# ROLE OF THE SAFE PATHWAY AND THE MITOCHONDRIA IN HDL CHOLESTEROL (AND ITS CONSTITUENT SPHINGOSINE-1-PHOSPHATE) INDUCED CARDIOPROTECTION

**Damian Hacking**



Thesis presented for the degree of:

**Masters in Medicine**

At the:

**Faculty of Health Sciences, University of Cape Town**

**Supervisor: Associate Professor Sandrine Lecour, PharmD, PhD**
(Hatter Cardiovascular Research Institute, Faculty of Health Sciences, University of Cape Town)

**Co-supervisor: Professor Lionel H. Opie MD, DPhil**
(Hatter Cardiovascular Research Institute, Faculty of Health Sciences, University of Cape Town)

**Co-supervisor: Dr Derek J Hausenloy MD, PhD**
(The Hatter Cardiovascular Institute, University College London)

# DECLARATION

I, Damian Hacking, hereby declare that the work on which this thesis is based is my original work (except where acknowledgement indicates otherwise), and that neither the whole work nor any part of it has been, is being, or is submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.



Signature

_____

Date

_____

# *Acknowledgements*

I would like to thank:

- All the staff and students at the Hatter Cardiovascular Research Unit at the University of Cape Town. In particular,
    - My supervisor Professor Sandrine Lecour for her guidance and support throughout this project. For always being able to make time to see me, and for supporting my various ambitions.
    - Dr Jonathan King for his collaborative work on the isolated rat heart model, specifically the infarct size and functional recovery data.
    - My co-supervisor Professor Lionel Opie for his advice throughout my thesis, and his valuable advice on the write-up process.

- The Hatter Institute at the University College London for agreeing to host me for 6 months. All the staff members who assisted me in learning new techniques in the lab and making me feel welcome. In particular,
    - Dr Derek Hausenloy, my co-supervisor, for agreeing to supervise my research at The Hatter Institute
    - Mr Ong Sang Bing and Dr Cara Hendry for their invaluable assistance and friendship, both in and out of the lab
    - Professor Derek Yellon for allowing me to spend time in his prestigious institution.

- Our collaborators at the Division of Endocrinology, Diabetology and Nutrition at the University Hospital, Geneva. In particular,
    - Dr Miguel Frias for his advice on mitochondrial extraction protocols

- My Sponsors for their financial assistance throughout my Masters degree.
    - The National Research Foundation
    - The Marion Beatrice Waddel Foundation
    - The KW Johnston Foundation
    - The University of Cape Town Postgraduate Funding Program
    - The Lionel H Opie scholarship

- Finally, my family for their continued support of my studies

# *Table of Contents*

# *Abstract*

**Introduction**

High density lipoprotein cholesterol (HDL) and its component sphingosine-1-phosphate (S1P) protect against myocardial infarction. Recently, the SAFE (survivor activating factor enhancement) pathway, involving tumour necrosis factor (TNF) and the transcription factor signal transducer and activator of transcription 3 (STAT-3), has been identified as a key signalling pathway in cardioprotection, although the end effector remains unclear. Opening of the mitochondrial Permeability Transition Pore (mPTP) is a crucial initiator of cell death in the setting of myocardial infarction. We therefore hypothesised that HDL/S1P protect the heart **by** the activation of the SAFE pathway, and the subsequent inhibition of mPTP opening.

**Methods**

Isolated adult rat hearts were pre-treated with 10nM S1P followed by either mitochondrial isolation or 30 minutes of regional ischaemia and 2 hours of reperfusion. Respiratory parameters of the mitochondria were measured using a Clarke-oxygen electrode. Infarct size was determined using triphenyltetrazolium chloride staining.

Isolated adult mouse cardiomyocytes (wildtype or STAT-3 cardiomyocyte deficient (KO)) were exposed to 2 hours of hypoxia with/without pre-treatment with native HDL (300mg/ml) or S1P (10nM). Cell viability was assessed, and mPTP opening using TetraMethylRhodamine Methylester (TMRM) (normalised to the normoxic control). Mitochondrial STAT-3 levels were assessed using **W**estern blotting.

**Results**

S1P improved respiratory control indices (state 3:state 4 respiration) in isolated rat heart mitochondria from 4.2±0.5 Arbitrary Units (AU) to 11.0±1.4 AU ($p<0.05$), and decreased infarct size from 25.8+/-3.1% to 4.6+/-1.4% of the area at risk ($p<0.05$).

Cardiomyocyte viability under normoxic conditions was 84.3±1.5%. Hypoxia reduced viability to 61.0±2.5% ($p< 0.05$ vs. control). Cardiomyocyte viability was restored with S1P (80.4±1.4%) or native HDL (76.8±1.8%) ($p<0.05$ vs. hypoxia). TMRM fluorescence following hypoxia was 77±6 AU ($p<0.05$ vs. control), and restored to 95±4 AU in the S1P group and 88.4±11.0 AU in the HDL group ($p<0.05$ vs. hypoxia). In the STAT-3 KO mice, no cardiomyocyte viability improvement or restoration of TMRM fluorescence was observed with either HDL or S1P. S1P also increased serine phosphorylation of mitochondrial STAT-3.

**Conclusions**

Both native HDL and S1P are cardioprotective, and our data strongly suggest that this effect is mediated via activation of the STAT3 component of the SAFE pathway, and subsequent inhibition of mPTP opening.

# *List of Figures*

# *List of Tables*

# 1: CARDIOVASCULAR DISEASE

## 1.1 GENERALITIES

Cardiovascular disease (CVD) is a broad term including cardiac pathologies, such as hypertrophy, atrophy and ischaemia, as well as vascular pathologies such as hypertension and arterial stiffening. Globally, it is the leading cause of death and accounts for 31.5% of all deaths in females and 26.8% in males (1). CVD has traditionally been viewed, firstly as a disease of the aged, and secondly as a problem of the developed world. However, CVD is becoming more and more a problem that spans both age and location (2). This shift in demographics is attributable to the urbanisation of those in the developing world and the increase in risk factors in younger individuals. According to the World Health Organisation (WHO), CVD varies between the 2nd and 3rd leading cause of death among individuals aged 15-59 in high income countries (2) (Figure 1).



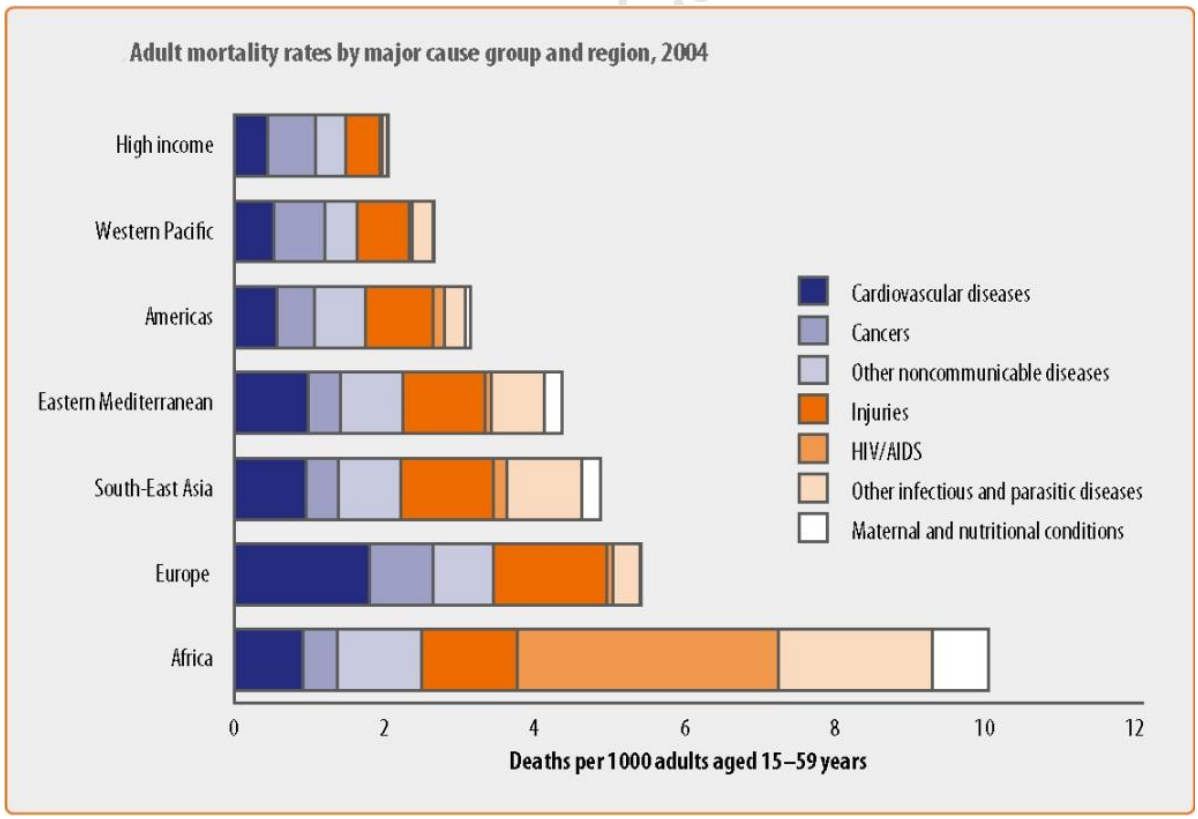


**Figure 1: Cause of death amongst individuals aged 15-59. High income countries are not separated by continent, and are used as a reference point for developing regions (as such 'Americas' and 'Europe' do not include high income countries from those regions)** (2).

The contribution of CVD to mortality among 15-59 year olds varies considerably in low and middle income countries. As can be seen in Figure 1, CVD is the largest contributor to mortality in low income countries in Europe, whilst it plays a substantially lesser role in Africa relative to its other burdens of disease. The burden of all diseases is strongly exaggerated in lower income regions, especially in Africa, and even though it may seem that CVD should be of little concern to Africa, it should be noted that 80% of the total deaths attributable to CVD occurred in developing countries. By way of example, in 2004 South Africa had 186 720 deaths attributable to CVD, whereas the UK (which has a slightly larger population) had only 105 000. This is strongly correlated with the increase in urbanisation in Sub-Saharan Africa and the risk factors associated therein (3).

Furthermore, many of the larger killers in Africa have been on the decline in recent years. The prevalence of HIV/AIDS in Sub-Saharan Africa (currently the leading cause of death in Sub-Saharan Africa) has been on the decrease since 2001, in line with the introduction of antiretroviral treatment (4). CVD on the other hand continues to rise at a rate of roughly 4.5% per year (3), and in the Western Cape area of South Africa it is already the leading cause of death (5).

## 1.2 ISCHEMIC HEART DISEASE

Of all the forms of CVD, ischemic heart disease (IHD) is the most lethal, accounting for 12.2% of all cause mortalities (over 7 million people) worldwide (Table 1). In middle income countries IHD is the leading cause of death, whilst in low income countries it is second only to lower respiratory infections (1).

Leading causes of death, all ages, 2004.

| | Disease or injury | Deaths (millions) | Per cent of total deaths |
|---|---|---|---|
| 1 | IHD | 7.2 | 12.2 |
| 2 | Cerebrovascular disease | 5.7 | 9.7 |
| 3 | Lower respiratory infections | 4.2 | 7.1 |
| 4 | COPD | 3.0 | 5.1 |
| 5 | Diarrhoeal diseases | 2.2 | 3.7 |
| 6 | HIV/AIDS | 2.0 | 3.5 |
| 7 | TB | 1.5 | 2.5 |
| 8 | Trachea, bronchus, lung cancers | 1.3 | 2.3 |
| 9 | Road traffic accidents | 1.3 | 2.2 |
| 10 | Prematurity and low birth weight | 1.2 | 2.0 |
| 11 | Neonatal infections* | 1.1 | 1.9 |
| 12 | Diabetes mellitus | 1.1 | 1.9 |
| 13 | Hypertensive heart disease | 1.0 | 1.7 |
| 14 | Malaria | 0.9 | 1.5 |
| 15 | Birth asphyxia and birth trauma | 0.9 | 1.5 |
| 16 | Self-inflicted injuries† | 0.8 | 1.4 |
| 17 | Stomach cancer | 0.8 | 1.4 |
| 18 | Cirrhosis of the liver | 0.8 | 1.3 |
| 19 | Nephritis and nephrosis | 0.7 | 1.3 |
| 20 | Colon and rectum cancers | 0.6 | 1.1 |
| | All causes | 58.8 | 100 |

COPD, chronic obstructive pulmonary disease; IHD, ischaemic heart disease; TB, tuberculosis.
*This category also includes other non-infectious causes arising in the perinatal period, apart from prematurity, low birth weight, birth trauma and asphyxia. These non-infectious causes are responsible for about 20% of deaths shown in this category.
†Self-inflicted injuries resulting in death can also be referred to as suicides.

**Table 1: Global impact of diseases, ranked by mortality. Ischemic heart disease (IHD) is the leading cause of death, having 1.5 million more deaths per year than the second biggest killer, cerebrovascular diseases** (1).



IHD is caused by a blockage in one of the coronary arteries supplying blood to the heart muscle. This can be caused by many factors, such as clot or spasm, and in all cases it results in the loss, or severe reduction, in supply of oxygen and nutrients to the affected tissue (Figure 2). This depletion initiates a series of cellular reactions known as the ischemic cascade (6). Briefly, adenosine triphosphate (ATP) synthesis fails due to the lack of oxygen. Without ATP, ion transport pumps cannot function adequately, causing an accumulation of calcium in the cell. Calcium excess causes oxidative damage and up regulates calcium dependent enzymes, such as phospholipases (7). These enzymes degrade cellular and mitochondrial membranes, which will result in either necrosis or apoptosis. Necrosis releases toxic cellular contents into the extracellular matrix and elicits a localised inflammatory response which induces further cell death in the surrounding tissue (8).

The current treatment for IHD is alleviation of the blockage, either via physical means (angioplasty) or pharmacological means (thrombolytics), and subsequent restoration of blood flow (reperfusion). However, reperfusion itself paradoxically leads to cellular damage and death, known as reperfusion injury.

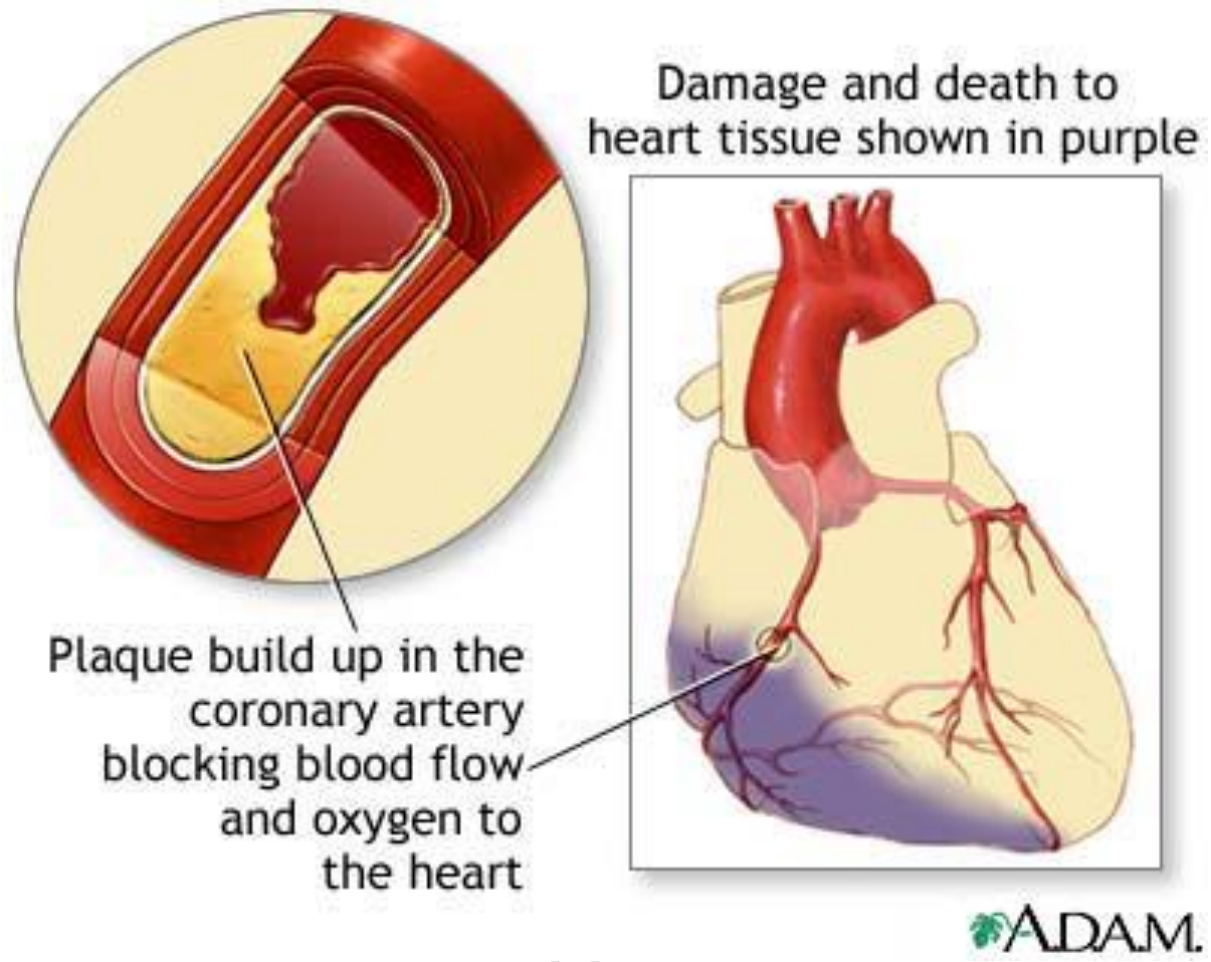


**Figure 2: Ischemic heart disease. Typical location and cause of ischemic heart disease is an atherosclerotic plaque in the coronary artery. Of note, the tissue highlighted in purple is at risk of death, but depending on the duration of the ischaemia only a proportion of it will infarct (irreplaceable death of tissue). (Picture sourced from (9)).**

### 1.2.1 Reperfusion Injury

The detrimental effect of reperfusion is caused by the rapid restoration of blood flow, which brings oxygen and nutrients to the tissue. This results in generation of reactive oxygen species (ROS) (10), excess calcium release (10) and an inflammatory response (11). The degree of damage is positively correlated with the severity of ischaemia and the speed of reperfusion (12). The damage from reperfusion injury may account for up to 50% of the infarct (dead tissue) following ischaemia (13). As such, there is a clinical need to reduce reperfusion injury, via pharmacological or physical means, to improve the benefit of reperfusion against ischemic heart disease. In particular, limiting reperfusion injury is important in the setting of percutaneous coronary intervention (PCI) as this is a very rapid form of reperfusion and hence susceptible to severe reperfusion injury.

# 2: PROTECTION AND PREVENTION OF CARDIOVASCULAR DISEASE

## 2.1 CONDITIONING

### 2.1.1 Ischemic conditioning

Two of the most powerful tools for acute protection against ischaemia-reperfusion injury are the phenomena known as ischemic preconditioning and ischemic postconditioning. Ischemic preconditioning was first discovered in the dog model in 1986, and involves the administration of short periods of sublethal ischaemia, followed by brief periods of reperfusion, prior to the index ischaemia (14). In ischemic postconditioning, short periods of sublethal ischaemia-reperfusion are delivered following the index ischaemia (15). Ischemic preconditioning, whilst counterintuitive, can create drastic reductions in infarct size (Figure 3).


**Figure 3: Protective effect of ischemic preconditioning (IPC). Cross sectional slices of hearts stained with triphenyltetrazolium chloride (TTC) reveal viable tissue (bright pink), and infarcted tissue (light yellow/white). A significant reduction in infarct size can be observed following IPC (orange bar) as compared to the control (red bar)** (16).

Ischemic conditioning can also be applied remotely, either to a separate section of the tissue, or to entirely different organ systems (17). This has allowed for the development of a third method of ischemic conditioning, known as perconditioning, wherein the sublethal ischaemia is administered to a separate organ during the ischemic attack. Figure 4 summarises the three main forms of ischemic conditioning,

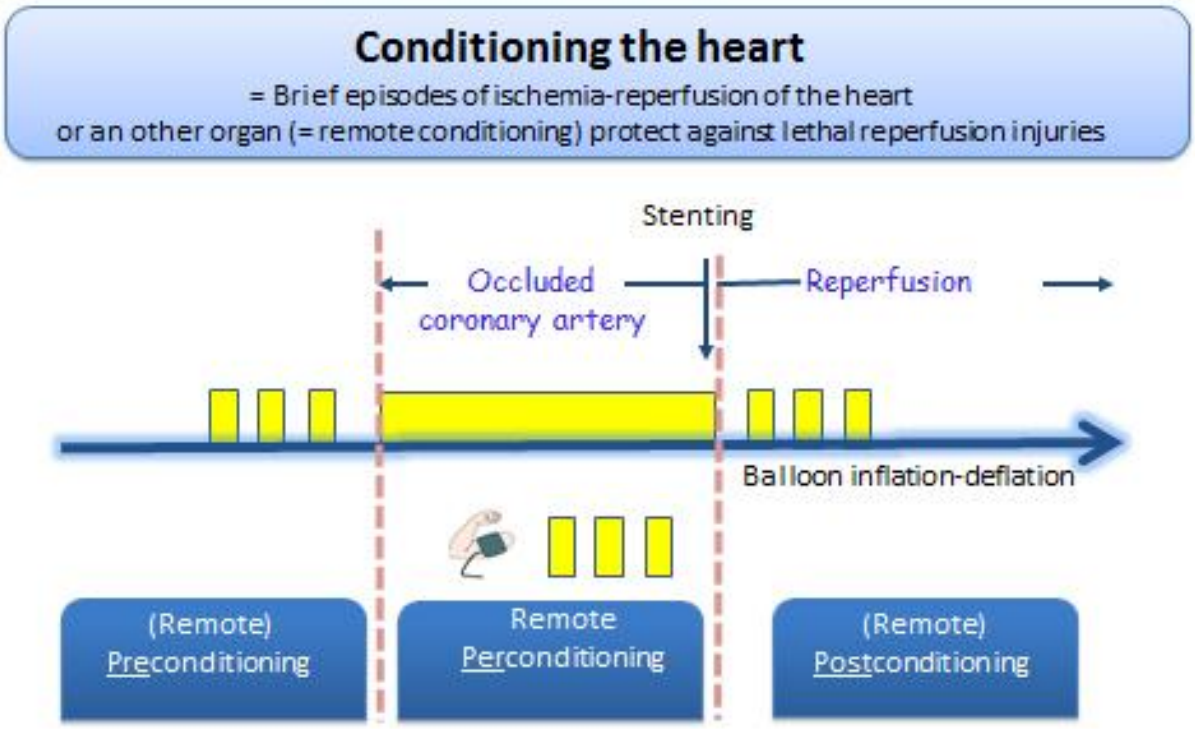


**Figure 4: Summary of methods for conditioning the heart with ischaemia.**

Ischemic preconditioning requires prior knowledge of the ischemic event. It can be applied to patients undergoing elective PCI, coronary artery bypass surgery, or heart transplant surgery (18).

Remote conditioning, performed by small episodes of ischaemia-reperfusion in an organ separate to the heart, can also protect against ischaemia-reperfusion injury, and it presents the advantage of not being invasive. Pilot studies with remote perconditioning in humans have shown promising results (19).

Similarly, ischemic postconditioning can be applied in patients with acute myocardial infarction, and this concept has been successfully tested in a clinical setting only two years after the discovery of ischemic postconditioning (20).

However, there are some limitations to ischemic conditioning. With the exception of remote conditioning, ischemic conditioning requires catheterisation in order to be performed; if conditioning is done incorrectly, it carries the risk of being more detrimental than protective. Therefore, it requires adequate access to medical facilities as well as trained staff and proper equipment. In 2006, the South African Medical Journal reviewed the hospital transfer times as well as the in-hospital waiting times of cardiovascular patients, and concluded that pre-hospital administration of thrombolytics (with PCI as a conjunctive therapy where necessary) could reduce treatment times by up to 130 minutes (21). As was mentioned in section 1.2.1 the duration of ischaemia has dramatic effects on patient outcome, and hence reducing time before treatment could make the difference between life or death. Therefore, pharmacological agents, mimicking ischemic conditioning, that can be prescribed to at risk individuals, either for acute or chronic periods of time, are much more desirable as it would be more practical and easier to apply worldwide.

### 2.1.2 Pharmacological conditioning

Most pharmacological conditioning strategies have been identified following the delineation of the signalling mechanisms in ischemic conditioning. Many mediators that have been identified in animal models are physiologically derived: adrenergic **antagonists** (22) ,opioids (23), bradykinin (24), phospholipids (25), inflammatory cytokines such as tumour necrosis factor alpha (26) and serum components such as erythropoietin (27). In a clinical setting, preconditioning with volatile anaesthetics has also shown to exert a marked benefit in patients undergoing off-pump cardiac procedures (28). Due to the physiological presence of many of these protective factors, an alternative target for treatment is to increase their basal levels, as opposed to a single bolus injection. As a consequence, they are less likely to present adverse side effects, especially if the agent remains within physiological ranges. This increase can be achieved either through medication or, in some cases, through changes to lifestyle.

## 2.2 LIFESTYLE CHANGES

Unhealthy lifestyles can contribute to up to 70% of cardiovascular deaths, whilst modest reductions in risk-associated behaviours can have exponential benefits (a 0.5% reduction in risk factors can result in as much as a 23% decrease in mortality) (29),(30). There is a further benefit to targeting lifestyles, as it is more cost-effective than medication (31) which makes it an ideal treatment strategy for low income countries. Positive lifestyle factors can either reduce the incidence of CVD or the severity of the attack, and in some cases it can do both. Ideal sleep duration (32), moderate consumption of alcohol (33), regular exercise (34) and a healthy diet all have protective effects. In particular, a diet high in green, leafy vegetables, low carbohydrates and polyunsaturated fats not only lowers detrimental Low Density Lipoprotein (LDL) cholesterol but may also increase protective High Density Lipoprotein (HDL) cholesterol levels (35).

## 2.3 HDL CHOLESTEROL

### 2.3.1 Structure and Function

HDL cholesterol has three major constituents: apolipoprotein A (ApoA), cholesterol and phospholipids (Figure 5). ApoA is synthesised predominantly in the liver and secreted into the bloodstream. It then associates with free cholesterol and phospholipids to form disc shaped micelles.

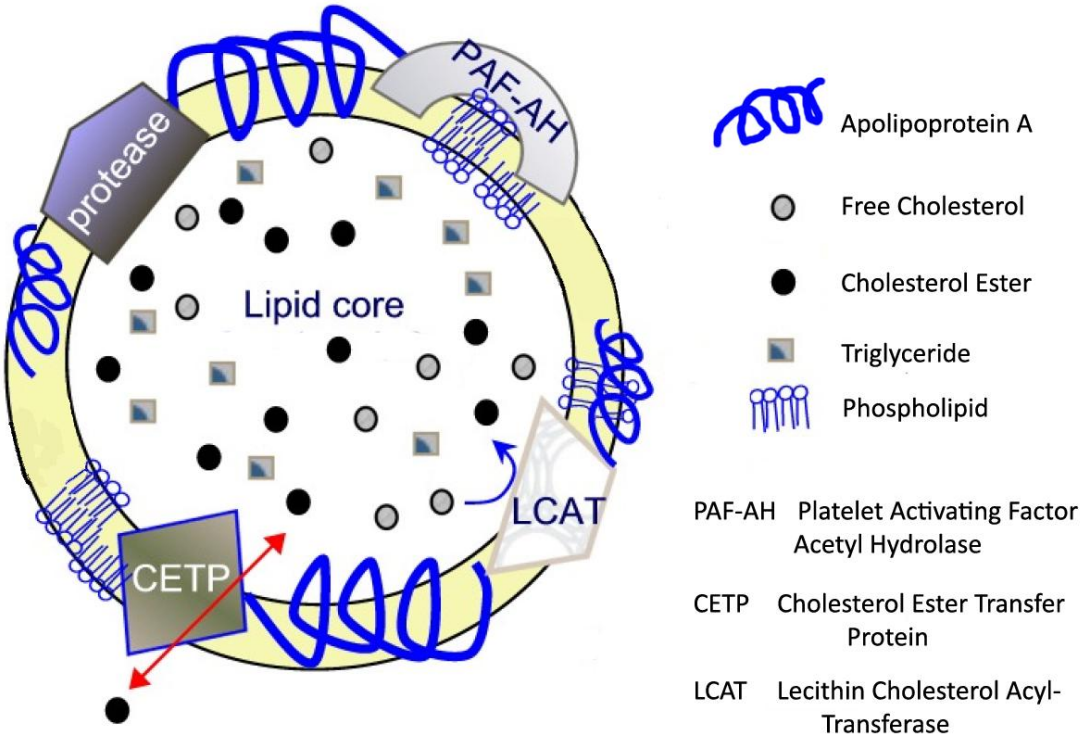


**Figure 5: Basic Structure of a mature HDL cholesterol molecule, adapted from** (36)**.**

In this form it is known as discoidal, or nascent HDL (Figure 6). There are key enzymes which then associate with the nascent HDL cholesterol that assist in its maturation. The nascent HDL continues to take up free cholesterol, while simultaneously converting the free cholesterol particles into cholesterol esters via an enzyme component, the Cholesterol Acyltransferase (LCAT). The phospholipids are the acyl donors in the esterification of the cholesterol, and allow for the binding of cholesterol to ApoA (37). When sufficient cholesterol has been esterified, the structure of HDL is converted into a spherical shape, allowing it to accommodate more cholesterol. Once it has reached this spherical shape it is referred to as mature, or spherical, HDL and is the most common form of HDL cholesterol found in the plasma.

Mature HDL cholesterol has two fates in the body, depending on the pathophysiological conditions. Under normal conditions, it is primarily involved in transport of cholesterol from the blood vessels and the tissues to the liver in order for it to be metabolised and excreted, in a process known as Reverse Cholesterol Transport. The cholesterol esters are transferred to apolipoprotein B via the **cholesteryl** ester transfer protein (CETP), resulting in the formation of low density, or very low density lipoproteins (LDL or VLDL). These molecules can then either be reabsorbed by the tissues, degraded by the liver or oxidised to form an atherosclerotic plaque. The cholesterol may also be recycled back into the bloodstream.
When cholesterol is in excess, either via dietary intake or from the peripheral tissues (such as adipose tissue or an atherosclerotic plaque), HDL is used as a transport mechanism to transport the excess cholesterol to the liver for degradation into bile salts or excretion in the faeces (36). Removal and prevention of plaque formation has obvious benefits for IHD, but

there are also other roles that HDL cholesterol, and its components, have in preventing CVD.

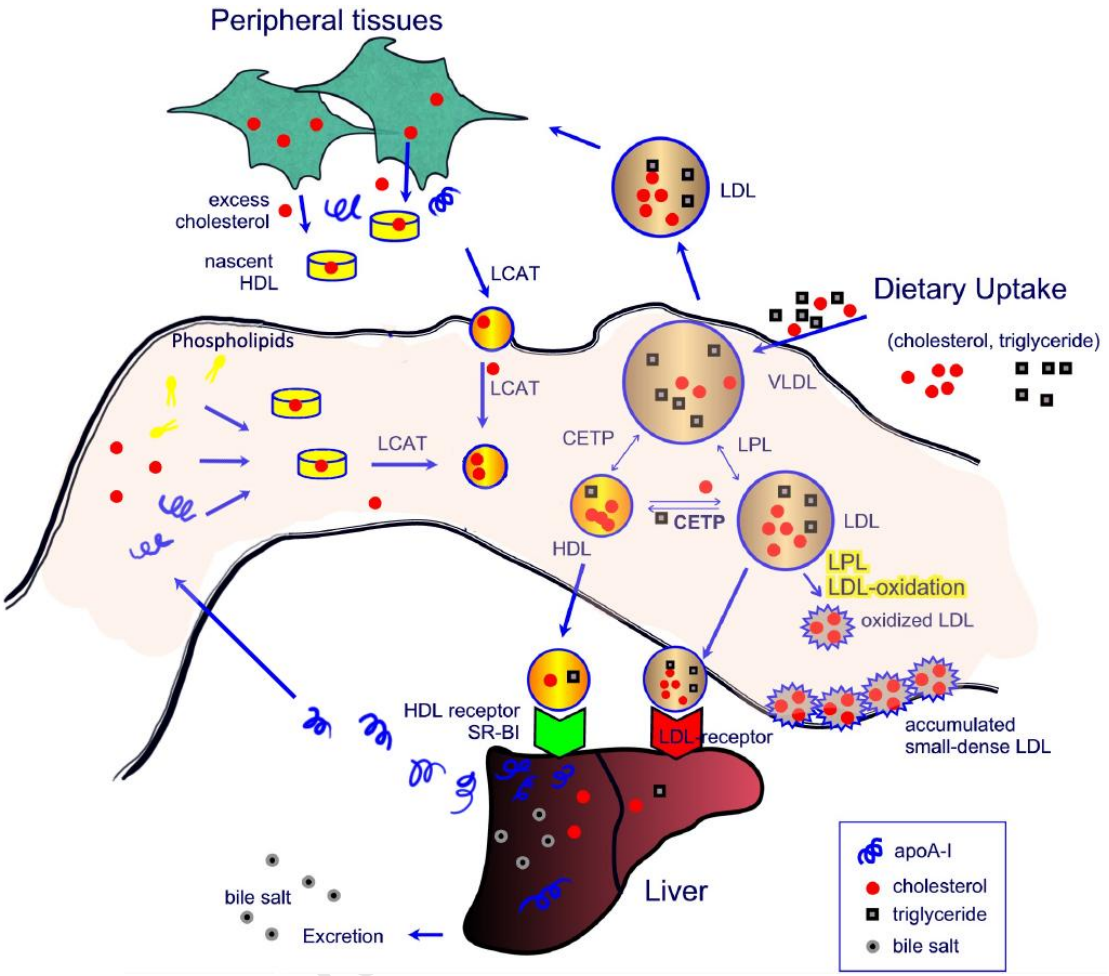


**Figure 6: Simplified version of the synthesis and various fates of HDL cholesterol in the body, modified from** (36)**.**

### 2.3.2 HDL and cardiovascular disease

The establishment of a link between high cholesterol level (hyperlipidaemia) and CVD has been known since the early 1950's (38) . There have been multiple studies exploring the composition, level and changes thereof in different races, countries and socioeconomic backgrounds. The general consensus of these studies is that whilst a low total cholesterol level is ideal, equally important is the ratio of HDL cholesterol relative to the total cholesterol, and triglyceride levels (Figure 7**Error! Reference source not found.**) (39).

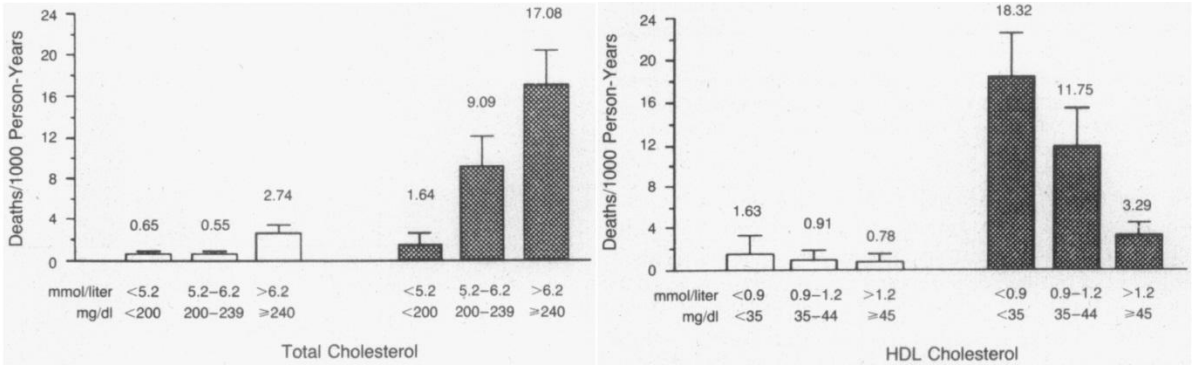


**Figure 7: Relationship between cholesterol levels and mortality. Total cholesterol, on the left, shows a positive correlation with deaths per 1000 people per year. White bars are men without any evidence of cardiovascular problems, whilst the shaded bars are men with existing cardiovascular problems. There is a greater sensitivity to higher total cholesterol levels in patients with pre-existing cardiovascular conditions. An inverse pattern is seen with HDL cholesterol, on the right, with increasing levels having a protective effect. Again, this seems to be more exaggerated amongst men with pre-existing cardiovascular conditions (shaded bar)** (39).

A high ratio of HDL cholesterol reduces overall mortality risk, and reduces the incidence of CVD. It is important to distinguish that although low HDL cholesterol is a very good risk indicator of developing CVD (40), it is not merely a marker for the disease but actually plays an active role in the prevention of cardiovascular incidents (41). In addition to its role in cholesterol transport and elimination, it can stimulate endothelial nitric oxide synthase, which produces **nitric oxide** and leads to vasodilation which attenuates hypertension and various forms of stroke. Also, HDL cholesterol stimulates fibrinolysis, stalling the progression of fibrosis of the heart. It has anti-inflammatory properties via the stimulation of the cyclooxygenase-2 pathway (41). It has antioxidant properties, preventing the formation of oxidised LDL particles and attenuating apoptosis in endothelial cells (41). Finally, it plays a crucial role in preventing IHD.

### 2.3.3 HDL and ischemic heart disease

Low relative levels of HDL cholesterol are strong predictors of ischemic events (42). This is likely due to HDL cholesterols anti-atherosclerotic effects (Figure 8). Atherosclerotic plaques form when oxidised LDL cholesterol damages the arterial wall via its phospholipase activity. This results in recruitment of macrophages which scavenge the LDL cholesterol. This scavenging process converts the macrophages into foam cells. Foam cells lose their migratory ability and hence form a plaque at the location of the arterial damage. The foam cells themselves often lyse, resulting in release of more oxidised LDL cholesterol and therefore increasing the plaque size. HDL cholesterol can scavenge the oxidised LDL cholesterol and can also remove LDL cholesterol from foam cells, eroding the plaque (43).

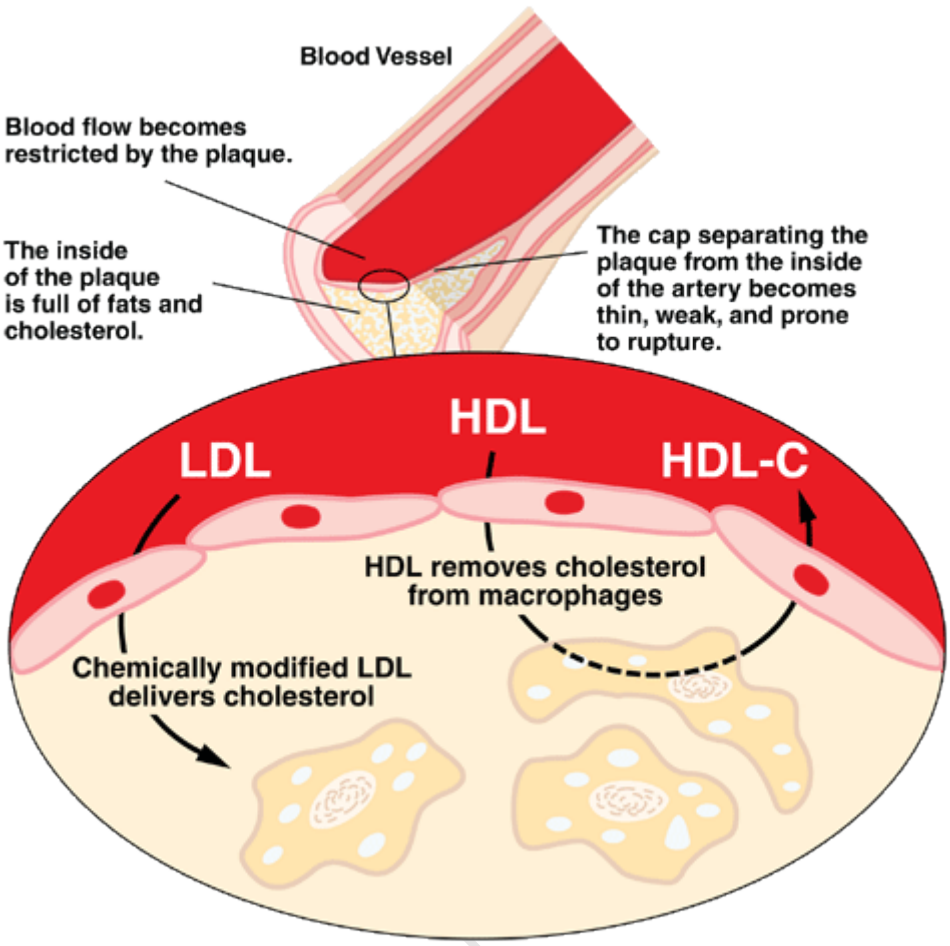


**Figure 8: Formation of an atherosclerotic plaque. Oxidised LDL cholesterol initiates plaque formation, whilst HDL cholesterol degrades foam cells and reduces plaque size** (44).

HDL cholesterol can also prevent plaque formation, through Platelet Activating Factor Acetyl Hydrolase (PAF-AH), one of its many enzymatic components (Figure 5). PAF-AH hydrolyses the phospholipase enzymes within oxidised LDL cholesterol, diminishing its capability to damage the arterial wall (45).

### 2.3.4 Mechanisms involved in HDL induced cardioprotection

HDL cholesterol improves prognosis following an ischemic event via its antioxidant, anti-apoptotic, anti-inflammatory and anti-atherosclerotic abilities. However, the exact mechanism of action of HDL cholesterol's protection against ischaemia remains unclear, although these effects may be mediated by its ApoA content (46). Another promising candidate is the lysophospholipid sphingosine-1-phosphate (S1P), a phospholipid component of HDL cholesterol (47). This is supported by evidence linking the protective effect of HDL cholesterol to activation of the S1P receptors (48) . Furthermore, it has been demonstrated that the levels of S1P found within HDL cholesterol inversely correlate with the occurrence of IHD (49), suggesting the pivotal role of S1P in HDL cholesterol mediated cardioprotection.

## 2.4 SPHINGOSINE-1-PHOSPHATE

### 2.4.1 Structure and Function

S1P is a sphingolipid comprised of an 18 length alkyl chain, phosphorylated at C1 and covalently bound to an amine group at C2 and a hydroxyl group at C3 (Figure 9).

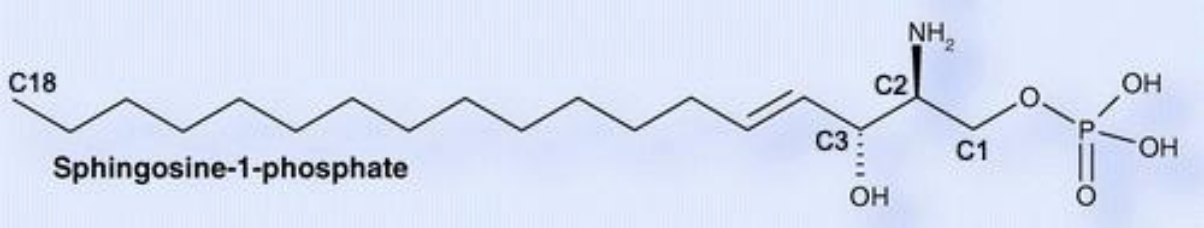


**Figure 9: Basic structure of S1P** (50).

It is a component of the ceramide pathway (Figure 10), which exists in all cells. As such S1P is produced intracellularly throughout the body. Once produced, it can either be involved in internal signalling, or be secreted and involved in paracrine/autocrine pathways via one or more of its five receptor subtypes.

The main secretors of S1P are the haematopoietic cells (platelets, erythrocytes, mast cells and leukocytes) and endothelial cells (51). Plasma S1P ranges in concentration from 200-1000nM. These concentrations are 20 to 100 fold higher than the binding coefficients of the S1P receptors (52), however the majority of plasma S1P is bound to HDL cholesterol (~60%), with the rest either bound to albumin or LDL cholesterol. Only a small percentage of the S1P remains in suspension (53). Thus, only 1-2% of plasma S1P is biologically active, the majority of which is located in suspension, although S1P bound to HDL cholesterol is not completely inactive, but does require the concurrent binding of HDL cholesterol to its srb1 receptor (54).

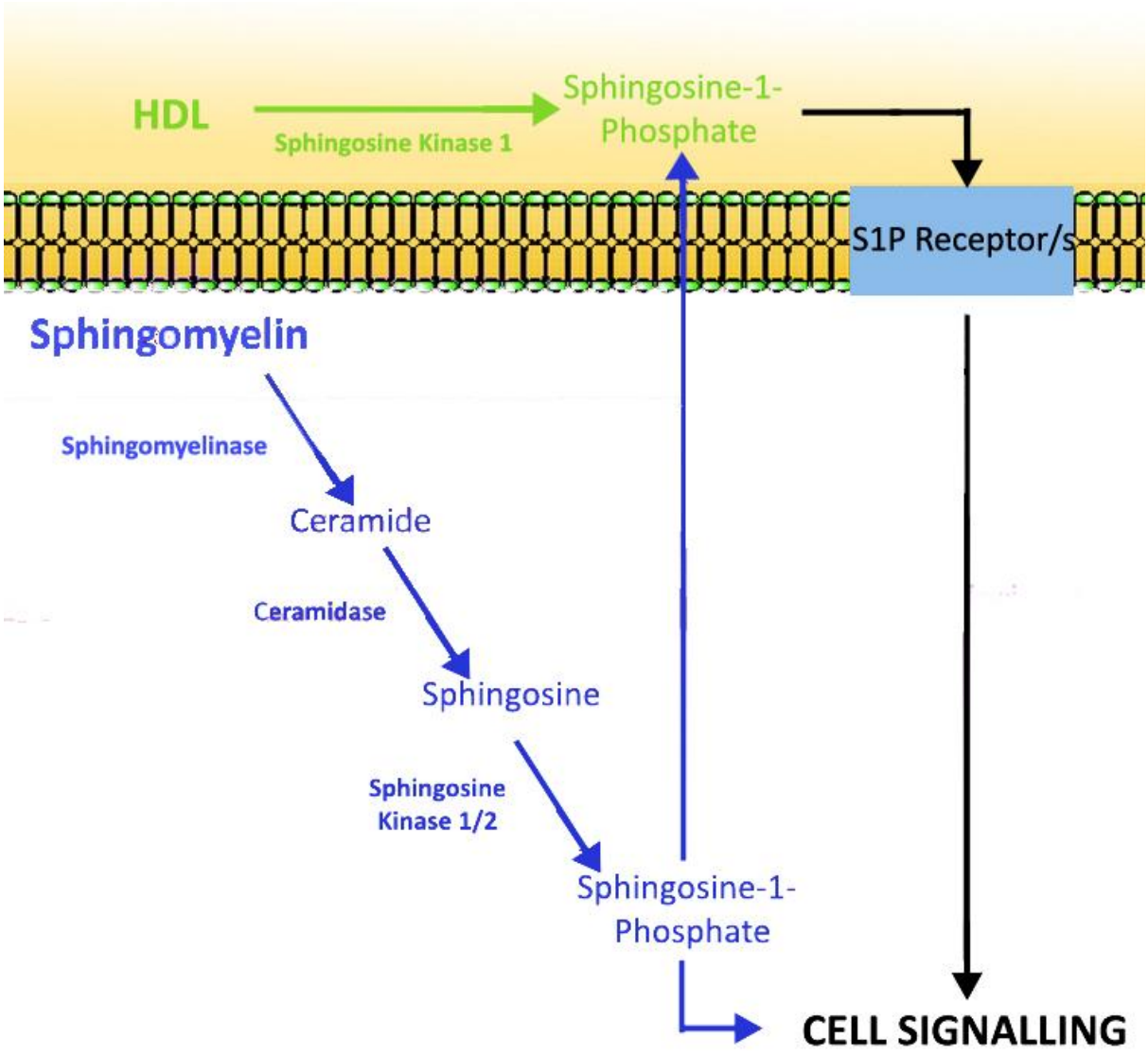


**Figure 10: Synthesis of Sphingosine-1-Phosphate (S1P) in the cell. S1P is derived from the cell membrane via a series of reversible enzymatic reactions. S1P may also be released from extracellular HDL via action of sphingosine kinase 1. Although not represented in this image, most of the enzymes involved in S1P synthesis are membrane bound. Extracellular S1P may enter the cell via binding to one of its 5 receptors, or may pass directly through the membrane due to its non-polar lipid composition.**

S1P is found intracellularly in cardiomyocytes, fibroblasts and endothelial cells with relative uniformity (55). However, the degree of expression of the S1P receptor subtypes (which have unique, even antagonistic, functions) vary considerably function to the cell type (Table 2) (56). Receptor expression level can also be induced to different degrees in these cells by external stimulating factors, such as beta-d-fructan, which promotes receptor 1 expression in fibroblasts (57).

| Tissue | Relative S1P receptor expression |
|---|---|
| Cardiac myocytes | $S1P_1 >> S1P_3 > S1P_2$ |
| Cardiac fibroblasts | $S1P_3 >> S1P_1 > S1P_2$ |
| Aortic smooth muscle cells | $S1P_2 > S1P_3 >> S1P_1$ |
| Vascular endothelial cells | $S1P_1 > S1P_3 >> S1P_2$ |

Comparison of S1P receptor subtype expression in various cell types found in the heart. Data for myocytes and fibroblasts are derived from studies of cells isolated from heart; those for vascular endothelium and smooth muscle are based on information from non-cardiac sources. >>, much greater than; >, greater than; =, similar.

**Table 2: Expression of S1P receptor subtypes in different cardiovascular tissue types** (56).



### 2.4.2 S1P and cardiovascular disease

In terms of the cardiovascular system, S1P is known to play four major roles: Mediation of cardiac electrophysiology, conflicting roles in hypertrophy, regulation of vascular function, and protection against ischemic insult (56).
Administration of S1P in vivo increases heart rate (chronotropy) and decreases the force of contraction (inotropy) (58). This has been suggested to be a novel mechanism, independent of the adrenergic pathway, regulated via the S1P receptors. Whether the electrophysiological role of S1P is positive or negative remains somewhat controversial, although it is likely that it may be beneficial or detrimental depending on the physiological circumstances. This may also be true for the role of S1P in hypertrophy, with some studies showing an increase in hypertrophy biomarkers following S1P treatment (59), whilst others showing no increase in other hypertrophy biomarkers (60).
The role of S1P in hypertrophy and electrophysiology of the heart is reported as both beneficial and detrimental, whereas the role of S1P in vascular function (it promotes vascular integrity and angiogenesis, and is crucial in migration and proliferation of endothelial cells (56)) and ischemic injury has only been reported as beneficial.

### 2.4.3 S1P and ischemic heart disease

Lecour et al first reported the cardioprotective effect of S1P against ischaemia-reperfusion injury in the isolated perfused rat heart (26), although S1P is also protective against ischemic injury in multiple organ systems besides the heart, including liver, kidney and lung (61). Both S1P and ceramide were reported as cardioprotective, and it was initially postulated that because they are intermediaries in sphingolipid signalling, they might converge on sphingosine as the active component. However, S1P is cardioprotective independent of sphingosine (62). Since then, strong evidence has accrued for an essential role of S1P in many protective pathways against ischemic injury: Antagonists of S1P receptors can abolish the protective effects of ischemic pre- and postconditioning (62). Furthermore, myocytes

release S1P following stimulus with ischemic preconditioning (63). Unlike ischemic pre and postconditioning, S1P has the added benefits of being non-invasive and easy to administer so it is an ideal candidate for therapeutic investigation. It should be noted that ischemic preconditioning and S1P administration have an additive effect (64), meaning that either ischemic preconditioning can act independent of S1P, or that it only taps into a small portion of the protective ability of S1P.

### 2.4.4 Mechanisms involved in S1P induced cardioprotection

The protective effect of S1P is mediated via specific receptor subtype activation (65), and this also holds true for the S1P contained within HDL cholesterol (48). In the cardiovascular system, S1P exerts its cardioprotective effect via binding to either receptor subtype 1 or 2. There is also evidence for S1P having direct intracellular actions independent of its receptors (66), although whether this plays a role in cardioprotection remains to be elucidated (evidence showing loss of protection using receptor antagonists would suggest it does not (67)). What remains unclear is the mode of action intracellularly. Recent evidence has suggested a role for two key cardioprotective signalling pathways, namely the Reperfusion Injury Salvage Kinase (RISK) pathway (68), and the Survivor Activating Factor Enhancement (SAFE) pathway (69), via the activation of Akt and STAT-3, respectively. However, it is unclear what the end effectors of S1P-induced activation of these signalling pathways are.

# 3: SIGNALLING PATHWAYS IN CARDIOPROTECTION

Two powerful prosurvival pathways exist in the heart: the Reperfusion Injury Salvage Kinase (RISK) pathway (70) and, the Survival Activating Factor Enhancement (SAFE) pathway (71). Whether these two pathways work in concert is still unclear in the literature, however both have been found to play a crucial role in ischemic preconditioning (70), (72) and postconditioning (73), (74), (75).

## 3.1 THE RISK PATHWAY

The RISK pathway was first described in 2004 (76), and is comprised of two important survival kinases (Figure 11). Firstly, protein kinase B (Akt), activated by phosphatidylinositol-3-OH kinase (PI3K). And secondly, the p42/p44 extracellular signal-regulated kinases (Erk 1/2), activated by mitogen-activated protein kinase kinases (MEK) (76).

The RISK pathway is only activated at reperfusion, however its components can also be activated before the index ischaemia. Prior to ischaemia, PI3K can be activated by various G-Protein Coupled Receptors (GPCR) (77), including insulin (78), bradykinin and opioid receptors (79). This sets off a phosphorylation cascade (Figure 11) that results in activation of endothelial Nitric Oxide Synthase (eNOS) and production of **Nitric Oxide** (NO). NO, via Guanylyl Cyclases (GC) and subsequent production of cyclic Guanylyl Monophosphate (cGMP), activates Protein Kinase G (PKG) which exerts a protective effect on the mitochondrion, via opening of the mitochondrial K ATP channel and low level production of reactive oxygen species (ROS) (80),(81). This low level production of ROS primes the system for the onset of index ischaemia.

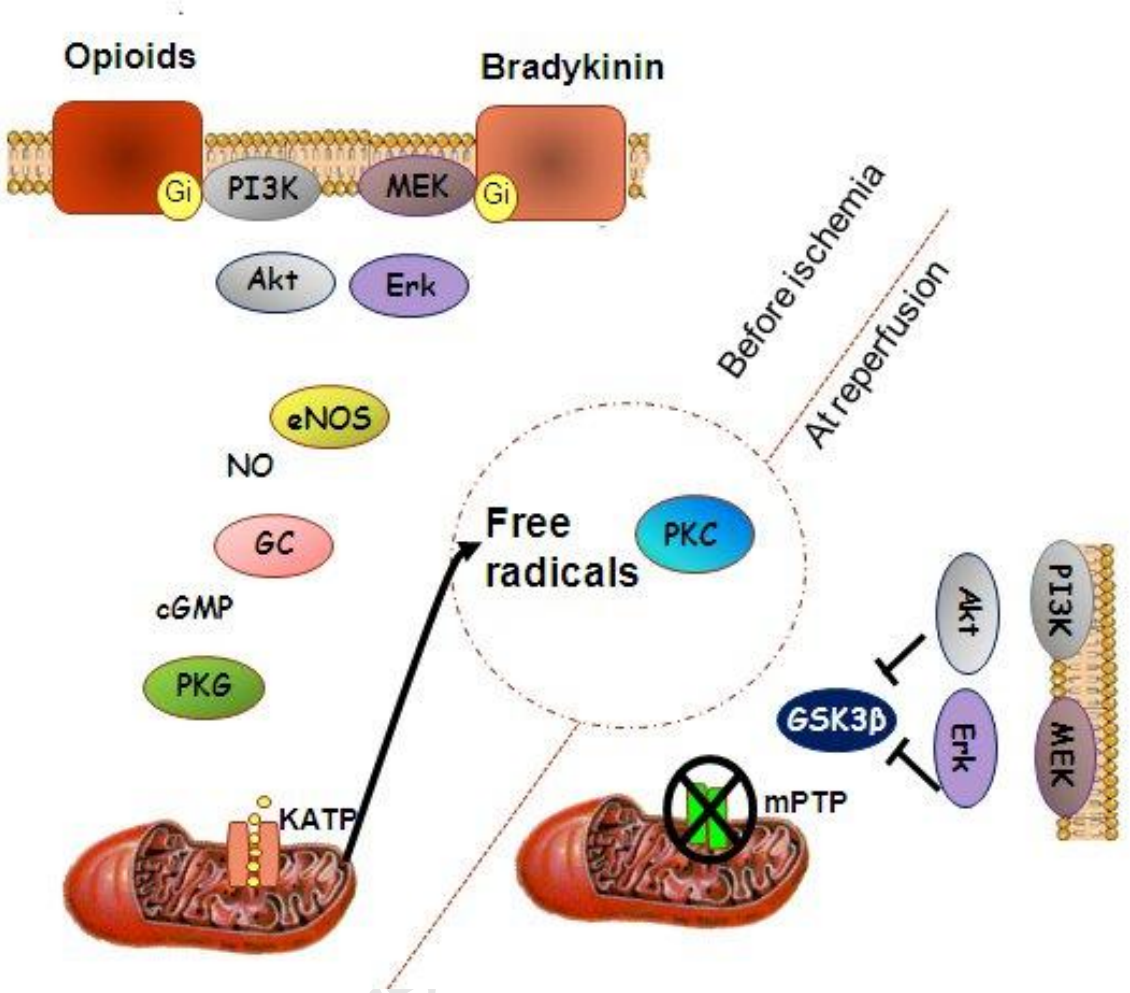


**Figure 11: The RISK pathway** (82)**. Abbreviations as described in the text.**

At reperfusion, the RISK pathway is activated, inhibiting Glycogen Synthase Kinase 3 beta (GSK3β) and the mitochondrial permeability transition pore (mPTP), via activation of the PI3K and MEK signalling cascades. The exact effect that the RISK pathway has on the mitochondrion will be detailed in section 4.
The RISK pathway can be activated by preconditioning (70), S1P (64) and HDL cholesterol. (However HDL cholesterol has only been shown in the context of cellular migration and proliferation, and not in cardiac cell lines) (83).

## 3.2 THE SAFE PATHWAY

The Survivor Activating Factor Enhancement (SAFE) pathway is a more recently discovered pathway (Figure 12), however some of its components are well established as prosurvival molecules and required for ischemic preconditioning (71). At the core of the SAFE pathway lie's the Signal Transducer and Activator of Transcription 3 (STAT-3) protein. STAT-3 is

activated upon phosphorylation, and in the case of the SAFE pathway this phosphorylation is induced by tumour necrosis factor alpha (TNF).

### 3.2.1 TNF

TNF is a pro-inflammatory cytokine component of the innate immune system. Ischaemia-reperfusion elicits an inflammatory response which results in the release of TNF from various cells, including macrophages and cardiac fibroblasts (84). As such, TNF is used as a prognostic marker for severity of ischaemia (85), and is associated with a poorer prognosis and larger infarct size (86). However, TNF can paradoxically be cardioprotective as a preconditioning agent (26). This paradox has been recently resolved by Lacerda et al (74), who demonstrated that TNF's effect is receptor dependent. At high concentrations, TNF binds to receptor type 1 and is detrimental, whereas at low concentrations it binds to receptor type 2 and is cardioprotective. Binding of TNF to its receptor type 2 initiates STAT-3 phosphorylation and is the first stage in the SAFE pathway (87) (Figure 12).

### 3.2.2. STAT-3

Two STAT isoforms are found in the heart, STAT-1 and STAT-3, and they have antagonistic effects; STAT-3 activation is anti-apoptotic, whilst STAT-1 promotes apoptosis (88). STAT-3 can be activated by various factors, such as ischemic conditioning (mice with a genetic depletion of STAT-3 cannot be preconditioned (89) nor postconditioned (74)), ethanolamine (90), the cannabinoid receptors (91), the interleukins (92) and potentially even the RISK pathway (93). STAT-3 may also be activated by both HDL cholesterol and S1P (69). However, it remains unclear as to whether the above stimulants are also mediated via TNF and its receptors. Regardless of the receptor type, ligand binding results in the activation of Janus Kinase 2 (JAK), a tyrosine kinase. JAK in turn phosphorylates STAT-3, activating it (71).

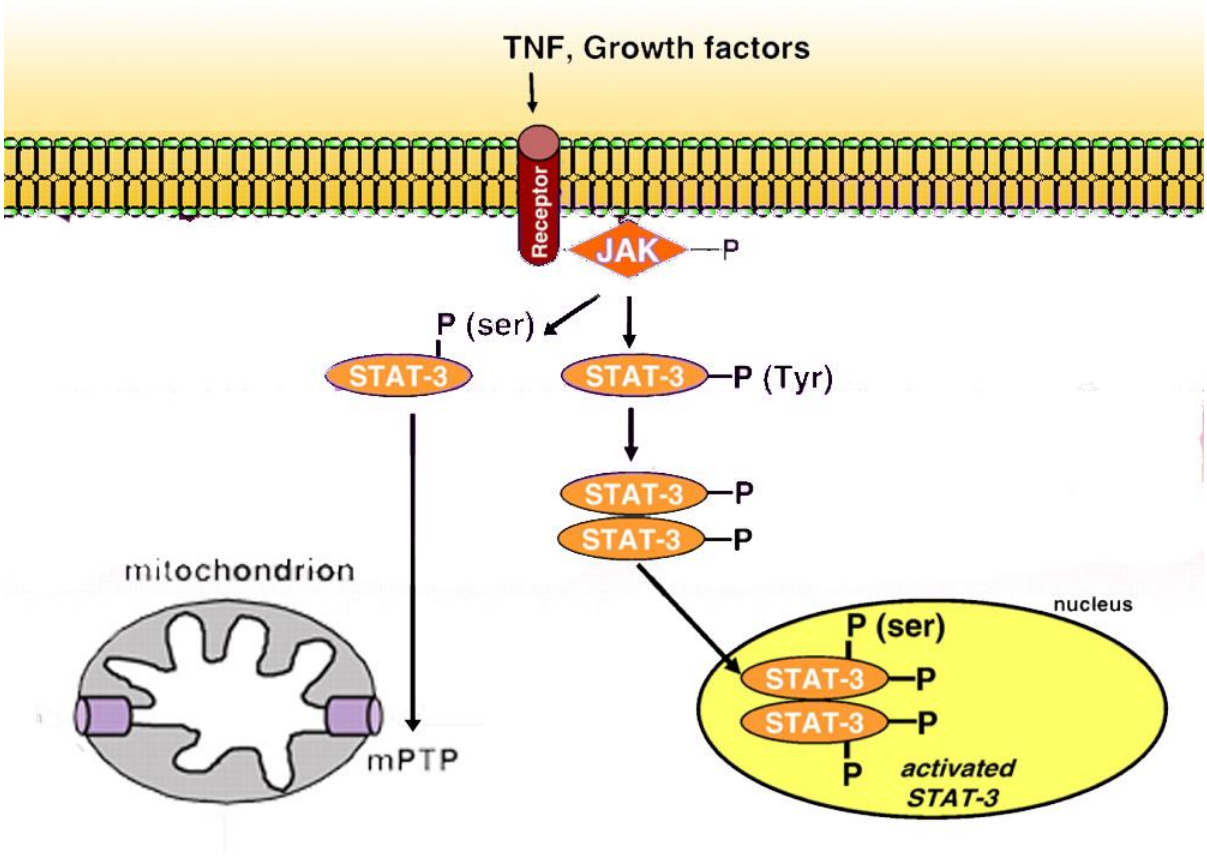


**Figure 12: the SAFE pathway. Tumour Necrosis Factor (TNF) binds to its type 2 receptor. This activates Janus Kinase 2 (JAK), which initiates the STAT-3 signalling cascade.**

### 3.2.3 Downstream targets of the SAFE pathway

There are two possible fates for STAT-3 following phosphorylation, depending largely on the residue that is phosphorylated. In the classic pathway, STAT-3 is phosphorylated at tyrosine 705 (pSTATTyr), which causes it to homodimerise (94). Homodimerisation exposes a nuclear translocation signal allowing it to enter the nucleus. Once in the nucleus it is phosphorylated further at serine 727 (pSTATSer) and becomes activated as a transcription factor for prosurvival genes such as Manganese Superoxide Dismutase (MnSOD) and inducible Nitric Oxide Synthase (iNOS) (95).

More recently, STAT-3 has also been discovered in the mitochondria (96), leading to the updated pathway which proposes that the location of STAT-3 phosphorylation may determine its fate: cytoplasmic phosphorylation of STAT-3 at its serine residue results in translocation to the mitochondria, whilst STAT-3 phosphorylated at the tyrosine residue follows a more classical nuclear route (97). Furthermore, recent evidence has suggested that STAT-3 may not even need to be phosphorylated or dimerised in order to enter the nucleus (98), although tyrosine phosphorylation is still required for transcriptional activity. Thus, in the new model, pSTATTyr translocates to the nucleus and acts as a transcription factor, whilst pSTATSer translocates to the mitochondria (99), where it has an anti-apoptotic effect, via inactivation of the proapoptotic molecule Bcl-2-associated death promoter (BAD) (87). What decides whether cytosolic STAT-3 is phosphorylated at its serine or tyrosine residue remains unclear, although it is likely to be receptor **dependent**.

# 4: THE MITOCHONDRION

The Mitochondrion is a distinct vesicle within the cell responsible primarily for ATP synthesis (100), although it also plays a vital role in calcium retention and regulation of apoptosis. Due to their high energy requirements, mitochondria are abundant in cardiomyocytes, accounting for roughly 30% of the total cell volume (101). The mitochondria within a cardiomyocyte are subdivided into 3 distinct populations: subsarcolemmal (SSM), interfibrillar (IFM) and perinuclear (102). SSM are located just below the cell membrane and are more responsive to cellular signalling and stresses (103), whilst IFM are stacked between the myofibrils and have higher respiration rates due to their proximity to the high energy demanding myofibrils (104). Perinuclear, as the name suggests, are located in proximity to the nucleus. Even though these noticeable differences exist between the different populations of mitochondria, their basic structure and function remain the same.

## 4.1 STRUCTURE AND FUNCTION

The mitochondrion is composed of two membranes, an outer membrane and an inner membrane (Figure 13). The outer membrane acts to compartmentalise the mitochondrion from the cytoplasmic elements, and excludes all molecules larger than 1500 Daltons (105). Between the outer membrane and the inner membrane lies the intermembrane space.

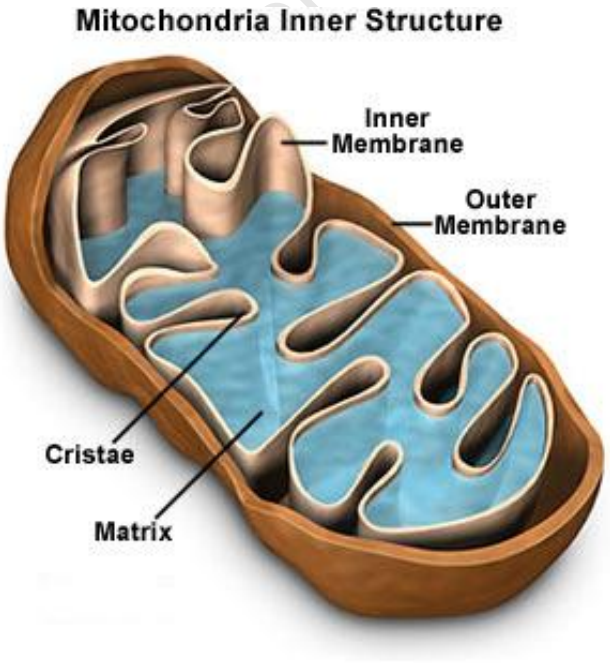


**Figure 13: Basic structure of a mitochondrion. The entire mitochondrion is encapsulated by the outer membrane. Within the mitochondrion, the inner membrane invaginates towards the central matrix to form cristae** (106)**.**

The inner membrane is divided into two regions: boundary regions which run parallel to the outer membrane and are involved in transport between the two membranes, and cristae that

invaginate into the matrix, or centre, of the mitochondrion and are the site of the electron transport chain (ETC) (101). Within the matrix metabolic enzymes, as well as mitochondrial DNA and RNA, are housed. The matrix is also used for calcium storage.

### 4.1.1 The electron transport chain

The ETC consists of 4 enzymatic subunits, bridged by 2 electron carriers, and an ATP generating hydrogen ion pump. The role of the 4 subunits is to actively transport hydrogen ions from the matrix to the intermembrane space (Figure 14), using energy provided via electrons donated by nicotinamide adenine dinucleotide hydroquinone (NADH) or flavin adenine dinucleotide hydroquinone ($FADH_2$) (sourced from the Krebs cycle).

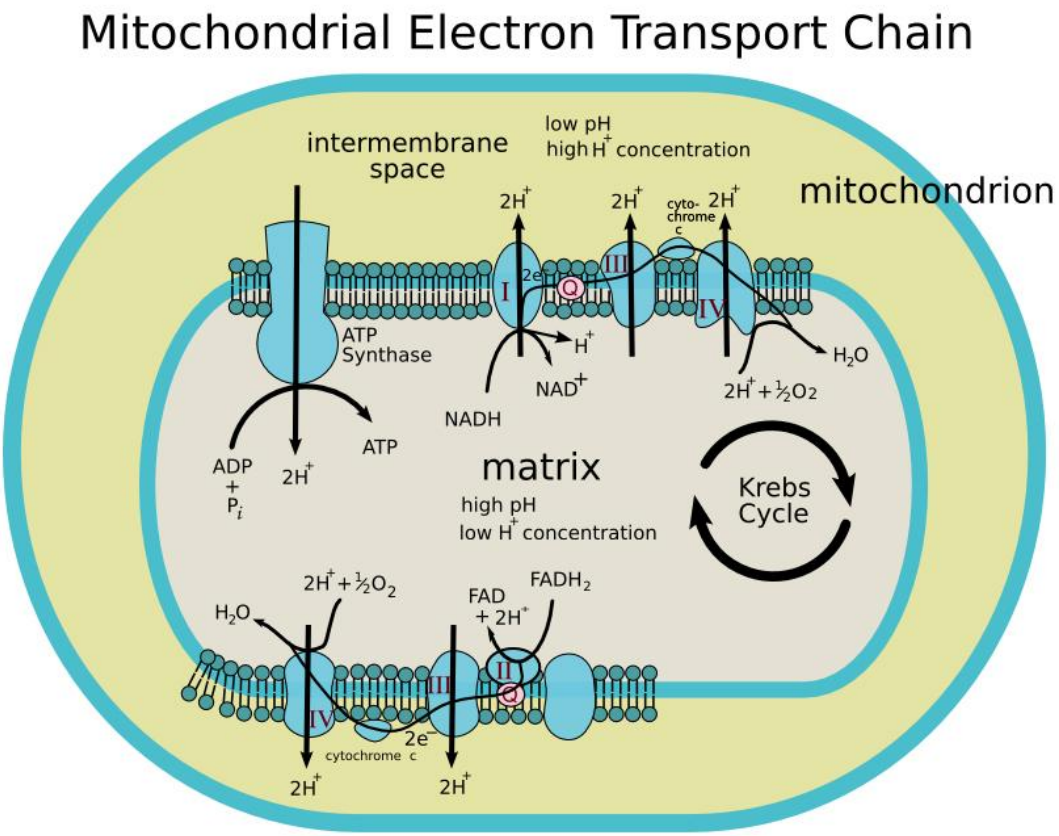


**Figure 14: The Electron Transport Chain. Energy is provided from the Krebs cycle in the form of NADH or FADH2. Electrons are donated at either Complex I or II depending on the substrate being utilised. Transferral of the electrons along the chain drives the proton pumps establishing a membrane potential. This in turn is used to drive the ATP synthase ion pump** (107).

This hydrogen gradient, also referred to as a proton motive force or membrane potential ($\Delta\upsilon$), powers ATP generation. Hydrogen ions flow through the ATP Synthase ion pump along their concentration gradient, which in turn drives the phosphorylation of ADP into ATP.

### 4.1.2 Calcium retention

Under normal physiological conditions there is a natural influx and efflux of calcium within the mitochondria in order to maintain homeostasis of the cell, and regulate ATP production. Calcium ions ($Ca^{2+}$) increase in cardiomyocytes when they are experiencing higher contractile force. Because the enzymes of the ETC are also dependent upon $Ca^{2+}$, there is a subsequent influx into the mitochondrion to drive their activity and increases their ATP output (108) in order to supply more energy for contraction. It is further hypothesised that this mechanism of $Ca^{2+}$ uptake and release may propagate the contractile signal along the cardiomyocyte (109). However in pathological situations, such as during ischemic injury, the mitochondria are also involved in $Ca^{2+}$ retention in response to stress (108). Most calcium is stored within the sarcoplasmic or endoplasmic reticulum of the cell. Mitochondria are positioned in proximity to $Ca^{2+}$ release sites and are able to absorb any $Ca^{2+}$ released from these reticula (110). Inside the mitochondrion the $Ca^{2+}$ is converted to calcium phosphate for storage in the matrix. Calcium phosphate precipitates within the matrix allowing for a greater degree of calcium storage than if it remained in solution (A single mitochondrion can store up to 1M of calcium, although the majority of this is in precipitate and hence has no effect on ion gradients (111)). Even though the precipitation of calcium is engineered to minimise interference with the ETC machinery, the role of mitochondria as calcium absorbers under stressful conditions will still take precedence over ATP production by the ETC (112). Mitochondria will continue to take up calcium to the point that overload occurs and the mitochondria eventually lyse, initiating cell death (113).

## 4.2 MITOCHONDRIA AND CELL DEATH

The mitochondria play a pivotal role in regulation of cell death; both apoptosis and necrosis. Cell death, mediated by mitochondria, can be activated by growth factors, hypoxia, oxidative stress and DNA damage (114). Mitochondria can induce cell death either via generation of ROS or opening of the mitochondrial permeability transition pore (mPTP). The consequences to the mitochondrion of opening of the mPTP are loss of membrane potential, swelling and eventual lysing (113). It is the mitochondrion's role in cell death that is targeted by the RISK pathway, and potentially by the SAFE pathway. Although, the components which regulate cell death are also integral parts of the mitochondria's native functions.

### 4.2.1 Oxidative stress

Mitochondria are the primary source of ROS (between 0.2 and 2% of oxygen consumed by mitochondria end up as superoxides (115)) and are usually fairly efficient at scavenging ROS via the enzymes MnSOD, catalase and glutathione peroxidase (116). Low levels of ROS production are important for ischemic preconditioning (117), and are a consequence of activation of the RISK pathway (118). In theory, low levels of ROS change the redox state of the cell, which results in structural and functional changes in certain proteins involved in cell survival (119), priming the system so that it is prepared for greater ROS exposure. However, in the absence of preconditioning and when a greater imbalance occurs, usually due to an increase in ROS production such as during reperfusion (120), the ROS scavenging enzymes can no longer cope with the excess ROS, and ROS become free to oxidise DNA and proteins, and also depolarise the mitochondrion via opening of the mPTP (121).

### 4.2.2 The mitochondrial permeability transition pore

The mPTP is a voltage-dependent pore which allows for the passage of molecules up to 1500 Daltons in size (122) into and out of the mitochondrion and the matrix. It is not known what proteins form the pore although there is evidence, summarised in Table 3, for the involvement of Cyclophilin D, the Voltage Dependent Anion Channel (VDAC) and Adenine Nucleotide Translocase (ANT), as well as complex 1 of the ETC (123).

| Structural Component | Evidence For | Evidence Against |
|---|---|---|
| **Cyclophilin D** | • Cyclophilin D knock outs are insensitive to mitochondrial swelling (124)<br>• Inhibition of Cyclophilin D with pharmacological agents inhibits mPTP activity (125)<br>• Reconstituted Cyclophilin D and ANT complexes can function as an mPTP (126)<br>• | • Cyclophilin D knock outs still contain a functioning mPTP, only less sensitive to external stimuli such as $Ca^{2+}$ (127) |
| **VDAC** | • Anti-VDAC antibodies inhibit mPTP opening (128)<br>• VDAC immunoprecipitates with Cyclophilin D (129)<br>• Increases in VDAC expression lead to increased mPTP sensitivity (130) | • VDAC is permeable to solutes of 1500 Daltons in its closed state (131)<br>• Reconstituted Cyclophilin D and ANT complexes can function as an mPTP (126) |
| **ANT** | • ANT inhibitors block mPTP opening (132)<br>• ANT interacts with Cyclophilin D in the mitochondrial membrane (133)<br>• Increases in ANT expression lead to increased mPTP sensitivity (130) | • ANT knock outs still exhibit normal mPTP responses (134) |

**Table 3: Proposed components of the mPTP.**

Under basal conditions the pore remains closed in order to maintain the membrane potential between the matrix and the intermembrane space of the mitochondrion. Upon stimulation, the pore opens and water floods into the matrix due to its hyperosmolarity. The matrix swells, **deforming** the cristae to account for the increased volume size and uncoupling the ETC. As the inner mitochondrial membrane expands, the more rigid outer membrane ruptures, releasing various proapoptotic factors (section 4.2.3) into the cytoplasm (114). Prevention of opening of the mPTP is seen as the endpoint for ischemic preconditioning, postconditioning (135) and the RISK pathway (118). It has recently been reported that ROS mediated opening of the Mitochondrial K ATP channel may delay pore opening, providing another mechanism for the paradoxical protective role played by ROS (136).

### 4.2.3 Proapoptotic factors

Once the mitochondria have lysed and the outer membrane permeabilised, an irreversible path towards cell death is set in motion (137). The disruption of the outer mitochondrial membrane has three consequences: disruption of the ETC, activation of the caspase apoptotic pathway and release of proapoptotic factors (independent of the caspase pathway).
The ETC is dependent upon integrity of the inner and outer mitochondrial membranes and subsequent maintenance of the hydrogen gradient (and accompanying pH gradient) in order to power the ATP Synthase ion pump (Figure 14). Disintegration of the outer mitochondrial membrane ablates the hydrogen gradient, reducing or even completely disabling the mitochondrion's ability to generate ATP for the cell. It also uncouples the crucial spatial orientation of the components of the ETC, such as Complexes I-III and cytochrome c.

Cytochrome c, an electron shuttle in the ETC (Figure 14), is thus released into the cytosol and activates the caspase apoptotic pathway via binding to caspase-9 and forming an 'apoptosome' (138). This activates caspase-9, which in turn activates caspase-3 (Figure 15). Caspase-3 then exerts proteolytic effects within the cell, dismantling and salvaging the components of the cell.

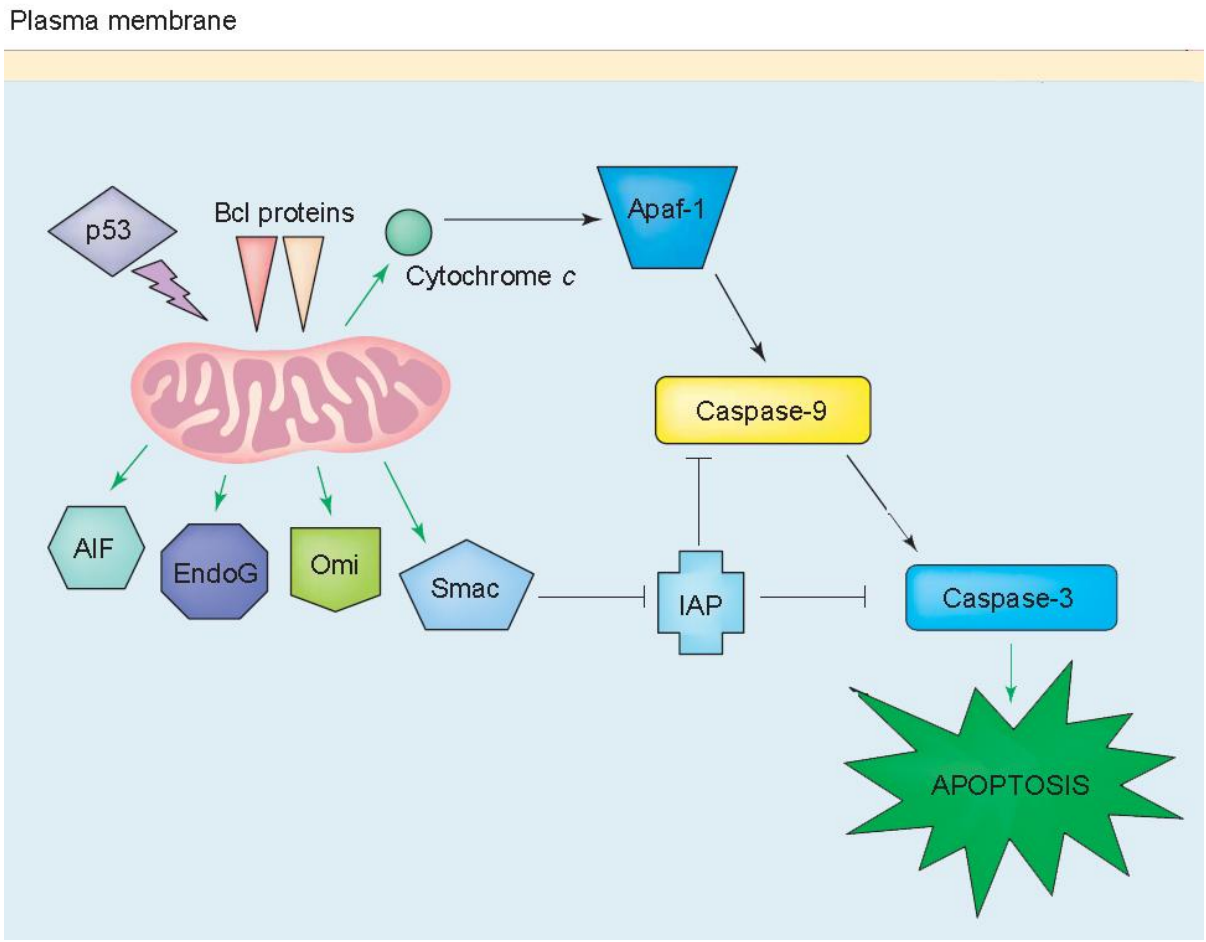


**Figure 15: The Caspase pathway. When the mitochondrion lyses, various proapoptotic factors are released (AIF=Apoptosis Inducing Factor, EndoG=EndonucleaseG). Some directly activate the caspase pathway, whilst others inhibit the inhibitors of the caspase pathway, ensuring its activation** (139)**. Abbreviations as described in the text.**

The caspase pathway is kept in a constant state of inhibition by the Inhibitors of Apoptosis (IAP) proteins. Dissolution of the mitochondrion also releases Smac and Htr2A proteins, which disable the IAPs and allow for activation of the caspase pathway (140)(141).

The mitochondrion also releases proapoptotic factors that are independent of the caspase pathway, most notably Apoptosis Inducing Factor (AIF) (142) and endonuclease G (EndoG). AIF is a misnomer as its native function is to scavenge ROS, however when released from the mitochondrion it translocates to the nucleus and initiates chromatin condensation and DNA fragmentation (143). Ischemic preconditioned hearts exhibit lower levels of AIF in the cytoplasm and nucleus (supporting the crucial role of maintenance of mitochondrial integrity in cardioprotection) (144). EndoG has a similar role to AIF, in that is involved in DNA fragmentation when released from the mitochondrion (145), whilst its native function is facilitation of mitochondrial DNA replication (146).

# *Chapter 2: Hypothesis and Objectives*

Ischemic heart disease is the leading cause of death worldwide. Multiple lifestyle factors contribute to the burden of disease, including smoking, diet, lack of exercise and alcohol consumption. These lifestyle factors can lead to alterations in the cholesterol profile, increasing LDL cholesterol and lowering HDL cholesterol levels. HDL cholesterol, and its major constituent S1P, can protect against ischaemia-reperfusion injury but the mechanisms of protection remain unknown. Our recent work suggests that S1P protects via the activation of the powerful prosurvival SAFE pathway, which involves the activation of STAT-3. Mitochondria are important constituents in the regulation of cell fate during myocardial infarction, and the inhibition of mPTP opening plays a critical role in limiting cell death from ischaemia-reperfusion injury. However, whether HDL cholesterol, S1P and the SAFE pathway promote cell survival by inhibiting opening of the mPTP is unknown.

## 1: HYPOTHESIS

We hypothesised that HDL cholesterol, and its component S1P, confer cardioprotection against ischaemia-reperfusion injury via the inhibition of mPTP opening (Figure 16). Furthermore, we proposed that this effect is mediated via the activation of STAT-3, an important constituent of the SAFE pathway.

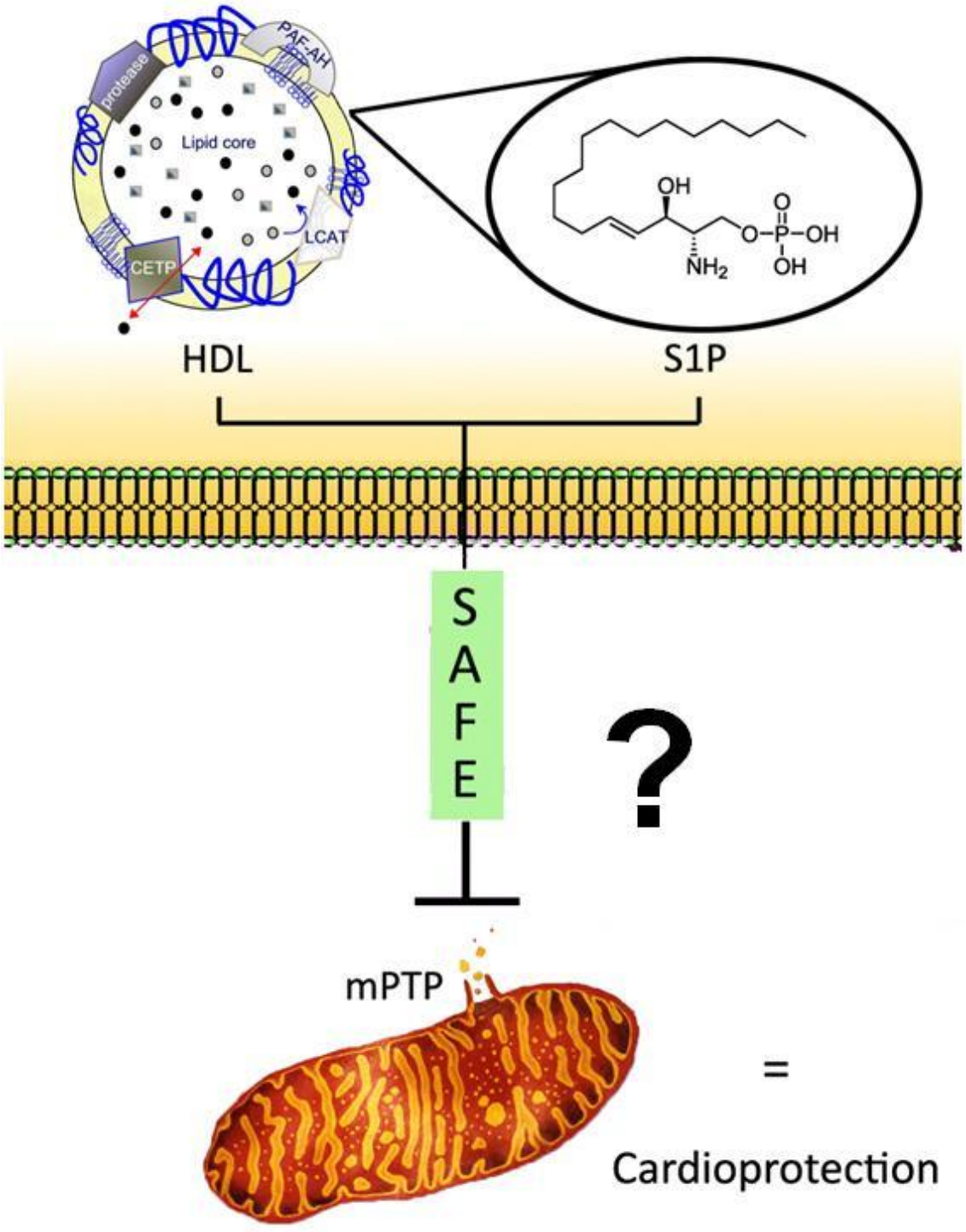


**Figure 16: Schematic diagram of the hypothesis (abbreviations as described in the text).**

# 2: OBJECTIVES

To answer this hypothesis, the following two objectives will be pursued:

## 2.1 DO HDL CHOLESTEROL AND S1P HAVE AN EFFECT ON THE MITOCHONDRIA?

To explore whether HDL cholesterol or its constituent SIP confer cardioprotection via the inhibition of mPTP opening **two** parameters will be assessed. Firstly, mitochondrial function will be measured using a Clarke-electrode in mitochondria extracted from the isolated rat heart pretreated with HDL cholesterol/S1P. Secondly, the opening of the mPTP will be assessed using flow cytometry or fluorescent microscopy techniques in isolated mouse cardiomyocytes subjected to a hypoxic insult, and pretreated with HDL cholesterol or S1P.

## 2.2 ARE THE EFFECTS OF HDL CHOLESTEROL AND S1P REGULATED VIA THE SAFE PATHWAY?

To answer this objective mPTP opening will be assessed in isolated cardiomyocytes from the cardiomyocyte specific STAT-3 deficient mouse. Similar to their wildtype controls, STAT-3 deficient cardiomyocytes will be isolated and subjected to a hypoxic insult with or without pretreatment with HDL cholesterol or S1P. Furthermore, STAT-3 activation in the mitochondria will be quantified using western blot techniques in the wildtype controls.

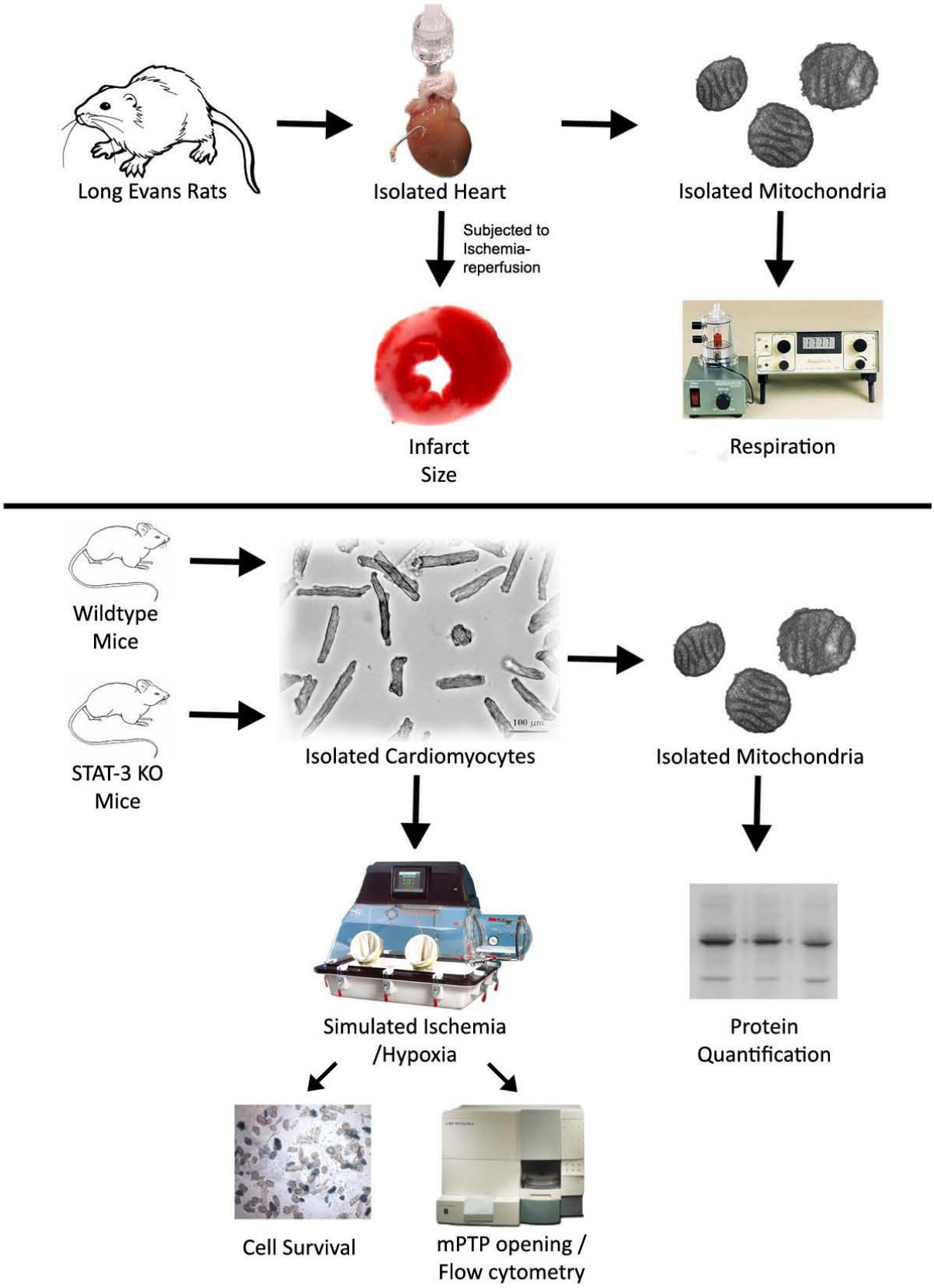


**Figure 17: Experimental Design of the project (abbreviations as described in the text).**

# Chapter 3: Materials and Methods

## 1: ANIMALS

All experiments involving animals were approved by the Health Science Faculty Animal Ethics Committee, University of Cape Town (Project numbers 009/015, 009/030 and 009/052). Animals were housed and treated in accordance with the Guide for Care and Use of laboratory Animals Eighth Edition, published by the US National Institute of Health Publication (147). Animals were bred in the Animal Unit of the University of Cape Town, and allowed access to food and water ad libitum.

Male Long-Evans rats weighing 250-300g were used for the isolated rat heart model. Rats were used because the isolation of mitochondria via the Percoll method does not yield a very high concentration of mitochondria. Hence, it would not have been feasible to isolate mitochondria from mouse hearts for analysis of respiratory parameters, as the yield would be too low.

In contrast, a mouse model was used in isolated cardiomyocyte studies as it allowed us to compare cardiomyocyte specific STAT-3 deficient (STAT-3 KO) mice versus their wildtype controls. Ideally, controls should be littermate controls, but due to availability and time constraints this was not possible. Thus, male c57black6 mice aged between 12-16 weeks were used as controls. STAT-3 KO mice were created from a c57black6 strain by crossing homozygous floxed STAT-3 mice with heterozygous Myosin Light Chain 2V (MLC2V) driven Cre recombinase mice, as previously described (148). MLC2V is only expressed in ventricular cardiomyocytes (149), and as such the resultant STAT-3 KO mice are ventricular cardiomyocyte specific.

## 2: ISOLATED RAT HEART MODEL

### 2.1 LANGENDORFF RAT HEART PERFUSION

Male Long-Evans rats were anaesthetised with 60mg/kg sodium pentobarbital via intraperitoneal injection. Rats were allowed to succumb to anaesthesia and were tested for sufficient depth of anaesthesia via the lack of the pedoreflex and changes in breathing rate. Thereafter, the chest cavity was opened, and the heart excised and mounted on the Langendorff retrograde perfusion apparatus (Figure 18) via cannulation of the aorta to a 20 gauge blunted needle (Figure 19). Because the buffer is entering via the aorta, the Langendorff system is a retrograde perfusion model (For a detailed review on retrograde perfusion models see (150)).

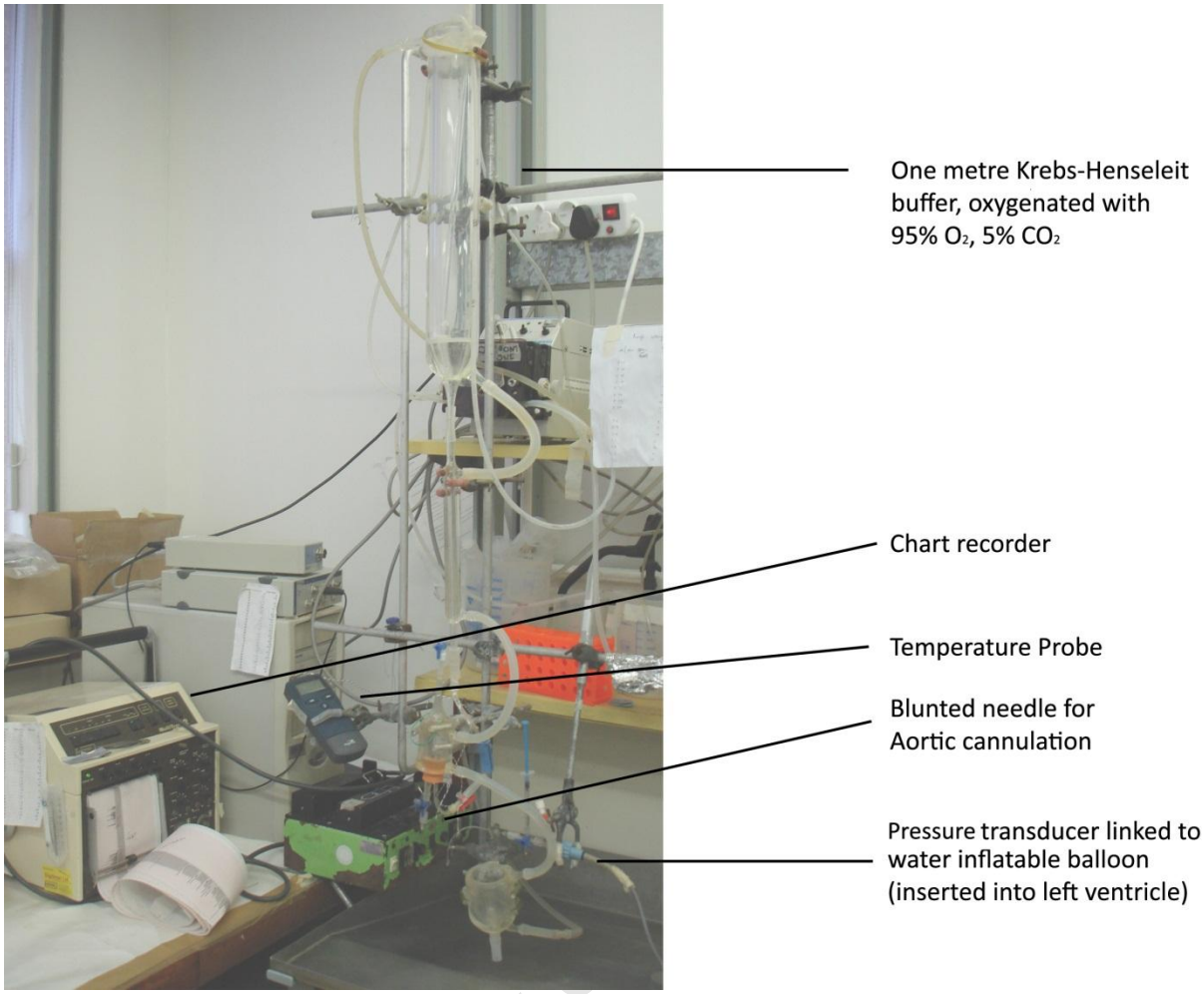


**Figure 18: Langendorff retrograde perfusion apparatus.**

The left atrium was surgically opened and an inflatable liquid-filled balloon was inserted into the left ventricle to measure functional parameters (Figure 19). The inflatable balloon was inflated using distilled water, and connected to a **pressure** transducer (Lasec). The pressure transducer converted the pressure changes in the balloon into electrical signals, which were in turn translated into functional parameters via a chart recorder (Lectromed). Hearts were perfused with Krebs-Henseleit buffer (appendix 1) and allowed to stabilise for 30 minutes. Pressure was maintained via a constant load of one metre high Krebs-Henseleit buffer (Figure 18). Temperature was measured with a thermocoupled wire (Physitemp, NJ, USA) inserted into the pulmonary artery, and connected  a Digitron 2600T temperature sensor (Torquay, UK). Temperature was controlled with a water jacket and a desk lamp.

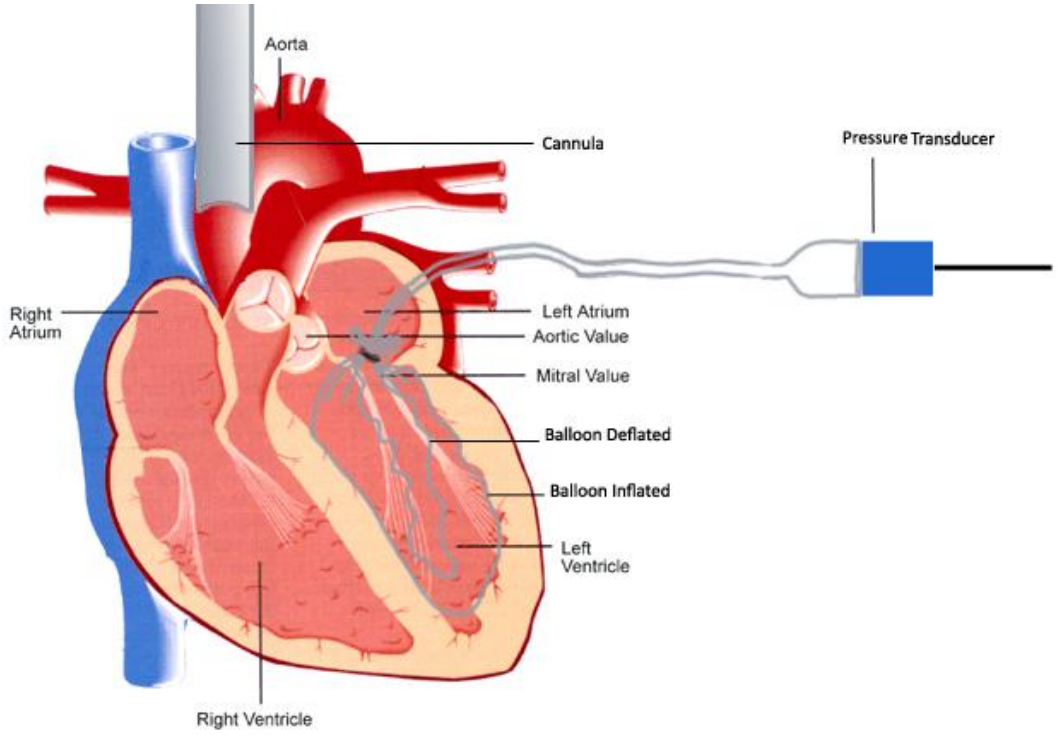


**Figure 19: Isolated rat heart mounted on a Langendorff apparatus. Hearts are perfused via the aorta. Ventricular contraction and heart rate are measured via the insertion of a balloon into the left ventricle. Flow rate is determined via collection of effluent from the pulmonary vessels. Temperature is monitored using a temperature probe inserted into the pulmonary artery (adapted from (151)).**

Functional parameters were measured at 5, 10, 20 and 30 minute intervals during the stabilisation period to ensure the inclusion criteria (as detailed in Table 4) were met. Functional parameters were also measured after 5 minutes, 30 minutes and 120 minutes of reperfusion. To evaluate the functional parameters of the heart, the left ventricular systolic pressure and the left ventricular diastolic pressure were measured, and expressed as the difference between the two, namely the left ventricular developed pressure (LVDP).

| Criterion | Inclusion Parameter |
|---|---|
| | |
| Heart Rate | 260-410 beats per minute |
| Left Ventricular Developed Pressure | 70-120 mmHg |
| Left Ventricular Diastolic Pressure | 2-10 mmHg |
| Coronary Flow | 8-18 ml per minute |
| Rate Pressure Product | 21 000 - 36 000 |

**Table 4: Inclusion Criteria for Langendorff perfused rat hearts. Hearts which did not fall within the inclusion parameters by the end of the 30 minute stabilisation period were excluded from the study.**

## 2.2 EXPERIMENTAL PROTOCOLS

Following the stabilisation period, hearts were perfused with Krebs-Henseleit buffer with or without the addition of 10nM S1P for 7 minutes, followed by a 10 minute washout period

(Figure 20). The concentration of 10nM was in line with the rest of the experiments in this thesis and was previously established as the optimum protective concentration in our laboratory (152). Immediately thereafter, some hearts were processed for Percoll mitochondrial isolation, whilst others underwent 30 minutes of regional ischaemia via occlusion of the left anterior descending artery. **3/0 silk ligature, attached to a 16mm curved atraumatic needle (Tyco healthcare), was threaded through the ventrical tissue behind the left anterior descending artery and tied tightly to induce ischaemia**. After 30 minutes, the ligature was released to allow for reperfusion for 120 minutes. These hearts were then removed and processed for infarct size analysis.

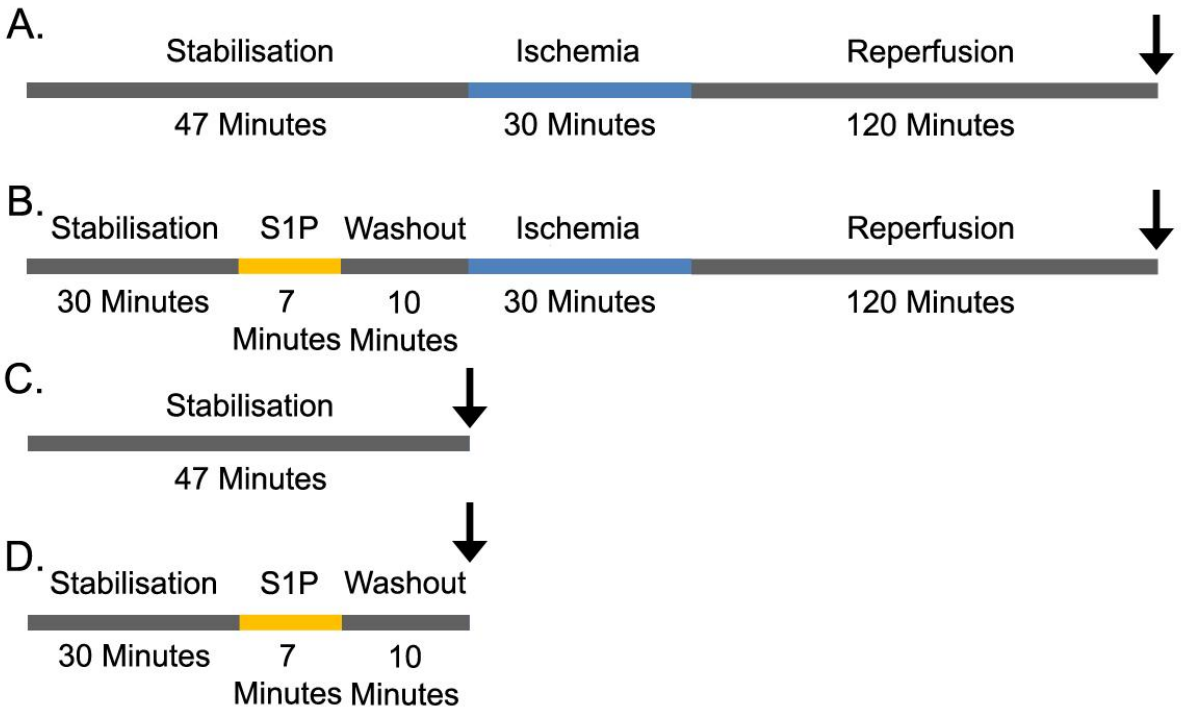


**Figure 20: Experimental protocols in the isolated heart. Arrows indicate points of removal for infarct size analysis in A and B and mitochondrial isolation processing in C and D. S1P = 10nM Sphingosine-1-phosphate.**

## 2.3 INFARCT SIZE ANALYSIS

Just prior to collection, the left coronary artery was re-ligated and the heart was perfused with 2% Evans blue. Non-ischemic tissue was stained blue, while the ischemic tissue remained unstained. Hearts were then immediately removed and frozen. After 24 hours, hearts were sectioned perpendicular to the septal wall using a surgical blade (5-6 slices per heart). Heart slices were incubated in sodium phosphate buffer (appendix 1) containing 1% w/v triphenyltetrazolium chloride (TTC) at 37°C, pH 7.4 for 15 minutes. TTC stains the viable tissue pink, and the infarcted tissue white/light yellow. Sections were then digitally scanned and analysed using planimetry. Infarct size was expressed as a percentage of tissue infarcted relative to the percentage of tissue that was ischemic (area at risk).

# 3: RESPIRATORY STUDIES IN ISOLATED RAT HEART MITOCHONDRIA

## 3.1 PERCOLL MITOCHONDRIAL ISOLATION

The Percoll mitochondrial isolation protocol was adapted from Costa and colleagues (153). Following isolation and perfusion (alone or with 10nM S1P), hearts were immediately removed from the Langendorff apparatus and resuspended in approximately 25ml of wash buffer (appendix 2) at 4°C. Hearts were minced lightly with surgical scissors. Wash buffer was then drained off and replaced 4 times to wash out any Krebs-Henseleit buffer, blood or other contaminants. Desiccated hearts were then resuspended in 2ml of Nagarse Type XXIV (at a concentration of 1mg/ml) and minced finely with scissors for 30-45 seconds at room temperature. Nagarse is a protease that allows for the extraction of both SSM and IFM mitochondrial fractions. Nagarse was then diluted out with the addition of 20ml bovine serum albumin buffer (appendix 2) in order to prevent damage to the mitochondrial proteins themselves. Hearts were homogenised with 4 upward and downward strokes of an automated Teflon pestle, on ice. Homogenate was centrifuged in 50ml Falcon centrifuge tubes (Adcock-Ingram) at 1700g for 2 minutes (All centrifugations were performed at 4°C). Time taken from removal of the heart from the Langendorff system to centrifugation was less than 4 minutes (a crucial time point in order to obtain viable mitochondria). Following centrifugation, the supernatant was retained and spun at 900g for a further 5 minutes. The crude mitochondrial pellet obtained may still contain cell membrane, peroxisome and lysosome contamination. Crude mitochondrial pellets were resuspended in 2ml of wash buffer. In order to get a purified mitochondrial fraction, this suspension was then spun through a Percoll gradient. Percoll is an inert density gradient that uses differences in size and buoyant density to fractionate cellular organelles (154). It is the gold standard of mitochondrial isolation, and produces the most pure form of isolated mitochondria currently possible. 40ml of 26% Percoll gradient (appendix 2) was transferred into a 50ml ultracentrifuge tube, and the resuspended crude mitochondrial preparation was then layered on top of this and centrifuged at 40 000g for 30 minutes. This resulted in the formation of two distinct bands (Figure 21). The lower band, which contained purified mitochondria, was extracted using a Pasteur pipette and resuspended in 40ml of EGTA-free buffer (appendix 2). Samples were then centrifuged at 10 000g for 10 minutes to wash out any remaining Percoll, as this would otherwise interfere with analysis of the respiration parameters. The pellet formed was removed with care as it contained the isolated mitochondrial fraction. This fraction was used for Clarke-electrode (oxygraph) analysis.

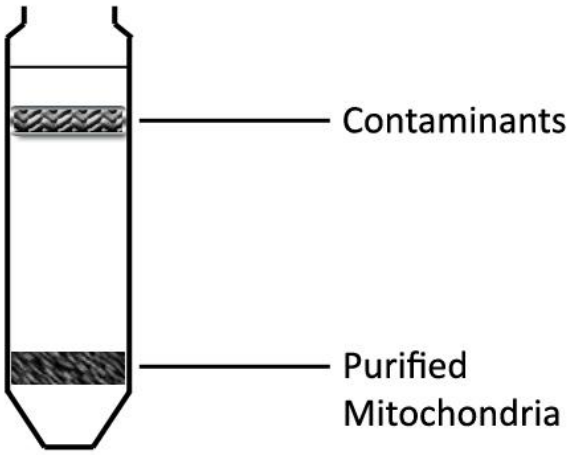


**Figure 21: Percoll mitochondrial isolation. Percoll ultracentrifugation fractionates mitochondria from membranous contaminants.**

## 3.2 BUIRET TEST FOR PROTEIN CONCENTRATION

In order to ensure that equal concentrations of mitochondria were added to the Clarke-electrode chamber, protein content was measured in each sample (as an indirect indicator of mitochondrial concentration) using the Buiret test (155). Briefly, Buiret reagent (appendix 3) was added to standard bovine serum albumin protein samples (0.63-10mg/ml) in a ratio of 1 sample:9 Buiret reagent and incubated at room temperature for 20 minutes. Samples were then read in a spectrophotometer at 540nm and a concentration-absorbance graph was plotted (Figure 22). Mitochondrial samples were assayed for protein content using the Buiret test, as described above. Absorbance was read at 540nm and corresponding protein concentration values were inferred from the trend line (Figure 22).

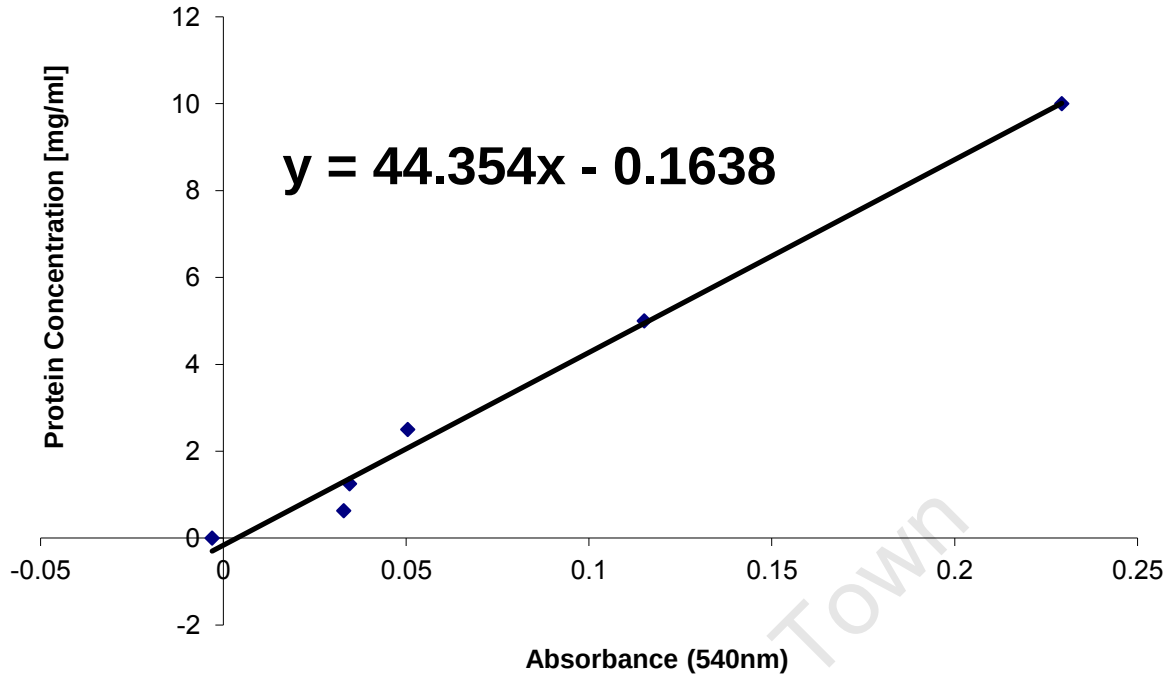


**Figure 22: Buiret concentration-absorbance plot. Standards of bovine serum albumin were plotted. A trend line was calculated, and the resulting equation was used for determination of mitochondrial protein concentration.**



## 3.3 MITOCHONDRIAL RESPIRATORY ANALYSIS

The Clarke-electrode was set up as depicted in Figure 23: A drop of saturated potassium chloride solution (appendix 4) was placed on top of the electrode [1], a roughly 2cm$^2$ square of cigarette paper, followed by equal sized membrane film, were layered on top of this [2] and sealed with a rubber O-ring. The outer O-ring was fitted and the space in between coated with saturated potassium chloride solution [3]. The electrode was then fitted to the chamber [4] and a magnetic stirrer bar added, stirring at a rate of 50 revolutions per minute (rpm). To calibrate the Clarke-electrode, sodium theothrioxide was added to approximately 400µl of potassium buffer (appendix 4) in the chamber in order to deplete the oxygen. The machine was zeroed and the chamber thoroughly washed with distilled water before use.

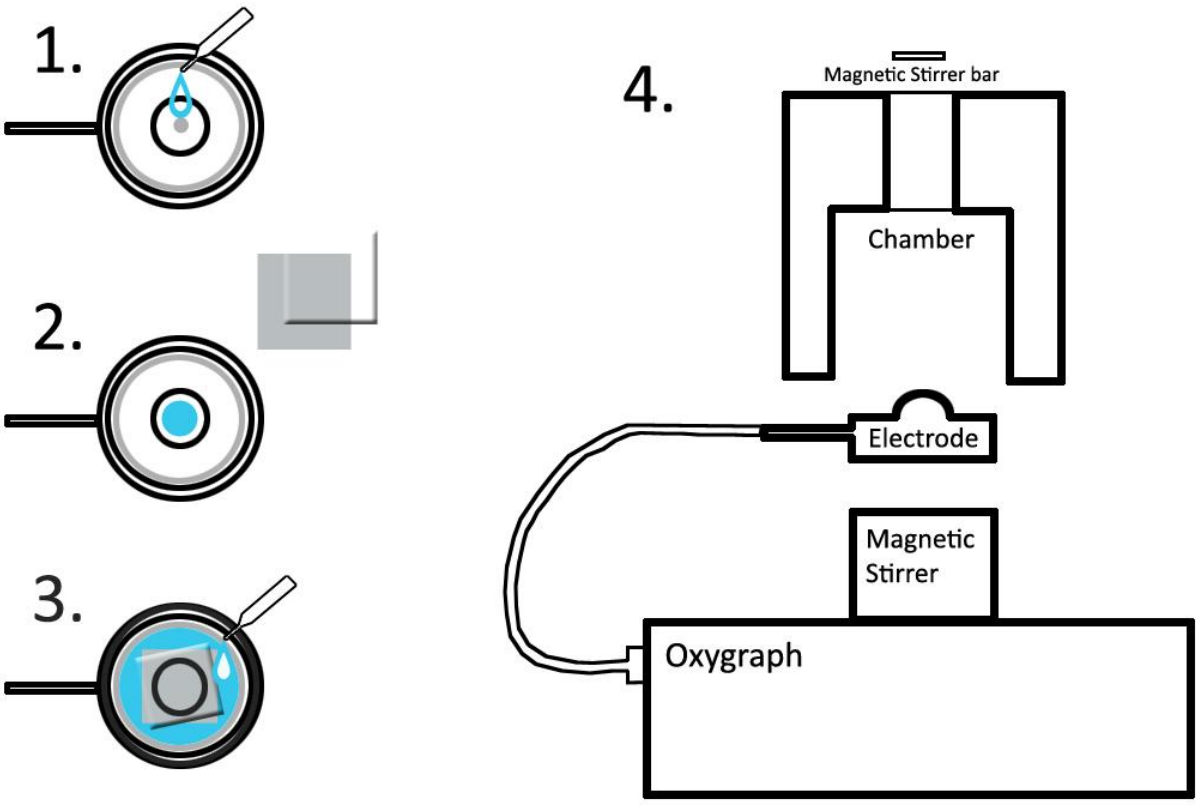


**Figure 23: Clarke-electrode setup. The electrode is assembled as detailed in pictures 1, 2 and 3. The electrode is then connected to the oxygraph and housed within the chamber.**

Mitochondria were evaluated for oxygen consumption as previously described (156). Briefly, mitochondrial samples were diluted to a protein concentration of 0.5mg/ml in potassium buffer, and allowed to respire in the chamber at basal levels. In order to stimulate state 2 respiration, a final concentration of 15nM glutamate was added to the chamber (Figure 24). Glutamate stimulates complexes I, III and IV, but not II of the ETC (157) and modest oxygen consumption rates are an indication of mitochondrial integrity and health. State 2 respiration was allowed to continue for 2 minutes. Thereafter, a final concentration of 0.5mM of ADP was added to stimulate the ETC and initiate state 3 respiration (158). This was allowed to continue until complete depletion of ADP and transition into state 4 respiration occurs.

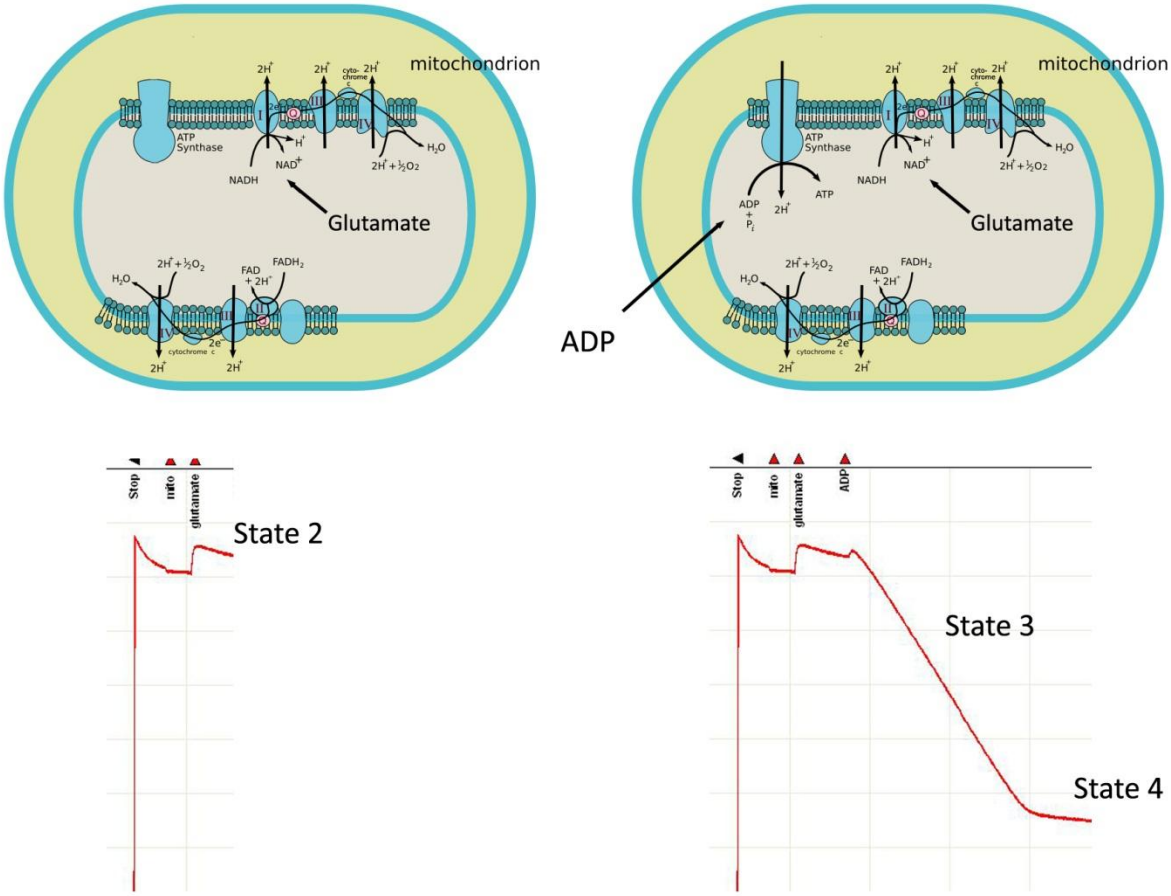


**Figure 24: Mitochondrial respiration in the Clarke-electrode apparatus. Trace line represents oxygen levels within the chamber, and is thus an indirect measurement of oxygen consumption by the mitochondria.**

Respiratory Control Indices (RCI) were measured by calculating the ratio of state 3 respiration to state 4 respiration (see Figure 24). RCI values give a good indication of ETC coupling and mitochondrial integrity. Any mitochondrial preparation with an RCI value of less than 2 was excluded from the study (159).

# 4: ISOLATED MOUSE CARDIOMYOCYTE MODEL

## 4.1 ISOLATED CARDIOMYOCYTES

12-14 week old male c57black6 mice were heparinised with 250 IU heparin intraperitoneally (160). After 30 minutes, the mice were anaesthetised via an intraperitoneal injection of 60mg/kg body weight sodium pentobarbital. Once anaesthesia was confirmed via the pedoreflex and changes in respiratory parameters (similar to those of the rats mentioned previously), the **chest cavity** was opened, the heart excised and mounted via the aorta on a

blunted 23 gauge cannula in less than 4 minutes. The apparatus used to mount the mouse heart was similar to the Langendorff apparatus (Figure 18) with the following exceptions: Flow rate was kept constant at 3ml/minute via a pump, functional parameters were not measured and hence no balloon was inserted into the ventricle and two independent chambers on the apparatus were used to separately house the perfusion buffer and the collagenase buffer. Hearts were perfused with oxygenated perfusion buffer (appendix 5) for 1 minute, followed by perfusion with collagenase buffer (appendix 5) for a further 11 minutes. Thereafter, collagenase effluent was collected for 2 minutes in a weigh boat. The heart was cut below the cannula and minced finely in the weigh boat with a pair of surgical scissors. This suspension was then transferred into a 50ml Falcon tube, attached to a steady supply of oxygen gas and shaken in an incubator at 180rpm for 10 minutes (at 37°C). Supernatant was then collected in a 15ml Falcon centrifuge tube. 10ml of fresh collagenase buffer was added to the remaining pellet and incubated for a further 8 minutes on the incubator/oxygen apparatus. Collected supernatant was neutralised with 250µl of foetal bovine serum in the interim. After 8 minutes of incubation the supernatant was pooled with the supernatant that had already been collected, bringing it to a total volume of roughly 14ml. Cardiomyocytes were pelleted via centrifugation at 600 rpm for 3 minutes in a Harrier MSE 18/80 centrifuge at room temperature. Supernatant was replaced with 10ml perfusion buffer 2 (appendix 5) and the pellet was resuspended with 2 or 3 vigorous taps to the base of the tube. Calcium was then reintroduced via the stepwise manner detailed in appendix 5 to prevent calcium shock, over a period of 20 minutes. Cells were left to settle via gravity sedimentation. Gravity sedimentation allows for particles in suspension to pellet via the force of gravity. Larger particles will travel faster down the tube through the solvent, whilst smaller particles will remain in suspension longer, due to the viscosity of the medium (provided by the bovine serum albumin). Because viable cardiomyocytes are much larger than dead cardiomyocytes, they will pellet prior to the dead cardiomyocytes. It was found that 10 minutes of gravity sedimentation resulted in a high yield of viable cells with optimal exclusion of nonviable cells. Following sedimentation, the supernatant was carefully removed and the pellet was gently resuspended in medium 199 (Appendix 5). Cells were plated onto 30mm 6 well plates precoated with 80µl of laminin (Appendix 5) per well. One hour of incubation was then allowed for adherence of viable cells to the wells. Wells were then gently washed twice with 1ml of fresh medium 199. At this point cells were evaluated under the light microscope for suitable phenotype and quantification. If found satisfactory (approximately >90% viable (Figure 25), and at least 10 000 cells per well), cardiomyocytes were then used for experimental procedures.

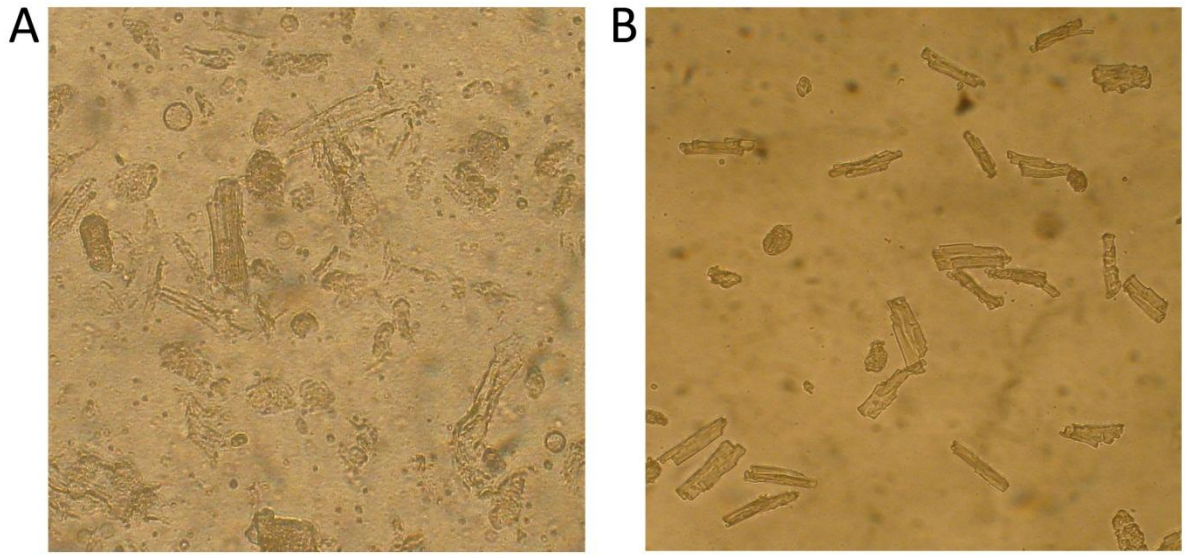


**Figure 25: Isolated adult mouse cardiomyocytes. Figure A displays initial crude isolation, and figure B shows cardiomyocytes which have been allowed to adhere to laminin coated wells.**



## 4.2 EXPERIMENTAL PROTOCOLS

For cell viability and hypoxia experiments, cardiomyocytes were exposed to 10nM S1P or 300mg/ml HDL cholesterol for 30 minutes, followed by a 30 minute washout period (Figure 26). This protocol was adapted from previous work performed in our laboratory or elsewhere on cell culture models (161). HDL cholesterol was kindly donated by Dr.Miguel Frias and Prof. Richard James (Division of Endocrinology, Diabetology and Nutrition, University Hospital, 24, rue Micheli-du-Crest, CH-1211 Geneva 14, Switzerland). Positive and negative controls were also included: CyclosporinA (CsA, 200nM) was administered as a powerful inhibitor of mPTP opening (162). Carbonyl Cyanide m-Chloro Phenyl hydrazone (CCCP) (10μM) on the other hand, rapidly opens the mPTP and was added at the beginning of reperfusion due to its potent cytotoxic effects (163). Normoxic and hypoxic controls were also included as baseline references. Following treatment as described above, medium 199 was removed from plated cardiomyocytes, washed once with either Simulated Ischaemia (SI) buffer (appendix 6) or medium 199 and then replaced with 1ml SI Buffer or medium 199. Cardiomyocytes were exposed to 2 hours total hypoxia, a time course derived from the literature, with a degree of optimisation (164),(165),(166) using a Gentronics $CO_2$ and $O_2$ Gas Controller (a sealed, temperature and gas controlled incubator). $O_2$ levels were maintained at 1%, with upper and lower limits of 0.5% and 1.5%. $CO_2$ levels were similarly maintained at 5% with upper and lower limits of 6% and 4%, respectively. Temperature was kept constant at 37°C.

For western blotting analysis, proteins were extracted after 7 minutes of drug treatment, as detailed in section 6.1 of the Methods chapter.

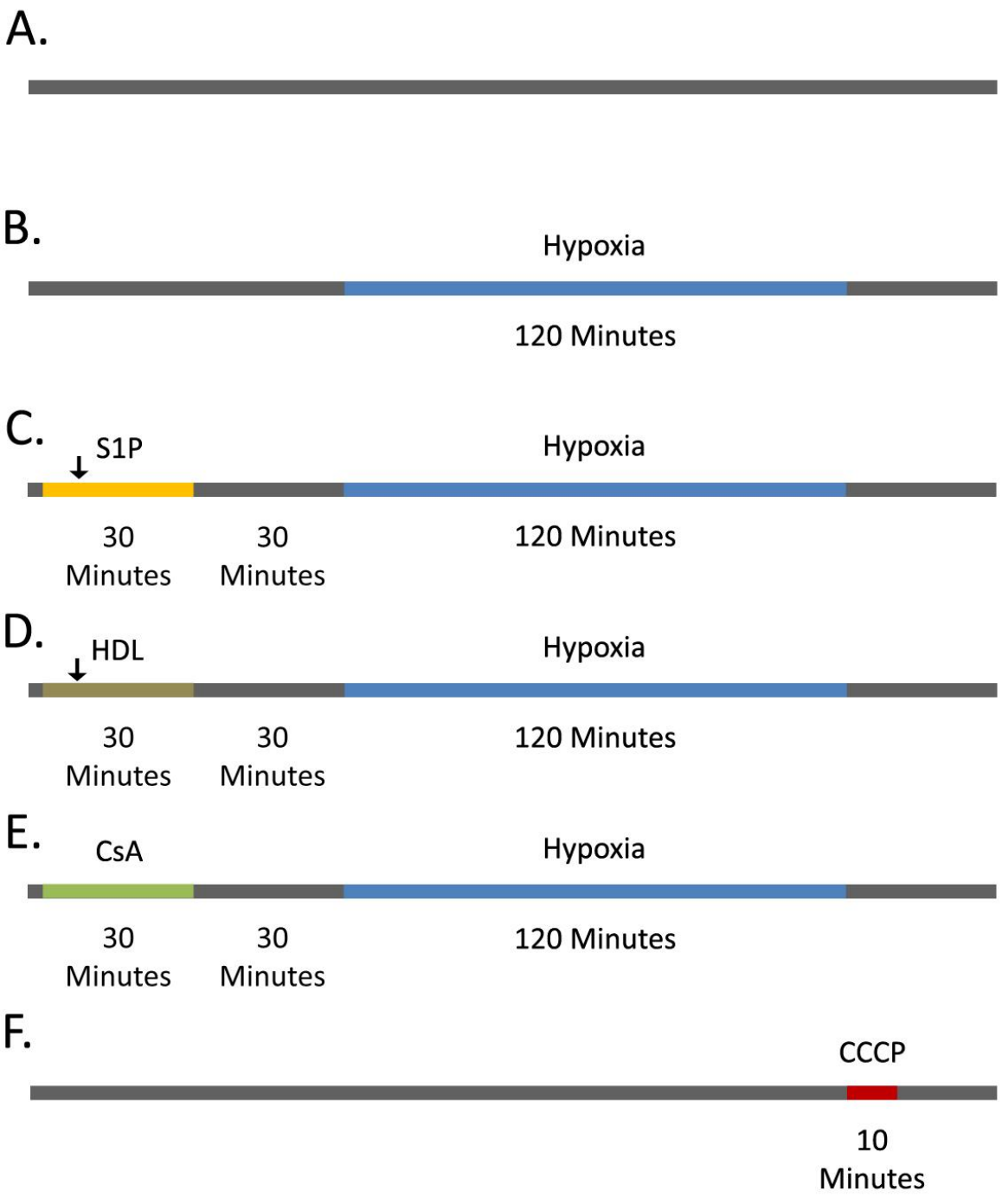


**Figure 26: Experimental protocols for isolated mouse cardiomyocytes. Arrows indicate time of mitochondrial protein isolation for western blot analysis (7 minutes since start of treatment). CsA = Cyclosporin A. CCCP= Carbonyl Cyanide m-Chloro Phenyl hydrazine.**

## 4.3 TRYPAN BLUE STAINING

Directly after hypoxia, cells were reoxygenated for 20 minutes with no change in medium. Cells were then loaded with 0.04% Trypan Blue (100µl of 0.4% Trypan Blue solution was added directly to the medium) and cell viability was immediately analysed using a light

microscope at 40x magnification. Trypan Blue is impermeable to cell membranes, and thus may only enter the cell if membrane integrity has been compromised (167). Thus dead cells (both apoptotic and necrotic) stain blue, whereas live cells remain unstained. Cell death could also be ascertained via morphological changes. Dead cells were round, while viable cells remained rod shaped. Trypan Blue was added directly to the well, and 4 randomly selected focal points, or a minimum of 100 cells each, were analysed.

# 5: ANALYSIS OF MPTP OPENING

mPTP opening was measured in isolated mouse cardiomyocytes subjected to hypoxia and pretreated with or without S1P or HDL cholesterol. mPTP opening was measured using the dye TetraMethylRhodamine Methylester (TMRM) (Invitrogen). TMRM is a lipophilic cation that accumulates in the mitochondrial inner membrane and matrix due to its charge and relative solubility in these regions (168). Importantly, TMRM has a low binding coefficient to proteins and hence will only enter mitochondria relative to their membrane potential, and will leave just as freely if the membrane depolarises. TMRM fluorescence was measured using both fluorescent microscopy methods and flow cytometry methods.

## 5.1. EXPERIMENTAL PROTOCOLS

Following hypoxia, cells were removed from the hypoxic chamber, and all cells were incubated with TMRM for 10 minutes (169). After incubation with TMRM, medium was replaced with fresh medium 199 for all cardiomyocytes and incubated for a further 10 minutes. For fluorescent microscopy, four focal points, or at least 100 cells, were immediately photographed. For flow cytometry, cardiomyocytes were lifted from the wells using a cell scraper, and transferred into a 10ml Falcon tube for flow cytometry analysis. 10 000 events were counted. Some researchers argue strongly for the treatment of cardiomyocytes with digitonin in order to allow for entry of TMRM into the cells (170), however this is not necessary, as evidenced by the obvious incorporation of TMRM into the cells when analysed using a fluorescent microscope (Figure 38). There were also concerns that digitonin treatment may adversely affect the membrane potential of the mitochondria, and hence it was omitted. Trypsin was not used to lift the cells from the wells because of similar fears, and so the cell scraping method was used instead.

## 5.2 FLUORESCENT MICROSCOPY

After 10 minutes of incubation in medium 199, cardiomyocytes were photographed using a green lit fluorescent microscope (Camera SPOT RT Colour 2.2.1. Images were taken using a Nikon Eclipse TE200 camera and captured with Spot Advanced imaging software). Fluorescent levels were then analysed using planimetric methods using ImageJ software. Because absolute fluorescent levels vary depending on cell number, microscopy focus, age of TMRM and various other factors, data was normalised to the fluorescent levels of the

normoxic cells analysed on the day of the experiment. The nature of TMRM is such that fluorescence levels are unlikely to ever dip below 50% of their baseline because the dye accumulates in the mitochondria based on proton gradients. With complete mPTP opening, this gradient is lost and the dye will passively diffuse until it reaches equilibrium with the cytosol and extracellular fluid. In addition to this, dead cells fluoresce at much higher levels than healthy cells (Figure 27) and thus are excluded from analysis by their morphological features.

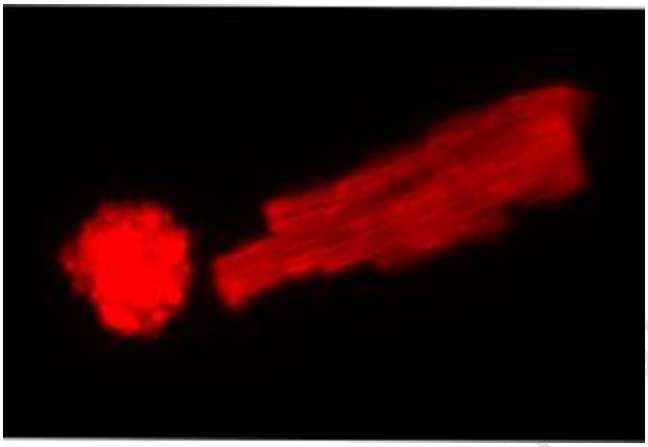

**Figure 27: TMRM Fluorescence of isolated mouse cardiomyocytes. A dead cell at left, clearly identifiable by its round morphology, fluoresces at much higher levels than a healthy cell at right.**



## 5.3 FLOW CYTOMETRY

Because The Hatter Cardiovascular Research Institute at the University of Cape Town does not have a fluorescent microscope, and the preliminary fluorescent microscopy studies were performed at The Hatter Institute at University College London, a subsection of this thesis consisted of set up of a flow cytometry method of TMRM measurement. Analysis of cardiomyocytes with a flow cytometer is well established (171), but was never used following a hypoxic stress which represented a unique set of challenges. Similarly, mPTP opening analysis has been performed using a flow cytometer in fibroblasts (172), but has not been applied in isolated cardiomyocytes. As such, this is was a novel technique that has not been detailed in the literature. The advantages of using a flow cytometer are that it allows for analysis of a larger numbers of cells, strengthening the statistical confidence of the results. It also objectively analyses the cells, unlike the more traditional methods of confocal or fluorescent microscopy analysis of mPTP opening in cardiomyocytes (173). It is however an expensive method as compared to fluorescent microscopy, but of equitable expense as compared to confocal microscopy. As such, significant calibration and optimisation of this methodology was required. Once the settings for the flow cytometer had been optimised, they remained constant throughout all experiments.

### 5.3.1 Calibration and Optimisation

The flow cytometer uses a laser to analyse individual cell characteristics. It can measure three aspects of the cell: Cell size (forward scatter), cell granularity (side scatter), and fluorescence intensity (FL-2 channel). Fluorescence can be a measurement of innate

autofluorescence of the cell, or it can measure antibody-linked or dye treated fluorescence. In this case, the dye TMRM was used as a measurement of mPTP opening. TMRM's absorption and emission spectra are detailed in Figure 28. Based on its absorption profile, an excitation laser of 488nm was used to stimulate the dye (which is the standard laser fitted to a FacsCalibur flow cytometer) (174).

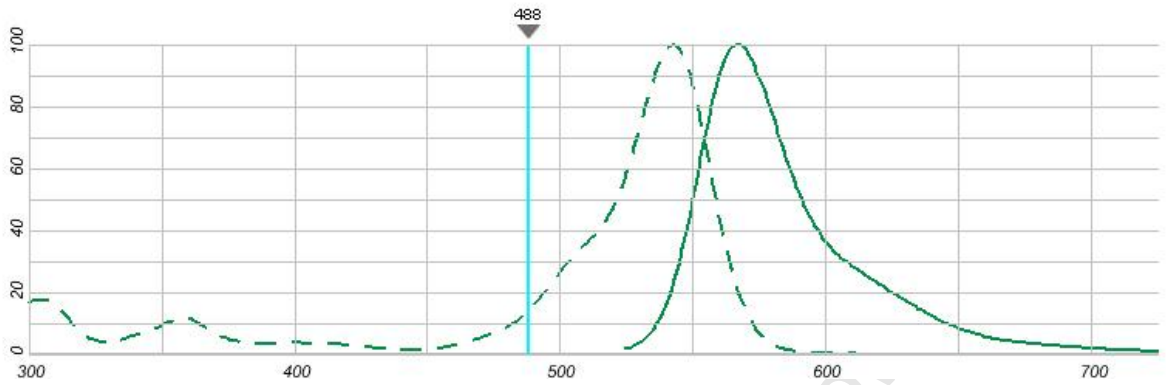


**Figure 28: Absorption/emission spectra of TMRM. Absorption is conveyed by the dotted green line, while the emission spectrum is portrayed by the solid green line. The blue line depicts a 488nm stimulus, equivalent to the 488nm laser equipped on the flow cytometer** (175).

The flow cytometer allows for modification of amplification of forward scatter (FSC), side scatter (SSC) and the FL-2 channel in order to accommodate variations in size and granularity between different cell types, and fluorescent intensities of different dyes. As a flow cytometry model of TMRM fluorescence in isolated mouse cardiomyocytes is a novel model, significant optimisation of all these settings was required (176) and the optimal TMRM dye concentration needed to be established (177).

In order to optimise the flow cytometer, during the gravity sedimentation component of cardiomyocyte isolation, the pellet, which contained majority rod shaped live cells was immediately analysed with no fluorescent dye treatment as a control of viable cells. It also allowed for the detection of any autofluorescence that may have been in play. Concurrently, the supernatant from the gravity sedimentation was reserved and spun at 1000g for 3 minutes. This resulted in a pellet of majority rod shaped, dead cells. These were also analysed on the flow cytometer as a control for dead cells. Furthermore, settings were optimised so as to prevent as many events as possible from being read off scale, as gating of populations following acquisition can be done at any stage. However, if the samples are recorded off scale, this cannot be remedied post-acquisition.

Fluorescent microscopy techniques use 200nM of TMRM, however flow cytometry techniques use a lower concentration of 20nM, most likely due to the intensity of the laser excitation. Initially a concentration of 200nM TMRM was used so as to deviate as little as possible from the fluorescent microscopy technique, however this proved to be unfeasible and resulted in too strong a signal for the FL-2 channel to detect. As such the TMRM concentration was reduced to 20nM and the FL-2 channel adjusted. The final amplification and detection settings for the flow cytometer are detailed in Table 5,

| Parameter | Setting | |
|---|---|---|
| | V | Amp |
| FSC | E-1 | 3.00 |
| SSC | 271 | 1.00 |
| FL-2 | 450 | 1.00 |

**Table 5: Flow cytometry settings for mPTP opening analysis. FSC= Forward Scatter, SSC=Side Scatter. Derived from (177), (178), (179) and (174) with significant optimisation. V = Voltage, Amp=Amplifiers**

### 5.3.2 Gating

The flow cytometer records all fragments that pass through its laser as events, and it cannot distinguish between viable cells, cellular detritus and other contaminants. As such, it is necessary to exclude these components from the analysis via gating of populations. Figure 29 shows typical TMRM fluorescence histograms for normoxic and hypoxic cells, with their accompanying FSC and SSC scatter plots.

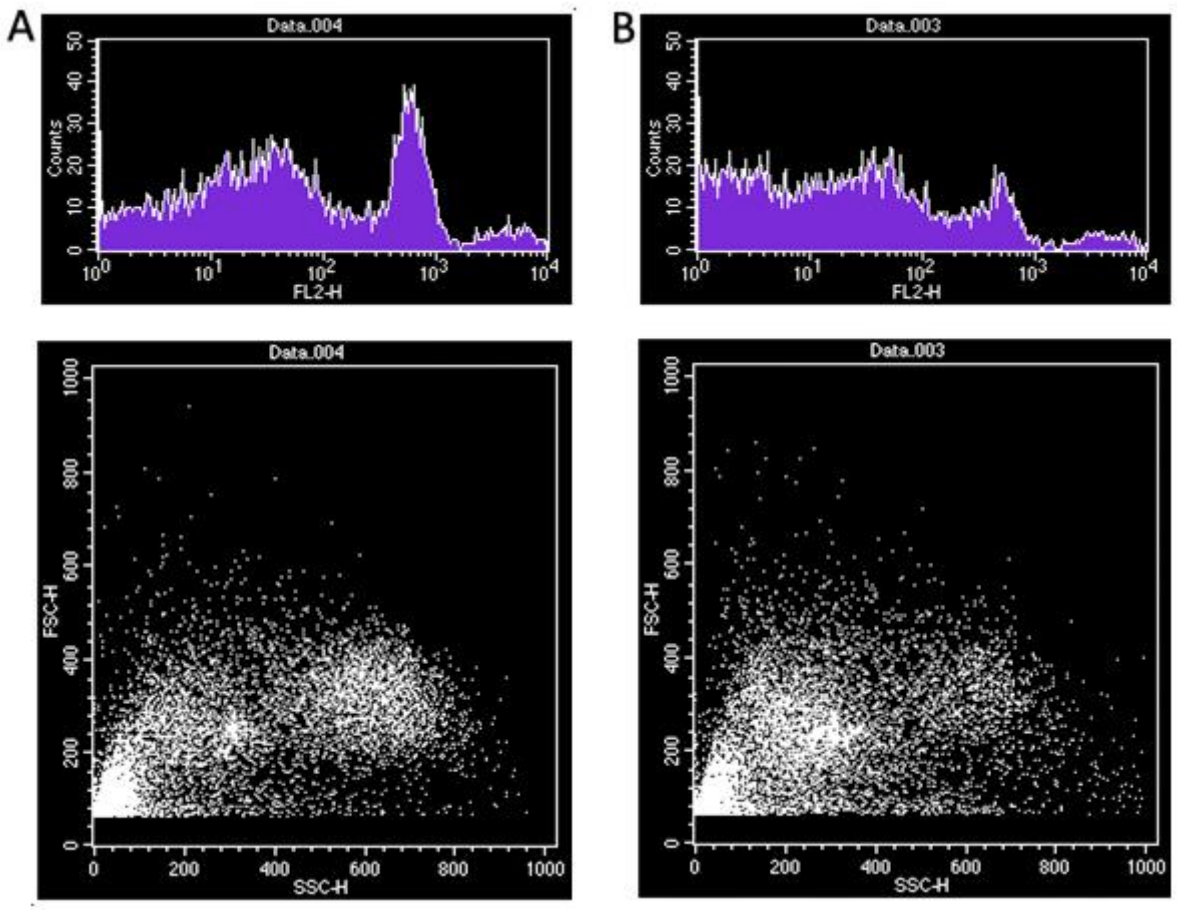


**Figure 29: FL-2 fluorescent histograms and FSC and SSC plots. A. Normoxic cells B. Hypoxic cells. FSC = Forward scatter, SSC = Side scatter**

Two clear populations are visible in the FSC and SSC plots, as well as a third lower population. The FL-2 histograms also show a clear decrease in fluorescence in the hypoxic group. When cells with virtually no TMRM fluorescence (0-10 units) are gated, they occupy the left population of the FSC and SSC plots (Figure 30), whereas the higher fluorescing cells occupy the right population (gating not shown). Due to the variability in size of individual cardiomyocytes FSC is not a good indication of cell viability (180), whereas SSC does remain a good indication. Furthermore, cellular detritus and other contaminants will have a very low FSC and SSC value and no fluorescence, and are evidenced by the third population found in the very lower left hand corner of the scatter plot (which is also retained when gated for low fluorescence). This is further supported by anecdotal evidence, where these low fluorescent events only occur right at the end of a run when sucking up contaminants floating on the surface of the sample in the test tube, if at all. As such, gating was performed to exclude all events with a FL-2 fluorescence of less than 10 units (Figure 30).

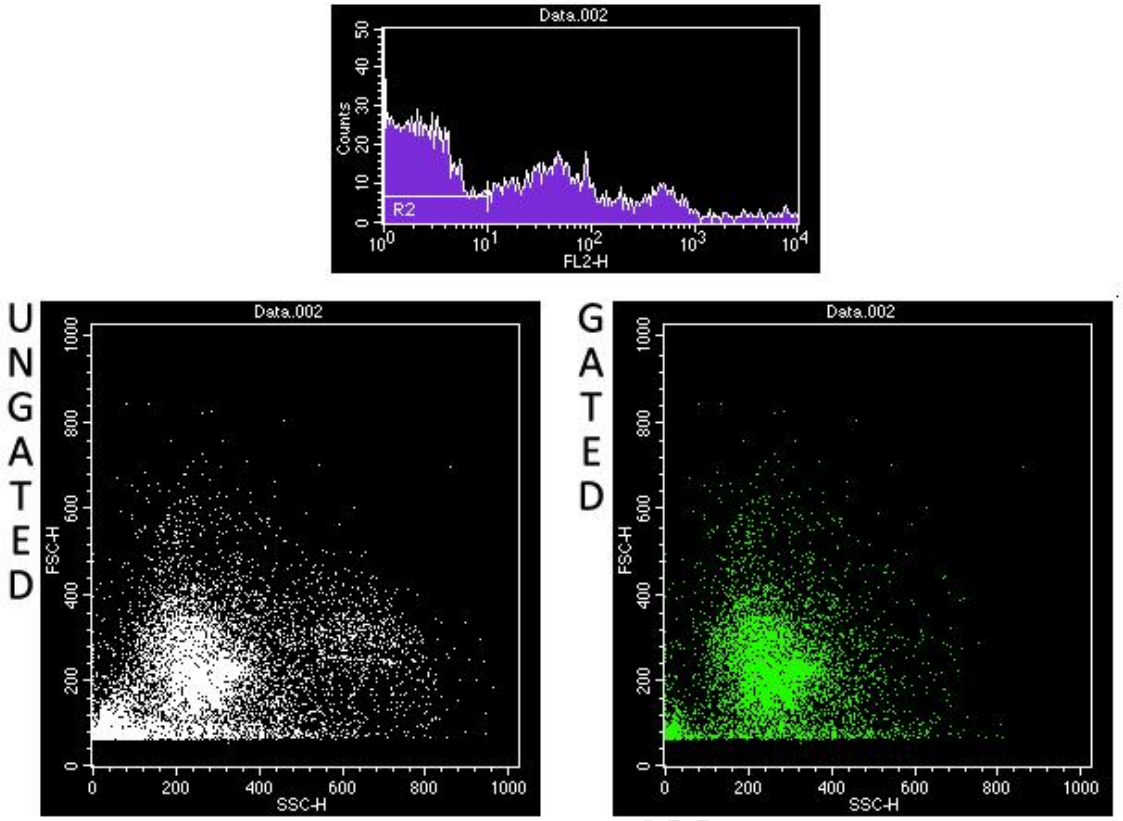


**Figure 30: Gated population (R2) of events registering little to no fluorescence.**

# 6: WESTERN BLOTTING

Western blot analysis was used to quantify mitochondrial protein levels, specifically total STAT-3 and its phosphorylated isoform at Serine 727 (pSTATSer), following HDL cholesterol or S1P treatment. All samples were also probed for Voltage Dependent Anion Channel 1 (VDAC), as a normalisation molecule to control for protein concentration and loading differences.

## 6.1 PROTEIN PREPARATION

Cardiomyocytes were isolated as previously described (section 4.1). Once isolated, the cardiomyocytes were resuspended in phosphate buffered saline (PBS), and treated with either 10nM S1P or 300mg/ml HDL cholesterol for 7 minutes at 37°C. Thereafter, a mitochondrial isolation kit (Sigma) for profiling cultured cells was used to isolate mitochondria. The procedure entailed homogenization with 10 strokes of a glass dounce homogenizer, incubation in supplied buffers at 4°C and various centrifugations as per the manufacturer's instructions.

In order to ensure that equal amounts of protein (60µg) were loaded into each well, a Buiret protein concentration test was performed as detailed in section 3.2. Corrected concentrations of isolated mitochondrial fractions were then added to a proportionate

amount of loading buffer (see Appendix 7) to a final volume of 20µl. Unlike typical western blotting protocols, mitochondrial fractions were not boiled, as this can result in dimerisation of the proteins (181).

## 6.2 GEL ELECTROPHORESIS

Gel electrophoresis 1.5mm glass plates were cleaned with 70% ethanol before being set up as per manufacturer's instructions (Biorad) (Figure 31). The system was checked with water for a watertight seal prior to pouring of the gel. Separating gel (see Appendix 7) was then added in-between the plates such that it ended approximately 1cm below the loading wells (as predetermined using the well-forming combs). A thin film of 70% ethanol was layered on the top to ensure a level line, remove potential air bubbles and to encourage setting of the gel by isolating it from atmospheric oxygen. Once the separating gel set, the ethanol was removed with blotting paper and the stacking gel (see Appendix 7) was added to the brim of the chamber. Any bubbles formed on the top were removed, using a needle and syringe, before fitting the 10 well combs and allowing the gel to set.

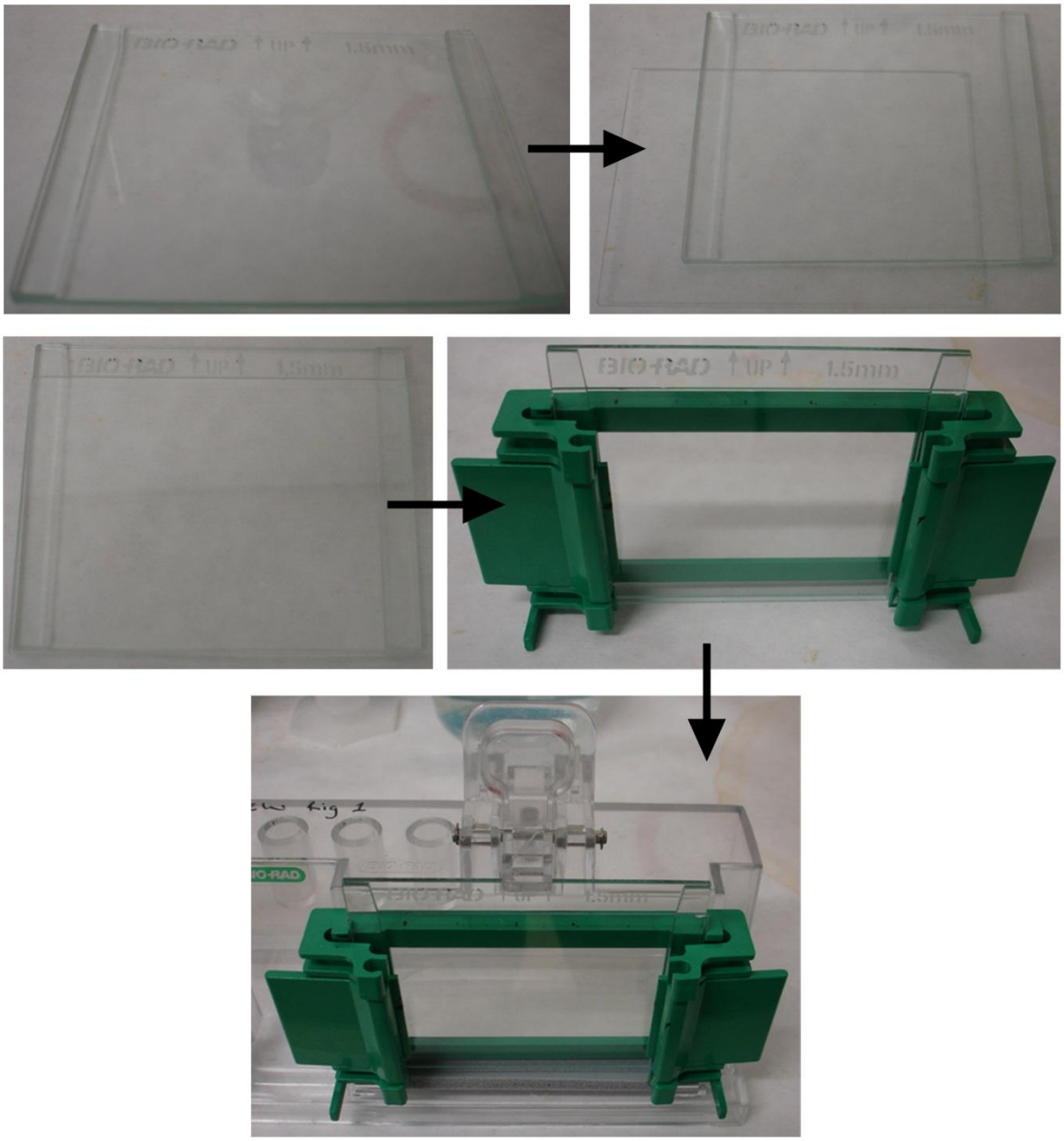


**Figure 31: Setup of Gel electrophoresis plates. A 15mm backing plate is covered with a glass front plate and sealed into place with a green clamp. This is then clamped onto a docking station to ensure the plates remain level and secure. A grey sponge base ensures water tightness of the system.**

Once the stacking gel was set, the combs were carefully removed and the gel plates were loaded into the running apparatus. The running apparatus was transferred into a running chamber. The chamber was flooded with 1x running buffer, prepared from a 10x stock solution (appendix 7). Each individual well was washed out using a syringe and fine needle prior to protein loading to remove any gel debris inside the well. Protein preparations were then loaded. 4µl of a molecular marker (PeqGold Prestained Protein Marker IV from PeqLab) was also included as a point of reference. The gel was run for 2 hours at 120 volts at 4°C.

## 6.3 MEMBRANE TRANSFER

Amersham Hybond-P membranes (GE Healthcare) were washed for 5 minutes in methanol, followed by 2 minutes in distilled water, and finally 15 minutes in transfer buffer (appendix 7). The gel was removed from the glass plates and prepared via the removal of the stacking gel. Immunoblot transfer apparatus was then set up as detailed below (Figure 32). This apparatus was immersed in transfer buffer during the set up process, ensuring any air bubbles between the gel and membrane were excluded. The immunoblot transfer apparatus was then inserted into a transfer chamber, along with an ice pack to prevent overheating during the transfer process. This was filled with transfer buffer and allowed to run at 0.02A overnight in a 4°C fridge.

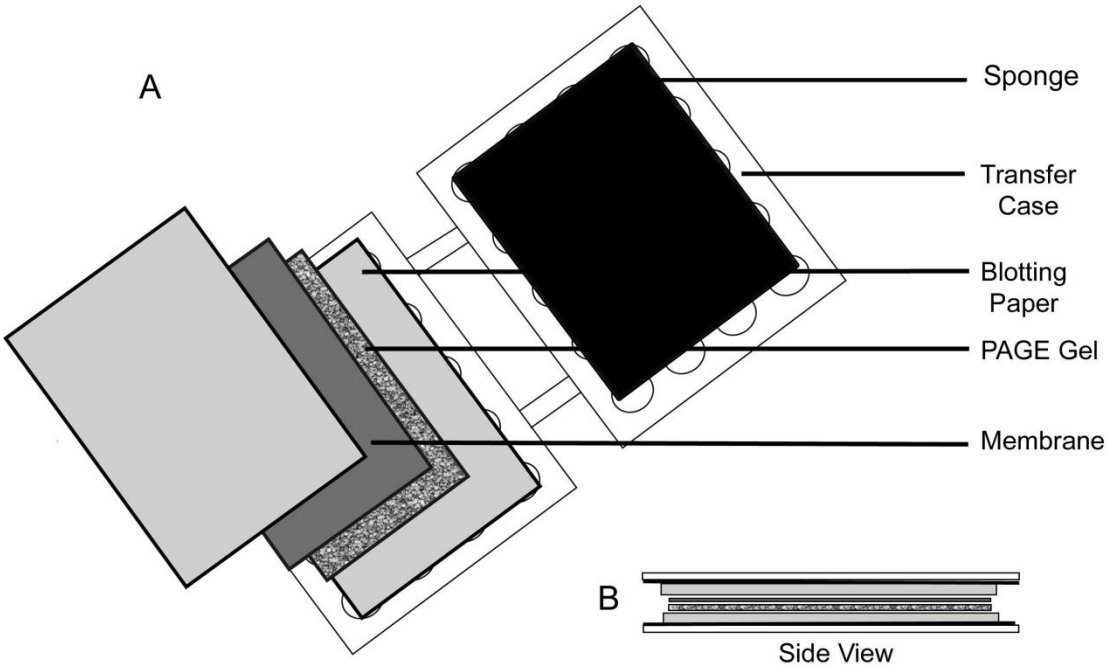


**Figure 32: A: Set up of transfer system for western blot analysis. It is important that the anode facing part of the transfer case (usually black) is at the bottom as the proteins migrate from the anode (-) to the cathode (+) lifting off the gel and transiently passing onto the membrane. The proteins adhere to the membrane and lose their charge, which stops the transfer process. B: Side view of set up of Transfer system. PAGE = Polyacrylamide Gel Electrophoresis**

## 6.4 PONCEAU STAINING

After transfer, the membrane was washed in methanol for 30 seconds, and allowed to dry completely. Membranes were then washed with Ponceau stain briefly, rinsed with water, sandwiched between two transparencies and digitally scanned (Figure 33). These scans were captured to confirm the presence of protein and equal protein loading amongst the samples. Ponceau stain was removed by washing three times in TBS-T (appendix 7), each for 5 minutes.

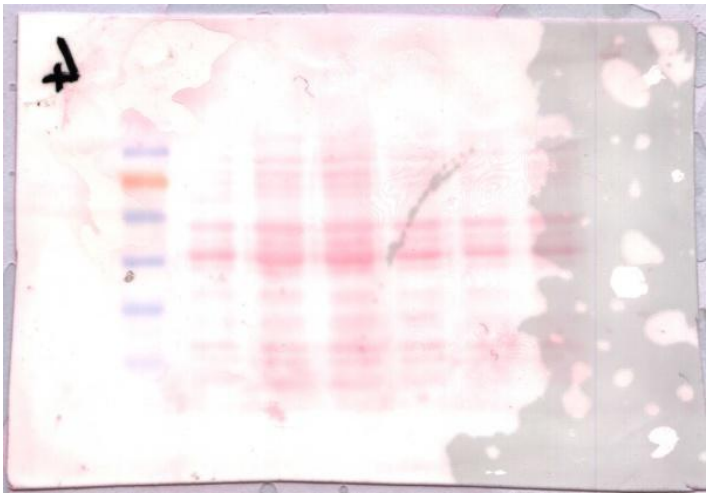

Figure 33: Ponceau staining of the membrane. All proteins stain light pink.



## 6.5 IMMUNOBLOTTING

Membranes were blocked with a 5% milk solution (appendix 7) for 2 hours to prevent non-specific binding of antibodies to the membrane. They were then washed three times with TBS-T, each for 5 minutes. Thereafter they were exposed to one of 2 primary antibodies: tSTAT or pSTATSer (appendix 7) overnight at 4°C on a rotating stand. Another washing step of 3 washes in TBS-T for 5 minutes each was performed, before being exposed to the secondary antibody (appendix 7) for 1 hour on a shaking tray. A final washing step of 3 washes in TBS-T for 5 minutes was performed before membranes were developed.

## 6.6 DEVELOPING

Membranes were incubated simultaneously in 2mls each of ECL developing reagents 1 and 2 (GE Healthcare) for 2 minutes. Membranes were then analyzed using the GENE Genome image capturing system (Syngene). A light image was also captured for the molecular weight marker, as it was not luminescent. A composite image was created in order to identify the correct bands for analysis.

## 6.7 STRIPPING

Following development, all membranes were washed twice for 5 minutes in $dH_2O$. Membranes were then stripped with 0.2M NaOH for 5 minutes on a shaker tray. Finally, they were further washed twice for another 5 minutes each with $dH_2O$, before being processed for VDAC development, following the same protocol as listed in sections 6.5 and 6.6 above, with the exception of the blocking solution containing only 3% milk.

# 7: DRUGS & ANALYSIS

## 7.1 DRUGS

Unless stated in the text, all reagents were supplied by Sigma-Aldrich.

## 7.2 ANALYSIS SOFTWARE

For infarct size analysis, heart sections were placed between two glass plates and scanned into the computer. Images were then printed out and a stylus was used to trace the perimeter of the heart, the area at risk and the infarcted tissue. This data was then analysed using Planimetry+ software.
For flow cytometry analysis, Gating and fluorescence levels were analysed using FlowJo 7.6. Mean fluorescence intensity was calculated, and normalised to the normoxic levels.
For fluorescent microscopy and western blot analysis, densitometry was performed using ImageJ version 1.44p.

## 7.3 STATISTICAL ANALYSIS

All statistical analysis was performed using Graphad Instat v3.01. All data is expressed as mean ± the Standard Error of the Mean (SEM).
Statistical significance between 2 groups (infarct size analysis, functional recovery data and RCI values) was determined by an unpaired, two-tailed t-test.
Statistical significance between more than 2 groups was determined by a one way analysis of variance (ANOVA), followed by a Student Newman-Keuls post hoc test, for isolated cardiomyocytes (flow cytometry, cell survival data and western blotting).

A p value of less than 0.05 was considered significant.

# Chapter 4: Results

## 1: S1P PROTECTS AGAINST ISCHAEMIA/REPERFUSION INJURY IN THE ISOLATED RAT HEART

### 1.1 EFFECT OF S1P ON FUNCTIONAL PARAMETERS.

Preischemic functional parameters (Table 6) for both the ischemic controls (IC) and the S1P treated hearts met all the inclusion criteria outlined in section 2.1 of the methods chapter. Postischemic values of the IC group displayed a significantly diminished LVDP (86.0±7.0mmHg preischaemia vs. 46.0±8.0mmHg postichemia, $p<0.05$). Coronary flow rate was also significantly decreased from 10.8±1.4 ml/minute to 7.8±1.9 ml/minute ($p<0.05$). These findings were consistent with those described in the literature, validating our model of regional ischaemia-reperfusion injury (182),(183). Pretreatment with 10nM S1P rescued neither LVDP nor coronary flow rate. No significant decrease following ischaemia was observed in heart rate for either IC or S1P groups

| Time point | LVDP | | Coronary Flow Rate | | Heart Rate | |
|---|---|---|---|---|---|---|
| | IC | S1P | IC | S1P | IC | S1P |
| **Preischemic** | 86±7 | 83±5 | 10.8±1.4 | 9.7±0.9 | 287±18 | 276±20 |
| **Reperfusion 5 min** | 69±8 | 71±7 | 11.2±1.7 | 9.8±0.8 | 270±14 | 288±42 |
| **Reperfusion 30 min** | 61±6 | 61±8 | 9.8±1.8 | 7.0±0.8 | 287±11 | 260±38 |
| **Reperfusion 120 min** | 46±8 | 45±7 | 7.8±1.9 | 5.9±0.8 | 293±11 | 268±28 |

**Table 6: Summary of functional recovery data. No significant differences were found between the IC or S1P groups at any time point.**

### 1.2 EFFECT OF S1P ON INFARCT SIZE

Isolated rat hearts subjected to 30 minutes of regional ischaemia and 2 hours of reperfusion (IC) had an infarct size of 25.8±3.1% of the area at risk (Figure 34). Pretreatment with S1P significantly decreased infarct size to 4.6±1.4% of the area at risk ($p<0.05$ vs. IC). Our data confirmed previous data reported in the literature (184) on the cardioprotective effect of S1P. Of note, there was no significant difference between the areas at risk for the two groups.

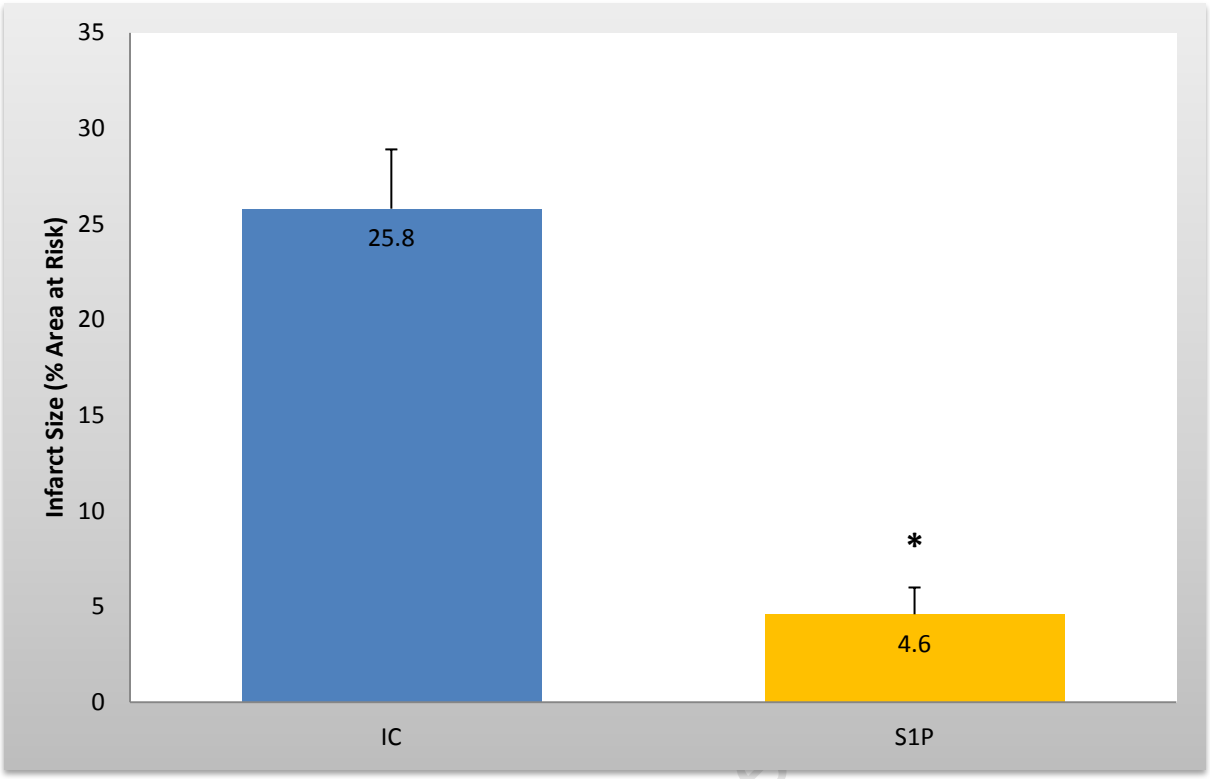


**Figure 34: Infarct size of isolated rat hearts subjected to regional ischaemia-reperfusion. Pretreatment with S1P (10nM) reduced infarct size from 25.8±3.1% to 4.6±1.4% of the area at risk. (*p<0.05 vs. IC, n=6 for all groups). IC = Ischemic Control.**



## 1.3 EFFECT OF S1P ON RESPIRATORY PARAMETERS

Untreated isolated rat heart mitochondria measured RCI values of 4.2±0.5 Arbitrary Units (AU), consistent with those cited for rat heart mitochondria in the literature (185),(159),(186) (Figure 35). Hearts treated with S1P prior to mitochondrial isolation, displayed significantly improved RCI values of 11.0±1.4 AU ($p<0.05$ vs. untreated).

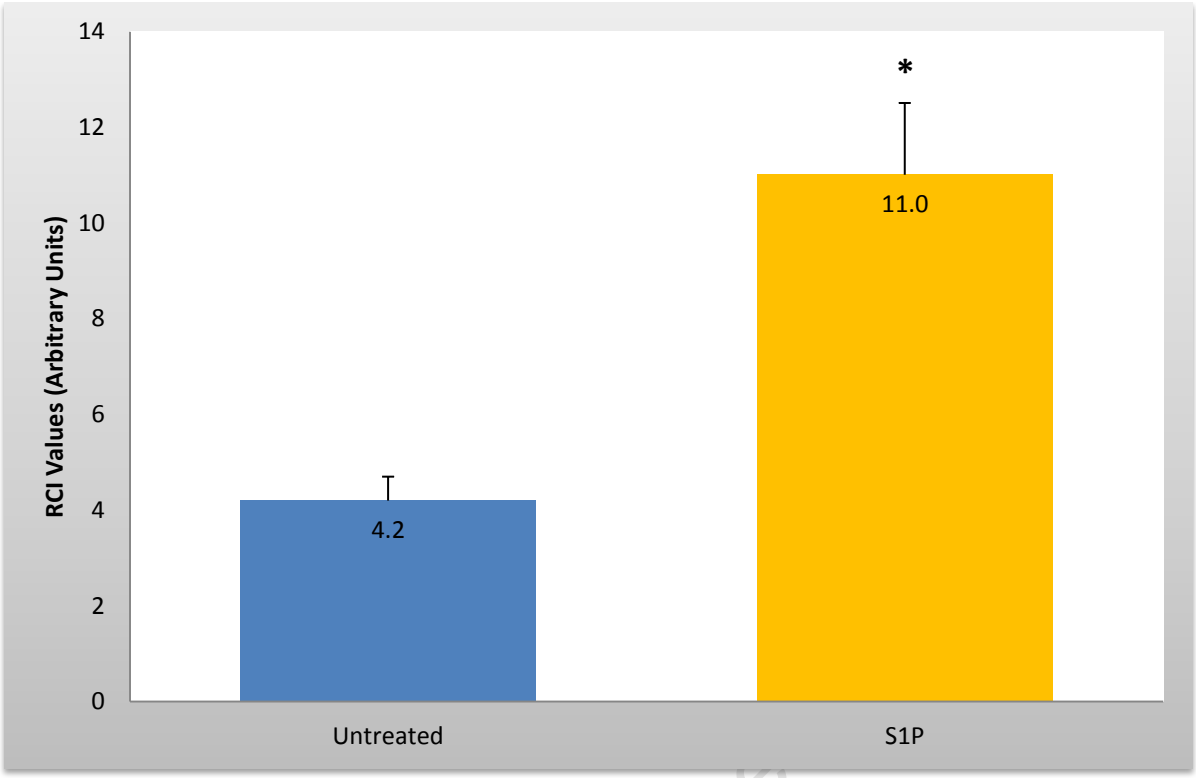


**Figure 35: Respiratory Control Index (RCI) values of isolated rat mitochondria. S1P (10nM) improves RCI values (*p<0.05 vs. Untreated, n=4 for all groups).**



# 2: S1P AND HDL CHOLESTEROL PROTECT AGAINST HYPOXIA IN ISOLATED WILDTYPE MOUSE CARDIOMYOCYTES

## 2.1 EFFECT OF S1P AND HDL CHOLESTEROL ON CELL VIABILITY

Normoxic cardiomyocytes had a **mean viability** of 84.4±1.6%, indicating that approximately 15% of cardiomyocytes did not survive the isolation and plating process (Figure 36). Exposure to 2 hours hypoxia reduced cardiomyocyte survival to 62.7±1.8% ($p<0.05$ vs. normoxia). S1P (10nM) pretreatment restored cell survival to 80.4±1.4% ($p<0.05$ vs. hypoxia).

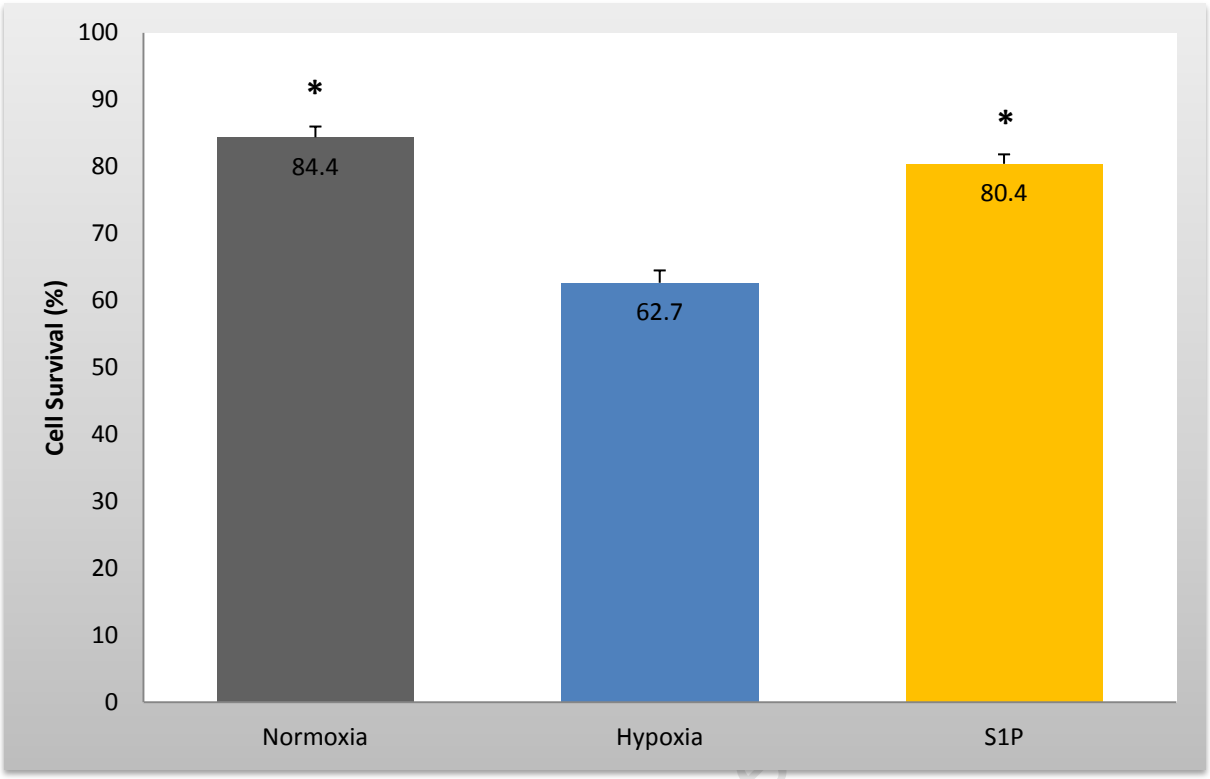


**Figure 36: Effect of S1P on cell viability in isolated wildtype cardiomyocytes subjected to 2 hours of hypoxia. S1P (10nM) improves cell survival. (*p<0.05 vs. Hypoxia, n=4 for all groups).**

Similarly for the HDL cholesterol experiments, untreated cardiomyocytes had a mean survival of 84.3±1.5% (indicating roughly 15% of cardiomyocytes did not survive the isolation and plating process) (Figure 37). Exposure to 2 hours of hypoxia significantly reduced cell viability to 59.6±2.5%. Administration of 10μM CCCP to normoxic cells (a potent mitochondrial membrane depolarizer) also resulted in a significant reduction in cell viability to 64.3±3.9%. Administration of HDL cholesterol prior to exposure to hypoxia abrogated the deleterious effect of hypoxia and increased cell viability to 76.7±1.8% ($p<0.05$ vs. hypoxia).

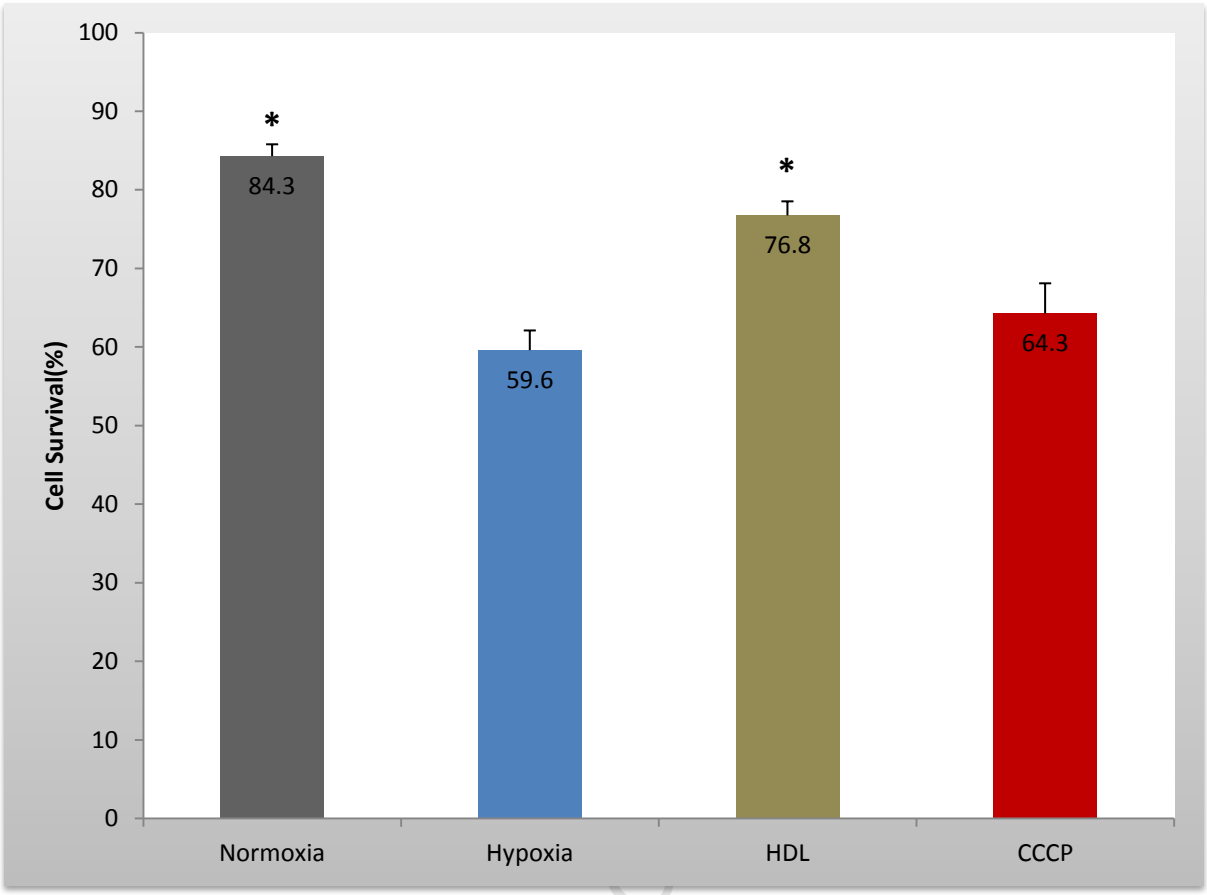


**Figure 37: Effect of HDL cholesterol on cell viability in isolated cardiomyocytes subjected to 2 hours of hypoxia. HDL cholesterol (300mg/ml) improved cell survival. (*p<0.05 vs. Hypoxia. n=8 for all groups, except CCCP which had an n=4). CCCP=Carbonyl Cyanide m-Chloro Phenyl hydrazone (10μM).**

## 2.2 EFFECT OF S1P AND HDL CHOLESTEROL ON MPTP OPENING

TMRM fluorescence was measured using two techniques: flow cytometry and fluorescent microscopy in the S1P group, while the HDL cholesterol group was only measured using flow cytometry.

In the fluorescent microscopy model, isolated mouse cardiomyocytes subjected to 2 hours hypoxia displayed a decrease in fluorescence to 77.8±2.0% of the normoxic controls ($p<0.05$ vs. Normoxia) (Figure 38). Cardiomyocytes treated with CsA had restored levels of fluorescence (94.3±1.0%), indicating inhibition of opening of the mPTP and validating the fluorescence microscopy model. Pretreatment of cells with 10nM S1P restored fluorescence to 95.0±4.0% of the normoxic cardiomyocytes.

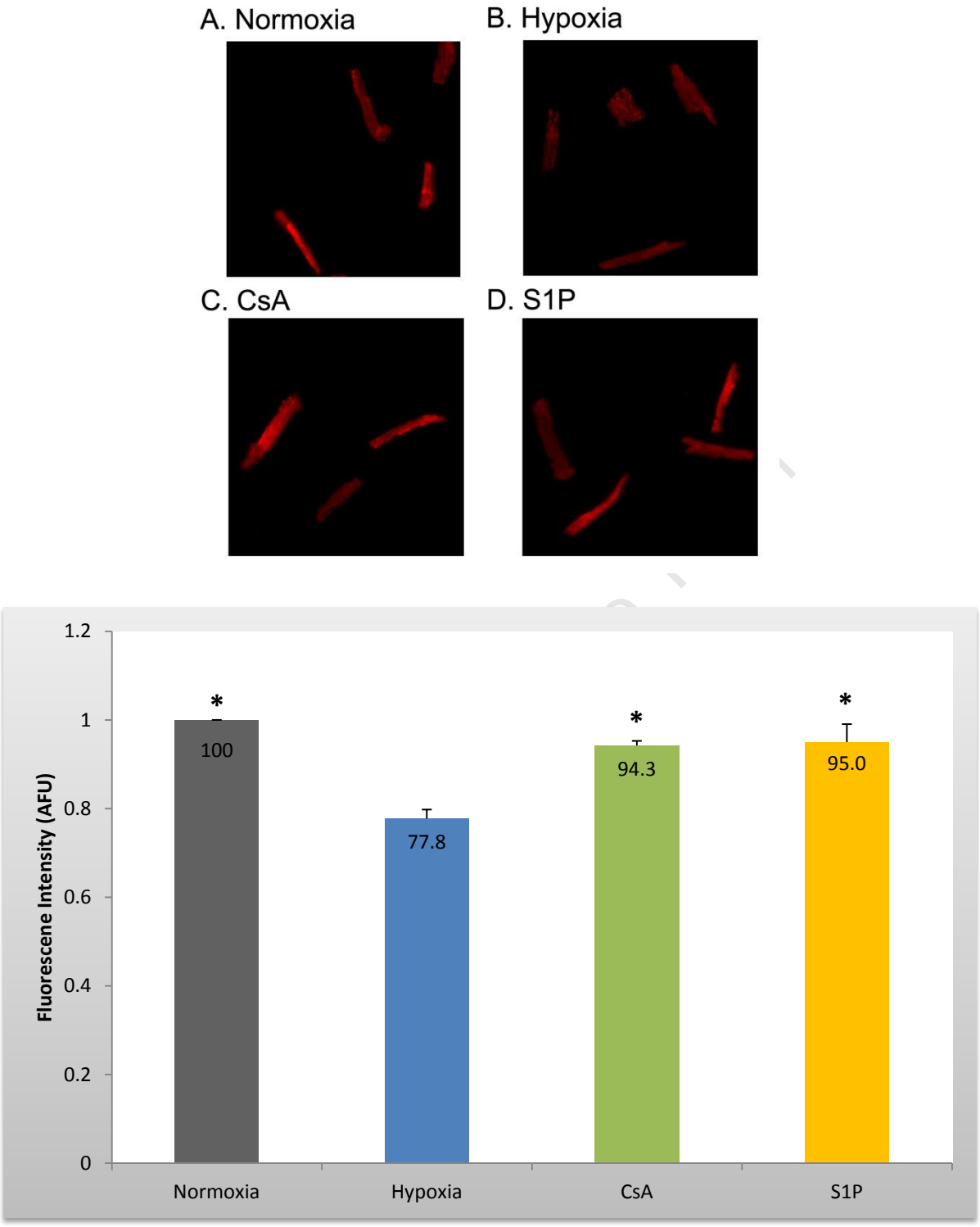


**Figure 38: Effect of S1P on TMRM fluorescence in isolated cardiomyocytes using a fluorescent microscope. Fluorescence is given as arbitrary fluorescent intensity units (AFU). Both S1P (10nM) and CsA (200nM) restored fluorescent levels to normoxic values. (*p<0.05 vs. Hypoxia. n=6 for all groups). CsA= Cyclosporin A.**

In the flow cytometry model, hypoxic cardiomyocytes experienced a decrease in fluorescence to 83.7±6.3% of the normoxic control level (p<0.05 vs. normoxia) (Figure 39). In this model pretreatment with S1P prior to hypoxia restored fluorescence levels to

95.5±4.3% of the normoxic control levels. These results for hypoxic and S1P treated groups were similar to the fluorescent microscopy results, which validated the use of the flow cytometry model for fluorescence analysis of TMRM.

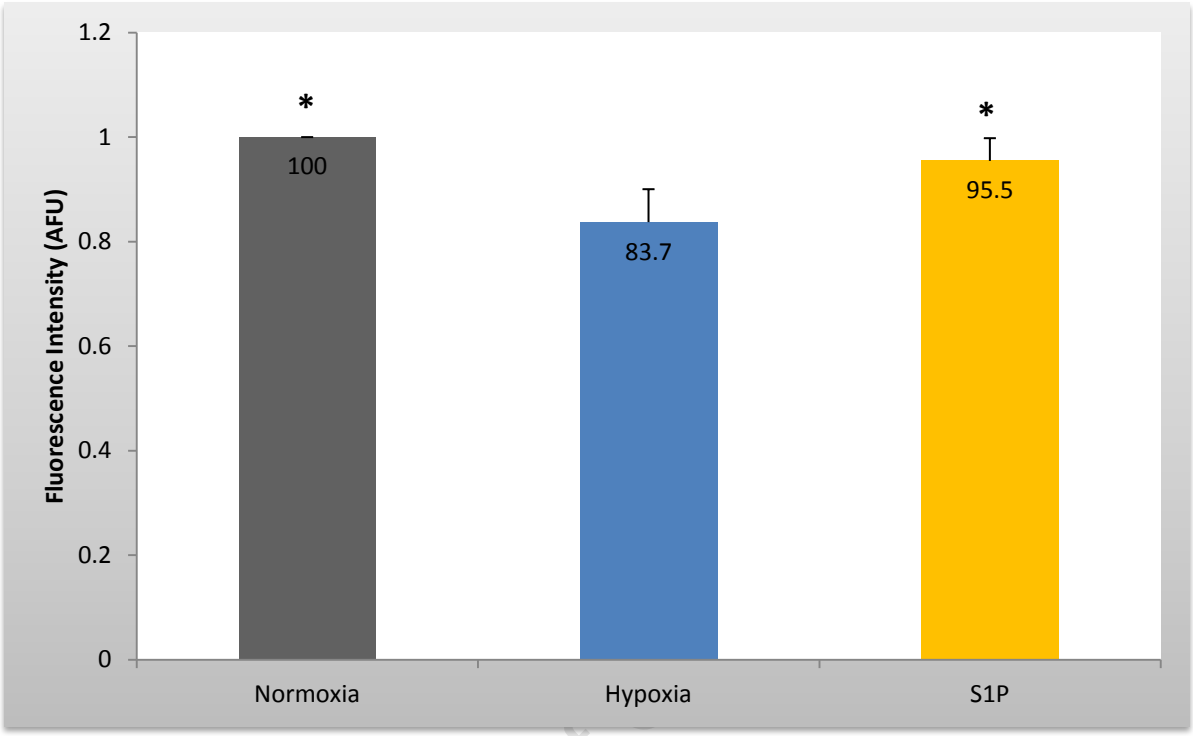


**Figure 39: Effect of S1P on TMRM fluorescence in cardiomyocytes using a flow cytometer. Pretreatment with S1P (10nM) prior to hypoxia restored fluorescence levels to normoxic values. (*p<0.05 vs. Hypoxia. n=9 for all groups).**

For the HDL cholesterol experiments, hypoxia reduced fluorescence intensity to 76.3±6.0% of the normoxic cardiomyocyte's value ($p<0.05$ vs. normoxia), indicating an opening of the mPTP (Figure 40). Similar to its effect on cell viability, pretreatment with HDL cholesterol significantly prevented fluorescence intensity loss (88.4±11.0%), indicating retention of membrane potential. Treatment with CCCP had the expected result of depolarising mitochondrial membranes, resulting in a substantial decrease in TMRM fluorescent levels; 56.5±14% of the normoxic levels ($p<0.05$).

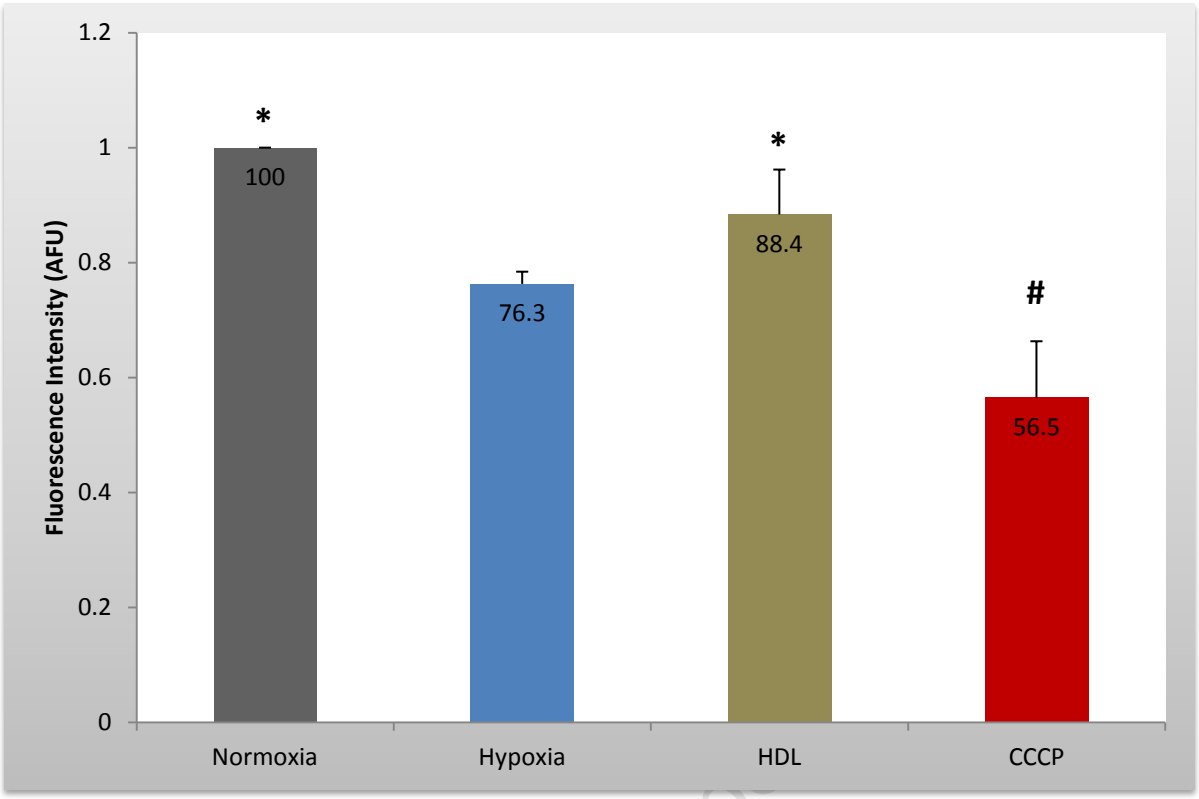


**Figure 40: Effect of HDL cholesterol on TMRM fluorescence levels in cardiomyocytes exposed to hypoxia using a flow cytometry. HDL cholesterol (300mg/ml) restored fluorescent levels to normoxic values. Treatment with CCCP alone reduced levels to 56.5+-14.0% of normoxic values. (*p<0.05 vs. Hypoxia. #p<0.05 vs. Normoxia. n=6 for all groups). CCCP= Carbonyl Cyanide m-Chloro Phenyl hydrazone (10μM).**

## 2.3 EFFECT OF S1P AND HDL CHOLESTEROL ON MITOCHONDRIAL STAT-3 LEVELS

Mitochondria were isolated from the cardiomyocytes of wildtype mice, following which they were probed for total STAT-3 levels, or phosphorylated STAT-3 (serine) levels. Blots were then stripped and re-probed for VDAC. All results were normalised to their respective VDAC levels.

### 2.3.1 Effect of S1P and HDL cholesterol on total STAT-3 levels

Untreated cardiomyocytes had total STAT (tSTAT) levels of 0.38±0.04 Arbitrary Units (AU) (Figure 41). Neither S1P (0.43±0.05 AU) nor HDL cholesterol (0.46±0.05 AU) pretreatment increased the total levels of STAT-3 protein within the mitochondria.

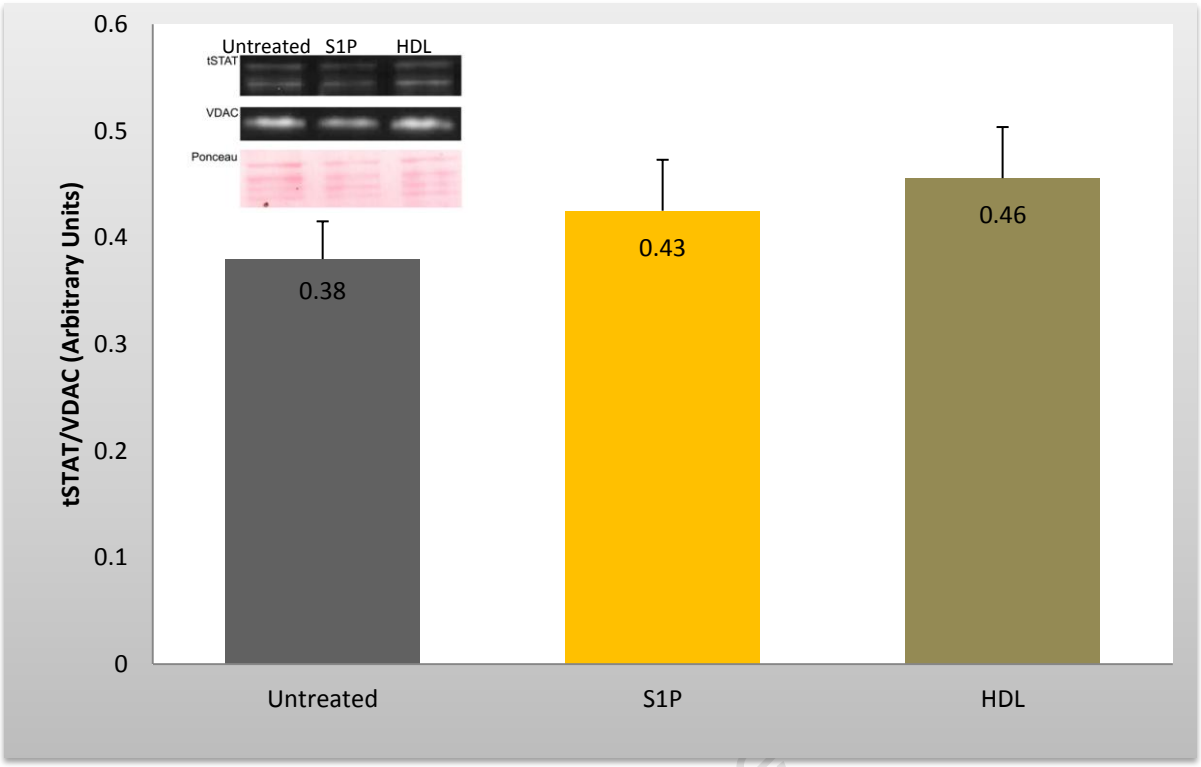


**Figure 41: Effect of HDL cholesterol (300mg/ml) or S1P (10nM) on total STAT-3 (tSTAT) levels in mitochondria. Data are normalised to VDAC levels, and expressed as arbitrary units (AU). Neither S1P nor HDL cholesterol increased tSTAT levels in the mitochondria (n=4).**

### 2.3.2 Effect of S1P and HDL cholesterol on pSTATSer levels

Untreated cardiomyocytes had phosphorylated levels of STAT-3 (serine) of 0.57±0.05 AU (Figure 42). Pretreatment with S1P significantly increased these levels to 1.13±0.25 AU ($p<0.05$ vs. untreated). However, HDL cholesterol pretreatment did not increase phosphorylation levels (0.63±0.01 AU).

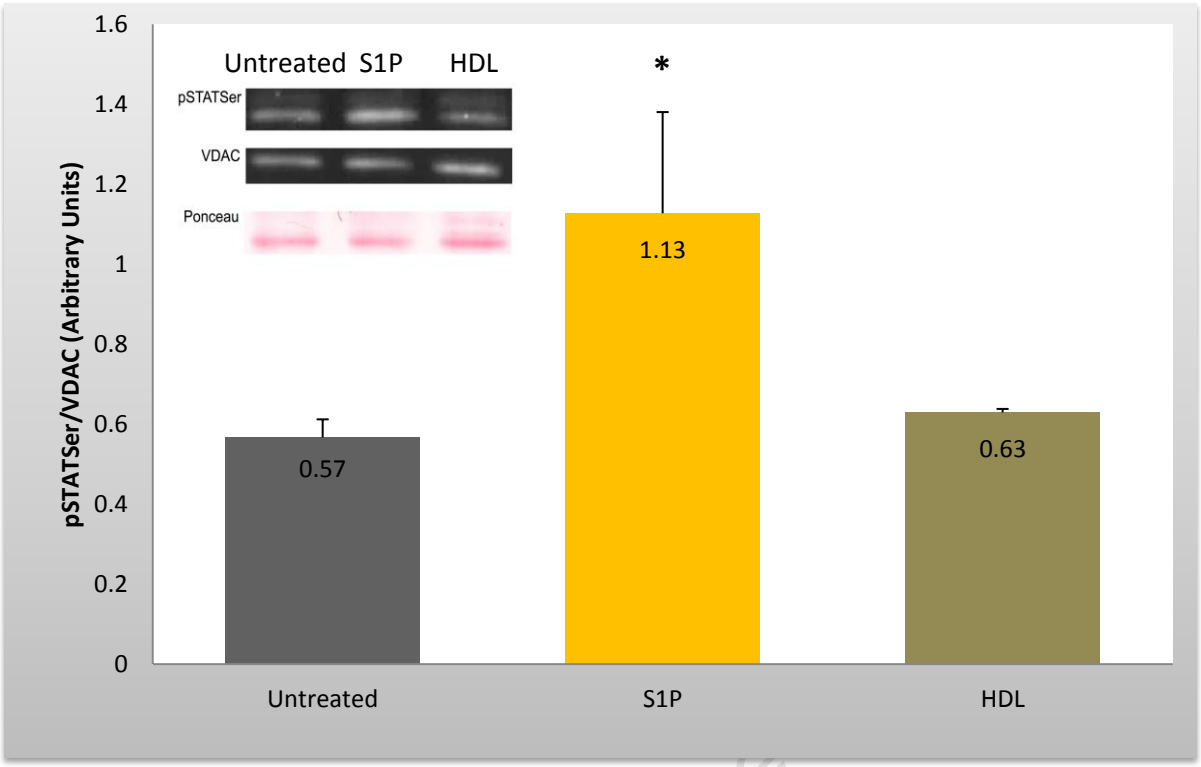


**Figure 42: Effect of HDL cholesterol (300mg/ml) or S1P (10nM) on serine phosphorylated STAT-3 (pSTATSer) levels in mitochondria. Data are normalised to VDAC levels, and expressed as arbitrary units (AU). S1P pretreatment increased levels of pSTATSer in the mitochondria (*p<0.05 vs. Untreated).HDL cholesterol had no effect (n=4).**



# 3: S1P AND HDL CHOLESTEROL FAIL TO PROTECT AGAINST HYPOXIA IN ISOLATED STAT-3 KO MOUSE CARDIOMYOCYTES

The relationship between S1P, HDL cholesterol and STAT-3 signalling to the mitochondria was investigated using transgenic STAT-3 KO mice subjected to hypoxia.

## 3.1 EFFECT OF S1P AND HDL CHOLESTEROL ON CELL VIABILITY

Normoxic cardiomyocytes had a mean viability of 78.7±3.3% survival, indicating that approximately 20% of cells did not survive the isolation process (Figure 43). Two hours of hypoxia decreased cell viability to 60.2±4.8 ($p<0.05$ vs. normoxia). Pretreatment with S1P conferred no protection against hypoxia, with a cell survival of 58.3±3.8% (not significant vs. hypoxia). HDL cholesterol pretreatment also did not improve cell viability in isolated cardiomyocytes exposed to hypoxia (60.7±2.8%).

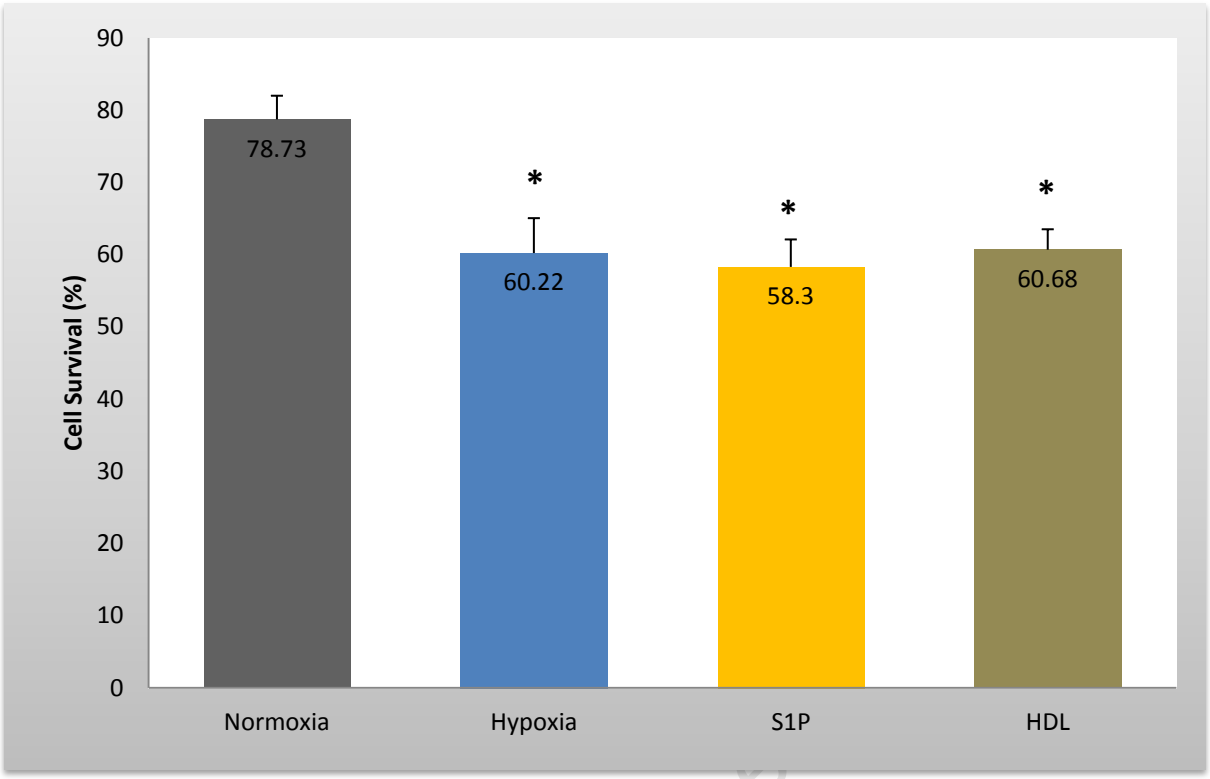


**Figure 43: HDL Cholesterol and S1P have no cardioprotective effect in STAT-3 KO mice. Neither HDL cholesterol (300mg/ml) nor S1P (10nM) enhanced cell survival following 2 hours exposure to hypoxia (*p<0.05 vs. Normoxia. n=6 for all groups).**



## 3.2 EFFECT OF S1P AND HDL CHOLESTEROL ON MPTP OPENING

Relative to fluorescence levels of normoxic controls, 2 hours of hypoxia reduced fluorescence to 80.7±11.9% ($p<0.05$ vs. normoxia) (Figure 44). Pretreatment with S1P did not restore fluorescence (73.8±6% of the normoxic control, not statistically different from the hypoxic group). Similarly, there was no significant difference in fluorescence levels between the hypoxic and HDL cholesterol group, which had a reduced fluorescence of 72.8%±7.7 relative to the normoxic controls.

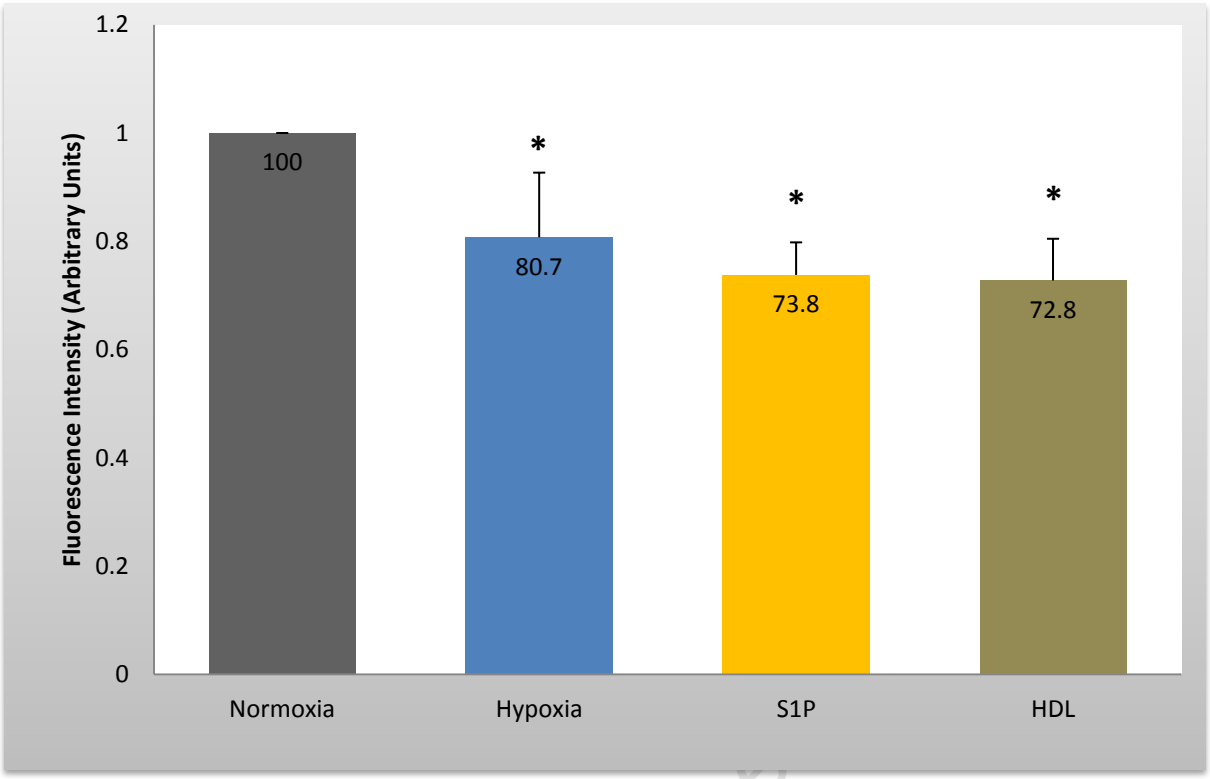


**Figure 44: Effect of HDL Cholesterol and S1P on mPTP opening in isolated cardiomyocytes from STAT-3 KO mice exposed to hypoxia. (*p<0.05 vs. Normoxia. n=4 for all groups).**

# Chapter 5: Discussion

## 1: SUMMARY OF RESULTS

Our data demonstrates that HDL cholesterol and its component S1P confer cardioprotection against ischaemia via the inhibition of opening of the mPTP. In brief, both native HDL cholesterol and S1P improved cardiomyocyte viability and inhibited opening of the mPTP. Furthermore, experiments repeated in STAT-3 deficient cardiomyocytes suggest that the effect of HDL cholesterol and S1P is mediated via the activity of STAT-3. This suggests a role of the SAFE pathway in HDL cholesterol and S1P cardioprotection. A summary of our experimental results is presented below.

| END POINT | S1P | HDL |
|---|---|---|
| *WILDTYPE* | | |
| INFARCT SIZE | ↓ | -- |
| RCI | ↑ | -- |
| CELL VIABILITY | ↑ | ↑ |
| MPTP OPENING | ↓ | ↓ |
| ACTIVATION OFSTAT-3 IN MITOCHONDRIA | ↑ | ↔ |
| | | |
| *STAT-3 KO* | | |
| CELL VIABILITY | ↓ | ↓ |
| MPTP OPENING | ↓ | ↓ |

**Table 7: Summary of Results. Green arrows indicate positive cardioprotective changes relative to the hypoxic control, whilst red arrows indicate negative changes relative to the normoxic control.**

- Isolated rat hearts treated with 10nM S1P prior to 30 minutes of regional ischaemia and 120 minutes of reperfusion displayed a significant decrease in infarct size. S1P did not improve functional parameters. Mitochondria isolated from rat hearts prior to the ischaemia displayed improved respiratory parameters if the hearts were treated with

S1P. This suggests a role for the conservation of mitochondrial function in the cardioprotective effect observed with S1P.

- Treatment of isolated mouse cardiomyocytes with either S1P (10nM) or HDL cholesterol (300mg/ml) conferred marked cytoprotection against 2 hours of hypoxic stress. Furthermore, both treatments reduced opening of the mPTP.
- In contrast, neither S1P nor HDL cholesterol improved cell viability in STAT-3 KO mice, nor could the opening of the mPTP be inhibited with either treatment.
- S1P increased phosphorylation of STAT-3 at its serine residue within the mitochondria, but did not increase total STAT-3 levels. HDL cholesterol had no effect on STAT-3 levels or phosphorylation.

# 2: CARDIOPROTECTIVE EFFECTS OF S1P AND HDL CHOLESTEROL

Using isolated perfused rat hearts and isolated adult mouse cardiomyocytes, our data demonstrated that 10nM S1P and 300mg/ml HDL cholesterol are cardioprotective against ischemic injury.

## 2.1 S1P

The isolated rat heart ischaemia-reperfusion model showed significant reduction in infarct size following treatment with S1P (Figure 34). **Infarct sizes of control hearts previously reported in the literature average approximately 35% (26). Our infarcts are smaller, and this could be attributed to the severity of the occlusion, which depends on how high up the occlusion is made on the coronary artery**. S1P did not improve functional recovery. Previous studies exploring the cardioprotective effect of various drugs in this model have not reported a change in functional recovery either (187). Treatment with S1P also protected isolated adult mouse cardiomyocytes against a hypoxic stress (Figure 36). This is the first time S1P has been administered to adult cardiomyocytes in a hypoxia model. Karliner et al. have administered S1P to neonatal rat cardiomyocytes and shown a protective effect against hypoxia, however the concentrations of S1P used were much higher (188).

The cardioprotective concentrations for S1P cited in the literature range from 10nM (189) to 10μM (188). Our choice of a low concentration of S1P was justified by the physiological concentrations of unbound S1P in the plasma, approximately 10nM (190). Furthermore, high concentrations of S1P have been associated with coronary vasoconstriction, bradycardia and decreased blood pressure (191),(192). Therefore, the use of a lower concentration of S1P is also justified in limiting the potential side effects of administration of S1P.

## 2.2 HDL CHOLESTEROL

HDL cholesterol protected isolated adult mouse cardiomyocytes from hypoxic cell death (Figure 37). HDL cholesterol has previously been shown to protect cardiomyocytes against doxorubicin induced apoptosis at similar concentrations (193). Our results are the first to show a cardioprotective effect against hypoxia in a cardiomyocyte model.

The degree of cardioprotection observed with HDL cholesterol was similar to that observed with S1P treatment. Our data suggest that S1P may be regulating the cardioprotective effect of HDL cholesterol. Many experimental studies have attributed the cardioprotection of HDL cholesterol to its apoA content, as the use of reconstituted HDL cholesterol has been shown to protect against ischaemia-reperfusion injury (194),(195). Reconstituted HDL cholesterol is composed of palmitoyloleoyl phosphatidylcholine, cholesterol and ApoA, and does not contain S1P (196). Our data strongly suggests that addition of S1P in reconstituted HDL cholesterol may enhance the protection against ischaemia-reperfusion injury. In fact, preliminary work done in our laboratory using an isolated mouse heart model demonstrated that reconstituted HDL cholesterol enriched with S1P is more cardioprotective than reconstituted HDL cholesterol containing ApoA alone (197). The use of reconstituted HDL cholesterol containing both ApoA and S1P, rather than S1P unbound to HDL cholesterol, may be a more physiological, and therefore safer, option in delivering S1P to patients. Recently, the presence of apolipoprotein M has been characterised in HDL cholesterol, and it is suggested that apolipoprotein M controls the bioavailability of S1P bound to HDL cholesterol (198). However, data from our group showing that reconstituted HDL cholesterol containing S1P alone, and no apolipoproteins, can confer cardioprotection. This suggests that S1P bound to HDL cholesterol is cardioprotective independent of the apolipoproteins in our setting (199).

## 2.3 ROLE OF THE SAFE PATHWAY

### 2.3.1 Changes in mitochondrial STAT-3

Recent literature suggests that STAT-3 phosphorylation is critical to mediate cardioprotection **(71).** However, it is not clear whether phosphorylation of STAT-3 occurs inside or outside the mitochondria. Our data showed no significant differences in levels of total STAT-3 protein in mitochondria isolated from wildtype mouse cardiomyocytes following treatment with either S1P or HDL cholesterol (Figure 41). This result suggests that there is no translocation of STAT-3 from the cytosol into the mitochondria, and STAT-3 phosphorylation may instead result from a phosphorylation cascade happening within the mitochondria. Surprisingly, phosphorylation of STAT-3 at the serine residue was increased in the mitochondria following treatment with S1P, but not with HDL cholesterol (Figure 42). There are various explanations for this apparent discrepancy.

Firstly, the time selected for analysing STAT-3 phosphorylation may not have been optimal. Here, mitochondria were harvested 7 minutes after treatment. Our laboratory has demonstrated a transient phosphorylation of STAT-3 signalling in mitochondria following treatment with S1P (200). In an isolated mouse heart model, protein extraction following 7 minutes of S1P treatment resulted in an increase in phosphorylated STAT-3, which disappeared at 15 minutes. It is reasonable to assume that the time point selected in our isolated cardiomyocyte model may not have been the optimal time point to detect phosphorylated STAT-3.

Secondly, unbound S1P might exert its effect quicker than the S1P bound to the HDL cholesterol molecule.

Thirdly, the phosphorylation of STAT-3 may not be required for the protection. Recent studies have demonstrated a pathophysiological role for unphosphorylated STAT-3 and this may be the case in our setting (201).

Lastly, there is also the possibility that the protective effect of STAT-3 is not the result of its translocation into the mitochondria. STAT-3 may instead translocate to the nucleus and subsequently activate unknown factors which influence the mitochondria. Alternatively, it may be part of the cytosolic signalling to the mitochondria, possibly as part of a signalosome as described by (202), and hence only gets as far as the outer mitochondrial membrane, but not get incorporated into the mitochondria. However, if HDL cholesterol is acting via a pathway independent of S1P, it would argue against our hypothesis that S1P is the active cardioprotective component of HDL cholesterol.

### 2.3.2 Cardioprotection in STAT-3 KO mice

Our data (Figure 43) clearly demonstrate a loss of S1P and HDL cholesterol cardioprotection in STAT-3 KO cardiomyocytes. Although S1P and HDL cholesterol have already been reported to activate STAT-3 (199),(203), our data demonstrate for the first time, to our knowledge, that activation of STAT-3 is required for S1P and HDL cholesterol induced cardioprotection against hypoxia. This suggests that STAT-3, a component of the SAFE pathway, is a crucial regulator of HDL cholesterol and S1P induced cardioprotection.

# 3: EFFECT OF S1P AND HDL CHOLESTEROL ON MITOCHONDRIAL FUNCTION

## 3.1 RESPIRATORY CONTROL INDICES

Treatment with S1P in isolated rat hearts resulted in a significant improvement of the RCI values of mitochondria (Figure 35). It is important to note that these are baseline levels, and not following exposure to any form of ischaemia. The validity of the treatment protocol and S1P concentration used was confirmed by those hearts which were exposed to ischaemia displaying a significant decrease in infarct size. There are two explanations for the difference in RCI values observed.

Firstly, there is evidence that S1P is present within the mitochondria and that it interacts directly with complex IV of the ETC, positively modulating respiration (204). Whether exogenous treatment with S1P increases mitochondrial levels of S1P remains unknown, but it is possible that in our model, administration of exogenous S1P increased levels of S1P in the mitochondria, and therefore improved respiratory function.

Alternatively, Percoll isolation may produce mild stress within the mitochondria, leading to mPTP opening and potential uncoupling of the ETC. S1P inhibits mPTP opening as demonstrated in this thesis, and this may preserve the respiratory function of the isolated mitochondria.

## 3.2 INHIBITION OF THE MPTP

Both S1P and HDL cholesterol pretreatment resulted in significant inhibition of mPTP opening (Figure 39 and Figure 40). Inhibition of mPTP opening was confirmed in two

separate models (albeit using the same fluorescent dye), therefore strengthening the evidence for S1P and HDL cholesterols effect on mPTP opening. The validity of the flow cytometer model in the analysis of TMRM fluorescence was confirmed using the fluorescent microscopy technique. The reduction in fluorescence in the hypoxic groups may seem modest, and the subsequent restoration by S1P and HDL cholesterol even more so, but it is important to bear in mind the limitations of the technique.
Our data demonstrate a role for HDL cholesterol and S1P on mPTP opening, and this is supported by the recent discovery that a knock-out model of sphingosine kinase 2 within the mitochondria is more susceptible to mPTP opening (205).

## 3.3 mPTP INHIBITION IN STAT-3 KO MICE

There was no inhibition of the mPTP by either HDL cholesterol or S1P in the STAT-3 KO cardiomyocytes (Figure 44). Combined with the cell viability data, our experiments suggest that HDL cholesterol and S1P are exerting their cardioprotective effects through the inhibition of mPTP opening, which is dependent on STAT-3 activation.

# 4: CLINICAL RELEVANCE

## 4.1 HDL CHOLESTEROL AS A THERAPEUTIC AGENT

Increases in baseline HDL cholesterol levels via lifestyle changes may confer chronic protection against incidence and severity of IHD. Acute administration of reconstituted HDL cholesterol is also protective in animal models of ischaemia-reperfusion (206) which makes it an ideal candidate as a therapeutic agent. In human studies, reconstituted HDL cholesterol has been tested in pilot studies, and resulted in a positive modulating effect on the lipid profiles of diabetic patients (207), and on atherosclerotic plaques (208), (209). Whether or not the cardioprotection against ischaemia-reperfusion injury observed with reconstituted HDL cholesterol in the animal models translates to human models remains to be proved.

## 4.2 S1P AS A THERAPEUTIC AGENT

In in-vivo animal models, acute administration of S1P reduces infarct size (48). No clinical trials for acute or chronic administration of S1P in humans currently exist. However, an S1P receptor agonist, Fingolimod, is an approved drug in the treatment of multiple sclerosis (210). As such, S1P, or a pharmacological mimetic, may be a potential candidate for therapeutic intervention; however more research in this area is still required. The main therapeutic benefit of S1P may lie in its administration as a component of reconstituted HDL cholesterol. Reconstituted HDL cholesterol that has been tested in a clinical setting does not contain S1P (211). However, the composition of HDL cholesterol, not merely the quantity, is important in cardioprotection (212) and recent evidence has suggested a pivotal role for S1P

in the antiatherosclerotic effect of HDL cholesterol (46). Our data show that S1P and HDL cholesterol protect to a similar degree, suggesting that S1P may be the active component in the cardioprotective effect of HDL cholesterol. Therefore, any therapeutic HDL cholesterol should be enriched with S1P in order to confer maximum protection.

# *Chapter 6: Limitations of the study, Further Work and Conclusion*

## 1: LIMITATIONS OF THE STUDY AND FURTHER WORK

- The isolated perfused rat heart model, which was used for infarct size analysis and mitochondrial respiration analysis, only received S1P pretreatment, and not HDL cholesterol pretreatment. These experiments were conducted at the beginning of the MSc dissertation and at this stage the native HDL cholesterol from our collaborators in Geneva was unavailable. Once the HDL cholesterol became available, it was incorporated into all subsequent experiments with the intention to repeat the initial rat heart experiments with HDL cholesterol. However, due to availability of equipment and time constraints this was not possible. The native HDL cholesterol used in these experiments has since been validated by Dr. Miguel Frias in an isolated mouse heart model, where it is shown to reduce infarct size (197).

- Respiratory function analysis of isolated rat heart mitochondria did not include ETC uncouplers, which would have given as a better idea of the maximal respiratory rates of the mitochondria. Further characterisation of isolated rat heart mitochondria would be beneficial in putting our results into context.

- Analysis of mPTP opening was restricted to use of TMRM dye. A different model could be used to confirm these results. There are other dyes which can be used to measure mitochondrial depolarization, such as Mitotracker Red, however TMRM is the current gold standard. Therefore, indirect ways to measure opening of the mPTP could be used, such as a calcium retention assay or probing for cytochrome c release into the cytoplasm.

- **CsA was used as a positive control for inhibition of the mPTP, while CCCP was used as a negative control for mPTP opening. However, as with most pharmacological agents, it has limitations in terms of specificity.**

- **The results obtained for S1P in the fluorescent microscopy model matched the results obtained with in the flow cytometer model. Unfortunately, a CsA group was not included in our experiments measuring the mPTP opening with the flow cytometer. The inclusion of a CsA group in this model is necessary to confirm its validity.**

- **Another limitation of our study is that we did not include a group with S1P nor HDL in normoxic conditions.**

- The HDL cholesterol used was native HDL, and the exact composition was unknown. Therefore one is unable to say whether or not it was the S1P component of HDL cholesterol which was responsible for its effects. Using reconstituted HDL cholesterol

with known concentrations of S1P is the focus of work done by our collaborators in Geneva (Dr Miguel Frias).

- Administration of a known cardioprotective agent to the STAT-3 KO mice, which does not act via the SAFE pathway, such as cyclosporinA may be desirable to confirm that STAT-3 KO mice are capable of being protected and that they do still have a functional mPTP.

- As with all animal models, there is a limit on physiological translation to humans. Furthermore, because isolated hearts and isolated cardiomyocytes were used, it is not a true representation of all physiological processes. For example, the liver plays a vital role in regulation of cholesterol levels, and it is unknown if supplementation with S1P or HDL cholesterol would be metabolized by the liver. Therefore, translating these results into an in vivo model is a potential area for further studies.

- S1P increased phosphorylation of STAT-3 at the serine residue in the mitochondria, yet HDL cholesterol had no effect. In order to resolve this discrepancy different time points of protein harvest could be investigated to get an idea of changes in STAT-3 state over time following treatment with S1P or HDL cholesterol. Furthermore, western blot analysis only looked at total STAT-3 and the serine phosphorylated isoform in the mitochondrial fraction. Expanding this to include cytosolic and nuclear fractions, as well as the phosphorylated tyrosine isoform, might illuminate HDL cholesterols effect on STAT-3 signalling.

- **Administration of both HDL cholesterol and S1P was followed by a wash out period in all models which does not represent a physiological model. Whether the 30 minutes of washout is truly necessary for the protection or whether 60 minutes of uninterrupted treatment would be equally protective merits further investigation.**



## 2: CONCLUSION

Our data confirms the cardioprotective effects of both S1P and HDL cholesterol against hypoxia (213). Furthermore, we demonstrated that both HDL cholesterol and S1P protect the cardiomyocytes via inhibition of mPTP opening, achieved via activation of the SAFE pathway. These results strongly suggest that both HDL cholesterol and S1P are ideal candidates for therapeutic treatment of CVD, either via acute administration or chronic changes in physiological levels.

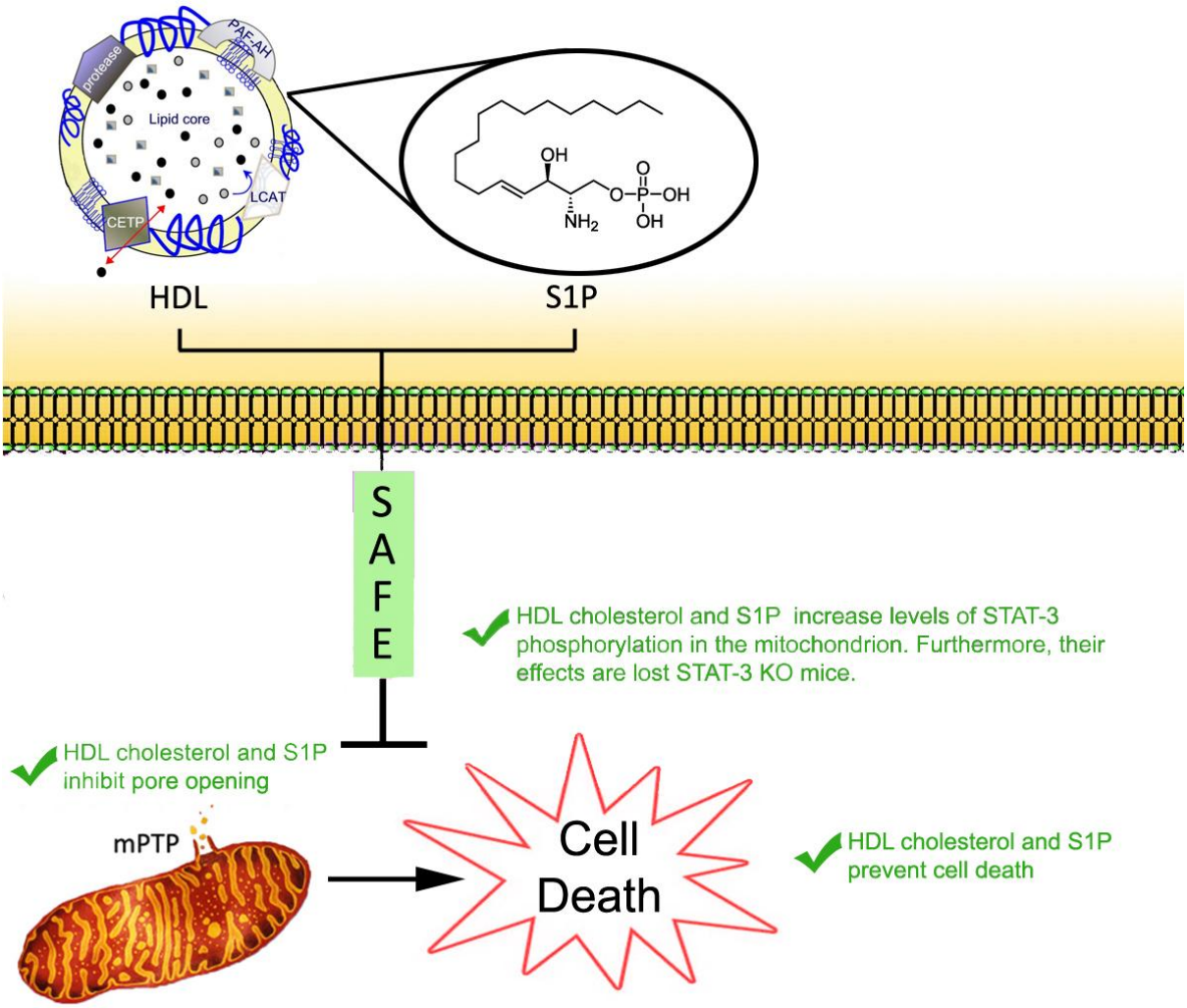


**Figure 45: Proposed signalling cascade in S1P and HDL cholesterol induced cardioprotection. Both S1P and HDL cholesterol are cytoprotective. This protection is mediated via the SAFE pathway. S1P and HDL cholesterol inhibit mPTP opening, which is also mediated via the SAFE pathway. Migration of STAT-3 into the mitochondria as the mechanism of action of the SAFE pathway remains unclear.**

# APPENDIX 1:

## Krebs-Henseleit Buffer

| | |
|---|---|
| NaCl | 118.8mM |
| $NaHCO_3$ | 25mM |
| Glucose | 11.1mM |
| KCl | 4.75mM |
| $MgSO_4.7H_2O$ | 1.19mM |
| $KH_2PO_4$ | 1.18mM |
| $CaCl_2.2H_2O$ | 1.35mM |

Oxygenate with 95% $O_2$ and 5% $CO_2$

## Sodium Phosphate Buffer

Solution 1:

| | |
|---|---|
| NaH2PO4.2H2O | 100mM |

Solution 2:

| | |
|---|---|
| Na2HPO4 | 100mM |

Mix 8 parts solution 2 with 2 parts solution 1

# APPENDIX 2:

## Wash Buffer

| | |
|---|---|
| Sucrose | 250mM |
| HEPES | 10mM |
| K-EGTA | 5mM |

## BSA Buffer

Wash buffer + 0.5% fatty acid free BSA

## Percoll Buffer

| | |
|---|---|
| Sucrose | 250mM |
| HEPES | 10mM |
| K-EGTA | 5mM |

Make up to 740ml instead of a litre (or 100-percent concentration of desired Percoll gradient)

## Percoll Gradient

| | |
|---|---|
| Percoll | 26ml |
| Percoll Buffer | 74ml |

## EGTA-free Buffer

| | |
|---|---|
| Sucrose | 250mM |
| HEPES | 10mM |

## Buffer A

| | |
|---|---|
| Sucrose | 250mM |
| TrisHCl | 20mM |
| EDTA | 1mM |

# APPENDIX 3:

## Biuret Reagent

0.2M NaOH made up to 400ml, instead of 1000ml
Add, in order:

| | |
|---|---|
| NaTartrate | 9g |
| $CuSO_4.5H_2O$ | 3g |
| KI | 5g |

Make up to 1L

# APPENDIX 4:

## Respiration Buffer

| | |
|---|---|
| KCl | 120mM |
| $KH_2PO_4$ | 5mM |
| MOPS | 5mM |
| EGTA | 1mM |

## Saturated KCl

Add KCl to $H_2O$ until no more crystals dissolve. Heat solution slightly to ensure saturation

# APPENDIX 5:

## Perfusion Buffer

| | |
|---|---|
| NaCl | 113mM |
| KCl | 4.7 mM |
| $KH_2PO_4$ | 0.6 mM |
| $Na_2HPO_4$ | 0.6 mM |
| $MgSO_4.7H_2O$ | 1.2 mM |
| $NaHCO_3$ | 12 mM |
| $KHCO_3$ | 10 mM |
| Taurine | 30 mM |
| HEPES | 10 mM |
| Glucose | 11 mM |
| 2,3 Butanedione monoxime | 10 mM |

## Perfusion Buffer 2

| | |
|---|---|
| Perfusion Buffer | 50ml |
| $CaCl_2$ | 12.5µM |
| Bovine Serum Albumin | 1% |

## Collagenase Buffer

| | |
|---|---|
| Perfusion Buffer 2 | 50ml |
| Collagenase (type 2, ~318U/mg) | 0.045g |

**NB:**May need to adjust collagenase according to the stock U/mg strength

## Calcium Reintroduction

Reintroduce calcium in a step wise fashion (lay the tube horizontal)
10ul of 0.1M $CaCl_2$ for 4 minutes
10ul of 0.1M $CaCl_2$ for 4 minutes
20ul of 0.1M $CaCl_2$ for 4 minutes
30ul of 0.1M $CaCl_2$ for 4 minutes
30ul of 0.1M $CaCl_2$ for 4 minutes

## Medium 199

| | |
|---|---|
| M-199 | 500ml |
| BSA | 1g |
| Creatine | 0.33g |
| Taurine | 0.31g |
| Carnitine | 0.16g |
| Pen/Strep | 1% |

## Laminin

Dissolve 1 vial of L2020 laminin in 30ml $H_2O$

# APPENDIX 6:

## SI Buffer

| | |
|---|---|
| NaCl | 137 mM |
| KCl | 12 mM |
| $MgCl_2.6H_2O$ | 0.5 mM |
| $CaCl_2.2H_2O$ | 0.9 mM |
| HEPES | 20 mM |
| 2-DG | 20 mM |

pH to 6.4
Calibrate pH meter at 24ºC and measure at 34ºC

# APPENDIX 7:

## Running Gel

| For 2 gels: | |
|---|---|
| $dH_2O$ | 9.8ml |
| 1.5M Tris HCl pH 8.8 | 5ml |
| 20% SDS | 100ul |
| Acrylamide | 5ml |
| 10% APS | 100ul |
| TEMED | 40ul |

## Stacking Gel

| For 2 gels: | |
|---|---|
| dH20 | 7.5ml |
| 0.5 M Tris Hcl pH 6.8 | 3ml |
| 20% SDS | 60ul |
| Acrylamide | 1.5ml |
| 10% APS | 80ul |
| TEMED | 40ul |

## 10x Running Buffer

| | |
|---|---|
| Tris | 60.6g |
| Glycine | 288g |
| SDS | 20g |
| $dH_2O$ | up to 2L |

## Transfer Buffer

| | |
|---|---|
| Tris | 3.03g |
| Glycine | 14.4g |
| Methanol | 200ml |
| dH20 | Make up to 1L |

## TBS-T

| | |
|---|---|
| Tris | 2.42g |
| NaCl | 8g |
| Tween20 | 1ml |
| $dH_20$ | make up to 1L |
| pH to 7.6 | |

## tSTAT Antibodies

**Primary**
rabbit 1:4000 in TBS-T with 5% milk
**Secondary**
1:3000 anti-rabbit in TBS-T

## pSTATSer Antibodies

**Primary**
1:3000 rabbit in TBS-T
**Secondary**
1:3000 anti-rabbit in TBS-T

## pSTATTyr Antibodies

**Primary**
1:2000 rabbit in TBS-T
**Secondary**
1:3000 anti-rabbit in TBS-T

## VDAC1 Antibodies

**Primary**
1:1000 Goat in TBS-T
**Secondar**y
1:3000 Anti-goat in TBS-T with 5% milk

# *Publications, Abstracts and Prizes*

## 1: PUBLICATIONS

**Ethanolamine is a novel STAT-3 dependent cardioprotective agent.**
2010
Kelly RF, Lamont KT, Somers S, **Hacking D**, Lacerda L, Thomas P, Opie LH, Lecour S.
Basic Research in Cardiology Nov;105(6):763-70

## 2: ABSTRACTS

**High density lipoprotein cholesterol and cardioprotection: role of sphingosine-1-phosphate on the mitochondrial function**
2010
**Damian Hacking**, Roisin Kelly, Derek Yellon, Lionel Opie, Derek Hausenloy and Sandrine Lecour
38th Annual Conference of the Physiology Society of Southern Africa
Abstract & Poster presentation

**Sphingosine-1-phosphate mediates cardioprotection by modulation of the mitochondrial function**
2010
**Damian Hacking**, Roisin Kelly, Derek Yellon, Lionel Opie, Derek Hausenloy and Sandrine Lecour
11th Annual South African Heart Congress (South Africa)
SA heart Journal Vol 7, No 3 (2010): Winter 2010
Abstract & Poster presentation



## 3: PRIZES

**Johnny VD Walt Poster Prize, First Place**
38th Annual Conference of the Physiology Society of Southern Africa
2010

**Silver Medal for Poster Presentation**
11th Annual South African Heart Congress
2010

# References


1. Mathers CD, Boerma T, Ma Fat D. Global and regional causes of death. [Internet]. British medical bulletin. 2009 Jan ;927-32.[cited 2010 Dec 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19776034

2. Mathers C, Boerma T, Ma Fat D. The global burden of disease 2004. 2004.

3. Maher D, Smeeth L, Sekajugo J. Health transition in Africa: practical policy proposals for primary care. [Internet]. Bulletin of the World Health Organization. 2010 Dec ;88(12):943-8.[cited 2010 Dec 18] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2995191&tool=pmcentrez&rendertype=abstract

4. UNAIDS report on the global AIDS epidemic 2010. 2010.

5. Bradshaw D, Nannan N, Groenewald P, Joubert J, Laubscher R, Nojilana B, et al. Provincial mortality in South Africa, 2000--priority-setting for now and a benchmark for the future. [Internet]. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde. 2005 Jul ;95(7):496-503.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16156448

6. Nesto RW, Kowalchuk GJ. The ischemic cascade: temporal sequence of hemodynamic, electrocardiographic and symptomatic expressions of ischemia. [Internet]. The American journal of cardiology. 1987 Mar 9;59(7):23C-30C.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/2950748

7. Jenkins CM, Cedars A, Gross RW. Eicosanoid signalling pathways in the heart. [Internet]. Cardiovascular research. 2009 May 1;82(2):240-9.[cited 2011 Jul 21] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2675928&tool=pmcentrez&rendertype=abstract

8. Burke AP, Virmani R. Pathophysiology of acute myocardial infarction. [Internet]. The Medical clinics of North America. 2007 Jul ;91(4):553-72; ix.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17640536

9. IHD picture [Internet]. [cited 2011 Jan 16] Available from: http://www.nlm.nih.gov/medlineplus/ency/imagepages/17004.htm

10. Yellon DM, Downey JM. Current research views on myocardial reperfusion and reperfusion injury. [Internet]. Cardioscience. 1990 Jun ;1(2):89-98.[cited 2011 Jan 6] Available from: http://www.ncbi.nlm.nih.gov/pubmed/2102803

11. Bucova M, Bernadic M, Buckingham T. C-reactive protein, cytokines and inflammation in cardiovascular diseases. [Internet]. Bratislavské lekárske listy. 2008

Jan ;109(8):333-40.[cited 2011 Jan 6] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18837239

12. Opie LH. Reperfusion injury and its pharmacologic modification. [Internet]. Circulation. 1989 Oct ;80(4):1049-62.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/2571429

13. Ferreira R. The reduction of infarct size--forty years of research. [Internet]. Revista portuguesa de cardiologia : orgão oficial da Sociedade Portuguesa de Cardiologia = Portuguese journal of cardiology : an official journal of the Portuguese Society of Cardiology. 2010 Jun ;29(6):1037-53.[cited 2011 Mar 1] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20964114

14. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. [Internet]. Circulation. 1986 Dec ;74(5):1124-36.[cited 2010 Oct 31] Available from: http://www.ncbi.nlm.nih.gov/pubmed/3769170

15. Zhao Z-Q, Corvera JS, Halkos ME, Kerendi F, Wang N-P, Guyton RA, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. [Internet]. American journal of physiology. Heart and circulatory physiology. 2003 Aug ;285(2):H579-88.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12860564

16. Wang Y, Ahmad N, Wang B, Ashraf M. Chronic preconditioning: a novel approach for cardiac protection. [Internet]. American journal of physiology. Heart and circulatory physiology. 2007 May ;292(5):H2300-5.[cited 2011 Feb 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17208991

17. Kerendi F, Kin H, Halkos ME, Jiang R, Zatta AJ, Zhao Z-Q, et al. Remote postconditioning. Brief renal ischemia and reperfusion applied before coronary artery reperfusion reduces myocardial infarct size via endogenous activation of adenosine receptors. [Internet]. Basic research in cardiology. 2005 Sep ;100(5):404-12.[cited 2011 Jul 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15965583

18. Walsh SR, Tang TY, Kullar P, Jenkins DP, Dutka DP, Gaunt ME. Ischaemic preconditioning during cardiac surgery: systematic review and meta-analysis of perioperative outcomes in randomised clinical trials. [Internet]. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery. 2008 Nov ;34(5):985-94.[cited 2011 Jan 16] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18783958

19. Munk K, Andersen NH, Schmidt MR, Nielsen SS, Terkelsen CJ, Sloth E, et al. Remote Ischemic Conditioning in Patients With Myocardial Infarction Treated With Primary Angioplasty: Impact on Left Ventricular Function Assessed by Comprehensive Echocardiography and Gated Single-Photon Emission CT. [Internet]. Circulation. Cardiovascular imaging. 2010 Nov 1;3(6):656-62.[cited 2011 Jul 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20826592

20. Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L□Huillier I, et al. Postconditioning the human heart. [Internet]. Circulation. 2005 Oct 4;112(14):2143-8.[cited 2011 Jul 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16186417

21. Castle N, Naidoo R, Owen R. Initiation of pre-hospital thrombolysis in South Africa. [Internet]. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde. 2006 Jan ;96(1):28-31.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16440106

22. Thomas M, Norris R, Opie L, Shulman G, Owen P. Beta-blockade in experimental myocardial ischaemia: metabolic and electrocardiographic effects. [Internet]. British heart journal. 1971 Jul ;33(4):609.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/4397629

23. Wong GTC, Li R, Jiang LL, Irwin MG. Remifentanil post-conditioning attenuates cardiac ischemia-reperfusion injury via kappa or delta opioid receptor activation. [Internet]. Acta anaesthesiologica Scandinavica. 2010 Apr ;54(4):510-8.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19878098

24. Manolis AJ, Marketou ME, Gavras I, Gavras H. Cardioprotective properties of bradykinin: role of the B(2) receptor. [Internet]. Hypertension research : official journal of the Japanese Society of Hypertension. 2010 Aug ;33(8):772-7.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20505673

25. Sano T, Izuishi K, Hossain MA, Kakinoki K, Okano K, Masaki T, et al. Protective effect of lipopolysaccharide preconditioning in hepatic ischaemia reperfusion injury. [Internet]. HPB : the official journal of the International Hepato Pancreato Biliary Association. 2010 Oct ;12(8):538-45.[cited 2011 Feb 20] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2997659&tool=pmcentrez&rendertype=abstract

26. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. [Internet]. Journal of molecular and cellular cardiology. 2002 May ;34(5):509-18.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12056855

27. Garg K, Yadav HN, Singh M, Sharma PL. Mechanism of cardioprotective effect of erythropoietin-induced preconditioning in rat heart. [Internet]. Indian journal of pharmacology. 2010 Aug ;42(4):219-23.[cited 2011 Feb 20] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2941611&tool=pmcentrez&rendertype=abstract

28. Landoni G, Figini F, Bignami E. Pharmacological preconditioning of the heart during surgery. [Internet]. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery. 2010 Apr ;37(4):982-3; author reply 983.[cited 2011 Feb 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19853466

29. Cheng J, Zhao D, Zeng Z, Critchley JA, Liu J, Wang W, et al. The impact of demographic and risk factor changes on coronary heart disease deaths in Beijing, 1999-2010. [Internet]. BMC public health. 2009 Jan ;930.[cited 2011 Mar 1] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2637858&tool=pmcentrez&rendertype=abstract

30. Panagiotakos DB, Pitsavos C, Stefanadis C. Short-term prognosis of patients with acute coronary syndromes through the evaluation of physical activity status, the adoption of Mediterranean diet and smoking habits: the Greek Acute Coronary Syndromes (GREECS) study. [Internet]. European journal of cardiovascular prevention and rehabilitation : official journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology. 2006 Dec ;13(6):901-8.[cited 2011 Mar 1] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17143121

31. Apor P. [Costs of health. Costs-effectiveness in case of lifestyle changes]. [Internet]. Orvosi hetilap. 2010 May ;151(19):788-94.[cited 2011 Mar 1] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20427262

32. Sabanayagam C, Shankar A. Sleep duration and cardiovascular disease: results from the National Health Interview Survey. [Internet]. Sleep. 2010 Aug ;33(8):1037-42.[cited 2011 Mar 1] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2910533&tool=pmcentrez&rendertype=abstract

33. Kabagambe EK, Baylin A, Ruiz-Narvaez E, Rimm EB, Campos H. Alcohol intake, drinking patterns, and risk of nonfatal acute myocardial infarction in Costa Rica. [Internet]. The American journal of clinical nutrition. 2005 Dec ;82(6):1336-45.[cited 2011 Mar 1] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16332668

34. Lavie CJ, Thomas RJ, Squires RW, Allison TG, Milani RV. Exercise training and cardiac rehabilitation in primary and secondary prevention of coronary heart disease. [Internet]. Mayo Clinic proceedings. Mayo Clinic. 2009 Apr ;84(4):373-83.[cited 2011 Mar 1] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2665984&tool=pmcentrez&rendertype=abstract

35. Sacks FM, Katan M. Randomized clinical trials on the effects of dietary fat and carbohydrate on plasma lipoproteins and cardiovascular disease. [Internet]. The American journal of medicine. 2002 Dec ;113 Suppl 13S-24S.[cited 2011 Mar 1] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12566134

36. Cho K-H. Biomedicinal implications of high-density lipoprotein: its composition, structure, functions, and clinical applications. [Internet]. BMB reports. 2009 Jul ;42(7):393-400.Available from: http://www.ncbi.nlm.nih.gov/pubmed/19643035

37. Tchoua U, Gillard BK, Pownall HJ. HDL superphospholipidation enhances key steps in reverse cholesterol transport. [Internet]. Atherosclerosis. 2010 Apr ;209(2):430-5.[cited 2010 Dec 25] Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2846204&tool=pmcentrez&rendertype=abstract

38. Gertler MM, Garn SM, Lerman J. The interrelationships of serum cholesterol, cholesterol esters and phospholipids in health and in coronary artery disease. [Internet]. Circulation. 1950 Aug ;2(2):205-14.[cited 2010 Dec 23] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15427208

39. Pekkanen J, Linn S, Heiss G, Suchindran CM, Leon A, Rifkind BM, et al. Ten-year mortality from cardiovascular disease in relation to cholesterol level among men with and without preexisting cardiovascular disease. [Internet]. The New England journal of medicine. 1990 Jun ;322(24):1700-7.[cited 2010 Dec 23] Available from: http://www.ncbi.nlm.nih.gov/pubmed/2342536

40. De Silva LDR, Kumar A, Sathian B. The significance of lipid profile and positive troponin in predicting cardiac event. [Internet]. Kathmandu University medical journal (KUMJ). 7(27):263-7.[cited 2010 Dec 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20071874

41. Toth PP, Davidson MH. High-density lipoproteins: marker of cardiovascular risk and therapeutic target. [Internet]. Journal of clinical lipidology. 2010 ;4(5):359-64.[cited 2010 Dec 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21122679

42. Zeljkovic A, Vekic J, Spasojevic-Kalimanovska V, Jelic-Ivanovic Z, Bogavac-Stanojevic N, Gulan B, et al. LDL and HDL subclasses in acute ischemic stroke: prediction of risk and short-term mortality. [Internet]. Atherosclerosis. 2010 Jun ;210(2):548-54.[cited 2010 Dec 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20022325

43. Lionel H. Opie. The Heart Physiology: from Cell to Circulation. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1998.

44. Gladstone Research Resource [Internet]. [cited 2011 Jul 20] Available from: http://www.gladstone.ucsf.edu/wp/2008/11/managingbloodlipids/

45. Tselepis AD, John Chapman M. Inflammation, bioactive lipids and atherosclerosis: potential roles of a lipoprotein-associated phospholipase A2, platelet activating factor-acetylhydrolase. [Internet]. Atherosclerosis. Supplements. 2002 Dec ;3(4):57-68.[cited 2011 Jul 23] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12573364

46. Okajima F, Sato K, Kimura T. Anti-atherogenic actions of high-density lipoprotein through sphingosine 1-phosphate receptors and scavenger receptor class B type I. [Internet]. Endocrine journal. 2009 Jan ;56(3):317-34.[cited 2011 Jul 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18753704

47. Sattler K, Levkau B. Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. [Internet]. Cardiovascular research. 2009 May ;82(2):201-11.[cited 2010 Dec 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19233866

48. Theilmeier G, Schmidt C, Herrmann J, Keul P, Schäfers M, Herrgott I, et al. High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. [Internet]. Circulation. 2006 Sep ;114(13):1403-9.[cited 2010 Dec 30] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16982942

49. Argraves KM, Sethi AA, Gazzolo PJ, Wilkerson BA, Remaley AT, Tybjaerg-Hansen A, et al. S1P, dihydro-S1P and C24:1-ceramide levels in the HDL-containing fraction of serum inversely correlate with occurrence of ischemic heart disease. [Internet]. Lipids in health and disease. 2011 Jan ;1070.[cited 2011 Jul 20] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3116499&tool=pmcentrez&rendertype=abstract

50. Horga A, Montalban X. FTY720 (fingolimod) for relapsing multiple sclerosis. [Internet]. Expert review of neurotherapeutics. 2008 May ;8(5):699-714.[cited 2011 Jun 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18457527

51. Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. [Internet]. Science (New York, N.Y.). 2007 Apr ;316(5822):295-8.[cited 2010 Dec 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17363629

52. Murata N, Sato K, Kon J, Tomura H, Yanagita M, Kuwabara A, et al. Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. [Internet]. The Biochemical journal. 2000 Dec ;352 Pt 3809-15.[cited 2010 Dec 25] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1221521&tool=pmcentrez&rendertype=abstract

53. Lucke S, Levkau B. Endothelial Functions of Sphingosine-1-phosphate. [Internet]. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2010 Jan ;26(1):87-96.Available from: http://www.ncbi.nlm.nih.gov/pubmed/20502008

54. Nofer J-R, Der Giet M van, Tölle M, Wolinska I, Wnuck Lipinski K von, Baba HA, et al. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. [Internet]. The Journal of clinical investigation. 2004 Feb ;113(4):569-81.[cited 2010 Dec 25] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=338256&tool=pmcentrez&rendertype=abstract

55. Weis T, Völker W, Holtwick R, Al Chahaf M, Schmidt A. Sphingosine 1-phosphate (S1P) induces expression of E-selectin and adhesion of monocytes via intracellular signalling pathways in vascular endothelial cells. [Internet]. European journal of cell biology. 2010 Oct ;89(10):733-41.[cited 2010 Dec 27] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20656374

56. Means CK, Brown JH. Sphingosine-1-phosphate receptor signalling in the heart. [Internet]. Cardiovascular research. 2009 May ;82(2):193-200.[cited 2010 Dec 27] Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2721649&tool=pmcentrez&rendertype=abstract

57. Wang S, Zhang Z, Lin X, Xu D-S, Feng Y, Ding K. A polysaccharide, MDG-1, induces S1P1 and bFGF expression and augments survival and angiogenesis in the ischemic heart. [Internet]. Glycobiology. 2010 Jan ;20(4):473-84.[cited 2011 Jul 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20008963

58. Sugiyama A, Yatomi Y, Ozaki Y, Hashimoto K. Sphingosine 1-phosphate induces sinus tachycardia and coronary vasoconstriction in the canine heart. [Internet]. Cardiovascular research. 2000 Apr ;46(1):119-25.[cited 2011 Jan 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/10727660

59. Robert P, Tsui P, Laville MP, Livi GP, Sarau HM, Bril A, et al. EDG1 receptor stimulation leads to cardiac hypertrophy in rat neonatal myocytes. [Internet]. Journal of molecular and cellular cardiology. 2001 Sep ;33(9):1589-606.[cited 2011 Jan 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11549339

60. Sekiguchi K, Yokoyama T, Kurabayashi M, Okajima F, Nagai R. Sphingosylphosphorylcholine induces a hypertrophic growth response through the mitogen-activated protein kinase signaling cascade in rat neonatal cardiac myocytes. [Internet]. Circulation research. 1999 Nov ;85(11):1000-8.[cited 2011 Jan 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/10571530

61. Park SW, Kim M, Chen SWC, Brown KM, D□Agati VD, Lee HT. Sphinganine-1-phosphate protects kidney and liver after hepatic ischemia and reperfusion in mice through S1P1 receptor activation. [Internet]. Laboratory investigation; a journal of technical methods and pathology. 2010 Aug ;90(8):1209-24.[cited 2010 Dec 30] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3007623&tool=pmcentrez&rendertype=abstract

62. Vessey DA, Li L, Kelley M, Zhang J, Karliner JS. Sphingosine can pre- and post-condition heart and utilizes a different mechanism from sphingosine 1-phosphate. [Internet]. Journal of biochemical and molecular toxicology. 22(2):113-8.[cited 2011 Jul 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18418901

63. Vessey DA, Li L, Honbo N, Karliner JS. Sphingosine 1-phosphate is an important endogenous cardioprotectant released by ischemic pre- and postconditioning. [Internet]. American journal of physiology. Heart and circulatory physiology. 2009 Oct ;297(4):H1429-35.[cited 2010 Dec 30] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2770755&tool=pmcentrez&rendertype=abstract

64. Vessey DA, Li L, Kelley M, Karliner JS. Combined sphingosine, S1P and ischemic postconditioning rescue the heart after protracted ischemia. [Internet]. Biochemical and biophysical research communications. 2008 Oct ;375(3):425-9.[cited 2010 Dec 30] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18706887

65. Hofmann U, Hu K, Walter F, Burkard N, Ertl G, Bauersachs J, et al. Pharmacological pre- and post-conditioning with the sphingosine-1-phosphate receptor modulator FTY720 after myocardial ischaemia-reperfusion. [Internet]. British journal of pharmacology. 2010 Jul ;160(5):1243-51.[cited 2010 Dec 30] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2936032&tool=pmcentrez&rendertype=abstract

66. Weis T, Völker W, Holtwick R, Al Chahaf M, Schmidt A. Sphingosine 1-phosphate (S1P) induces expression of E-selectin and adhesion of monocytes via intracellular signalling pathways in vascular endothelial cells. [Internet]. European journal of cell biology. 2010 Oct ;89(10):733-41.[cited 2011 Mar 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20656374

67. Sekine Y, Suzuki K, Remaley AT. HDL and sphingosine-1-phosphate activate stat3 in prostate cancer DU145 cells via ERK1/2 and S1P receptors, and promote cell migration and invasion. [Internet]. The Prostate. 2010 Oct ;[cited 2011 Mar 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20979115

68. Means CK, Xiao C-Y, Li Z, Zhang T, Omens JH, Ishii I, et al. Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. [Internet]. American journal of physiology. Heart and circulatory physiology. 2007 Jun ;292(6):H2944-51.[cited 2011 Mar 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17293497

69. Frias MA, James RW, Gerber-Wicht C, Lang U. Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-1-phosphate. [Internet]. Cardiovascular research. 2009 May ;82(2):313-23.[cited 2011 Mar 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19151362

70. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. [Internet]. American journal of physiology. Heart and circulatory physiology. 2005 Feb ;288(2):H971-6.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15358610

71. Lecour S. Multiple protective pathways against reperfusion injury: a SAFE path without Aktion? [Internet]. Journal of molecular and cellular cardiology. 2009 May ;46(5):607-9.[cited 2011 Jan 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19318238

72. Suleman N, Somers S, Smith R, Opie LH, Lecour SC. Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning. [Internet]. Cardiovascular research. 2008 Jul ;79(1):127-33.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18339648

73. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. [Internet]. Circulation research. 2004 Aug ;95(3):230-2.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15242972

74. Lacerda L, Somers S, Opie LH, Lecour S. Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. [Internet]. Cardiovascular research. 2009 Nov ;84(2):201-8.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19666677

75. Hausenloy DJ, Lecour S, Yellon DM. Reperfusion Injury Salvage Kinase and Survivor Activating Factor Enhancement Prosurvival Signaling Pathways in Ischemic Postconditioning: Two Sides of the Same Coin. [Internet]. Antioxidants & redox signaling. 2010 Oct ;[cited 2011 Jan 24] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20615076

76. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. [Internet]. Cardiovascular research. 2004 Mar ;61(3):448-60.[cited 2010 Oct 30] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14962476

77. Yellon DM, Baxter GF. Reperfusion injury revisited: is there a role for growth factor signaling in limiting lethal reperfusion injury? [Internet]. Trends in cardiovascular medicine. 1999 Nov ;9(8):245-9.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11094333

78. Jonassen AK, Sack MN, Mjøs OD, Yellon DM. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. [Internet]. Circulation research. 2001 Dec ;89(12):1191-8.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11739285

79. Bell RM, Yellon DM. Bradykinin limits infarction when administered as an adjunct to reperfusion in mouse heart: the role of PI3K, Akt and eNOS. [Internet]. Journal of molecular and cellular cardiology. 2003 Feb ;35(2):185-93.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12606259

80. Oldenburg O, Qin Q, Krieg T, Yang X-M, Philipp S, Critz SD, et al. Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoKATP channel opening and leads to cardioprotection. [Internet]. American journal of physiology. Heart and circulatory physiology. 2004 Jan ;286(1):H468-76.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12958031

81. Krieg T, Qin Q, Philipp S, Alexeyev MF, Cohen MV, Downey JM. Acetylcholine and bradykinin trigger preconditioning in the heart through a pathway that includes Akt and NOS. [Internet]. American journal of physiology. Heart and circulatory physiology. 2004 Dec ;287(6):H2606-11.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15331366

82. Costa ADT, Pierre SV, Cohen MV, Downey JM, Garlid KD. cGMP signalling in pre- and post-conditioning: the role of mitochondria. [Internet]. Cardiovascular research. 2008 Jan ;77(2):344-52.[cited 2011 Jul 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18006449

83. Zhang Q, Yin H, Liu P, Zhang H, She M. Essential role of HDL on endothelial progenitor cell proliferation with PI3K/Akt/cyclin D1 as the signal pathway. [Internet].

Experimental biology and medicine (Maywood, N.J.). 2010 Sep ;235(9):1082-92.[cited 2011 Mar 4] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20724534

84. Gilles S, Zahler S, Welsch U, Sommerhoff CP, Becker BF. Release of TNF-alpha during myocardial reperfusion depends on oxidative stress and is prevented by mast cell stabilizers. [Internet]. Cardiovascular research. 2003 Dec 1;60(3):608-16.[cited 2011 Jul 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14659806

85. Halawa B, Salomon P, Jołda-Mydłowska B, Zyśko D. [Levels of tumor necrosis factor (TNF-alpha) and interleukin 6 (IL-6) in serum of patients with acute myocardial infarction]. [Internet]. Polskie Archiwum Medycyny Wewnętrznej. 1999 Mar ;101(3):197-203.[cited 2011 Jul 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/10697395

86. Kleinbongard P, Schulz R, Heusch G. TNFα in myocardial ischemia/reperfusion, remodeling and heart failure. [Internet]. Heart failure reviews. 2011 Jan ;16(1):49-69.[cited 2011 Jul 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20571888

87. Lecour S, Suleman N, Deuchar GA, Somers S, Lacerda L, Huisamen B, et al. Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). [Internet]. Circulation. 2005 Dec ;112(25):3911-8.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16344382

88. Barry SP, Townsend PA, Latchman DS, Stephanou A. Role of the JAK-STAT pathway in myocardial injury. [Internet]. Trends in molecular medicine. 2007 Feb ;13(2):82-9.[cited 2011 Jan 5] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17194625

89. Smith RM, Suleman N, Lacerda L, Opie LH, Akira S, Chien KR, et al. Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. [Internet]. Cardiovascular research. 2004 Sep 1;63(4):611-6.[cited 2011 Jul 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15306216

90. Kelly RF, Lamont KT, Somers S, Hacking D, Lacerda L, Thomas P, et al. Ethanolamine is a novel STAT-3 dependent cardioprotective agent. [Internet]. Basic research in cardiology. 2010 Nov ;105(6):763-70.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20938668

91. Montecucco F, Lenglet S, Braunersreuther V, Burger F, Pelli G, Bertolotto M, et al. CB(2) cannabinoid receptor activation is cardioprotective in a mouse model of ischemia/reperfusion. [Internet]. Journal of molecular and cellular cardiology. 2009 May ;46(5):612-20.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19162037

92. Kurdi M, Booz GW. Can the protective actions of JAK-STAT in the heart be exploited therapeutically? Parsing the regulation of interleukin-6-type cytokine signaling.

[Internet]. Journal of cardiovascular pharmacology. 2007 Aug ;50(2):126-41.[cited 2010 Nov 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17703129

93. Pfeffer LM, Mullersman JE, Pfeffer SR, Murti A, Shi W, Yang CH. STAT3 as an adapter to couple phosphatidylinositol 3-kinase to the IFNAR1 chain of the type I interferon receptor. [Internet]. Science (New York, N.Y.). 1997 May ;276(5317):1418-20.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/9162009

94. Lecour S, Suleman N, Deuchar GA, Somers S, Lacerda L, Huisamen B, et al. Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). [Internet]. Circulation. 2005 Dec ;112(25):3911-8.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16344382

95. Yu X, Kennedy RH, Liu SJ. JAK2/STAT3, not ERK1/2, mediates interleukin-6-induced activation of inducible nitric-oxide synthase and decrease in contractility of adult ventricular myocytes. [Internet]. The Journal of biological chemistry. 2003 May ;278(18):16304-9.[cited 2011 Jan 5] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12595539

96. Wegrzyn J, Potla R, Chwae Y-J, Sepuri NBV, Zhang Q, Koeck T, et al. Function of mitochondrial Stat3 in cellular respiration. [Internet]. Science (New York, N.Y.). 2009 Feb ;323(5915):793-7.Available from: http://www.ncbi.nlm.nih.gov/pubmed/19131594

97. Reich NC. STAT3 revs up the powerhouse. [Internet]. Science signaling. 2009 Jan ;2(90):pe61.[cited 2011 Jan 5] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19797267

98. Liu L, McBride KM, Reich NC. STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3. [Internet]. Proceedings of the National Academy of Sciences of the United States of America. 2005 Jun ;102(23):8150-5.[cited 2011 Jan 5] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1149424&tool=pmcentrez&rendertype=abstract

99. Boengler K, Hilfiker-Kleiner D, Heusch G, Schulz R. Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. [Internet]. Basic research in cardiology. 2010 Nov ;105(6):771-85.[cited 2011 Jan 6] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2978889&tool=pmcentrez&rendertype=abstract

100. Mitchell P, Moyle J. Chemiosmotic hypothesis of oxidative phosphorylation. [Internet]. Nature. 1967 Jan ;213(5072):137-9.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/4291593

101. Skárka L, Ostádal B. Mitochondrial membrane potential in cardiac myocytes. [Internet]. Physiological research / Academia Scientiarum Bohemoslovaca. 2002 Jan

;51(5):425-34.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12470194

102. Palmer JW, Tandler B, Hoppel CL. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. [Internet]. The Journal of biological chemistry. 1977 Dec ;252(23):8731-9.Available from: http://www.ncbi.nlm.nih.gov/pubmed/925018

103. Karmazyn M. The 1990 Merck Frosst Award. Ischemic and reperfusion injury in the heart. Cellular mechanisms and pharmacological interventions. [Internet]. Canadian journal of physiology and pharmacology. 1991 Jun ;69(6):719-30.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/1913318

104. Palmer JW, Tandler B, Hoppel CL. Biochemical differences between subsarcolemmal and interfibrillar mitochondria from rat cardiac muscle: effects of procedural manipulations. [Internet]. Archives of biochemistry and biophysics. 1985 Mar ;236(2):691-702.[cited 2010 Dec 29] Available from: http://www.ncbi.nlm.nih.gov/pubmed/2982322

105. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. [Internet]. Journal of molecular and cellular cardiology. 2001 Jun ;33(6):1065-89.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11444914

106. Picture of a Mitochondrion [Internet]. [cited 2011 Feb 26] Available from: http://www.greetin.gs/phoebecell/photogallery/mitochondria.jpg

107. User:Rozzychan. Picture of ETC [Internet]. 2006 ;[cited 2011 Feb 25] Available from: http://commons.wikimedia.org/wiki/File:Mitochondrial_electron_transport_chain.png

108. Duchen MR. Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. [Internet]. The Journal of physiology. 1999 Apr ;516 ( Pt 11-17.[cited 2010 Sep 28] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2269224&tool=pmcentrez&rendertype=abstract

109. Ichas F, Mazat JP. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. [Internet]. Biochimica et biophysica acta. 1998 Aug ;1366(1-2):33-50.[cited 2011 Jan 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/9714722

110. Rizzuto R, Brini M, Murgia M, Pozzan T. Microdomains with high Ca2+ close to IP3-sensitive channels that are sensed by neighboring mitochondria. [Internet]. Science (New York, N.Y.). 1993 Oct ;262(5134):744-7.[cited 2011 Jan 13] Available from: http://www.ncbi.nlm.nih.gov/pubmed/8235595

111. Chinopoulos C, Adam-Vizi V. Mitochondrial Ca2+ sequestration and precipitation revisited. [Internet]. The FEBS journal. 2010 Sep ;277(18):3637-51.[cited 2011 Jan 13] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20659160

112. Rossi CS, Lehninger AL. Stoiciometry of respiratory stimulation, accumulation of Ca++ and phosphate, and oxidative phosphorylation in rat liver mitochondria. [Internet]. The Journal of biological chemistry. 1964 Nov ;2393971-80.[cited 2011 Jan 13] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14257633

113. Halestrap AP. Calcium, mitochondria and reperfusion injury: a pore way to die. [Internet]. Biochemical Society transactions. 2006 Apr ;34(Pt 2):232-7.[cited 2011 Jan 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16545083

114. Gustafsson AB, Gottlieb RA. Heart mitochondria: gates of life and death. [Internet]. Cardiovascular research. 2008 Jan ;77(2):334-43.[cited 2011 Jan 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18006487

115. Turrens JF. Mitochondrial formation of reactive oxygen species. [Internet]. The Journal of physiology. 2003 Oct ;552(Pt 2):335-44.[cited 2010 Oct 25] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2343396&tool=pmcentrez&rendertype=abstract

116. Nordberg J, Arnér ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. [Internet]. Free radical biology & medicine. 2001 Dec ;31(11):1287-312.[cited 2010 Jul 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11728801

117. Otani H. Reactive oxygen species as mediators of signal transduction in ischemic preconditioning. [Internet]. Antioxidants & redox signaling. 2004 Apr ;6(2):449-69.[cited 2011 Jan 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15025947

118. Hausenloy DJ, Tsang A, Yellon DM. The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. [Internet]. Trends in cardiovascular medicine. 2005 Feb ;15(2):69-75.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15885573

119. Chen W, Gabel S, Steenbergen C, Murphy E. A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. [Internet]. Circulation research. 1995 Aug ;77(2):424-9.[cited 2011 Jan 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/7614726

120. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K, et al. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. [Internet]. Circulation research. 2001 Mar ;88(5):529-35.[cited 2010 Aug 16] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11249877

121. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. [Internet]. The Journal of experimental medicine. 2000 Oct ;192(7):1001-14.[cited 2011 Jan 11] Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2193314&tool=pmcentrez&rendertype=abstract

122. Zoratti M, Szabò I. The mitochondrial permeability transition. [Internet]. Biochimica et biophysica acta. 1995 Jul ;1241(2):139-76.Available from: http://www.ncbi.nlm.nih.gov/pubmed/7640294

123. Fontaine E, Eriksson O, Ichas F, Bernardi P. Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation By electron flow through the respiratory chain complex i. [Internet]. The Journal of biological chemistry. 1998 May ;273(20):12662-8.[cited 2011 Jan 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/9575229

124. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. [Internet]. Nature. 2005 Mar ;434(7033):658-62.[cited 2011 Jan 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15800627

125. Connern CP, Halestrap AP. Purification and N-terminal sequencing of peptidyl-prolyl cis-trans-isomerase from rat liver mitochondrial matrix reveals the existence of a distinct mitochondrial cyclophilin. [Internet]. The Biochemical journal. 1992 Jun ;284 ( Pt 2381-5.[cited 2011 Jun 11] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1132649&tool=pmcentrez&rendertype=abstract

126. Woodfield K, Rück A, Brdiczka D, Halestrap AP. Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. [Internet]. The Biochemical journal. 1998 Dec ;336 ( Pt 2287-90.[cited 2011 Jun 11] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1219869&tool=pmcentrez&rendertype=abstract

127. Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P. Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. [Internet]. The Journal of biological chemistry. 2005 May ;280(19):18558-61.[cited 2011 Jun 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15792954

128. Zheng Y, Shi Y, Tian C, Jiang C, Jin H, Chen J, et al. Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome c release induced by arsenic trioxide. [Internet]. Oncogene. 2004 Feb ;23(6):1239-47.[cited 2011 Jun 11] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2913247&tool=pmcentrez&rendertype=abstract

129. Crompton M, Virji S, Ward JM. Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. [Internet]. European journal of biochemistry / FEBS. 1998 Dec ;258(2):729-35.[cited 2011 Jun 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/9874241

130. Chorna SV, Dosenko VI, Strutyns□ka NA, Vavilova HL, Sahach VF. [Increased expression of voltage-dependent anion channel and adenine nucleotide translocase and the sensitivity of calcium-induced mitochondrial permeability transition opening pore in the old rat heart]. [Internet]. Fiziolohichnyĭ zhurnal (Kiev, Ukraine : 1994). 2010 Jan ;56(4):19-25.[cited 2011 Jun 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20968034

131. Rostovtseva TK, Tan W, Colombini M. On the role of VDAC in apoptosis: fact and fiction. [Internet]. Journal of bioenergetics and biomembranes. 2005 Jun ;37(3):129-42.[cited 2011 Jun 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16167170

132. Halestrap AP, Davidson AM. Inhibition of Ca2(+)-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nuc [Internet]. The Biochemical journal. 1990 May ;268(1):153-60.[cited 2011 Jun 11] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1131405&tool=pmcentrez&rendertype=abstract

133. Vyssokikh MY, Katz A, Rueck A, Wuensch C, Dörner A, Zorov DB, et al. Adenine nucleotide translocator isoforms 1 and 2 are differently distributed in the mitochondrial inner membrane and have distinct affinities to cyclophilin D. [Internet]. The Biochemical journal. 2001 Sep ;358(Pt 2):349-58.[cited 2011 Jun 11] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1222067&tool=pmcentrez&rendertype=abstract

134. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, et al. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. [Internet]. Nature. 2004 Jan ;427(6973):461-5.[cited 2010 Oct 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14749836

135. Hausenloy DJ, Ong S-B, Yellon DM. The mitochondrial permeability transition pore as a target for preconditioning and postconditioning. [Internet]. Basic research in cardiology. 2009 Mar ;104(2):189-202.[cited 2011 Jan 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19242644

136. Penna C, Mancardi D, Rastaldo R, Pagliaro P. Cardioprotection: a radical view Free radicals in pre and postconditioning. [Internet]. Biochimica et biophysica acta. 2009 Jul ;1787(7):781-93.[cited 2010 Jul 29] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19248760

137. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. [Internet]. Science (New York, N.Y.). 2004 Jul ;305(5684):626-9.[cited 2010 Sep 9] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15286356

138. Zou H, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. [Internet]. The Journal of biological chemistry. 1999 Apr ;274(17):11549-56.[cited 2010 Jul 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/10206961

139. Shiozaki EN, Shi Y. Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. [Internet]. Trends in biochemical sciences. 2004 Sep ;29(9):486-94.[cited 2010 Jun 29] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15337122

140. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. [Internet]. Cell. 2000 Jul ;102(1):33-42.Available from: http://www.ncbi.nlm.nih.gov/pubmed/10929711

141. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. [Internet]. Molecular cell. 2001 Sep ;8(3):613-21.[cited 2011 Jan 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11583623

142. Empel VPM van, Bertrand AT, Der Nagel R van, Kostin S, Doevendans PA, Crijns HJ, et al. Downregulation of apoptosis-inducing factor in harlequin mutant mice sensitizes the myocardium to oxidative stress-related cell death and pressure overload-induced decompensation. [Internet]. Circulation research. 2005 Jun ;96(12):e92-e101.[cited 2010 Oct 4] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15933268

143. Candé C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N, et al. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. [Internet]. Biochimie. 84(2-3):215-22.[cited 2011 Jan 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12022952

144. Kim G-T, Chun Y-S, Park J-W, Kim M-S. Role of apoptosis-inducing factor in myocardial cell death by ischemia-reperfusion. [Internet]. Biochemical and biophysical research communications. 2003 Sep ;309(3):619-24.[cited 2011 Jan 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12963035

145. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. [Internet]. Nature. 2001 Jul ;412(6842):95-9.Available from: http://www.ncbi.nlm.nih.gov/pubmed/11452314

146. Côté J, Ruiz-Carrillo A. Primers for mitochondrial DNA replication generated by endonuclease G. [Internet]. Science (New York, N.Y.). 1993 Aug ;261(5122):765-9.[cited 2011 Jan 12] Available from: http://www.ncbi.nlm.nih.gov/pubmed/7688144

147. National Research Council. Guide for the Care and use of Laboratory Animals [Internet]. Eighth Edi. National Academies Press; 2011. Available from: www.nap.edu

148. Smith RM, Suleman N, Lacerda L, Opie LH, Akira S, Chien KR, et al. Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. [Internet]. Cardiovascular research. 2004 Sep 1;63(4):611-6.[cited 2011 Jul 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15306216

149. Boecker W, Bernecker OY, Wu JC, Zhu X, Sawa T, Grazette L, et al. Cardiac-specific gene expression facilitated by an enhanced myosin light chain promoter. [Internet].

Molecular imaging. 2004 Apr ;3(2):69-75.[cited 2011 Jul 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15296671

150. Bell RM, Mocanu MM, Yellon DM. Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. [Internet]. Journal of molecular and cellular cardiology. 2011 Jun ;50(6):940-50.[cited 2011 Jul 8] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21385587

151. Isolated Heart [Internet]. 2010 ;[cited 2011 Jul 8] Available from: http://doctorexclusive.com/?p=2156

152. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. [Internet]. Journal of molecular and cellular cardiology. 2002 May ;34(5):509-18.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12056855

153. Costa ADT, Quinlan CL, Andrukhiv A, West IC, Jaburek M, Garlid KD. The direct physiological effects of mitoKATP opening on heart mitochondria [Internet]. American Journal of Physiology- Heart and Circulatory Physiology. 2006 ;290(1):H406.[cited 2011 Jan 27] Available from: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:The+direct+physiological+effects+of+mitoK+ATP+opening+on+heart+mitochondria#0

154. Pertoft H. Fractionation of cells and subcellular particles with Percoll. [Internet]. Journal of biochemical and biophysical methods. 2000 Jul ;44(1-2):1-30.[cited 2010 Oct 6] Available from: http://www.ncbi.nlm.nih.gov/pubmed/10889273

155. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. [Internet]. The Journal of biological chemistry. 1951 Nov ;193(1):265-75.[cited 2010 Dec 20] Available from: http://www.ncbi.nlm.nih.gov/pubmed/14907713

156. Lacerda L, McCarthy J, Mungly SFK, Lynn EG, Sack MN, Opie LH, et al. TNFα protects cardiac mitochondria independently of its cell surface receptors. [Internet]. Basic research in cardiology. 2010 Nov ;105(6):751-62.[cited 2011 Jan 27] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20680307

157. Mujahid A, Sato K, Akiba Y, Toyomizu M. Acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, possibly via downregulation of uncoupling protein content. [Internet]. Poultry science. 2006 Jul ;85(7):1259-65.[cited 2011 Feb 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16830867

158. Lucas DT, Szweda LI. Declines in mitochondrial respiration during cardiac reperfusion: age-dependent inactivation of alpha-ketoglutarate dehydrogenase. [Internet]. Proceedings of the National Academy of Sciences of the United States of America. 1999 Jun ;96(12):6689-93.Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=21976&tool=pmcentrez&rendertype=abstract

159. Khaliulin I, Schwalb H, Wang P, Houminer E, Grinberg L, Katzeff H, et al. Preconditioning improves postischemic mitochondrial function and diminishes oxidation of mitochondrial proteins [Internet]. Free Radical Biology and Medicine. 2004 ;37(1):1–9.[cited 2011 Jan 27] Available from: http://linkinghub.elsevier.com/retrieve/pii/S0891584904003387

160. Lim SY, Davidson SM, Paramanathan AJ, Smith CCT, Yellon DM, Hausenloy DJ. The novel adipocytokine visfatin exerts direct cardioprotective effects. [Internet]. Journal of cellular and molecular medicine. 2008 Aug ;12(4):1395-403.[cited 2011 Jan 27] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2905617&tool=pmcentrez&rendertype=abstract

161. Adam T, Opie L, Faadiel E. Transcriptional Regulation of Cardiac Acetyl-Coenzyme A Carboxylase Gene by Nuclear Respiratory Factor-1. SA Heart Journal. 2008 ;5(4):192-249.

162. Sharov VG, Todor AV, Imai M, Sabbah HN. Inhibition of mitochondrial permeability transition pores by cyclosporine A improves cytochrome C oxidase function and increases rate of ATP synthesis in failing cardiomyocytes. [Internet]. Heart failure reviews. 2005 Dec ;10(4):305-10.[cited 2011 Feb 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/16583179

163. Ganote CE, Armstrong SC. Effects of CCCP-induced mitochondrial uncoupling and cyclosporin A on cell volume, cell injury and preconditioning protection of isolated rabbit cardiomyocytes. [Internet]. Journal of molecular and cellular cardiology. 2003 Jul ;35(7):749-59.[cited 2011 Feb 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12818565

164. Ong S-B, Subrayan S, Lim SY, Yellon DM, Davidson SM, Hausenloy DJ. Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. [Internet]. Circulation. 2010 May ;121(18):2012-22.[cited 2011 Feb 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20421521

165. Zhang J, Honbo N, Goetzl EJ, Chatterjee K, Karliner JS, Gray MO. Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia. [Internet]. American journal of physiology. Heart and circulatory physiology. 2007 Nov ;293(5):H3150-8.[cited 2011 Feb 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17766476

166. Hausenloy DJ, Lim SY, Ong S-G, Davidson SM, Yellon DM. Mitochondrial cyclophilin-D as a critical mediator of ischaemic preconditioning. [Internet]. Cardiovascular research. 2010 Oct ;88(1):67-74.[cited 2011 Feb 3] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2936122&tool=pmcentrez&rendertype=abstract

167. Strober W. Trypan blue exclusion test of cell viability. [Internet]. Current protocols in immunology / edited by John E. Coligan ... [et al.]. 2001 May ;Appendix 3Appendix 3B.Available from: http://www.ncbi.nlm.nih.gov/pubmed/18432654

168. Neuromics. TMRE/TMRM Mitochondrial Membrane Potential Assessment Kit [Internet]. Test. Available from: www.neuromics.com

169. Ward MW. Quantitative analysis of membrane potentials. [Internet]. Methods in molecular biology (Clifton, N.J.). 2010 Jan ;591335-51.[cited 2011 Feb 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19957140

170. Ruiz-Meana M, Abellán A, Miró-Casas E, Agulló E, Garcia-Dorado D. Role of sarcoplasmic reticulum in mitochondrial permeability transition and cardiomyocyte death during reperfusion. [Internet]. American journal of physiology. Heart and circulatory physiology. 2009 Oct ;297(4):H1281-9.[cited 2011 Jul 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19684187

171. Yamada S, Zhang XQ, Kadono T, Matsuoka N, Rollins D, Badger T, et al. Direct toxic effects of aqueous extract of cigarette smoke on cardiac myocytes at clinically relevant concentrations. [Internet]. Toxicology and applied pharmacology. 2009 Apr ;236(1):71-7.[cited 2011 Feb 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19371621

172. Kvetny J, Bomholt T, Pedersen P, Wilms L, Anthonsen S, Larsen J. Thyroid hormone effect on human mitochondria measured by flow cytometry. [Internet]. Scandinavian journal of clinical and laboratory investigation. 2009 Jan ;69(7):772-6.[cited 2011 Feb 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19929720

173. Davidson SM, Yellon D, Duchen MR. Assessing mitochondrial potential, calcium, and redox state in isolated mammalian cells using confocal microscopy. [Internet]. Methods in molecular biology (Clifton, N.J.). 2007 Jan ;372421-30.[cited 2011 Feb 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18314743

174. Exner N, Treske B, Paquet D, Holmström K, Schiesling C, Gispert S, et al. Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. [Internet]. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2007 Nov ;27(45):12413-8.[cited 2010 Sep 25] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17989306

175. Invitrogen Spectraviewer [Internet]. [cited 2011 Jan 12] Available from: http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html?fileId1=6121meoh

176. Tao R, Zhang J, Vessey D a, Honbo N, Karliner JS. Deletion of the sphingosine kinase-1 gene influences cell fate during hypoxia and glucose deprivation in adult mouse cardiomyocytes. [Internet]. Cardiovascular research. 2007 Apr ;74(1):56-63.[cited 2011 Feb 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17320845

177. Plásek J, Vojtísková a, Houstek J. Flow-cytometric monitoring of mitochondrial depolarisation: from fluorescence intensities to millivolts. [Internet]. Journal of photochemistry and photobiology. B, Biology. 2005 Feb ;78(2):99-108.[cited 2010 Aug 26] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15664496

178. Mattiasson G. Flow cytometric analysis of isolated liver mitochondria to detect changes relevant to cell death. [Internet]. Cytometry. Part A : the journal of the International Society for Analytical Cytology. 2004 Aug ;60(2):145-54.[cited 2010 Dec 17] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15290715

179. Strijdom H, Muller C, Lochner A. Direct intracellular nitric oxide detection in isolated adult cardiomyocytes: flow cytometric analysis using the fluorescent probe, diaminofluorescein. [Internet]. Journal of molecular and cellular cardiology. 2004 Oct ;37(4):897-902.[cited 2011 Feb 27] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15380680

180. Strijdom H, Muller C, Lochner A. Direct intracellular nitric oxide detection in isolated adult cardiomyocytes: flow cytometric analysis using the fluorescent probe, diaminofluorescein. [Internet]. Journal of molecular and cellular cardiology. 2004 Oct ;37(4):897-902.[cited 2011 Jun 14] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15380680

181. Bugger H, Guzman C, Zechner C, Palmeri M, Russell KS, Russell RR. Uncoupling protein downregulation in doxorubicin-induced heart failure improves mitochondrial coupling but increases reactive oxygen species generation. [Internet]. Cancer chemotherapy and pharmacology. 2010 Aug ;[cited 2011 Feb 17] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20809120

182. Lamont KT, Somers S, Lacerda L, Opie LH, Lecour S. Is red wine a SAFE sip away from cardioprotection? Mechanisms involved in resveratrol- and melatonin-induced cardioprotection. [Internet]. Journal of pineal research. 2011 May ;50(4):374-80.[cited 2011 Jun 11] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21342247

183. Bester DJ, Kupai K, Csont T, Szucs G, Csonka C, Esterhuyse AJ, et al. Dietary red palm oil supplementation reduces myocardial infarct size in an isolated perfused rat heart model. [Internet]. Lipids in health and disease. 2010 Jan ;964.[cited 2011 Jun 28] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2906443&tool=pmcentrez&rendertype=abstract

184. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. [Internet]. Journal of molecular and cellular cardiology. 2002 May ;34(5):509-18.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12056855

185. Heinen A, Winning A, Schlack W, Hollmann MW, Preckel B, Frässdorf J, et al. The regulation of mitochondrial respiration by opening of mKCa channels is age-dependent. [Internet]. European journal of pharmacology. 2008 Jan ;578(2-3):108-13.[cited 2011 Jan 27] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17936270

186. Liem D a, Manintveld OC, Schoonderwoerd K, McFalls EO, Heinen A, Verdouw PD, et al. Ischemic preconditioning modulates mitochondrial respiration, irrespective of the employed signal transduction pathway. [Internet]. Translational research : the journal

of laboratory and clinical medicine. 2008 Jan ;151(1):17-26.[cited 2011 Jan 27] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18061124

187. Alizadeh AM, Faghihi M, Sadeghipour HR, Mohammadghasemi F, Khori V. Role of endogenous oxytocin in cardiac ischemic preconditioning. [Internet]. Regulatory peptides. 2011 Feb 25;167(1):86-90.[cited 2011 Jul 14] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21130120

188. Karliner JS, Honbo N, Summers K, Gray MO, Goetzl EJ. The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. [Internet]. Journal of molecular and cellular cardiology. 2001 Sep ;33(9):1713-7.[cited 2011 Jul 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/11549349

189. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. [Internet]. Journal of molecular and cellular cardiology. 2002 May ;34(5):509-18.[cited 2011 Jan 10] Available from: http://www.ncbi.nlm.nih.gov/pubmed/12056855

190. Lucke S, Levkau B. Endothelial functions of sphingosine-1-phosphate. [Internet]. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2010 Jan ;26(1):87-96.[cited 2011 Jun 17] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20502008

191. Hoefer J, Azam MA, Kroetsch JTE, Leong-Poi H, Momen MA, Voigtlaender-Bolz J, et al. Sphingosine-1-phosphate-dependent activation of p38 MAPK maintains elevated peripheral resistance in heart failure through increased myogenic vasoconstriction. [Internet]. Circulation research. 2010 Oct ;107(7):923-33.[cited 2011 Jul 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20671234

192. Alewijnse AE, Peters SLM, Michel MC. Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites. [Internet]. British journal of pharmacology. 2004 Nov ;143(6):666-84.[cited 2011 Jul 22] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1575924&tool=pmcentrez&rendertype=abstract

193. Frias MA, Lang U, Gerber-Wicht C, James RW. Native and reconstituted HDL protect cardiomyocytes from doxorubicin-induced apoptosis. [Internet]. Cardiovascular research. 2010 Jan 1;85(1):118-26.[cited 2011 Jul 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19700468

194. Tabet F, Lambert G, Cuesta Torres LF, Hou L, Sotirchos I, Touyz RM, et al. Lipid-free apolipoprotein A-I and discoidal reconstituted high-density lipoproteins differentially inhibit glucose-induced oxidative stress in human macrophages. [Internet]. Arteriosclerosis, thrombosis, and vascular biology. 2011 May ;31(5):1192-200.[cited 2011 Jul 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21330603

195. Shi N, Wu M-P. Apolipoprotein A-I attenuates renal ischemia/reperfusion injury in rats. [Internet]. Journal of biomedical science. 2008 Sep ;15(5):577-83.[cited 2011 Jul 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18535924

196. Park K-H, Cho K-H. High-density lipoprotein (HDL) from elderly and reconstituted HDL containing glycated apolipoproteins A-I share proatherosclerotic and prosenescent properties with increased cholesterol influx. [Internet]. The journals of gerontology. Series A, Biological sciences and medical sciences. 2011 May ;66(5):511-20.[cited 2011 Jul 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21415260

197. Frias M, Somers S, Lacerda L, James R, Lecour S. HDL protects against lethal reperfusion injury via the SAFE pathway. SA Heart Journal. 2010 ;7(3):202.

198. Christoffersen C, Obinata H, Kumaraswamy SB, Galvani S, Ahnström J, Sevvana M, et al. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. [Internet]. Proceedings of the National Academy of Sciences of the United States of America. 2011 Jun ;108(23):9613-8.[cited 2011 Jul 22] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3111292&tool=pmcentrez&rendertype=abstract

199. Frias MA, James RW, Gerber-Wicht C, Lang U. Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-1-phosphate. [Internet]. Cardiovascular research. 2009 May 1;82(2):313-23.[cited 2011 Jul 3] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19151362

200. Suleman N, Somers S, Smith R, Opie LH, Lecour SC. Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning. [Internet]. Cardiovascular research. 2008 Jul 1;79(1):127-33.[cited 2011 Jan 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18339648

201. Sehgal PB. Paradigm shifts in the cell biology of STAT signaling. [Internet]. Seminars in cell & developmental biology. 2008 Aug ;19(4):329-40.[cited 2011 Jul 24] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2597702&tool=pmcentrez&rendertype=abstract

202. Quinlan CL, Costa ADT, Costa CL, Pierre SV, Dos Santos P, Garlid KD. Conditioning the heart induces formation of signalosomes that interact with mitochondria to open mitoKATP channels. [Internet]. American journal of physiology. Heart and circulatory physiology. 2008 Sep ;295(3):H953-H961.[cited 2011 Jul 5] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2544503&tool=pmcentrez&rendertype=abstract

203. Sekine Y, Suzuki K, Remaley AT. HDL and sphingosine-1-phosphate activate stat3 in prostate cancer DU145 cells via ERK1/2 and S1P receptors, and promote cell migration and invasion. [Internet]. The Prostate. 2010 Oct 26;[cited 2011 Mar 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20979115

204. Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, et al. Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. [Internet]. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2011 Feb ;25(2):600-12.[cited 2011 Feb 23] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3023391&tool=pmcentrez&rendertype=abstract

205. Gomez L. New mechanisms and targets in ischemia [Internet]. 2011 ;[cited 2011 Jul 22] Available from: http://spo.escardio.org/Presenterdetails.aspx

206. Imaizumi S, Miura S-ichiro, Nakamura K, Kiya Y, Uehara Y, Zhang B, et al. Antiarrhythmogenic effect of reconstituted high-density lipoprotein against ischemia/reperfusion in rats. [Internet]. Journal of the American College of Cardiology. 2008 Apr 22;51(16):1604-12.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18420105

207. Patel S, Drew BG, Nakhla S, Duffy SJ, Murphy AJ, Barter PJ, et al. Reconstituted high-density lipoprotein increases plasma high-density lipoprotein anti-inflammatory properties and cholesterol efflux capacity in patients with type 2 diabetes. [Internet]. Journal of the American College of Cardiology. 2009 Mar 17;53(11):962-71.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19281927

208. Shaw JA, Bobik A, Murphy A, Kanellakis P, Blombery P, Mukhamedova N, et al. Infusion of reconstituted high-density lipoprotein leads to acute changes in human atherosclerotic plaque. [Internet]. Circulation research. 2008 Nov 7;103(10):1084-91.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/18832751

209. Tardif J-C, Grégoire J, L□Allier PL, Ibrahim R, Lespérance J, Heinonen TM, et al. Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial. [Internet]. JAMA : the journal of the American Medical Association. 2007 Apr 18;297(15):1675-82.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17387133

210. Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. [Internet]. Nature reviews. Drug discovery. 2010 Nov ;9(11):883-97.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21031003

211. Calkin AC, Drew BG, Ono A, Duffy SJ, Gordon MV, Schoenwaelder SM, et al. Reconstituted high-density lipoprotein attenuates platelet function in individuals with type 2 diabetes mellitus by promoting cholesterol efflux. [Internet]. Circulation. 2009 Nov ;120(21):2095-104.[cited 2011 Jul 22] Available from: http://www.ncbi.nlm.nih.gov/pubmed/19901191

212. Asztalos BF, Tani M, Schaefer EJ. Metabolic and functional relevance of HDL subspecies. [Internet]. Current opinion in lipidology. 2011 Jun ;22(3):176-85.[cited 2011 Jul 19] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21537175

213. Tao R, Hoover HE, Honbo N, Kalinowski M, Alano CC, Karliner JS, et al. High-density lipoprotein determines adult mouse cardiomyocyte fate after hypoxia-reoxygenation through lipoprotein-associated sphingosine 1-phosphate. [Internet]. American journal of physiology. Heart and circulatory physiology. 2010 Mar ;298(3):H1022-8.[cited 2011 Feb 23] Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2838562&tool=pmcentrez &rendertype=abstract