear DNA
NF-κB	Nuclear Factor-Kappa B
NFP	N-formyl peptides
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NYHA	New York heart association
OPCAB	Off pump coronary artery bypass
PAMPs	Pathogen associated molecular patterns
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
POAF	Post-operative atrial fibrillation
POSR	Post-operative sinus rhythm
PRR	Pattern Recognition Receptors
RLR	Retinoid acid-inducible gene-I (RIG-I)-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
TGF	Transforming growth factor
TICAM	TIR domain-Containing Adaptor Molecule
TIR	Toll/Interleukin-1 Receptors
TIRAP	TIR domain-Containing Adapter Protein
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TRAF	TNF-Receptor Associated Factor
TRAM	TRIF-Related Adapter Molecules
TRIF	TIR domain containing adapter-inducing INF- β
VRE	Vancomycin resistance enterococci
Vs.	Versus

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Dedication

I dedicate this work to my family including my late parents and to my lovely wife Najla and my boys Adam, Amin and Aban.

Statement of originality

This is to certify that this is my original work, performed within the Imperial College London and does not overlap with any other institutes or departments and has not submitted for any other degree or other purposes. To the best of my knowledge, my thesis does not infringe copyright of anyone and does not contain any material previously published except where due references are made. Part of this research has been presented in the Society of Cardiothoracic Surgery in Great Britain and Ireland conference and published in The Journal of Thoracic and Cardiovascular Surgery.

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Presentations and publications arising from this study

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Publications

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1 CHAPTER I: Introduction

1.1 Inflammatory response

1.1.1 Inflammation and inflammatory response

Inflammation as an entity has been recognized from the prehistoric times. There have been studies of this process and its clinical implications for over 3,000 years (1). Inflammation in Latin means “I ignite, or set a fire”, it is part of complex biological response to harmful injury or infection to the body. These harmful stimuli can be pathogens, damaged cells or irritant (2). Inflammation is the key component of the immune system in that it has a significant role in defence mechanism as well as maintaining the dynamic homeostasis of the host organism (3). This process is an adaptive response allowing organisms to adapt to a variety of injuries or infectious agents (4). Inflammation is triggered by intracellular molecules released from dying cells, as well as from microbes that have lost integrity of their plasma membrane.

Inflammatory process has different triggers and different aim to overcome this pathological condition. However, they can share variable consequences. Auto-regulation of the inflammatory signaling cascade is important to stop at the host defence and tissue repair level avoiding subsequent complication (Figure 1.1). Inflammation plays a significant role in the eradication of debris and pathogens. Moreover, it plays an important role in the initiation of the healing process; hence controlled inflammatory response is beneficial with a positive protective effect against infection or injury. However, its downstream effects can be unpredictable and furthermore detrimental effects can be engendered if the regulatory effect is lost, including the development of septic shock (5). Each cell in the organs possesses both pro-inflammatory and anti-inflammatory mechanism. The interaction between these two systems determine the inflammatory status. This means that they can either exhibit

a protective or damaging effect according to this dynamic equilibrium, hence this inflammatory homeostasis should be under strict regulatory control (3).

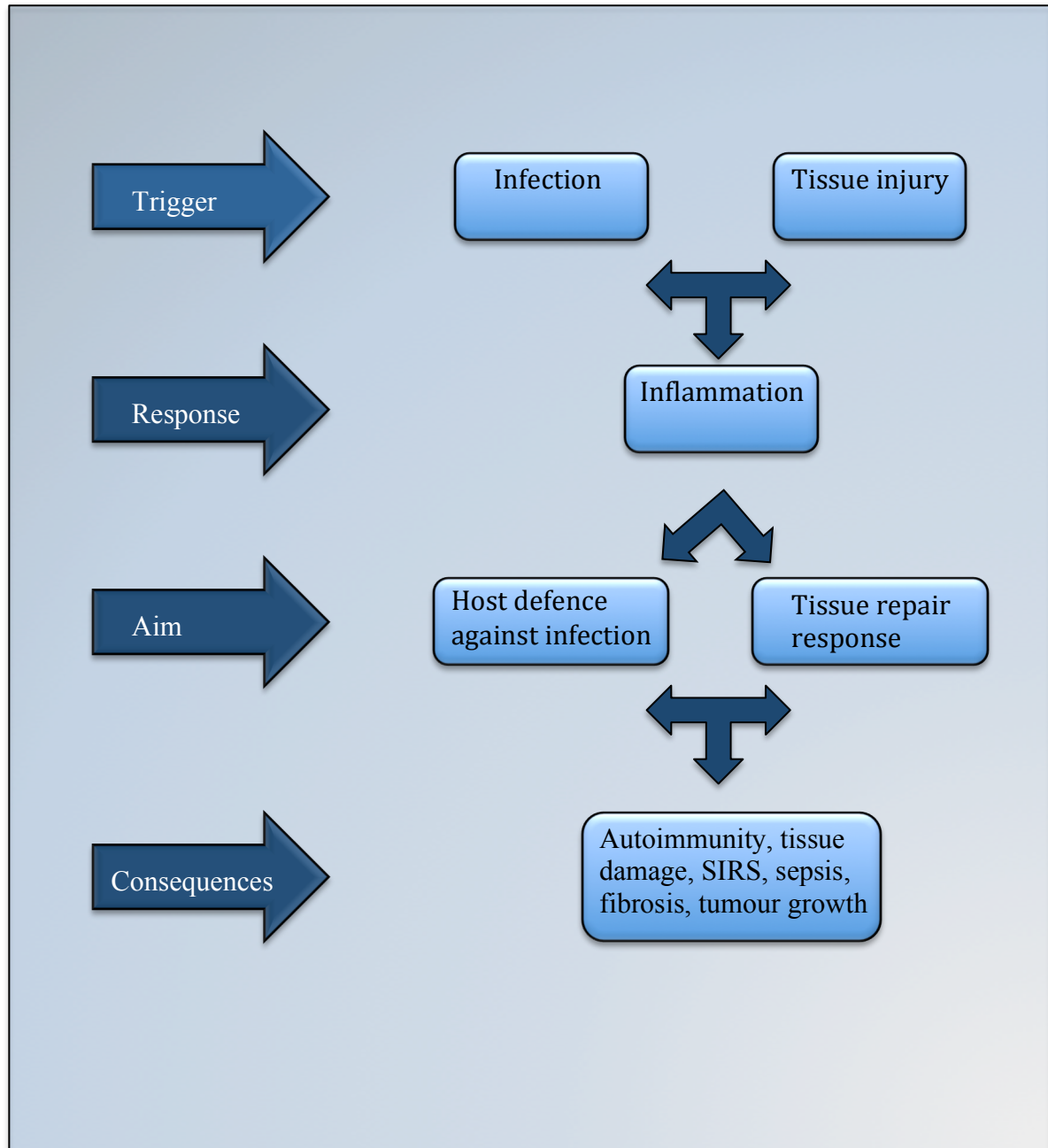


Figure 1.1 Inflammatory response

Inflammatory response course and outcome may vary according to the trigger. Auto-regulation of the inflammatory process is important to be maintained at the host defence and tissue repair level to subsequent complication.

Acute inflammation is a response to pathogens or tissue injury, which results in the elimination of infectious microorganism or injurious material. This is followed by a

period of resolution (6). This inflammatory process produces characteristic symptoms and usually resolves spontaneously. These symptoms are referred to as the cardinal signs of inflammation, which include: pain, swelling, tenderness, redness and hotness. Failure to eliminate the triggers will lead to persistence of the inflammatory process and continued release of macrophages or T- cell to replace the neutrophils for further support. However, if this mechanism is still insufficient, then chronic inflammation occurs with formation of tertiary lymphoid tissue and granuloma (7).

In contrast to acute inflammation, chronic inflammation contributes to a major factor in the development of degenerative diseases and loss of functionality. It is for this precise reason that it is imperative to closely regulate and actively terminate inflammation as to avoid any unnecessary damage to tissues (8). Failure to achieve this control will result in subsequent chronic inflammation and cellular destruction with further tissue damage.

The pathways which help in the termination of inflammation includes (8, 9): Short half-life of the inflammatory mediators, Transforming growth factor (TGF) beta production and release from macrophages (10-12), anti inflammatory Interleukin 10 (IL-10) production and release (13), anti-inflammatory lipoxins production (14), pro-inflammatory molecules down-regulation such as leukotrienes, anti-inflammatory molecules up-regulation such as the soluble tumour necrosis factor (TNF) receptors and the IL-1 receptor antagonist or in apoptosis of pro-inflammatory cells (15). Receptors desensitization increases survival of cells in the regions of inflammation due to their interaction with the extracellular matrix (ECM) (16, 17), receptor activity down-regulation by ligands increased concentration level and chemokines cleavage by matrix metalloproteinases (MMPs) will possibly lead to anti-inflammatory factors

production (18). When injury is recognized in any biological structure, an attempt to eliminate this will take place followed by period of resolution and repair. If the damaged tissue is beyond repair this will lead to apoptosis at the cellular level with subsequent elimination of those particular cells (19). Moreover, this can also lead to further infection when the dead cells become necrotized leading to subsequent development of inflammation. Death of the cell can also occur due to autophagy (20).

1.1.2 Inflammatory pathway

Generally the inflammatory response is maintained and regulated by wide range of mediators. There are several inflammatory inducers, which can be either endogenous or exogenous to initiate the systemic inflammatory response. In turn this can activate specific sensors that subsequently produce specific mediators, which will cause alteration in the functional state of the tissue i.e. effector of inflammation. The type of the inflammatory response depends on the component of each inducer, sensor, mediator and effector pathway (5) (Figure 1.2).

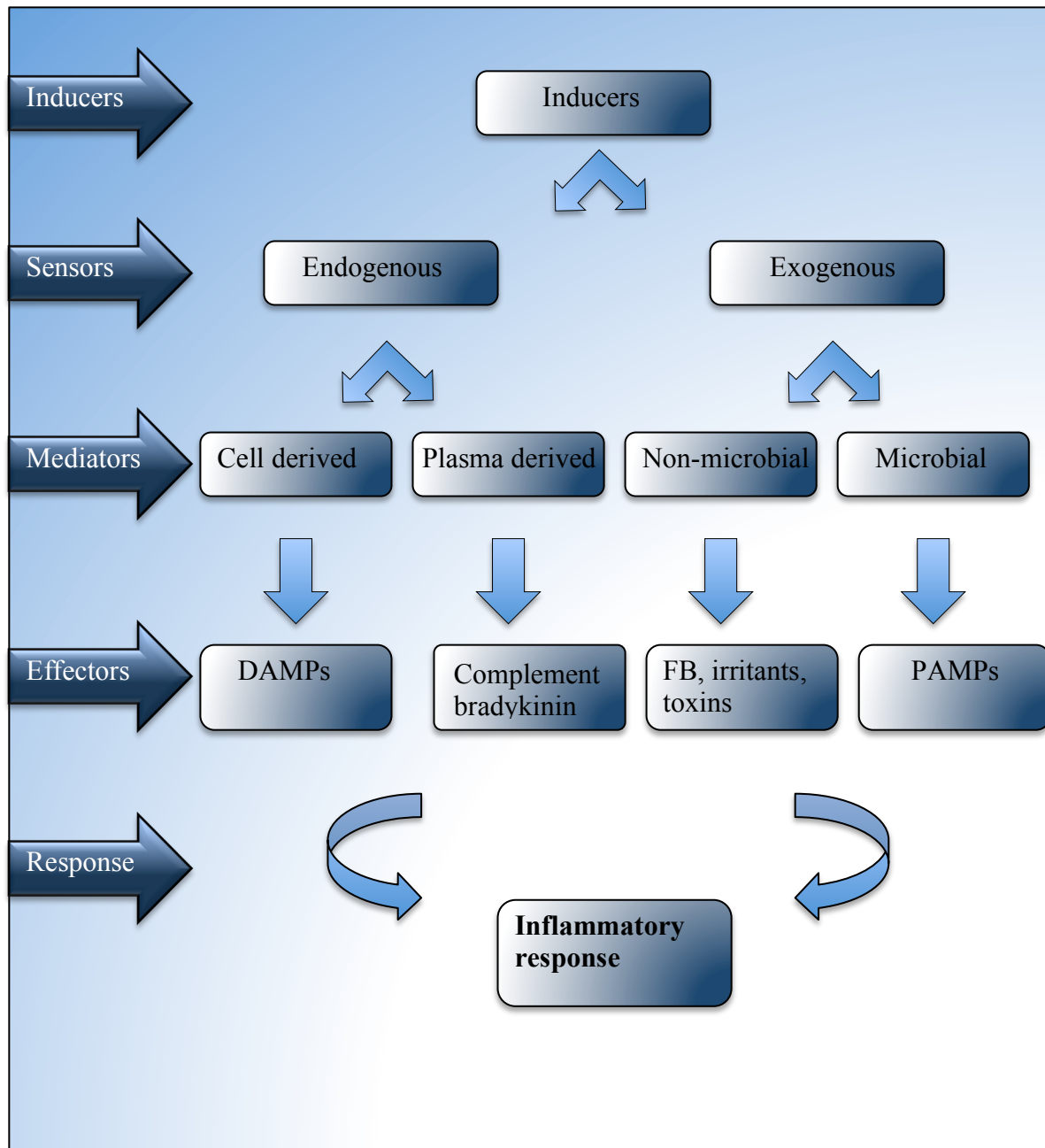


Figure 1.2 Inflammatory pathway

Wide range of mediators regulates inflammatory response. Inducers can be either endogenous or exogenous for the inflammatory response initiation, which activate a specific sensors that later produces specific mediators, which in turn will apply the alteration in the tissue functional state (effector of inflammation). The type of the inflammatory response will depend on the component of each inducer, sensor, mediator and effector pathway. (DAMPs-Damage associated molecular pattern, PAMPs- Pathogens associated molecular pattern, and FB-Foreign bodies).

1.1.3 Sterile inflammation

Sterile inflammatory processes are triggered in response to non-microbial stimuli causing tissue insult such as trauma, heart attack, ischemia, cell death, hyperoxia, hypoxia and chemical stimuli (21). When the cell dies in vivo it stimulates an acute inflammatory response (22, 23). The sterile inflammatory response often follows similar pattern without paying attention to the nature of the initial insult. If a tissue suffers an injury, it reacts by utilizing the localized immune mechanisms leading to the development of localized inflammation. In the following given scenario, if the local control of the injury was lost, a generalized inflammatory response can be initiated and number of innate immunity receptors will be activated. Activation of these receptors leads to production and release of variety of pro-inflammatory mediators from the stimulated cells such as chemokines and cytokines. One set of these receptors that participate in sterile inflammatory response are the Toll-like receptors (TLR). These receptors are the key component of the innate immune system inducing expression of various inflammatory genes promoting systemic inflammatory and innate immune response (24). These receptors will be discussed in details in the next chapter.

1.1.4 Inflammatory response in cardiovascular system

1.1.4.1 Vascular changes in inflammation

Acute inflammation is associated with significant changes in the vasculature. This includes vasodilatation due to arteriolar smooth muscle relaxation. Increased blood flow leading to increase in the hydrostatic pressure and vessels permeability (4) and these can be induced by the actions of different inflammatory mediators. The arteriolar vasodilatation is initiated then progresses into the capillary level leading to escalation in the amount of blood causing redness and hotness of the inflamed site. The increase

in the vessel permeability results in the movement of plasma into the tissues leading to stasis due to the increase concentration of the cells within blood. This condition characterized by enlarged vessels packed with cells, stasis encourages leukocytes to marginate along the endothelium, a process critical to their recruitment into the tissues. After activation of the leukocytes it will exhibit margination followed by rolling and adhesion into vascular endothelium before transmigration between endothelial cells into the surrounding tissue where the inflammatory response can start in earnest (Figure 1.3).

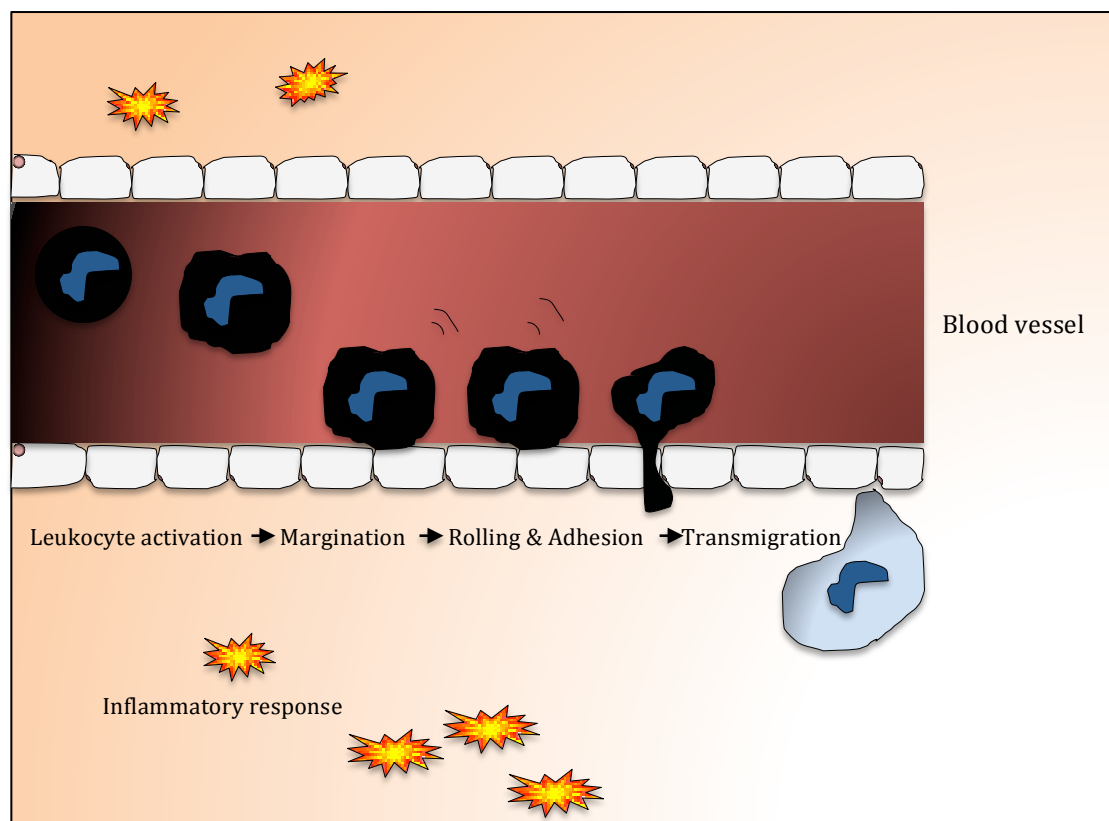


Figure 1.3 Leukocyte transmigration

Inflammatory response triggers and recruits leukocytes through its activation. Activated leukocyte will marginate, rolling into endothelial surface followed by adhesion prior to transmigration into surrounding tissue to begin the inflammatory process.

The inflammatory response in acute inflammation is characterized by a series of local cellular and vascular responses that are triggered by our bodies when the harmful injury occurs following either trauma or invasion by antigens. A particular reaction is pain due to swollen tissue causing pressure on the nerve endings alerting us to the injury. Redness and hotness as part of the cardinal sign of inflammation can occur from release of number of chemical signals such as histamine, cytokines and prostaglandins, which can lead to the increase of blood flow to the injured area through local vasodilatation. Hence, both of these signs are associated with acute inflammation (3). Some of the chemicals attract specific type of leukocytes phagocytic neutrophils and monocytes; others stimulate capillary permeability leading to plasma and leukocytes leaks into the tissue leading to oedema of the affected area. Presence of injurious material can potentially activate the complement protein, which will help to promote phagocytosis (3).

1.1.4.2 The role of inflammation in heart disease

Inflammatory heart disease relates to inflammation because of microorganism invasion or due to individual idiosyncrasy. The type of disease associated with this inflammatory response differs with each tissue of the heart, namely pericardium, myocardium, endocardium, coronary vessels and heart valves. These inflammatory reactions contribute to variety of significant and important heart diseases and its treatment is significantly dependent on our understanding of its cause and the pathway of the disease progress. Now we will discuss each in turn.

1.1.4.2.1 Pericarditis

This disease is caused by inflammation of the pericardium, most common cause is idiopathic but it can be related to various contributing factors including direct micro-organismal infection (bacterial, viral, fungal or parasitic), systemic disease such as autoimmune disorder (SLE, sarcoidosis, rheumatoid arthritis, Behçet disease), metabolic disorder (hypothyroidism, uremia or renal failure). It can also be as consequences from myocardial infarction, post-surgical or traumatic pericarditis (Dressler syndrome), drug related (procainamide, hydralazine, phenytoin, penicillin), neoplastic (primary as pericardial mesothelioma or secondary as breast cancer) and its related radiotherapy treatments (25-27).

1.1.4.2.2 Myocarditis

Inflammatory disease involves the myocardium characterized by inflammatory cellular infiltration and injury to myocardial tissue a condition may associate with myocytes necrosis (28). Clinical symptoms vary from self-limiting mild shortness of breath and chest pain to the extreme of presence cardiogenic shock and death. It accounts for one-fifth sudden deaths in those adults younger than forty years old of age and athletes (29-31). Causes can be infective such as viral (e.g. adenovirus, parvovirus, coxsackie B virus), bacterial (Brucella, H. influenza), protozoan (Trypanosoma cruzi “Chagas disease”) parasitic or fungal infection or due to their toxins (32), or due to non-infective causes such as autoimmune (e.g. SLE, sarcoidosis, vasculitis), toxin (arsenic, snake venom). The long-term complication can include dilated cardiac myopathy and heart failure.

1.1.4.2.3 Endocarditis

A disease with infection of the endocardium with subsequent dysfunction of the heart valve leaflets due formation of vegetation (plugs of bacteria, debris, platelets and fibrin), which may lead to thickening, perforation and deformity of the affected valve leaflets. It is mainly infective in origin most commonly bacterial (staphylococcus aureus, streptococcus viridance and other streptococci and enterococci) but can result from other organisms (33). It presents a challenge to clinicians especially when it is an antibiotic resistant (e.g. vancomycin resistance enterococci-VRE) or attributable to a fungal infection. Risk factor include rheumatic heart disease, immune compromised patients, IV drug abusers due to damaged valve. Endocarditis can also be due to non-infective causes such as in Marantic endocarditis a non-bacterial thrombotic endocarditis related to plugs of platelets on damaged valve due to hypercoagulability or advanced cancer (34).

1.2 Atherosclerosis and heart diseases

Ischemic heart disease is commonly related to atherosclerosis of the coronary arteries. In recent years it is becoming a significant health burden worldwide, accounting for a significant number of deaths each year (35). In the UK alone it accounts for approximately a third of all deaths. Testament to this fact is that between the years 1961 to 2012, cardiovascular diseases were the most common cause of death in UK. The recent statistics showed it has become second for the first time after cancer (28% and 29%, respectively), however coronary heart disease still is the biggest single cause of death in the UK with a total of 74,000 deaths of 16% male and 10% female. It is considered to be the highest cost of all diseases in UK of £7.06 billion estimated cost in 1999 (36), increased to £8.7 billion in 2012. (Cardiovascular disease statistics 2014,

BHF. Weissberg P.). Atherosclerosis development has been related to numerous contributing factors including sedentary life style, smoking, diabetes, hypertension, hypercholesterolemia, age related, sex, obesity and family history. However, chronic inflammation is common motivator for atherosclerosis (37). The mechanism involved the inflammatory cytokines stimulating the vascular generation of endothelial molecule adhesion with accumulation of lipid, macrophages secreting metalloproteinase (38), leading to thickening of the blood vessel wall with subsequent plaque and slowly progression lesion formation leading to narrowing of the arterial lumen and subsequent ischemia (39, 40) (Figure 1.4).

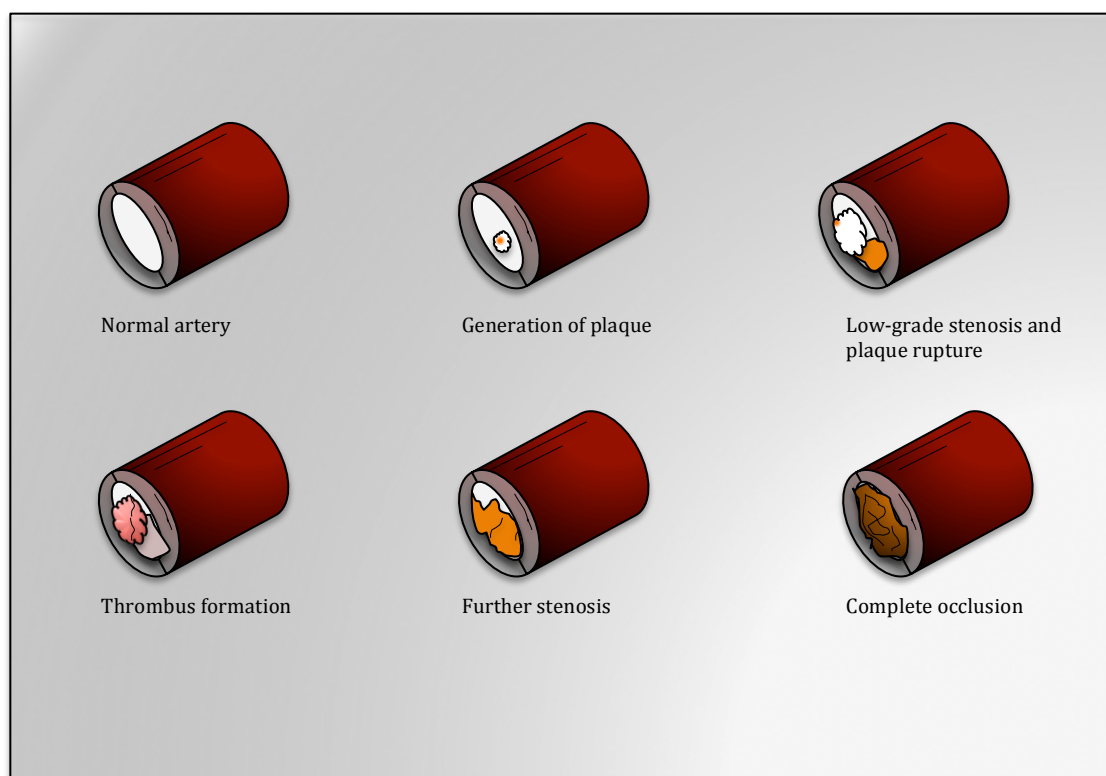


Figure 1.4 Vascular ischemia

The mechanism involved the inflammatory cytokines stimulating the vascular generation of endothelial molecule adhesion with accumulation of lipid, macrophages secreting metalloproteinase leading to thickening of the blood vessel wall with subsequent plaque and thrombus and slowly progression lesion formation leading to narrowing of the arterial lumen and subsequent ischemia.

1.2.1 Coronary artery ischemia

The vascular system for the coronary artery is a unique system, which supplies the myocardium. The plaques formation are mainly driven by inflammation leading to the narrowing and blockage of the coronary arteries causing ischemia of the myocardium, which is clinically referred as ischemic heart disease (IHD) (41). The clinical manifestations of IHD differ in relation to the extent, severity and the site of the affected coronary vessels. It varies from mild chest discomfort on exertion to severe chest pain and shortness of breath at rest and fatigue related to the reduction in heart function due to necrosis and infarction of the affected myocardium (42).

Additionally, treatment modalities of IHD also depend on the extent and severity of the disease. The options range from non-invasive module including implementing changes in the life style and weight control along with medical treatment such as anti-platelets, cholesterol lowering agents, nitrates, β -blockers and calcium channel antagonist. The invasive module includes percutaneous coronary intervention, surgical revascularization of the coronary arteries or both.

1.2.2 Percutaneous coronary intervention (PCI)

Andreas Gruenzig first introduced PCI in 1977 in the United States (43). It is an invasive non-surgical percutaneous wiring of the coronary arteries with diagnostic and therapeutic values, which involves the insertion of percutaneous arterial guide-wire usually through radial, femoral or brachial arteries passed into coronary arteries. Firstly a dye is injected followed by balloon inflation or stent catheter insertion in order to dilate or stent the narrowed coronary artery (44).

Different types of stents are available, most common are the bare metal stent (BMS) and the drug eluting stent (DES). DES has shown to have certain advantages over BMS in reducing re-stenosis rate as it is coated with a drug that aims to prevent intimal cell proliferation (45). PCI is a rising practice and has evolved dramatically in the last decade. In the UK alone 92,445 PCIs were performed in 2012 in 118 centers, the majority (92%) of these procedures involved stent insertion as recommended by current NICE guidelines (46). PCI however, is not without complications; in addition to the risk of failure of the procedure it can carry serious complication risks such as coronary perforation that has high morbidity and mortality rate, which in most cases will require emergency surgical intervention.

1.2.3 Surgical revascularisation

PCI is usually utilized as a first choice for coronary revascularization. However, the definitive surgical revascularization option in form of coronary artery bypass graft (CABG) is performed if failed PCI or for multiple coronary vessels disease, left main stem coronary artery disease or diffuse coronary disease not amenable for PCI (47). In the long-term revascularization outcome, CABG is still superior to PCI especially in those with high complex anatomy (48). Additionally, there has been evidence reported improvement in the heart function in response to CABG in patients with poor heart function preoperatively (49).

1.3 Coronary artery bypass graft surgery (CABG)

1.3.1 The surgery

Various surgical attempts to treat the coronary disease symptoms have been implicated before the definitive surgical coronary revascularisation flourished. Indirect surgical

approach to the coronary arteries to reduce the sympathetic drive to the heart was performed by sympathectomy in 1916 and total thyroidectomy in 1934 by Jonnesco and Weinstein respectively. This approach has achieved marginal symptomatic improvement (50). In 1961 DeBakey and Henley (51) performed their first bypass graft in dogs using Dacron graft. Sabiston (52) in 1962 was the first to perform coronary revascularization using vein graft in human and in 1968 Green (53) reported to use left internal mammary artery as a conduit.

1.3.2 Principle of CABG (The technique)

CABG is a surgical procedure, which defines the placement of vascular conduit (graft) upstream and downstream of the narrowed or blocked coronary arteries to bridge or bypass the lesions in order to restore optimum myocardial perfusion. However, restoring normal myocardial perfusion will not be achieved if the effected myocardium is infarcted with subsequent scarring. The most commonly used grafts are: the left internal mammary artery (LIMA), the long saphenous vein (with 10-years patency of 90.3% and 67%, respectively) (54) and the radial artery (commonly used in young patients and in total arterial revascularization). Short saphenous, cephalic veins and right gastro-epiploic artery are less commonly used due to their surgical harvesting difficulties.

During conventional CABG, the heart is accessed via performing median sternotomy incision and separation of the sternal bone using electric reciprocating blade saw. This followed by pericardium separation exposing the heart to identify the proposed targets for revascularisation, at this stage or before according to the operator preference, the LIMA will be harvested and mobilized providing sufficient length enabling reaching

the proposed target. (Coronary artery bypass surgery; principle and choice of documents: Mr Aonghus O'Donnell) (Figure 1.5).

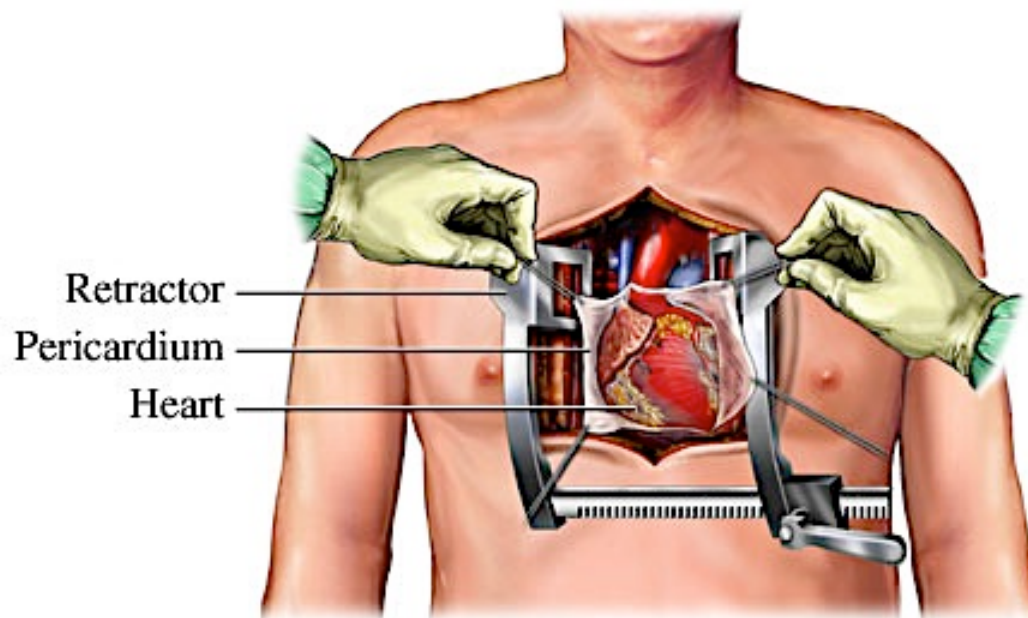


Figure 1.5 Median sternotomy

Heart exposure requires midline skin incision and sternotomy exposing the pericardium, which needs to be excised to fully expose the heart. Figure adopted from (<http://www.heart-valve-surgery.com/surgeon-database/median-sternotomy.jpg>).

Once the heart is exposed sufficiently and the LIMA conduit harvested, the aorta and the right atrium are cannulated using their appropriate size cannulea, then connected to cardiopulmonary bypass circuit (heart-lung machine) to provide bloodless and motionless field for coronary arteries anastomosis. The current preferred practice implicates grafting the LIMA to the left anterior descending artery (LAD) and garfting the long saphenous vein to the remaining diseased vessels) (Figure 1.6).

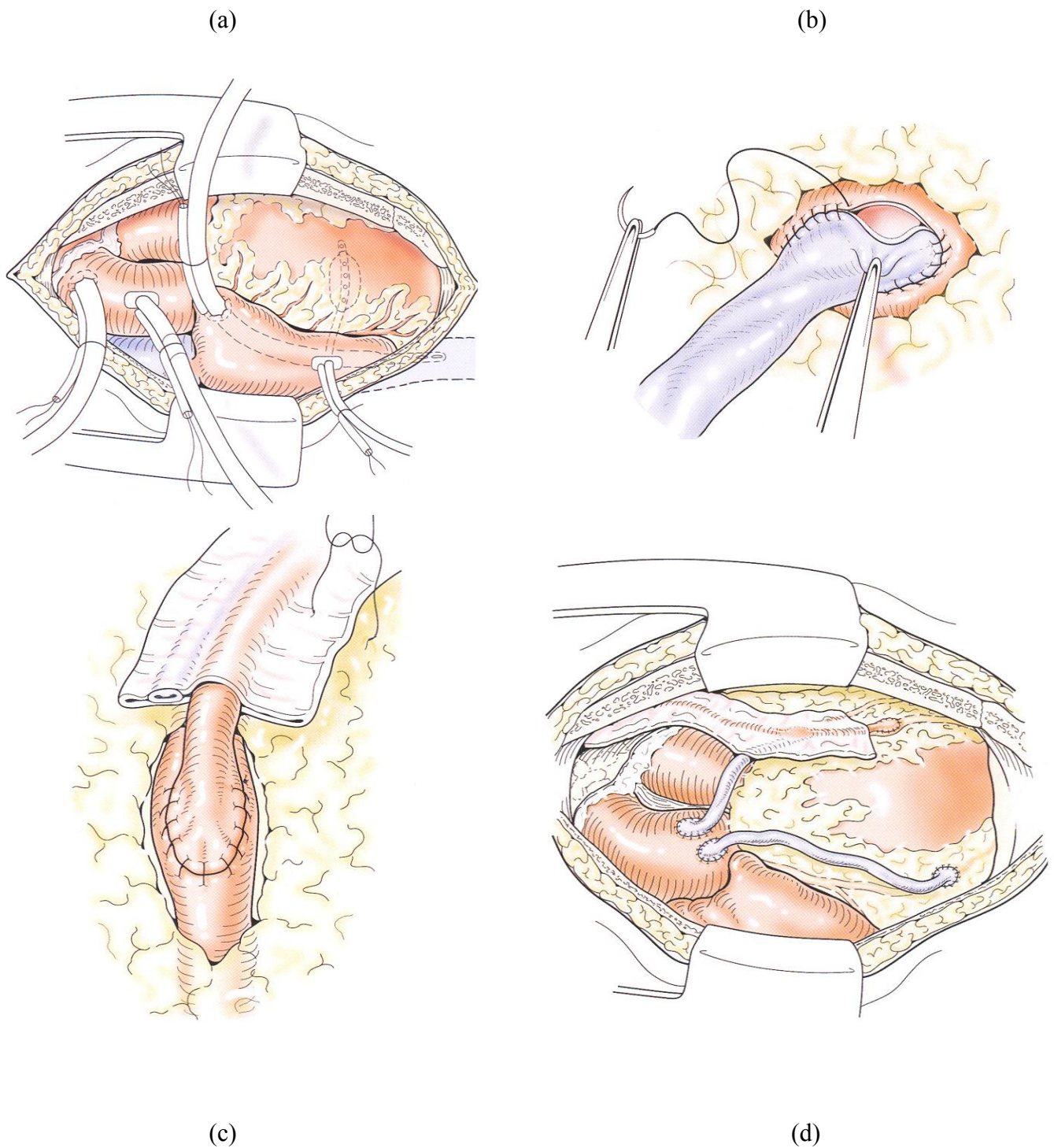


Figure 1.6 Coronary artery bypass graft (CABG)

The surgery showing aortic (arterial) and atria (venous) cannulation (a), distal venous (b) and arterial (c) anastomosis to the coronary artery, Final anastomosis of proximal ends to the aorta (d). Figures adopted from <http://www.wcccd.edu/dept/pdf/AF/LectureCABG.pd>.

1.3.3 Cardio pulmonary bypass (CPB)

1.3.3.1 Overview of CPB

CPB is a process of an artificial perfusion and is typically carried out during major vascular or cardiac surgery in order to provide a motionless and bloodless operating field. Maximilian von Frey (1852-1932) and Max Gruber (1853-1927) together were the first to develop artificial lung apparatus in 1885 (55). Clarence Dennis experimentally reported this technique in 1951. He implemented the heart-lung machine in nine dogs in four years period performing open cardiectomy. Only two survived for a few days whilst the others died due to pump failure or faulty surgical technique. On the 5th of April 1951 Dennis was the first to perform an open-heart operation on 6 years old girl at the University of Minnesota Hospital but unfortunately the patient did not survive due to uncontrolled blood loss (56, 57).

In 1953, John H. Gibbon (1903-1973) reported the first successful intra cardiac operation utilising CPB with a pump oxygenator performing atrial septal defect repair operation on an eighteen years old woman with successful 26 minutes of CPB time at Thomas Jefferson University Hospital in Philadelphia (58, 59). However, the same procedure was not successful in a few following patients that later made him to abandoned further operations. Dr John Kirklin in 1955 has made additional modifications to John Gibbon apparatus, he was the first to perform successful series of heart operations on eight patients using extra corporal circulation (60). Even though the mortality was 50% in a small number of patients, it has opened the way and motivated others for further development in his technique. A year later, Richard DeWall developed simpler and safer circuit with a disposable bubble oxygenator (61).

From then onward, considerable development were required to accomplish safer CPB. The extracorporeal oxygenation devices improved, which replaces the lung by extracting carbon dioxide from and delivering oxygen to the blood, leading to large number of cardiac procedures performed.

1.3.3.2 The CPB (The technique)

To establish a safe CPB the blood should be heparinized effectively (300 IU/kg) prior to arterial cannulation reaching appropriate activating clotting time (ACT) for CPB circuit (i.e. more than 480 seconds). To achieve satisfactory ACT, it is required to be checked every 20-30 minutes to maintain this appropriate level. The basic principle in CPB is to drain venous blood from the right atrium. This is through large venous cannula to allow adequate drainage with high flow rate and low systemic resistance. By gravity this venous blood goes to the oxygenator where carbon dioxide is extracted and oxygen administered. The oxygenated blood is then pumped through a filter to the aorta resembling heart-lung function whilst excluding them from the circulation.

The blood in the CPB circuit is cooled down depending on the type of the operation (28-34°C) to provide brain protection. Some of the challenging operation required deep hypothermic circulatory arrest with temperature less than 20°C arresting the circulation for short period as in aortic arch operation and other complex congenital heart procedure (62, 63). Additionally, the contractility of the heart will be then stopped by applying a cross clamp on the ascending aorta between the aortic valve and the aortic cannula. Subsequently high potassium solution perfusion of the coronary arteries is required to completely arrest the heart with a myocardial protection solution (cardioplegia) to provide a non-beating heart for all intra cardiac procedures and most of CABG operations to be performed.

1.3.3.3 The CPB circuit

While the heart and lungs are excluded from the circulation, the CPB circuit is designed to provide adequate perfusion to the body organs whilst the heart is in a static position. The pump and the oxygenator are the two main functional units in CPB. Their function is to remove oxygen-deprived blood and replace it with oxygen-rich blood by different tubes and pumps. The basic of CPB comprises the following component (Figure 1.7).

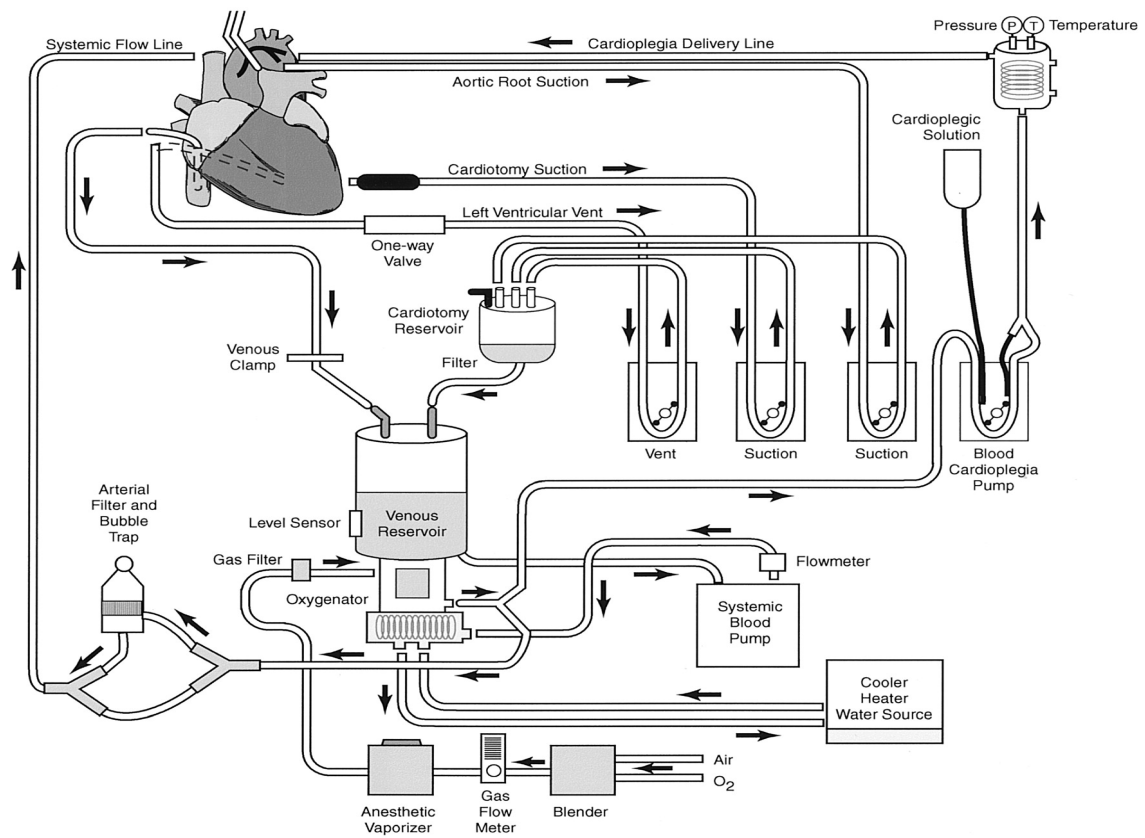


Figure 1.7 The basic CPB circuit

Venous blood drained from the right atrium to a reservoir for oxygenation and temperature regulation then returns to the systemic circulation through arterial cannula inserted most commonly through the aorta. (http://www.mpoullis.net/bscpb/cpb/blank.htm#CPB_Setup), reproduced from (Cohn 2007).

1.3.3.3.1 The pumps

The pump is primed with a balanced electrolyte solution, usually Ringer's lactate or Hartmann's solution with priming volume of approximately 1.5-2 liters. Ideally, the pumps should be able to provide blood pump flow at 7 liters per minutes against 500mmgH pressure resistance with minimal blood contact activation cellular damage and lastly should be operated manually in case of power failure (57). There are different type of pumps; the roller or peristaltic pump, which have been developed for decades. The mechanism involve expressing numerous rotatory-driven pumps movements resembling massaging the tubing leading to drive the blood through gently. The centrifugal pump is another type of pumps, which is more advanced and expensive. It was developed in 1976, The blood flow is achieved by centrifugal forces that change the speed of revolution (RPM). Its advantage over the roller pump is the association with less air embolism incidence and greater tolerance to excessive line pressure and less blood damage and heamolysis (57, 59).

1.3.3.3.2 The cannulae

The venous cannula drains blood from the right atrium to the venous reservoir with gravity driving force hence they are larger than the arterial cannula to provide the required drainage to the reservoir. Venous cannula can be either single or double "two stages or bi-caval", cannulating both the superior and the inferior vena cava. Arterial cannula is the narrowest part of the CPB circuit but has a high flow rate. It come in different sizes and tip shapes (right angle, tapered or flanged) to adapt the size and shape of the aorta (57). It was designed to reduce exit velocity and to avoid the direct sheer stress effect on the aortic wall (64). Standard CPB cannulation for most of the heart operations is via the aortic arterial cannulation and right atrial venous cannulation with antegrade and retrograde cardioplagia catherters (Figure 1.8).

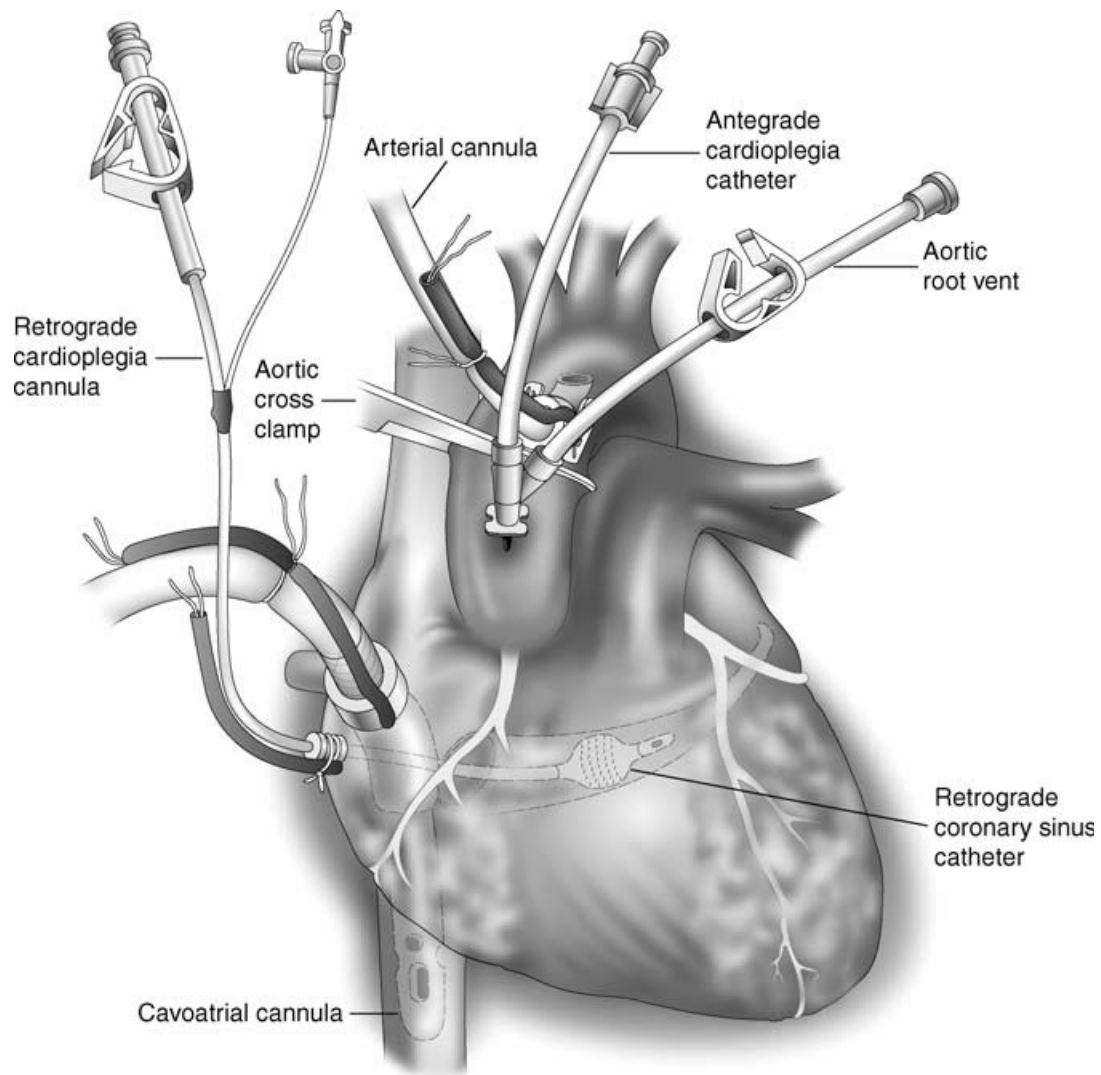


Figure 1.8 Cannulation for cardiopulmonary bypass circuit

Cannulation and clamping during a routine on-pump coronary bypass operation. Note aortic and venous cannulation, antegrade and retrograde cardioplegia catheters, and the aortic cross-clamp just proximal to the aortic cannulation site. (Reproduced from Bojar 2011 ISBN: 978-1-444-33143-1).

1.3.3.3 The venous reservoir

The reservoir must be placed below the level of the patient to allow adequate return of the venous blood. In addition to its reservoir function, it has an important role in filtration functions and preventing air embolism. They can be rigid or soft plastic the latter has less risk of air embolism because they collapse when emptying (65).

1.3.3.3.4 The oxygenator

Receives blood from the reservoir to the arterial reservoir where blood and gas are separated by permeable membrane and allows oxygen and carbon dioxide exchange by diffusion. There are two type of oxygenator; bubble and membranous oxygenators. The arterial filter removes the gaseous and micro particle and passes through a last safety device before the blood enters into the patient (66).

1.3.3.3.5 The heat exchanger

Designed to regulate the blood temperature within the CPB circuit for optimal metabolic conditions using either hot or cold water system by counter current system mechanism flowing in opposite blood current direction. They are usually positioned adjacent to the gas exchanger to avoid micro air bubble when the blood is warmed after saturated with gas (67).

1.3.3.4 Adverse effects of CPB

CPB is extremely essential tool in major cardiovascular surgery, and has enabled many repairs of intra cardiac and intravascular lesions in addition to CABG surgery. However, CPB is associated with morbidity and mortality despite significant improvement in the material and the technique (68). These associated complications led to some surgeon to avoid CPB when feasible in some of the cardiac operations such as in off-pump CABG (OPCAB). The adverse effect from the CPB can result from mechanical issues such as from cannulation or decannulation of the aorta, which may result in emboli and subsequent stroke, kinking, blockage, dislodgement of the cannula or disconnection of the circuit tubing leading to catastrophic exsanguination, or malfunction of any part of the CPB circuit (e.g. heater, cooler, pump). Moreover, CPB also has a role in participating in hypo perfusion, hypoxia, and organ dysfunction.

CPB haematologically can cause platelet depletion and decreased function with severe postoperative haemorrhage, fibrinolysis and activation of numerous cascades including the kallikrein, coagulation or complement system (69, 70). Coagulopathy also can occur as a result of hypothermia. Additionally, haemolysis can arise as consequence of the high sheer stress of the roller pump leading to red blood cells membrane rupture with subsequent release of haemoglobin (71).

CPB is responsible for the short-term neuro-cognitive disturbance following cardiac surgery, which refer as post perfusion syndrome or “pump head”. The post perfusion syndrome characterised by lack of concentration, attention with short memory loss, which is transient but may take weeks to resolve and has no permanent neurological dysfunction (72, 73). However, elderly and obese group of patients carry an additional risk for the development of neurological complications following CPB as compared to off-pump cardiac surgery. This demonstrates the potential neuro-protective effect of off-pump surgery in these particular groups (74, 75). Many studies demonstrated deterioration in cognitive outcome in relation to post cardiac surgery with the use of CPB (76-80), which is higher in valve related surgery than just isolated CABG (81). These cognitive dysfunctions include short-term (33-83%), long-term (29-60%) cognitive changes and delirium (10-30%) (81).

1.4 The inflammatory response to CPB

The surgical trauma has potential synergetic contributing role in the inflammatory response process in cardiac surgery. However, the major role is played by the CPB mechanism. The contact activation of circulating blood to artificial, non-physiological and non-endothelialised surfaces in response to the CPB biomaterials involves inflammatory and coagulation changes with release of several inflammatory mediators.

The cellular inflammatory pathway including neutrophils, monocytes, platelets and endothelial cells, or the humoral inflammatory pathway including complement, cytokines, adhesion molecules, proteolytic enzymes and plasmin (69, 82) are involved in immunity and subsequent release of potent vasoactive substance. The inflammatory response in CPB initiated in two phases; early through blood contact to artificial surface (83) and late due to ischemic-reperfusion injury, change in the body temperature and release of the endotoxins, both phases coupling together can lead to activation of inflammatory cascades (84).

Inflammatory cascade activation leads to abnormal pathophysiological phenomena, which may be manifested clinically as the systemic inflammatory response syndrome (SIRS), which can occur in quarter to third of patients receiving CPB (85). The severity ranges from organ-specific complications to multiple organs failure (MOF) (86, 87). There are certain criteria defining SIRS development, these include two or more of the followings; Temperature ($<36^{\circ}\text{C}$ or $>38^{\circ}\text{C}$), heart rate (> 90 beat per minute), respiratory rate (> 20 breath per minute) or hyperventilation with arterial CO_2 (< 4.3 kPa) and white blood cell count ($< 4 \times 10^9$ or $> 12 \times 10^9$ or 10 % of immature neutrophils) (88).

SIRS has very similar clinical picture to sepsis, which is a condition characterised by sever SIRS with additional suspected or proven infection (89). Sepsis and SIRS are clinical syndromes; they can complicate infectious and non-infectious inflammatory response, respectively (90). Generally, stimulating the inflammatory response may lead to increase production and release of vasoactive and cytotoxic substance leading to increase in capillary permeability and interstitial fluid with subsequent inflammatory cell infiltrate to various organs (82).

The mechanism of inflammatory response to CPB may involve several pathways (Figure 1.9):

- Complement cascade activation and production of active form complement (C3a, C5a and membrane attack complex C5b-9) activating neutrophil leading to direct endothelial cell damage (91-93).
- Neutrophils activation along with adhesion molecule expression (E-selectin & integrin) leads to adherence of leukocytes to endothelial wall with release of damaging product causing tissue injury (94, 95).
- Release of cytokines TNF- α and interleukins (IL1, IL-2, IL-4, IL-6, IL-8 & IL-10) (96), which implement further neutrophils chemo-attraction. Their imbalance may lead to development of SIRS and MOF (97, 98).
- Activation of coagulation cascade, fibrinolysis cascade and platelet activation factor can attract neutrophils and also amplifies the inflammatory process (84, 99).
- Production of oxygen-free radical or reactive oxygen species (ROS) which results polyunsaturated fatty acid oxidation or increases of lipid peroxidation leading to damage to many cellular molecules such as lipid, protein and DNA with subsequent cell damage (96, 100).
- Endotoxemia, which may result from splanchnic vasoconstriction and gut mucosal ischemia causing translocation of endotoxin (101). It also can amplify complement activation and cytokines release (102) as well as neutrophil activation and nitric oxide induction (103).

Inflammatory response to CPB

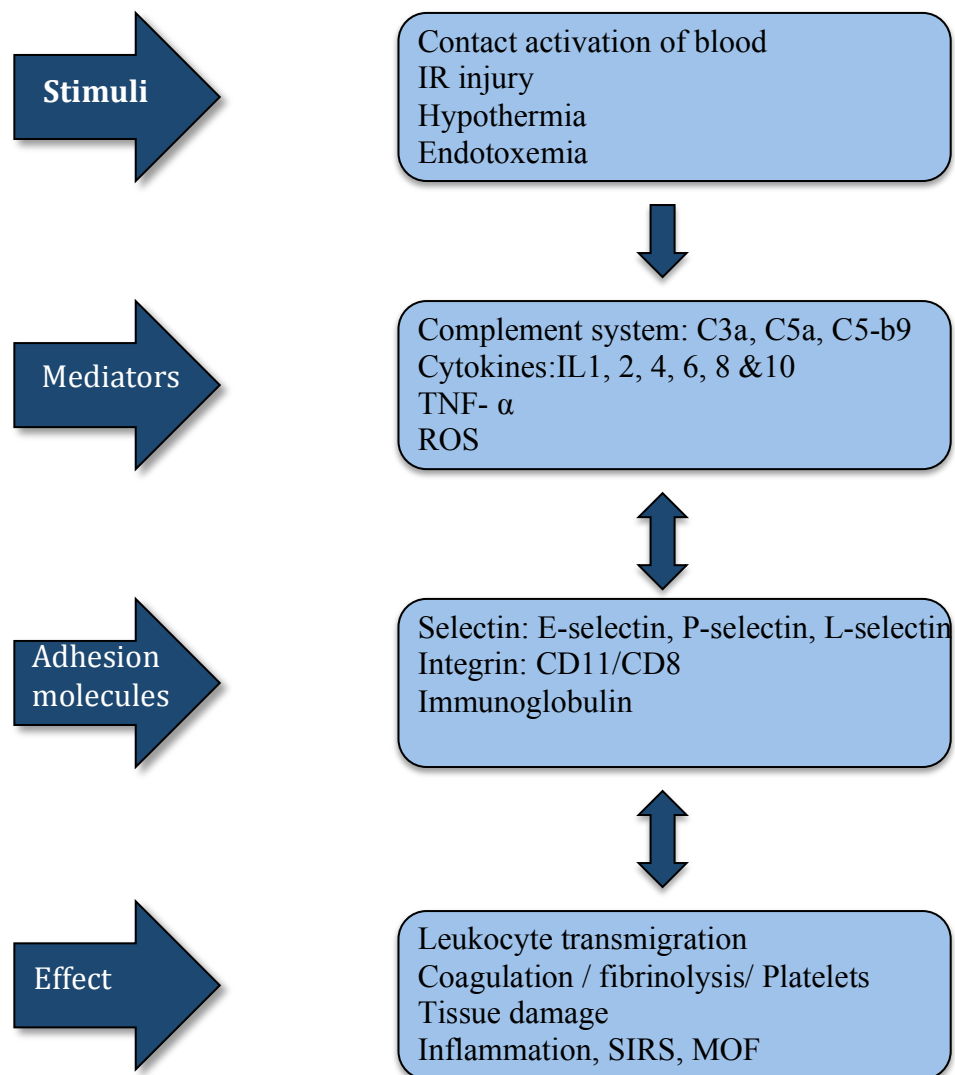


Figure 1.9 Inflammatory process in CPB

Schematic diagram of the inflammatory process induced by CPB with potential of developing SIRS and MOF. (ROS: Reactive oxygen species, SIRS: systemic inflammatory response syndrome, MOF: Multi organ failure).

1.4.1 Strategies to attenuate the inflammatory response to CPB

The inflammatory response in cardiac surgery can start from the time of intubating the patient coupled by the surgical trauma, but continuous circulation of heparinised blood into CPB circuit coupled with ischemic-reperfusion injury magnifies this response. The management of this inflammatory response is uneasy due to the fact of complex interplay of inflammatory cascade indicating the potential of failure with single therapy to attenuate the systemic inflammatory response. Many strategies have been implemented to reduce this effect in relation to CPB. This has included the use of pharmacological agents, biomaterial coating or treatment of the CBP circuit, hemofiltration or use of alternative option to a conventional CPB. The aim is to target the stimulator of the inflammatory cascades whether intercellular, molecular or receptor signalling.

1.4.1.1 Pharmacological strategy

1.4.1.1.1 Glucocorticoid

The use of steroids in CPB was first implemented in 1971(104). Steroids decrease the release of endotoxins, cytokines and TNF- α from macrophage (105). However, other studies showed reduction of TNF- α production along with aprotinin (106) in relation to steroid pre-treatment prior to CPB. Also steroids reduce the expression of adhesion molecules (107, 108). There are some studies that demonstrate reduction in complement and interleukin level with steroids (109).

1.4.1.1.2 Aprotinin

Aprotinin is a serine protease inhibitor and in addition to its antifibrinolytic and platelet sparing role in control bleeding in cardiac surgery (110). It has also anti-inflammatory effect, strongly inhibit plasmin and has partial role in inhibition of kallikrein

production (111). It reduces the up-regulation of adhesion molecule integrin and inhibits leukocyte transmigration and extravasation (112). Complement activation also was reduced with subsequent reduction in cytokine (105). Testing broncho-alveolar lavage of patients received aprotinin in cardiac surgery with CPB, showed reductions in the level of IL-8 and neutrophil accumulation with aprotinin (113).

1.4.1.1.3 Complement inhibitors

The main role of complement is in the acute phase of the inflammatory response to CPB. Complement recombinant protein inhibitor to C5 (h5G1.1-scFv) showed total blockage of C5b-9 in patients undergoing cardiac surgery with CPB as demonstrated by Fitch et al. (114) Additionally anti-C5 monoclonal antibody showed reduction in CD11b integrin expression by leukocyte during CPB (115).

1.4.1.1.4 Monoclonal antibodies against adhesion molecules

Adhesion molecules interact between neutrophil and vascular endothelial cell amplifying acute inflammatory response via this interaction of carbohydrate ligand in neutrophil and selectin family (116). Targeting blocking of adhesion molecules can prevent endothelial activation during CPB (117). P-selectin monoclonal antibodies administrations to prime prior to CPB in the rat model have shown significant reduction the in pro-inflammatory interleukin (IL-6, IL-8) (118). Previous studies demonstrate the use of anti C5 monoclonal antibody protect against myocardial infarction and showed down-regulation of CD11/CD18 expression on leukocyte after CPB (114).

1.4.1.1.5 Anti-oxidants

These agents are able to neutralise free radicals either by accepting or giving electrons, which will likely counteract reactive oxygen species (ROS) effect. Broccoli is a rich

natural source of anti-oxidant sulforaphane. A recent study by our group showed broccoli consumption induce antioxidant enzyme in leukocytes of healthy volunteers and sulforaphane administration reduces leukocytes activation and protection against renal injury in response to CPB in pig model (119).

1.4.1.1.6 Other pharmacological agents

Sodium nitroprosside: This agent acts as a nitric acid analogue, which has shown to lower pro-inflammatory cytokines and reduces activation of leukocytes and platelet in relation to CABG with CPB (120, 121).

Dipyridamole: Decreases the superoxide anion generation and adhesion of the neutrophils in relation to CPB reducing the inflammatory response (122).

Indomethacin: It blocks the down-regulation of prostaglandin E2 reducing the interleukin and TNF- α level (123).

1.4.1.2 Mechanical strategy

1.4.1.2.1 Heparin coated circuit

In addition to its anti-thrombotic effect of the heparin, it has also anti-inflammatory effect. It has noted to reduce complement activation and leukocytes-adhesion molecule response (124), reduce production of TNF- α and neutrophil activation (125) and reduce IL-8 and C3a level in paediatric CPB improving the lung function (126).

1.4.1.2.2 Leukocyte filtration

A leukocyte depleting filters (LDF) has been developed due to the significant role of the neutrophil in acute inflammatory response to CPB. LDF has been used for different field successfully as in prevention of HLA-alloimmunization and refractoriness to

allogeneic platelet transfusion blood transfusion and graft versus host diseases (127). Their concept is to capture activated leukocyte before entering the patient's body from CPB circuit. However, many studies have been conflicting regarding the use of leukocyte-depleting filters as in cardiac surgery with CPB but overall there was no clear benefit for its routine use (128, 129).

1.4.1.2.3 Modified ultrafiltration

It consists of removal of fluid overload and inflammatory mediators substance from intravascular and extravascular tissue. It has shown its benefit in paediatric cardiac surgery (130), but no major benefit in adults as a routine practice, only post-operatively with development of renal insufficiency (131).

1.4.1.2.4 Alternate to CPB

This includes avoiding CPB by operating on beating heart (off-pump) or using shorter CPB circuit as in paediatric cardiac surgery (miniaturized CPB).

1.4.2 Off-pump cardiac surgery

In many years the off- pump coronary artery bypass grafting (OPCAB) is becoming the main practice to many surgeons in order to avoid the potential adverse effect of the CPB. There are numerous studies comparing these two practices. The concept of OPCAB surgery implies performing an operation on a beating heart under a normothermic condition without the use of CPB. OPCAB requires the use of special flexible stabilizing devise providing temporarily localized stability between its two arms with suction on the surface (Octopus tissue stabilizer) to the specific part required to perform the coronary anastomosis or by capturing the target artery using vessel loops stabilizer (Figure 1.10).

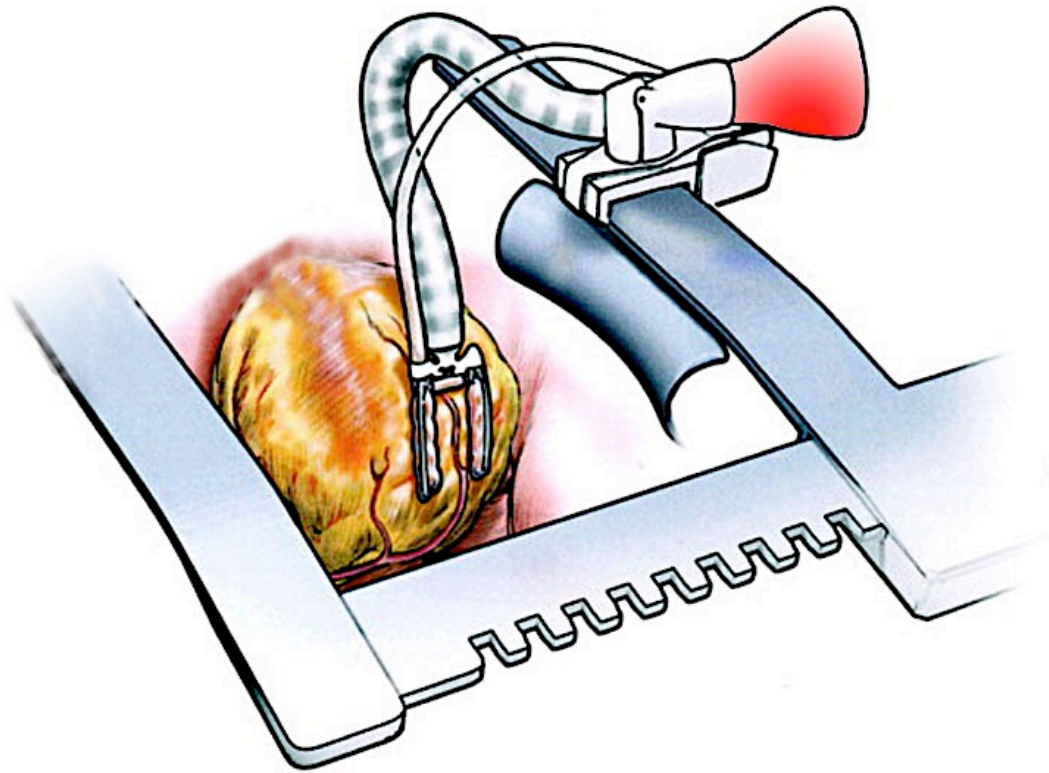


Figure 1.10 Off-pump coronary revascularisation

The concept involves stabilizing and capturing the area of heart contains the targeted coronary artery, which needed revascularisation, the Octopus tissue stabilizer system achieving local stabilization by suction onto the heart surface.

Several studies comparing the on-pump and off-pump CABG showed that myocardial revascularization with OPCAB has less magnitude of acute inflammatory response, however, does not prevent it (132, 133). It requires third amount of the anticoagulation needed for the conventional CPB. The limitation comprise in that it is technically challenging due to the difficult heart manipulation whilst beating. Additionally, has no role in the intra-cardiac operation such as in valve surgeries.

1.4.3 Miniaturised cardiopulmonary bypass (mini-CPB)

Mini-CPB is another form of conventional CPB but with less surface area exposed to the blood components with shorter circuit and smaller priming volume. No cardiotomy suction or venous reservoir leading to the prevention of air-blood contact (Figure 1.11). In relation to inflammatory response, Mini-CBP has advantage over the conventional CPB in attenuating cytokine release and leukocyte activation (134). Additionally, our research group found both ROS and activated P38-MAPK were attenuated after using mini-CPB as compared to conventional CPB (135). Other advantages includes less blood loss, stroke and renal injury (136).

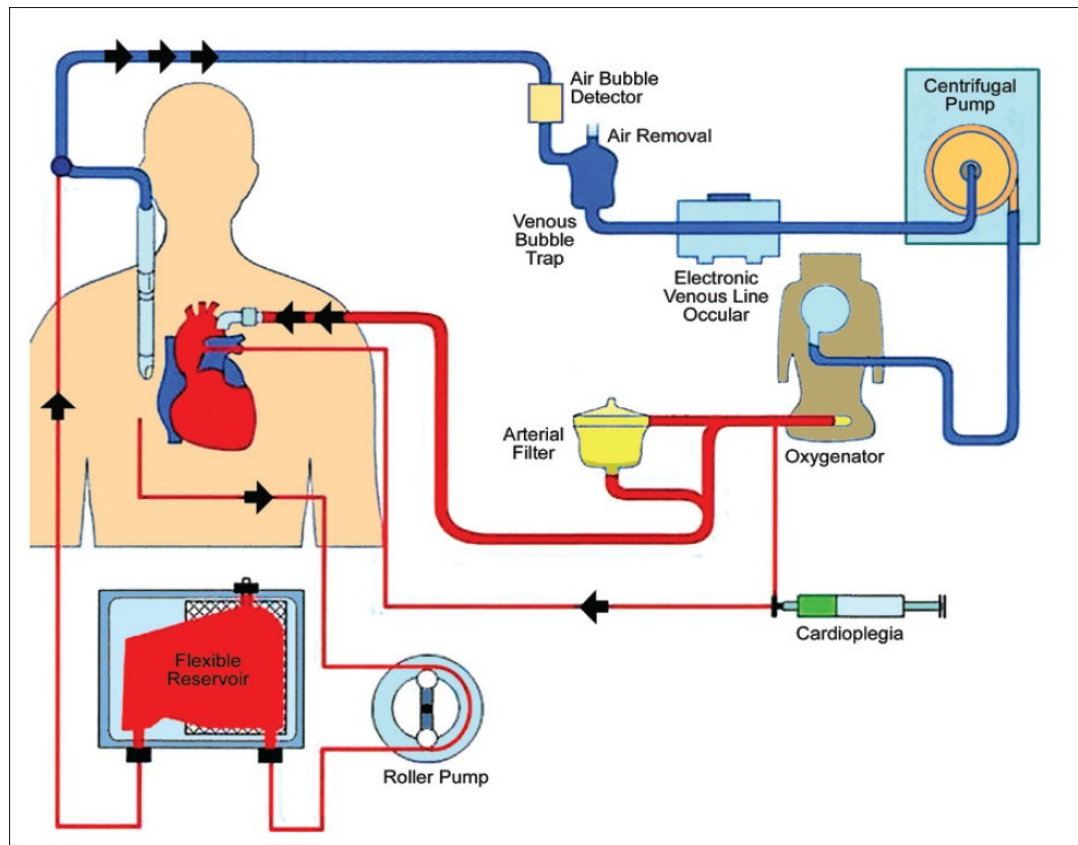


Figure 1.11 Miniaturised Cardiopulmonary Bypass

Mini CPB has smaller circuit and less surface area exposed to the blood component within the CPB circuit (Figure adopted from <http://www.aeronline.org/article.asp?issn=0259-1162;year=2012;volume=6;issue=1;spage=10;epage=13;aulast=Alsatli>).

1.5 Hypothesis

The magnitude of cellular injury as a result from cardiac surgery especially with utilization of CPB can result in the enhancement of DAMPs such as mitochondria DNA (mtDNA), which is similar to bacterial DNA can act as a ligand to signal the TLR9 with potential activation of the systemic inflammatory response. However, the contributions of DAMPs to inflammatory responses to CPB are unknown. Here in this study we addressed the following hypotheses:

- i. The systemic inflammatory response to cardiac surgery is promoted via TLR9 through binding to its ligand ‘the mitochondrial DNA’, leading to induction of numerous cytokines and chemokines. This can increase postoperative inflammatory related complications such as atrial fibrillation.
- ii. Blocking the TLR9 can potentially attenuate the inflammatory response in cardiac surgery. Additionally, utilization of antioxidant agents such as sulforaphane can potentially reduce apoptosis and mitochondrial damage leading to subsequent reduction the release of the mtDNA, which in turn can attenuate TLR9 signalling.

1.6 Aim of the study

This study was aimed to identify the potential inflammation associated risks in cardiac surgery and to reduce related morbidity and mortality. This approached by comprehensive understanding the signalling pathways of the TLRs activation in cardiac surgery, the course of the systemic inflammatory response associated with CPB at the cellular level and the molecular mechanism behind it, which can be assessed by:

- i. Identifying the effect of the release of mitochondrial DAMPs such as mtDNA and TLR9 signalling on the activation of the immune response in cardiac surgery.
- ii. Evaluating the downstream signalling of TLR9 and identifying potential TLR9 antagonist to attenuate this response.
- iii. Identifying possible predictors for the development of POAF, which may be contributed to the inflammatory response.

2 CHAPTER II: Toll-Like Receptors Review, Disease & Therapeutic value

2.1 Introduction

2.1.1 Chapter outline

Toll-like receptors (TLRs) are specific receptors that are able to recognise exogenous pathogen or endogenous damage associated molecular pattern, which will potentially stimulate cytokine production through different stimulatory pro-inflammatory pathways. TLR discovery has provided better understanding of the innate immune system. They are unique complex regulatory system that have an important protective role. If TLRs become dysregulated their protective role will become rather detrimental with subsequent tissue damage.

Despite many researchs on TLR system, it is still not completely understood. Here in this review we addressed the importance of the TLR signalling, understanding their various pathways mediating the systemic inflammatory response through binding to their ligands. Moreover, highlighting overview of previous studies of the TLRs role played in the cardiovascular system and in their contributions to various illnesses and their potential therapeutic values.

2.1.2 Inflammation and innate immunity

The immune system is comprised of innate and acquired immunity. Many experimental studies were done on inflammation in order to understand the inflammatory response. In particular recognizing the inflammatory and anti-inflammatory mediators and the inflammatory regulation on the cellular level including involvement of the genes analysis, which have recognized inflammation as the key factor for the innate immunity as well as the acquired immunity (19).

The major role of the immune system includes recognition and removal of any invading organism or foreign substance that are not normally present within the host body. Innate immunity is the first line of defence in the host activating phagocytes such as macrophage, leucocytes and dendritic cells with additional response of synthesis of variety of cytokines and inflammatory mediators (137, 138).

Both acquired and adaptive immunity has memory and specificity through B and T lymphocytes, which may take several days to appear (138). Recognition of microorganisms invasion such as bacteria or virus can lead to the stimulation of the innate immunity mechanism, mainly inflammation (139). This recognition is carried out by pattern recognition receptors (PRRs) (140-145).

2.1.3 Pattern recognition receptors (PRRs)

These receptors are Germline-encoded PPRs, where the inflammatory cascade initiated by recognizing exogenous microorganism-derived pathogen-associated molecular pattern (PAMPs) and host cell derived endogenous damaged-associated molecular pattern (DAMPs) from damaged cell (139, 146). They both can act synergistically to provoke more effective systemic immune response than would result from either alone (147). PRRs can be expressed in macrophage, dendritic cells and in some other immune cells. There are four different classes of PRR families including toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which are expressed in the cell surface and or in the cytoplasm as a cytoplasmic protein such as NOD-like receptors (NLRs) and RIG-like receptors (RLRs) (148, 149).

2.1.4 Pathogen-associated molecular pattern (PAMPs)

PAMPs are small molecular motifs that are associated with the presence of pathogens, which can be recognized by the cell of the innate immunity mechanism. These molecules recognized by the Toll-like receptors (TLRs) and other PRRs as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoid acid-inducible gene-I (RIG-I)-like receptors (RLRs) (149-152).

PAMPs can be presented as flagellin of the bacterial flagella, lipopolysaccharide (LPS) an endotoxin in the bacterial cell membrane, peptidoglycan associated with viruses or Gram-positive bacterial, unmethylated DNA and single or double stranded RNA. These are widely expressed by microbial pathogens, which are not present on the host tissue. Each PAMPs accordingly will be recognized by its specific receptor (141). They are utilized by PRRs to differentiate between pathogen and self-antigen (147).

2.1.5 Damage-associated molecular pattern (DAMPs)

In contrast to infection, tissue damage leads to non-physiological unscheduled cell death, which can enhance the activation of the immune system. Cell damage will release small endogenous danger signals molecules alerting for an early cell death. This activation can be provoked through molecules derived from the cell but not normally found within the extracellular space (147). These molecules are danger signals also known as “alarmins”. They are associated with the stimulation of immune response in non-infectious or sterile inflammatory response. Mainly released by stressed cells in relation to tissue injury or following cell death or necrosis (153).

DAMPs can be classified as protein (e.g. S100 proteins, heat shock proteins (HSP), high mobility group box-1 (HMGB1) or non-protein (e.g. ATP, uric acid, heparin sulphate, RNA, DNA, mitochondrial fragments). High serum level of these DAMPs

has been associated with variety of inflammatory diseases such as SLE, arthritis, inflammatory bowel diseases, sepsis and also cancer, therefore therapeutic strategies has been established in many studies to modulate the expression of these DAMPs for disease treatment (154-159).

2.2 Toll-like receptors (TLRs)

TLRs are class of proteins with single, membrane-spanning and non-catalytic receptors, acting by recognizing structurally conserved molecules derived from microbes and host cells. They were discovered in *Drosophila melanogaster* (a fruit fly) in 1985 by Christiane Nüsslein-Volhard. TLRs obtained their name due to their structural similarity to the protein coded by the Toll genes found in *Drosophila* (160, 161). In mid 1990s Jules Hoffmann found that TLRs plays very important role in the fly's immunity to fungal infection (162). They were first described in 1994 (163) and mapped to chromosome in 1996 (164).

TLRs are the key component of innate immune system. They form the first line of defense against pathogens and injurious stimuli recognized as PAMPs and DAMPs respectively activating innate immunity through pattern recognition receptors (PRRs). Their recognition facilitated by the N-terminal outer membranous domain called leucine-rich repeat (LRR) of TLRs (149). TLRs triggering during injury induces the expression of numerous inflammatory genes, which plays significant role in the pathogenesis of the inflammation, cell immune regulation and proliferation (165-168). Negative regulation of TLR-mediated immune response is very important to avoid autoimmunity (168).

TLRs are part of a superfamily of PRRs, comprised of 11 members characterized in humans (TLR1-11), however, TLR11 is pseudo-genome in humans (169)(170). Together TLRs with interleukin-1 receptors form "Interleukin-1 Receptor/Toll-Like Receptor Superfamily" sharing the name of Toll/Interleukin-1 Receptors (TIR) domain (161, 171). TIR domain will recruit the TIR-containing adaptor myeloid differentiation factor 88 (MyD88), TIR domain containing adapter-inducing INF- β (TRIF) also known as TIR domain-Containing Adaptor Molecule (TICAM1) (172), TIR domain-Containing Adapter Protein (TIRAP), TRIF-Related Adapter Molecules (TRAM) also known as (TICAM2) leading to activation of Nuclear Factor-Kappa B (NF- κ B) and Mitogen-Activated Protein Kinases (MAPK) (173) (Figure 2.1).

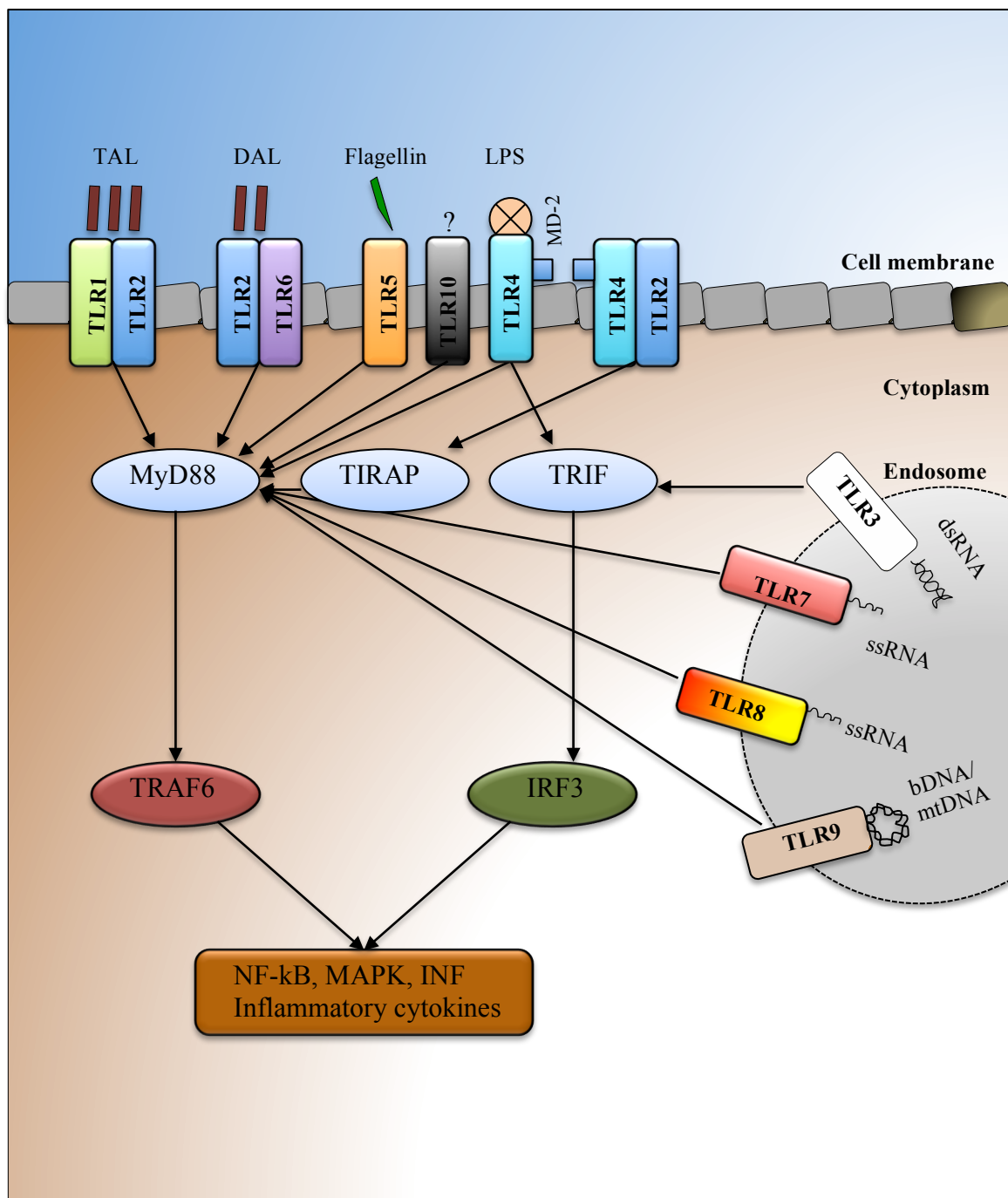


Figure 2.1 TLRs signalling pathway

All TLRs act to activate MyD88-dependant pathway except TLR3 activate TRIF-dependent pathway and TLR4 (with its MD-2 co-receptor) shares both pathways. (TAL: Triacyl lipoprotein, DAL: Diacyl lipoprotein, LPS: Lipopolysaccharide, ssRNA: Single stranded RNA, dsRNA: Double stranded RNA, bDNA: Bacterial DNA, mtDNA: Mitochondrial DNA).

2.2.1 TLRs and their ligands

TLR's ligands have been recognized to be lipoproteins, lipids and nucleic acids. Each TLR recognise and binds to a specific ligand (Table 2.1); this recognition defines the specific reaction of TLR-mediated immune responses with production and release of pro-inflammatory precursors such as cytokines (168, 174).

Toll-like receptor	Specific ligand	Notes
TLR1	Triacyl lipopeptides	Homodimer
TLR2	TLR2/TLR1: Triacyl peptides. TLR2/TLR6: Diacyl peptides	Pairs with TLR1/TLR6 forming heterophilic dimers recognizing different PAMPs
TLR3	Double stranded DNA	Only signals through TRIF-dependent pathway
TLR4	Lipopolysaccharide	MD-2 act as co-receptor
TLR5	Flagellin	Homodimer
TLR6	Diacyl peptides	Heterodimer
TLR7	Single stranded RNA	Homodimer
TLR8	Single stranded RNA	Homodimer
TLR9	Unmethylated CpG DNA	Homodimer (Viral, bacterial or mitochondrial DNA)
TLR10	Unknown ligand	Clusters with TLR1, TLR2 & TLR6
TLR11	Uropathogenic bacteria Profilin-like protein	Pseudo-gene in human

Table 2.1 TLRs and their binding ligands

The Toll-like receptors and some of their important specific ligands required for their activation and signalling pathways.

Most of the TLRs form homodimers when binding to their ligands. However, some ligands need another TLR cooperation for signalling such as TLR1/TLR2 and TLR2/TLR6. They are heterodimers recognizing triacylated lipopeptide and diacyl lipopeptide respectively (175, 176). TLR1, TLR2, TLR4, TLR5, and TLR6 are placed on the cell surfaces; these subgroups can identify bacterial and fungal cell wall component and to some extent some viral protein (177). The other subgroup includes TLR3, TLR7, TLR8, and TLR9 are intracellular located at the endosomal compartment and binds with viral and bacterial nucleic acid (173, 177, 178). Engagement of these TLRs to their specific PAMP or DAMPs ligand causing signals to propagate into the cell leading to activation of NF- κ B and IRF transcription factors which results in expression of numerous cytokines promoting inflammatory response.

2.2.1.1 TLR1

Located on the cell surface, TLR1 also known as (TIL, cluster of differentiation 281 (CD281); rsc786; TIL. LPRS5) (179), TLR1 forms heterodimer with TLR2 dimer recognizing lipoprotein or triacylated lipopeptide such as Pam3CSK4 facilitates innate immune response when this complex bind to these bacterial cell wall component (175, 180-182). Qi et al. (183) has conducted first case-control paediatric study in Chinese children related TLR1 single nucleotide polymorphism and tuberculosis. They concluded that G allele of rs5743618 distribution in TLR1 was more susceptible to TB, which related to decrease in surface expression of TLR1 in monocyte and granulocytes. Also in relation to Lyme disease vaccination, Alexopoulou L et al. (184) showed TLR1 recognizes outer surface lipoprotein of *Borrelia burgdorferi* when tested TLR1 knock out mice.

2.2.1.2 TLR2

TLR2 located on the cell surface, also known as CD282, it recognizes a wide spectrum of microbial products. It pairs with TLR1 or TLR6 forming heterophilic dimers recognizing different PAMPs (175, 185). TLR1/TLR2 heterodimer responds to triacyl lipopeptides while TLR2/6 heterodimer responds to diacyl lipopeptides and peptidoglycan (174). Moreover, TLR2 also can cooperate with structurally related or unrelated proteins to recognize that wide range variety of microbial components.

Gantner et al. (186) showed TLR2 has collaborated with dectin-1 receptors recognizing fungal cell wall-derived β -glucan with enhancement of TLR2-mediated NF-KB activation and cytokines production initiating inflammatory response. In autoimmune disease TLR2 polymorphism has been linked to be more prevalent in acute reactive arthritis following *S enteritidis* bacterial infection (187). Also overexpression of TLR2 may contribute to more damage and injury as in sepsis where over activation can contribute to development of septic acute kidney injury (188, 189). TLR2 along with TLR4 have been associated with the initiation of the inflammatory response to cardiopulmonary bypass in cardiac surgery (190-192).

2.2.1.3 TLR3

TLR3 are endosomal receptors, they recognize viral double stranded RNA (dsRNA), which is a molecular pattern associated with viral infection produced during viral replication (193). The dsRNA interact with N-terminal and C-terminal on TLR3 surface (174) activates NF-kB. Alexopoulou et al (178) has shown that a TLR3-deficient mouse has impaired response to dsRNA.

All TLRs depends on MyD88 adaptor protein for their signalling with exception to TLR3 where their signalling pathway induced by recruitment of Toll/IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF) activating TRIF-dependent pathways via TRAF3 degradation with subsequent production and release of type-1 IFN- β and inflammatory cytokines. Their role in vivo is promoting cross priming for initiation of CD8+ T cell response against viruses (194-196).

2.2.1.4 TLR4

Also known as CD284, present in the cell membrane. It has extracellular luecine-rich repeat domain and TIR domain (197). TLR4 with its co-receptor MD-2 can recognize lipopolysaccharides (LPS) (198, 199) after transferred to MD-2, which is molecule that confers LPS responsiveness to TLR4. MD-2 binds to extracellular part of TLR4 with subsequent oligomerization of TLR4 (198, 200, 201). LPS is a common component of Gram-negative bacterial cell wall, which is recognized by the immune system (202). TLR4 exceptionally is the only TLR that can activate two separate downstream pathways such as TRIF-dependent inducing the expression of type-1 interferon and MyD88-dependant inducing the expression of inflammatory cytokines (149).

TLR4 over expression has been related to the pathogenesis of atherosclerosis and atherosclerotic plaque instability through activation of the oxidized lipids 27-hydroxycholesterol and aldehyde 4-hydroxynonenal when they accumulate in the atherosclerotic plaque (203). Some of the autoimmune disease has linked to TLR4 overexpression. Abdollahi-Roodsaz et al. (204) demonstrated that, inhibition of TLR4 has led to degree of suppression of the severity of an experimental arthritis with decrease IL-1 expression. TLR4 alone (205, 206) or TLR4 along with TLR2 had been implicated in relation to initiation of the inflammatory response in cardiac surgery

utilizing the cardiopulmonary bypass (190-192). However, some studies showed little effect of bypass on TLR2/4 expression (207). Moreover, TLR4 plays an important role in myocardial ischemic-reperfusion injury associated inflammatory response, such as after percutaneous coronary arteries intervention (PCI) for IHD or in CPB (208). Some animal models were tested modulating the ischemic-reperfusion injury in the heart (209, 210), lung (211, 212), liver (213, 214), kidneys (215) and brain (216). Their results indicate the significant role of TLR4 in IR-injury when inhibited.

2.2.1.5 TLR5

TLR5 are cell surface receptors, also known as (SLE1; TIL3; SLEB1; MELIOS) (217). It is expressed in the innate immunity when recognizes flagellin, a protein component of bacterial flagellum and an important structure in bacterial motility (175, 218). Flagellin acts as a PAMP for TLR5. It is a potent activator of the cells involved in both innate and adaptive immunity promoting cytokines production for both gram positive and negative motile bacteria (219, 220). TLR5 can be expressed in the epithelial cell of the intestine or lung and play an important role in defence mechanism in the intestine and respiratory tract (149).

It signals through MyD88-dependant pathway leading to the induction of the pro inflammatory mediators (221). Their activation will mobilize NF- κ B stimulating the production of tumour necrosis factor- α (TNF- α) (220). Some studies suggest that TLR5 can potentially show pro or anti-tumour activities when expressed in response to different types of tumours (222). Kauppila et al. (223) demonstrated the over expression of TLR5 in squamous cell carcinoma of the tongue associated with poor prognosis with potential carcinogenic role. In contrast, Zhou et al. (224) concluded a

high expression of TLR5 was associated with better prognosis and can potentially exhibit therapeutic target in non-small cell lung cancer patient.

2.2.1.6 TLR6

TLR6 referred as CD286 (225). It is located and expressed on the cell membrane. TLR6 functionally interact with TLR2 exhibiting cellular response to bacterial lipoprotein. TLR2/TLR6 heterodimer responds to diacyl lipopeptide and peptidoglycan with subsequent signalling activating transcription factor NF- κ B and up regulation of gene expression of pro-inflammatory mediators (174, 226, 227).

Takeuchi et al. (176) has demonstrated that TLR6-deficient mice were unable to produce pro-inflammatory cytokines in response to diacylated mycoplasma macrophage-activating lipopeptides-2 (MALP-2) while showed normal response to triacylated lipopeptide derived from Gram-negative bacteria suggesting TLR6 cooperate with TLR2 for recognition of MALP-2 a diacylated protein.

2.2.1.7 TLR7

TLR7 are endosomal intra cellular receptors expressed by variety of cells including B-cells, T-cells, plasmacytoid and myeloid dendritic cells (228). They recognize single stranded RNA (ssRNA) a common feature of viral genomes. TLR7 largely expressed in dendritic cells inducing large quantity of INF- α and pro-inflammatory cytokines (229). TLR7 stimulation leads to the dimerization of the receptor stimulating the recruitment of TIRAP, MyD88-dependant pathway. This will lead to activation of NF- κ B and IFN regulatory factor 7 (IRF7) inducing large quantity of INF- α and other pro-inflammatory cytokines such as TNF- α , IL-6 & IL-12 (230).

TLR7 is also involved in autoimmunity and autoantibody production in human. It has shown increased level of TLR7 gene expression in patients with systemic lupus erythematosus (SLE) (231-233). Subramanian et al. demonstrated that the translocation of TLR7 gene from X-chromosome to Y-chromosome led to overexpression of TLR7 and development of fatal lupus in mice (234).

2.2.1.8 TLR8

TLR8 identified as CD288 (235). They are intracellular endosomal highly conserved protein homo-dimer receptors, recognising viral and bacterial ssRNA ligand in human (236). Even though ssRNA is abundant in host it is not detected by TLR8 or TLR7 due to the fact that they are expressed in the endosome while host-derived ssRNA not delivered there (237). TLR8 acts via MyD88-dependant pathway with activation of NF-kB and cytokines secretion inducing the inflammatory response. It locates in proximity to TLR7 on chromosome X. TLR8 is expressed in leukocytes and CD4+ regulatory T cell (Treg), the latter involved in preventing autoimmunity by actively suppressing host immune response. Peng et al. (238) showed that the activation of TLR8 signalling pathway leads to the inhibition of the Treg cell's function.

2.2.1.9 TLR9

Can be identifying as CD289. TLR9 are intracellular endosomal homo-dimer receptors. Recognise unmethylated CpG sequences in DNA molecules, which is a hallmark of viral and bacterial genomes and recognized as PAMP. Mitochondrial DNA in turn contains CpG DNA repeats, which retains molecular motifs similar to bacteria and recognized as DAMPs for TLR9 (154, 158). TLR9 is expressed in variety of immune system cells including dendritic cell, B-lymphocytes, monocytes and natural

killer cells (236). Binding to its specific ligand will lead to its activation mediated through MyD88-dependant pathway with subsequent activation of kinases.

MyD88 interact with IL-1 receptor-associated kinases (IRAK-1 and IRAK-4) (239) and TRAF6 forming signalling complex ultimately leading to activation and recruitment of transcription factors such as IRF to the complex activating other signalling cascade, with subsequent TLR gene induction (240). Activation of TLR9 signalling pathway will lead to activation of NF- κ B and AP-1 inducing diverse immunity related gene with secretion of different pro-inflammatory cytokines and chemokines (239-241). In particular TLR9 can induce the antiviral response through inducing type-1 INF (IFN- β & IFN- α) (241).

TLR9 has been implicated in the pathogenesis of several autoimmune diseases through recognition of chromatin structure where co-engagement of chromatin immune complex (IgG2a-chromatin) to TLR9 will stimulate B-cell receptor. Subsequently, produce rheumatoid factor by auto reactive B-cells implicating autoimmune response such as in SLE or rheumatoid arthritis (242, 243). Chloroquine medication was used for one of autoimmune disease treatment modality. It has the potential of blocking the TLR9 along with TLR7 (244). On the other hand synergizing TLR9 can have therapeutic potential as in the current on going phase one trial studying the effect of TLR9 agonist (MGN1703) in metastatic solid tumours (245).

2.2.1.10 TLR10

Recently identified, it is the only TLR without known specific ligand and function (246). Hasan et al. (247) revealed that TLR10 gene clusters with TLR1 and TLR6 both of which known to function along with TLR2. Hence, TLR10 may be contributed to recognize TLR2 ligand (248). Therefore, it is believed to be signal through MyD88-

dependant pathway as well. Oosting et al. concluded that TLR10 is the only inhibitory receptor as compared to other TLRs with the main role of activation and production of the proinflammatory cytokines (246). The author suggested that this inhibitory effect is highly likely from TLR10-TLR2 interaction. Mulla et al. on the other hand proved that TLR10 induces apoptosis through activation caspase-3 triggered by TLR2 ligand (249).

2.2.2 Diseases related and the therapeutic value of TLRs

Genetic single nucleotide polymorphism (SNP), over expression or down regulation of the TLRs and its adaptor protein pathways may contribute to variety of disorders (173). Hence, its regulation plays an important role in avoidance of variety of diseases. TLR-related molecules dysregulation or signalling, can contribute to infection, sepsis, allergy, cancer (250, 251), autoimmune disease or immune deficiency such as in HIV (252-254).

In sepsis the TLRs pathways and the adapter protein can lead to the synthesis of pro-inflammatory cytokines and chemokines. It implicates a crucial role in sepsis control and pathogenesis. Ghosh et al. demonstrated that LPS from Gram-negative bacteria induce up-regulation of TLR4 (255). Additionally, Weighardt et al. showed TLR4 and its MyD88-dependant pathway activation plays an important role in the pathogenesis of sepsis in acute Gram-negative bacterial infection when tested MyD88 knockout mice, they were protected from septic peritonitis (256). On the other hand TLRs deficiency and defect in their signalling pathway contribute to primary immune deficiency diseases leading to severe infection and sepsis (173, 257, 258).

TLR has fundamental role in tumorigenesis and expression in cancer cells. Multiple studies for gastric cancer have shown genetic polymorphism and gene expression of

various TLRs (TLR2, TLR4 & TLR10) can influence *H. pylori*-induced gastric carcinogenesis (257, 259-261). TLR2, TLR4 and TLR10 also have been linked to colorectal, epithelial ovarian and nasopharyngeal cancer, respectively. When they over expressed this will activate MyD88 pathway signalling, which has significant role in inducing tumour development (262-264).

In contrast, Feng et al. suggested that TLR signalling facilitate selective tumour cell phagocytosis by macrophage synergizing with anti-CD47 blockade (265). Over expression of these receptor can prevent tumour cell phagocytosis, which gives negative prognosis in variety of cancers (266). Stevens et al. identified that the TLR gene cluster (TLR10, TLR1 & TLR6) has protective effect against prostate cancer, which can be potentially of therapeutic value (251). Also the phase I trial study the effect of TLR9 agonist (MGN1703) on solid metastatic tumours showed potential therapeutic outcome if giving in regular low doses twice weekly (245).

TLRs dysregulation with subsequent activation of MyD88 or TRIF pathway and production of pro-inflammatory cytokines may induce variety of autoimmune diseases. As explained earlier SLE was linked to over expression of TLR7 and TLR9 as well as the rheumatoid arthritis (231-233, 242, 243). In Sjögren's syndrome, which affects the salivary and lacrimal glands causing dry mouth and eyes, there was over expression of TLR2, TLR3 and TLR4 (267). TLR2 and TLR4 also were linked to the development of systemic sclerosis (268, 269). Theoretically, TLR was thought to have a link to multiple sclerosis (MS) development even though there is still lack of proper understanding of its pathogenesis. However, Gooshe et al. suggested that the activation of TRIF-dependent or inhibition of MyD88-dependent pathways could be potentially a therapeutic target in the treatment modality of MS (270).

2.2.3 TLRs and cardiovascular diseases

It is known clinically that the inflammation has poor implication on the cardiovascular system. Also known is the important role of the TLRs in inflammation; therefore the correlation between TLRs and the cardiovascular system was an interesting subject for many researches. All human TLRs were found in the heart (especially TLR2, 3, 4 & 9) with different levels of expressions providing an important role in protecting the heart (271). They were expressed 10 times more than other TLRs in cardiac myocytes (272). However, TLRs on the other hand can potentially implicate harmful effect if they become dysregulated.

Several studies showed correlations between TLRs (especially TLR2 and TLR4) and the pathogenesis and progression of atherosclerosis (273-276), the main contributing factor for coronary artery disease development. This was supported by Mizoguchi, who reported expression of TLR2 and TLR4 in monocytes, increases the severity of coronary artery diseases in patients with stable angina (277). TLR2 also have been contributed to the aggravation of the aortic valve stenosis leading to increased accumulation of biglycan and phospholipid in aortic valve (278, 279). Moreover, expression of both TLR2 and TLR4 in human aortic valve interstitial cells has played a role in aortic valve inflammation and subsequent stenosis (280).

In viral myocarditis, mice experimental models were injected with coxsackie virus B3 (CVB3) known to cause human myocarditis, results showed mice with MyD88-adaptor protein knock-out (downstream signalling pathway for all TLRs except TLR3) has protective effect against the virus (281). Furthermore, TRIF knock-out (downstream for TLR3 & TLR4) has higher susceptibility to CVB3 infection (271), suggesting

harmful effect and protective effect of MyD88 and TRIF pathways on CVB3 myocarditis respectively as a result of TLRs signalling.

2.2.4 Role of TLRs in cardiac surgery

Understanding the TLRs effect on the heart is of important value in cardiac surgery. Cardiac surgery with cardio pulmonary bypass (CPB) can activate the systemic inflammatory response and have been associated with systemic inflammatory response syndrome (SIRS) with potential morbidity and mortality. The mechanism of the CPB modulating the inflammatory response still not fully understood. However, it is likely to be multi factorial, which involve the exposure of circulating blood to artificial surfaces of extracorporeal circuit, non-physiological shear stress of the pump and the hypothermia (83, 87) coupled with the conditions of obligatory ischemia-reperfusion activating inflammatory cascade (84).

Flier et al. (191) and Chalk et al. (192), both suggested decrease monocyte expression of TLR4 and identified an association between decrease in TLR2 expression and development of SIRS and pneumonia in patients underwent coronary artery bypass graft surgery (CABG). Similar finding were reported in patients who underwent aortic and mitral double valve replacement where early expression of TLR4 leads to the early onset ventilator-associated pneumonia (206).

Dybdahi et al. demonstrated signalling of TLR4 after immediate release of heat shock protein 70 (HSP70) in response to CABG with CPB (155). HSP70 can act as a cytokine promoting the inflammatory signalling cascade in monocytes leading to up-regulation of IL-1 β , IL-6, NF-kB and TNF- α mediating the inflammatory response (282).

Trop et al. also suggested increase expression of TLR2 and TLR4 with exacerbated systemic inflammatory response in response to CABG with CPB, even though it was more at certain ethnic group than other (South Asians vs. Caucasians) (283). Some studies suggested transient overexpression of TLR2 and TLR4 on monocytes and neutrophils (190). However, other studies suggested minimal contribution of TLR2 and TLR4 expression in response to CPB (207).

Kaczorowski et al. has indicated that TLR4 has an important link between the myocardial ischemic-reperfusion (IR) injury and the inflammatory response (208). Additionally, Lu et al. showed in mouse model the significant role of TLR3 in myocardial injury after IR injury when compared to TLR3 knockout mice (284). Methe et al. also concluded that chronic rejection of transplanted heart is related to the activation of innate immunity contributed by TLR4 signalling where also IR-injury can be contributed (285). We have performed different experiments studying the effect of TLR9 in systemic inflammatory response related to cardiac surgery, which will be discussed thoroughly in the next chapters.

2.3 Overview

Since the discovery of the TLRs, they were a main focus of attention for many researches. They are important for understanding the pathomechanism that drives and underpins diseases. Targeting TLRs or the signals generated by them has potential therapeutic value with more background knowledge of TLR family individually. Their different signalling pathways were studied and data were published but yet there is still lack of their complete understanding.

Clearly, TLRs have an important role in innate immune system and can collaborate with NOD-like receptors synergetically to initiate immune response (286). However, their role exceed beyond just immunity to non-immune function, their signaling has relavent physiological and developmental contributing factor. Physiological role in signalling includes many TLRs, for example TLR2 and TLR4 has role in regulating hippocampal nuerogenesis (287). TLR3 and TLR4 associated with cognition and memory (288, 289). TLR2 has found to have role in metabolism, TLR2 and TLR4 associated with the regulation of cardiovascular function (290), and TLR9 is involved in the autonomic cardiac and baroreflex control of the arterial pressure (291).

TLRs expression and activation of its downstream signalling pathway has shown earlier to be related to several diseases, mainly related to inbalance in cytokines homestasis either due to low or overwhelming release and production. Disabling these pathways can be an attarctive therapuetic target. However, disabling of these pathways can also compromise the immune defence system from the endogenous molecules released from tissue injury, invading pathogenic microorganisms or cancer cells. This suggests that the therapuetic modality in TLR-related diseases can be inhibitory or stimulatory by TLR antagonist or TLR agonist respectively.

TLRs system is unique, differential compartmentalization generate complex regulatory system by which ligands are to be recognized and what signals are to be generated, allowing balance to prevent any subsequent consequences of either immune deficiency or autoimmunity (236). Hence, many research studies have focused on ligand-signal relationship with the aim to identify protien, which positively or negatively regulate TLR signalling especially in those with TLR genetic polymorphism and defect.

TLRs in the cardiovascular system has fundamental protective role against infection or tissue injury. This role will be lost if dysregulated with over expression leading to cardiovascular hemodynamic instability. Some trials showed positive response as in eritoran tetrasodium (E5565), a synthetic TLR4 antagonist showed tolerance and beneficial influence on CVS in patients with severe sepsis attenuating cytokine expression in the vascular wall (292, 293).

In the rat CPB model monitoring lung inflammatory injury, utilisation of curcumin a natural extracted from rhizome and turmeric has inhibited TLR4 expression and activation of NF- κ B attenuating inflammatory lung injury (205). We have tested blocking TLR9 in mouse model in relation to sternotomy, which will be discussed later in Chapter 4.

Taken together, TLRs are delicate and complicated system. A well controlled regulation will provide protection from infectious and injurious material, while if became dysregulated their effect become harmful. Therefore understanding their different activation pathways and the potential SNP has valuable clinical implication in variety of diseases, an area whereby researches are progressively growing to provide successful TLR-based therapy with careful selection of the therapeutic target in the TLR-signaling cascade.

3 CHAPTER III: Evaluation Of Mitochondrial DNA In Inflammatory Response To Cardiopulmonary Bypass

3.1 Abstract

Background: Cardiopulmonary bypass (CPB) can lead to a spectrum of complications due to activation of systemic inflammatory responses. Cellular injury can lead to the release of damage-associated molecular patterns (DAMPs) such as mitochondria DNA (mtDNA), which can activate leukocytes and endothelial cells via innate immunity receptors. The contribution of DAMPs to inflammatory responses to CPB is unknown.

Aim: Firstly, we aimed to identify that Ischemic heart disease (IHD) can potentially influence the release of the mtDNA contributing to inflammation. Secondly, to test the hypothesis that CPB initiates a sterile systemic inflammatory response via the release mtDNA into the circulation signalling the TLR9.

Methods: 66 patients underwent coronary artery bypass graft (CABG) surgeries were recruited.). Circulating mtDNA levels were tested at different time-points related to CPB and off-pump CABG (OPCAB). This include preoperatively and after anaesthetic induction followed by at the onset of CPB (CPB0) and equivalent time in OPCAB, 30 (CPB30), 60 (CPB60), 120 (CPB120), 300 minutes (CPB300) and 24 hours (CPB24). Real-time PCR was performed to detect the number of thermal cycle (Ct) required to generate mtDNA. The level of mtDNA was measured relatively to Ct value. Additionally, to study the effect of IHD on mtDNA release we recruited another 22 patients undergoing aortic valve replacement (AVR) with normal coronary vessels.

Results: The preoperative levels of mtDNA were significantly elevated in IHD compared to non-IHD ($p < 0.01$). The mtDNA has significantly increased in response to surgery alone and with CPB. However, it was significantly higher in CPB compared to OPCAB (mean $\pm$ SD) in the following pattern: CPB0 (4.85 ± 3.48 and 3.48 ± 1.06 -fold, $p < 0.01$), CPB30 (41.63 ± 8.53 and 4.22 ± 1.45 -fold, $p < 0.001$), CPB60 (43.26 ± 9.17 and 3.93 ± 1.54 -fold, $p < 0.001$), CPB120 (15.18 ± 8.91 and 3.46 ± 1.64 -fold, $p < 0.001$), CPB300 (4.02 ± 3.14 and 2.56 ± 1.15 -fold, $p < 0.05$) and CPB24 (2.12 ± 1.26 and 1.5 ± 1 -fold, $p < 0.05$).

Conclusion: Elevation in the level of circulating mtDNA is related to the extent of the IHD, which is a novel finding. This can be related to the fact that inflammation can induce atherosclerosis, a main contributor for IHD. CPB and to the less extent the cardiac surgical trauma leads to the release of free, circulating mtDNA, which may contribute to the initiation of a sterile systemic inflammatory response in cardiac surgery.

3.2 Introduction

3.2.1 Mitochondria

Mitochondria are evolutionary derived from bacteria and likely able to bear the bacterial molecular motifs (158). Mitochondria are double membrane organelles within cells. They exist in hundreds and thousands per each cell related to the cell type and provide vital function. They are able to supply energy to the cell from food and are involved in the metabolic regulation. Furthermore, they play an important role in the calcium homeostasis, apoptosis (self-cell destruction) and in the production of cholesterol, heme and most of cell's reactive oxygen species (ROS) (294-297).

3.2.2 Mitochondrial DAMPs

During cell injury or death, the mitochondrion disruption can lead to the release of its contents. These include endogenous molecules, which act as a danger signals to stimulate the immune response. These molecules known as damage associated molecular pattern (DAMPs), include mitochondrial DNA (mtDNA), N-formyl peptides (NFP), adenosine tri-phosphate (ATP), cytochrome oxidase C and cardiolopin (CL) (Figure 3.1).

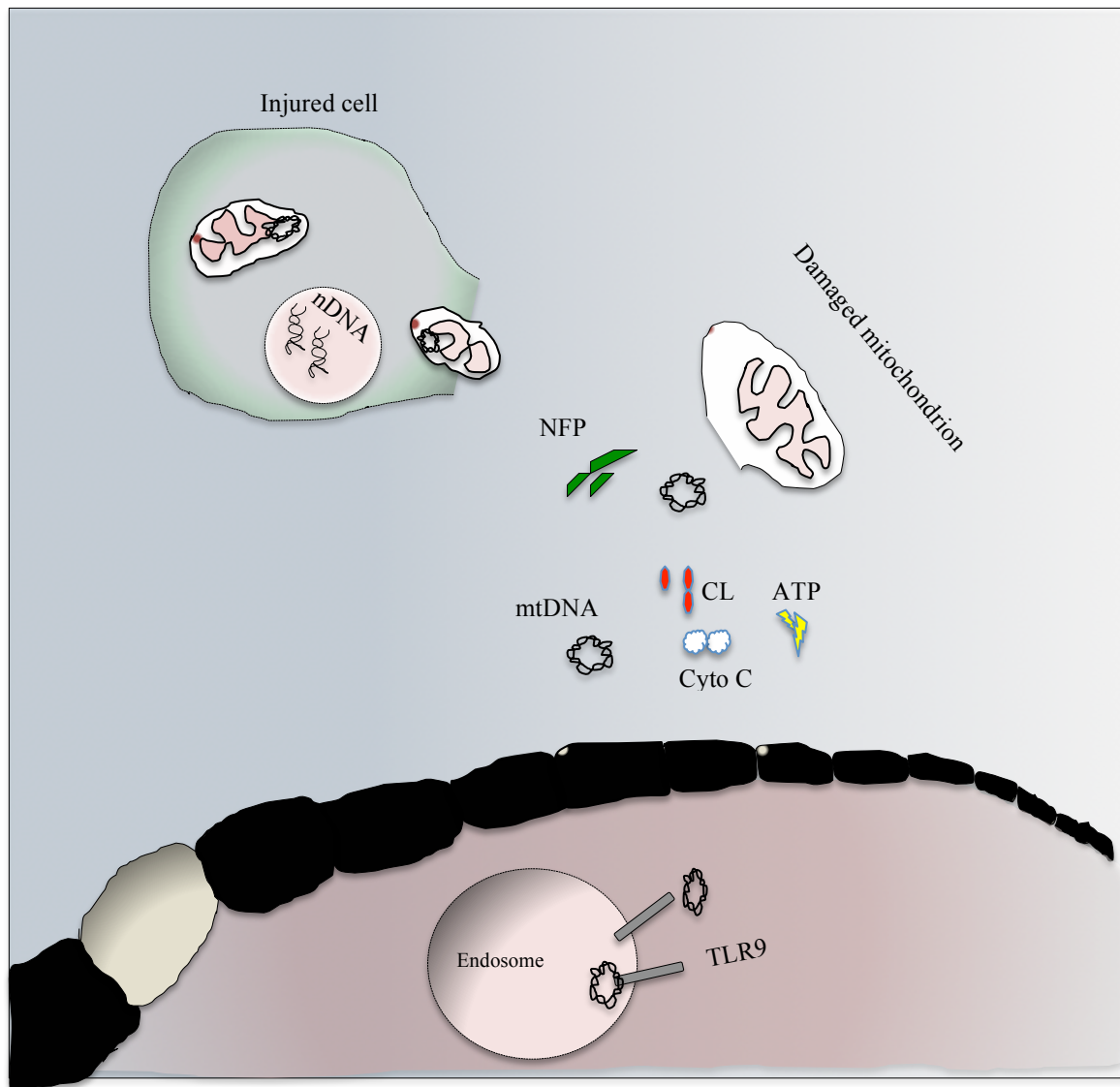


Figure 3.1 Mitochondrial damage associated molecular patterns (DAMPs)

Due to cell injury, damaged mitochondria will release its DAMPs into extra cellular space, which are potent immune activators. These DAMPs include mtDNA, NFP, ATP, Cyto C & CL. mtDNA acts as a ligand to endosomal TLR9 promoting it's signalling. (TLR9: Toll-like receptor 9, nDNA: nuclear DNA, mtDNA: mitochondrial DNA, NFP: N-formyl peptides, ATP: Adenosine tri-phosphate, Cyto C: Cytochrome oxidase C, CL: Cardiolipin).

The mitochondrial DAMPs have been demonstrated as an inflammatory modulators leading to tissue damage in variety of pathological conditions including SIRS, connective tissue diseases, myocardial infarction, vascular dysfunction and in individuals receiving chemotherapy or hemodialysis (298-301). One important molecule of these DAMPs is the mtDNA.

Mitochondria are the only organelles that have their own DNA, which carries its own genetic material. Similar to bacteria, the mtDNA is rich in unmethylated cytosine-phosphate-guanine (CpG) motifs (302, 303). The mtDNA is double stranded circular DNA with 16,500 DNA building block (base pair) (304). It contains 37 genes, which has an important role in mitochondrial function. These genes include; 2 ribosomal RNA (rRNA), 22 transfer RNA (tRNA) and 13 structural genes where ATP is generated involved in oxidative phosphorylation process (305). Each mitochondrion contains several identical copies of mtDNA and has 10-17 times higher mutation rate than other nuclear DNA (306).

The respiratory chain in the mitochondrion is a mtDNA-specific protein complex involved in the mitochondrial function, its deficiency may lead to variety of diseases, these respiratory chains include; complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome C oxidoreductase), complex IV (cytochrome C oxidase) the main mtDNA primer used in this study and the last step required to for oxidation phosphorylation forming complex V (ATP synthase) (307).

3.2.3 Mitochondrial DNA related diseases

Previously mitochondrial diseases were wrongly thought to be rare, but identification of the mitochondrial genome by sequencing mtDNA and discovering the pathological mtDNA mutation, have shown this can affect any body organs (308). Mitochondrial diseases have become one of the important common specific genetic disorders nationally (309) and highly related to the abnormality in mtDNA and its mutation (310). The mtDNA mutation can affect either mitochondrial protein synthesis or respiratory chain proteins (309).

There are different diseases developed in relation to particular mitochondrial gene mutation, these include:

- **Cancers:** Some cancers linked to mtDNA mutation potentially increase cell proliferation in response to increase in reactive oxygen species (ROS) production leading to cancer such as colorectal, pulmonary, breast and prostate cancer (311-314).
- **Cyclic vomiting syndrome:** Whereby changes in mtDNA disabling the mitochondria to produce energy (315).
- **Cytochrome C oxidase (COX) or complex VI deficiency:** COX is responsible for creating ATP in the last step of the oxidative phosphorylation, which may cause cell death of tissue energy dependent as the brain if COX deficiency (316).
- **Complex III deficiency:** Lack of protein cytochrome B due to mutation in mtDNA gene leading to muscle weakness but also can affect other organs (317-319).
- **Miscellaneous mtDNA mutation disorders:** chronic progressive external ophthalmoplegia and Kearns-Sayre syndrome due to mtDNA rearrangement mutation (duplication or deletion respectively), Leber hereditary optic neuropathy and neurogenic weakness due to single nucleotide variant mutation, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) due to tRNA gene mutation and aminoglycoside-induced deafness due to rRNA gene mutation (304, 309, 320-323).

3.2.4 Cross-talk between mtDNA and innate immunity

The mtDNA is similar to bacterial DNA (bDNA) in structure and share its unmethylated CpG motifs which is a potent innate immune inducer (324). The bDNA is known microbial pathogen-associated molecular pattern (PAMPs) to pattern recognition receptors (PRR) including toll-like receptors (TLRs). TLRs signalled by many PAMPs, TLR9 in particular respond to bDNA as its known ligand (324-326).

Signalling TLR9 activates the inflammatory cascade through MyD88 pathway, activating innate immune response with production of pro-inflammatory cytokines (239, 327). Similarly, mtDNA is a DAMP, which when released into the circulation it will potentially signal TLR9 following the same signalling pathway, activating the systemic inflammatory response with downstream release of pro-inflammatory cytokines (158) (Figure 3.2).

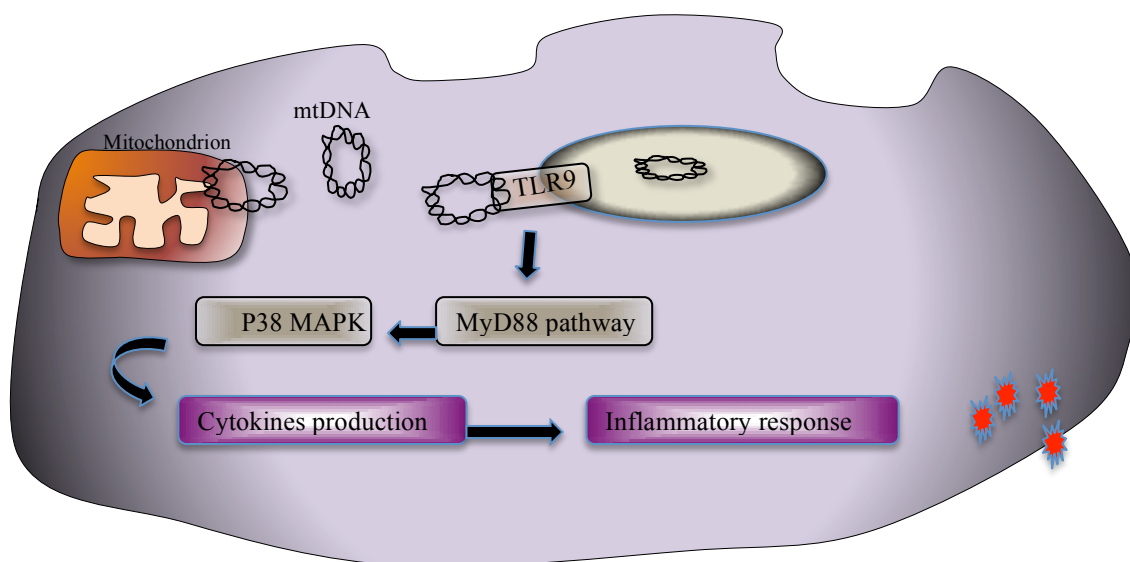


Figure 3.2 Mitochondrial DNA, a ligand for TLR9

During cell injury, mtDNA is recognized as DAMPs and when released into circulation it becomes ligand for TLR9 which binds to it leading to its signalling which in turn through the MyD88 pathway it will activate PMN p38 MAPK and other cytokines inducing an inflammatory response.

3.3 Hypothesis

- We hypothesize here that cell injury with spillage of the mitochondrial DAMPs following cardiac surgery trauma has a role in systemic inflammatory response, this involves the release of mtDNA promoting inflammatory activation by signaling through TLRs.
- Coronary artery bypass grafting surgery has potential to release more mtDNA when using the cardiopulmonary bypass machine with subsequent initiation of a sterile systemic inflammatory response via the release mitochondrial DAMPs into the circulation.

3.4 Material and methods

In order to study the significance difference between all the study variables, we developed a study model that enabled us to focus on physiological inflammatory changes in relation to duration of CPB according to a specific time points. We aimed for the similarity between the study groups to rule out any difference that could affect the study outcome.

All studies were performed in compliance with the institutional review board of Imperial College and the Imperial Healthcare NHS Trust. Collection of human peripheral blood samples and the other studies were reviewed and approved by the institutional review board and the ethical committee granted the ethical approval (Reference 08/H0708/67).

3.4.1 Patients selection

3.4.1.1 Inclusion criteria

The main groups of patients involved in this study are those with known ischemic heart disease undergoing elective CABG either on or off-pump operated at Hammersmith Hospital, London, UK (Table 3.1). The control groups consist of patients with aortic valve pathology with normal coronary angiogram undergoing aortic valve replacement (AVR) also operated at Hammersmith Hospital and healthy volunteers. The age of the patients were between 60-80 years old to avoid variation in body physiology that could affect the outcome due to age difference. However, the healthy volunteers were much younger (mean age 34 ± 6 years).

We recruited 66 patients received CABG, 44 patients of those received CABG with standard CPB (mean age 64 ± 11 years) and 22 patients received CABG without CPB “off-pump” (OPCAB) (mean age 64 ± 7 years). Baseline samples from 25 patients undergoing AVR surgery (mean age 71 ± 5 years) and 10 healthy individuals (mean age 34 ± 6 years) were studied as control groups.

3.4.1.2 Exclusion criteria

We excluded those patients who did not consent or unable to consent. Additionally, any patients received combined cardiac surgical procedure other than isolated CABG (e.g. CABG and valve surgery), patients with suspected or proven infection, emergency CABG, redo CABG or redo sternotomy, high risk group and those who had unstable angina or myocardial infarction within six weeks before the surgical intervention, any pre-existing peripheral vascular diseases, liver or renal diseases and any recent neurological abnormality to eliminate any significant difference between the groups.

Table of patient's demographics

Variables	Group 1 (n=44) CABG-on pump	Group 2 (n=22) OPCAB	P-value
Age	64 ± 11	64 ± 7	0.72
Sex	M = 35 (79.5%) F = 9 (20.5%)	M = 18 (82%) F = 4 (18%)	0.55
BMI	29 ± 6	29 ± 4	0.76
Ejection fraction (%)	53.4 ± 8.4	54.1 ± 8.9	0.45
Logistic Euroscore (%)	3.47 ± 2.84	2.91 ± 4.61	0.9
No. of coronary diseases:			
1	1 (2.3%)	3 (14%)	0.11
2	16 (36%)	8 (36%)	
3	25 (57%)	10 (45%)	
4	2 (4.5%)	1 (4.5%)	
ANGINA:			
CCS 0	3 (6.8%)	2 (9%)	0.34
CCS I	24 (54.5%)	15 (68%)	
CCS II	16 (36%)	4 (18%)	
CCS III	1 (2.3%)	1 (4.5%)	
CCS IV	0 (0%)	0 (0%)	
Previous MI	17 (39%)	11 (50%)	0.1
DM: No DM	21 (48%)	6 (27%)	0.11
Diet control	7 (16%)	7 (32%)	
Oral therapy	12 (27%)	8 (36%)	
Insulin therapy	4 (9%)	1 (4.5%)	
HT	41 (93%)	20 (91%)	0.54
Hypercholesterolemia	44(100%)	22(100%)	0.33
NYHA: Class I	9 (20.5%)	5 (23%)	0.19
Class II	23 (52%)	11 (50%)	
Class III	10 (23%)	5 (23%)	
Class IV	2 (4.5%)	1 (4.5%)	
Pulmonary disease	4 (9%)	4 (18%)	0.16
Renal disease	0 (0%)	0 (0%)	NA
Liver disease	0 (0%)	0 (0%)	NA
Neurological disease	0 (0%)	0 (0%)	NA
Peripheral vascular diseases	5 (11.4%)	3 (13.6)	0.22
Heart Rhythm: SR	42 (97.7%)	20 (91%)	0.47
AF	1 (2.3%)	1 (4.5%)	
Others	0 (0%)	0 (0%)	
Smoking History:			
Never smoked	16 (36%)	8 (36%)	0.5
Ex-smoker	20 (45%)	12 (54.5%)	
Current smoker	8 (18%)	2 (9%)	

Table 3.1 Demographic of patients undergoing CABG

The preoperative and postoperative demographic data for patients underwent CABG utilizing CPB (group 1, n=44) and CABG without utilizing CPB (group 2, n=22). Data showed absolute value, SD and percentage. Significant statistical difference if $p < 0.05$.

3.4.2 Study design

3.4.2.1 Anaesthetic parameters

All patients recruited received similar anaesthetic protocol; anaesthesia was induced with 1-3mg/kg thiopentone and 3-5mg/kg fentanyl followed by propofol infusion (3mg/kg/hour) as maintenance in addition to intermittent muscle relaxant pancuronium bromide 0.1mg/kg. Sodium heparin anticoagulation was given immediately after harvesting the left internal mammary artery (LIMA) and prior to cannulation for CPB group at dose of 3mg/kg aiming for active clotting time (ACT) above 480 seconds and supplemented regularly according to ACT results. Half dose of the sodium heparin was given for the off-pump group aiming for ACT around 300 seconds.

3.4.2.2 CPB circuit

Standard conventional CPB circuit was used in all on-pump patients, with non-heparin bonded coated circuit, roller pump and membrane oxygenator. Arterial cannulation performed through ascending aorta and venous cannulation performed through right atrium. ACT was maintained at 480 seconds or above, ACT was checked every 20-30 minutes with supplemented sodium heparin when required. Heparin was reversed at the end of CPB by protamine sulphate. Postoperative blood loss was measured from heparin reversal time and until removal of the chest drains.

3.4.2.3 Blood sampling (time-points)

Venous blood samples (5 ml) were taken at different time-points from the admission of the patients until 24 hours from the initiation of the CPB and with corresponding time-points to OPCAB surgery. Blood was taken pre-operatively on admission, at the time of anaesthetic induction for both groups, after sternotomy at commencement of CPB in group 1, which correspond to 45 minutes after sternotomy in OPCAB group 2.

Subsequently sampling then was taken from both groups at varying time points in relation to the initiation of CPB (CPB0) at 30 (CPB30), 60 (CPB60), 120 (CPB120), 300 minutes (CPB300) respectively and after 24 hours (CPB24) (Figure 3.3). Pre operatively and at anaesthetic induction blood sampling was taken peripherally, afterward samples were taken via the internal jugular central line.

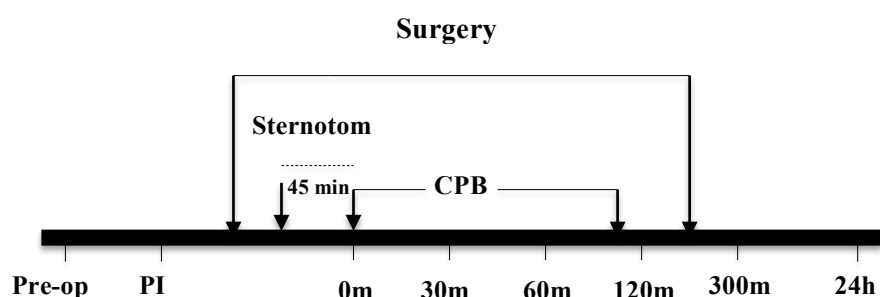


Figure 3.3 The study design

Blood sampling was taken on admission, at anaesthetic induction, on commencement of CPB (CPB0) in group 1 and 45 minutes after sternotomy in OPCAB group 2 (equivalent estimated time to initiate CPB in group 1), then both groups at varying time points in relation to CPB0 at 30, 60, 120 & 300 minutes respectively followed by after 24 hours.

3.4.2.4 Plasma separation

All samples were collected in heparin filled tube (EDTA), immediately they were centrifuged at 6000x g for 10 minutes. Plasma was collected in 2ml polypropylene tubes and stored at -80°C for later DNA extraction.

3.4.2.5 Purification of mtDNA

3.4.2.5.1 From cultured cells

DNA was purified from Human umbilical vein endothelial cell (HUVEC) using specific kit (DNeasy Blood & tissue kit QIAGEN). Cells were collected by centrifugation at 300x g for 5 min in a 1.5 ml sample tube at room temperature (15-25°C); the supernatant was then discarded taking care not to disturb the cell pellet.

Cells were stored at -80°C for DNA extraction later using the above kit. Extraction of DNA was performed using: “Purification of Total DNA from Animal Blood or Cells (Spin-Column) Protocol”, the steps were performed according to manufacturer guidelines. Once the DNA extracted it was used as a positive control.

3.4.2.5.2 From plasma

For purification of total mitochondrial DNA from plasma we used: DNA Purification from Blood or Body Fluids Spin Protocol, all centrifugation steps were carried out at room temperature ($15-25^{\circ}\text{C}$) using (QIAamp DNA Blood Mini Kit-from Qiagen®). Each sample contains 200 μl of plasma; the extracted DNA was double eluted with 50 μl of buffer AE to yield more concentrated DNA. The steps of purification were done according to the manufacturer guidelines. To detect DNA purity for any protein contamination, the $A_{260/280}$ ratio of the DNA was tested with spectrophotometer (NanoDrop®) and was between 1.7-1.9 excluding any contamination.

3.4.2.6 Quantitative PCR

To enable us to detect and quantify the extracted targeted DNA molecule, we used Quantitative real-time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) or kinetic polymerase chain reaction (KPCR). qPCR can detect the quantity either as an absolute number of copies or as a relative amount when normalized to DNA input or additional normalizing genes (328). Quantitative PCR using specific primers was used to measure mtDNA and genomic DNA levels. The reaction was prepared using 1 μl of DNA in each tested well plus 12.5 μl of SYBR® Green PCR Master Mix (From Life-Technology), 0.5 μl of mtDNA primer and 10.5 μl of water.

The mitochondrial primers used include human cytochrome B (Forward 5'-ATGACCCCAATACGCAAAAT-3', Reverse 5'-CGAAGTTTCATCATGC GGAG-3'), human cytochrome C oxidase subunit III (forward 5'-ATGACCCACCAATCACATGC-3', Reverse 5'-ATCACATGGCTAGGCCGGAG-3') and human NADH dehydrogenase (forward 5'-ATACCCATGGCCAACCTCCT-3' , Reverse 5'-GGGCCTTTGCGTAGTTGTAT-3'). We used water as a negative control and mtDNA extracted from HUVEC as a positive control.

3.4.3 Statistical analysis

We have used some other studies utilizing the CPB as guide using power calculator, this gave us adequate of sample size of 52 based on anticipated baseline incidence and type I/II error rate. We calculated the mean and standard deviation for each time point in the study and we tested the homogeneity of variance for equal variances across samples using Levene test to verify that assumption. An intergroup analysis between the CPB time points used the Mann-Whitney *U* test, in accordance with the nonparametric nature of the data.

Similarly, statistical analysis was performed to enable comparisons between the groups (off and on pump CABG). Variation in circulating mtDNA was measured repeatedly. These measures assumed to be related to baseline samples and different CPB/OPCAB related time points. Models used in this analysis were Bonferroni correction for multiple comparisons of the CPB and CPB related time points. We also used ANOVA test to assess the explanatory variables when comparing between and within the groups in a random effect model. Statistical Package for the Social Sciences (SPSS) version 20 software package (IBM Corporation, Armonk, NY, USA). Differences were considered statistically significant when p-value less than 0.05.

3.5 Results

The results showed no significant difference in the early postoperative outcome between both groups (CABG and OPCAB) as shown in table 3.2

Variables	Group 1 (n=44) CABG-on pump	Group 2 (n=22) OPCAB	P-value
Age	64 ± 11	64 ± 7	0.72
Sex	M = 35 (79.5%) F = 9 (20.5%)	M = 18 (82%) F = 4 (18%)	0.55
CPB time	73.6 ± 20.1	NA	0.000
Number of coronary grafts:			
CABG X1	0 (0%)	3 (14%)	0.45
CABG X2	19 (43%)	8 (36%)	
CABG X3	23 (52%)	10 (45%)	
CABG X4	2 (4.5%)	1 (4.5%)	
Post operative drainage	1081 ± 578	856 ± 390	0.11
Blood transfusion:			
0 unit	29 (66%)	16 (73%)	0.24
1 unit	2 (4.5%)	2 (9%)	
2 units	7 (16%)	3 (14%)	
3 units	2 (4.5%)	0 (0%)	
4 units	3 (6.8%)	0 (0%)	
7 units	1 (2.3%)	0 (0%)	
Length of ITU stay (days)	1	1	0.11
Length of hospital stay (days)	10 ± 7	7 ± 2	0.13
Post-operative complication:			
AF	10 (22.7%)	6 (27.3%)	0.68
Heart block	1 (2.3%)	0 (0%)	0.5
Wound infection	5 (11.4%)	1 (4.5%)	0.34
Chest infection	6 (14%)	4 (18%)	0.43
Renal hemofiltration	4 (9%)	0 (0%)	0.18
Stroke	0 (0%)	0 (0%)	NA
Re-opening	3 (6.8%)	0 (0%)	0.34
IABP	1 (2.3%)	0 (0%)	0.5
Inotropes	11 (25%)	4 (18.2%)	0.36

Table 3.2 Demographic of postoperative data

The postoperative demographic data for patients underwent CABG utilizing CPB (group 1, n=44) and CABG without utilizing CPB (group 2, n=22). Data showed absolute value, SD and percentage. Significant statistical difference if $p < 0.05$.

3.5.1 Level of mtDNA in patients with ischemic heart disease

Comparing samples from young healthy individuals (mean age 34 ± 6 years) to baseline samples from patients with known ischemic heart disease (IHD) (mean age 64 ± 11 years) revealed that the levels of circulating mtDNA were significantly higher in IHD group, approximately 31-fold compared to healthy control group ($p < 0.001$; paired student T test) confirming the significant differences applied between these two age groups.

However, here the comparison was biased, as there were more than one contributing factor including age, IHD and other comorbidity such as diabetes, hypertension, hypercholesterolemia and other age-related issues. Therefore, we selected similar age group with similar comorbidities, this group include those patients undergoing aortic valve replacement (AVR) but without any evidence of IHD and normally reported coronary angiogram (non-IHD group) (mean age 71 ± 8 years).

Interestingly, when comparing all groups together even though there is still significant difference in the circulating mtDNA from baseline samples between the healthy group and the non-IHD group. However, the trend was much less when compared them to the IHD group. Interestingly, the circulating mtDNA was significantly higher in the IHD group compared to the non-IHD group, which is a novel finding ($p < 0.001$; ANOVA), the correlation coefficient between age and mtDNA was 0.5 ($p < 0.001$), and the Beta coefficient of their linear regression was 0.49 ($p < 0.001$) (Figure 3.4).

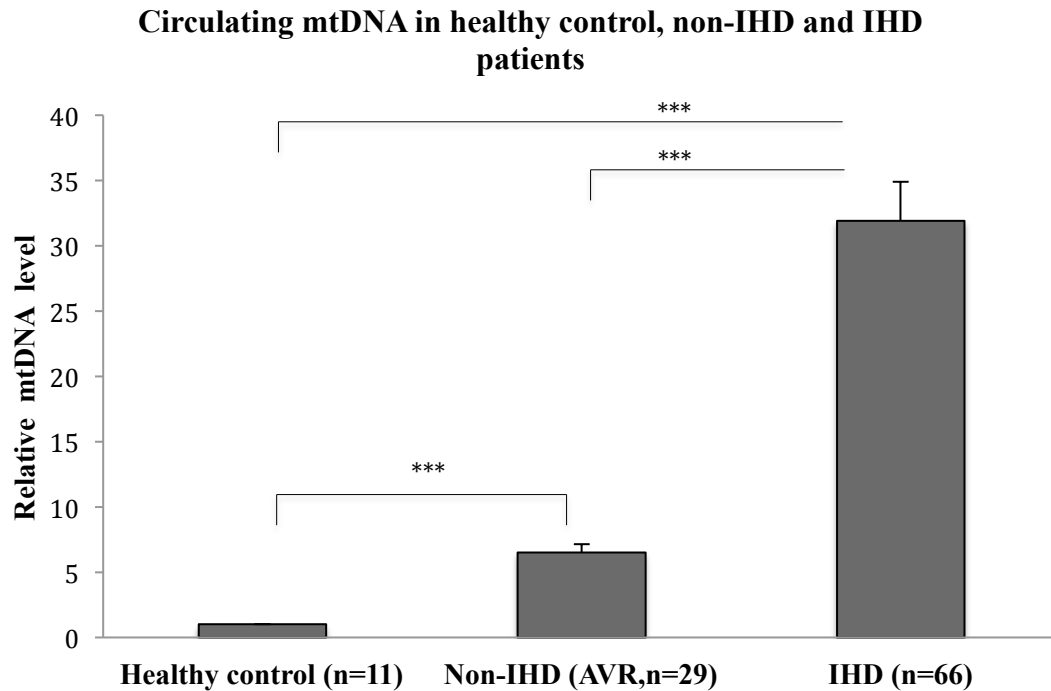


Figure 3.4 Circulating mtDNA in control and test groups

Levels of mtDNA were measured in 10 healthy volunteers (mean age 34 ± 5 years), 66 patients undergoing CABG (mean age 64 ± 11 years) and in 29 patients undergoing AVR (non-IHD, mean age 72 ± 6 years). There were significant differences among the groups, the level of mtDNA in non-IHD and IHD groups were 6.5 and 31.9-folds higher, respectively than the healthy control group ($p < 0.001$), also significantly higher in IHD group compared to non-IHD ($p < 0.001$). (* = $p < 0.05$, ** = $p < 0.01$ & *** = $p < 0.001$).

3.5.2 CABG with CPB enhanced circulating level of mtDNA

The total mean time for the CPB was (73.6 ± 20.1 minutes) and for the full CABG operation from skin incision to skin closure was (190 ± 35 minutes). Analysis with qPCR revealed that CABG with CPB led to changes in the plasma level of mtDNA in certain time-points. The result has shown significant increase in mtDNA circulating level mainly at CPB30, CPB60 and CPB120 minutes in relation to other time points as demonstrated in tables 3.2. These are the time-points where most of the patients remain in CPB machine, which can justify that increase in the mtDNA levels in these particular time-points.

Significant statistical differences in relevant CPB time-points

CPB time-points	mtDNA (Mean $\pm$ SD)	Significant relation to other time-points	P – value
Pre-op	1	CPB0 CPB30 CPB60 CPB120	0.016* 0.000*** 0.000*** 0.000***
Post induction (PI)	1.93 $\pm$ 1.19	CPB30 CPB60 CPB120	0.000*** 0.000*** 0.000***
Start of CPB (CPB0)	5.18 $\pm$ 2.32	Pre-op CPB30 CPB60 CPB120	0.016* 0.000*** 0.000*** 0.000***
CPB after 30 min (CPB30)	41.63 $\pm$ 8.53	Pre-op PI CPB0 CPB120 CPB300 CPB24	0.000*** 0.000*** 0.000*** 0.000*** 0.000*** 0.000***
CPB after 60 min (CPB60)	43.26 $\pm$ 9.17	Pre-op PI CPB0 CPB120 CPB300 CPB24	0.000*** 0.000*** 0.000*** 0.000*** 0.000*** 0.000***
CPB after 120 min (CPB120)	15.18 $\pm$ 8.91	All CPB time-points	0.000***
CPB after 300 min (CPB300)	4.02 $\pm$ 3.14	CPB30 CPB60 CPB120	0.000*** 0.000*** 0.000***
CPB after 24 hours (CPB24)	2.11 $\pm$ 1.25	CPB30 CPB60 CPB120	0.000*** 0.000*** 0.000***

Table 3.3 Data summary for significant differences ($p < 0.05$ or less) in on-pump CABG

There are significant differences among the different time-points in those patients had CABG with CPB, the table shows only the mtDNA level that differs significantly ($p < 0.05$ or less) between the time-points. This analysis confirms that mtDNA release into plasma occurs maximally at 30-60 minutes following commencement of CPB (41.63 $\pm$ 8.53 and 43.26 $\pm$ 9.17 folds from pre-operative level respectively) and gradually declines afterward. (*= $p < 0.05$, **= $p < 0.01$ & ***= $p < 0.001$).

The mtDNA is at its highest level after 60 minutes from CPB (CPB60), which is the longest time-point where the patients remains on by bypass, then starts to decline as the patients starting to come off CPB in the next time-point (CPB120), followed by drop nearly to the level of pre-op after 24 hours indicating the transient effect related to the usage of CPB (Figure 3.5).

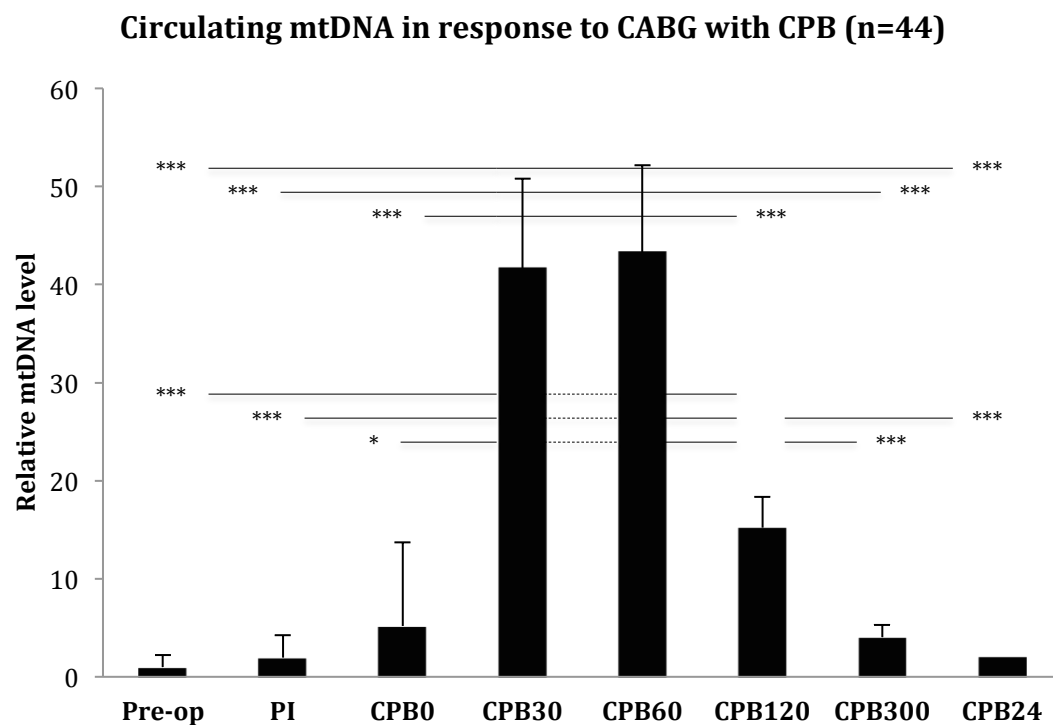


Figure 3.5 Circulating mtDNA level in on-pump CABG

The mtDNA level in 44 patients underwent CABG with CPB; it reveals the transient mode of elevation in response to CPB, the highest peak is at 30-60 minutes (CPB30-CPB60) in comparison to the other time points, (41.63 ± 8.53 and 43.26 ± 9 nearly 17-folds from pre-operative level, respectively) then the level drop in 24 hours (*= $p < 0.05$, **= $p < 0.01$ & ***= $p < 0.001$).

3.5.3 Off-Pump CABG (OPCAB)

The potential effect of absence of CPB on mtDNA release, or whether it was released in response to surgical trauma and stress alone was tested on OPCAB with similar fashion to on-pump group. The difference only at collecting CPB0, which was here

estimated at 45 minutes from sternotomy. The result demonstrates significant transient increase in the circulating mtDNA in response to surgery with similar pattern to the on-pump surgery (Table 3.3).

Significant statistical differences in relevant time-points in OPCAB

CPB time-points	mtDNA (Mean $\pm$ SD)	Significant relation to other equivalent (eq) time-points	P – value
Pre-op	1	CPB0eq CPB30eq CPB60eq CPB120eq CPB300eq	0.000*** 0.000*** 0.000*** 0.000*** 0.001 **
Post induction (PI)	2.17 $\pm$ 1.10	CPB30eq CPB60eq CPB120eq CPB300eq	0.013* 0.000*** 0.000*** 0.015*
CPB0eq= (Equivalent to 45 min from sternotomy)	3.48 $\pm$ 1.06	Pre-op PI CPB24eq	0.000*** 0.013* 0.000***
CPB30 equivalent	4.22 $\pm$ 1.45	Pre-op PI CPB300eq CPB24eq	0.000*** 0.000*** 0.000*** 0.000***
CPB60 equivalent	3.93 $\pm$ 1.54	Pre-op PI CPB300eq CPB24eq	0.000*** 0.000*** 0.007** 0.000***
CPB120 equivalent	3.46 $\pm$ 1.64	Pre-op PI CPB24eq	0.000*** 0.015* 0.000***
CPB300 equivalent	2.56 $\pm$ 1.15	Pre-op CPB30eq CPB60eq	0.001** 0.000*** 0.007**
CPB24 equivalent	1.50 $\pm$ 1.00	CPB0eq CPB30eq CPB60eq CPB120eq	0.000*** 0.000*** 0.000*** 0.000***

Table 3.4 Data summary for significant differences ($p < 0.05$ or less) in OPCAB

There were significant differences among the different time-points in OPCAB. The table shows only the mtDNA level that differs significantly ($p < 0.05$) between the time-points. Plasma mtDNA level peaked at 75 minutes from sternotomy, which is equivalent to CPB30 (3.92 $\pm$ 1.53 fold from the pre-operative level) and gradually declines, lowest after 24 hours (*= $p < 0.05$, **= $p < 0.01$ & ***= $p < 0.001$).

There was similar transient pattern in the elevation of the mtDNA level to surgical trauma alone in the OPCAB group. However, the magnitude of this elevation was much less than the CABG utilising CPB circuit (Figure 3.6).

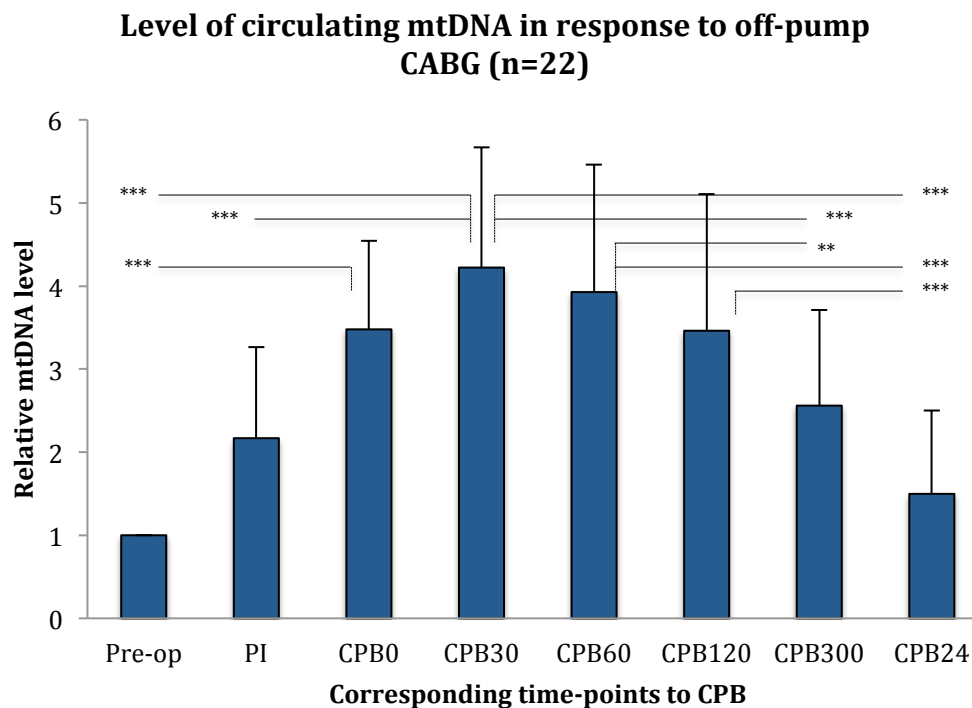


Figure 3.6 Circulating mtDNA level in off-pump CABG

mtDNA level measured in 22 patients underwent OPCAB; it reveals the transient mode of elevation in response to surgical trauma, the highest peak is at 30 minutes (4.22 ± 1.45 fold from the pre-operative level) in comparison to the other time points, then the level drop in 24 hours (*= $p < 0.05$, **= $p < 0.01$ & ***= $p < 0.001$).

3.5.4 Comparison between mtDNA in on-pump CABG & OPCAB

Even though the pattern of the enhancement in circulating mtDNA level was nearly similar. However, it was clear that the amount of mtDNA released was more related to the effect of the CPB other than the surgical trauma it self even though it is still statistically significant in both group as compared to the base line.

When circulating mtDNA level was compared between the on-pump and the off-pump groups, the results showed significant increase in mtDNA level when utilized CPB. This elevation in on-pump CABG vs. OPCAB was as follows: CPB0 (4.85 ± 3.48 vs. 3.48 ± 1.06 -fold), CPB30 (41.63 ± 8.53 vs. 4.22 ± 1.45 -fold), CPB60 (43.26 ± 9.17 vs. 3.93 ± 1.54 -fold), CPB120 (15.18 ± 8.91 vs. 3.46 ± 1.6 -fold), CPB300 (4.02 ± 3.14 vs. 2.56 ± 1.15 -fold), which is highly significant at all times peaking at (CPB30, CPB60 & CPB120. $p < 0.001$). These effects remain significant even after 24 hours ($p < 0.05$) (Figure 3.7).

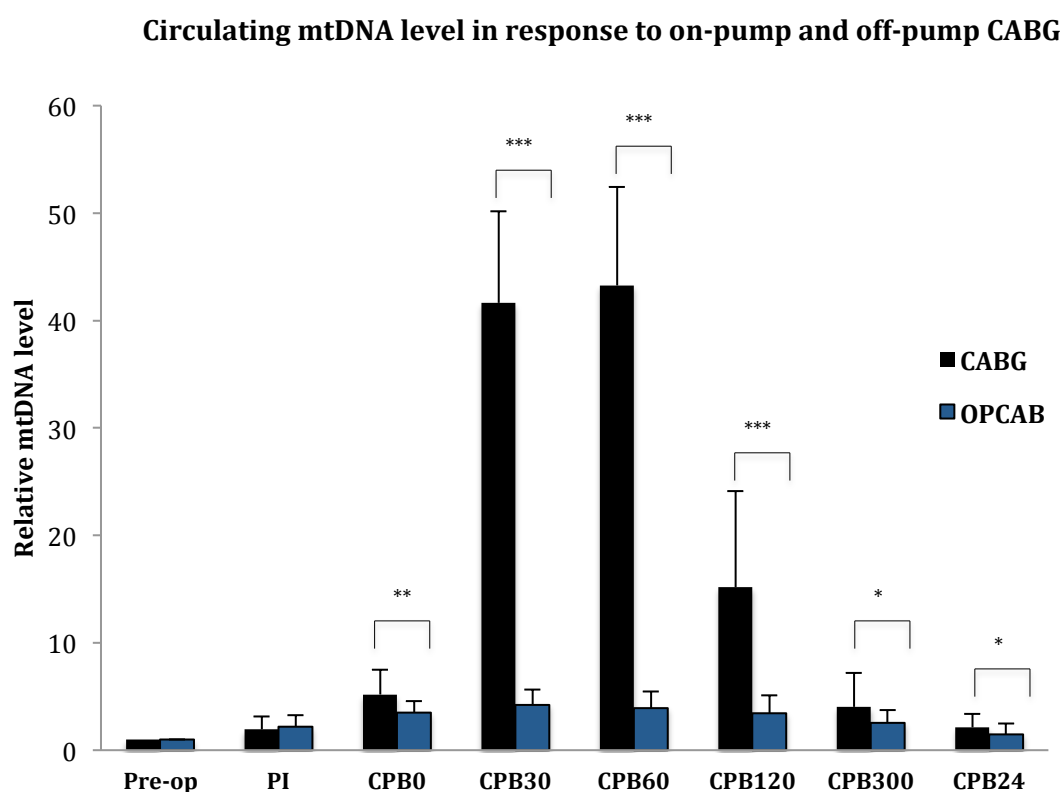


Figure 3.7 Data comparison between on and off-pump CABG

Circulating mtDNA level was compared between the on-pump (CABG) and the off-pump CABG (OPCAB) groups ($n=44$ and $n=22$ respectively); the comparison has revealed the significant increase in the mtDNA when utilizing CPB, which is statistically highly significant at its peak time (CPB30, CPB60 & CPB120. $P < 0.001$), the effect still remain high after 24 hours of CPB but less significant ($* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$).

3.5.5 CPB did not influence levels of circulating genomic DNA

Despite following the steps of isolation of the mtDNA from plasma according to manufacturer protocol and to eliminate any doubt of nuclear genomic DNA contamination to ensure mtDNA purity; we used mitochondrial genes cytochrome B, cytochrome C and NADPH dehydrogenase to detect mtDNA and used GAPDH and B-actin to detect any nuclear genomic DNA. We used the highest four plasma samples eluted the highest mtDNA from four different patients to measure genomic DNA using all mentioned genes above. The result of this experiment observed that CABG with CPB had little or no effect on genomic DNA level in plasma (calculated less than 0.2% of total mtDNA detected by qPCR) compared to mtDNA (Figure 3.8).

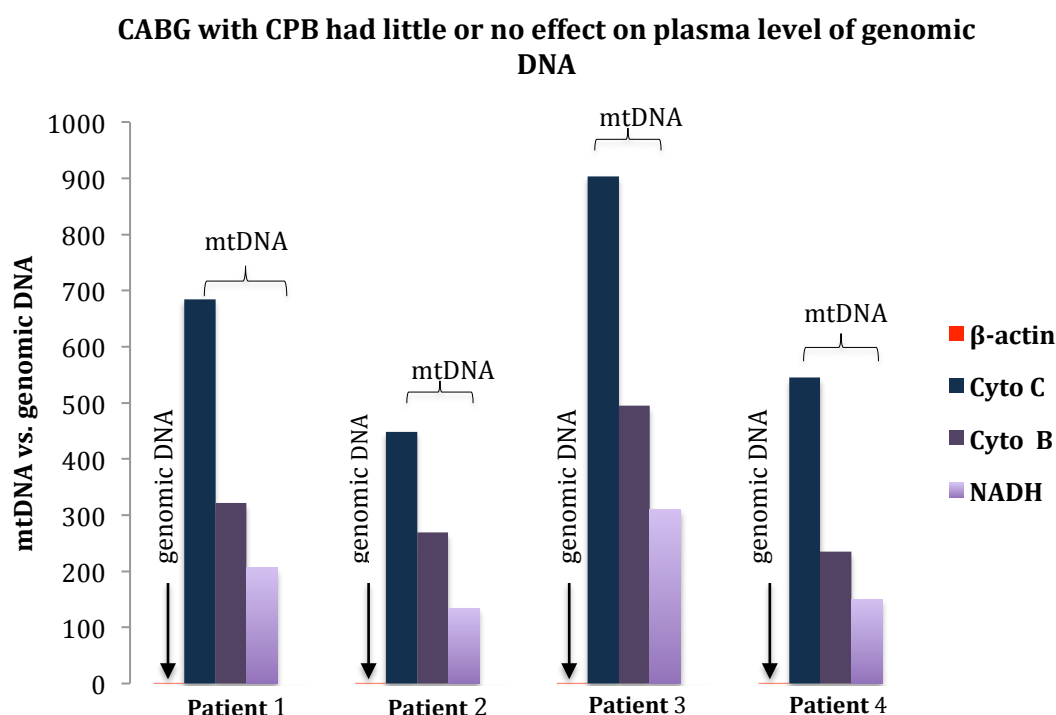


Figure 3.8 Genomic DNA has no effect on CPB

Four samples with highest level of mtDNA were tested in parallel with genomic DNA, cytochrome oxidase C (Cyto C), cytochrome oxidase B (Cyto B) and NADPH dehydrogenase (NADH) were used as specific primers for mtDNA. β -actin used as primer for genomic DNA; the results showed very low level of genomic DNA indicating that CABG with CPB had little or no effect on the release of genomic DNA in plasma while it has significant effect on mtDNA release.

3.6 Discussion

Toll-like receptors (TLRs) are specific receptors that have an important role in innate immune system. They are part of pathogen recognition receptors (PRR), which are able to recognise pathogen associated molecular pattern (PAMPs) or non pathogenic endogenous “danger” signals materials known as damage associated molecular patten (DAMPs). These are released by dead or injured cells leading to cytokine production.

Cytokines produced through different stimulatory pro-inflammatory pathways (149, 166, 329). There are different types of DAMPs that can act as a ligands binding to a specific receptor leading to its signalling and activation contributing to inflammatory response with potential SIRS development following a different types of an injury (Table 3.4).

DAMPs	Receptors
Protein DAMPs	
Heat shock protein 70 (HSP70)	Triggering receptor expressed on myeloid cell 1 (TREM-1) & TLR4 (330)
High mobility group box 1 (HMGB1)	Receptor for advanced glycation end product (RAGE), TLR2 & TLR4 (159, 331).
Serum amyloid A (SAA)	TLR2, TLR4 (332, 333).
S100	RAGE (334).
Non-protein DAMPs	
ATP (nucleotides)	P2XR, P2YR (335, 336).
Uric acid	NALP inflammasome (337).
mtDNA	TLR9 (158, 299, 302, 338).

Table 3.5 Types of DAMPs and their recognised receptors

In this study we demonstrate the variations in the levels of circulating mtDNA in response to CABG and the utilization of the CPB circuit as well as in response to each specific CPB time-point. For the mitochondrial DNA to reach the circulation, it will need to leak from the mitochondrion due to cell injury or death (300), which is through its specific signalling pathway will initiate the inflammatory response as part of defence mechanism. Therefore, the more circulating mtDNA level, the higher the cells damage. This damage can be related to either mechanical as in trauma (158), ischemic-reperfusion injury or any kind of injury including the one related to CPB, or also can be related to the ageing cell and apoptosis (339, 340).

When comparing younger age group to the other groups. Our finding demonstrated that being young and healthy would have less potential for cell injury and less impact on leakage of the mtDNA from the mitochondrion in the absence of mechanical insult. However this could be related to the theory by which ageing may cause apoptosis deregulation, a process important for tissue haemostasis where the mitochondria plays an important role in its regulation through cytochrome C as one of the mitochondrial pro-apoptotic factors (341, 342). The theory links ageing to apoptosis deregulation with oxidative stress, mitochondrial ROS production and mitochondrial disruption with release and accumulation of mtDNA (343, 344). Therefore, providing another comparison with similar age group undergoing AVR was logical especially when they share similar morbidity and environment.

In comparing the IHD group with the non-IHD group, we explore for the first time the novel finding of the direct relationship between the increase in the level of circulating mtDNA in the blood and the extent of the ischemic heart disease. This can be related to

the fact that atherosclerosis is an important contributing factor to IHD and also inflammation can induce atherosclerosis in IHD (345).

Mitochondrial DNA is an important mitochondrial DAMPs during injury. It acts as a ligand for TLR9 (158, 299, 302, 338). Activation of TLR9 can initiate the systemic inflammatory response through MyD88 pathway leading to production of the pro-inflammatory cytokines (239, 327). Our results showed clearly that coronary revascularization surgery using CPB circuit transiently increases the circulating mtDNA, which peaked at 30-60 minutes following commencement of CPB. Here we can argue that, this elevation in mtDNA may activate signalling of TLR9 following CABG with CPB, which can potentially play an important role in initiation of systemic inflammatory response leading to subsequent CPB related complications.

To ensure purity of mtDNA and to exclude any contamination of undetected nuclear genomic DNA which is genetically differs from mtDNA (346). We tested the samples contains the highest level of mtDNA for the presence of any genomic DNA. The results showed significant elevation of the circulating mtDNA level in these samples compared to genomic DNA confirming purity of mtDNA detected ($p<0.001$)(Figure 4.8). Absence of genomic DNA can be explained by understanding that during cell injury in relation to CPB, injured mitochondria will release its mtDNA into cytoplasm, the mtDNA is smaller and larger in number than genomic DNA that is contained in the nucleus.

In the OPCAB surgery results, there was increase in mtDNA in different correlated time-points to CPB (Figure 3.6). However, even though the difference was significant but the trend was much lower than with CPB. This increase mainly related to the surgical trauma it self as we provisionally hypothesized.

When we compared both groups together, there were markedly significant differences between each time-point (Figure 3.7). Here it becomes apparent that mtDNA release is far more strongly related to CPB than surgical trauma, and the CPB with blood component exposure to the artificial surfaces could have a direct effect on the release of the mtDNA with subsequent activation of the inflammatory cascade.

Taken together, the elevation of mtDNA has potential to signal TLR9 leading to initiation of inflammatory response. Our findings give us a valuable clinical relevance in accordance to the potential direct effect of CPB on enhancing the mtDNA level in cardiac surgery. Clinically we could suggest that minimal invasive surgery and OPCAB may attenuate the inflammatory response. Moreover, using TLR9 antagonist may have potential effect in attenuating systemic inflammation.

4 CHAPTER IV: Elucidating The Role Of TLR9 In The Systemic Inflammatory Response Due To Cardiac Surgery Trauma And Cardiopulmonary Bypass - The Animal Model

4.1 Abstract

Background: Major surgical trauma and cardiopulmonary bypass (CPB) in cardiac surgery can lead to a spectrum of post-operative end-organ complications due to the activation of systemic inflammatory response.

Aim: The study tested the hypothesis that surgical trauma or CPB initiates a systemic inflammatory response via the release of mitochondrial DNA (mtDNA) signalling the Toll-like receptors 9 (TLR9) and interleukin-6 (IL-6) production as a downstream activation of TLR9.

Methods: We studied small and large animal models at different types of the cardiac surgical interventions such as: sternotomy, CPB and ischemia-reperfusion (IR) injury. The mtDNA and IL-6 were measured with and without TLR9-antagonists. To study IR injury, we utilized ex-vivo porcine kidney model. mtDNA and IL-6 were assessed by quantitative PCR using their specific gene primers. Statistical comparison of two or multiple groups was undertaken accordingly. We validated the downstream signalling of TLR9 by measuring IL-6 in HUVEC samples treated with increasing dose of TLR9-antagonist IRS954 and fixed dose of TLR9-agonist ODN1668.

Results: The IL-6 expression in the HUVEC was inversely related to the TLR9-antagonist concentrations ($P=0.1$ to 0.003) in comparison to TLR9-agonist alone. In the rodent model, the circulating mtDNA level (mean $\pm$ SE) significantly increased to 19 fold (19.29 ± 3.31) of pre-sternotomy ($P < 0.001$) at 1-minute then dropped nearly to half at 10-minutes (9.79 ± 1.61 , $P < 0.001$). Tissue mtDNA in heart, kidney, liver and lung was significantly higher than plasma mtDNA at 10-minutes, P -value < 0.001 for all. In the murine model, the IL-6 in the TLR9-antagonist group was significantly lower than the controls at sternotomy (59.06 ± 14.98 vs. 5.25 ± 1.08 , $P = 0.01$). In the porcine model, the CPB group had significantly higher level of circulating mtDNA at the end of CPB than the sham group (29.89 ± 4.78 vs. 2.4 ± 0.42 , $P < 0.001$). In the ex-vivo IR model, there was significant increase in the mtDNA ($p = 0.001-0.007$) and in IL-6 ($p < 0.001-0.05$) at all time-points of reperfusion in relation to pre-reperfusion level.

Conclusion: CPB and to the less extent the surgical incision trauma precipitate the release of circulating mtDNA and subsequently the release of IL-6, which may contribute to the initiation of systemic inflammatory response.

4.2 Introduction

Cardiac surgery with the use of cardiopulmonary bypass (CPB) has been associated with systemic inflammatory response syndrome (SIRS); this further can subsequently upsurge the risk of morbidity and mortality. SIRS development is multi factorial, can be due to exposure of circulating blood to artificial surfaces of the extracorporeal circuit, non-physiological shear stress of the CPB roller pump machine and the associated hypothermia (83, 87), and a potentially inevitable ischemia-reperfusion (IR) injury.

These factors may all contribute in inducing the activation of a series of inflammatory cascades (84). This inflammatory response could be initiated and exacerbated by significant tissue injury incurred during CPB, and worsened by the release of endogenous inflammatory mediators. This pathophysiological condition results in a hyper-dynamic circulation, with an increase in the cardiac output and decrease in the systemic vascular resistance, prompting the need for inotropic and vasoconstrictive support (347).

Despite several significant improvements in the CPB circuit, the complications secondary to tissue damage and SIRS still remain and can have a profound impact on post-operative outcomes (348). Miniaturized CPB circuits have been used with some success, especially in congenital cardiac surgery, with the concept being minimization of contact between the circulating volume and artificial surfaces in order to reduce SIRS and the change in the microcirculation (349-351).

The magnitude of the inflammatory response varies but it is still potentially harmful nonetheless. The inflammatory response can be initiated through signalling the TLRs as first-line in the defense against pathogens, recognizing both PAMPs and DAMPs,

playing a significant role in inflammation, cell immune regulation and proliferation (165-168).

TLR9 in particular is an intracellular endosomal homo-dimer receptor, recognizing unmethylated CpG sequences in DNA molecules, which is a hallmark of viral and bacterial genomes (239). Sources of DAMPs in sterile inflammation can be found in the mitochondrion, specifically the mitochondrial DNA (mtDNA), which also contains CpG-DNA repeats (154, 158). During mechanical injury or ischemia, in which the processes of necrosis or apoptosis occur, mitochondrial damage leads to the leak of mitochondrial contents into the cytosol and then the extracellular space, and ultimately into the bloodstream (158, 313, 352, 353). This sequence leads to the potential signalling of TLR9.

Here in this study we investigated for the first time the relationship between potential TLR9 signalling in cardiac surgery with CPB and the systemic inflammatory response. We hypothesize that due to the traumatic nature of cardiac surgery there is a great potential for the induction of SIRS. This trauma can arise from the operative procedure itself, the effects of CPB, or the IR injury to the tissues. Mitochondrial DAMPs (mtDNA) are the main contributors in initiating sterile systemic inflammatory response when signalling the TLR9.

4.3 Material & methods

We aimed to identify the relationship between the surgical trauma, CPB and/or IR-injury, and the acute sterile systemic inflammatory response, measuring the downstream activation of the TLR9. We have used small and large animal models in our experiments. In the rat and mice model we performed midline sternotomy, mimicking the surgical approach in human cardiac surgery, in the pig model we performed midline sternotomy and initiated CPB, using the same CPB circuit used in human. Finally to study the effect of tissue IR-injury we collaborated with the transplant team at Imperial College London, performing ex-vivo renal IR-injury models in pigs.

4.3.1 Validating the downstream of TLR9 signalling

We prepared cultured human umbilical cord vein endothelial cells (HUVEC) following aseptic technique and good cell culture practice. All HUVEC experimental settings were done in triplicates. In order to test the effect of the TLR9-agonist oligodeoxynucleotides (ODN-1668) and TLR9-antagonist oligodeoxynucleotides with immunoregulatory sequences (IRS-954).

We prepared a complete media then added to the passage and incubated for 24 hours. HUVEC were then initially treated with TLR9-agonist at different concentrations and time points (1,2 & 4 hours) in order to identify the appropriate dose to be utilized to stimulate the cell's TLR9. We optimized at a dose of 1 μ m of ODN-1668. New sets of cells were pre-incubated with different doses of TLR9-antagonists (IRS-954) for one hour with concentrations of 2 μ m, 4 μ m, 6 μ m and 8 μ m as recommended by InvivoGen® to use concentration from 1:1 to 10:1 antagonist:agonist ratio. This was followed by stimulation with 1 μ m ODN-1668 for 1 hour. At the end of the incubation

period the culture media was collected. RNA was extracted from each set of cells using RNeasy mini Kit (Qiagen®) according to the manufacturer's instruction protocol. cDNA was then formed by reverse transcription PCR, followed by quantitative real-time PCR for IL-6 gene expression as a downstream activation for TLR9 signalling (173, 354, 355).

4.3.2 Animal models

All animals received humane care in compliance with the Principles of Laboratory Animal Care and according to UK Home Office regulations.

4.3.2.1 Rodent model

Adult male Sprague–Dawley rats (n=15) (400-450g) were anaesthetized using IP ketamine (50mg/kg)/xylazine (2mg/kg), tracheostomy and endotracheal intubation with 14G cannula and mechanical ventilation for 15-20 minutes. To detect the circulating level of the mtDNA through mimicking the cardiac surgical access, we performed midline sternotomy.

Tail vein blood sample was collected at pre-sternotomy, 1 minute and 10 minutes after procedure. Extraction of DNA was performed from 200µl of plasma using QIAamp DNA Blood Mini kit (Qiagen®) as per the manufacturer's protocol. At the end of the experiment we harvested heart, lung, kidneys and liver. DNA was extracted from the tissues using DNeasy Blood & tissue kit (Qiagen®). Rat mitochondrial gene cytochrome C oxidase subunit III (forward 5'-ACATACCAAGGCCACCAAC-3', Reverse 5'-CAGAAAAATCCGGCAAAGAA-3') was used as mitochondrial primer (Invitrogen®).

Real-Time PCR was performed using SYBR Green Master Mix, using forward and reverse cytochrome C mtDNA primer marker. The mtDNA level was measured, relative to the number of the thermal cycles required to produce the product using the following protocol: 95°C for 2-3 minutes; 35-40 cycles of 95°C for 10-15 seconds; 55-65°C for 39-45 seconds; then 68-72°C for 30 seconds.

4.3.2.2 Murine model

After measuring circulating mtDNA in response to sternotomy, with potential signalling of the TLR9 activating the inflammatory cascade and in order to test the effect of suppressing the TLR9 in an animal model, we applied a mouse-preferred synthetic TLR9-antagonist oligodeoxynucleotide (ODN2088) (InvivoGene®) (356, 357). This was similar to our previous experiment on HUVEC, however in this case the TLR9 stimulator is the surgical trauma performing sternotomy.

We acclimatized 11 male C57BL/6, 9-10 weeks old mice divided into 6 control and 5 in the experimental group. The test mice were injected with intra-peritoneal ODN2088 (100µg/25g body weight) prior to induction of anaesthesia; saline was used for the control group. Mice were anaesthetized using ketamine (50mg/kg)/xylazine (2mg/kg) and intubation was done through neck incision. Midline sternotomy was performed. Two pre-sternotomy blood samples were collected, before and after intubation. A third sample taken shortly after sternotomy, where the mtDNA was found at its peak in the rat model (Figure 4.2).

To test the downstream activation of TLR9 we measured the circulating level of IL-6. Firstly mRNA was extracted using RNeasy mini kit (Qiagen®). The cDNA was formed by reverse transcription using qScript cDNA supermix (Quanta BioSciences),

CFX96 RT-PCR for gene specific primer for mice IL-6. The level measurement then was calculated relative to the number of the thermal cycles required to generate IL-6.

4.3.2.3 Porcine model

In the large animal setting we proceeded with performing CPB with the same bypass circuit systems that are used in humans. In the first part of the experiment we collaborated with the University of Bristol to identify the effect of circulating mtDNA in pigs in relation to CPB. We received plasma samples from pigs, which underwent CPB through median sternotomy (n=5), at different time-points in relation to CPB commencement (pre, end of CPB, and 90-min after CPB); and sham mechanically ventilated pigs without any further intervention (n=5). The mtDNA was extracted as per the previous experiments, RT-PCR with forward and reverse cytochrome C primer.

The second part of the experiment was to assess the effect of antioxidant (sulforaphane) on the mtDNA release in response to CPB. The experiment was carried out in the Imperial College London laboratory at the Hammersmith Hospital Campus, where the animals were acclimatized. Ten female Landrace pigs (50-60 kg) were used according to UK Home Office regulations and Directive 2010/63/EU of the European Parliament and in compliance with the Guide for the Care and Use of Laboratory Animals.

Ketamine (20mg/kg)/ xylazine (2mg/kg) was used for sedation prior to induction of anaesthesia with 5% isoflurane, followed by standard endotracheal intubation. Five pigs were then treated immediately with IV injection of Saline and the other 5 with IV sulforaphane (2mg/kg). Another set of results related to sulforaphane in this study were published by our groups (Nguyen et al.) (119).

Surgical approach was through midline sternotomy using a Gigli saw and standard systemic heparinisation was performed (300IU/kg). The arterial and venous CPB circuit tubing were cannulated to the ascending aorta and right atrium respectively, maintaining CPB for 2 hours with flow of 2 to 4 L/min with disconnected lung ventilation. Activated clotting time of greater than 400 seconds was maintained during CPB. Blood sampling was performed at 4 different time-points in relation to CPB commencement (pre, at onset of CPB, at 60-min and 120-min of CPB). mtDNA was extracted from plasma as per the previous experiment, RT-PCR performed using each samples in triplicate with analysis of their mean.

4.3.3 Ischemic-reperfusion injury model

As the effect of IR-injury consists of a series of pathophysiological events that involve deprivation of blood and oxygen followed by their restoration, we assessed the effect of IR-injury on the release of both mtDNA and IL-6 by using an ex-vivo renal reperfusion model. In collaboration with the transplant team in our institution, experimental ex-vivo renal reperfusion models were used. Autologous whole blood and perfusate were used to simulate the cold ischemia prior to implantation, followed by warm reperfusion. This simulation closely resembles the ischemia and reperfusion when the aorta is cross-clamped and then released during CPB.

Thirteen porcine kidneys were retrieved from cadaveric pigs at an abattoir, flushed with cold storage University of Wisconsin (UW) solution and placed on ice for transport back to the laboratory. Kidneys were then perfused on a modified Waters Medical (RM3) perfusion machine at 4°C with UW solution for 6-hours and then underwent autologous whole blood normothermic perfusion for 6-hours. DNA was extracted from plasma at different time-points of reperfusion (pre, 5-minutes, 20-

minutes, 2-hours, 4-hours and 6-hours). RT-PCR with cytochrome C mitochondrial primer was employed to detect the level of circulating mtDNA. We also measured plasma IL-6 in these samples using the same methodology explained in the above murine model.

4.3.4 Statistical analysis

Statistical analysis was conducted using SPSS version 20 software package (IBM Corporation, Armonk, NY, USA). Results are expressed as means $\pm$ SE (standard error) at each time point for each group, *P*-values < 0.05 were considered statistically significant. Due to the nature of the experiments with small number of animals the homogeneity and normality of the data were not tested. However, comparative analysis between any two groups was carried out using non-parametric test (Mann–Whitney U test), if no value computed due to small number, independent samples t-test was then used.

Changes within each group over different time points, such as pre-procedure and post-procedure were calculated with non-parametric test (Wilcoxon test), if no value computed due to small number, paired samples t-test was then used. One-way analysis of variance (ANOVA) was employed to compare results between more than two groups, with Bonferroni multiple-comparison method to identify any significant result between any two groups.

4.4 Results

4.4.1 Validating the downstream of TLR9 signalling

Our experiment showed increase expression of IL-6 in HUVEC (mean $\pm$ SE) when stimulated with TLR9-agonist ODN-1668 for 1 hour (12.8 ± 3.18 -fold, $P= 0.003$). In contrary, when stimulated with TLR9-antagonist (IRS-954) alone the IL-6 expression level decreased below the control level (0.66 ± 0.07 -fold). When HUVEC samples were treated with increasing doses of IRS-954 and a fixed dose of TLR9-agonist (ODN-1668), the IL-6 level was inversely related to the IRS-954 concentration of $2\mu\text{m}$, $4\mu\text{m}$, $6\mu\text{m}$ and $8\mu\text{m}$ ($p= 0.1, 0.01, 0.004$ & 0.003 , respectively) in comparison to ODN-1668 alone (Figure 4.1).

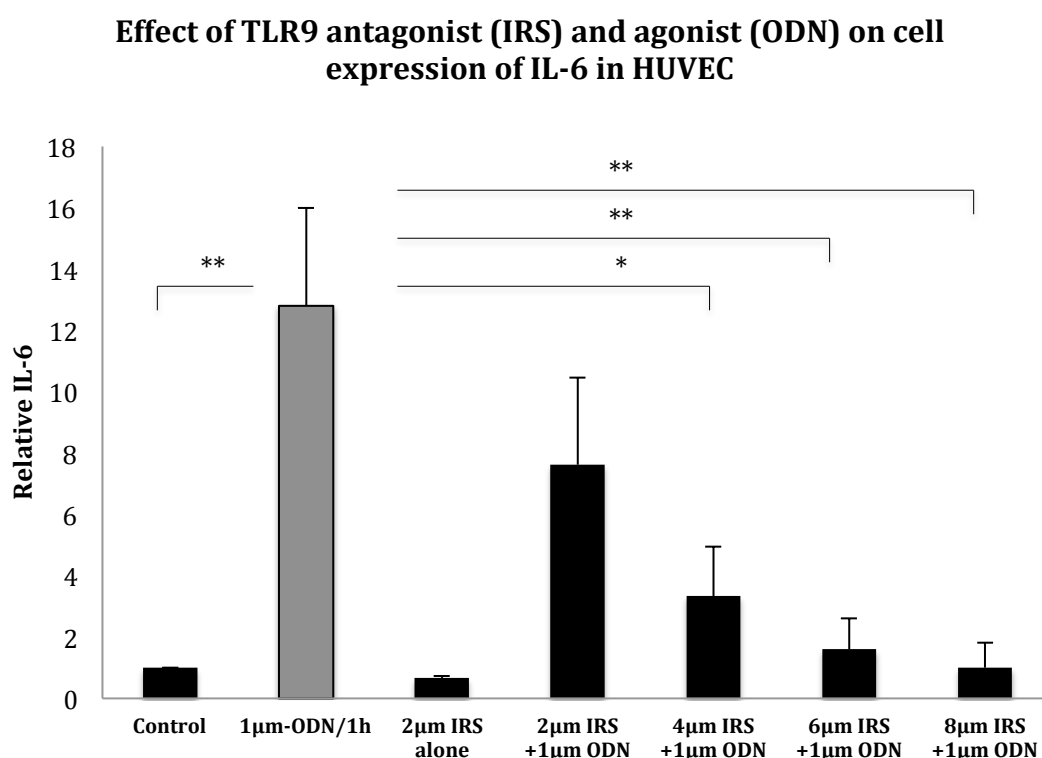


Figure 4.1 Effect of TLR9 agonist and antagonist on IL-6 expression

Stimulation of HUVEC with established dose of $2\mu\text{m}$ TLR9 agonist (ODN-1668) cause increase in IL-6 production. Pre-treated of HUVEC with TLR9 antagonist (IRS-954) showed significant reduction in IL-6 inversely related to IRS-954 dose ($*= p<0.05$, $**= p<0.01$).

4.4.2 Rodent model

Immediately after exposure to sternotomy (1-minute), the circulating mtDNA level (mean $\pm$ SE) significantly increased to 19-fold (19.29 ± 3.31) of pre-sternotomy ($p < 0.001$). The mtDNA level nearly halved at 10-minutes post-sternotomy (9.79 ± 1.61) but still remained statistically significant ($p < 0.001$) in relation to pre-sternotomy and 1-minute level. This results suggesting significant response of plasma mtDNA to the surgical trauma (Figure 4.2).

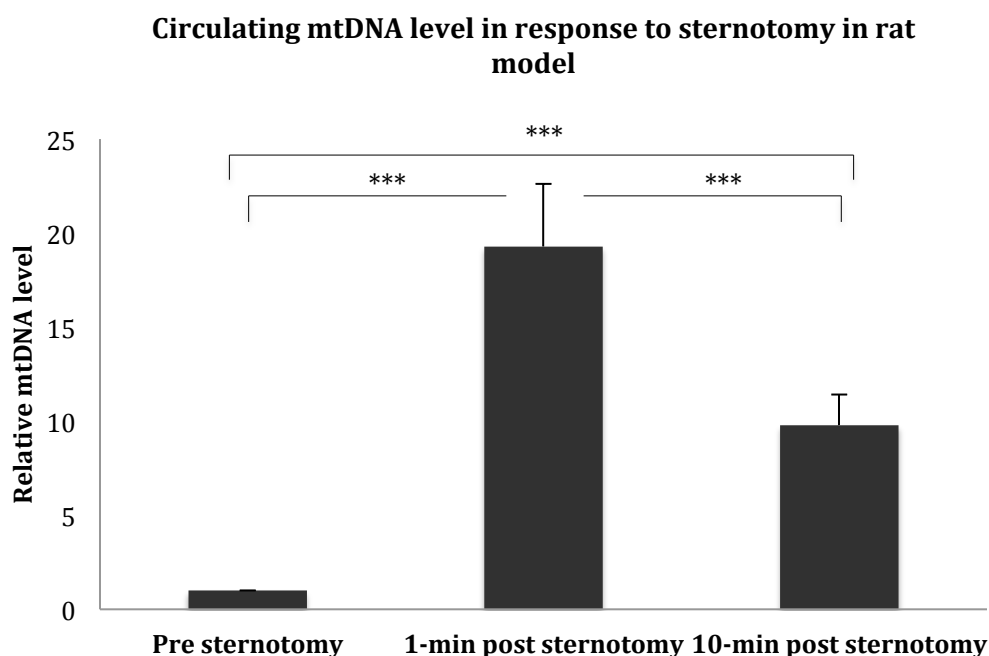


Figure 4.2 Effect of sternotomy on mtDNA in rats model

Plasma levels of circulating mtDNA in the rodent model undergoing median sternotomy in different time point. Data are mean $\pm$ SE showed the level of mtDNA peaked immediately after sternotomy (***) ($p < 0.001$).

Increased level of mtDNA was found in various harvested organ tissues (at 10-minutes post-sternotomy) but at larger scale. Tissue mtDNA level in heart, lung, liver and kidney were significantly higher than plasma mtDNA (10-minutes), $p < 0.001$ for all. Interestingly, the mtDNA level was significantly different between various organs

($p < 0.001$, ANOVA), Bonferroni analysis showed a significant difference between these organs, except between the kidney and liver ($p = 1.0$). It was notable that the heart mtDNA was 37-fold of the plasma (367.85 ± 1.01 vs. 9.79 ± 1.61) followed by lung, liver then kidney (155.40 ± 16.61 , 136 ± 16.74 and 50.14 ± 4.02 , respectively) (Figure 4.3).

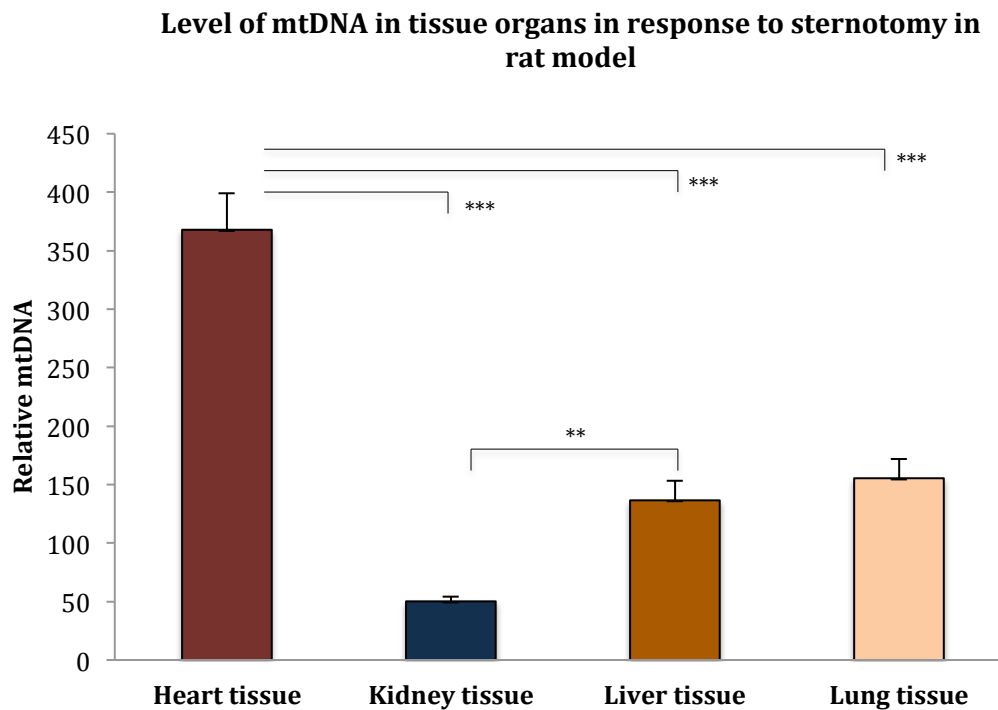


Figure 4.3 mtDNA levels in different organs after sternotomy in rat model

The level of mtDNA in various tissue organs harvested at 10-minutes after sternotomy was significantly different ($p < 0.001$) between different organs apart from between liver and kidney tissue ($p = 1.0$).

4.4.3 Murine model

Pre-induction levels of IL-6 were used to normalize the subsequent time-points in both groups (control vs. TLR9-antagonist pre-treated group). At intubation, both groups showed significant elevation in IL-6 compared to pre-induction ($p = 0.02$ and 0.03 , respectively). The scale was significantly higher in the control group compared to TLR9-antagonist group (22.76 ± 6.27 vs. 3.22 ± 0.68 -fold, respectively. $p = 0.02$).

Similar pattern after sternotomy showed significant elevation in IL-6 levels compared to pre-induction in both groups ($p=0.01$). Moreover, the scale here of IL-6 was more significantly elevated in control than in the TLR9-antagonist group (59.06 ± 14.98 vs. 5.25 ± 1.08 -fold, $p=0.01$). The levels of IL-6 were significantly high after sternotomy in both groups compared to at intubation ($p=0.01$) (Figure 4.4).

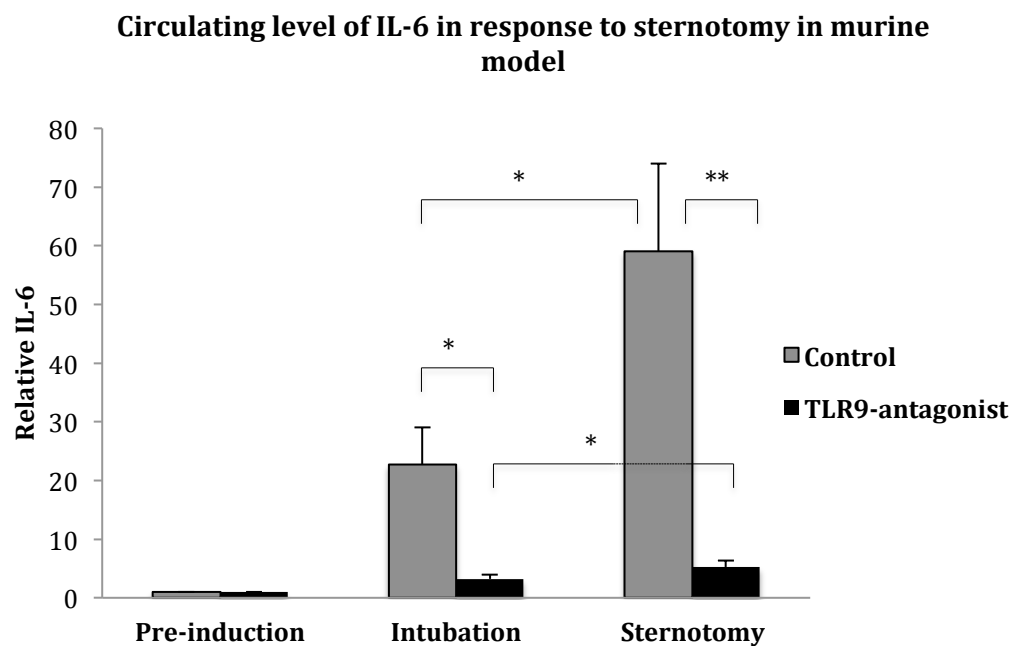


Figure 4.4 Levels of IL-6 after sternotomy in mouse model

Plasma levels of IL-6 in murine model undergoing median sternotomy in different time-points with and without pre-treatment with TLR9-antagonist (ODN2088). Data (mean $\pm$ SE) showed the levels of IL-6 have significantly decreased when TLR9 blocked by ODN2088 (*= $p<0.05$, **= $p<0.01$).

4.4.4 Porcine model

The result of the CPB group showed significant increase in circulating mtDNA at the end of CPB (29.89 ± 4.78 -fold, $p=0.004$), followed by a significant reduction at 90-minutes post-CPB (6.46 ± 0.56 -fold, $p=0.001$) compared to pre-op group. The reduction in the mtDNA between the end of CPB and 90-minutes post-CPB was also significant ($p=0.007$), suggesting transient response to CPB. In comparison, the sham group

showed minimal but significant increase in mtDNA levels at equivalent time to the end of CPB time-point (1.68 ± 0.22 -fold, $p = 0.04$), but with further significant increase at the 90-minute post-CPB time-point equivalent (2.4 ± 0.42 -fold, $p = 0.03$) compared to baseline. The increase in the mtDNA between end of CPB and 90-minute post-CPB time-point equivalent was not significant ($p = 0.2$), (Figure 4.5).

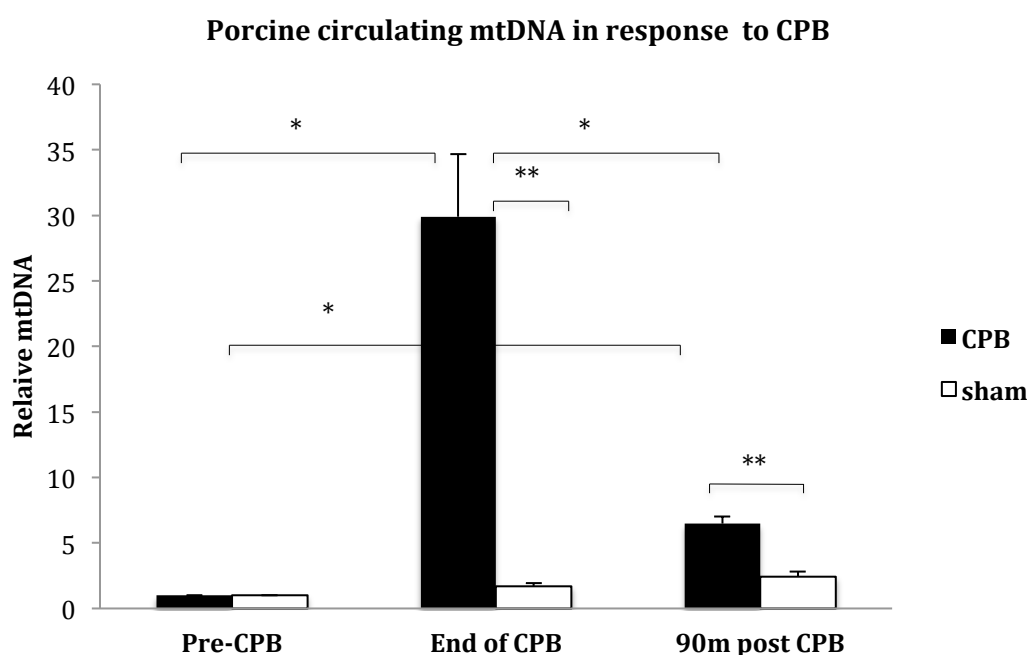


Figure 4.5 Levels of mtDNA in response to CPB in pigs model

The circulating levels of the mtDNA in porcine model underwent median sternotomy with CPB in different time-points vs. sham. Data are mean $\pm$ SE. There was significant elevation in mtDNA levels in pigs received CPB (*= $p < 0.05$, **= $p < 0.001$).

In the sulforaphane experiment, the control group (saline) revealed significant increase in their circulating mtDNA level to nearly 30 and 50-fold at CPB60 and CPB120, respectively compared to pre-CPB ($p = 0.002$ and 0.005 , respectively). The sulforaphane group also showed significant elevation of mtDNA levels at CPB60 and CPB120 with nearly 7 and 13-fold, respectively ($p < 0.001$ for both). However, the magnitude of this elevation was much less than the control group ($p < 0.001$ at CPB 60

and $p=0.003$ at CPB120). There was no significant difference between the two groups prior to CPB or at CPB0 (Figure 4.6).

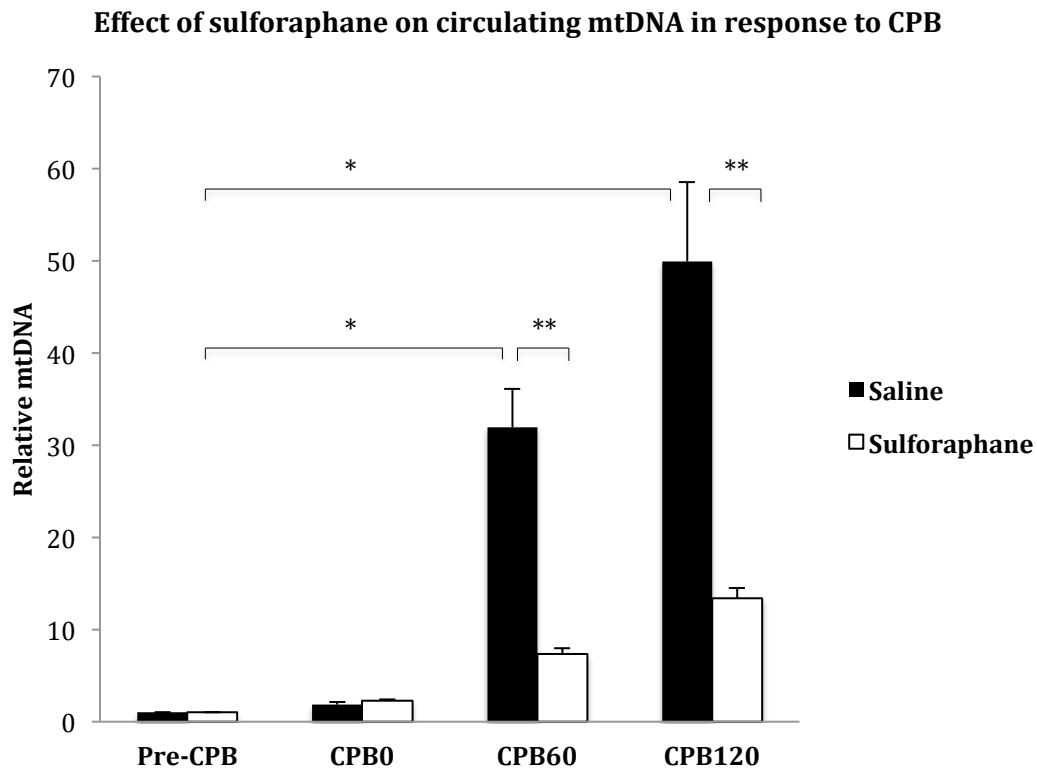


Figure 4.6 Effect of sulforaphane on mtDNA levels in CPB in pig model

Plasma levels of circulating mtDNA (mean $\pm$ SE) in porcine model underwent CPB at different time-points with or without sulforaphane pre-treatment. There was significant reduction in mtDNA levels in response to sulforaphane pre-treatment (*= $p < 0.05$, **= $p < 0.001$).

The general trend of mtDNA and IL-6 response in the ex-vivo ischemic-reperfusion model was proportional to the reperfusion time. Interestingly, while the IL-6 was constantly rising with reperfusion time, the level of circulating mtDNA showed period of constant increase up to 4 hours, followed by a decline, which was not significant ($P=0.79$). There was significant increase in the mtDNA ($p=0.001-0.007$) and in IL-6 ($p<0.001-0.05$) at all time-points of reperfusion in relation to pre-reperfusion level (Figure 4.7).

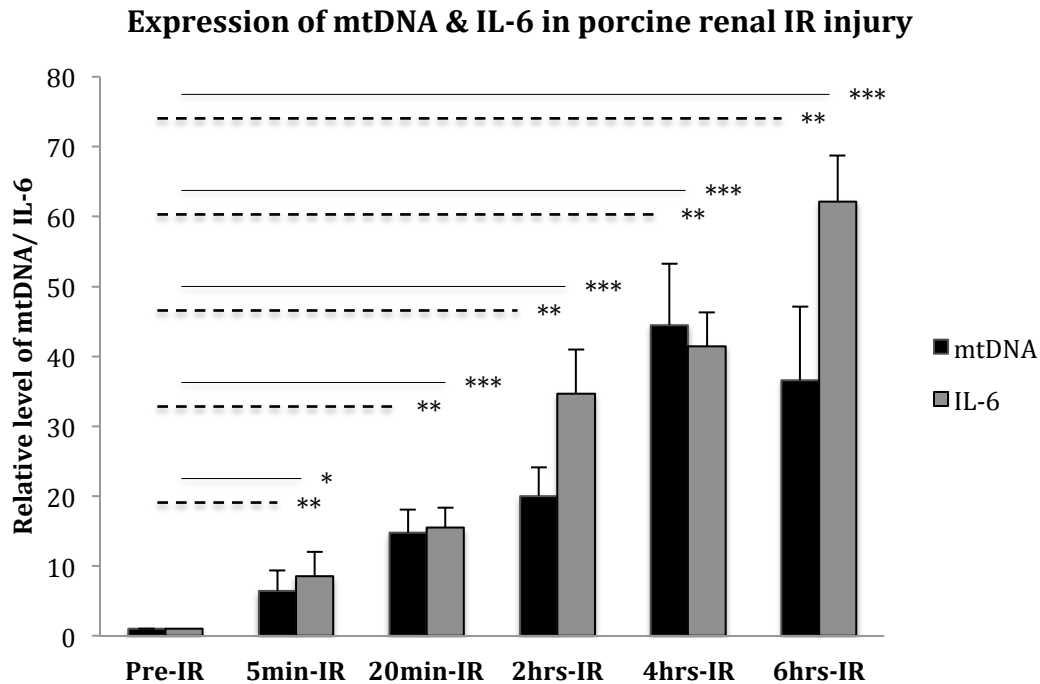


Figure 4.7 Effect of ischemic-perfusion on mtDNA nad IL-6 in porcine kidneys

Plasma level of circulating mtDNA and IL-6 in ex-vivo porcine kidney model underwent period of ischemia then reperfusion at different time points (Pre IR, 5 min, 20 min, 2h, 4h & 6h of IR). Data are mean $\pm$ SE (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$). (Dotted lines are for mtDNA and solid lines for IL-6).

4.5 Discussion

Cardiac surgery is known to have a significant effect on the systemic inflammatory response (87, 358-360). CPB is currently used routinely in most major cardiac procedures. The exposure of blood to the artificial surface of the bypass circuit may activate inflammatory cascades, potentially leading to systemic inflammatory response syndrome (SIRS) with significant morbidity (361). However the involved mechanisms and molecular pathways are still not completely understood.

The other likely contributors for SIRS in cardiac surgery, in addition to CPB, include the surgical trauma and the associated IR-injury. In this study we aimed to evaluate the effect of these three components separately in different experimental settings using

small and large animal models. Several animal models were used for systemic inflammatory response modulation in response to mitochondrial DAMPs (MTD) (158, 362-366). MTD have been demonstrated as inflammatory modulators leading to tissue damage in variety of pathological conditions including SIRS, connective tissue diseases, myocardial infarction, vascular dysfunction and in those individuals receiving either chemotherapy and hemodialysis (298-301).

Here we evaluated whether the mitochondrial substances and DAMP would potentially contribute to SIRS if tissue injury during cell necrosis and apoptosis leads to spillage of the mitochondrial content into the circulation (298). We selectively chose to detect mtDNA as the main key link for potential signalling of the TLR9 in a sterile environment due to the fact that TLR9 recognise bacterial DNA motifs (239) and the mtDNA has a similar molecular signature to bacterial DNA (367).

The question that was raised was whether the utilization of CPB plays an important role in the development of SIRS. The results from our study demonstrate that the mtDNA seems to be released in all three of the surgical components hypothesized above. However, when comparing surgical trauma to CPB, it becomes apparent that mtDNA release is far more strongly related to CPB than surgical trauma, observed when these factors merge together in one experimental setting, as in the pig model (Figure 4.4 & 4.5).

This study suggests that the greater, or specifically the longer the exposure to the CPB circuit results in more cell injury and apoptosis. This leads to the spillage of the mitochondrial contents into the circulation, thereby signalling the TLR9 through recognizing the mtDNA CPG motifs, leading to activation of the inflammatory cascade and production of pro-inflammatory cytokine IL-6 (368).

The ex-vivo renal IR-injury as applied in the described model leads to an increase of the mtDNA and pro-inflammatory cytokine IL-6, which can potentially activate intracellular signalling and leukocyte-endothelial interaction followed by migration of activated leukocytes of the re-perfused tissue (369).

Our group has previously published the results of the antioxidant sulforaphane pre-treatment in this study. It was found to reduce pro-inflammatory p38 and nuclear factor- κ B activation and suppressed inflammatory cytokine expression in a porcine model on CPB (Nguyen et al.) (119). Although the kinetics are not fully understood, interestingly we found the mtDNA circulating level was decreased with sulforaphane pre-treatment, and we hypothesize that this is due to the anti-apoptotic effect of sulforaphane and protection against mitochondrial depolarization and DNA fragmentation (370). Similarly, as we assumed that TLR9 would be signalled in our experiment, when blocking this receptor with selective TLR9 antagonists IRS-954 and ODN-2088 (in HUVEC and murine model respectively), interestingly the result showed significant statistical reduction in the level of circulating IL-6, which confirms our assumption.

Therefore to attenuate the harmful effect of the inflammatory response to CPB and open-heart surgery as a result of signalling TLR9 as our result suggested, one can argue that therapeutic blocking of TLR9 as a mode of prophylactic therapy in CPB can be of valuable clinical relevance. This proposition does however require further investigation in large animal model with CPB using relevant TLR9 antagonist to attenuate undesired immune response with potential clinical application, similar to a current phase one clinical trial using TLR7, 8 and 9 antagonist (IMO-8400) in treatment of autoimmune disease (371).

The one major limitation of our study is the inability to test the antagonization of TLR9 in the CPB setting with the application of myocardial ischemia and reperfusion. This experiment will need future exploration.

4.6 Conclusion

Considering our findings in this study, we conclude that the enhancement of circulating mtDNA will activate and signal TLR9 in CPB, with subsequent release of IL-6, which will in turn leads to endothelial activation and leukocyte migration.

Therefore avoidance of the release of cytokines and endothelial activation using relevant TLR antagonists is likely to be beneficial in minimizing or perhaps preventing the significant systemic inflammatory response observed with cardiopulmonary bypass. This is investigated in the next chapter.

5 CHAPTER V: Effect Of Downstream Activation Of TLR9 On Cardiac Surgery

5.1 Abstract

Background: Coronary artery bypass graft (CABG) with cardiopulmonary bypass (CPB) is associated systemic inflammatory responses. This response can result from the signalling of some toll-like receptors (TLRs) such as TLR9, which is specifically signalled via MyD88-dependent pathway leading to the subsequent production of proinflammatory cytokines and chemokines.

Aim: We aim to identify that CPB initiates a sterile systemic inflammatory response leading to over production of proinflammatory cytokines and chemokines. This is done through the MyD88-pathway as a result of TLR9 signalling by binding to the mitochondrial DNA (mtDNA) ligand, which was tested in previous chapters.

Methods: Twenty-nine patients underwent coronary artery bypass graft (CABG) surgeries were recruited. 17 patients utilized CPB and 12 had off-pump CABG (OPCAB). Blood samples were taken at 24 hours period with various time-points. Multiple cytokines and chemokines using MyD88- pathway and other pathway for comparison were measured. This done by PCR array detecting the number of thermal cycles (Ct) required generating the product. The level of cytokines and chemokines were measured relatively to Ct value.

Results: The cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, INF- α , TNF- α , NF-kB, MAPK1, MAPK14, TRAF3 and TRAF6) and chemokines (CXCL9) using the MyD88-dependent pathway were markedly increased in response to CPB compared to OPCAB (p between <0.001 to <0.05). In contrast, the cytokines (TRIF3 and IRF7) using TRIF-dependent pathway were not affected by CPB and showed no difference in all time-point between CABG and OPCAB ($p > 0.05$).

Conclusion: CPB can initiate systemic inflammatory response via MyD88-dependent pathway due to TLR signalling mediated through binding to mtDNA, which has increased during CPB. This signalling leads to over production of various proinflammatory cytokines and chemokines.

5.2 Introduction

Cardiac surgery especially when using the CPB has long been recognized for having a major role in the initiation of acute inflammatory response. These ranges from minor local inflammation adjacent to the surgical wound site to the most severe systemic inflammatory response syndrome (SIRS) with potential serious early or late postoperative complications, which are characterized by over production of pro-inflammatory cytokines (87, 360, 372). However, few studies have contributed the inflammatory response in cardiac surgery is more related to surgical trauma and pulmonary or myocardial reperfusion injury rather than CPB alone (373).

In inflammation, recruitment of pro-inflammatory mediators will initiate inflammatory reactions, which may destroy the organ tissues leading to organ dysfunction. Different recruited cells during inflammation will respond to specific stimulus and will release a specific inflammatory mediator. Cytokines for example are small secreted proteins released by specific recruited cells which can be named according to the cells that are made by, for example the interleukins are cytokines made by leukocytes, lymphokines by lymphocytes or monokines are made by monocytes (374). Different cells in immunity such as B-cells, T-cells, macrophages and leukocytes can communicate with other immune cells by releasing these cytokines or chemokines with chemotactic effect (374) (Figure 5.1).

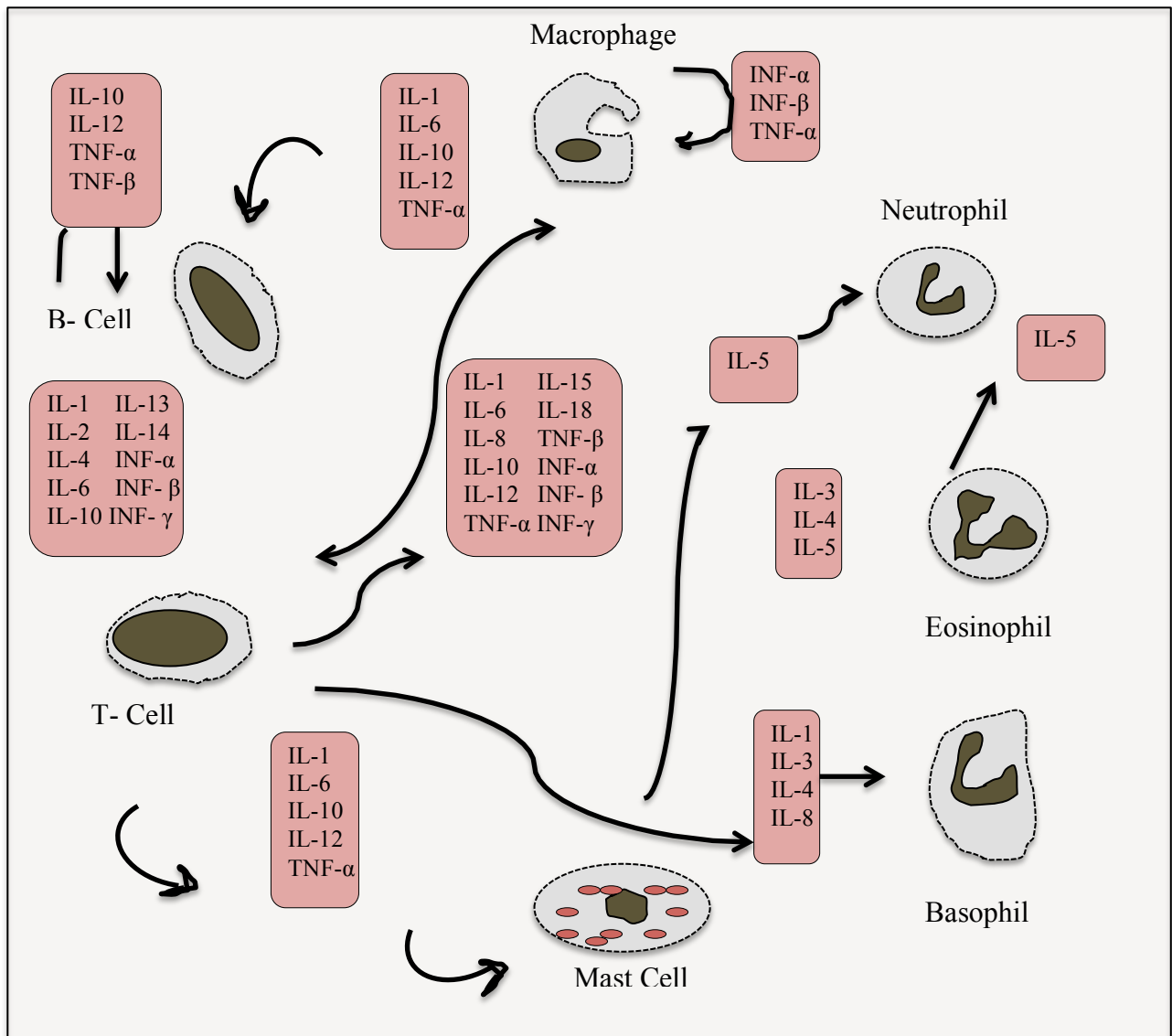


Figure 5.1 Cytokines network

Different types of cells will secrete different types of cytokines and chemokines as well as communicate and coordinate with the other immune cells, these immune system cells include B-cells, T-cells, macrophage and different types of leukocytes.

Cytokines can be released due to signalling of various TLRs. TLR9 for example is an intracellular receptor found in the endoplasmic reticulum in the resting cell prior to stimulation such as in infection or trauma (375). Furthermore, when stimulated it become distributed and translocated into the endosomal compartment to bind its DNA ligand (376). TLR9 mostly expressed in B-lymphocytes, plasmacytoid dendritic cells, monocytes and natural killer cells, with subsequent production of variety of cytokines and chemokines such as INF- α and other inducible related genes (236, 245).

TLR9 is the only immune stimulatory DNA receptor recognizing unmethylated CpG sequences in DNA molecules, which is a hallmark of microorganisms such as bacterial and viral genomes to be recognized as PAMPs (377, 378), or the mitochondrial DNA that also contain CpG DNA repeats similar to bacteria that can be recognized as DAMPs (154, 158). Binding to its specific ligand such bacterial or mitochondrial DNA, TLR9 will lead to its activation mediated through myeloid differentiation primary response gene-88 (MyD88) dependent pathway.

As explained in chapter 2, MyD88 interact with Interleukin-1 Receptor-Associated Kinases (IRAK) (239) and TNF-Receptor Associated Factor 6 (TRAF6) forming signalling complex. This complex ultimately leads to the activation and recruitment of transcription factors such as Interferon Regulatory Factor-7 (IRF7). Together this complex activates other signalling cascades, with subsequent TLR genes induction. In particular, TLR9 signalling pathway activation leads to activation of Nuclear Factor-Kappa B (NF- κ B) and Activator Protein 1 (AP-1) inducing various immunity related gene with secretion of different pro-inflammatory cytokines and chemokines (239-241). Eventually, TLR9 can also induce the antiviral response through inducing type-1 INF (IFN- β & IFN- α) (241) (Figure 5.2).

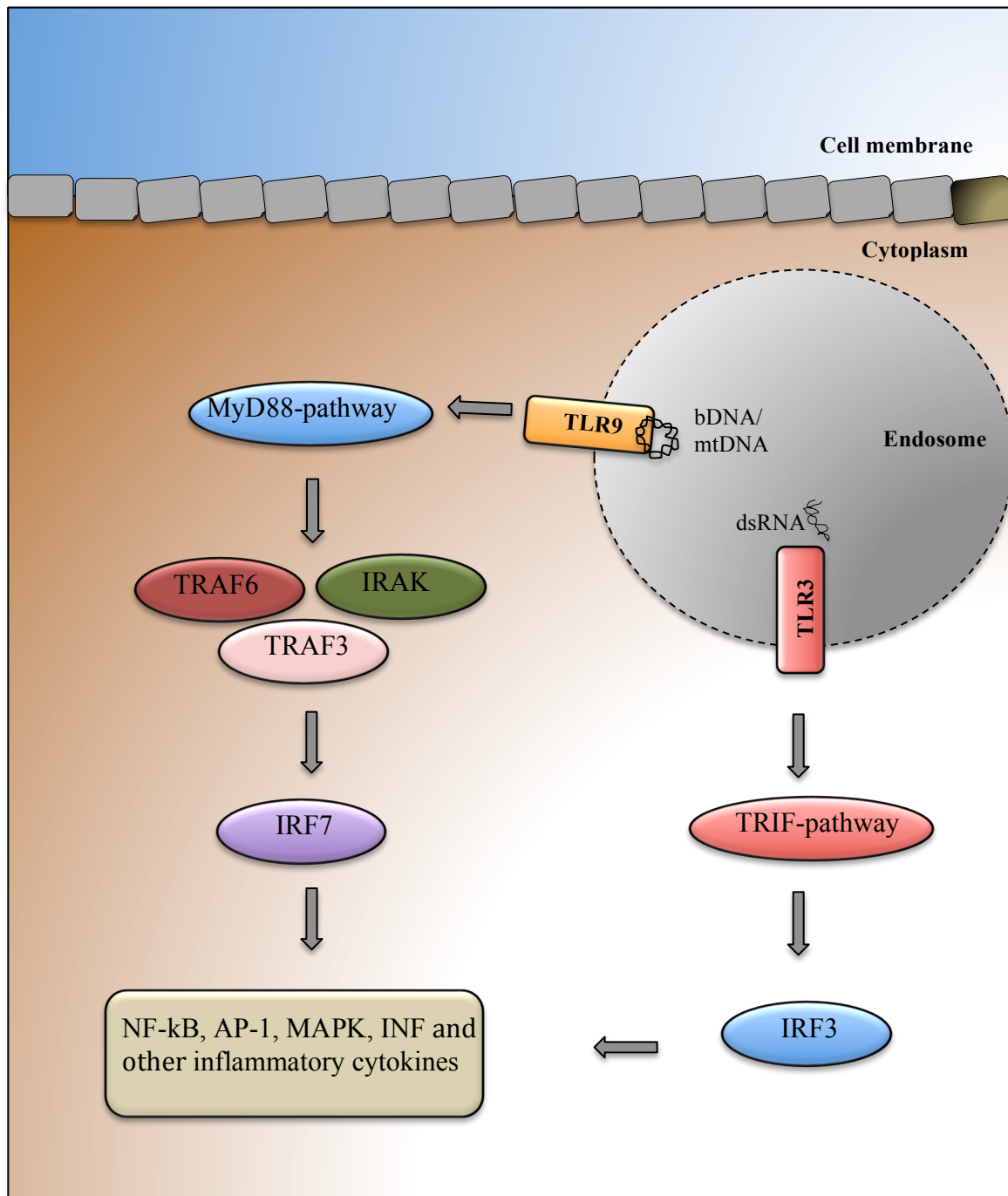


Figure 5.2 TLR9 signalling pathway

TLR9 signals through MyD88-dependant pathway interacting with IRAK and TRAF6 forming signalling complex, which activates IRF7 leading to activation of NF-kB and AP-1 inducing other pro-inflammatory cytokines. TLR9 does not signal through TRIF-dependent pathway, it is predominantly by TLR3 and TLR4. (MyD88: Myeloid Differentiation primary response gene 88, TRAF: TNF-Receptor Associated Factor, TRIF: TIR-domain-containing adaptor Inducing INF, IRAK: Interleukin-1 Associated Kinases, IRF: Interferon Regulatory Factor, AP-1: Activator Protein 1).

We have previously identified circulating mtDNA significantly increased in response to CPB in human (Chapter 3) and in animal model (Chapter 4). Also we mentioned previously that TLR9 is the only receptor involved in immune stimulatory response recognizing unmethylated CpG sequences in DNA. Therefore, to identify the signalling and activation of TLR9 through its only MyD88-dependent pathway, we monitored this by measuring MyD88 pathway downstream activation.

The MyD88-dependant pathway activation leads to downstream production of various cytokines and chemokines including; Interferons (INF), Interleukins (ILs), Mitogen Activated Protein (MAP) Kinases, Tumour necrosis factors (TNF), Nuclear Factor kappa B (NF- κ B) and Chemokine (C-X-C motif) Ligands (CXCLs) (240, 379-383), as a result of activation of TNF-Receptor Associated Factors (TRAF3, TRAF6) in this pathway. However, the TIR-domain-containing adaptor Inducing INF (TRIF) and Interferon Regulatory Factor-3 (IRF3) are not part of the MyD88 pathway, instead they use TRIF-dependent pathway which is exclusively activated by TLR3 or TLR4 signalling but not TLR9 (240) (Figure 5.2).

5.3 Hypothesis

- Cardiac surgery will lead to activation of various proinflammatory cytokines and chemokines, this is due to the rapid induction of TLR9 signaling through MyD88-pathway only as a result from increased circulating mtDNA level in relation to cardiopulmonary bypass, Which was previously tested (chapter 3).
- TRIF-dependent pathway is activated only by TLR3 and TLR4 leading to IRF3 production, and plays no role in the systemic inflammatory response to cardiopulmonary bypass.

5.4 Material and methods

5.4.1 Study protocol

We have included in this study patients previously tested for the circulating levels of the mtDNA in relation to coronary artery bypass grafting (CABG) surgery with and without the use of CPB (chapter 3). Among these patients we selected those with the highest levels of circulating mtDNA to establish the maximum levels of their downstream activation. 29 patients were involved, 17 on-pump (CABG-group) and 12 off-pump (OPCAB-group).

We used the same plasma samples stored at -80°C in previous experiment. 7 samples for each patient with varying time points and 17 different genes were tested for each sample. The time-points tested includes preoperatively, at initiation of CPB and its corresponding time point for OPCAB (CPB0), followed by after 30 minutes (CPB30), 60 minutes (CPB60), 120 minutes (CPB120), 300 minutes (CPB300) and after 24 hours (CPB24) (Figure 5.3).

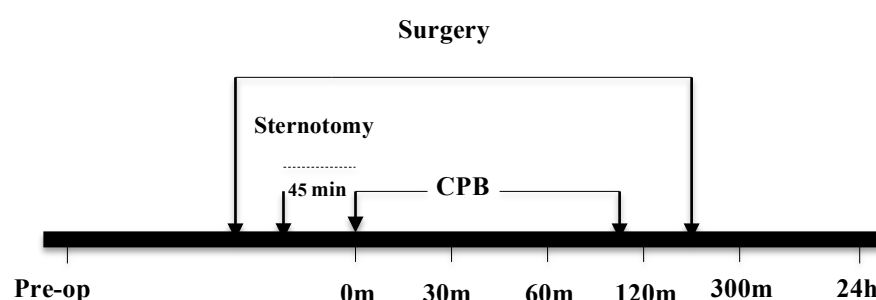


Figure 5.3 The study design

Plasma samples tested for mtDNA downstream activation taken pre-operatively, at commencement of CPB (CPB0) in CABG-group, which is equivalent to 45 minutes after sternotomy in OPCAB-group, then both groups at varying time points in relation to the initiation of CPB at 30, 60, 120 & 300 minutes respectively followed by after 24 hours.

5.4.2 PCR arrays

PCR arrays are the most convenient and sensitive pathway to analyse panel of focused multiple genes expression. It is a method for a named designed targeted genes. Each PCR array can be modified according to the experiment requirement, here we customized the PCR array plate with the genes relevant to the downstream activation of the TLR9 (developed by Qiagen®).

Each PCR plate accommodates 4 samples with 17 different designed genes, the genes are: IL-2, IL-4, IL-6, IL-8, IL-10, IL-12RB1, INF- α , INF- γ , TNF, CXCL9, IRF3, NF- κ B, MAPK14, MAPK1, TICAM1 (TRIF), TRAF3 & TRAF6 as well as house keeping gene control (ACTB, GAPDH, B2M), genomic DNA control (HGDC), reverse transcriptase controls (RTC) and positive PCR controls (PPC) (Figure 5.4). Genomic DNA less than 30 thermal cycle (Ct) in PCR array was considered as contaminated and needed to be addressed for further RNA sample purification as explained later. The results from the PCR array determined by the relative expression of the Ct values using the delta-delta CT ($\Delta\Delta$ Ct) method.

Designed PCR Array Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	IL-2	TNF- α	TRAF6	IL-2	TNF- α	TRAF6	IL-2	TNF- α	TRAF6	IL-2	TNF- α	TRAF6
B	IL-4	CXCL9	RPLP0	IL-4	CXCL9	RPLP0	IL-4	CXCL9	RPLP0	IL-4	CXCL9	RPLP0
C	IL-6	IRF3	ACTB	IL-6	IRF3	ACTB	IL-6	IRF3	ACTB	IL-6	IRF3	ACTB
D	IL-8	NFKB1	GAPDH	IL-8	NFKB1	GAPDH	IL-8	NFKB1	GAPDH	IL-8	NFKB1	GAPDH
E	IL-10	MAPK14	B2M	IL-10	MAPK14	B2M	IL-10	MAPK14	B2M	IL-10	MAPK14	B2M
F	IL12 RB1	MAPK1	HGDC	IL12 RB1	MAPK1	HGDC	IL12 RB1	MAPK1	HGDC	IL12 RB1	MAPK1	HGDC
G	INF- α	TICAM1 (TRIF)	RTC	INF- α	TICAM1 (TRIF)	RTC	INF- α	TICAM1 (TRIF)	RTC	INF- α	TICAM1 (TRIF)	RTC
H	INF- β	TRAF3	PPC	INF- β	TRAF3	PPC	INF- β	TRAF3	PPC	INF- β	TRAF3	PPC

Figure 5.4 Designed PCR plate

The plate was pre incubated with targeted genes as downstream of TLR9 activation through MyD88-dependent pathway but not TICAM1 (TRIF) and IRF3 as they are activated through TRIF-dependent pathway operated by TLR3 and TLR4 (IL: interleukin, INF: Interferon, TNF: Tumour necrosis factor, CXCL9: Chemokine (C-X-C motif) ligand 9, IRF3: Interferon regulatory factor 3, NFKB: Nuclear factor kabba B, MAPK: Mitogen activating protein kinase, TRIF: TIR-domain-containing adaptor Inducing INF, TRAF: TNF-Receptor Associated Factor, RPLP0: Ribosomal protein large P0, ACTB: β -actin, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, B2M: β -2 microglobulin, HGDC: Human genome DNA control, RTC: Revers transcription control and PPC: Positive PCR control).

5.4.3 RNA extraction

200µl of the plasma samples were thawed at room temperature and used to prepare mRNA using miRNeasy Serum/Plasma Kit (Qiagen®). The purification of mRNA was done according to the manufacturer guidelines using five times plasma volumes (1000µl) of QIAzol lysis reagent, incubated at room temperature for 5 minutes taking care minimizing any risk of RNA contamination. Next, 200µl of chloroform solution was added, similarly incubated at room temperature for 2-3 minutes then centrifuged at 4°C and 12,000 x g for 15 minutes. The top aqueous phase of the mixture was then transferred to a new tube and 300µl of 100% ethanol was added. After that RWT and RPE buffer were added and centrifuged for 15 seconds each at 8000 x g respectively then washed with 80% ethanol before the final stage using RNAase-free water to elute the final RNA followed by quantification using spectrophotometry and stored at -80 °C for subsequent analysis.

5.4.4 Revers transcription PCR

The revers transcription procedure comprises two main steps including genomic DNA (gDNA) elimination followed by revers transcription. After purification of the RNA from plasma, the concentration measured by NanoDrop®, 0.5µg of total RNA was used at each reaction to generate cDNA using QuantiTect® Reverse Transcription Kit.

5.4.4.1 Elimination of genomic DNA (gDNA)

Purified RNA samples were incubated in gDNA wipeout buffer at 42°C for two minutes to remove any contaminated gDNA before proceeding to revers transcription reaction.

5.4.4.2 Revers transcription

0.5µg of total RNA was added to a revers transcription master mix (Quantiscript revers transcriptase, MgCl₂, dNTPs, RNase inhibitor protein, RT primer mix) followed by period of incubation as per manufacturer protocol.

The protocol of incubation includes; Annealing: for 5 minutes at 25 °C, extension: for 30 minutes at 42°C, revers transcriptase inactivation: for 5 minutes at 85°C. The products were then stored at -20°C to be used later. The cDNA products then were amplified by real-time PCR for all genes expression as a downstream activation for TLR9 signalling.

5.4.5 Statistical analysis

Statistical analysis was conducted using SPSS version 20 software package (IBM Corporation, Armonk, NY, USA). Results are expressed as means ± SD (standard deviation) at each time point for each group, $p < 0.05$ were considered statistically significant. Comparative analysis between any two groups was carried out using non-parametric test (Mann–Whitney U test). Changes within each group over different time points were calculated with non-parametric test (Wilcoxon test). One-way analysis of variance (ANOVA) was employed to compare results between more than two groups, with Bonferroni multiple-comparison method to identify any significant result between any two groups.

5.5 Results

5.5.1 Preliminary patient's demographic data

The demographics of the patients involved are summarized in table 5.1. The mean age for on-pump group (CABG) and off-pump groups (OPCAB) were very similar of 64 ± 11 and 64 ± 7 years old respectively. The body mass index was slightly higher in OPCAB than CABG group of 30.3 ± 3.9 and 27 ± 3.8 , respectively. Logistic Euroscore for CABG and OPCAB were $3.3\pm3.25\%$ and $2.92\pm2.43\%$, respectively. The post-operative incidence of atrial fibrillation and sternal wound infection was higher in CABG group than OPCAB group ($p < 0.05$). All the other parameters were comparable between both groups with no significant difference.

Table of patient's demographics

Variables	Group 1 (n = 17) CABG- on pump	Group 2 (n = 12) OPCAB	P-value
Age	64±11	64±7	0.81
Sex	M = 13 (76.5%) F = 4 (23.5%)	M = 11 (91.7%) F = 1 (8.3%)	0.28
BMI	27.7 ± 3.8	30.3 ± 3.9	0.08
Ejection fraction (%)	55.6 ± 7.6	54.1 ± 6.7	0.58
Logistic Euroscore (%)	3.3 ± 3.25	2.92 ± 2.43	0.77
Number of coronary diseases:			0.73
1	0 (0%)	0 (0%)	
2	4 (23.5%)	3 (25%)	
3	12 (70.5%)	9 (75%)	
4	1 (6%)	0 (0%)	
Smoking: No smoking	10 (58.8%)	6 (50%)	0.88
Ex-smoker	5 (29.4%)	4 (33.3%)	
Current smoker	2 (11.8%)	2 (16.7%)	
Previous MI	4 (23.5%)	5 (41.7%)	0.23
DM: No DM	11 (64.7%)	4 (33.3 %)	0.14
Diet control	1 (5.9%)	4 (33.3 %)	
Oral therapy	4 (23.5%)	4 (33.3 %)	
Insulin therapy	1 (5.9%)	0 (0.0 %)	
Hypertension	15 (88.2%)	10 (83.3%)	0.70
Hypercholesterolemia	17 (100%)	11 (91.7%)	0.22
NYHA: Class I	6 (35%)	3 (25%)	ns
Class II	6 (35%)	6 (50%)	ns
Class III	4 (24%)	2 (17%)	ns
Class IV	1 (6%)	1 (8%)	ns
Pulmonary disease	2 (11.7%)	1 (8%)	0.22
Renal disease	0 (0%)	0 (0%)	na
Liver disease	0 (0%)	0 (0%)	ns
Neurological disease	1 (5.9%)	2 (16.7%)	0.34
Peripheral vascular diseases	3 (25%)	2 (17%)	ns
Smoking History:			
Never smoked	9 (53%)	4 (33.33%)	ns
Ex-smoker	7 (41%)	7 (58.33%)	ns
Current smoker	1 (6%)	1 (8.33%)	ns
Heart Rhythm: SR	17 (100%)	12 (100%)	ns
AF	0 (6%)	0 (8%)	ns
Others	0 (0%)	0 (0%)	ns

Variables	Group 1 (n = 17) CABG- on pump	Group 2 (n = 12) OPCAB	P-value
CPB time	73.6 ± 20.1	NA	< 0.001*
Number of coronary grafts:			
CABG X1	0 (0%)	0 (8.3%)	0.51
CABG X2	6 (35.3%)	5 (41.7%)	
CABG X3	10 (58.8%)	6 (50%)	
CABG X4	1 (5.9%)	0 (0%)	
Post operative drainage	991 ± 459	808 ± 336	0.28
Blood transfusion:			
0 unit	10 (58.8%)	12 (100%)	0.16
1 unit	1 (5.9%)	0 (0.0%)	
2 units	4 (23.5%)	0 (0.0%)	
3 units	1 (5.9%)	0 (0.0%)	
4 units	1 (5.9%)	0 (0.0%)	
7 units	0 (0.0%)	0 (0.0%)	
Length of ITU stay (days)	1	1	0.34
Length of hospital stay (days)	8.5 ± 6.5	6 ± 1	0.47
Post-operative complication:			
AF	5 (29%)	1 (8%)	<i>p</i> < 0.05*
Heart block	1 (6%)	0 (0.0%)	ns
Wound infection	2 (11.5%)	0 (0.0%)	<i>p</i> < 0.05*
Chest infection	2 (11.5%)	2 (17%)	ns
Renal hemofiltration	1 (6%)	1 (8%)	ns
Stroke	0 (0.0%)	0 (0.0%)	ns
Re-opening	1 (6%)	0 (0.0%)	ns
IABP	0 (0.0%)	0 (0.0%)	0.08

Table 5.1 Pre and postoperative demographic data

Preoperative and postoperative demographic data for patients underwent CABG utilizing CPB (group 1, n=17) and CABG without utilizing CPB (group 2, n=12). Data showed absolute value, SD and percentage. Significant statistical difference if *p* < 0.05.

5.5.2 Interleukin Assay

5.5.2.1 Interleukin-2 (IL-2)

In the CABG groups, results showed a significant increase in IL-2 circulating level mainly at 30, 60 and 120 minutes after initiation of CPB in relation to the other time points. The IL-2 peaked at 60 minutes followed by a drop after 24 hours but was still statistically higher than the pre-operative baseline level (p -value in relation to pre-operative baseline is < 0.001 at all time points) (Figure 5.5 a)

There were significant differences among all time points with varying p values (from $p < 0.001$ to < 0.05), except between CPB60 and 120 (those with the highest IL-2 levels amongst all) where no significant difference was detected ($p > 0.05$). The same pattern was found in the OPCAB group, however the trend was much smaller but still significant in all of the corresponding time points to the baseline level ($p < 0.01$) (Figure 5.5 b).

When comparing both groups together, the CABG group showed significant elevation in the circulating IL-2 level in response to CPB as compared to the OPCAB group at all time points from the beginning of the operation until the next 24 hours (p - value for CABG vs. OPCAB was < 0.001 at all time points) (Figure 5.5 c).

The increase in the IL-2 level was higher in on-pump (CABG) until 24 hours later compared to OPCAB as follows: CPB0 (11 ± 3 vs. 5 ± 2 -fold, $p < 0.001$), CPB30 (72 ± 40 vs. 8 ± 3 -fold, $p < 0.001$), CPB60 (134 ± 73 vs. 10 ± 2 -fold, $p < 0.001$), CPB120 (125 ± 39 vs. 12 ± 2 -fold, $p < 0.001$), CPB300 (95 ± 19 vs. 9 ± 2 -fold, $p < 0.001$) and CPB24 (32 ± 17 vs. 2 ± 1 -fold, $p < 0.001$).

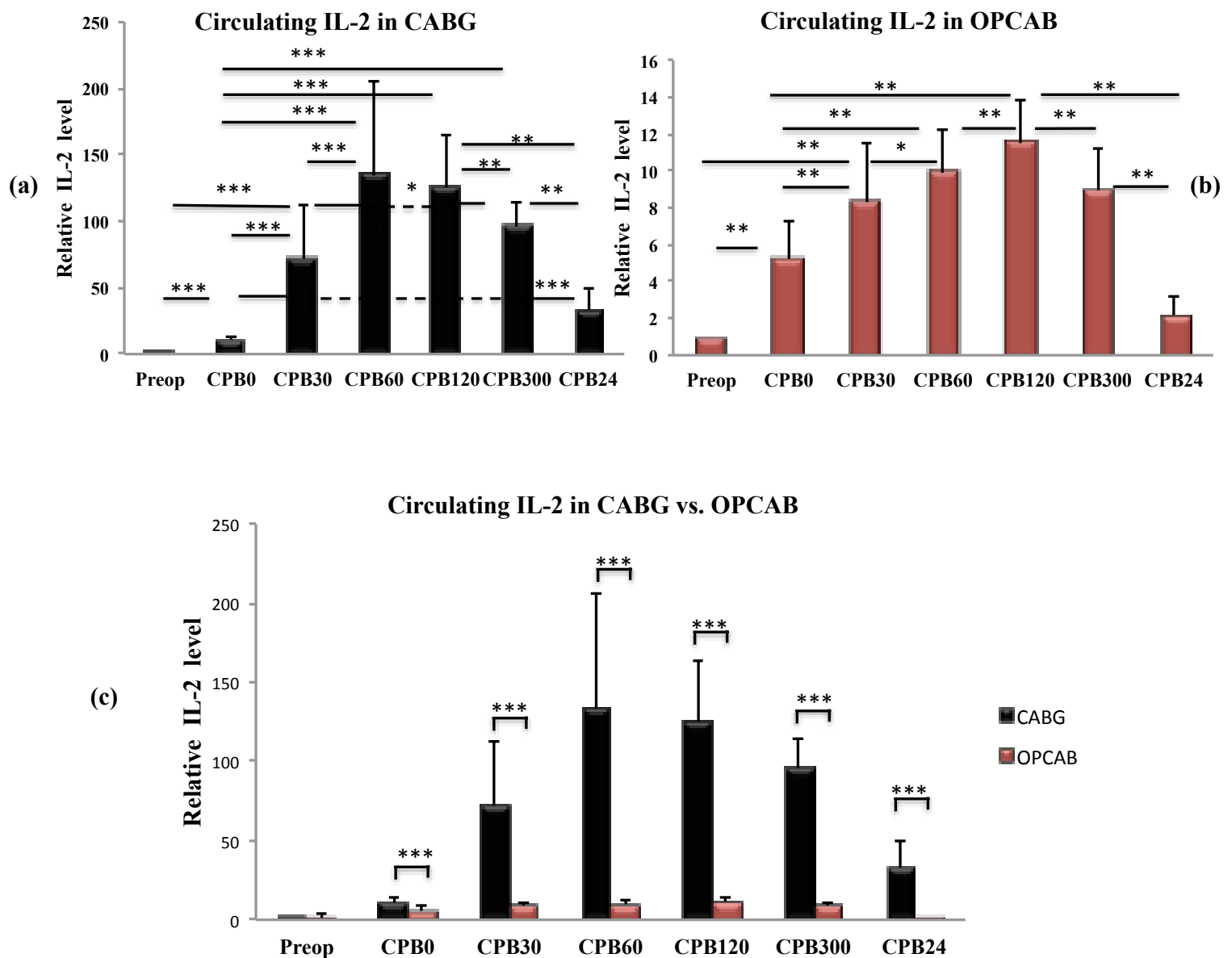


Figure 5.5 The circulating levels of IL-2 in response to CABG

There was a significant increase in circulating IL-2 levels in the CABG group, more prominent at CPB60, CPB120 in relation to other time points however they were all statically significant to the baseline (a), same pattern was found in the OPCAB group, however the trend was much less (b). When comparing both groups together (c), the CABG group showed signification elevation in IL-2 levels in response to CPB as compared to the OPCAB group. (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.2.2 Interleukin-4 (IL-4)

Circulating IL-4 levels showed transient enhancement. This is similar to IL-2 pattern for both CABG and OPCAB groups, peaking at CPB60 on-pump (Figure 5.6 a) and at CPB120 corresponding time point for the OPCAB group (Figure 5.6 b). However, their levels declined nearly to the baseline level after 24 hours from the onset of the CPB.

Even though the level of IL-4 declines after 24 hours but still significantly different to the preoperative baseline in both groups. There was significant difference to all other time points with varying significant p -values (<0.001 to <0.05) with the exception between CBP30 and CPB120 time points where there was no difference ($p = 0.3$).

When comparing both groups together, the CABG group showed significant increase in circulating IL-4 levels from the initiation of CPB as compared to the corresponding time point in OPCAB until 5 hours later (CPB300). P -value was <0.001 between time points CPB0 to CPB120 and was < 0.05 in CPB300. There was no significant difference after 24 hours ($p = 0.12$) (Figure 5.6 c).

The increase in IL-4 levels in the CABG group was much higher from the onset of CPB until 300 minutes compared to the OPCAB group as follows: CPB0 (13 ± 2 vs. 9 ± 3 -fold, $p < 0.001$), CPB30 (117 ± 50 vs. 12 ± 4 -fold, $p < 0.001$), CBP60 (137 ± 34 vs. 17 ± 5 -fold, $p < 0.001$), CPB120 (115 ± 15 vs. 22 ± 7 -fold, $p < 0.001$), CPB300 (42 ± 46 vs. 13 ± 16 -fold, $p < 0.05$) and CPB24 (3 ± 2 vs. 2 ± 2 -fold, $p = 0.12$).

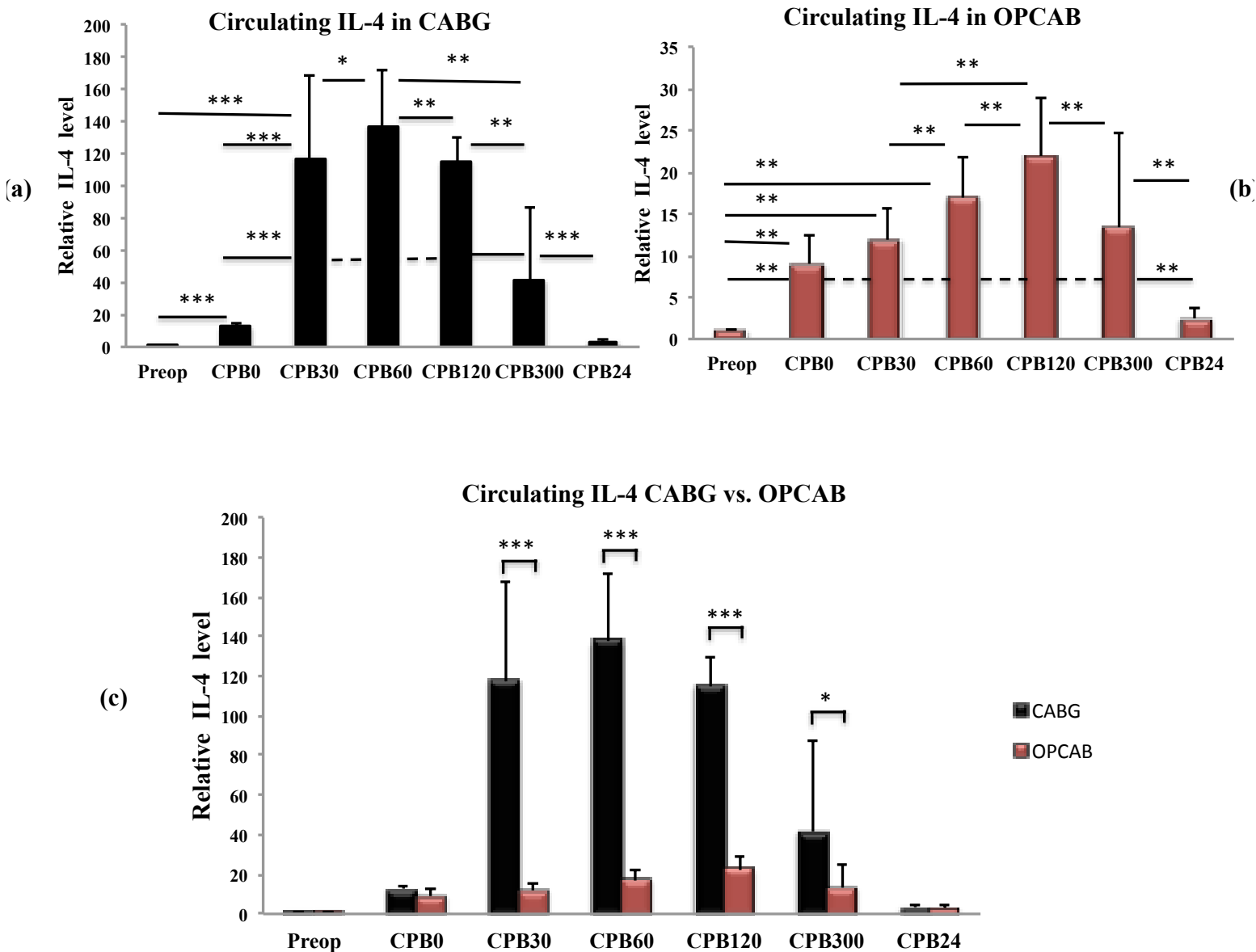


Figure 5.6 The circulating levels of IL-4 in response to CABG

There was significant increase in circulating IL-4 levels mainly at CPB30, CPB60 and CPB120 minutes in relation to other time points (a), same pattern was found in the OPCAB group (b), however the trend was much less. When comparing both groups together (c), the CABG group showed significant elevation in IL-4 level in response to CPB as compared to the OPCAB group from time point CPB0 to CPB300 then become insignificant after 24 hours (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.2.3 Interleukin-6 (IL-6)

IL-6 showed increase in circulating level with a similar pattern of IL-2 where the increase in IL-6 level persist for both groups for more than 24 hours from the onset of the CPB in CABG group and its corresponding time in OPCAB. IL-6 peaked at CPB60 and CPB120 time point and there was a significant difference at all time points in comparison to the baseline, (p -value in relation to preoperative baseline is < 0.001 at all time points). There was no difference between CPB30 and CPB24 or between the peaked IL-6 level at CPB60 and CPB120 ($p= 0.11$ and 0.30 , respectively) (Figure 5.7 a).

Even though the pattern is similar in the two groups but the IL-6 level was much smaller in the OPCAB group (p -value in relation to preoperative baseline is < 0.01 at all time points) (Figure 5.7 b). Again when comparing both groups together, the CABG group showed significant increase in circulating IL-6 level from the initiation of CPB and the corresponding time point at all times ($p < 0.001$ at all time points) (Figure 5.7 c).

The increase in IL-6 level in the CABG group was much higher from the onset of CPB to the next 24 hours compared to the OPCAB group as follows: CPB0 (13 ± 3 vs. 7 ± 3 -fold, $p < 0.001$), CPB30 (135 ± 47 vs. 13 ± 3 -fold, $p < 0.001$), CBP60 (250 ± 58 vs. 18 ± 3 -fold, $p < 0.001$), CPB120 (261 ± 50 vs. 25 ± 6 -fold, $p < 0.001$), CPB300 (187 ± 38 vs. 15 ± 5 -fold, $p < 0.001$) and CPB24 (107 ± 38 vs. 15 ± 5 -fold, $p < 0.001$).

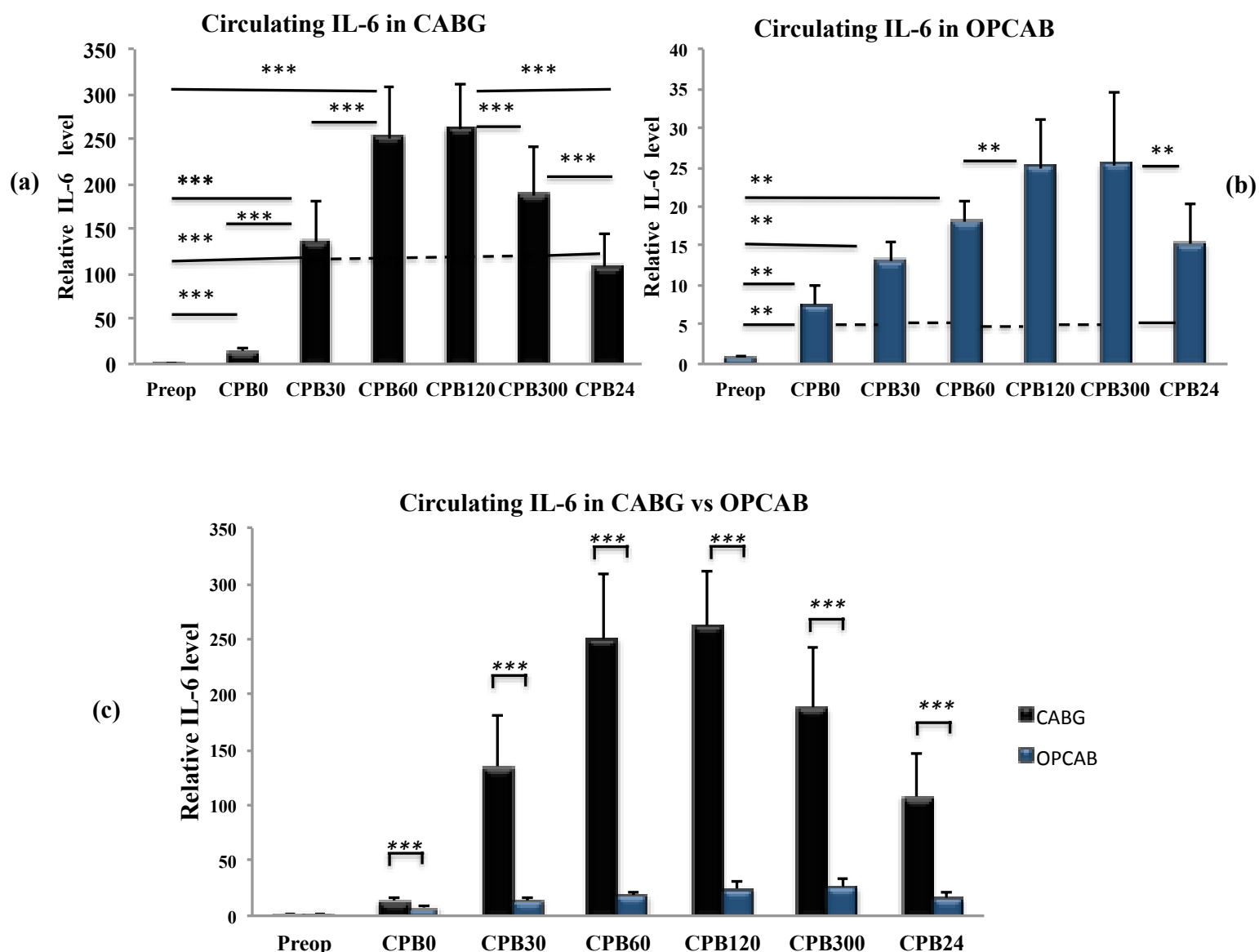


Figure 5.7 The circulating levels of IL-6 in response to CABG

There was significant increase in circulating IL-6 levels for 24 hours from the initiation of the CPB as compared to preoperative baseline (a), however same pattern of increase in IL-6 level was found in the OPCAB group but with much smaller scale (b). When comparing both groups together (c), the CABG group showed significant elevation in IL-6 level in response to CPB as compared to the OPCAB group in all time-points (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.2.4 Interleukin-8 (IL-8)

The results for circulating IL-8 levels showed its transient enhancement in the similar pattern of previous interleukins for both CABG and OPCAB groups, peaked at 60 minutes on-pump (CPB60) (Figure 5.8 a) and at 120 minutes corresponding time to CPB for the OPCAB group (CPB120) but with lower scale (Figure 5.8 b). However, their levels declined nearly to the baseline after 24 hours but still highly significant in CABG and OPCAB groups ($p < 0.001$ and < 0.01 , respectively).

When comparing both groups together, the CABG group showed significant increase in circulating IL-8 level from the initiation of CPB as compared OPCAB, only until 1 hours (CPB60) then declined with no significant difference from CPB120 to 24 hour later (Figure 5.8 c).

The level of increase in IL-8 levels between the CABG and the OPCAB groups were as follows: CPB0 (8 ± 3 vs. 6 ± 3 -fold, $p < 0.01$), CPB30 (35 ± 26 vs. 7 ± 7 -fold, $p < 0.01$), CBP60 (44 ± 23 vs. 13 ± 8 -fold, $p < 0.001$), CPB120 (16 ± 23 vs. 15 ± 11 -fold, $p = 0.88$), CPB300 (10 ± 14 vs. 10 ± 9 -fold, $p = 0.1$) and CPB24 (3 ± 1 vs. 3 ± 2 -fold, $p = 0.5$).

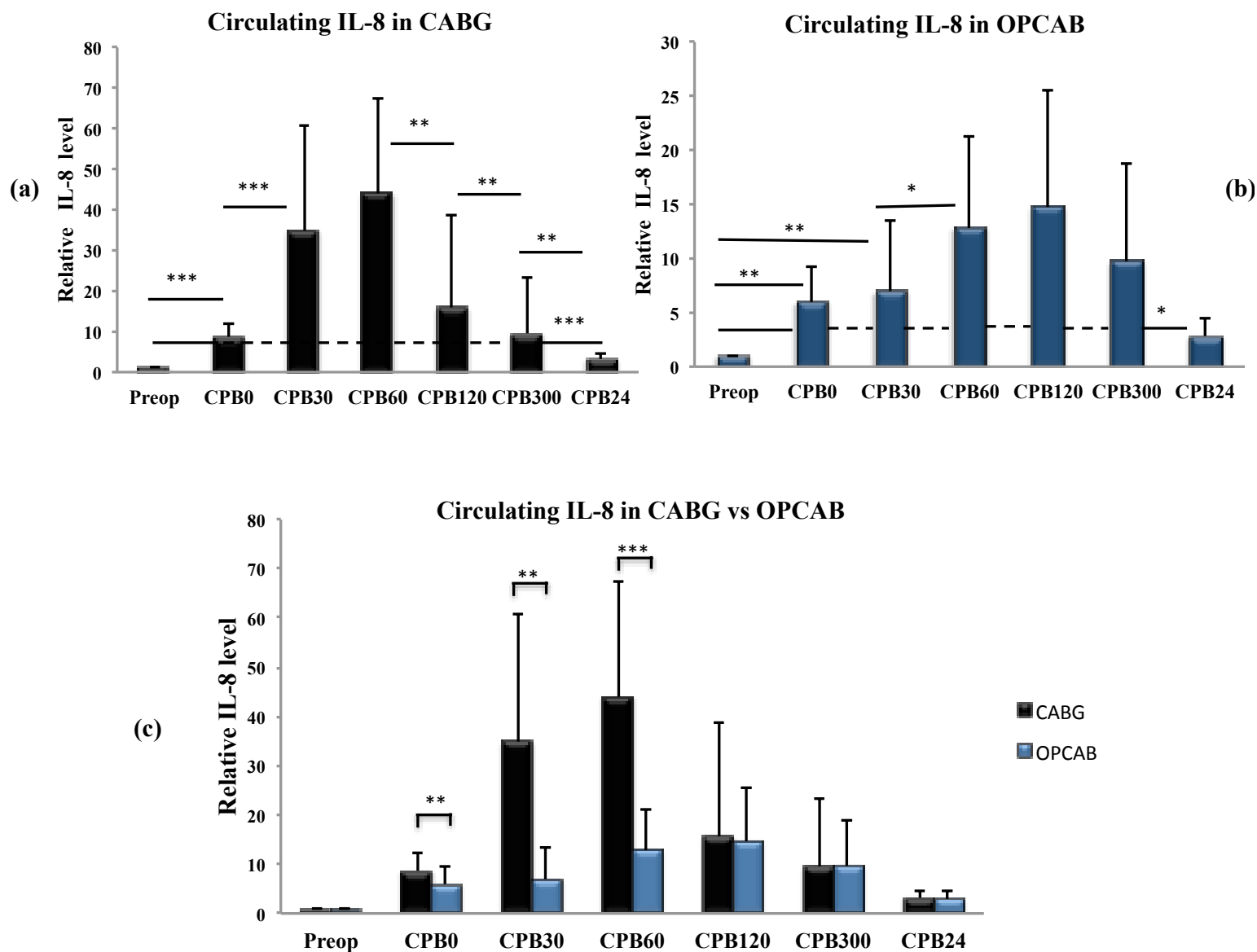


Figure 5.8 The circulating levels of IL-8 in response to CABG

There was significant increase in circulating IL-8 levels for 24 hours from the initiation of the CPB as compared to preoperative baseline (a), however same pattern of increase in IL-8 level was found in the OPCAB group but with smaller scale (b). When comparing both groups together (c), the CABG group showed significant elevation in IL-8 level only in response to CPB0, CPB30 and CPB60 as compared to the OPCAB (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.2.5 Interleukin-10 (IL-10)

Circulating IL-10 levels showed transient elevation in similar pattern to previous interleukins for both CABG and OPCAB groups, peaked at 60 minutes on-pump (Figure 5.9 a) and at 120 minutes off-pump (Figure 5.9 b). However, their levels reduced nearly to the baseline level after 24 hours from the onset of the CPB but remain statistically significant ($p < 0.001$ and < 0.01 , respectively).

In the on-pump group there was a significant difference to all other time points with varying significant p - values (< 0.001 to < 0.05) with the exception between CBP0 and both CPB120 and CPB300 ($p = 0.11$ and 0.38 respectively) and between those with the highest level at CPB30 and CPB60 ($p = 0.06$).

When comparing both groups together, the CABG group showed significant increase in circulating IL-10 level 30 minutes after the initiation of CPB as compared to the corresponding time point in OPCAB until 5 hours later (CPB300). P-value was < 0.001 between time points CPB30 and CPB60 and was < 0.05 in CPB120 and CPB300. There was no significant difference after 24 hours ($p = 0.056$) (Figure 5.9 c).

The increase in the IL-10 levels in both groups were as follows: CPB0 (3 ± 2 vs. 3 ± 2 -fold, $p = 0.74$), CPB30 (50 ± 36 vs. 7 ± 7 -fold, $p < 0.001$), CBP60 (69 ± 37 vs. 9 ± 7 -fold, $p < 0.001$), CPB120 (39 ± 36 vs. 12 ± 8 -fold, $p < 0.01$), CPB300 (16 ± 16 vs. 6 ± 5 -fold, $p < 0.05$) and CPB24 (3 ± 2 vs. 2 ± 1 -fold, $p = 0.056$).

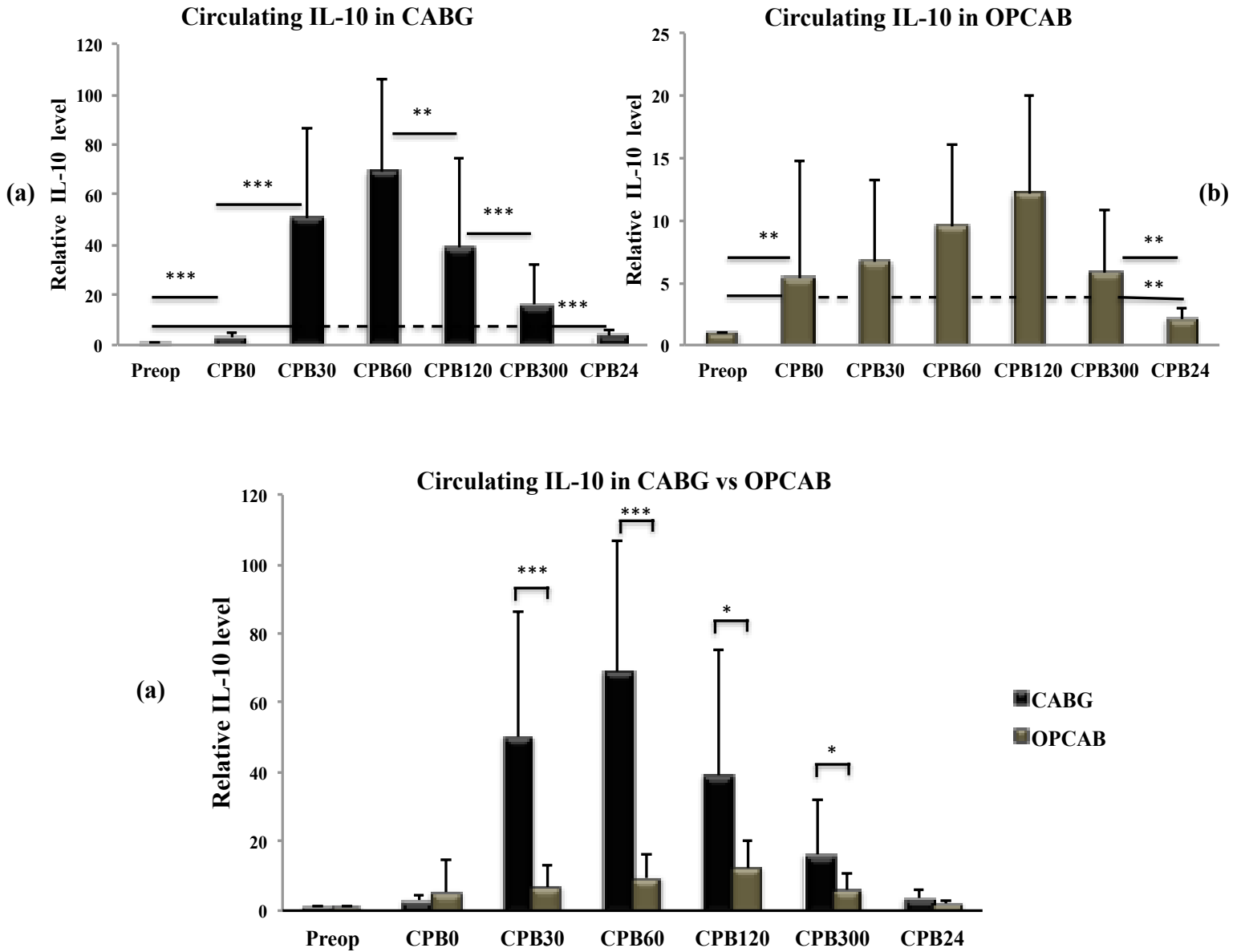


Figure 5.9 The circulating levels of IL-10 in response to CABG

There was significant increase in circulating IL-10 levels mainly at CPB30 and CPB60 minutes in relation to other time points (a), same pattern of transient increase of IL-10 was found in the OPCAB group (b), however the trend was much less. When comparing both groups together (c), the CABG group showed significant elevation in IL-10 level in response to CPB until 300 minutes later then become insignificant as compared to the OPCAB group (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.2.6 Interleukin-12RB1 (IL-12RB1)

Circulating IL-12RB1 level results, showed transient enhancement in the similar pattern to other interleukins for both CABG and OPCAB groups with highest level after 60 minutes and subsequent decrease after 24 hours. However, remain statistically significant to the baseline. There was significant difference to all other time points with varying significant p - values (< 0.001 to < 0.01) with exception between CBP0 and CPB24 ($p = 0.5$) and between CPB30 and both CPB120 and 300 ($p = 0.6$ and 0.12 , respectively) in on-pump CABG group (Figure 5.10 a).

OPCAB group also showed significant difference among their time points except with corresponding times CPB0 and both CPB300 and 24 ($p = 0.6$ and 0.9 respectively), between CPB30 and both CPB60 and 120 ($p = 0.07$ and 0.15 , respectively) and between CPB60 and CBP120 ($p = 0.06$) (Figure 5.10 b).

When comparing both groups together, the CABG group showed significant increase in circulating IL-12RB1 level after 30 minutes of CPB as compared to the corresponding time point in OPCAB until 5 hours later (CPB300). P-values were <0.001 between time points CPB30 – CPB120 and < 0.01 in CPB300. There was no significant difference at beginning of CPB or after 24 hours ($p = 0.12$) (Figure 5.10 c).

The increase in the IL-12RB1 levels in both groups were as follows: CPB0 (4 ± 3 vs. 4 ± 1 -fold, $p = 0.94$), CPB30 (36 ± 21 vs. 9 ± 2 -fold, $p < 0.001$), CBP60 (56 ± 27 vs. $7 \pm$ -fold, $p < 0.001$), CPB120 (40 ± 25 vs. 7 ± 2 -fold, $p < 0.001$), CPB300 (29 ± 24 vs. 4 ± 1 -fold, $p < 0.01$) and CPB24 (3 ± 3 vs. 3 ± 0.5 -fold, $p = 0.43$).

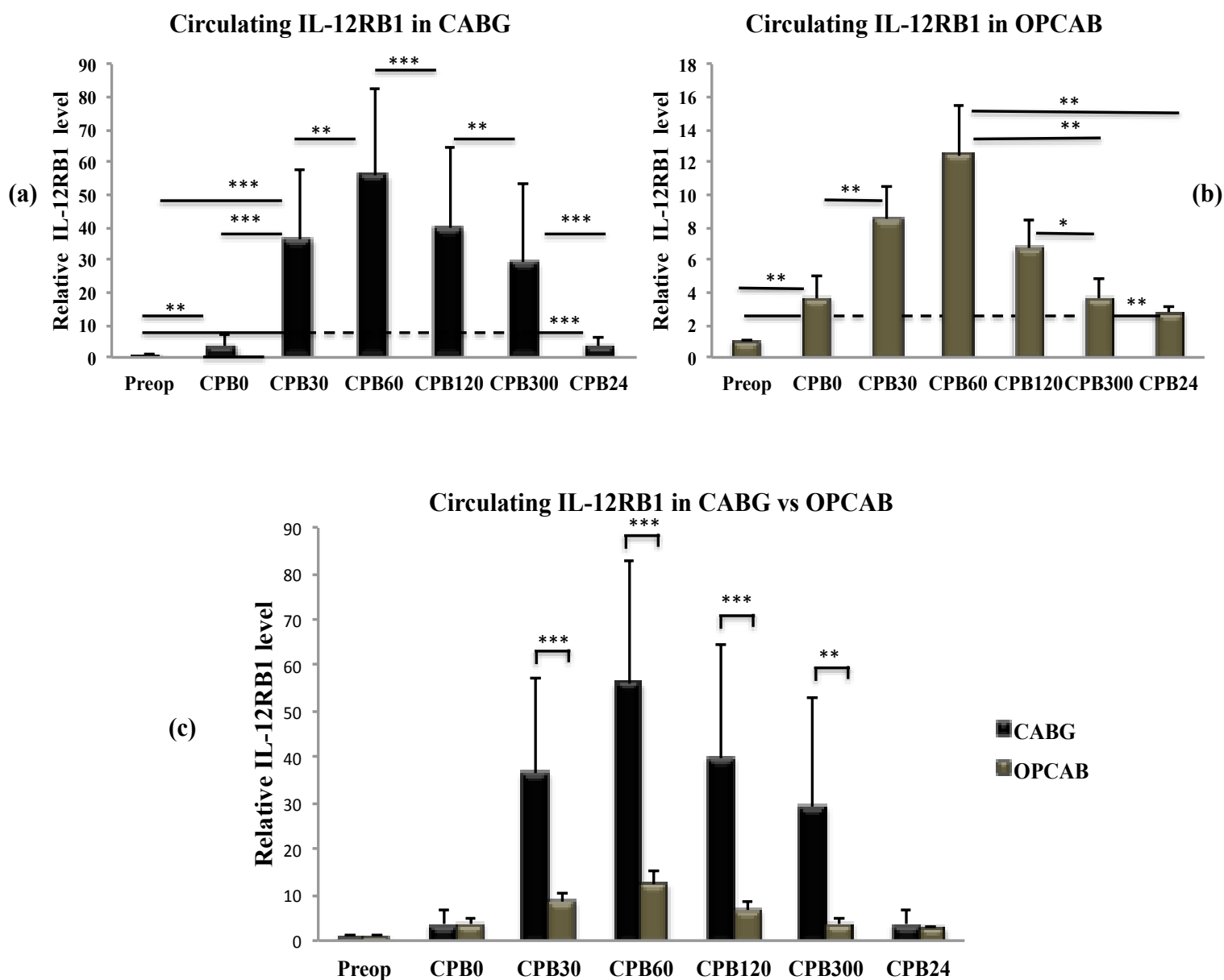


Figure 5.10 The circulating levels of IL-12RB1 in response to CABG

There was significant increase in circulating IL-12 levels mainly at CPB30 and CPB60 minutes in relation to other time points (a), same pattern of transient increase of IL-12 was found in the OPCAB group (b), however the trend pattern was much less. When comparing both groups together, the CABG group showed significant elevation in IL-12 level in response to CPB as compared to the OPCAB group (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.3 Interferon assay

5.5.3.1 Interferon-Alpha (INF- α)

INF- α circulating levels were significantly elevated in both CABG and OPCAB groups. The elevation was transient and declined after 24 hours but remain significantly different from the baseline for both groups. The pattern of elevation was similar in both groups but with much smaller trend in the OPCAB group.

There was significant increase in the INF- α level throughout all time points from the initiation of the CPB until 24 hours later ($p < 0.001$ to < 0.01) and among each time point in the CABG on –pump group with exception between CPB30 and CPB300 were not significant ($p = 0.09$) (Figure 5.11 a). Same result to OPCAB group but with much smaller scale and with exception between corresponding time points CPB300 with both CPB60 and 120 were not significant ($p = 0.4$ and 0.06 , respectively) (figure 5.11 b).

When comparing both groups together, the CABG group showed significant increase in circulating INF- α level at all time points ($p < 0.001$ to < 0.05). The increase in the INF- α levels in both groups were as follows: CPB0 (13 ± 5 vs. 9 ± 3 -fold, $p < 0.05$), CPB30 (132 ± 46 vs. 18 ± 6 -fold, $p < 0.001$), CBP60 (321 ± 74 vs. 28 ± 6 -fold, $p < 0.001$), CPB120 (277 ± 71 vs. 36 ± 4 -fold, $p < 0.001$), CPB300 (167 ± 64 vs. 31 ± 10 -fold, $p < 0.001$) and CPB24 (50 ± 20 vs. 5 ± 2 -fold, $p < 0.001$) (Figure 5.11 c).

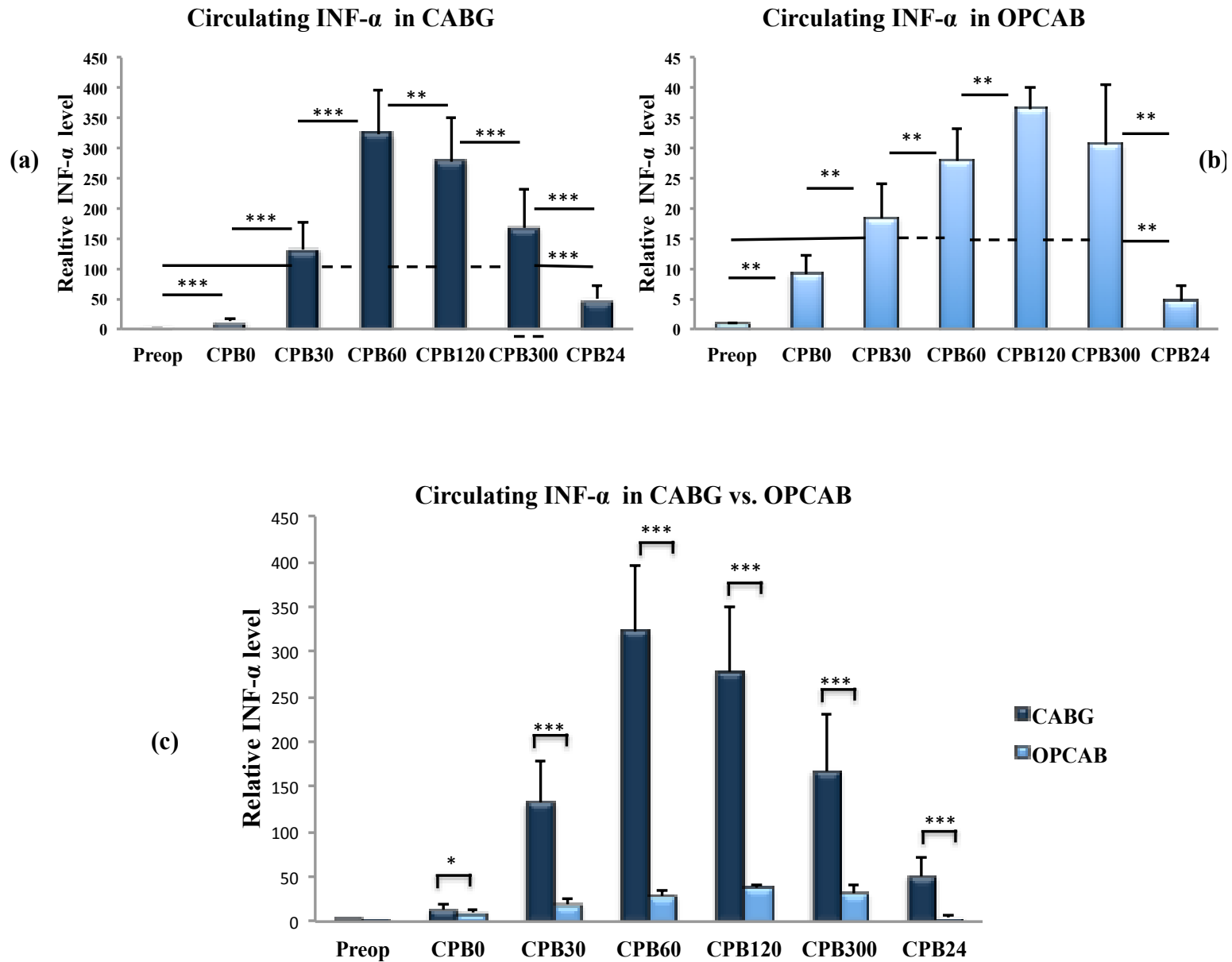


Figure 5.11 The circulating levels of INF- α in response to CABG

There was significant increase in circulating INF- α levels, the highest at 60 and 120 minutes in relation to other time points (a), same pattern of transient increase of INF- α was found in the OPCAB group with much smaller scale (b). When comparing both groups together, the CABG group showed significant elevation in INF- α level in response to CPB as compared to the OPCAB group which is significant at all time points (c) (*= p <0.05, **= p <0.01, ***= p <0.001).

5.5.3.2 Interferon-gamma (INF- γ)

Circulating INF- γ level showed also transient increase with similar transient increase pattern to INF- α , which decreases after 24 hours but still remain significant in both CABG and OPCAB groups ($p < 0.001$ and < 0.01 , respectively). The highest level at on-pump CABG group was noticed at 60 minutes from CPB and at corresponding 120 minutes of the off-pump.

There was significant difference in INF- γ level at all time points ($p < 0.001$ to < 0.01) in on-pump group except between CPB0 and 300 and between CPB30 and 120 ($p = 0.2$ and 0.5 , respectively) (Figure 5.12 a). For the OPCAB group, the significant difference was among all the time points ($p < 0.01$ to < 0.05) the exception was between CPB0 and both CPB30 and 300 ($p = 0.06$ and 0.6 , respectively) and between CPB30 and 300 ($p = 0.8$) (Figure 5.12 b).

Comparing both groups together, the CABG group showed significant increase in circulating INF- γ level from the commencement of CPB until 5 hours later (CPB300) ($p < 0.001$ to < 0.05). The increase in the INF- α levels in both groups were as follows: CPB0 (11 ± 3 vs. 3 ± 2 -fold, $p < 0.001$), CPB30 (47 ± 21 vs. 8 ± 4 -fold, $p < 0.001$), CPB60 (75 ± 36 vs. 16 ± 7 -fold, $p < 0.001$), CPB120 (5 ± 28 vs. 23 ± 8 -fold, $p < 0.01$), CPB300 (14 ± 10 vs. 7 ± 5 -fold, $p < 0.05$) and CPB24 (3 ± 2 vs. 2 ± 1 -fold, $p = 0.3$) (Figure 5.12 c).

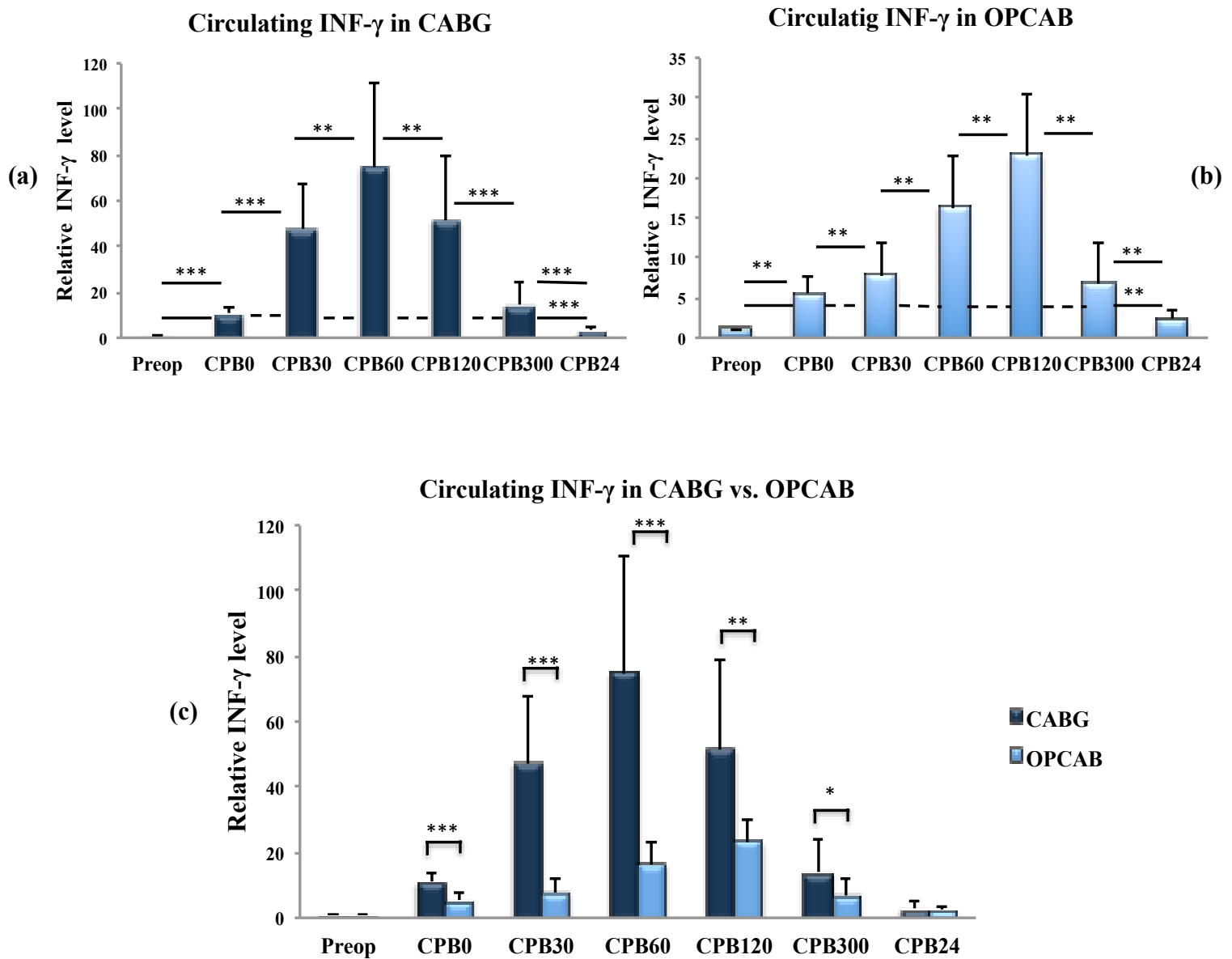


Figure 5.12 The circulating levels of INF- γ in response to CABG

There was significant increase in circulating INF- γ levels more prominent at 60 minutes from CPB (a) and at 120 minutes in OPCAB group (b) with similar pattern and smaller scale. When comparing both groups together, the CABG group showed significant elevation in INF- γ level in response to CPB as compared to the OPCAB group then become insignificant after 24 hours (c), however both groups showed significant increase to their baseline after 24 hours but not when compared together (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.4 Tumour Necrosis Factor-alpha (TNF- α)

The circulating levels of TNF- α also demonstrated transient increase in coronary revascularization surgery for both on and off-pump groups with similar pattern but with much higher trend for the on-pump CABG group. They both showed increase followed by decrease in TNF- α level after 24 hours peaked at the peak of CPB; nevertheless both groups remain statistically significant from their baseline level ($p < 0.001$ for CABG and < 0.05 for OPCAB).

There was significant difference to the all other time points in on-pump CABG group with varying significant p - values ($p = 0.001$ or less) with exception between CPB0 and 300 and between CPB30 and 120 were no difference detected ($p = 0.05$ and 0.8 respectively) (Figure 5.13 a). Similarly for OPCAB p -value varies between < 0.01 to < 0.05 with exception CPB0 and both CPB 300 and 24 ($p = 0.7$ and 0.09 respectively) and between CPB30 and 300 ($p = 0.05$) (Figure 5.13 b).

On comparison between both groups, the CABG group showed significantly higher circulating TNF- α level from the beginning of CPB until 5 hours later (CPB300) than the OPCAB group. The increase in the TNF- α levels in both groups were as follows: CPB0 (7 ± 4 vs. 4 ± 3 -fold, $p < 0.05$), CPB30 (47 ± 17 vs. 7 ± 4 -fold, $P < 0.001$), CPB60 (111 ± 36 vs. 13 ± 9 -fold, $p < 0.001$), CPB120 (50 ± 25 vs. 17 ± 9 -fold, $p < 0.001$), CPB300 (12 ± 11 vs. 4 ± 2 -fold, $p < 0.05$) and CPB24 (2 ± 2 vs. 1 ± 1 -fold, $p = 0.06$) (Figure 5.13 c).

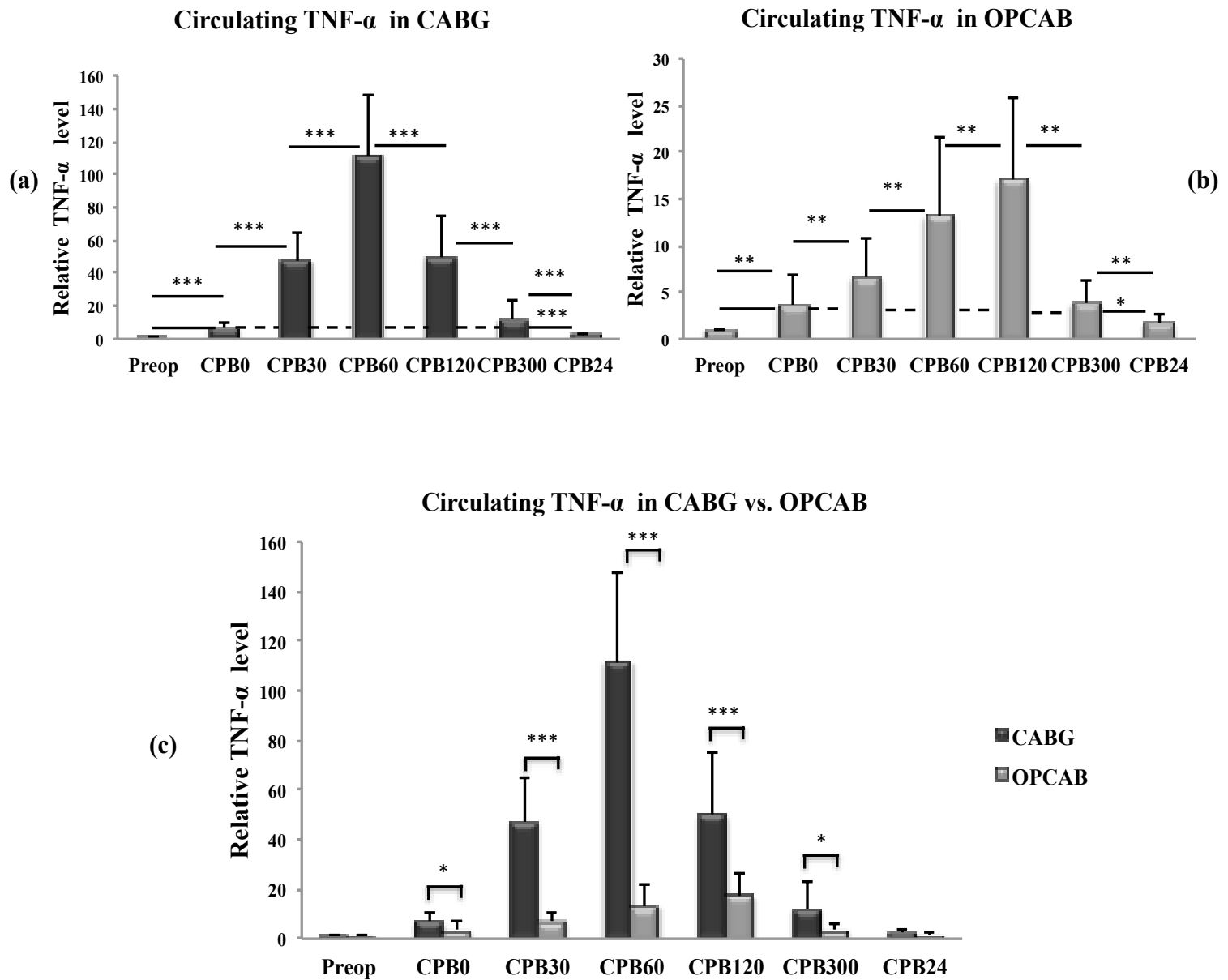


Figure 5.13 The circulating levels of TNF- α in response to CABG

There was significant increase in circulating TNF- α levels mainly at CPB60 minutes in relation to other time points (a), same pattern of transient increase of TNF- α but with significant smaller scale was found in the OPCAB group (b). When comparing both groups together, the CABG group showed significant elevation in TNF- α level in response to CPB as compared to the OPCAB group, no significant difference after 24 hours between the groups (c), (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.5 Chemokine (C-X-C motif) Ligand 9 (CXCL9)

The result here also showed that the CXCL9 is elevated in response to cardiac surgery in both on and off-pump groups however the scale is much higher in the on-pump (CABG) group as expected. Their both results also showed decrease in CXCL9 level after 24 hours, which is also remain significantly different from the pre operative baseline level.

Level of CXCL9 among the time points of each group also significantly different, p value varies from < 0.001 to < 0.01 in CABG group with exception between CPB60 and 120 ($p = 0.14$) (Figure 5.14 a). Also p value ranged from < 0.01 to < 0.05 in OPCAB group with exception between corresponding time point CPB0 and 24 hour later ($p = 0.43$) and between CPB30 and 300 ($p = 0.75$) (Figure 5.14 b).

On comparison between both groups, the CABG group showed higher significant increase in circulating CXCL9 level from the onset of the CPB until 5 hours later (CPB300). The increase in the CXCL9 levels in both groups were as follows: CPB0 (6 ± 2 vs. 4 ± 1 -fold, $p < 0.001$), CPB30 (42 ± 18 vs. 7 ± 1 -fold, $p < 0.001$), CPB60 (86 ± 30 vs. 12 ± 2 -fold, $p < 0.001$), CPB120 (90 ± 25 vs. 15 ± 2 -fold, $p < 0.001$), CPB300 (22 ± 15 vs. 7 ± 1 -fold, $p < 0.01$) and CPB24 (2 ± 2 vs. 1 ± 0.4 -fold, $p = 0.25$) (Figure 5.14 c).

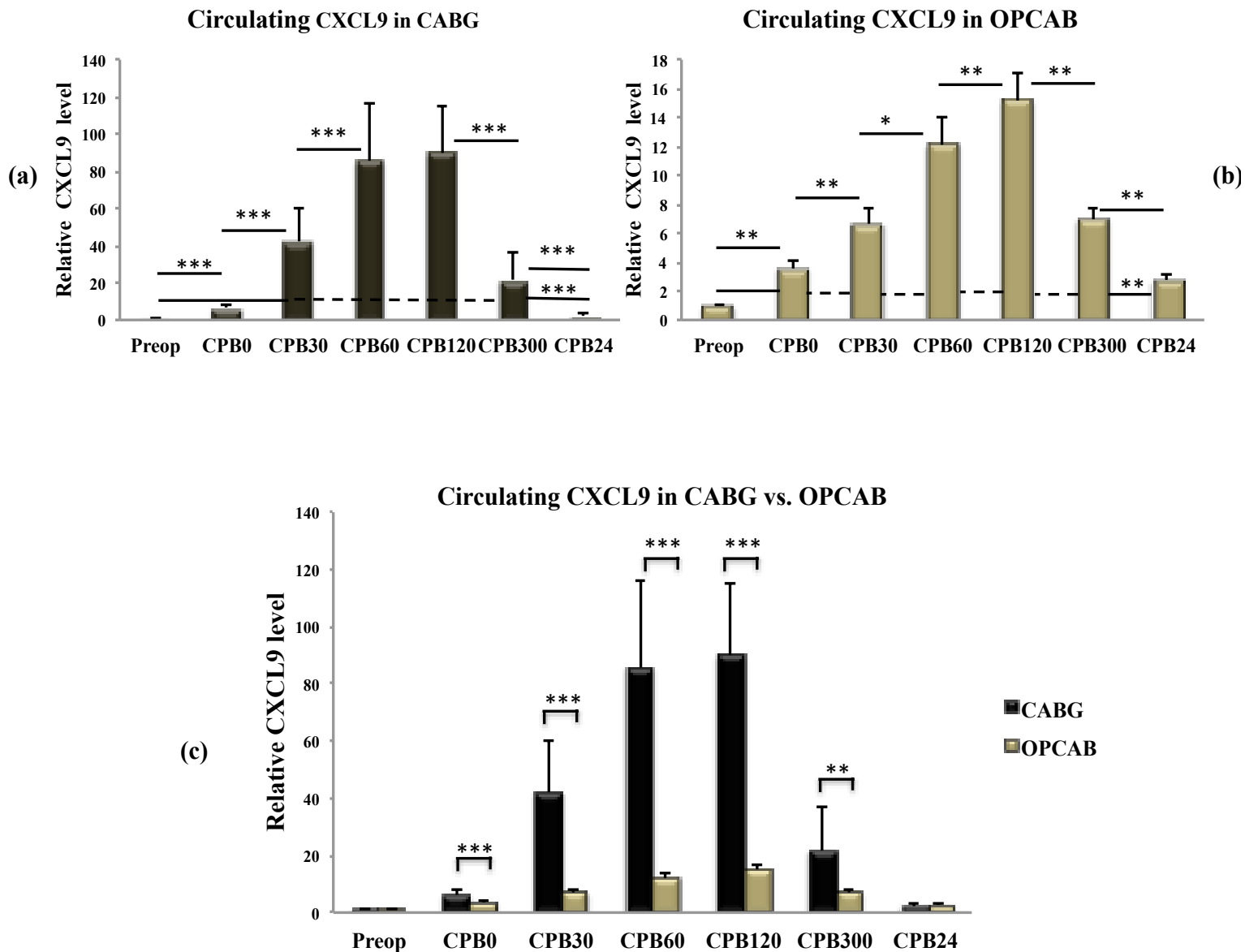


Figure 5.14 The circulating levels of CXCL9 in response to CABG

There was significant increase in circulating CXCL9 levels in all time points, more prominent at CPB60 and CPB120 minutes in CABG group (a), same pattern of transient increase of CXCL9 was found in the OPCAB group, however the trend was much less (b). When comparing both groups together (c), the CABG group showed significant elevation in CXCL9 level in response to CPB as compared to the OPCAB group, which became insignificant after 24 hours from initiation of CPB (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.6 Nuclear Factor Kappa-of B cells (NF-kB)

NF-kB data showed increase of its circulating level in relation to CPB time points peaked at 60 minutes of CPB, then starts to decline after termination of CPB until measured after 24 however still statistically different from the baseline ($p < 0.001$). Similar pattern for the OPCAB group showed rise in NF-kB level followed by declining in 24 hours, which is also still significant ($p < 0.01$)

There was significant difference in NF-kB level at all time points ($p < 0.001$ to < 0.05) in on-pump group except between CPB300 and both CPB60 and 120 ($p = 0.17$ & 0.58 respectively) and between CPB30 and 24 hours later ($p = 0.06$) (Figure 5.15 a). In the OPCAB group, the significant difference was among the all time points ($p < 0.01$ to < 0.05) the exception was between CPB0 and 120 ($p = 0.03$) and between CPB300 and 24 hour later ($p = 0.6$) (Figure 5.15 b).

Comparing both groups together, the CABG group showed significant increase in circulating NF-kB level at all time points from the preoperative baseline until 24 hours later ($p < 0.001$). The increase in the INF- α levels in both groups were as follows: CPB0 (18 ± 5 vs. 9 ± 4 -fold, $p < 0.001$), CPB30 (86 ± 28 vs. 20 ± 7 -fold, $p < 0.001$), CBP60 (253 ± 80 vs. 29 ± 8 -fold, $p < 0.001$), CPB120 (287 ± 74 vs. 9 ± 9 -fold, $p < 0.001$), CPB300 (278 ± 41 vs. 4 ± 2 -fold, $p < 0.001$) and CPB24 (115 ± 41 vs. 3 ± 1 -fold, $p < 0.001$) (Figure 5.15 c).

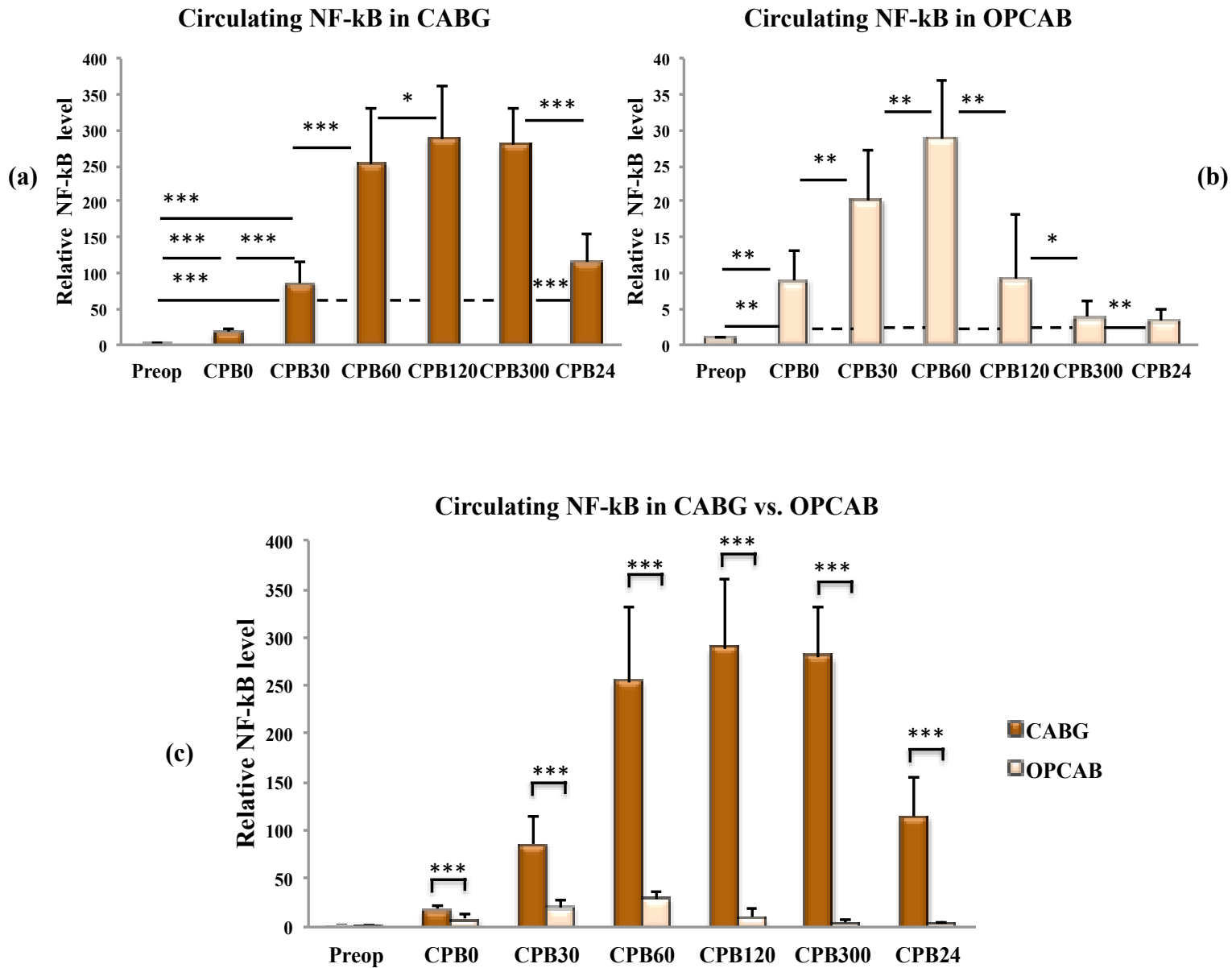


Figure 5.15 The circulating levels of NF-kB in response to CABG

The increase in circulating NF-kB levels was at its highest level after 120 minutes from CPB in relation to other time points (a) and was markedly higher than OPCAB group, however they showed similar pattern of increase of NF-kB (b). When comparing both groups together, the CABG group showed significant elevation in NF-kB level in response to CPB as compared to the OPCAB group at all time points (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

5.5.7 Mitogen-Activated Protein Kinase (MAPK)

5.5.7.1 MAPK1= P42-MAPK

MAPK1 or P40-MAPK circulating level exhibited elevation in response to cardiac surgery in both on and off-pump groups. However, the scale is much higher in the on-pump (CABG) group as anticipated. Both groups showed decrease in MAPK1 level after 24 hours, which is also remain significantly different from the preoperative baseline level ($p < 0.001$ for CABG and < 0.01 for OPCAB) but not between the groups ($p = 0.5$) suggesting transient increase to CPB.

Among the time points of each group also significantly different, p value varies from < 0.001 to < 0.05 in CABG group with the exception between CPB30 and 300 ($p = 0.68$) and between CBP60 and 120 ($p = 0.21$) (Figure 5.16 a). In OPCAB group the p value ranged from < 0.01 to < 0.05 with the exception between corresponding time point CPB0 and CPB24 ($p = 0.11$) and between CPB30 and 300 ($p = 0.69$) (Figure 5.16 b).

When compared both groups together at each time point, the CABG group showed larger significant increase in circulating MAPK1 levels from the onset of the CPB until 5 hours later (CPB300) compared to OPCAB group, which they were as follows: CPB0 (6 ± 2 vs. 4 ± 2 -fold, $p < 0.05$), CPB30 (42 ± 20 vs. 11 ± 4 -fold, $p < 0.001$), CBP60 (128 ± 42 vs. 16 ± 5 -fold, $p < 0.001$), CPB120 (136 ± 50 vs. 22 ± 7 -fold, $p < 0.001$), CPB300 (48 ± 36 vs. 11 ± 4 -fold, $p < 0.01$) and CPB24 (4 ± 3 vs. 3 ± 2 -fold, $p = 0.5$) (Figure 5.16 c).

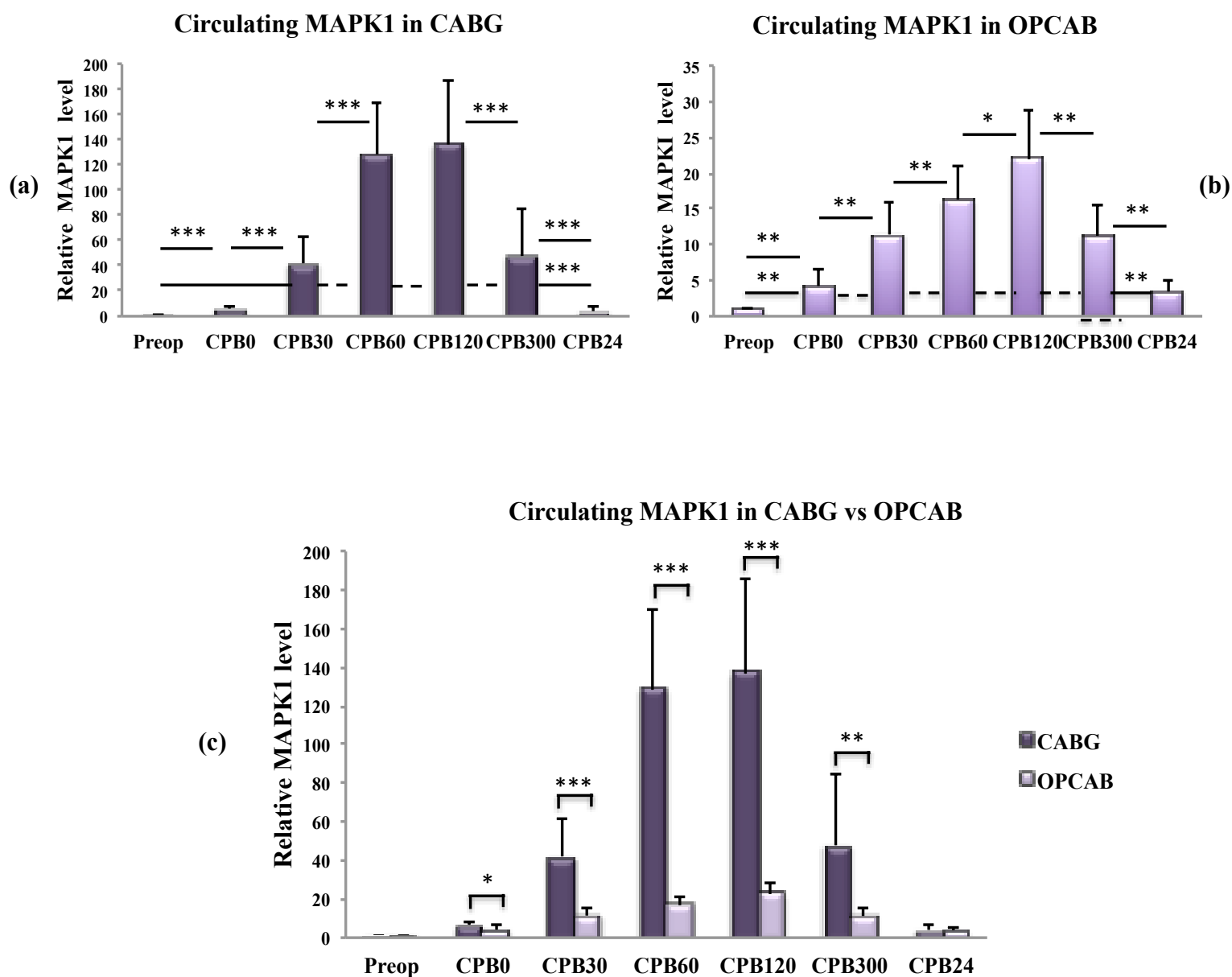


Figure 5.16 The circulating levels of MAPK1 in response to CABG

There was significant increase in circulating MAPK1 levels mainly at CPB60 and CPB120 in relation to other time points (a), same pattern of transient increase of MAPK1 was found in the OPCAB group (b), however the trend was much less. When comparing both groups together, the CABG group showed significant elevation in MAPK1 level in response to CPB as compared to the OPCAB group (c) points (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.7.2 MAPK14= P38-MAPK

MAPK14 or P38-MAPK results showed similar result to MAPK1 where circulating level elevated in response to cardiac surgery in both on and off-pump groups with smaller scale of OPCAB compared to the on-pump (CABG) group. Also with decrease in MAPK14 level after 24 hours in both groups but still remain significantly different from the pre operative baseline level ($p < 0.001$ for CABG and < 0.01 for OPCAB) but not between the groups ($p = 0.46$).

There was significant difference among the time points of each group; p value varies from < 0.001 to < 0.01 in CABG group with the exception between CPB30 and 300 ($p = 0.46$) and between CBP60 and 120 ($p = 0.79$) (Figure 5.17 a). P value ranged from < 0.01 to < 0.05 in OPCAB group, with the only exception between corresponding time point CPB0 and 24 hour later ($p = 0.58$) (Figure 5.17 b).

When compared both groups together, the CABG group showed significant increase in circulating MAPK14 (P38-MAKP) levels from the onset of the CPB0 to CPB300, compared to OPCAB group as follows: CPB0 (6 ± 4 vs. 4 ± 2 -fold, $p < 0.05$), CPB30 (50 ± 26 vs. 8 ± 3 -fold, $p < 0.001$), CBP60 (164 ± 61 vs. 16 ± 5 -fold, $p < 0.001$), CPB120 (159 ± 44 vs. 23 ± 6 -fold, $p < 0.001$), CPB300 (58 ± 44 vs. 12 ± 4 -fold, $p < 0.01$) and CPB24 (4 ± 2 vs. 3 ± 2 -fold, $p = 0.46$) (Figure 5.17 c).

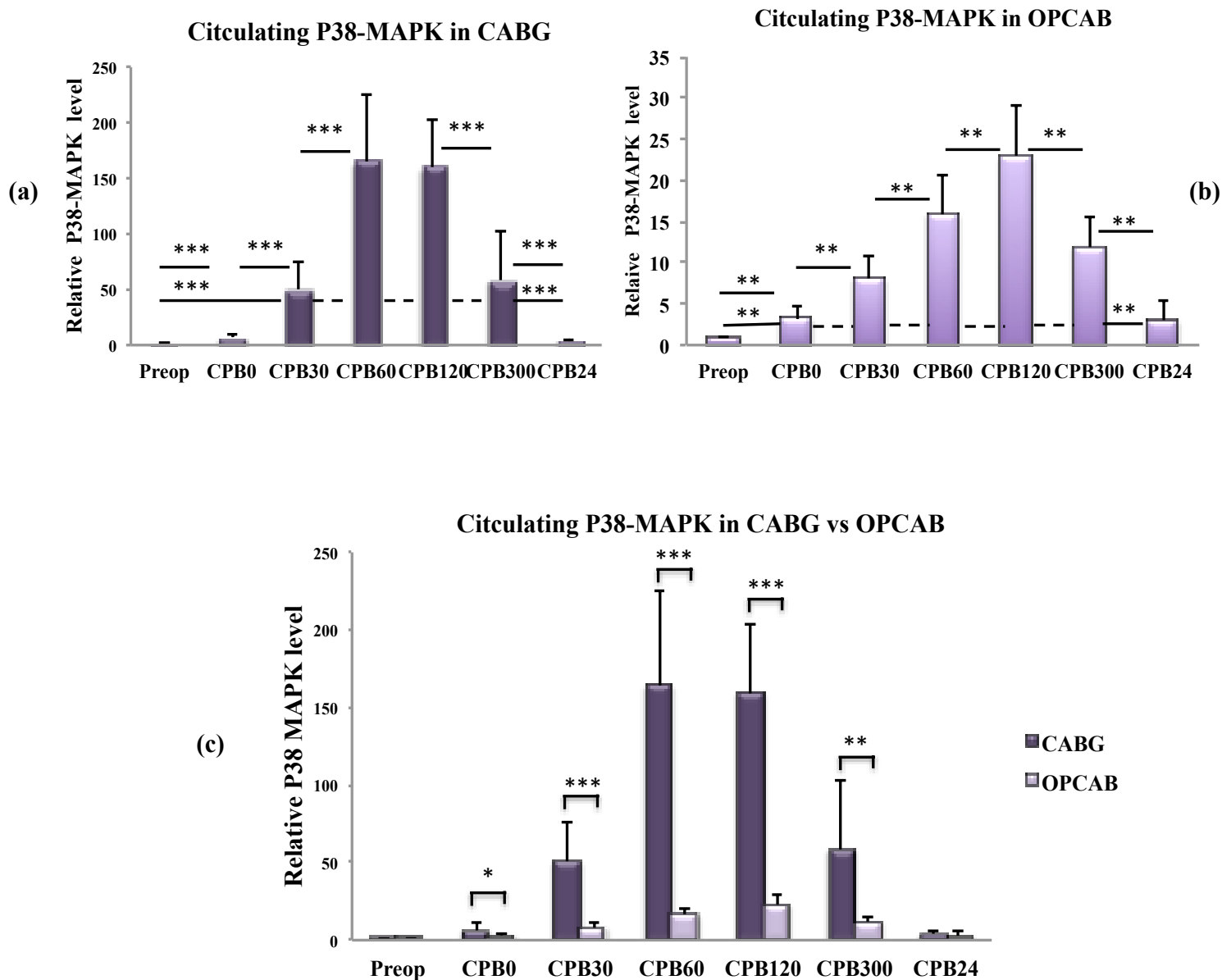


Figure 5.17 The circulating levels of MAPK14 (P38-MAPK) in response to CABG

There was significant increase in circulating MAPK14 levels mainly at CPB30 and CPB60 minutes in relation to other time points, same pattern of transient increase of MAPK14 was found in the OPCAB group, however the trend was much less. When comparing both groups together, the CABG group showed significant elevation in MAPK14 level in response to CPB as compared to the OPCAB group (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.8 Tumour Necrosis Factor Receptor-Associated Factor (TRAF)

5.5.8.1 TRAF3

With no change in the pattern from previously tested cytokines and chemokines, circulating TRAF3 levels also showed transient elevation for both CABG and OPCAB groups, peaked at 120 minutes on-pump and at 60 minutes off-pump. However their levels declines after 24 hours from the onset time of the CPB but remain statistically significant ($p < 0.001$ and < 0.01 respectively)

In on-pump group (CABG) there was significant difference to the all other time points with varying significant p - values (<0.001 to <0.01) with exception between CPB30 and 300 and between CPB 60 and 120 ($p = 0.98$ and 0.43 respectively) and between those with highest level CPB30 and CPB60 ($p = 0.06$) (Figure 5.18 a). Similarly in OPCAB group the exceptions were between CPB0 and 24 hours and between CPB30 and 300 ($p = 0.87$ and 0.06 respectively) (Figure 5.18 b).

When comparing both groups together, the CABG group showed marked increase from the onset of CPB until 5 hours (CPB300) compared to OPCAB group, the trend of this elevation was as follows: CPB0 (6 ± 3 vs. 3 ± 1 -fold, $p < 0.01$), CPB30 (51 ± 31 vs. 7 ± 3 -fold, $p < 0.001$), CBP60 (166 ± 64 vs. 18 ± 6 -fold, $p < 0.001$), CPB120 (158 ± 63 vs. 26 ± 7 -fold, $p < 0.001$), CPB300 (56 ± 51 vs. 5 ± 2 –fold, $p < 0.01$) and CPB24 (4 ± 2 vs. 3 ± 1 -fold, $p = 0.49$) (Figure 5.18 c).

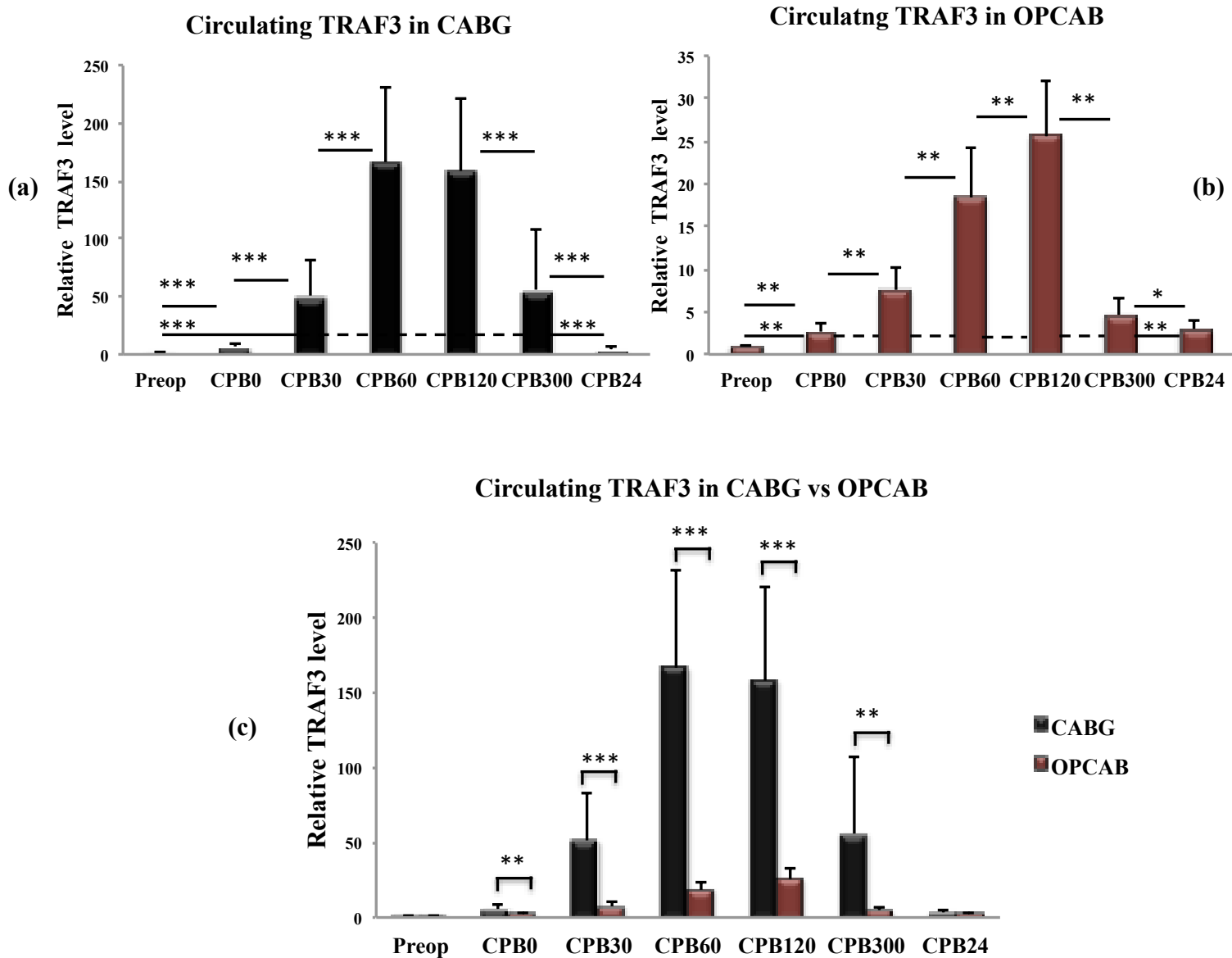


Figure 5.18 The circulating levels of TRAF3 in response to CABG

There was significant increase in circulating TRAF3 levels mainly at CPB30 and CPB60 minutes in relation to other time points, same pattern of transient increase of TRAF3 was found in the OPCAB group, however the trend was much less. When comparing both groups together, the CABG group showed significant elevation in TRAF3 level in response to CPB as compared to the OPCAB group (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

5.5.8.2 TRAF6

Circulating TRAF6 levels similar to TRAF3 showed transient enhancement, which peaked after 60 minutes from of CPB (CPB60) then starts to decline, however remain significant after 24 hours ($p < 0.001$) (Figure 5.19 a). In the similar pattern, the OPCAB groups showed decrease after period of elevation in response to surgery, which is also remain significant to the base line after 24 hours (Figure 5.19 b).

There was significant difference to all time points with varying significant p - values (<0.001 to <0.05) with exception between CBP60 and CPB120 time points where there was no difference ($p = 0.12$). When comparing both groups, the CABG group showed significant increase in circulating TRAF6 level from 30 minutes until 120 minutes of CPB (CPB120) as compared to the corresponding time point in OPCAB. The differences become insignificant between the two groups after 120 minutes.

The levels of elevation of TRAF6 in both groups were as follows: CPB0 (4 ± 3 vs. 4 ± 4 -fold, $p = 0.91$), CPB30 (146 ± 37 vs. 56 ± 31 -fold, $p < 0.001$), CBP60 (233 ± 52 vs. 67 ± 30 -fold, $p < 0.001$), CPB120 (198 ± 73 vs. 83 ± 36 -fold, $p < 0.001$), CPB300 (63 ± 35 vs. 65 ± 27 -fold, $p = 0.82$) and CPB24 (5 ± 3 vs. 3 ± 3 -fold, $p = 0.61$) (Figure 5.19 c).

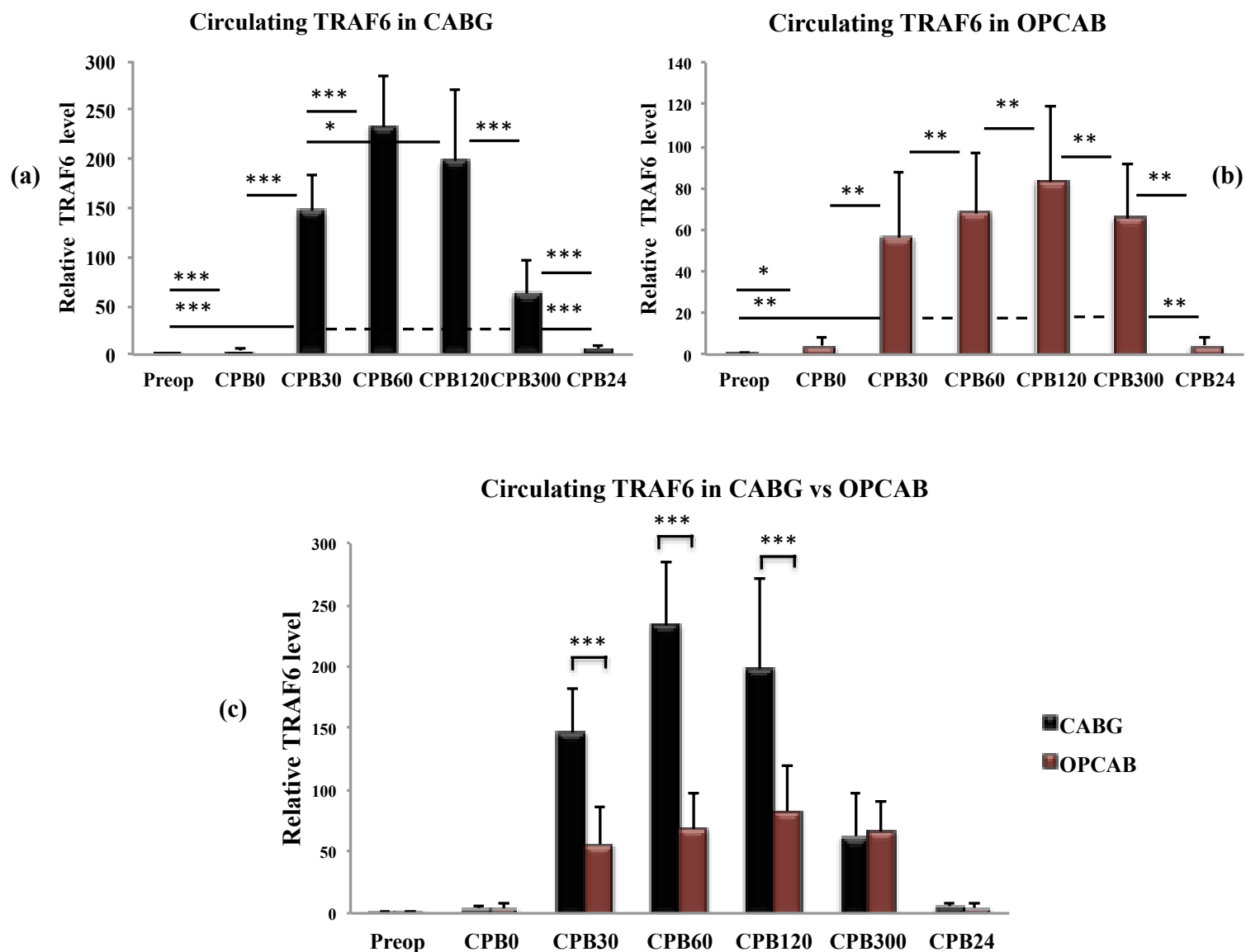


Figure 5.19 The circulating levels of TRAF6 in response to CABG

There was significant increase in circulating TRAF6 levels mainly at CPB30 and CPB60 minutes in relation to other time points, same pattern of transient increase of TRAF6 was found in the OPCAB group, however the trend was much less. When comparing both groups together, the CABG group showed significant elevation in TRAF6 level in response to CPB as compared to the OPCAB group (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.9 TRIF-dependent pathway genes

TRIF is the main pathway for TLR3 and TLR4 signalling but not with the other TLRs, The results here showed different patterns to the other previous cytokines and chemokines involved, even though their levels were significantly increased in response to surgery at different time points, but this was not related to the utilization of CPB and no difference at any time point in both CABG and the OPCAB groups.

5.5.9.1 TLR-Adaptor Molecule 1 (TICAM1=TRIF)

The result for the circulating TRIF levels were transiently increased in both groups with similar pattern to each other, peaking at 60 minutes of CPB (Figures 5.20 a) and at 120 minutes in OPCAB (Figures 5.20 b), and then starts to decline. However it remains significant after 24 hours compared to the baseline level. Both groups demonstrated similar significant difference between their time points with the exception between CPB0 and 24 ($p = 0.98$ for CABG and 0.38 for OPCAB) and between CPB60 and 120 ($p = 0.17$ for CABG and 0.93 for OPCAB), additionally borderline difference between CPB30 and 300 in the OPCAB group ($p = 0.05$)

There was no difference at any time point when compared both group together only at CPB300 where surprisingly for the first time the level was higher in OPCAB ($p = 0.045$). The level of elevation of circulating TRIF to the baseline for CABG and OPCAB groups were as follows: CPB0 (3 ± 2 vs. 4 ± 2 -fold, $p = 0.21$), CPB30 (17 ± 6 vs. 15 ± 4 -fold, $p = 0.43$), CPB60 (25 ± 5 vs. 22 ± 4 -fold, $p = 0.1$), CPB120 (22 ± 8 vs. 23 ± 8 -fold, $p = 0.91$), CPB300 (8 ± 4 vs. 11 ± 4 -fold, $p < 0.05$) and CPB24 (3 ± 3 vs. 1 ± 2 -fold, $p = 0.9$) (Figure 5.20 c).

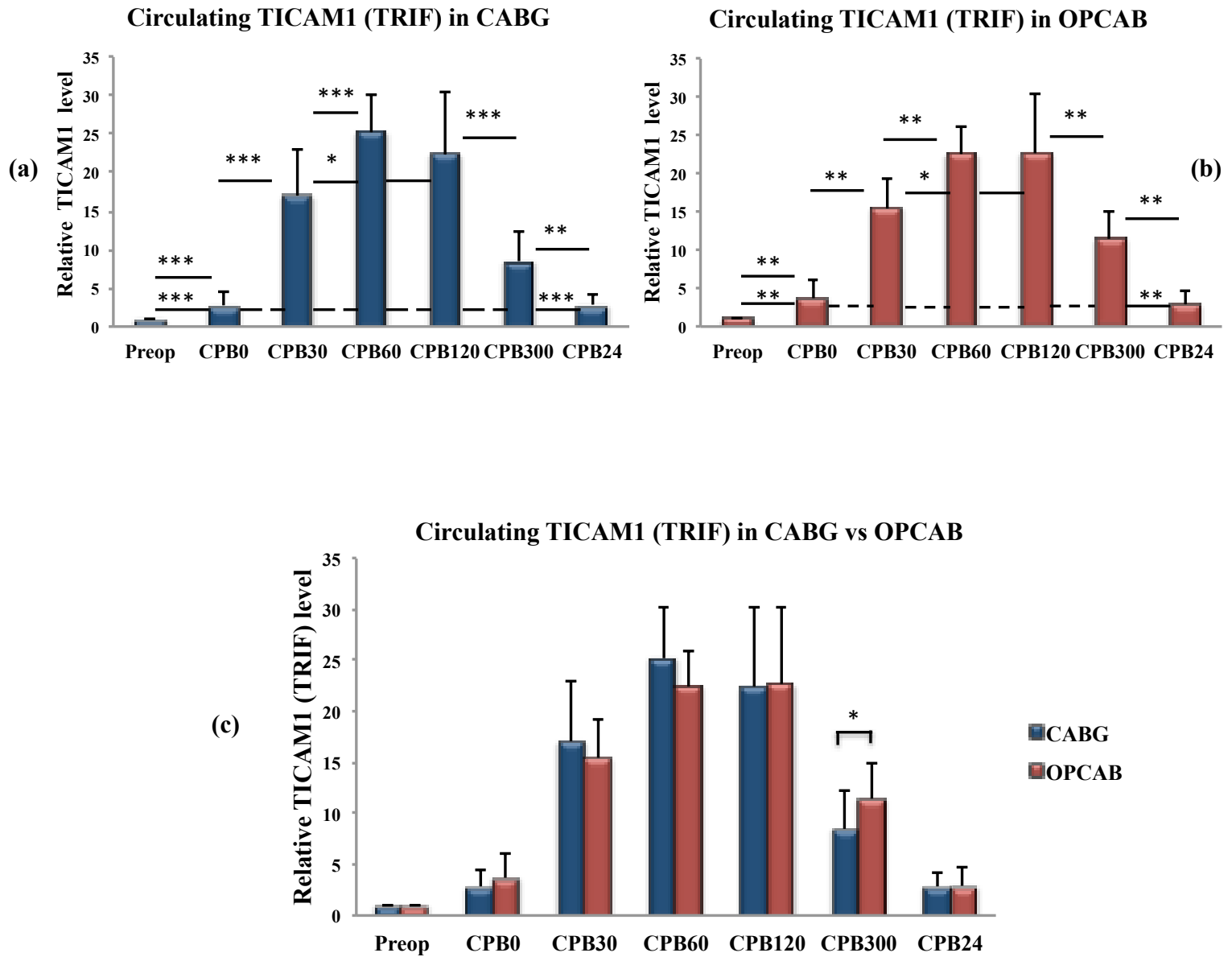


Figure 5.20 The circulating levels of TRIF in response to CABG

There was significant increase among time points in followed by decrease after 24 hours but still remain significantly different in both CABG (a) and OPCAB (b) groups. There is different patterns to the other previous cytokines tested when compared both group together (c), where there was no difference at any time point except for CPB300 time point with borderline difference ($p = 0.045$) (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

5.5.9.2 Interferon Regulatory Factor 3 (IRF3)

IRF3 is a downstream of TRIF-dependent pathway. The results showed similar pattern to TRIF, which applied to both on-pump CABG and OPCAB groups with small difference among their time points. Both shows transient elevation response to surgery and CPB, this elevation then decline after 24 hours but remains significant ($p < 0.001$ and < 0.01 , respectively).

Both groups expressed significant difference between time points ($p < 0.001$ to < 0.05) with the exception between CPB0 and 300 ($p = 0.72$) in CABG group (Figures 5.21 a) and between CPB0 and both CPB 300 and 24 ($p = 0.81$ & 0.09 , respectively) and also between CPB60 and 120 ($p = 0.53$) and between CPB300 and 24 ($p = 0.08$) in the OPCAB group (Figures 5.21 b).

Interestingly, there were no significant statistical differences at any time points from the beginning of CPB until 24 hours later. The levels of the circulating IRF3 in response to CPB and surgery alone (CABG vs. OPCAB) were as follows: CPB0 (3 ± 1 vs. 3 ± 1 -fold, $p = 0.68$), CPB30 (6 ± 2 vs. 7 ± 3 -fold, $p = 0.56$), CBP60 (9 ± 2 vs. 10 ± 3 -fold, $p = 0.1$), CPB120 (11 ± 3 vs. 10 ± 3 -fold, $p = 0.73$), CPB300 (3 ± 2 vs. 4 ± 3 -fold, $p = 0.81$) and CPB24 (2 ± 1 vs. 2 ± 2 -fold, $p = 0.75$) (Figure 5.21 c).

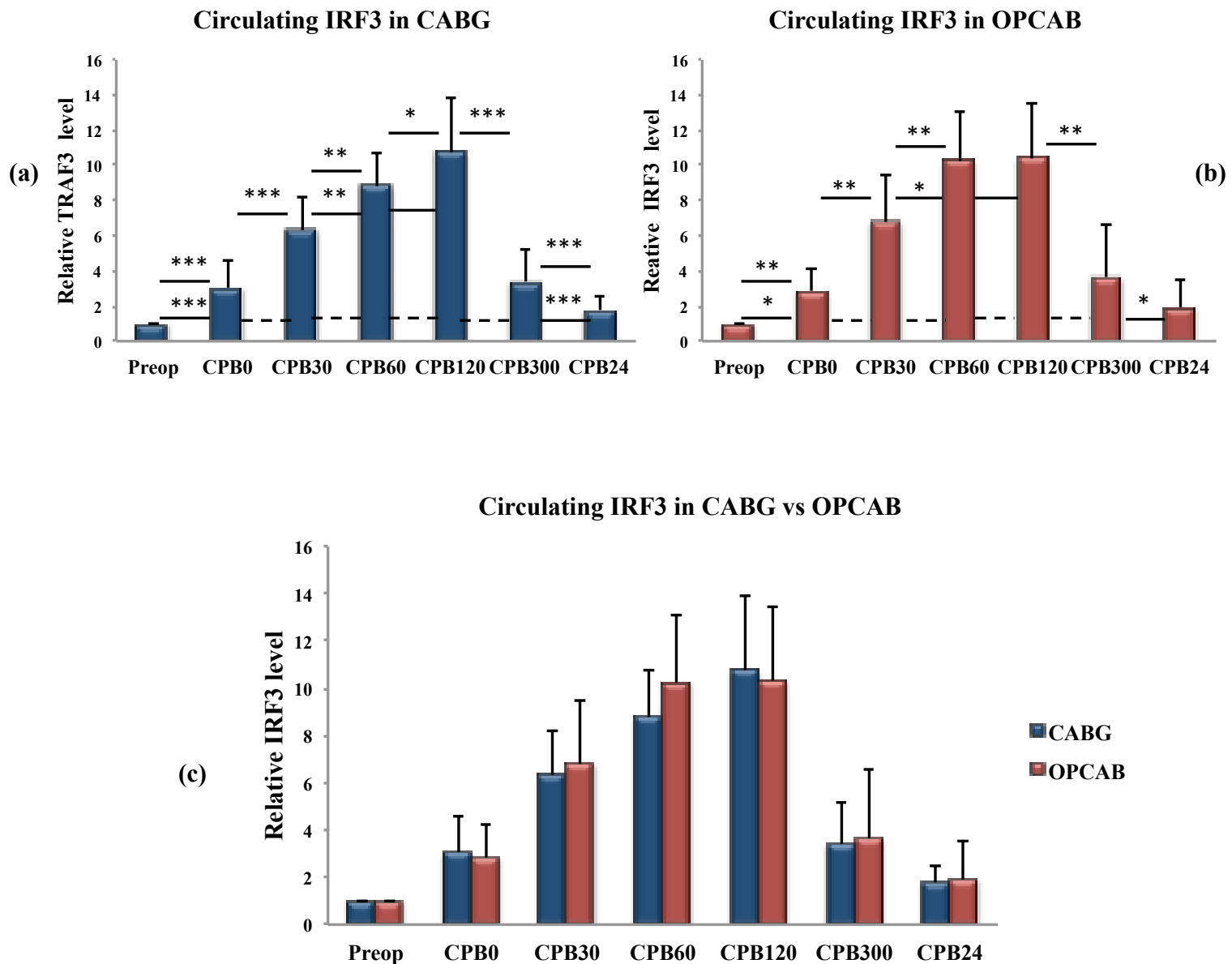


Figure 5.21 The circulating levels of IRF3 in response to CABG

The circulating IRF3 level results here showed different patterns to the other previous cytokines tested, even though their level showed significant increase among their different time points in CABG (a) or in OPCAB (b) groups, but there was no significant difference at any time point between the both CABG and OPCAB groups (c) (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

**Summary Of The Levels Of Cytokines And Chemokines Expression In Coronary
Revascularization Surgery With And Without CPB**

Gene expression in CPB (in folds) to the pre-operative level		CPB0 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB30 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB60 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB120 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB300 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB24 Mean $\pm$ SD-fold (<i>p</i> -value)
IL-2	CABG	11 $\pm$ 3 (< 0.05)	72 $\pm$ 40 (< 0.001)	133 $\pm$ 72 (< 0.001)	125 $\pm$ 39 (< 0.001)	96 $\pm$ 19 (< 0.001)	32 $\pm$ 17 (< 0.001)
	OPCAB	5.2 $\pm$ 2 (<0.01)	8 $\pm$ 3 (<0.01)	10 $\pm$ 2 (<0.05)	11 $\pm$ 2 (<0.01)	19 $\pm$ 2 (<0.01)	17 $\pm$ 1 (<0.01)
IL-4	CABG	13 $\pm$ 2 (< 0.001)	117 $\pm$ 50 (< 0.001)	137 $\pm$ 34 (< 0.001)	115 $\pm$ 15 (< 0.001)	42 $\pm$ 46 (< 0.001)	3 $\pm$ 2 (< 0.001)
	OPCAB	9 $\pm$ 4 (<0.01)	12 $\pm$ 4 (<0.01)	17 $\pm$ 5 (<0.01)	22 $\pm$ 7 (<0.01)	13 $\pm$ 12 (<0.01)	2 $\pm$ 1 (<0.01)
IL-6	CABG	13 $\pm$ 3 (< 0.001)	135 $\pm$ 47 (< 0.001)	250 $\pm$ 58 (< 0.001)	261 $\pm$ 50 (< 0.001)	187 $\pm$ 55 (< 0.001)	108 $\pm$ 38 (< 0.001)
	OPCAB	8 $\pm$ 3 (<0.01)	13 $\pm$ 3 (<0.01)	18 $\pm$ 3 (<0.01)	25 $\pm$ 6 (<0.01)	25 $\pm$ 9 (<0.01)	15 $\pm$ 5 (<0.01)
IL-8	CABG	9 $\pm$ 3 (< 0.001)	35 $\pm$ 26 (< 0.001)	44 $\pm$ 23 (< 0.001)	16 $\pm$ 23 (< 0.001)	10 $\pm$ 14 (< 0.001)	3 $\pm$ 1 (< 0.001)
	OPCAB	6 $\pm$ 3 (<0.01)	7 $\pm$ 7 (<0.01)	13 $\pm$ 8 (<0.01)	14 $\pm$ 10 (<0.01)	10 $\pm$ 9 (<0.01)	3 $\pm$ 2 (<0.01)
IL-10	CABG	3 $\pm$ 2 (< 0.001)	50 $\pm$ 36 (< 0.001)	69 $\pm$ 37 (< 0.001)	39 $\pm$ 36 (< 0.001)	16 $\pm$ 16 (< 0.001)	3 $\pm$ 2 (< 0.001)
	OPCAB	3 $\pm$ 2 (<0.01)	7 $\pm$ 7 (<0.001)	9 $\pm$ 7 (<0.01)	12 $\pm$ 8 (<0.01)	6 $\pm$ 5 (<0.01)	2 $\pm$ 1 (<0.01)
IL-12	CABG	4 $\pm$ 3 (< 0.01)	36 $\pm$ 21 (< 0.001)	56 $\pm$ 27 (< 0.001)	40 $\pm$ 25 (< 0.001)	29 $\pm$ 24 (< 0.001)	3 $\pm$ 3 (< 0.001)
	OPCAB	4 $\pm$ 1 (<0.01)	9 $\pm$ 2 (<0.01)	12 $\pm$ 3 (<0.01)	7 $\pm$ 2 (<0.01)	4 $\pm$ 1 (<0.05)	3 $\pm$ 1 (<0.01)
INF-α	CABG	13 $\pm$ 5 (< 0.001)	132 $\pm$ 46 (< 0.001)	321 $\pm$ 74 (< 0.001)	277 $\pm$ 71 (< 0.001)	167 $\pm$ 64 (< 0.001)	50 $\pm$ 20 (< 0.001)
	OPCAB	9 $\pm$ 3 (<0.01)	18 $\pm$ 6 (<0.01)	28 $\pm$ 6 (<0.01)	31 $\pm$ 10 (<0.01)	31 $\pm$ 10 (<0.01)	5 $\pm$ 2 (<0.01)
INF-γ	CABG	11 $\pm$ 3 (< 0.001)	47 $\pm$ 8 (< 0.001)	75 $\pm$ 36 (< 0.001)	51 $\pm$ 28 (< 0.001)	14 $\pm$ 10 (< 0.001)	3 $\pm$ 2 (< 0.001)
	OPCAB	5 $\pm$ (<0.01)	8 $\pm$ 4 (<0.01)	16 $\pm$ 7 (<0.01)	23 $\pm$ 8 (<0.01)	7 $\pm$ 4 (<0.01)	2 $\pm$ 2 (<0.01)
TNF-α	CABG	7 $\pm$ 4 (< 0.001)	47 $\pm$ 18 (< 0.001)	111 $\pm$ 40 (< 0.001)	50 $\pm$ 25 (< 0.001)	12 $\pm$ 11 (< 0.001)	2 $\pm$ 1 (< 0.001)
	OPCAB	4 $\pm$ 3 (<0.01)	7 $\pm$ 4 (<0.01)	13 $\pm$ 9 (<0.01)	17 $\pm$ 9 (<0.01)	4 $\pm$ 2 (<0.01)	2 $\pm$ 1 (<0.05)
CXCL9	CABG	6 $\pm$ 2 (< 0.001)	42 $\pm$ 18 (< 0.001)	86 $\pm$ 30 (< 0.001)	90 $\pm$ 25 (< 0.001)	22 $\pm$ 15 (< 0.001)	2 $\pm$ 1 (< 0.001)
	OPCAB	4 $\pm$ 1 (<0.01)	7 $\pm$ 1 (<0.01)	15 $\pm$ 2 (<0.01)	15 $\pm$ 2 (<0.01)	7 $\pm$ 1 (<0.01)	3 $\pm$ 1 (<0.01)
NF-kB	CABG	18 $\pm$ 5 (< 0.001)	86 $\pm$ 28 (< 0.001)	253 $\pm$ 80 (< 0.001)	287 $\pm$ 74 (< 0.001)	278 $\pm$ 53 (< 0.001)	115 $\pm$ 41 (< 0.001)
	OPCAB	9 $\pm$ 4 (< 0.01)	20 $\pm$ 7 (< 0.01)	29 $\pm$ 9 (< 0.01)	9 $\pm$ 9 (< 0.01)	4 $\pm$ 2 (< 0.01)	3 $\pm$ 1 (< 0.01)

Gene expression in CPB (in folds) to the pre-operative level		CPB0 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB30 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB60 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB120 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB300 Mean $\pm$ SD-fold (<i>p</i> -value)	CPB24 Mean $\pm$ SD-fold (<i>p</i> -value)
MAPK1	CABG	6 $\pm$ 2 (< 0.001)	42 $\pm$ 20 (< 0.001)	128 $\pm$ 42 (< 0.001)	136 $\pm$ 50 (< 0.001)	48 $\pm$ 36 (< 0.001)	4 $\pm$ 3 (< 0.001)
	OPCAB	4 $\pm$ 2 (< 0.01)	11 $\pm$ 4 (< 0.01)	16 $\pm$ 5 (< 0.01)	22 $\pm$ 7 (< 0.01)	11 $\pm$ 4 (< 0.01)	3 $\pm$ 2 (< 0.01)
MAPK14	CABG	6 $\pm$ 4 (< 0.001)	50 $\pm$ 26 (< 0.001)	164 $\pm$ 61 (< 0.001)	159 $\pm$ 44 (< 0.001)	58 $\pm$ 44 (< 0.001)	4 $\pm$ 2 (< 0.001)
	OPCAB	3 $\pm$ 2 (< 0.01)	8 $\pm$ 3 (< 0.01)	16 $\pm$ 5 (< 0.01)	23 $\pm$ 6 (< 0.01)	12 $\pm$ 4 (< 0.01)	3 $\pm$ 2 (< 0.01)
TRAF3	CABG	6 $\pm$ 3 (< 0.001)	51 $\pm$ 31 (< 0.001)	166 $\pm$ 64 (< 0.001)	158 $\pm$ 63 (< 0.001)	56 $\pm$ 51 (< 0.001)	3 $\pm$ 2 (< 0.001)
	OPCAB	3 $\pm$ 1 (< 0.01)	7 $\pm$ 3 (< 0.01)	18 $\pm$ 6 (< 0.01)	26 $\pm$ 7 (< 0.01)	5 $\pm$ 2 (< 0.01)	3 $\pm$ 1 (< 0.01)
TRAF6	CABG	4 $\pm$ 3 (< 0.001)	146 $\pm$ 37 (< 0.001)	233 $\pm$ 52 (< 0.001)	198 $\pm$ 73 (< 0.001)	63 $\pm$ 35 (< 0.001)	5 $\pm$ 3 (< 0.001)
	OPCAB	4 $\pm$ 4 (< 0.01)	56 $\pm$ 31 (< 0.01)	67 $\pm$ 30 (< 0.01)	83 $\pm$ 36 (< 0.01)	65 $\pm$ 27 (< 0.01)	5 $\pm$ 3 (< 0.01)
TRIF	CABG	3 $\pm$ 2 (< 0.001)	17 $\pm$ 6 (< 0.001)	25 $\pm$ 5 (< 0.001)	22 $\pm$ 8 (< 0.001)	8 $\pm$ 4 (< 0.001)	3 $\pm$ 1 (< 0.001)
	OPCAB	4 $\pm$ 2 (< 0.01)	15 $\pm$ 4 (< 0.01)	22 $\pm$ 4 (< 0.01)	23 $\pm$ 8 (< 0.01)	11 $\pm$ 4 (< 0.01)	3 $\pm$ 2 (< 0.01)
IRF3	CABG	3 $\pm$ 1 (< 0.001)	6 $\pm$ 2 (< 0.001)	9 $\pm$ 2 (< 0.001)	11 $\pm$ 3 (< 0.001)	3 $\pm$ 2 (< 0.001)	2 $\pm$ 1 (< 0.001)
	OPCAB	3 $\pm$ 1 (< 0.01)	7 $\pm$ 3 (< 0.01)	10 $\pm$ 3 (< 0.01)	10 $\pm$ 3 (< 0.01)	4 $\pm$ 3 (< 0.01)	2 $\pm$ 2 (< 0.05)

Table 5.2 Summary of PCR array

Genes expression and downstream production of various cytokines and chemokines in relation to cardiac surgery with and without utilization of the CPB. Results are in folds of enhancement related to the preoperative baseline level. All cytokines and chemokines are increased in all time points compared to the baseline (*p*-value: from < 0.001 to < 0.05), however the trend is much higher in CABG group than the OPCAB group. (*p* < 0.05 is considered significant).

5.6 Discussion

There are wide variety of cytokines and chemokines, which are released in response to different inflammatory stimuli. In this study we assessed the intracellular signalling pathways that leads to the initiation of the systemic inflammatory response to cardiac surgery with and without the utilization of CPB. An important observation we found is that cardiac surgery indeed has increased different circulatory cytokines and chemokines level independently regardless the use of the CPB circuit.

Cardiac surgery also induces both TLR signalling pathways, the MyD88-dependent pathway and the TRIF-dependent pathway leading to their activation; this was detected by measuring the downstream of both pathways. However, using the CPB in cardiac surgery leads to a further release of the pro-inflammatory cytokines and mainly those involved in MyD88-dependant pathway, this potential leads to a further inflammatory response. The level of enhancement of circulating cytokines was tens and hundreds times higher in relation to CPB than in cardiac surgery alone depending on which cytokine was released, with the exception of the cytokines released through TRIF-dependent pathway such as IRF3 and TRIF, where utilisation of CPB made no difference on their level.

It is known that CPB has a potential role in systemic inflammatory response and in agreement with previous findings, but yet the mechanism still not fully understood. We have previously identified the positive relationship between the increase in the level of circulating mtDNA and the use of CPB (Chapters 3 and 4). The mtDNA signals the TLR9, whereby it acts as ligand leading to its activation with subsequent release of a variety of cytokines and chemokines as downstream activation (154, 158, 338).

In our animal model experiments (Chapter 4) we identified that sternotomy in mice has increased mtDNA and subsequently IL-6 level as downstream of TLR9 activation. When we blocked TLR9, the levels of IL-6 were significantly reduced. Similarly the results here also showed IL-6 has the highest level amongst other interleukins (260-fold in CPB and 25-fold in surgery alone) which may contribute to TLR9 signalling as shown in the previous experiments, especially when the same plasma samples from the same patients (Chapter 3) revealed similar patterns of elevation in circulating mtDNA (known ligand for TLR9) in relation to CPB.

Binding of the TLR9 to its specific ligand leads to activation and signalling only through the MyD88-pathway (Chapter 2-TLR9). Furthermore, to study both TLRs signalling pathways (MyD88-dependent and TRIF-dependent pathway), we measured various downstream released cytokines and chemokine. We established that the cytokines and chemokines using the MyD88 pathway were significantly elevated in response to CPB these include (IL2-12, INF- α , INF- γ , TNF, CXCL9, NF-kB, P38-MAPK, MAPK1, TRAF3 & TRAF6). On the other hand the cytokines using TRIF pathway in their activation (IRF3 and TRIF) were not affected by utilization of the CPB, which excluded TLR3 and to a certain extent TLR4 activation.

Taken together, we can conclude that CPB initiates systemic inflammatory response through MyD88-dependent pathway, which is likely to have contributed to the TLR9 activation mediated through binding to mtDNA with elevated levels in CPB leading to the subsequent release of various inflammatory mediators such as interleukin (158, 384) interferon (241), MAP kinases (385), NF-kB and other cytokines using the above pathways (239-241).

6 CHAPTER VI: Circulating Mitochondrial DNA as Predictor For Development of Atrial Fibrillation After Cardiac Surgery

6.1 Abstract

Background: Postoperative atrial fibrillation (POAF) is a common complication in cardiac surgery and associated with increased morbidity and mortality. It is multifactorial but the pathophysiology is still not fully understood. However, inflammation can play an important role in the POAF pathogenesis. Understanding this pathomechanism of the upstream and downstream of inflammatory response activation can help us in the early management of this unwanted complication following cardiac surgery. Mitochondrial DNA (mtDNA) is a ligand for TLR9. Elevation in mtDNA circulating level can potentially activate the inflammatory response via TLR9 signalling increasing the chance of the development of POAF.

Aim: To test the hypothesis that mtDNA and downstream-activated specific inflammatory biomarker can potentially be predictive for the development of POAF

Methods: Prospectively, 88 patients without history of atrial fibrillation who underwent coronary artery bypass graft (CABG) or aortic valve replacement (AVR) were included. We measured the level of the mtDNA preoperatively in relation to POAF development. To determine the effect of cardiopulmonary bypass on POAF, a subgroup of 29 patients divided into on-pump and off-pump CABG were selected. Here we measured the preoperative level of several proinflammatory cytokines (includes IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, INF- α , TNF- α , NF-kB and P38 MAPK) and their relation to the development of POAF.

Results: The prevalence of POAF in AVR, on-pump and off-pump CABG were 50%, 22.7% and 27.3%, respectively. CRP, mtDNA and all the cytokines tested were significantly elevated preoperatively in patients developed POAF. However, mtDNA and INF- α were the only independent predictors for the development of POAF on logistic regression analysis. No difference between on-pump and off-pump CABG on POAF development ($p = 0.51$).

Conclusion: AF is common postoperative complication. Increase in the circulating levels of either mtDNA or INF- α can predict POAF development. This supports the inflammation theory in AF development. Therefore, prophylactic management of AF with possibly anti-inflammatory and rate control can be beneficial.

6.2 Introduction

6.2.1 Atrial fibrillation overview

Atrial fibrillation (AF) is the most common irregular heart rhythm, which can be manifested in the general populations with a prevalence of 2% in individuals above the age of 40. Prevalence increases to 9% above 80 years old (386, 387). It can be presented alone “lone AF” without known underlying cause, which can be contributed to the electrophysiological abnormality, or it can occur in association with variety of cardiac or non-cardiac diseases such as hyperthyroidism, alcohol toxicity, inflammatory diseases or even genetically predisposed (388).

AF is a frequent complication after cardiac surgery. It affects nearly third of the patients (389), whilst other studies have suggested nearly 50% of the patients can be affected (390). However, it varies according to the type of the procedure and the patient’s demographics. Overall, more percentage of this incidence occurs in valve related surgery (391) than those with coronary artery bypass grafting (CABG) surgery alone, the highest incidence reported after combined CABG and valve procedure (392, 393). On the other hand, heart transplantation carries much lower incidence of AF (394). AF can also occur in non-cardiac thoracic operations (395) such as in lung resections (396, 397), oesophagectomies (398). Additionally, can occur in some major abdominal surgeries such as in major colorectal procedures and in liver transplant (399, 400).

6.2.2 Course of postoperative AF (POAF)

POAF occurs usually within the first few days but it can also present late after weeks. It is usually manifested in the first 72 hours, in majority peaks at second postoperative day (401) and it considers one of the common causes for readmission in cardiac

surgery considering as a late complication. It varies from a symptomatic self-limiting benign arrhythmia with controlled heart rate (<100 beat per minute) to an uncontrolled symptomatic and complicated arrhythmia (>100 beat per minute) affecting the hemodynamic stability of the patient with subsequent high comorbidity or mortality (402). POAF is a predictor for longer hospital admission combined with more morbidity, this requiring further management and use of more hospital resources with subsequent higher cost as compared to those remains in sinus rhythm postoperatively (393, 401).

6.2.3 Pathophysiology of POAF

The pathomechanism of AF after cardiac surgery is still not fully understood. It can be contributed to the slowing of the atrial conduction or re-entry in small excitation waves to the atria (403). Some suggested that, the disruption of the right coronary system possibly interferes with the heart conduction system leading to dysrhythmias (404). However, some studies found age is an independent risk factor for AF (401, 405, 406), which can be contributed to age related pathological changes in the atrium such as atrial dilatation, fibrosis or hypertrophy (407, 408).

On the other hand, some clinical studies associate development of inflammation and the pathogenesis of POAF (409-412). In general the development of POAF is multifactorial including surgical trauma, local or systemic inflammation and disturbance in the electrolyte balance especially potassium and magnesium. Additionally, pharmacological stimulation by inotropes during or after the operation can provoke POAF development.

6.2.4 Cross-talk between POAF, inflammation and immune response

Ischemic heart disease expected to have some degree of myocardial damage and atrial inflammation especially in the healing process, which subsequently may participate in the development of the AF especially after surgery (413). Inflammation and immune response are synergistic factors in initiation and maintaining AF. Moreover, AF also can provoke inflammation giving the clinical phenomenon ‘ AF begets AF’ whereby the pre-existing inflammation promote AF that subsequently promote the inflammatory response, leading to further increase in the remodelling of the atria and propagates the AF (414).

All human TLRs were found in the heart (especially TLR2, 3, 4 & 9) with different levels of expression providing an important role in protecting the heart (271), they are expressed 10 times more than other TLRs in cardiac myocytes (272). However, TLRs on the other hand can potentially implicate harmful effect when dysregulated. The other argument is that in cardiac surgery, cardiac injury leads to release DAMPs with potential initiation of cardiac inflammation through activation of the TLRs leading to expression and production of proinflammatory cytokines. Which in turn promotes the arrhythmia and POAF.

Moreover, manifestation of local or systemic inflammation can predict AF occurrence in the general population and in those undergoing cardiac surgery (414). Several studies correlate the elevation in the circulating level of some inflammatory biomarker and the presence of the AF postoperatively such as C-reactive proteins (CRP) (412, 415, 416), interleukins (ILs) (417-419) and tumour necrosis factor (TNF) (409, 420). Furthermore, oxidative stress is also a potential predictor for POAF confirmed when atrial biopsies showed oxidative damage to the atrial myocardium (421, 422).

In support of the inflammation theory, various methods aimed to attenuate the inflammatory response in cardiac surgery such as off-pump CABG (423), leukofiltration (424), cardioplegia temperature (425), posteriorly pericardiotomy technique to avoid pericardial effusion (426, 427). All have been associated with reduced incidence of POAF.

6.2.5 Adverse effect of AF

AF has been associated with a two-fold increase in the cardiovascular morbidity and mortality (428). The contractility of the left atrial appendage decreases in AF, where the atrial thrombi usually originate (429), are an ideal site for blood stasis and thrombus formation and account for the majority of subsequent thromboembolic events and stroke (430). Many studies showed short term complications related to AF includes myocardial infarction with potential increase the need for intra aortic balloon pump, ventricular arrhythmia, heart failure, stroke and respiratory or renal failure (401).

6.2.6 Prevention and management of POAF

Prophylactic pharmacological treatment (431, 432) or atrial overdrive pacing (433, 434) have been tried with varying results, and the question whether it is reliable to predispose the patients to the risk of the prophylactic treatment side effect despite large percentage will still not develop AF postoperatively. In the clinical practice, the majority of centres treat POAF when it arises. However, rate control with beta-blocker is commonly used in practice if not contraindicated, which can be considering as prophylaxis. The use of medications with anti-inflammatory properties has been tried as prophylaxis preoperatively, which has shown reduction in the prevalence of POAF (435). The treatment modalities of POAF include correction of any electrolyte imbalance, pharmacological rate control such as with beta-blockers, and if no response,

pharmacological cardioversion such as with amiodarone or electrical cardioversion with consideration of anticoagulation therapy if persistent.

6.3 Hypothesis

Elevated level of the circulating mitochondrial DNA and/or pro-inflammatory cytokines can be a potential predictor for the development of POAF in cardiac surgery.

6.4 Material and methods

6.4.1 Inclusion criteria

Patients undergoing cardiac surgery including coronary artery bypass graft (CABG) or aortic valve replacement (AVR) were involved in the study total of 88 patients, 44 of them had on-pump CABG, mean age 64 ± 11 years, 22 OPCAB, mean age 64 ± 7 years and 22 AVR, mean age 73 ± 6 years. Confirmation of POAF development required being persistent for more than 10 minutes on a cardiac monitoring.

To compare also the effect of CPB on development of AF, patients who had the highest level of the mtDNA and involved previously in PCR array in comparison study between on and off-pump CABG (Chapter 5) were studied, total of 29 patients, 17 with on-pump CABG and 12 with off-pump CABG (OPCAB).

6.4.2 Exclusion criteria

Any patients who were unable or did not consent were excluded from the study. Additionally, those who had preoperative AF, suspected or proven infection, emergency operation, unstable angina or myocardial infarction within six weeks before surgical intervention, any pre-existing liver or renal diseases, any recent neurological abnormality were excluded from the study to eliminate any significant difference between the groups.

6.4.3 Experimental protocol

Only the preoperative blood samples were processed. Purification of mtDNA as described in chapter 3, purification of RNA and cDNA synthesis as described in chapter 4. Real time PCR prepared and ran as in chapter 3 to detect the mtDNA and cytokines level. The cytokines tested includes: IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, INF- α , TNF- α and NF-kB. Their levels were measured according to the relative thermal cycle generated by PCR. All levels were normalized to the mean of all sinus rhythm samples.

6.4.4 Statistical analysis

Statistical analysis was conducted using SPSS version 20 software package (IBM Corporation, Armonk, NY, USA). All data were expressed as means $\pm$ SD (standard deviation). Normality of the continuous variables distribution was tested by Shapiro-Wilk test. Logarithmic transformation of skewed data was used for the logistic regression. Differences in continuous and categorized variables were tested by unpaired Student's t test and Chi square (χ^2) test, respectively. Correlation between variable conducted using bivariate correlation analysis. Binary logistic regression was used to identify potential predictors between endpoint and other significant variables in the previous correlation analysis.

6.5 Results

6.5.1 Mitochondrial DNA assay

6.5.1.1 Descriptive results

The demographic and clinical characteristics of the patients participated in the study were total of 88 patients, 27 patients developed POAF and 61 remained in postoperative sinus rhythm (POSR) with mean $\pm$ SD (age of 68 $\pm$ 9 and 66 $\pm$ 10,

respectively). No significant difference in age, gender or size of the patients. No statistical difference (but clinical difference) related to the development of POAF and the increase in the logistic Euroscore ($p = 0.05$). No major difference between the patients developed POAF and POSR related to the preoperative comorbidity parameters. However, there was significant difference related to the use of the surgical procedure ($p < 0.05$), length of hospital stay ($p < 0.05$) and the preoperative level of both CRP and circulating mtDNA ($p < 0.01$ and $p < 0.001$, respectively) (Table 6.1).

Parameter	Group 1 (n=27) POAF (30.7%)	Group 2 (n=61) POSR (69.3%)	P -value
Age	68 ± 9	66 ± 10	0.22
Sex: Male	22 (81.5%)	47 (77%)	0.64
Female	5 (18.5%)	14 (23%)	
BMI	28.64 ± 7.36	28.59 ± 3.99	0.40
Ejection fraction (%)	54.75 ± 10.18	53.28 ± 7.99	0.80
Logistic Euroscore (%)	5.89 ± 6.42	3.76 ± 3.33	0.05
Operation: CABG	10 (27.7%)	34 (77.3%)	0.04*
OPCAB	6 (27.3%)	16 (77.7%)	
AVR	11 (50%)	11 (50%)	
No previous MI	20 (74%)	43 (70.5%)	0.80
MI > 6 weeks	7 (25.9%)	18 (29.5%)	
No coronary disease	11 (40.7%)	11 (18%)	0.03*
Single coronary vessel	1 (3.7%)	0 (0.0%)	
Two coronary vessels	3 (11.1%)	11 (18%)	
Three coronary vessels	11 (40.7%)	39 (63.9%)	
Four coronary vessels	1 (3.7%)	0 (0.0%)	
DM: No	18 (66.7%)	28 (45.9%)	0.088
Diet control	5 (18.5%)	9 (14.8%)	
Oral therapy	2 (7.4%)	20 (32.8%)	
Insulin therapy	2 (7.4%)	4 (6.6%)	
Hypertension	23 (85.2%)	52 (85.2%)	0.99
Hypercholesterolemia	24 (88.9%)	56 (91.8)	0.66
Pulmonary disease	3 (11.1%)	3 (5%)	0.29
Mild renal disease	1 (3.7%)	2 (3.3%)	0.92
Neurological disease	4 (14.8%)	7 (11.5%)	0.66

Parameter (cont'd)	Group 1 (n=27) POAF (30.7%)	Group 2 (n=61) POSR (69.3%)	P -value
No. of grafts: CABG X0	11 (40.7%)	11 (18%)	0.078
CABG X1	2 (7.4%)	1 (1.6%)	
CABG X2	7 (25.9%)	20 (32.8%)	
CABG X3	6 (22.2%)	27 (44.3%)	
CABG X4	1 (3.7%)	2 (3.3%)	
CPB time (minutes)	63 ± 42	56 ± 36	0.46
Postoperative drainage	886 ± 921	697 ± 453	0.61
Smoking: Never smoked	14 (51.9%)	22 (36.1%)	0.35
Ex-smoker	11 (40.7%)	31 (50.8%)	
Current smoker	2 (7.4%)	8 (13.1%)	
Postoperative inotropes	12 (44.4%)	13 (21.3%)	0.027*
Length of ITU stay (days)	1.44 ± 1.21	1.12 ± 0.55	0.08
Length of hospital stay (days)	10.44 ± 7.91	7.23 ± 3.57	0.018*
Preoperative Hb	13.43 ± 1.45	13.7 ± 1.48	0.5
Preoperative CRP	6.26 ± 4.12	3.36 ± 2.80	0.003**
Preoperative WBC	7.46 ± 1.53	7.88 ± 2.25	0.66
Preoperative Creatinin	93.03 ± 73.62	83.86 ± 26.88	0.87
Preoperative mtDNA	7.32 ± 7.37	1.29 ± 1.02	<0.001***
Blood transfusion: 0 unit	19 (70.4%)	51 (83.6%)	0.29
1 unit	3 (11.1%)	2 (3.3%)	
2 units	3 (11.1%)	4 (6.6%)	
3 units	0 (0.0%)	2 (3.3%)	
4 units	1 (3.7%)	2 (3.3%)	
≥ 5 units	1 (3.7%)	0 (00%)	

Table 6.1 Pre and postoperative patients characteristics in relation to POAF development

Characteristics of patients had different type cardiac surgery categorized according to the development of postoperative AF. Values are shown as mean ± SD for continuous variables and as n (%) for categorical variables. Significant statistical difference if $p < 0.05$. (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$). (CABG: on-pump coronary artery bypass graft, OPCAB: Off-pump coronary artery bypass graft, AVR: Aortic valve replacement, POAF: postoperative atrial fibrillation, POSR: Postoperative sinus rhythm, Hb: Haemoglobin, CRP: C-reactive protein, WBC: White blood cells, mtDNA: Mitochondrial DNA).

To identify any potential predictor for POAF development, we performed bivariate correlation test between different variables listed in tables 7.1. The test showed significant correlation between the development of POAF and performing the operation with the number of bypass grafted, length of hospital stay and the preoperative levels of both CRP and mtDNA (Table 6.2).

Variable	POAF Correlation coefficient P - value
Group	1
Age	0.132 0.22
Sex	0.05 0.64
Operation	0.221* 0.039*
Number of grafts	-.258* 0.015
Preoperative CRP	0.322** 0.002
Preoperative mtDNA	0.509** 0.000
Postoperative inotropes	0.237* 0.026
Length of hospital stay	0.256* 0.017

Table 6.2 Bivariate correlation

Spearman rank-order correlation for postoperative AF (POAF) development and correlated variables related. Only significant variables, in addition to age and gender were included in this table. *Correlation is significant at the 0.05 level (2-tailed); ** correlation is significant at the 0.01 level (2-tailed).

Furthermore, binary logistic regressions analysis was adjusted for only significant variables in the bivariate correlation including operation, preoperative CRP and mtDNA levels, number of grafts used, the use of postoperative inotropes and the length of hospital stay. The test has confirmed that, in addition to the surgery, the elevated level of mtDNA preoperatively is the only independent predictor for development of POAF (Table 6.3).

Variable	Independent predictor P - value
Operation	0.012*
Number of grafts	0.42
Preoperative CRP	0.082
Preoperative mtDNA	0.005**
Postoperative inotropes	0.908
Length of hospital stay	0.088

Table 6.3 Binary logistic regression

Only significant variable from bivariate correlation were tested. Surgery and increase level of mitochondrial DNA (mtDNA) are independent predictors for development of postoperative AF (POAF) (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

The mtDNA levels and the onset of the POAF vary according to the type of operation. The preoperative levels of mtDNA were higher in patients undergoing coronary artery bypass graft (CABG) than with aortic valve replacement (AVR). However, the overall incidence of developing POAF was higher in AVR than CABG (50% for AVR. 27.7% for on-pump CABG and 27.3% for OPCAB). The onset of the POAF was noted from the same day of the operation (day 0) to day 4 (Table 6.4).

Type of operation	Mean age ± SD	POSR (n)	Pre-op mtDNA in POSR	POAF (n)	Pre-op mtDNA in POAF	Onset of POAF (Days)
CABG	64 ± 11	34 (77.3%)	1.45-fold	10 (22.7%)	11.15-fold	1.7 ± 0.15
OPCAB	64 ± 7	16 (72.7%)	1.37-fold	6 (27.3%)	7.26-fold	1.5 ± 1.04
AVR	73 ± 6	11 (50%)	0.84-fold	11 (50%)	2.7-fold	2.2 ± 1.1
TOTAL	76 ± 10	61	1	27	6.14-fold	1.88 ± 1.12

Table 6.4 Summary of mtDNA level and POAF development

This table shows the level of mtDNA in each surgery before and after the procedure and the onset of the POAF, CABG has higher level of mtDNA preoperatively than AVR, however the incidence of POAF is higher in AVR than CABG. The level of mtDNA postoperatively was the highest with on-pump CABG (11.15-fold), followed by OPCAB (7.26-fold) and AVR (2.7-fold).

Over all, the preoperative mtDNA levels were markedly elevated in patients developed POAF; the highest was among patients with ischemic heart disease undergoing CABG operation (Figure 6.1).

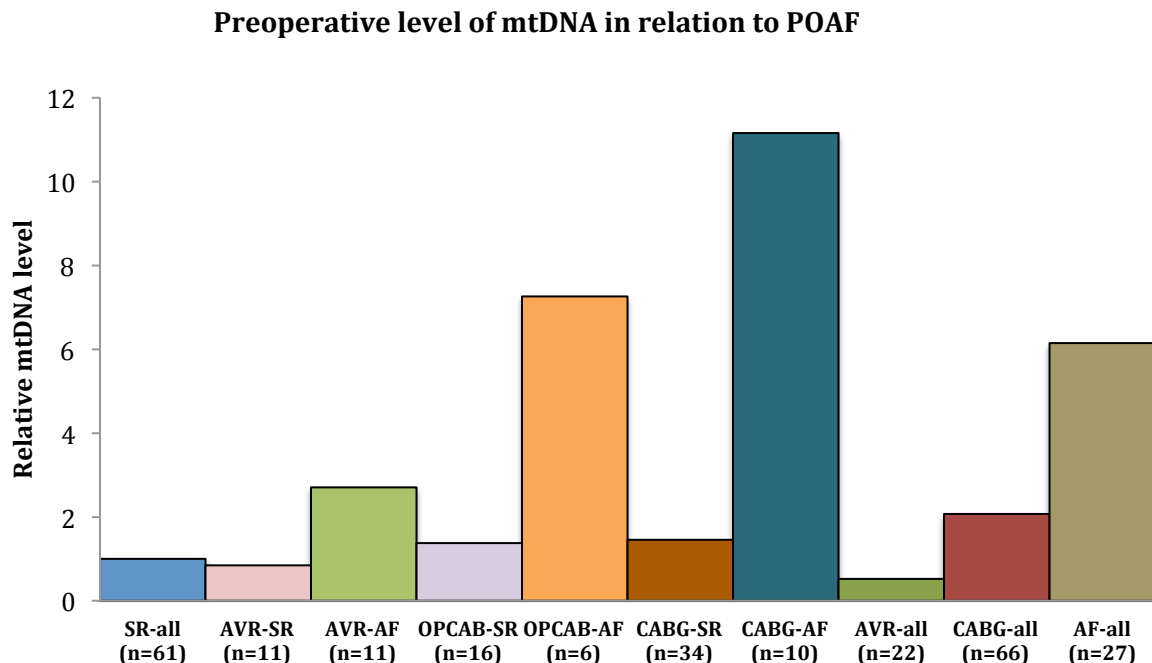


Figure 6.1 Pre-operative level of mtDNA in response to POAF

The circulating level of mtDNA is at highest in the patients undergoing CABG than AVR operation, and was overall higher in those developed POAF.

6.5.2 Cytokines assay

For the evaluation of the circulating levels of the proinflammatory cytokines and the effect of the CPB on the development POAF, we involved patients who had the highest mtDNA levels from previous experiment but only those had CABG surgery. 29 patients were involved, 17 had CABG with CPB and 12 without CPB. Total of 6 patients developed POAF (4 for CABG-group and 2 for OPCAB-group).

6.5.2.1 Descriptive results

The demographic and clinical characteristics of the patients participated in the study were total of 29 patients, 6 patients developed POAF and 23 remained in POSR with mean $\pm$ SD age of 67 ± 7 and 61 ± 11 , respectively. No significant difference in age or gender of the patients. No statistical difference related to the development of POAF and the increase in the logistic Euroscore or related comorbidity parameters. However, there was significant difference related to pre-existing pulmonary disease ($p < 0.05$) and the level of preoperative CRP ($p < 0.05$) (Table 6.5).

Variables	Group 1 (n=6) POAF	Group 2 (n=23) POSR	p-value
Age	67 ± 7	61 ± 11	0.33
Sex	M = 6 (100%) F = 0 (0.0%)	M = 18 (78.3%) F = 5 (21.7%)	0.2
BMI	27.7 ± 3.5	29 ± 4.2	0.41
Ejection fraction (%)	56 ± 8.36	54.7 ± 6.9	0.99
Logistic Euroscore (%)	4.78 ± 3.81	2.72 ± 2.55	0.48
No. of coronary diseases: 2 3 4	1 (16.7%) 4 (66.7%) 1 (16.7%)	6 (26.1%) 17 (73.9%) 0 (0.0%)	0.13
Smoking: No smoking Ex-smoker Current smoker	2 (33.3%) 3 (50%) 1 (16.7%)	14 (60.9%) 6 (26.1%) 3 (13%)	0.45
Previous MI	2 (33.3%)	7 (30.4%)	0.87
DM: No DM Diet control Oral therapy Insulin therapy	4 (66.7%) 2 (33.3%) 0 (0.0%) 0 (0.0%)	11 (47.8%) 3 (13%) 8 (34.8%) 1 (4.3%)	0.28
HT	6 (100%)	19 (82.6%)	0.27
Hypercholesterolemia	6 (100%)	22 (95.7%)	0.6
Pulmonary disease	1 (16.7%)	0 (0.0%)	0.04*
Renal disease	0 (0.0%)	0 (0.0%)	NA
Neurological disease	1 (16.7%)	2 (8.7%)	0.56
Preoperative Hb	13.7 ± 0.96	13.7 ± 1.3	0.26
Preoperative CRP	7.3 ± 5.1	5.5 ± 7.5	0.03*
Preoperative WBC	6.9 ± 1.2	6.8 ± 1.8	0.88
Preoperative Creatinin	68.3 ± 14.8	81.5 ± 16.4	0.93
Preoperative EGFR	69.3 ± 15	68.5 ± 12	0.6
CPB time On-pump Off-pump	49.16 ± 42.37 4 (23.5%) 2 (16.7%)	46.82 ± 45.89 13 (76.5%) 10 (83.5%)	0.51
Number of coronary grafts	2.5 ± 0.8	2.6 ± 0.5	0.25
Post operative drainage (ml)	1265 ± 585	837 ± 301	0.28
Blood transfusion: 0 unit 1 unit 2 units 3 units 4 units	4 (66.7%) 0 (0.0%) 2 (33.3%) 0 (0.0%) 0 (0.0%)	18 (78.3%) 1 (4.3%) 2 (8.7%) 1 (4.3%) 1 (4.3%)	0.56
Postoperative inotropes	3 (50%)	6 (26.1%)	0.26
Length of ITU stay (days)	1.8 ± 2	1 ± 0.2	0.34
Length of hospital stay (days)	11.8 ± 8.5	6.4 ± 3.3	0.47

Table 6.5 Patients demographic data in relation to POAF

Comparison in the preoperative and postoperative demographic data between patients developed POAF and those remained in POSR after CABG). Data showed absolute values, SD and percentage. Significant statistical difference if $p < 0.05$ (*= $p < 0.05$).

To identify if any of the cytokines are potential predictors for POAF development, we conducted bivariate correlation test between different variables listed in tables 7.5. The test showed significant correlation between the development of POAF and pre-existing lung disease, amount of chest drainage, length of hospital stay and elevated levels of all the cytokines tested with the exception of IL-10, which was not correlated to POAF (Table 6.6).

Variables	POAF Correlation coefficient P - value
IL-2	0.594** 0.001
IL-4	0.559** 0.002
IL-6	0.863 <0.001
IL-8	0.395* 0.03
IL-10	0.302 0.11
IL-12	0.626** <0.001
INF- α	0.651** <0.001
TNF- α	0.707** <0.001
NF-kB	0.737** <0.001
P38MAPK	0.776** <0.001
Pulmonary disease	0.373* 0.04
Chest drainage	0.436* 0.018
Length of hospital stay	0.432* 0.019

Table 6.6 Bivariate correlation

Spearman rank-order was used for the correlations of POAF development and the variables. Only significant correlated variables (except IL-10 was not significant) were included in this table. *Correlation is significant at the 0.05 level (2-tailed); ** correlation is significant at the 0.01 level (2-tailed).

Furthermore, binary logistic regressions analysis was adjusted for only significant cytokines and other significant variables in the bivariate correlation including pre-existing pulmonary diseases, chest drainage and length of hospital stay. The test has shown that the increase in INF- α levels preoperatively was the only independent predictor for development of POAF ($p < 0.05$) (Table 6.7).

Variables	POAF Correlation coefficient P - value
IL-2	0.15
IL-4	0.05
IL-6	0.99
IL-8	0.14
IL-12	0.97
INF- α	0.04*
TNF- α	0.99
NF-kB	0.15
P38MAPK	0.3
Pulmonary disease	0.99
Chest drainage	0.05
Length of hospital stay	0.25

Table 6.7 Binary logistic regression

Only significant variable from bivariate correlation were tested. INF- α is the only independent predictor for development of POAF ($p < 0.05$) (* = $p < 0.05$).

6.5.2.2 Interleukins

All the interleukins were not independent predictors for POAF. However, they were significantly elevated preoperatively in patients developed POAF with varying p values (< 0.001 to < 0.05). Comparing the preoperative levels of interleukins in POAF to those remain in sinus rhythm (POSR), all interleukins showed significant elevation as follows: 1.4 - fold for IL-2 ($p < 0.01$), 1.43 - fold for IL-4 ($p < 0.01$), 1.68 - fold for IL-6 ($p < 0.001$), 1.14 - fold for IL-8 ($p < 0.05$), 2 - fold for IL-10 ($p < 0.05$) and 2.47 - fold for IL-12 ($p < 0.01$) (Figure 6.2).

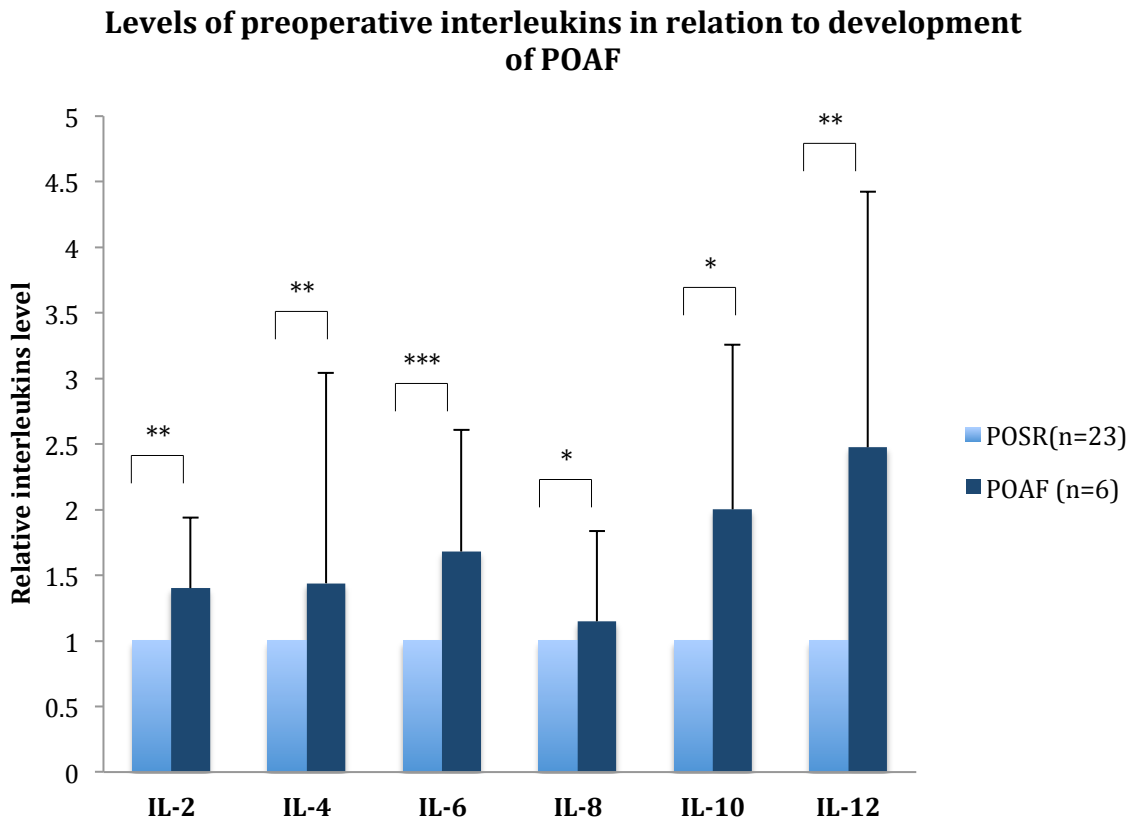


Figure 6.2 Interleukins levels in relation to POAF

The interleukins (IL-2, IL-4, IL-6, IL-8, IL-10, IL-12) levels preoperatively were significantly higher in patients developed POAF (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

6.5.2.3 INF- α , TNF- α , NF-kB and P38MAPK

Only elevation in the INF- α level was independent predictor for POAF. However, all these cytokines tested were significantly elevated preoperatively in patients developed POAF compared to POSR with p values < 0.001 , which was as follows: 2.26-fold for INF- α ($p < 0.001$), 2.72-fold for TNF- α ($p < 0.001$), 2.48-fold for NF-kB ($p < 0.001$) and 3.02-fold for P38MAPK ($p < 0.001$) (Figure 6.3).

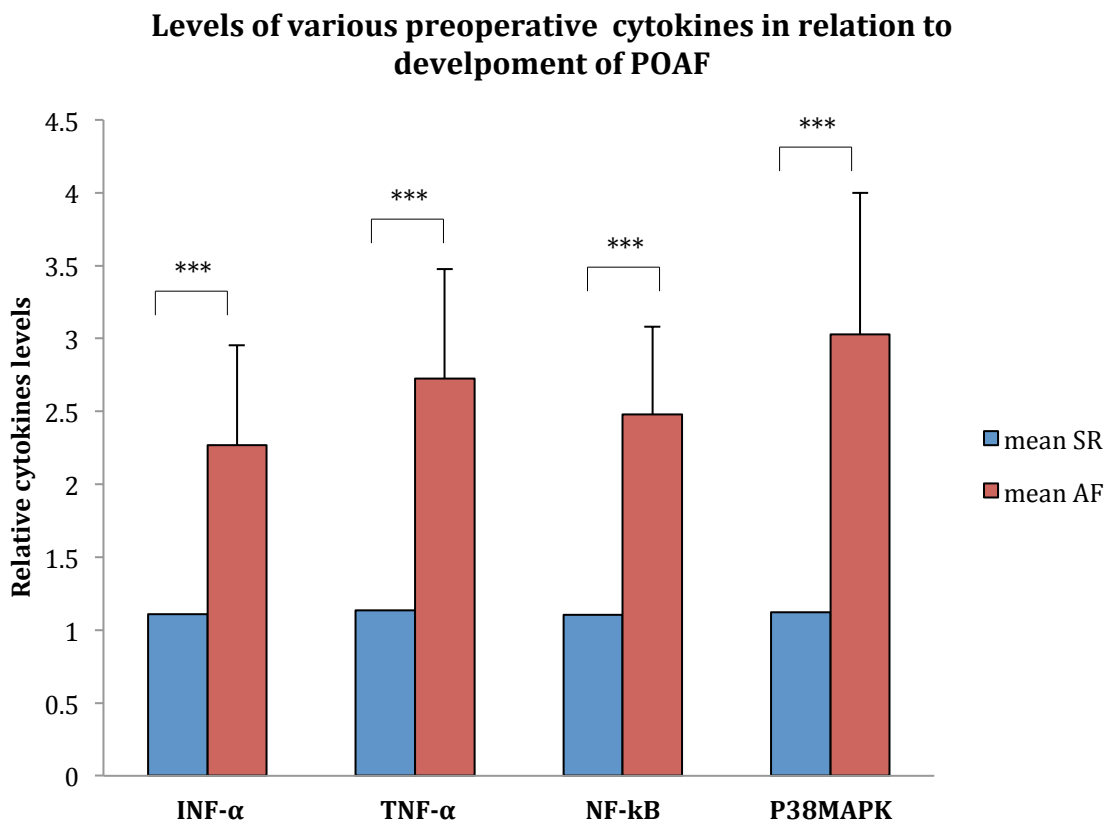


Figure 6.3 Cytokines levels in relation to POAF

The cytokines (INF- α , TNF- α , NF-kB and P38MAPK) levels preoperatively were significantly elevated in patients developed POAF (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

6.6 Discussion

Cardiac surgery has evolved with enormous advancement in both surgical and non-surgical care. However, despite this improvement, AF still remain a common complication after surgery, which has an impact on the recovery with associated higher risk of stroke, systemic embolism and prolonged hospitalisation with the use of greater hospital resources. Several studies have showed that the background inflammation have been associated with the predisposition of AF in non-surgical patients (436-439), therefore we need to consider the implication of this finding on the cardiac surgery, including its role in different types of operations including AVR and CABG with and without the utilization of the CPB.

To summarize, this study was a prospective study, data were collected only preoperatively from patients undergoing cardiac surgery. The principle aim of this chapter was to identify predictive values for the development of AF after cardiac surgery and whether the increase in the levels of some inflammatory biomarkers would be potentially one of these predictive values. We investigated the hypothesis that non-invasive evaluation of elevated circulating levels of mtDNA and cytokines might facilitate prediction of POAF in patients undergoing cardiac surgery.

The results showed that the levels of preoperative CRP and the mitochondrial DNA (mtDNA) were significantly higher in those patients that developed POAF compared to POSR. Furthermore, they were also both correlated to the POAF when using bivariate correlation analysis. However, when performing binary logistic regression analysis, we identified that the mtDNA is the only independent predictor for POAF development.

Similarly, when studying the effect of CPB on development of POAF, even though the incidence was higher in CPB group (23.5%) compared to OPCAB (16.7%), it was not

statistically significant ($p = 0.51$). Moreover, we already have identified that mtDNA is an independent predictor for POAF; therefore we proceed to evaluate the level of the cytokine in the same way. The result showed significant elevation in all the ten tested cytokines in POAF group, with significant correlation (except with IL-10) to POAF development on bivariate correlation analysis. However, on logistic regression analysis, INF- α was the only independent predictor for the development of POAF. Therefore, the most relevant predictors for POAF here were the elevation of the preoperative circulating levels of both mtDNA and INF- α .

The development of POAF is multifactorial and many potential contributors were reported and one them is the inflammatory theory whereby inflammation plays role in the pathogenesis of POAF (414). Number of case control studies has demonstrated an increase in the inflammatory biomarker such as (CRP, IL-2, IL-6, IL-8 and TNF- α) in AF patients (440, 441). However, they were mainly associated with AF rather than predictors with the exception of the CRP, which was also a predictor in some studies (412, 415). Our finding to some extent points more toward the inflammatory theory, whereby the inflammation is an important trigger for POAF, which may require further assessment preoperatively with potential of prophylactic treatment directed to attenuate the inflammatory burden and the AF in this particular group.

7 CHAPTER VII: Discussion Summary And Concluding Remarks

7.1 Overview

For more than six decades, cardiac surgery and the use of the cardiopulmonary bypass (CPB) has evolved with significant development and improvement. Nevertheless, despite this advancement, cardiac surgery still carries risk of complications for patients with varying degrees of severity. The advancement in cardiac surgery involves continuous improvement in the surgical setup and it's techniques, optimizing the perioperative care plan and finally understanding the molecular pathways and the mechanisms involved in the course of cardiac surgery.

Cardiac surgery plays a potential role in the initiation of the inflammatory responses with subsequent related complications such as systemic inflammatory response syndrome (SIRS), which is characterized by hyper production of proinflammatory cytokines. The responsibility of the development of systemic inflammatory response can be shared between the surgical trauma with heart manipulation, the CPB and the ischemic-reperfusion injury, which are usually unavoidable in many of the heart operations.

7.2 General context of the study

The work performed in this study was to identify the risk in cardiac surgery and to reduce related morbidity and mortality. This approached by understanding the course of the systemic inflammatory response mainly those associated with CPB at the cellular level and the molecular mechanism behind it. This was assessed by (1) identifying the relationship between the mitochondrial DNA (mtDNA) and the TLR9 signalling with subsequent activation of the immune response in CPB; (2) Identifying potential TLR9 antagonist to attenuate this response; (3) Evaluate the downstream

activation of TLR9 and (4) Identifying the predictors for the development of postoperative atrial fibrillation (POAF). Here we have tested the followings:

- The systemic inflammatory response to cardiac surgery is promoted via TLR9 through binding to its ligand “the mitochondrial DNA”, with subsequent induction of numerous cytokines and chemokines.
- Blocking TLR9 will attenuate the inflammatory response in cardiac surgery.
- Rise in the preoperative mtDNA level and some cytokines can be predictive for the development of postoperative atrial fibrillation (POAF).

7.3 Mitochondrial DNA a novel marker

7.3.1 Mitochondrial DNA and TLR9 overview summary

TLR9 is an endosomal receptor; it binds to its DNA ligand for activation and signalling. The DNA can be either bacterial DNA acting as a pathogen associated molecular pattern (PAMP) in sepsis or mtDNA acting as damage associated molecular pattern (DAMP) in a sterile environment (158, 298, 363). Binding of mtDNA to TLR9 leads to signalling of the inflammatory cascade with subsequent downstream release of various cytokine and chemokines initiating the inflammatory response (158, 324). We then proceed to discuss the mtDNA whilst referring to the key role-played by signalling of the TLR9.

7.3.2 Mitochondrial DNA and aging

We demonstrated in this study being young and healthy in the absence of mechanical injury would have less potential for the cell injury and less impact on leakage of the mtDNA from the mitochondrion, which is a new finding and in agreement with ageing

theory, which links ageing to apoptosis deregulation with oxidative stress, mitochondrial disruption and accumulation of mtDNA (343, 344).

7.3.3 Mitochondrial DNA and ischemic heart disease

A novel finding was identified in this study, which is the direct relationship between elevation in the circulating mtDNA level and the extent of the ischemic heart disease (IHD). The levels were significantly higher in patients with IHD compared to similar age group patients but with normal coronary arteries. This can be related to the fact that inflammation can induce atherosclerosis, which is in turn an important contributing factor in the pathogenesis of IHD (345). This subject will need further evaluation in the future in due course of IHD treatment.

7.4 Inflammatory responses and the CPB

We demonstrated that the level of the mtDNA was significantly elevated in relation to the surgical trauma alone in the off-pump surgery. However, it was markedly higher when using CPB, these levels were peaking at the maximum duration of CPB. Furthermore, it was markedly higher when compared to the off-pump group (**Figure 3.7**). These findings have shown us the direct relationship between the circulating mtDNA level and the duration of the CPB utilization. Furthermore, knowing that the mtDNA is a TLR9 ligand. This finding can arguably be linked to the activation of the inflammatory cascade mechanism mediated by TLR9.

Additionally, when measuring the potential downstream activation of the TLR9, we found those patients with highest levels of mtDNA, also had higher production of numerous proinflammatory cytokines, with the same pattern of significant elevation in their levels in relation to CPB. These analyses indicate that off-pump surgery reduces the inflammatory response compared to on-pump but does not prevent it totally.

Indeed, this is in agreement with best based evidence of randomized controlled trial (Grade A / level Ib) (442).

7.5 Effect of antagonizing the TLR9

We firstly tested the antagonism of the TLR9 in ex-vivo cultured cells (HUVEC), which has shown reduction in the IL-6 in relation to TLR9 blockage. Moreover, in the mice model experiment, data showed elevation in the circulating mtDNA level and subsequently downstream IL-6 level in relation to sternotomy. IL-6 level was significantly reduced when the mice were treated with intra-peritoneal TLR9 antagonist (ODN 2088).

7.6 Novel predictors for postoperative atrial fibrillation

POAF is a common complication in cardiac surgery. The pathogenesis is multifactorial. However, inflammation has been mentioned as one of the factors involved in AF pathogenesis (409). Several studies correlate the rise in the level of some inflammatory biomarker and the presence of the POAF such as CRP, interleukins or tumour necrosis factors (440, 441).

The data presented in this study shows that CRP and some of the cytokines were significantly higher preoperatively in the patients developed POAF afterward. However, they have not been proven to be predictors for the development of POAF in logistic regression analysis, which is in agreement with number of similar studies (443, 444). In contrast, increase in the levels of the mtDNA and INF- α preoperatively were the only independent predictors for the POAF development, which is another new finding of our study. These findings are more suggestive of the inflammation as a potential contributing factor for development of POAF. This may need to be taken in

consideration for preoperative prophylactic treatment directed to attenuate the inflammation and AF.

7.7 Concluding remarks

Taking together our findings presented in thesis in totality, we conclude the following:

- Ischemic heart disease and increase in age are associated with the increase in the circulating level of the mtDNA and are more susceptible to inflammation. This is in agreement with the aging theory suggesting age may cause apoptosis de-regulation, which is important for tissue haemostasis.
- Cardiac surgery with cardiopulmonary bypass leads to increase in the circulating mtDNA level and subsequent TLR9 signalling with potential initiation of the immune and the systemic inflammatory response and subsequent related complication.
- Off-pump cardiac surgery has less influence on systemic inflammatory response but not without any risk due to the significant response in the proinflammatory biomarker and mtDNA related to surgery alone. However, it has much less effect when compared to on-pump surgery.
- Using relevant TLR9 antagonists is likely to be beneficial in minimizing or perhaps preventing the significant systemic inflammatory response observed with cardiopulmonary bypass.
- Sulforaphane in addition to its anti oxidant effect, it has also an anti-apoptotic effect with protection against mitochondrial depolarization and DNA fragmentation

- Rise in the mtDNA or in the INF- α levels before cardiac surgery can be considered as independent predictors for the development of atrial fibrillation after surgery. Prophylactic treatment for atrial fibrillation in patients with pre-existing high level of mtDNA or INF- α can be beneficial.

7.8 Future work

- Evaluation of the role of the mitochondrial DNA in IHD with assessment of effect of the treatment of IHD on its level.
- Similar study in response to minimal invasive cardiac procedures such as minimal invasive direct coronary bypass (MIDCAB), mini-sternotomy AVR and minimal invasive mitral valve repair or replacement.
- Optimizing TLR9 antagonist in large animal with potential clinical implication

References

1. Majno G. The healing hand : man and wound in the ancient world. Cambridge, Mass.: Harvard University Press; 1975. xxiii, 571 p., 6 leaves of plates p.
2. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. *Clinical and experimental immunology*. 2007;147(2):227-35.
3. Ferencik M, Stvrtinova V, Hulin I, Novak M. Inflammation--a lifelong companion. Attempt at a non-analytical holistic view. *Folia microbiologica*. 2007;52(2):159-73.
4. Majno G, Joris I. Cells, tissues, and disease : principles of general pathology. 2nd ed. New York: Oxford University Press; 2004. xxviii, 1005 p. p.
5. Medzhitov R. Origin and physiological roles of inflammation. *Nature*. 2008;454(7203):428-35.
6. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. *Nature immunology*. 2005;6(12):1191-7.
7. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. *Nature immunology*. 2006;7(4):344-53.
8. Cotran RS, Kumar V, Collins T, Robbins SL. Robbins pathologic basis of disease. 6th ed. Philadelphia: Saunders; 1999. xv, 1424 p. p.
9. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *The Journal of investigative dermatology*. 2007;127(3):514-25.
10. Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nature cell biology*. 1999;1(5):260-6.
11. Ashcroft GS. Bidirectional regulation of macrophage function by TGF-beta. *Microbes and infection / Institut Pasteur*. 1999;1(15):1275-82.
12. Werner F, Jain MK, Feinberg MW, Sibinga NE, Pellacani A, Wiesel P, et al. Transforming growth factor-beta 1 inhibition of macrophage activation is mediated via Smad3. *The Journal of biological chemistry*. 2000;275(47):36653-8.
13. Sato Y, Ohshima T, Kondo T. Regulatory role of endogenous interleukin-10 in cutaneous inflammatory response of murine wound healing. *Biochemical and biophysical research communications*. 1999;265(1):194-9.
14. Serhan CN. Controlling the resolution of acute inflammation: a new genus of dual anti-inflammatory and proresolving mediators. *Journal of periodontology*. 2008;79(8 Suppl):1520-6.
15. Greenhalgh DG. The role of apoptosis in wound healing. *The international journal of biochemistry & cell biology*. 1998;30(9):1019-30.
16. Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nature medicine*. 2005;11(11):1173-9.
17. Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, Pure E, et al. Resolution of lung inflammation by CD44. *Science*. 2002;296(5565):155-8.
18. McQuibban GA, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science*. 2000;289(5482):1202-6.

19. Henson PM. Dampening inflammation. *Nature immunology*. 2005;6(12):1179-81.
20. Van Cruchten S, Van Den Broeck W. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anatomia, histologia, embryologia*. 2002;31(4):214-23.
21. Libby P, Braunwald E. *Braunwald's heart disease : a textbook of cardiovascular medicine*. 8th ed. Philadelphia: Saunders/Elsevier; 2008.
22. Majno G, La Gattuta M, Thompson TE. Cellular death and necrosis: chemical, physical and morphologic changes in rat liver. *Virchows Archiv fur pathologische Anatomie und Physiologie und fur klinische Medizin*. 1960;333:421-65.
23. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *The American journal of pathology*. 1995;146(1):3-15.
24. Nan Y, Nan G, Zhang YJ. Interferon Induction by RNA Viruses and Antagonism by Viral Pathogens. *Viruses*. 2014;6(12):4999-5027.
25. Imazio M, Gaita F. Diagnosis and treatment of pericarditis. *Heart*. 2015.
26. Kabbani SS, LeWinter MM. Pericardial Diseases. Current treatment options in cardiovascular medicine. 2002;4(6):487-95.
27. Imazio M. Pericardial involvement in systemic inflammatory diseases. *Heart*. 2011;97(22):1882-92.
28. Aretz HT, Billingham ME, Edwards WD, Factor SM, Fallon JT, Fenoglio JJ, Jr., et al. Myocarditis. A histopathologic definition and classification. *The American journal of cardiovascular pathology*. 1987;1(1):3-14.
29. Drory Y, Turetz Y, Hiss Y, Lev B, Fisman EZ, Pines A, et al. Sudden unexpected death in persons less than 40 years of age. *The American journal of cardiology*. 1991;68(13):1388-92.
30. McCaffrey FM, Braden DS, Strong WB. Sudden cardiac death in young athletes. A review. *American journal of diseases of children*. 1991;145(2):177-83.
31. Eckart RE, Scoville SL, Campbell CL, Shry EA, Stajduhar KC, Potter RN, et al. Sudden death in young adults: a 25-year review of autopsies in military recruits. *Annals of internal medicine*. 2004;141(11):829-34.
32. Cooper LT, Jr. Myocarditis. *The New England journal of medicine*. 2009;360(15):1526-38.
33. Josephson L. Infective endocarditis: a review for nurses. *Dimensions of critical care nursing : DCCN*. 2014;33(6):327-40.
34. Mazokopakis EE, Syros PK, Starakis IK. Nonbacterial thrombotic endocarditis (marantic endocarditis) in cancer patients. *Cardiovascular & hematological disorders drug targets*. 2010;10(2):84-6.
35. Wong ND. Epidemiological studies of CHD and the evolution of preventive cardiology. *Nature reviews Cardiology*. 2014;11(5):276-89.
36. Liu JL, Maniadakis N, Gray A, Rayner M. The economic burden of coronary heart disease in the UK. *Heart*. 2002;88(6):597-603.
37. Siasos G, Tsigkou V, Oikonomou E, Zaromitidou M, Tsalamandris S, Mourouzis K, et al. Circulating Biomarkers Determining Inflammation in Atherosclerosis Progression. *Current medicinal chemistry*. 2015.
38. Yayan J. Emerging families of biomarkers for coronary artery disease: inflammatory mediators. *Vascular health and risk management*. 2013;9:435-56.
39. Packard RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clinical chemistry*. 2008;54(1):24-38.
40. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nature medicine*. 2011;17(11):1410-22.

41. Zakynthinos E, Pappa N. Inflammatory biomarkers in coronary artery disease. *Journal of cardiology*. 2009;53(3):317-33.
42. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, et al. Third universal definition of myocardial infarction. *Nature reviews Cardiology*. 2012;9(11):620-33.
43. Gruntzig AR, Senning A, Siegenthaler WE. Nonoperative dilatation of coronary-artery stenosis: percutaneous transluminal coronary angioplasty. *The New England journal of medicine*. 1979;301(2):61-8.
44. Barbieri L, Verdoia M, Schaffer A, Marino P, De Luca G. [Two catheters for one coronary perforation]. *Giornale italiano di cardiologia*. 2015;16(2):112-5.
45. Chandwani P, Prajapati J, Porwal S, Khambhati B, Thakkar A. Evaluation of clinical outcomes in patients undergoing dual vessel percutaneous coronary intervention using sirolimus-eluting coronary stent system in India. *Journal of clinical and diagnostic research : JCDR*. 2015;9(2):OC05-9.
46. Ludman PF. National audit of percutaneous coronary artery interventional procedures public report. 2012 2012. Report No.
47. Chen HL, Liu K. Timing of coronary artery bypass graft surgery for acute myocardial infarction patients: a meta-analysis. *International journal of cardiology*. 2014;177(1):53-6.
48. Shiomi H, Morimoto T, Furukawa Y, Nakagawa Y, Sakata R, Okabayashi H, et al. Comparison of Percutaneous Coronary Intervention With Coronary Artery Bypass Grafting in Unprotected Left Main Coronary Artery Disease- 5-Year Outcome From CREDO-Kyoto PCI/CABG Registry Cohort-2. *Circulation journal : official journal of the Japanese Circulation Society*. 2015.
49. Haxhibeqiri-Karabdic I, Hasanovic A, Kabil E, Straus S. Improvement of ejection fraction after coronary artery bypass grafting surgery in patients with impaired left ventricular function. *Medical archives*. 2014;68(5):332-4.
50. Noon GP. Evolution of surgical treatment of coronary artery occlusive disease. *Jama*. 2009;301(9):970-1.
51. De Bakey ME, Henly WS. Surgical treatment of angina pectoris. *Circulation*. 1961;23:111-20.
52. Sabiston DC, Jr. The William F. Rienhoff, Jr. lecture. The coronary circulation. *The Johns Hopkins medical journal*. 1974;134(6):314-29.
53. Green GE, Stertzer SH, Reppert EH. Coronary arterial bypass grafts. *The Annals of thoracic surgery*. 1968;5(5):443-50.
54. Kitamura S, Kawachi K, Kawata T, Kobayashi S, Mizuguchi K, Kameda Y, et al. [Ten-year survival and cardiac event-free rates in Japanese patients with the left anterior descending artery revascularized with internal thoracic artery or saphenous vein graft: a comparative study]. *Nihon Geka Gakkai zasshi*. 1996;97(3):202-9.
55. Zimmer HG. The heart-lung machine was invented twice--the first time by Max von Frey. *Clinical cardiology*. 2003;26(9):443-5.
56. Dennis C, Spreng DS, Jr., Nelson GE, Karlson KE, Nelson RM, Thomas JV, et al. Development of a pump-oxygenator to replace the heart and lungs; an apparatus applicable to human patients, and application to one case. *Annals of surgery*. 1951;134(4):709-21.
57. Gravlee GP. *Cardiopulmonary bypass : principles and practice*. 3rd ed. Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2008. xv, 783 p. p.
58. Cohn LH. Fifty years of open-heart surgery. *Circulation*. 2003;107(17):2168-70.

59. Lynch MF, Peterson D, Baker V. Centrifugal blood pumping for open heart surgery. *Minnesota medicine*. 1978;61(9):536-8.
60. Kirklin JW, Dushane JW, Patrick RT, Donald DE, Hetzel PS, Harshbarger HG, et al. Intracardiac surgery with the aid of a mechanical pump-oxygenator system (gibbon type): report of eight cases. *Proc Staff Meet Mayo Clin*. 1955;30(10):201-6.
61. Dewall RA, Gott VL, Lillehei CW, Read RC, Varco RL, Warden HE, et al. A simple, expendable, artificial oxygenator for open heart surgery. *Surg Clin North Am*. 1956;1025-34.
62. Vallabhajosyula P, Jassar AS, Menon RS, Komlo C, Gutsche J, Desai ND, et al. Moderate Versus Deep Hypothermic Circulatory Arrest for Elective Aortic Transverse Hemiarch Reconstruction. *The Annals of thoracic surgery*. 2015.
63. Gega A, Rizzo JA, Johnson MH, Tranquilli M, Farkas EA, Elefteriades JA. Straight deep hypothermic arrest: experience in 394 patients supports its effectiveness as a sole means of brain preservation. *The Annals of thoracic surgery*. 2007;84(3):759-66; discussion 66-7.
64. Grooters RK, Ver Steeg DA, Stewart MJ, Thieman KC, Schneider RF. Echocardiographic comparison of the standard end-hole cannula, the soft-flow cannula, and the dispersion cannula during perfusion into the aortic arch. *The Annals of thoracic surgery*. 2003;75(6):1919-23.
65. Schonberger JP, Everts PA, Hoffmann JJ. Systemic blood activation with open and closed venous reservoirs. *The Annals of thoracic surgery*. 1995;59(6):1549-55.
66. Pascale F. Removal of gaseous microemboli from extracorporeal circulation. *Medical instrumentation*. 1985;19(2):70-2.
67. Consolo F, Fiore GB, Pelosi A, Reggiani S, Redaelli A. A numerical performance assessment of a commercial cardiopulmonary by-pass blood heat exchanger. *Medical engineering & physics*. 2015.
68. Alat I, Yuksel M, Buket S, Nalbantgil S, Askar F, Bayindir U, et al. The side-effects of cardiopulmonary bypass on the lungs: changes in bronchoalveolar lavage fluids. *Perfusion*. 2001;16(2):121-8.
69. Levy JH, Tanaka KA. Inflammatory response to cardiopulmonary bypass. *The Annals of thoracic surgery*. 2003;75(2):S715-20.
70. Shann KG, Likosky DS, Murkin JM, Baker RA, Baribeau YR, DeFoe GR, et al. An evidence-based review of the practice of cardiopulmonary bypass in adults: a focus on neurologic injury, glycemic control, hemodilution, and the inflammatory response. *The Journal of thoracic and cardiovascular surgery*. 2006;132(2):283-90.
71. Hansbro SD, Sharpe DA, Catchpole R, Welsh KR, Munsch CM, McGoldrick JP, et al. Haemolysis during cardiopulmonary bypass: an in vivo comparison of standard roller pumps, nonocclusive roller pumps and centrifugal pumps. *Perfusion*. 1999;14(1):3-10.
72. Jensen BO, Hughes P, Rasmussen LS, Pedersen PU, Steinbruchel DA. Cognitive outcomes in elderly high-risk patients after off-pump versus conventional coronary artery bypass grafting: a randomized trial. *Circulation*. 2006;113(24):2790-5.
73. Van Dijk D, Jansen EW, Hijman R, Nierich AP, Diephuis JC, Moons KG, et al. Cognitive outcome after off-pump and on-pump coronary artery bypass graft surgery: a randomized trial. *Jama*. 2002;287(11):1405-12.
74. Ricci M, Karamanoukian HL, Abraham R, Von Fricken K, D'Ancona G, Choi S, et al. Stroke in octogenarians undergoing coronary artery surgery with and without cardiopulmonary bypass. *The Annals of thoracic surgery*. 2000;69(5):1471-5.

75. Ascione R, Reeves BC, Rees K, Angelini GD. Effectiveness of coronary artery bypass grafting with or without cardiopulmonary bypass in overweight patients. *Circulation*. 2002;106(14):1764-70.
76. Bartels K, McDonagh DL, Newman MF, Mathew JP. Neurocognitive outcomes after cardiac surgery. *Curr Opin Anaesthesiol*. 2013;26(1):91-7.
77. Messerotti Benvenuti S, Zanatta P, Valfre C, Polesel E, Palomba D. Preliminary evidence for reduced preoperative cerebral blood flow velocity as a risk factor for cognitive decline three months after cardiac surgery: an extension study. *Perfusion*. 2012;27(6):486-92.
78. Plaschke K, Hauth S, Jansen C, Bruckner T, Schramm C, Karck M, et al. The influence of preoperative serum anticholinergic activity and other risk factors for the development of postoperative cognitive dysfunction after cardiac surgery. *The Journal of thoracic and cardiovascular surgery*. 2013;145(3):805-11.
79. Newman MF, Kirchner JL, Phillips-Bute B, Gaver V, Grocott H, Jones RH, et al. Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. *The New England journal of medicine*. 2001;344(6):395-402.
80. Hudetz JA, Gandhi SD, Iqbal Z, Patterson KM, Pagel PS. Elevated postoperative inflammatory biomarkers are associated with short- and medium-term cognitive dysfunction after coronary artery surgery. *J Anesth*. 2011;25(1):1-9.
81. Gao L, Taha R, Gauvin D, Othmen LB, Wang Y, Blaise G. Postoperative cognitive dysfunction after cardiac surgery. *Chest*. 2005;128(5):3664-70.
82. Edmunds LH, Jr. Inflammatory response to cardiopulmonary bypass. *The Annals of thoracic surgery*. 1998;66(5 Suppl):S12-6; discussion S25-8.
83. Gu YJ, Boonstra PW, Graaff R, Rijnsburger AA, Mungroop H, van Oeveren W. Pressure drop, shear stress, and activation of leukocytes during cardiopulmonary bypass: a comparison between hollow fiber and flat sheet membrane oxygenators. *Artificial organs*. 2000;24(1):43-8.
84. Kirklin JK, Blackstone EH, Kirklin JW. Cardiopulmonary bypass: studies on its damaging effects. *Blood purification*. 1987;5(2-3):168-78.
85. Amanullah MM, Hamid M, Hanif HM, Muzaffar M, Siddiqui MT, Adhi F, et al. Effect of steroids on inflammatory markers and clinical parameters in congenital open heart surgery: a randomised controlled trial. *Cardiol Young*. 2015:1-10.
86. Asimakopoulos G. Systemic inflammation and cardiac surgery: an update. *Perfusion*. 2001;16(5):353-60.
87. Day JR, Taylor KM. The systemic inflammatory response syndrome and cardiopulmonary bypass. *International journal of surgery*. 2005;3(2):129-40.
88. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest*. 1992;101(6):1644-55.
89. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Critical care medicine*. 2001;29(7):1303-10.
90. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992. *Chest*. 2009;136(5 Suppl):e28.

91. McGuinness J, Bouchier-Hayes D, Redmond JM. Understanding the inflammatory response to cardiac surgery. *The surgeon : journal of the Royal Colleges of Surgeons of Edinburgh and Ireland*. 2008;6(3):162-71.
92. Moat NE, Shore DF, Evans TW. Organ dysfunction and cardiopulmonary bypass: the role of complement and complement regulatory proteins. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 1993;7(11):563-73.
93. Mojciak CF, Levy JH. Aprotinin and the systemic inflammatory response after cardiopulmonary bypass. *The Annals of thoracic surgery*. 2001;71(2):745-54.
94. Kirklin JK, Westaby S, Blackstone EH, Kirklin JW, Chenoweth DE, Pacifico AD. Complement and the damaging effects of cardiopulmonary bypass. *The Journal of thoracic and cardiovascular surgery*. 1983;86(6):845-57.
95. Gourlay T, Stefanou DC, Asimakopoulos G, Taylor KM. The effect of circuit surface area on CD11b(mac-1) expression in a rat recirculation model. *Artificial organs*. 2001;25(6):475-9.
96. Wan S, LeClerc JL, Vincent JL. Inflammatory response to cardiopulmonary bypass: mechanisms involved and possible therapeutic strategies. *Chest*. 1997;112(3):676-92.
97. Partrick DA, Moore FA, Moore EE, Biffl WL, Sauaia A, Barnett CC, Jr. Jack A. Barney Resident Research Award winner. The inflammatory profile of interleukin-6, interleukin-8, and soluble intercellular adhesion molecule-1 in postinjury multiple organ failure. *American journal of surgery*. 1996;172(5):425-9; discussed 9-31.
98. Zakkar M, Guida G, Suleiman MS, Angelini GD. Cardiopulmonary bypass and oxidative stress. *Oxidative medicine and cellular longevity*. 2015;2015:189863.
99. Hunt BJ, Parratt RN, Segal HC, Sheikh S, Kallis P, Yacoub M. Activation of coagulation and fibrinolysis during cardiothoracic operations. *The Annals of thoracic surgery*. 1998;65(3):712-8.
100. Prasad K, Kalra J, Bharadwaj B, Chaudhary AK. Increased oxygen free radical activity in patients on cardiopulmonary bypass undergoing aortocoronary bypass surgery. *American heart journal*. 1992;123(1):37-45.
101. Landow L, Andersen LW. Splanchnic ischaemia and its role in multiple organ failure. *Acta anaesthesiologica Scandinavica*. 1994;38(7):626-39.
102. Tao W, Zwischenberger JB, Nguyen TT, Vertrees RA, McDaniel LB, Nutt LK, et al. Gut mucosal ischemia during normothermic cardiopulmonary bypass results from blood flow redistribution and increased oxygen demand. *The Journal of thoracic and cardiovascular surgery*. 1995;110(3):819-28.
103. Asimakopoulos G. Mechanisms of the systemic inflammatory response. *Perfusion*. 1999;14(4):269-77.
104. Chaney MA. Corticosteroids and cardiopulmonary bypass : a review of clinical investigations. *Chest*. 2002;121(3):921-31.
105. Hall RI, Smith MS, Rocker G. The systemic inflammatory response to cardiopulmonary bypass: pathophysiological, therapeutic, and pharmacological considerations. *Anesthesia and analgesia*. 1997;85(4):766-82.
106. Hill GE, Alonso A, Spurzem JR, Stammers AH, Robbins RA. Aprotinin and methylprednisolone equally blunt cardiopulmonary bypass-induced inflammation in humans. *The Journal of thoracic and cardiovascular surgery*. 1995;110(6):1658-62.
107. Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1.

- Proceedings of the National Academy of Sciences of the United States of America. 1992;89(21):9991-5.
108. Hill GE, Alonso A, Thiele GM, Robbins RA. Glucocorticoids blunt neutrophil CD11b surface glycoprotein upregulation during cardiopulmonary bypass in humans. *Anesthesia and analgesia*. 1994;79(1):23-7.
 109. Engelman RM, Rousou JA, Flack JE, 3rd, Deaton DW, Kalfin R, Das DK. Influence of steroids on complement and cytokine generation after cardiopulmonary bypass. *The Annals of thoracic surgery*. 1995;60(3):801-4.
 110. Lu H, Du Buit C, Soria J, Touchot B, Chollet B, Commin PL, et al. Postoperative hemostasis and fibrinolysis in patients undergoing cardiopulmonary bypass with or without aprotinin therapy. *Thrombosis and haemostasis*. 1994;72(3):438-43.
 111. Gallimore MJ, Fuhrer G, Heller W, Hoffmeister HE. Augmentation of kallikrein and plasmin inhibition capacity by aprotinin using a new assay to monitor therapy. *Advances in experimental medicine and biology*. 1989;247B:55-60.
 112. Asimakopoulos G, Thompson R, Nourshargh S, Lidington EA, Mason JC, Ratnatunga CP, et al. An anti-inflammatory property of aprotinin detected at the level of leukocyte extravasation. *The Journal of thoracic and cardiovascular surgery*. 2000;120(2):361-9.
 113. Hill GE, Pohorecki R, Alonso A, Rennard SI, Robbins RA. Aprotinin reduces interleukin-8 production and lung neutrophil accumulation after cardiopulmonary bypass. *Anesthesia and analgesia*. 1996;83(4):696-700.
 114. Fitch JC, Rollins S, Matis L, Alford B, Aranki S, Collard CD, et al. Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. *Circulation*. 1999;100(25):2499-506.
 115. Bhole D, Stahl GL. Therapeutic potential of targeting the complement cascade in critical care medicine. *Critical care medicine*. 2003;31(1 Suppl):S97-104.
 116. Hayashi Y, Sawa Y, Nishimura M, Tojo SJ, Ichikawa H, Satoh H, et al. P-selectin monoclonal antibody may attenuate the whole body inflammatory response induced by cardiopulmonary bypass. *ASAIO journal*. 2000;46(3):334-7.
 117. Boyle EM, Jr., Morgan EN, Kovacich JC, Canty TG, Jr., Verrier ED. Microvascular responses to cardiopulmonary bypass. *Journal of cardiothoracic and vascular anesthesia*. 1999;13(4 Suppl 1):30-5; discussion 6-7.
 118. Hayashi Y, Sawa Y, Nishimura M, Tojo SJ, Fukuyama N, Nakazawa H, et al. P-selectin participates in cardiopulmonary bypass-induced inflammatory response in association with nitric oxide and peroxynitrite production. *The Journal of thoracic and cardiovascular surgery*. 2000;120(3):558-65.
 119. Nguyen B, Luong L, Naase H, Vives M, Jakaj G, Finch J, et al. Sulforaphane pretreatment prevents systemic inflammation and renal injury in response to cardiopulmonary bypass. *The Journal of thoracic and cardiovascular surgery*. 2014;148(2):690-7 e3.
 120. Freyholdt T, Massoudy P, Zahler S, Henze R, Barankay A, Becker BF, et al. Beneficial effect of sodium nitroprusside after coronary artery bypass surgery: pump function correlates inversely with cardiac release of proinflammatory cytokines. *Journal of cardiovascular pharmacology*. 2003;42(3):372-8.
 121. Massoudy P, Zahler S, Freyholdt T, Henze R, Barankay A, Becker BF, et al. Sodium nitroprusside in patients with compromised left ventricular function undergoing coronary bypass: reduction of cardiac proinflammatory substances. *The Journal of thoracic and cardiovascular surgery*. 2000;119(3):566-74.

122. Chello M, Mastroroberto P, Malta E, Cirillo F, Celi V. Inhibition by dipyridamole of neutrophil adhesion to vascular endothelium during coronary bypass surgery. *The Annals of thoracic surgery*. 1999;67(5):1277-82.
123. Markewitz A, Faist E, Lang S, Endres S, Hultner L, Reichart B. Regulation of acute phase response after cardiopulmonary bypass by immunomodulation. *The Annals of thoracic surgery*. 1993;55(2):389-94.
124. Moen O, Hogasen K, Fosse E, Dregelid E, Brockmeier V, Venge P, et al. Attenuation of changes in leukocyte surface markers and complement activation with heparin-coated cardiopulmonary bypass. *The Annals of thoracic surgery*. 1997;63(1):105-11.
125. Yamada H, Kudoh I, Hirose Y, Toyoshima M, Abe H, Kurahashi K. Heparin-coated circuits reduce the formation of TNF alpha during cardiopulmonary bypass. *Acta anaesthesiologica Scandinavica*. 1996;40(3):311-7.
126. Grossi EA, Kallenbach K, Chau S, Derivaux CC, Aguinaga MG, Steinberg BM, et al. Impact of heparin bonding on pediatric cardiopulmonary bypass: a prospective randomized study. *The Annals of thoracic surgery*. 2000;70(1):191-6.
127. Heddle NM, Blajchman MA. The leukodepletion of cellular blood products in the prevention of HLA-alloimmunization and refractoriness to allogeneic platelet transfusions. *Blood*. 1995;85(3):603-6.
128. Boodram S, Evans E. Use of leukocyte-depleting filters during cardiac surgery with cardiopulmonary bypass: a review. *The Journal of extra-corporeal technology*. 2008;40(1):27-42.
129. Bechtel JF, Muhlenbein S, Eichler W, Marx M, Sievers HH. Leukocyte depletion during cardiopulmonary bypass in routine adult cardiac surgery. *Interactive cardiovascular and thoracic surgery*. 2011;12(2):207-12.
130. Wang MJ, Chiu IS, Hsu CM, Wang CM, Lin PL, Chang CI, et al. Efficacy of ultrafiltration in removing inflammatory mediators during pediatric cardiac operations. *The Annals of thoracic surgery*. 1996;61(2):651-6.
131. Luciani GB, Menon T, Vecchi B, Auriemma S, Mazzucco A. Modified ultrafiltration reduces morbidity after adult cardiac operations: a prospective, randomized clinical trial. *Circulation*. 2001;104(12 Suppl 1):I253-9.
132. Dhurandhar V, Saxena A, Parikh R, Vallely MP, Wilson MK, Butcher JK, et al. Outcomes of On-Pump versus Off-Pump Coronary Artery Bypass Graft Surgery in the High Risk (AusSCORE > 5). *Heart, lung & circulation*. 2015.
133. Ghorbel MT, Cherif M, Mokhtari A, Bruno VD, Caputo M, Angelini GD. Off-pump coronary artery bypass surgery is associated with fewer gene expression changes in the human myocardium in comparison with on-pump surgery. *Physiological genomics*. 2010;42(1):67-75.
134. Ohata T, Mitsuno M, Yamamura M, Tanaka H, Kobayashi Y, Ryomoto M, et al. Minimal cardiopulmonary bypass attenuates neutrophil activation and cytokine release in coronary artery bypass grafting. *Journal of artificial organs : the official journal of the Japanese Society for Artificial Organs*. 2007;10(2):92-5.
135. Nguyen BA, Fiorentino F, Reeves BC, Baig K, Athanasiou T, Anderson JR, et al. Mini Bypass and Proinflammatory Leukocyte Activation: A Randomized Controlled Trial. *The Annals of thoracic surgery*. 2015.
136. Biancari F, Rimpilainen R. Meta-analysis of randomised trials comparing the effectiveness of miniaturised versus conventional cardiopulmonary bypass in adult cardiac surgery. *Heart*. 2009;95(12):964-9.
137. Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by TLR family. *Immunology letters*. 2003;85(2):85-95.

138. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annual review of immunology*. 1999;17:593-623.
139. Medzhitov R, Janeway C, Jr. Innate immunity. *The New England journal of medicine*. 2000;343(5):338-44.
140. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. *Mediators of inflammation*. 2010;2010.
141. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signals that spur autophagy and immunity. *Immunological reviews*. 2012;249(1):158-75.
142. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature*. 2006;442(7098):39-44.
143. Zhao L, Lee JY, Hwang DH. The phosphatidylinositol 3-kinase/Akt pathway negatively regulates Nod2-mediated NF-kappaB pathway. *Biochemical pharmacology*. 2008;75(7):1515-25.
144. Gavins FN. Are formyl peptide receptors novel targets for therapeutic intervention in ischaemia-reperfusion injury? *Trends in pharmacological sciences*. 2010;31(6):266-76.
145. Dufton N, Perretti M. Therapeutic anti-inflammatory potential of formyl-peptide receptor agonists. *Pharmacology & therapeutics*. 2010;127(2):175-88.
146. Palmai-Pallag T, Bachrati CZ. Inflammation-induced DNA damage and damage-induced inflammation: a vicious cycle. *Microbes and infection / Institut Pasteur*. 2014;16(10):822-32.
147. Delves PJ, Roitt IM. *Roitt's essential immunology*. 12th ed. Chichester, West Sussex ; Hoboken, NJ: Wiley-Blackwell; 2011. xiii, 546 p. p.
148. Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *International immunology*. 2009;21(4):317-37.
149. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124(4):783-801.
150. Ausubel FM. Are innate immune signaling pathways in plants and animals conserved? *Nature immunology*. 2005;6(10):973-9.
151. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annual review of immunology*. 2002;20:197-216.
152. Creagh EM, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends in immunology*. 2006;27(8):352-7.
153. Farkas AM, Kilgore TM, Lotze MT. Detecting DNA: getting and begetting cancer. *Current opinion in investigational drugs*. 2007;8(12):981-6.
154. Zhang Q, Itagaki K, Hauser CJ. Mitochondrial DNA is released by shock and activates neutrophils via p38 map kinase. *Shock*. 2010;34(1):55-9.
155. Dybdahl B, Wahba A, Lien E, Flo TH, Waage A, Qureshi N, et al. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. *Circulation*. 2002;105(6):685-90.
156. Pisetsky DS, Erlandsson-Harris H, Andersson U. High-mobility group box protein 1 (HMGB1): an alarmin mediating the pathogenesis of rheumatic disease. *Arthritis research & therapy*. 2008;10(3):209.
157. Stoecklein VM, Osuka A, Lederer JA. Trauma equals danger--damage control by the immune system. *Journal of leukocyte biology*. 2012;92(3):539-51.
158. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature*. 2010;464(7285):104-7.
159. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nature reviews Immunology*. 2009;9(5):353-63.

160. Hansson GK, Edfeldt K. Toll to be paid at the gateway to the vessel wall. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25(6):1085-7.
161. Akira S, Takeda K. Toll-like receptor signalling. *Nature reviews Immunology*. 2004;4(7):499-511.
162. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell*. 1996;86(6):973-83.
163. Nomura N, Miyajima N, Sazuka T, Tanaka A, Kawarabayasi Y, Sato S, et al. Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. *DNA research : an international journal for rapid publication of reports on genes and genomes*. 1994;1(1):27-35.
164. Taguchi T, Mitcham JL, Dower SK, Sims JE, Testa JR. Chromosomal localization of TIL, a gene encoding a protein related to the *Drosophila* transmembrane receptor Toll, to human chromosome 4p14. *Genomics*. 1996;32(3):486-8.
165. Ceccarelli S, Nobili V, Alisi A. Toll-like receptor-mediated signaling cascade as a regulator of the inflammation network during alcoholic liver disease. *World journal of gastroenterology : WJG*. 2014;20(44):16443-51.
166. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology*. 2010;11(5):373-84.
167. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*. 2010;140(6):805-20.
168. Sasai M, Yamamoto M. Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors. *International reviews of immunology*. 2013;32(2):116-33.
169. Yarovinsky F. Innate immunity to *Toxoplasma gondii* infection. *Nature reviews Immunology*. 2014;14(2):109-21.
170. Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, et al. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(10):3516-21.
171. O'Neill LA. How Toll-like receptors signal: what we know and what we don't know. *Current opinion in immunology*. 2006;18(1):3-9.
172. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nature immunology*. 2003;4(2):161-7.
173. Frazao JB, Errante PR, Condino-Neto A. Toll-like receptors' pathway disturbances are associated with increased susceptibility to infections in humans. *Archivum immunologiae et therapiae experimentalis*. 2013;61(6):427-43.
174. Jin MS, Lee JO. Structures of the toll-like receptor family and its ligand complexes. *Immunity*. 2008;29(2):182-91.
175. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *Journal of immunology*. 2002;169(1):10-4.
176. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *International immunology*. 2001;13(7):933-40.

177. Prinz M, Heikenwalder M, Schwarz P, Takeda K, Akira S, Aguzzi A. Prion pathogenesis in the absence of Toll-like receptor signalling. *EMBO reports*. 2003;4(2):195-9.
178. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. 2001;413(6857):732-8.
179. Committee HGN. TLR1 toll-like receptor 1 [Homo sapiens (human)] [updated 4-Jan-2015]. Available from: <http://www.ncbi.nlm.nih.gov/gene/7096>.
180. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nature reviews Immunology*. 2006;6(1):33-43.
181. Shimizu T, Kida Y, Kuwano K. Triacylated lipoproteins derived from *Mycoplasma pneumoniae* activate nuclear factor-kappaB through toll-like receptors 1 and 2. *Immunology*. 2007;121(4):473-83.
182. Chantratita N, Tandhavanant S, Myers ND, Chierakul W, Wuthiekanun V, Mahavanakul W, et al. Common TLR1 genetic variation is not associated with death from melioidosis, a common cause of sepsis in rural Thailand. *PloS one*. 2014;9(1):e83285.
183. Qi H, Sun L, Wu X, Jin Y, Xiao J, Wang S, et al. Toll-like receptor 1(TLR1) Gene SNP rs5743618 is associated with increased risk for tuberculosis in Han Chinese children. *Tuberculosis*. 2014.
184. Alexopoulou L, Thomas V, Schnare M, Lobet Y, Anguita J, Schoen RT, et al. Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice. *Nature medicine*. 2002;8(8):878-84.
185. Tunis MC, Marshall JS. Toll-like receptor 2 as a regulator of oral tolerance in the gastrointestinal tract. *Mediators of inflammation*. 2014;2014:606383.
186. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *The Journal of experimental medicine*. 2003;197(9):1107-17.
187. Tsui FW, Xi N, Rohekar S, Riarh R, Bilotta R, Tsui HW, et al. Toll-like receptor 2 variants are associated with acute reactive arthritis. *Arthritis and rheumatism*. 2008;58(11):3436-8.
188. Gluba A, Banach M, Hannam S, Mikhailidis DP, Sakowicz A, Rysz J. The role of Toll-like receptors in renal diseases. *Nature reviews Nephrology*. 2010;6(4):224-35.
189. Alves-Filho JC, Freitas A, Souto FO, Spiller F, Paula-Neto H, Silva JS, et al. Regulation of chemokine receptor by Toll-like receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(10):4018-23.
190. Hadley JS, Wang JE, Michaels LC, Dempsey CM, Foster SJ, Thiemermann C, et al. Alterations in inflammatory capacity and TLR expression on monocytes and neutrophils after cardiopulmonary bypass. *Shock*. 2007;27(5):466-73.
191. Flier S, Concepcion AN, Versteeg D, Kappen TH, Hoefer IE, de Lange DW, et al. Monocyte hyporesponsiveness and Toll-like receptor expression profiles in coronary artery bypass grafting and its clinical implications for postoperative inflammatory response and pneumonia: An observational cohort study. *European journal of anaesthesiology*. 2015;32(3):177-88.
192. Chalk K, Meisel C, Spies C, Volk T, Thuenemann K, Linneweber J, et al. Dysfunction of alveolar macrophages after cardiac surgery and postoperative pneumonia?--An observational study. *Critical care (London, England)*. 2013;17(6):R285.

193. Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. *Virology*. 1996;219(2):339-49.
194. Schulz O, Diebold SS, Chen M, Naslund TI, Nolte MA, Alexopoulou L, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature*. 2005;433(7028):887-92.
195. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science*. 2003;301(5633):640-3.
196. Bell JK, Askins J, Hall PR, Davies DR, Segal DM. The dsRNA binding site of human Toll-like receptor 3. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(23):8792-7.
197. Keshava Prasad TS, Goel R, Kandasamy K, Keerthikumar S, Kumar S, Mathivanan S, et al. Human Protein Reference Database--2009 update. *Nucleic acids research*. 2009;37(Database issue):D767-72.
198. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine*. 1999;189(11):1777-82.
199. Viriyakosol S, Tobias PS, Kitchens RL, Kirkland TN. MD-2 binds to bacterial lipopolysaccharide. *The Journal of biological chemistry*. 2001;276(41):38044-51.
200. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282(5396):2085-8.
201. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. *The Journal of biological chemistry*. 2000;275(4):2251-4.
202. Nielsen TG, Nordestgaard BG, von Jessen F, Andreassen JJ, Wiik A, Heegaard NH, et al. Antibodies to cardiolipin may increase the risk of failure of peripheral vein bypasses. *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery*. 1997;14(3):177-84.
203. Gargiulo S, Gamba P, Testa G, Rossin D, Biasi F, Poli G, et al. Relation between TLR4/NF-kappaB signaling pathway activation by 27-hydroxycholesterol and 4-hydroxynonenal, and atherosclerotic plaque instability. *Aging cell*. 2015.
204. Abdollahi-Roodsaz S, Joosten LA, Roelofs MF, Radstake TR, Matera G, Popa C, et al. Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. *Arthritis and rheumatism*. 2007;56(9):2957-67.
205. Liu K, Shen L, Wang J, Dong G, Wu H, Shao H, et al. The preventative role of curcumin on the lung inflammatory response induced by cardiopulmonary bypass in rats. *The Journal of surgical research*. 2012;174(1):73-82.
206. Liu X, Wu Y, Tang Y, Geng Z, Han Y, Zhang D. The development of early-onset ventilator-associated pneumonia after cardiac surgery with cardiopulmonary bypass is associated with toll-like receptor 4 signal transduction pathways. *Inflammation*. 2015;38(1):187-94.
207. Krejsek J, Kolackova M, Mand'ak J, Kunes P, Holubcova Z, Holmannova D, et al. TLR2 and TLR4 expression on blood monocytes and granulocytes of cardiac surgical patients is not affected by the use of cardiopulmonary bypass. *Acta medica*. 2013;56(2):57-66.
208. Kaczorowski DJ, Nakao A, McCurry KR, Billiar TR. Toll-like receptors and myocardial ischemia/reperfusion, inflammation, and injury. *Curr Cardiol Rev*. 2009;5(3):196-202.

209. Chong AJ, Shimamoto A, Hampton CR, Takayama H, Spring DJ, Rothnie CL, et al. Toll-like receptor 4 mediates ischemia/reperfusion injury of the heart. *The Journal of thoracic and cardiovascular surgery*. 2004;128(2):170-9.
210. Oyama J, Blais C, Jr., Liu X, Pu M, Kobzik L, Kelly RA, et al. Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. *Circulation*. 2004;109(6):784-9.
211. Merry HE, Phelan P, Doak MR, Zhao M, Hwang B, Mulligan MS. Role of toll-like receptor-4 in lung ischemia-reperfusion injury. *The Annals of thoracic surgery*. 2015;99(4):1193-9.
212. Shimamoto A, Pohlman TH, Shomura S, Tarukawa T, Takao M, Shimpō H. Toll-like receptor 4 mediates lung ischemia-reperfusion injury. *The Annals of thoracic surgery*. 2006;82(6):2017-23.
213. Wu HS, Zhang JX, Wang L, Tian Y, Wang H, Rotstein O. Toll-like receptor 4 involvement in hepatic ischemia/reperfusion injury in mice. *Hepatobiliary Pancreat Dis Int*. 2004;3(2):250-3.
214. Tsung A, Hoffman RA, Izuishi K, Critchlow ND, Nakao A, Chan MH, et al. Hepatic ischemia/reperfusion injury involves functional TLR4 signaling in nonparenchymal cells. *Journal of immunology*. 2005;175(11):7661-8.
215. Kim BS, Lim SW, Li C, Kim JS, Sun BK, Ahn KO, et al. Ischemia-reperfusion injury activates innate immunity in rat kidneys. *Transplantation*. 2005;79(10):1370-7.
216. Li XQ, Wang J, Fang B, Tan WF, Ma H. Intrathecal antagonism of microglial TLR4 reduces inflammatory damage to blood-spinal cord barrier following ischemia/reperfusion injury in rats. *Mol Brain*. 2014;7:28.
217. Committe HGN. Toll-like receptor 5 [updated 4-Jan-2015]. Available from: <http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=7100>.
218. Sebastiani G, Leveque G, Lariviere L, Laroche L, Skamene E, Gros P, et al. Cloning and characterization of the murine toll-like receptor 5 (Tlr5) gene: sequence and mRNA expression studies in Salmonella-susceptible MOLF/Ei mice. *Genomics*. 2000;64(3):230-40.
219. Mizel SB, Bates JT. Flagellin as an adjuvant: cellular mechanisms and potential. *Journal of immunology*. 2010;185(10):5677-82.
220. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*. 2001;410(6832):1099-103.
221. Burdelya LG, Krivokrysenko VI, Tallant TC, Strom E, Gleiberman AS, Gupta D, et al. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science*. 2008;320(5873):226-30.
222. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. *Nature reviews Cancer*. 2009;9(1):57-63.
223. Kauppila JH, Mattila AE, Karttunen TJ, Salo T. Toll-like receptor 5 (TLR5) expression is a novel predictive marker for recurrence and survival in squamous cell carcinoma of the tongue. *British journal of cancer*. 2013;108(3):638-43.
224. Zhou H, Chen JH, Hu J, Luo YZ, Li F, Xiao L, et al. High expression of Toll-like receptor 5 correlates with better prognosis in non-small-cell lung cancer: an anti-tumor effect of TLR5 signaling in non-small cell lung cancer. *Journal of cancer research and clinical oncology*. 2014;140(4):633-43.
225. Committe HGN. Toll-like receptor 6 [updated 7-Jan-2015]. Available from: <http://www.ncbi.nlm.nih.gov/gene/10333>.

226. Takeuchi O, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, et al. TLR6: A novel member of an expanding toll-like receptor family. *Gene*. 1999;231(1-2):59-65.
227. Winters AH, Levan TD, Vogel SN, Chesko KL, Pollin TI, Viscardi RM. Single nucleotide polymorphism in toll-like receptor 6 is associated with a decreased risk for ureaplasma respiratory tract colonization and bronchopulmonary dysplasia in preterm infants. *The Pediatric infectious disease journal*. 2013;32(8):898-904.
228. Wang JP, Liu P, Latz E, Golenbock DT, Finberg RW, Libraty DH. Flavivirus activation of plasmacytoid dendritic cells delineates key elements of TLR7 signaling beyond endosomal recognition. *Journal of immunology*. 2006;177(10):7114-21.
229. Kawai T, Akira S. Innate immune recognition of viral infection. *Nature immunology*. 2006;7(2):131-7.
230. Funk E, Kottlil S, Gilliam B, Talwani R. Tickling the TLR7 to cure viral hepatitis. *Journal of translational medicine*. 2014;12:129.
231. Komatsuda A, Wakui H, Iwamoto K, Ozawa M, Togashi M, Masai R, et al. Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Clinical and experimental immunology*. 2008;152(3):482-7.
232. Rubtsova K, Marrack P, Rubtsov AV. TLR7, IFN γ , and T-bet: Their roles in the development of ABCs in female-biased autoimmunity. *Cellular immunology*. 2014.
233. Lau CM, Broughton C, Tabor AS, Akira S, Flavell RA, Mamula MJ, et al. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *The Journal of experimental medicine*. 2005;202(9):1171-7.
234. Subramanian S, Tus K, Li QZ, Wang A, Tian XH, Zhou J, et al. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(26):9970-5.
235. Committe HGN. Toll-like receptor 8 [updated 4-Jan-2015]. Available from: <http://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=51311>.
236. Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity*. 2010;32(3):305-15.
237. Takeda K, Akira S. Toll-like receptors in innate immunity. *International immunology*. 2005;17(1):1-14.
238. Peng G, Guo Z, Kiniwa Y, Voo KS, Peng W, Fu T, et al. Toll-like receptor 8-mediated reversal of CD4⁺ regulatory T cell function. *Science*. 2005;309(5739):1380-4.
239. Kumagai Y, Takeuchi O, Akira S. TLR9 as a key receptor for the recognition of DNA. *Advanced drug delivery reviews*. 2008;60(7):795-804.
240. Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, et al. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nature immunology*. 2004;5(10):1061-8.
241. Kawai T, Akira S. TLR signaling. *Cell death and differentiation*. 2006;13(5):816-25.
242. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*. 2002;416(6881):603-7.
243. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nature reviews Immunology*. 2006;6(11):823-35.

244. Hacker H, Mischak H, Miethke T, Liptay S, Schmid R, Sparwasser T, et al. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *The EMBO journal*. 1998;17(21):6230-40.
245. Weihrauch MR, Richly H, von Bergwelt-Baildon MS, Becker HJ, Schmidt M, Hacker UT, et al. Phase I clinical study of the toll-like receptor 9 agonist MGN1703 in patients with metastatic solid tumours. *Eur J Cancer*. 2015;51(2):146-56.
246. Oosting M, Cheng SC, Bolscher JM, Vestering-Stenger R, Plantinga TS, Verschuere IC, et al. Human TLR10 is an anti-inflammatory pattern-recognition receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111(42):E4478-84.
247. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *Journal of immunology*. 2005;174(5):2942-50.
248. Takeda K, Akira S. Toll-like receptors. *Current protocols in immunology* / edited by John E Coligan [et al]. 2007;Chapter 14:Unit 14 2.
249. Mulla MJ, Myrtolli K, Tadesse S, Stanwood NL, Garipey A, Guller S, et al. Cutting-edge report: TLR10 plays a role in mediating bacterial peptidoglycan-induced trophoblast apoptosis. *American journal of reproductive immunology*. 2013;69(5):449-53.
250. Fan Y, Liu B. Expression of Toll-like receptors in the mucosa of patients with ulcerative colitis. *Experimental and therapeutic medicine*. 2015;9(4):1455-9.
251. Stevens VL, Hsing AW, Talbot JT, Zheng SL, Sun J, Chen J, et al. Genetic variation in the toll-like receptor gene cluster (TLR10-TLR1-TLR6) and prostate cancer risk. *International journal of cancer Journal international du cancer*. 2008;123(11):2644-50.
252. Baenziger S, Heikenwalder M, Johansen P, Schlaepfer E, Hofer U, Miller RC, et al. Triggering TLR7 in mice induces immune activation and lymphoid system disruption, resembling HIV-mediated pathology. *Blood*. 2009;113(2):377-88.
253. Bafica A, Scanga CA, Schito M, Chaussabel D, Sher A. Influence of coinfecting pathogens on HIV expression: evidence for a role of Toll-like receptors. *Journal of immunology*. 2004;172(12):7229-34.
254. Cohen KW, Dugast AS, Alter G, McElrath MJ, Stamatatos L. HIV-1 Single-Stranded RNA Induces CXCL13 Secretion in Human Monocytes via TLR7 Activation and Plasmacytoid Dendritic Cell-Derived Type I IFN. *Journal of immunology*. 2015;194(6):2769-75.
255. Ghosh AK, Sinha D, Mukherjee S, Biswas R, Biswas T. LPS stimulates and Hsp70 down-regulates TLR4 to orchestrate differential cytokine response of culture-differentiated innate memory CD8 T cells. *Cytokine*. 2015;73(1):44-52.
256. Weighardt H, Kaiser-Moore S, Vabulas RM, Kirschning CJ, Wagner H, Holzmann B. Cutting edge: myeloid differentiation factor 88 deficiency improves resistance against sepsis caused by polymicrobial infection. *Journal of immunology*. 2002;169(6):2823-7.
257. Netea MG, Wijmenga C, O'Neill LA. Genetic variation in Toll-like receptors and disease susceptibility. *Nature immunology*. 2012;13(6):535-42.
258. Picard C, von Bernuth H, Ghandil P, Chrabieh M, Levy O, Arkwright PD, et al. Clinical features and outcome of patients with IRAK-4 and MyD88 deficiency. *Medicine*. 2010;89(6):403-25.

259. Castano-Rodriguez N, Kaakoush NO, Mitchell HM. Pattern-recognition receptors and gastric cancer. *Frontiers in immunology*. 2014;5:336.
260. Schmausser B, Andrulis M, Endrich S, Muller-Hermelink HK, Eck M. Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with *Helicobacter pylori*. *International journal of medical microbiology* : IJMM. 2005;295(3):179-85.
261. Tang FB, Li ZX, Wang YM, Zhang L, Ma JL, Zhou T, et al. Toll-like receptor 1 and 10 polymorphisms, *Helicobacter pylori* susceptibility and risk of gastric lesions in a high-risk Chinese population. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*. 2015;31:263-9.
262. Boraska Jelavic T, Barisic M, Drmic Hofman I, Boraska V, Vrdoljak E, Peruzovic M, et al. Microsatellite GT polymorphism in the toll-like receptor 2 is associated with colorectal cancer. *Clinical genetics*. 2006;70(2):156-60.
263. Kelly MG, Alvero AB, Chen R, Silasi DA, Abrahams VM, Chan S, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. *Cancer research*. 2006;66(7):3859-68.
264. Zhou XX, Jia WH, Shen GP, Qin HD, Yu XJ, Chen LZ, et al. Sequence variants in toll-like receptor 10 are associated with nasopharyngeal carcinoma risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2006;15(5):862-6.
265. Feng M, Chen JY, Weissman-Tsukamoto R, Volkmer JP, Ho PY, McKenna KM, et al. Macrophages eat cancer cells using their own calreticulin as a guide: Roles of TLR and Btk. *Proceedings of the National Academy of Sciences of the United States of America*. 2015;112(7):2145-50.
266. Roberts DD, Kaur S, Soto-Pantoja DR. Therapeutic targeting of the thrombospondin-1 receptor CD47 to treat liver cancer. *Journal of cell communication and signaling*. 2015.
267. Chen JQ, Szodoray P, Zeher M. Toll-Like Receptor Pathways in Autoimmune Diseases. *Clinical reviews in allergy & immunology*. 2015.
268. Broen JC, Bossini-Castillo L, van Bon L, Vonk MC, Knaapen H, Beretta L, et al. A rare polymorphism in the gene for Toll-like receptor 2 is associated with systemic sclerosis phenotype and increases the production of inflammatory mediators. *Arthritis and rheumatism*. 2012;64(1):264-71.
269. van Lieshout AW, Vonk MC, Bredie SJ, Joosten LB, Netea MG, van Riel PL, et al. Enhanced interleukin-10 production by dendritic cells upon stimulation with Toll-like receptor 4 agonists in systemic sclerosis that is possibly implicated in CCL18 secretion. *Scandinavian journal of rheumatology*. 2009;38(4):282-90.
270. Gooshe M, Aleyasin AR, Abdolghaffari AH, Rezaei N. Toll like receptors: a new hope on the horizon to treat multiple sclerosis. *Expert review of clinical immunology*. 2014;10(10):1277-9.
271. Wagner KB, Felix SB, Riad A. Innate immune receptors in heart failure: Side effect or potential therapeutic target? *World journal of cardiology*. 2014;6(8):791-801.
272. Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. *Biological & pharmaceutical bulletin*. 2005;28(5):886-92.
273. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002;105(9):1135-43.

274. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation*. 2002;105(10):1158-61.
275. Shinohara M, Hirata K, Yamashita T, Takaya T, Sasaki N, Shiraki R, et al. Local overexpression of toll-like receptors at the vessel wall induces atherosclerotic lesion formation: synergism of TLR2 and TLR4. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27(11):2384-91.
276. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. *Nature reviews Immunology*. 2015;15(2):104-16.
277. Mizoguchi E, Orihara K, Hamasaki S, Ishida S, Kataoka T, Ogawa M, et al. Association between Toll-like receptors and the extent and severity of coronary artery disease in patients with stable angina. *Coronary artery disease*. 2007;18(1):31-8.
278. Derbali H, Bosse Y, Cote N, Pibarot P, Audet A, Pepin A, et al. Increased biglycan in aortic valve stenosis leads to the overexpression of phospholipid transfer protein via Toll-like receptor 2. *The American journal of pathology*. 2010;176(6):2638-45.
279. Song R, Zeng Q, Ao L, Yu JA, Cleveland JC, Zhao KS, et al. Biglycan induces the expression of osteogenic factors in human aortic valve interstitial cells via Toll-like receptor-2. *Arteriosclerosis, thrombosis, and vascular biology*. 2012;32(11):2711-20.
280. Meng X, Ao L, Song Y, Babu A, Yang X, Wang M, et al. Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: potential roles in aortic valve inflammation and stenosis. *Am J Physiol Cell Physiol*. 2008;294(1):C29-35.
281. Fuse K, Chan G, Liu Y, Gudgeon P, Husain M, Chen M, et al. Myeloid differentiation factor-88 plays a crucial role in the pathogenesis of Coxsackievirus B3-induced myocarditis and influences type I interferon production. *Circulation*. 2005;112(15):2276-85.
282. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, et al. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nature medicine*. 2000;6(4):435-42.
283. Trop S, Marshall JC, Mazer CD, Gupta M, Dumont DJ, Bourdeau A, et al. Perioperative cardiovascular system failure in South Asians undergoing cardiopulmonary bypass is associated with prolonged inflammation and increased Toll-like receptor signaling in inflammatory monocytes. *The Journal of surgical research*. 2014;187(1):43-52.
284. Lu C, Ren D, Wang X, Ha T, Liu L, Lee EJ, et al. Toll-like receptor 3 plays a role in myocardial infarction and ischemia/reperfusion injury. *Biochimica et biophysica acta*. 2014;1842(1):22-31.
285. Methe H, Zimmer E, Grimm C, Nabauer M, Koglin J. Evidence for a role of toll-like receptor 4 in development of chronic allograft rejection after cardiac transplantation. *Transplantation*. 2004;78(9):1324-31.
286. Foley NM, Wang J, Redmond HP, Wang JH. Current knowledge and future directions of TLR and NOD signaling in sepsis. *Military Medical Research*. 2015;2:1.
287. Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, et al. Toll-like receptors modulate adult hippocampal neurogenesis. *Nature cell biology*. 2007;9(9):1081-8.
288. Okun E, Griffioen K, Barak B, Roberts NJ, Castro K, Pita MA, et al. Toll-like receptor 3 inhibits memory retention and constrains adult hippocampal neurogenesis.

Proceedings of the National Academy of Sciences of the United States of America. 2010;107(35):15625-30.

289. Okun E, Barak B, Saada-Madar R, Rothman SM, Griffioen KJ, Roberts N, et al. Evidence for a developmental role for TLR4 in learning and memory. *PloS one*. 2012;7(10):e47522.

290. Okun E, Griffioen KJ, Rothman S, Wan R, Cong WN, De Cabo R, et al. Toll-like receptors 2 and 4 modulate autonomic control of heart rate and energy metabolism. *Brain, behavior, and immunity*. 2014;36:90-100.

291. Rodrigues FL, Silva LE, Hott SC, Bomfim GF, da Silva CA, Fazan R, Jr., et al. Toll-like Receptor 9 (TLR9) Plays a Key Role in the Autonomic Cardiac and Baroreflex Control of Arterial Pressure. *American journal of physiology Regulatory, integrative and comparative physiology*. 2015:ajpregu 00150 2014.

292. Ehrentraut S, Lohner R, Schwederski M, Ehrentraut H, Boehm O, Noga S, et al. In vivo Toll-like receptor 4 antagonism restores cardiac function during endotoxemia. *Shock*. 2011;36(6):613-20.

293. Tidswell M, Tillis W, Larosa SP, Lynn M, Wittek AE, Kao R, et al. Phase 2 trial of eritoran tetrasodium (E5564), a toll-like receptor 4 antagonist, in patients with severe sepsis. *Critical care medicine*. 2010;38(1):72-83.

294. Oh-hama T. Evolutionary consideration on 5-aminolevulinate synthase in nature. *Orig Life Evol Biosph*. 1997;27(4):405-12.

295. Kepp O, Galluzzi L, Kroemer G. Mitochondrial control of the NLRP3 inflammasome. *Nature immunology*. 2011;12(3):199-200.

296. Rowland AA, Voeltz GK. Endoplasmic reticulum-mitochondria contacts: function of the junction. *Nat Rev Mol Cell Biol*. 2012;13(10):607-25.

297. Hajnoczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, et al. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca²⁺ uptake in apoptosis. *Cell Calcium*. 2006;40(5-6):553-60.

298. Krysko DV, Agostinis P, Krysko O, Garg AD, Bachert C, Lambrecht BN, et al. Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. *Trends in immunology*. 2011;32(4):157-64.

299. McCarthy CG, Wenceslau CF, Gouloupoulou S, Ogbi S, Baban B, Sullivan JC, et al. Circulating mitochondrial DNA and Toll-like receptor 9 are associated with vascular dysfunction in spontaneously hypertensive rats. *Cardiovascular research*. 2015.

300. Wenceslau CF, McCarthy CG, Szasz T, Spitler K, Gouloupoulou S, Webb RC, et al. Mitochondrial damage-associated molecular patterns and vascular function. *European heart journal*. 2014;35(18):1172-7.

301. Eleftheriadis T, Pissas G, Antoniadi G, Liakopoulos V, Stefanidis I. Damage-associated molecular patterns derived from mitochondria may contribute to the hemodialysis-associated inflammation. *International urology and nephrology*. 2014;46(1):107-12.

302. Gu X, Wu G, Yao Y, Zeng J, Shi D, Lv T, et al. Intratracheal administration of mitochondrial DNA directly provokes lung inflammation through the TLR9-p38 MAPK pathway. *Free radical biology & medicine*. 2015;83:149-58.

303. Cardon LR, Burge C, Clayton DA, Karlin S. Pervasive CpG suppression in animal mitochondrial genomes. *Proceedings of the National Academy of Sciences of the United States of America*. 1994;91(9):3799-803.

304. Graeber MB, Muller U. Recent developments in the molecular genetics of mitochondrial disorders. *Journal of the neurological sciences*. 1998;153(2):251-63.

305. DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. *The New England journal of medicine*. 2003;348(26):2656-68.
306. Piganeau G, Eyre-Walker A. A reanalysis of the indirect evidence for recombination in human mitochondrial DNA. *Heredity*. 2004;92(4):282-8.
307. Gray MW, Burger G, Lang BF. Mitochondrial evolution. *Science*. 1999;283(5407):1476-81.
308. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. *Nature*. 1981;290(5806):457-65.
309. Dimauro S, Davidzon G. Mitochondrial DNA and disease. *Annals of medicine*. 2005;37(3):222-32.
310. Schaefer AM, Taylor RW, Turnbull DM, Chinnery PF. The epidemiology of mitochondrial disorders--past, present and future. *Biochimica et biophysica acta*. 2004;1659(2-3):115-20.
311. Kamata H, Hirata H. Redox regulation of cellular signalling. *Cellular signalling*. 1999;11(1):1-14.
312. Hochhauser D. Relevance of mitochondrial DNA in cancer. *Lancet*. 2000;356(9225):181-2.
313. Kohler C, Radpour R, Barekati Z, Asadollahi R, Bitzer J, Wight E, et al. Levels of plasma circulating cell free nuclear and mitochondrial DNA as potential biomarkers for breast tumors. *Molecular cancer*. 2009;8:105.
314. Mehra N, Penning M, Maas J, van Daal N, Giles RH, Voest EE. Circulating mitochondrial nucleic acids have prognostic value for survival in patients with advanced prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2007;13(2 Pt 1):421-6.
315. Boles RG, Williams JC. Mitochondrial disease and cyclic vomiting syndrome. *Digestive diseases and sciences*. 1999;44(8 Suppl):103S-7S.
316. Dennerlein S, Rehling P. Human mitochondrial COX1 assembly into cytochrome c oxidase at a glance. *Journal of cell science*. 2015;128(5):833-7.
317. Andreu AL, Hanna MG, Reichmann H, Bruno C, Penn AS, Tanji K, et al. Exercise intolerance due to mutations in the cytochrome b gene of mitochondrial DNA. *The New England journal of medicine*. 1999;341(14):1037-44.
318. Benit P, Lebon S, Rustin P. Respiratory-chain diseases related to complex III deficiency. *Biochimica et biophysica acta*. 2009;1793(1):181-5.
319. Gil Borlado MC, Moreno Lastres D, Gonzalez Hoyuela M, Moran M, Blazquez A, Pello R, et al. Impact of the mitochondrial genetic background in complex III deficiency. *PloS one*. 2010;5(9).
320. Holt IJ, Harding AE, Morgan-Hughes JA. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature*. 1988;331(6158):717-9.
321. Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, et al. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science*. 1988;242(4884):1427-30.
322. Finsterer J, Harbo HF, Baets J, Van Broeckhoven C, Di Donato S, Fontaine B, et al. EFNS guidelines on the molecular diagnosis of mitochondrial disorders. *Eur J Neurol*. 2009;16(12):1255-64.
323. Chinnery PF. Mitochondrial Disorders Overview. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, et al., editors. *GeneReviews(R)*. Seattle (WA)1993.
324. Ashkar AA, Rosenthal KL. Toll-like receptor 9, CpG DNA and innate immunity. *Current molecular medicine*. 2002;2(6):545-56.

325. Wagner H. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. *Current opinion in microbiology*. 2002;5(1):62-9.
326. Chuang TH, Lee J, Kline L, Mathison JC, Ulevitch RJ. Toll-like receptor 9 mediates CpG-DNA signaling. *Journal of leukocyte biology*. 2002;71(3):538-44.
327. Goulopoulou S, Matsumoto T, Bomfim GF, Webb RC. Toll-like receptor 9 activation: a novel mechanism linking placenta-derived mitochondrial DNA and vascular dysfunction in pre-eclampsia. *Clin Sci (Lond)*. 2012;123(7):429-35.
328. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques*. 2008;44(5):619-26.
329. Li M, Carpio DF, Zheng Y, Bruzzo P, Singh V, Ouaz F, et al. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. *Journal of immunology*. 2001;166(12):7128-35.
330. El Mezayen R, El Gazzar M, Seeds MC, McCall CE, Dreskin SC, Nicolls MR. Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin. *Immunology letters*. 2007;111(1):36-44.
331. Thorburn J, Horita H, Redzic J, Hansen K, Frankel AE, Thorburn A. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. *Cell death and differentiation*. 2009;16(1):175-83.
332. Sandri S, Rodriguez D, Gomes E, Monteiro HP, Russo M, Campa A. Is serum amyloid A an endogenous TLR4 agonist? *Journal of leukocyte biology*. 2008;83(5):1174-80.
333. He RL, Zhou J, Hanson CZ, Chen J, Cheng N, Ye RD. Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2. *Blood*. 2009;113(2):429-37.
334. Huang J, Xie Y, Sun X, Zeh HJ, 3rd, Kang R, Lotze MT, et al. DAMPs, ageing, and cancer: The 'DAMP Hypothesis'. *Ageing Res Rev*. 2014.
335. Saez-Orellana F, Godoy PA, Bastidas CY, Silva-Grecchi T, Guzman L, Aguayo LG, et al. ATP leakage induces P2XR activation and contributes to acute synaptic excitotoxicity induced by soluble oligomers of beta-amyloid peptide in hippocampal neurons. *Neuropharmacology*. 2015.
336. Song Z, Vijayaraghavan S, Sladek CD. ATP increases intracellular calcium in supraoptic neurons by activation of both P2X and P2Y purinergic receptors. *American journal of physiology Regulatory, integrative and comparative physiology*. 2007;292(1):R423-31.
337. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*. 2003;425(6957):516-21.
338. Zhang JZ, Liu Z, Liu J, Ren JX, Sun TS. Mitochondrial DNA induces inflammation and increases TLR9/NF-kappaB expression in lung tissue. *Int J Mol Med*. 2014;33(4):817-24.
339. Chalah A, Khosravi-Far R. The mitochondrial death pathway. *Advances in experimental medicine and biology*. 2008;615:25-45.
340. Barja G. The mitochondrial free radical theory of aging. *Prog Mol Biol Transl Sci*. 2014;127:1-27.
341. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*. 1996;86(1):147-57.
342. Orrenius S, Zhivotovsky B. Cardiolipin oxidation sets cytochrome c free. *Nat Chem Biol*. 2005;1(4):188-9.

343. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, et al. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell*. 2005;122(2):221-33.
344. Chen Y, Parker WD, Chen H, Yang K. Aberrant mitochondrial RNA in the role of aging and aging associated diseases. *Med Hypotheses*. 2015.
345. Tousoulis D, Davies G, Stefanadis C, Toutouzas P, Ambrose JA. Inflammatory and thrombotic mechanisms in coronary atherosclerosis. *Heart*. 2003;89(9):993-7.
346. Margulis L, Bermudes D. Symbiosis as a mechanism of evolution: status of cell symbiosis theory. *Symbiosis*. 1985;1:101-24.
347. Cremer J, Martin M, Redl H, Bahrami S, Abraham C, Graeter T, et al. Systemic inflammatory response syndrome after cardiac operations. *The Annals of thoracic surgery*. 1996;61(6):1714-20.
348. Liguori GR, Kanas AF, Moreira LF. Managing the inflammatory response after cardiopulmonary bypass: review of the studies in animal models. *Revista brasileira de cirurgia cardiovascular : orgao oficial da Sociedade Brasileira de Cirurgia Cardiovascular*. 2014;29(1):93-102.
349. Schnoering H, Arens J, Terrada E, Sachweh JS, Runge M, Schmitz-Rode T, et al. A newly developed miniaturized heart-lung machine--expression of inflammation in a small animal model. *Artificial organs*. 2010;34(11):911-7.
350. Ugaki S, Honjo O, Nakakura M, Douguchi T, Itagaki A, Yokoyama N, et al. Transfusion-free neonatal cardiopulmonary bypass using a TinyPump. *The Annals of thoracic surgery*. 2010;90(5):1615-21.
351. Ng R, Chew S, Liu W, Ong P, Caleb M, Ti L. The inflammatory response between miniaturised and conventional cardiopulmonary bypass after cardiac surgery in an Asian population. *Perfusion*. 2014.
352. Garrabou G, Moren C, Lopez S, Tobias E, Cardellach F, Miro O, et al. The effects of sepsis on mitochondria. *The Journal of infectious diseases*. 2012;205(3):392-400.
353. Cossarizza A, Pinti M, Nasi M, Gibellini L, Manzini S, Roat E, et al. Increased plasma levels of extracellular mitochondrial DNA during HIV infection: a new role for mitochondrial damage-associated molecular patterns during inflammation. *Mitochondrion*. 2011;11(5):750-5.
354. Duramad O, Fearon KL, Chang B, Chan JH, Gregorio J, Coffman RL, et al. Inhibitors of TLR-9 act on multiple cell subsets in mouse and man in vitro and prevent death in vivo from systemic inflammation. *Journal of immunology*. 2005;174(9):5193-200.
355. Kandimalla ER, Bhagat L, Wang D, Yu D, Sullivan T, La Monica N, et al. Design, synthesis and biological evaluation of novel antagonist compounds of Toll-like receptors 7, 8 and 9. *Nucleic acids research*. 2013;41(6):3947-61.
356. David BT, Ratnayake A, Amarante MA, Reddy NP, Dong W, Sampath S, et al. A toll-like receptor 9 antagonist reduces pain hypersensitivity and the inflammatory response in spinal cord injury. *Neurobiology of disease*. 2013;54:194-205.
357. Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology*. 2011;54(3):999-1008.
358. Guvener M, Korun O, Demirturk OS. Risk factors for systemic inflammatory response after congenital cardiac surgery. *Journal of cardiac surgery*. 2015;30(1):92-6.
359. Butler J, Rocker GM, Westaby S. Inflammatory response to cardiopulmonary bypass. *The Annals of thoracic surgery*. 1993;55(2):552-9.

360. Holmes JHt, Connolly NC, Paull DL, Hill ME, Guyton SW, Ziegler SF, et al. Magnitude of the inflammatory response to cardiopulmonary bypass and its relation to adverse clinical outcomes. *Inflammation research : official journal of the European Histamine Research Society* [et al]. 2002;51(12):579-86.
361. Boeken U, Feindt P, Zimmermann N, Kalweit G, Petzold T, Gams E. Increased preoperative C-reactive protein (CRP)-values without signs of an infection and complicated course after cardiopulmonary bypass (CPB)-operations. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 1998;13(5):541-5.
362. Kuck JL, Obiako BO, Gorodnya OM, Pastukh VM, Kua J, Simmons JD, et al. Mitochondrial DNA Damage-associated Molecular Patterns Mediate a Feed-Forward Cycle of Bacteria-Induced Vascular Injury in Perfused Rat Lungs. *American journal of physiology Lung cellular and molecular physiology*. 2015:ajplung 00015 2015.
363. He J, Lu Y, Xia H, Liang Y, Wang X, Bao W, et al. Circulating Mitochondrial DAMPs Are Not Effective Inducers of Proteinuria and Kidney Injury in Rodents. *PloS one*. 2015;10(4):e0124469.
364. Yao X, Wigginton JG, Maass DL, Ma L, Carlson D, Wolf SE, et al. Estrogen-provided cardiac protection following burn trauma is mediated through a reduction in mitochondria-derived DAMPs. *American journal of physiology Heart and circulatory physiology*. 2014;306(6):H882-94.
365. Pazmandi K, Agod Z, Kumar BV, Szabo A, Fekete T, Sogor V, et al. Oxidative modification enhances the immunostimulatory effects of extracellular mitochondrial DNA on plasmacytoid dendritic cells. *Free radical biology & medicine*. 2014;77:281-90.
366. Liu D, Xu M, Ding LH, Lv LL, Liu H, Ma KL, et al. Activation of the Nlrp3 inflammasome by mitochondrial reactive oxygen species: a novel mechanism of albumin-induced tubulointerstitial inflammation. *The international journal of biochemistry & cell biology*. 2014;57:7-19.
367. Andersson SG, Karlberg O, Canback B, Kurland CG. On the origin of mitochondria: a genomics perspective. *Philosophical transactions of the Royal Society of London Series B, Biological sciences*. 2003;358(1429):165-77; discussion 77-9.
368. Damm J, Wiegand F, Harden LM, Wenisch S, Gerstberger R, Rummel C, et al. Intraperitoneal and subcutaneous injections of the TLR9 agonist ODN 1668 in rats: Brain inflammatory responses are related to peripheral IL-6 rather than interferons. *Journal of neuroimmunology*. 2014;277(1-2):105-17.
369. Kunes P, Lonsky V, Mand'ak J, Brtko M, Kolackova M, Andrys C, et al. The inflammatory response in cardiac surgery. An up-to-date overview with the emphasis on the role of heat shock proteins (HSPs) 60 and 70. *Acta medica*. 2007;50(2):93-9.
370. Tarozzi A, Morroni F, Merlicco A, Hrelia S, Angeloni C, Cantelli-Forti G, et al. Sulforaphane as an inducer of glutathione prevents oxidative stress-induced cell death in a dopaminergic-like neuroblastoma cell line. *Journal of neurochemistry*. 2009;111(5):1161-71.
371. Bhagat L JW, Yu D, Arbeit R, Sullivan T. Phase 1 clinical trial of IMO-8400, an antagonist of toll-like 7, 8 and 9: Pharmacodynamic activity. 2013:22.
372. Rubens FD, Mesana T. The inflammatory response to cardiopulmonary bypass: a therapeutic overview. *Perfusion*. 2004;19 Suppl 1:S5-12.
373. Franke A, Lante W, Fackeldey V, Becker HP, Kurig E, Zoller LG, et al. Pro-inflammatory cytokines after different kinds of cardio-thoracic surgical procedures: is what we see what we know? *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 2005;28(4):569-75.

374. Zhang JM, An J. Cytokines, inflammation, and pain. *Int Anesthesiol Clin*. 2007;45(2):27-37.
375. Leifer CA, Kennedy MN, Mazzoni A, Lee C, Kruhlak MJ, Segal DM. TLR9 is localized in the endoplasmic reticulum prior to stimulation. *Journal of immunology*. 2004;173(2):1179-83.
376. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature immunology*. 2004;5(2):190-8.
377. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. 1995;374(6522):546-9.
378. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature*. 2000;408(6813):740-5.
379. Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, et al. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature*. 2005;434(7036):1035-40.
380. Qiu Y, Zeltzer S, Zhang Y, Wang F, Chen GH, Dayrit J, et al. Early induction of CCL7 downstream of TLR9 signaling promotes the development of robust immunity to cryptococcal infection. *Journal of immunology*. 2012;188(8):3940-8.
381. Kandimalla ER, Bhagat L, Li Y, Yu D, Wang D, Cong YP, et al. Immunomodulatory oligonucleotides containing a cytosine-phosphate-2'-deoxy-7-deazaguanosine motif as potent toll-like receptor 9 agonists. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(19):6925-30.
382. Hsu K, Chung YM, Endoh Y, Geczy CL. TLR9 ligands induce S100A8 in macrophages via a STAT3-dependent pathway which requires IL-10 and PGE2. *PloS one*. 2014;9(8):e103629.
383. Nakamura K, Miyazato A, Xiao G, Hatta M, Inden K, Aoyagi T, et al. Deoxynucleic acids from *Cryptococcus neoformans* activate myeloid dendritic cells via a TLR9-dependent pathway. *Journal of immunology*. 2008;180(6):4067-74.
384. Wan S, Izzat MB, Lee TW, Wan IY, Tang NL, Yim AP. Avoiding cardiopulmonary bypass in multivessel CABG reduces cytokine response and myocardial injury. *The Annals of thoracic surgery*. 1999;68(1):52-6; discussion 6-7.
385. Lee SH, Lee JG, Kim JR, Baek SH. Toll-like receptor 9-mediated cytosolic phospholipase A2 activation regulates expression of inducible nitric oxide synthase. *Biochemical and biophysical research communications*. 2007;364(4):996-1001.
386. Feinberg WM, Blackshear JL, Laupacis A, Kronmal R, Hart RG. Prevalence, age distribution, and gender of patients with atrial fibrillation. Analysis and implications. *Archives of internal medicine*. 1995;155(5):469-73.
387. Ryder KM, Benjamin EJ. Epidemiology and significance of atrial fibrillation. *The American journal of cardiology*. 1999;84(9A):131R-8R.
388. Tsai CT, Lai LP, Hwang JJ, Lin JL, Chiang FT. Molecular genetics of atrial fibrillation. *Journal of the American College of Cardiology*. 2008;52(4):241-50.
389. Almassi GH, Schowalter T, Nicolosi AC, Aggarwal A, Moritz TE, Henderson WG, et al. Atrial fibrillation after cardiac surgery: a major morbid event? *Annals of surgery*. 1997;226(4):501-11; discussion 11-3.
390. Maesen B, Nijs J, Maessen J, Allessie M, Schotten U. Post-operative atrial fibrillation: a maze of mechanisms. *Europace : European pacing, arrhythmias, and cardiac electrophysiology : journal of the working groups on cardiac pacing,*

arrhythmias, and cardiac cellular electrophysiology of the European Society of Cardiology. 2012;14(2):159-74.

391. Auer J, Weber T, Berent R, Ng CK, Lamm G, Eber B. Risk factors of postoperative atrial fibrillation after cardiac surgery. *Journal of cardiac surgery*. 2005;20(5):425-31.

392. Siebert J, Anisimowicz L, Lango R, Rogowski J, Pawlaczyk R, Brzezinski M, et al. Atrial fibrillation after coronary artery bypass grafting: does the type of procedure influence the early postoperative incidence? *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 2001;19(4):455-9.

393. Creswell LL, Schuessler RB, Rosenbloom M, Cox JL. Hazards of postoperative atrial arrhythmias. *The Annals of thoracic surgery*. 1993;56(3):539-49.

394. Vaseghi M, Boyle NG, Kedia R, Patel JK, Cesario DA, Wiener I, et al. Supraventricular tachycardia after orthotopic cardiac transplantation. *Journal of the American College of Cardiology*. 2008;51(23):2241-9.

395. Elrakhawy HM, Alassal MA, Elsadeck N, Shaalan A, Ezeldin TH, Shalabi A. Predictive factors of supraventricular arrhythmias after noncardiac thoracic surgery: a multicenter study. *The heart surgery forum*. 2014;17(6):E308-12.

396. Curtis JJ, Parker BM, McKenney CA, Wagner-Mann CC, Walls JT, Demmy TL, et al. Incidence and predictors of supraventricular dysrhythmias after pulmonary resection. *The Annals of thoracic surgery*. 1998;66(5):1766-71.

397. Amar D, Roistacher N, Burt M, Reinsel RA, Ginsberg RJ, Wilson RS. Clinical and echocardiographic correlates of symptomatic tachydysrhythmias after noncardiac thoracic surgery. *Chest*. 1995;108(2):349-54.

398. Ma JY, Wang Y, Zhao YF, Wu Z, Liu LX, Kou YL, et al. Atrial fibrillation after surgery for esophageal carcinoma: clinical and prognostic significance. *World journal of gastroenterology : WJG*. 2006;12(3):449-52.

399. Walsh SR, Oates JE, Anderson JA, Blair SD, Makin CA, Walsh CJ. Postoperative arrhythmias in colorectal surgical patients: incidence and clinical correlates. *Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland*. 2006;8(3):212-6.

400. Bargehr J, Trejo-Gutierrez JF, Patel T, Rosser B, Aranda-Michel J, Yataco ML, et al. Preexisting atrial fibrillation and cardiac complications after liver transplantation. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society*. 2015;21(3):314-20.

401. Mathew JP, Fontes ML, Tudor IC, Ramsay J, Duke P, Mazer CD, et al. A multicenter risk index for atrial fibrillation after cardiac surgery. *Jama*. 2004;291(14):1720-9.

402. Lip GY, Tse HF, Lane DA. Atrial fibrillation. *Lancet*. 2012;379(9816):648-61.

403. Hogue CW, Jr., Creswell LL, Gutterman DD, Fleisher LA, American College of Chest P. Epidemiology, mechanisms, and risks: American College of Chest Physicians guidelines for the prevention and management of postoperative atrial fibrillation after cardiac surgery. *Chest*. 2005;128(2 Suppl):9S-16S.

404. Futami C, Tanuma K, Tanuma Y, Saito T. The arterial blood supply of the conducting system in normal human hearts. *Surgical and radiologic anatomy : SRA*. 2003;25(1):42-9.

405. Asher CR, Miller DP, Grimm RA, Cosgrove DM, 3rd, Chung MK. Analysis of risk factors for development of atrial fibrillation early after cardiac valvular surgery. *The American journal of cardiology*. 1998;82(7):892-5.

406. Aranki SF, Shaw DP, Adams DH, Rizzo RJ, Couper GS, VanderVliet M, et al. Predictors of atrial fibrillation after coronary artery surgery. Current trends and impact on hospital resources. *Circulation*. 1996;94(3):390-7.
407. Kitzman DW, Edwards WD. Age-related changes in the anatomy of the normal human heart. *Journal of gerontology*. 1990;45(2):M33-9.
408. Lie JT, Hammond PI. Pathology of the senescent heart: anatomic observations on 237 autopsy studies of patients 90 to 105 years old. *Mayo Clinic proceedings*. 1988;63(6):552-64.
409. Boos CJ, Anderson RA, Lip GY. Is atrial fibrillation an inflammatory disorder? *European heart journal*. 2006;27(2):136-49.
410. Roldan V, Marin F, Martinez JG, Garcia-Herola A, Sogorb F, Lip GY. Relation of interleukin-6 levels and prothrombin fragment 1+2 to a point-based score for stroke risk in atrial fibrillation. *The American journal of cardiology*. 2005;95(7):881-2.
411. Sata N, Hamada N, Horinouchi T, Amitani S, Yamashita T, Moriyama Y, et al. C-reactive protein and atrial fibrillation. Is inflammation a consequence or a cause of atrial fibrillation? *Japanese heart journal*. 2004;45(3):441-5.
412. Lo B, Fijnheer R, Nierich AP, Bruins P, Kalkman CJ. C-reactive protein is a risk indicator for atrial fibrillation after myocardial revascularization. *The Annals of thoracic surgery*. 2005;79(5):1530-5.
413. Sinno H, Derakhchan K, Libersan D, Merhi Y, Leung TK, Nattel S. Atrial ischemia promotes atrial fibrillation in dogs. *Circulation*. 2003;107(14):1930-6.
414. Hu YF, Chen YJ, Lin YJ, Chen SA. Inflammation and the pathogenesis of atrial fibrillation. *Nature reviews Cardiology*. 2015;12(4):230-43.
415. Kinoshita T, Asai T, Takashima N, Hosoba S, Suzuki T, Kambara A, et al. Preoperative C-reactive protein and atrial fibrillation after off-pump coronary bypass surgery. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 2011;40(6):1298-303.
416. Choi YS, Shim JK, Hong SW, Kim DH, Kim JC, Kwak YL. Risk factors of atrial fibrillation following off-pump coronary artery bypass graft surgery: predictive value of C-reactive protein and transfusion requirement. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 2009;36(5):838-43.
417. Ucar HI, Tok M, Atalar E, Dogan OF, Oc M, Farsak B, et al. Predictive significance of plasma levels of interleukin-6 and high-sensitivity C-reactive protein in atrial fibrillation after coronary artery bypass surgery. *The heart surgery forum*. 2007;10(2):E131-5.
418. Pretorius M, Donahue BS, Yu C, Greelish JP, Roden DM, Brown NJ. Plasminogen activator inhibitor-1 as a predictor of postoperative atrial fibrillation after cardiopulmonary bypass. *Circulation*. 2007;116(11 Suppl):I1-7.
419. Wu N, Xu B, Xiang Y, Wu L, Zhang Y, Ma X, et al. Association of inflammatory factors with occurrence and recurrence of atrial fibrillation: a meta-analysis. *International journal of cardiology*. 2013;169(1):62-72.
420. Leftheriotis DI, Fountoulaki KT, Flevari PG, Parissis JT, Panou FK, Andreadou IT, et al. The predictive value of inflammatory and oxidative markers following the successful cardioversion of persistent lone atrial fibrillation. *International journal of cardiology*. 2009;135(3):361-9.
421. Ramlawi B, Otu H, Mieno S, Boodhwani M, Sodha NR, Clements RT, et al. Oxidative stress and atrial fibrillation after cardiac surgery: a case-control study. *The Annals of thoracic surgery*. 2007;84(4):1166-72; discussion 72-3.

422. Kim YM, Kattach H, Ratnatunga C, Pillai R, Channon KM, Casadei B. Association of atrial nicotinamide adenine dinucleotide phosphate oxidase activity with the development of atrial fibrillation after cardiac surgery. *Journal of the American College of Cardiology*. 2008;51(1):68-74.
423. Abreu JE, Reilly J, Salzano RP, Khachane VB, Jekel JF, Clyne CA. Comparison of frequencies of atrial fibrillation after coronary artery bypass grafting with and without the use of cardiopulmonary bypass. *The American journal of cardiology*. 1999;83(5):775-6, A9.
424. Gunaydin S, Ayrancioglu K, Dikmen E, McCusker K, Vijay V, Sari T, et al. Clinical effects of leukofiltration and surface modification on post-cardiopulmonary bypass atrial fibrillation in different risk cohorts. *Perfusion*. 2007;22(4):279-88.
425. Adams DC, Heyer EJ, Simon AE, Delphin E, Rose EA, Oz MC, et al. Incidence of atrial fibrillation after mild or moderate hypothermic cardiopulmonary bypass. *Critical care medicine*. 2000;28(2):309-11.
426. Biancari F, Mahar MA. Meta-analysis of randomized trials on the efficacy of posterior pericardiotomy in preventing atrial fibrillation after coronary artery bypass surgery. *The Journal of thoracic and cardiovascular surgery*. 2010;139(5):1158-61.
427. Kaya M, Utkusavas A, Erkanli K, Guler S, Kyaruzi M, Birant A, et al. The Preventive Effects of Posterior Pericardiotomy with Intrapericardial Tube on the Development of Pericardial Effusion, Atrial Fibrillation, and Acute Kidney Injury after Coronary Artery Surgery: A Prospective, Randomized, Controlled Trial. *The Thoracic and cardiovascular surgeon*. 2015.
428. Benjamin EJ, Wolf PA, D'Agostino RB, Silbershatz H, Kannel WB, Levy D. Impact of atrial fibrillation on the risk of death: the Framingham Heart Study. *Circulation*. 1998;98(10):946-52.
429. Goldman ME, Pearce LA, Hart RG, Zabalgoitia M, Asinger RW, Safford R, et al. Pathophysiologic correlates of thromboembolism in nonvalvular atrial fibrillation: I. Reduced flow velocity in the left atrial appendage (The Stroke Prevention in Atrial Fibrillation [SPAF-III] study). *Journal of the American Society of Echocardiography : official publication of the American Society of Echocardiography*. 1999;12(12):1080-7.
430. Hart RG, Halperin JL. Atrial fibrillation and stroke : concepts and controversies. *Stroke; a journal of cerebral circulation*. 2001;32(3):803-8.
431. Sedrakyan A, Treasure T, Browne J, Krumholz H, Sharpin C, van der Meulen J. Pharmacologic prophylaxis for postoperative atrial tachyarrhythmia in general thoracic surgery: evidence from randomized clinical trials. *The Journal of thoracic and cardiovascular surgery*. 2005;129(5):997-1005.
432. Dobrilovic N, Vadlamani L, Buchert B, Wright CB. Diltiazem prophylaxis reduces incidence of atrial fibrillation after coronary artery bypass grafting. *The Journal of cardiovascular surgery*. 2005;46(5):457-61.
433. Blommaert D, Gonzalez M, Mucumbitsi J, Gurne O, Evrard P, Buche M, et al. Effective prevention of atrial fibrillation by continuous atrial overdrive pacing after coronary artery bypass surgery. *Journal of the American College of Cardiology*. 2000;35(6):1411-5.
434. Hakala T, Valtola AJ, Turpeinen AK, Hedman AE, Vuorenniemi RE, Karjalainen JM, et al. Right atrial overdrive pacing does not prevent atrial fibrillation after coronary artery bypass surgery. *Europace : European pacing, arrhythmias, and cardiac electrophysiology : journal of the working groups on cardiac pacing, arrhythmias, and cardiac cellular electrophysiology of the European Society of Cardiology*. 2005;7(2):170-4.

435. Jacob KA, Nathoe HM, Dieleman JM, van Osch D, Kluin J, van Dijk D. Inflammation in new-onset atrial fibrillation after cardiac surgery: a systematic review. *European journal of clinical investigation*. 2014;44(4):402-28.
436. Aviles RJ, Martin DO, Apperson-Hansen C, Houghtaling PL, Rautaharju P, Kronmal RA, et al. Inflammation as a risk factor for atrial fibrillation. *Circulation*. 2003;108(24):3006-10.
437. Chung MK, Martin DO, Sprecher D, Wazni O, Kanderian A, Carnes CA, et al. C-reactive protein elevation in patients with atrial arrhythmias: inflammatory mechanisms and persistence of atrial fibrillation. *Circulation*. 2001;104(24):2886-91.
438. Engelmann MD, Svendsen JH. Inflammation in the genesis and perpetuation of atrial fibrillation. *European heart journal*. 2005;26(20):2083-92.
439. Malouf JF, Kanagala R, Al Atawi FO, Rosales AG, Davison DE, Murali NS, et al. High sensitivity C-reactive protein: a novel predictor for recurrence of atrial fibrillation after successful cardioversion. *Journal of the American College of Cardiology*. 2005;46(7):1284-7.
440. Guo Y, Lip GY, Apostolakis S. Inflammation in atrial fibrillation. *Journal of the American College of Cardiology*. 2012;60(22):2263-70.
441. Patel P, Dokainish H, Tsai P, Lakkis N. Update on the association of inflammation and atrial fibrillation. *Journal of cardiovascular electrophysiology*. 2010;21(9):1064-70.
442. Raja SG, Berg GA. Impact of off-pump coronary artery bypass surgery on systemic inflammation: current best available evidence. *Journal of cardiac surgery*. 2007;22(5):445-55.
443. Pilatis ND, Anyfantakis ZA, Spiliopoulos K, Degiannis D, Chaidaroglou A, Vergou G, et al. The Role of BNP and CRP in Predicting the Development of Atrial Fibrillation in Patients Undergoing Isolated Coronary Artery Bypass Surgery. *ISRN cardiology*. 2013;2013:235018.
444. Fontes ML, Amar D, Kulak A, Koval K, Zhang H, Shi W, et al. Increased preoperative white blood cell count predicts postoperative atrial fibrillation after coronary artery bypass surgery. *Journal of cardiothoracic and vascular anesthesia*. 2009;23(4):484-7.