UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Human Development and Health

Effects of acute early age statin treatment on rat vasculature

by

Piia Heidi Marjatta Keskiväli-Bond

Thesis for the degree of Doctor of Philosophy

October 2013

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Cardiovascular Physiology

Thesis for the degree of Doctor of Philosophy

EFFECTS OF ACUTE EARLY AGE STATIN TREATMENT ON RAT VASCULATURE

Piia Heidi Marjatta Keskiväli-Bond

A poor developmental environment increases the risk of cardiovascular disease in humans; this is modelled by protein restriction (PR) during pregnancy in rats. Chronic statin treatment in the PR model from an early age restores endothelial function while an acute early age angiotensin blockade reduces blood pressure long term. The aim of this study was to investigate if acute early age statin treatment could improve vascular function long term in the PR offspring.

Female Wistar rats were fed control (18 % protein) or PR (9% casein) diet throughout pregnancy. Subgroups of the offspring from both maternal groups received atorvastatin (10 mg/kg/day) in their drinking water from three to five weeks of age. Vascular reactivity was assessed at five and sixteen weeks. Components of the vasoreactivity signalling pathways were investigated with real-time PCR and Western blotting.

Maternal PR did not alter vascular function in the offspring and the effects of statin treatment were independent of maternal diet. At five weeks of age maximal constriction to phenylephrine was reduced in the statin treated male offspring aortae ($p<0.01$). Conversely, at sixteen weeks maximal constriction was increased of male ($p<0.05$) and female ($p<0.01$) statin treated offspring mesenteric arteries. RhoA and ROCK1 mRNA levels were increased in statin treated offspring mesenteric arteries at sixteen weeks ($p<0.05$). Statin treatment had no effect on NO-mediated vasorelaxation.

This study shows that early age acute statin treatment has both short and long term effects on cardiovascular function and molecular markers in rats. The long term effects appear to have arisen as a consequence of the withdrawal rather than being a direct effect of the statin treatment adding to the emerging evidence of a rebound effect after statin withdrawal. The RhoA pathway is indicated as the mechanisms behind the effects of statin withdrawal in this study.

Contents

List of tables

List of figures

DECLARATION OF AUTHORSHIP

I, Piia Heidi Marjatta Keskiväli-Bond declare that the thesis entitled

Effects of early age acute statin treatment on rat vasculatrure

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission, or [delete as appropriate] parts of this work have been published as: [please list references]

Signed: ……..

Date:…….

Acknowledgements

Dr Christopher Torrens, Prof. Geraldine Clough & Prof Nick Curzen: Thank you to all my supervisors for all the guidance and support they've given me.

Dr Christopher Kelsall: For all the help with myogrphy and any random queries I have had. Also for great lunch and coffee breaks.

Nicola Irvine: For the times in and out of the lab. A good friend to puzzle over any difficulties with.

Melissa Doherty: For all the guidance and help in the vascular lab and later in the molecular lab.

Kerry Hyde: For the help getting me started with molecular work and help around the molecular lab.

Dr Kate Bull: For the help with the aorta myography.

Emma Lofthouse, Claire Simner, Katy Gould, Sarah Finn & Kasia Kuliga: For the friendship that has helped me through small and some bigger problems

Dr Rohan Lewis: For all the extra advice in science and some interesting conversations.

Dr Jane Cleal: For teaching me Western blotting and being a great example on how to deal with reps.

James Atherton: Last but not least, for all the laughs and balanced perspective. You will always live in our memories.

My husband Matthew Bond, parents, Paula Karlsson and Kalle Keskiväli and brothers Teemu and Juha Keskiväli: For being there for me all these years and helping me to get here.

Definitions and Abbreviations

ACh	acetylcholine
ANOVA	analysis of variance
ApoE	Apolipoprotein E
BH4	tetrahydrobiopterin
BMI	body mass index
BSA	bovine serum albumin
C	control diet (18% casein)
cDNA	complimentary DNA
cGMP	cyclic guanidine monophosphate
COX	cyclo-oxygenase
CRP	c-reactive protein
CS	control diet (18 % casein) + statin (10mg/kg)
C_t	threshold cycle
CVD	cardiovascular disease
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC_{50}	concentration producing 50 % of maximal response
EC_{80}	concentration producing 80 % of maximal response
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
eNOS	endothelial nitric oxide synthase
ET	endothelin
GPCR	G-protein coupled receptor
HDAC	histone deacetylase
HDL	high density lipoprotein
HKG	housekeeping gene
HMG-Co A	3-hydroxy-3-methylglutaryl coenzyme A
HR	heart rate
HRP	horse radish peroxidase
IC_{100}	internal circumference corresponding to 100 mmHg of intramural pressure
INDO	indomethacin
IP_3	inositol trisphosphate
IUGR	intrauterine growth restriction
KPSS	PSS with equimolar substitution of K^+ for Na^+
LDL	low density lipoprotein
L-NAME	N_ω-nitro-L-arginine methyl ester
MAP	mean arterial pressure
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phophatase
mRNA	messenger ribonucleic acid
NaKATPase	sodium potassium ATPase
NO	nitric oxide
O_2-	superoxide
PE	phenylephrine
PLC	phospholipase C
PR	protein restricted diet (9% casein)
PRS	protein restricted diet (9% casein) + statin (10 mg/kg)
PSS	physiological salt solution
PVDF	polyvinylidene fluoride
RhoA	Ras homologue gene family, member A
RNA	ribonucleic acid
ROCK	Rho kinase
ROS	reactive oxygen species
rtPCR	real-time PCR
SBP	systolic blood pressure
SDS	sodium dodecyl sufate
SDS-PAGE	sodium dodecyl sufate poly agrylamide gel electrophoresis
SEM	standard error of mean
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SMC	smooth muscle cells
SNP	sodium nitroprusside

Chapter 1 Introduction

1.1 Cardiovascular function and disease

The cardiovascular system consists of the heart and vasculature and functions as the transportation system of the body. Cardiovascular disease (CVD) is an umbrella term covering all the conditions where the proper function of the heart and vasculature are impaired. CVD has severe long term implications with a high health burden and it accounts for a third of all deaths in the UK (Scarborough P *et al.*, 2011) as well as worldwide (World Health Organization, 2004). Understanding the aspects of normal cardiovascular function and the processes involved in the disease is crucial for the prevention of the disease.

1.1.1 Structure of blood vessels

The vascular wall is formed of three layers; tunica intima, tunica media and tunica adventitia (Figure 1.1). The adventitia is the outermost layer and mainly formed of fibroblasts and connective tissue. The media is formed of smooth muscle cells (SMC) which are responsible for maintaining the vascular tone. The smooth muscle is flanked by the internal and external elastic lamina. The media is thicker in the arteries than in the veins allowing better control of the vascular tone. The endothelium forms the intima.

1.1.1.1 Vascular smooth muscle

The SMC have a central nucleus and are positioned around the vessel wall. The level of constriction of the SMC determines the luminal diameter of the vessel and the relative amount of smooth muscle in the vessel wall varies depending on the role of the vessel. Conduit arteries, responsible for distributing large volumes of blood, are bigger and have more elastin in the arterial wall, layered in with the smooth muscle, and thus are more flexible. By contrast, the media in the resistance arteries, small arteries and arterioles, contains proportionately more smooth muscle to allow efficient control over local blood flow.

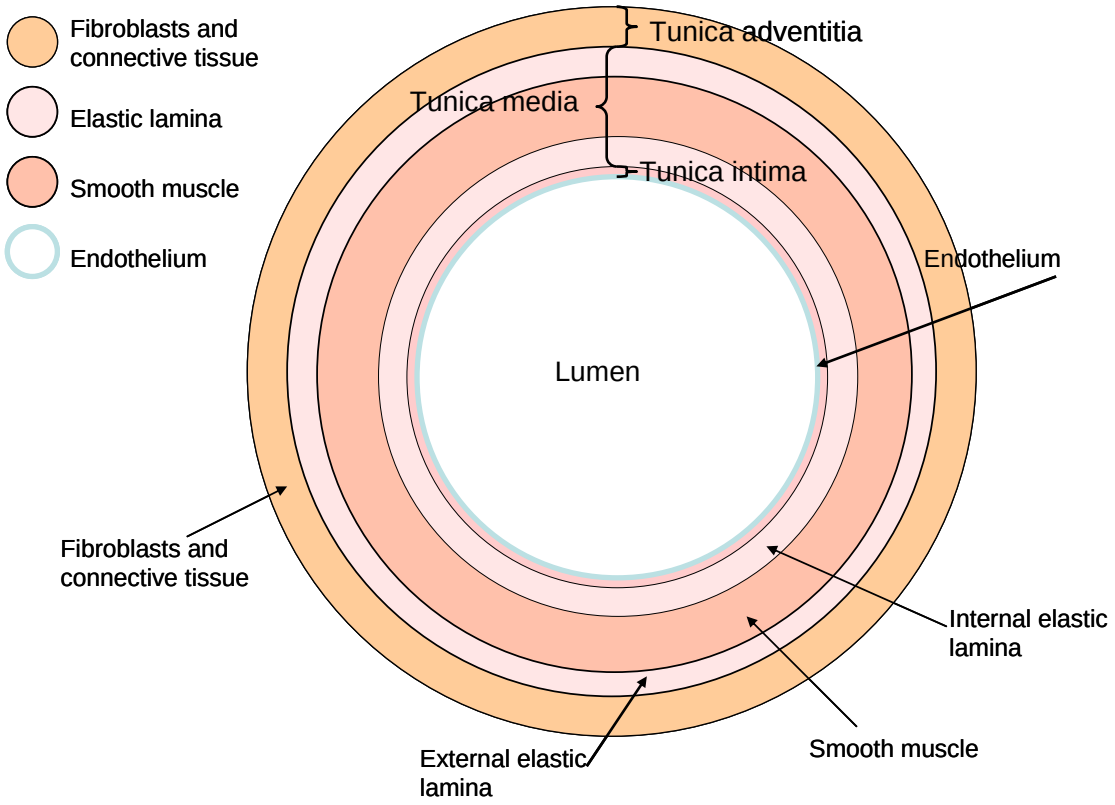


Figure 1.1 Cross section of a vessel

1.1.1.2 Endothelium

The endothelium is a single layer lining the whole of the vascular tree forming a barrier between the blood and the rest of the vessel wall. The endothelium is important for the proper function of the vasculature, contributing to processes both in the arterial wall and the lumen. The endothelium contributes to control of local blood flow through the production and release of vasodilators and constrictors (Bellien *et al.*, 2010;Furchgott & Zawadzki, 1980;Raymenants *et al.*, 1993;Shimokawa *et al.*, 1987;Vanhoutte, 1989). It is also important in several other mechanisms such as inflammation, oxidative stress and platelet function (Brown & Hu, 2001;Duran *et al.*, 2010;Feletou & Vanhoutte, 2009;Kubes *et al.*, 1991;Muzaffar *et al.*, 2008;Radomski *et al.*, 1987;Sitia *et al.*, 2010). The endothelium maintains the homeostasis of these factors in response to pressure, shear stress and platelet products among several other stimuli (Vanhoutte *et al.*, 2009;Vanhoutte & Mombouli, 1996).

1.1.2 Control of vascular tone

Vascular tone is the degree of constriction of the vessel relative to its maximal diameter. This is determined by the balance between vasodilators and vasoconstrictors. Vascular

tone is the main determinant of vascular resistance. Partial constriction of the vessels is maintained under normal circumstances thus allowing both relaxation and further constriction as needed. This partial constriction is due to the Bayliss effect: blood pressure, hydrostatic pressure exerted by the flowing blood on the vessel walls, stretches the arterial wall inducing depolarisation and constriction via stretch activated channels (Setoguchi *et al.*, 1997). The differences in blood pressure maintain blood flow, as it flows from high to low pressure. Cardiac output and peripheral resistance determine blood pressure. Local blood flow is controlled by the changes in vascular tone. Some aspects of control of vascular tone will be discussed below

1.1.2.1 Vasoconstriction

Vasoconstriction occurs in response to increase in intracellular Ca^{2+} in the SMC. Increase in Ca^{2+} allows the formation of Ca^{2+}-calmodulin complex which goes on to activate myosin light chain kinase (MLCK) (Zhou & Liao, 2009) leading to phosphorylation of myosin light chain and constriction (Kauffenstein *et al.*, 2012;Zhou & Liao, 2009).

In receptor mediated constriction the Ca^{2+} is released from the intracellular stores. Vasoconstrictors such as endothelin (ET), phenylephrine (PE), angiotensin II and thromboxane bind to G-protein coupled receptors (GPCR; Figure 1.2) allowing Ca^{2+} release from the intracellular stores by activating inositol trisphosphate (IP_3) (Kauffenstein *et al.*, 2012). The GPCR can also promote constriction independently of the Ca^{2+} release pathway via RhoA activation (Figure 1.2). Activation of membrane bound RhoA induces Rho kinases (ROCK) which inhibit myosin light chain phosphatase (MLCP) thus preventing dephosphorylation of myosin light chain (MLC) (Kauffenstein *et al.*, 2012;Khalil, 2010;Wang *et al.*, 2009;Zhou & Liao, 2009).

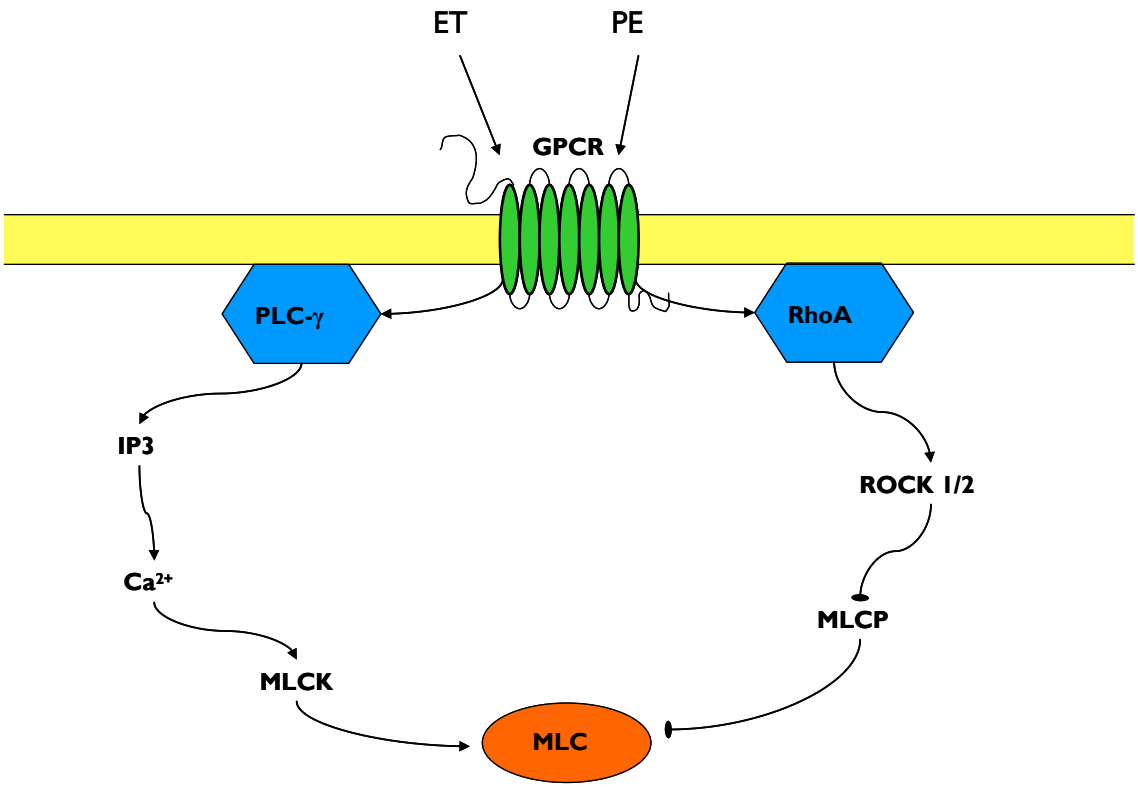


Figure 1.2 Receptor induced smooth muscle cell constriction. Activation of G-protein coupled receptors (GPCR) by constrictor agents such as endothelin (ET) and phenylephrine (PE) leads to inositol trisphosphate (IP_3) mediated Ca^{2+} release via phospholipase (PLC) activation. Intracellular calcium Ca^{2+} induces activation of myosin light chain kinase (MLCK) which phosphorylates myosin light chain (MLC) activating it. GPCR also activate RhoA which inhibits myosin light chain phosphates (MLCP) via ROCK 1 and 2 thus promoting constriction.

1.1.2.2 Endothelium-dependent vasorelaxation

For vasorelaxation to occur Ca^{2+} needs to be depleted from the smooth muscle cells either by removing it to the extracellular space or transferring it to the intracellular stores reducing activation of MLCK(Earley & Nelson, 2006;McDaniel *et al.*, 1991). Also activation of MLCP promotes relaxation (Hashiba *et al.*, 2007).

Range of vasodilators can be produced by the endothelium and these then induce relaxation via receptors on the SMC or diffusing into the SMC (Figure 1.3). Nitric oxide (NO) is one of the endothelial vasodilators. It is produced in the endothelial cells via conversion of L-arginine to L-citrulline (Palmer et al 1988) by nitric oxide synthase of which there are three isoforms: endothelial,(eNOS), neural and inducible. NO then diffuses into the smooth muscle leading to production of cyclic guonosine monophosphate (cGMP) (Caballero, 2005;Priviero *et al.*, 2009) which activates protein kinase G (Munzel *et al.*, 2005). This leads to activation of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) which moves Ca^{2+} into the intracellular stores (Lau et al 2003)

thus reducing MLCK activation. NO has also been shown to reduce MLCP inhibition (Kitazawa et al 2009) thus contributing to MLC dephosphorylation. Substances such as bradykinin and acetylcholine (ACh) can induce NO synthesis although it is unlikely that ACh can reach the endothelial cells *in vivo* and thus is not physiologically significant at least in large and middle sized arteries (Vanhoutte, 1989). Also blood flow properties such as shear stress, have been shown to increase NO synthesis (Ali *et al.*, 2009;Vanhoutte, 1989) Other endothelial dilators include prostanoids such as prostacyclin (PGI_2). Prostanoids are synthesised by cyclo-oxyganese (COX) in the endothelial cells and they work through GPCR on the smooth muscle and from there on elicit their effects in a similar manner to NO via cyclic adenosine monophosphate and protein kinase A (Arnal *et al.*, 1999;Vanhoutte & Mombouli, 1996). Endothlium can also induce relaxation through hyperpolarisation via an agent termed endothelium-dependent hyperpolarisation factor (EDHF), the nature of which is not fully understood, In response to EDHF the open probability of voltage dependent Ca^{2+} channels is reduced as is the activation of sarcoplasmic reticulum leading to relaxation. EDHF relaxation is identified as the relaxation remaining after NO and prostanoid relaxation is blocked. The relative contribution of the different vasodilators varies depending on the vascular bed and alterations can occur under pathophysiological conditions (Arnal *et al.*, 1999).

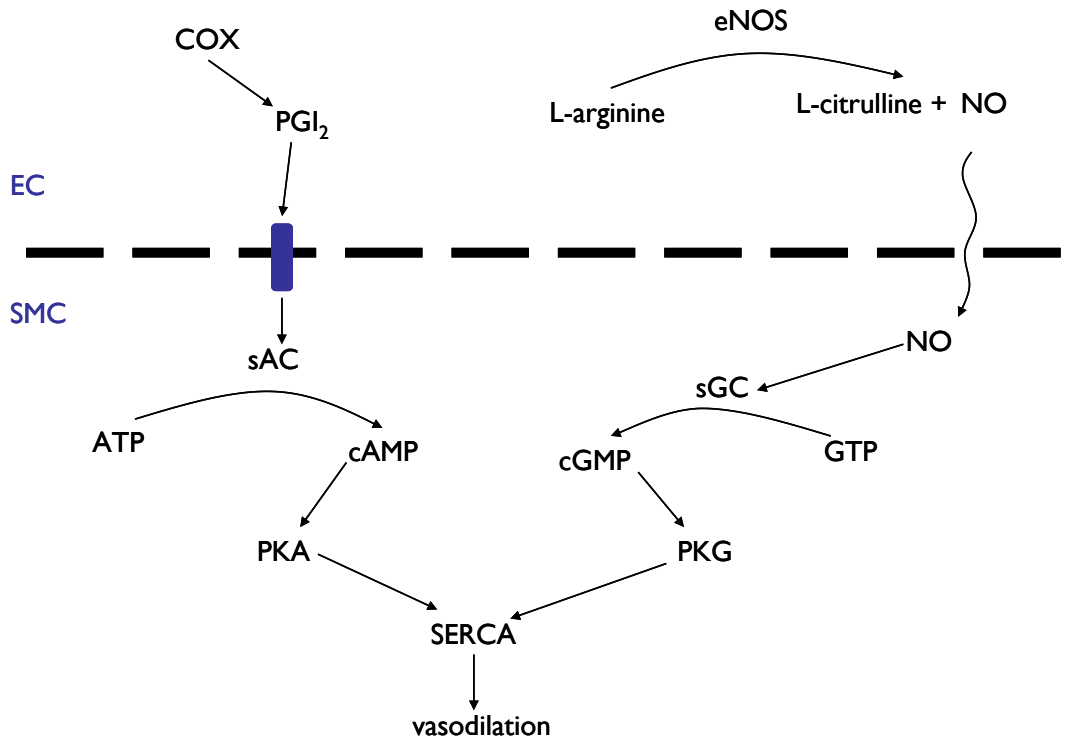


Figure 1.3 Endothelium-dependent relaxation pathways. Vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2) are synthesised in the endothelial cells (EC) and induce relaxation in the smooth muscle cells (SMC) through receptors on the SMC (PGI_2) or by diffusing into the SMC (NO). This leads to activation of sarco/endoplasmic reticulum $^{Ca2+}$-ATPase (SERCA) which moves Ca^{2+} into the intracellular stores thus allowing constriction.

1.1.3 Cardiovascular disease

CVD is an inflammatory disease though several other factors, such as endothelial dysfunction and increased cholesterol levels, are also involved. Driving factors of CVD include athersclerosis, hypertension and diabetes. The imbalance in the mechanisms involved in CVD lead to stiffening of the arteries, reduced vasorelaxation and plaque formation. These contribute to reduced luminal diameter and thus reduction or complete block of the blood flow leading to cardiovascular events.

1.1.3.1 Inflammation

Increased inflammation has been associated with increased cardiovascular risk factors as well as manifest disease (Codoner-Franch *et al.*, 2011;Huang *et al.*, 2009;Liu *et al.*, 2011;Sardo *et al.*, 2008;Swirski & Nahrendorf, 2013;Tzoulaki *et al.*, 2008). Inflammatory marker levels can also predict the risk of CVD events as has been shown with interleukin 6 (Ridker *et al.*, 2000b) and C-reactive protein (CRP) (Ridker *et al.*, 1997;Ridker *et al.*, 2000a;Zebrack *et al.*, 2002a;Zebrack *et al.*, 2002b). Though inflammatory response is beneficial to start with it becomes a problem when the

inflammatory agents do not clear leading to chronic inflammation. Increased inflammation affects atherosclerotic plaque characteristics as leukocytes adhere to the endothelium and macrophages migrate into the arterial wall and phagocytose oxidised LDL becoming foam cells and this leads to more vulnerable plaque composition (Davies, 2000;Ross, 1999). Also inflammation can contribute to accumulation of cholesterol in general as inflammatory markers have been shown to inhibit negative low density lipoprotein (LDL) feedback loop (Chen *et al.*, 2007)

1.1.3.2 Oxidative stress

Oxidative stress has recently been associated with CVD. In oxidative stress there is an increase in production of reactive oxygen species (ROS) though it can also involve increased production of reactive nitrogen species. Under normal conditions cellular homeostasis is maintained by continually produced ROS stimulating antioxidant pathways but during oxidative stress production of ROS overwhelms the antioxidant system (Gao & Mann, 2009;Nuyt, 2008). Oxidative stress allows the oxidation of biological molecules, such as LDL (Gao & Mann, 2009;Nuyt, 2008), and can also lead to reduction in NO bioavailability (Brandes, 2010;Gao & Mann, 2009;Landmesser *et al.*, 2003;Leduc *et al.*, 2010;Sussan *et al.*, 2008;Tong *et al.*, 2010). At normal levels though, ROS can contribute to NO synthesis (Feng *et al.*, 2010). It has also been indicated that oxidative stress can impair relaxation independent of NO (Priviero *et al.*, 2009).

1.1.3.3 Endothelial dysfunction

Endothelial dysfunction has been associated with CVD and it is one of the first steps in the development of atherosclerosis (Caballero, 2005;Lind *et al.*, 2011;Ross, 1999). It can be detected in people with increased CVD risk factors and altered vascular properties before the disease is manifest (Celermajer *et al.*, 1994;Leeson *et al.*, 2001;Martin *et al.*, 2000;Sorensen *et al.*, 1994). Endothelial dysfunction is also associated with hypertension (Giannotti *et al.*, 2010;Panza *et al.*, 1990;Rizzoni *et al.*, 1998;Wallace *et al.*, 2007) though whether one induces the other is not certain. Impaired endothelial dysfunction can be detected in smokers prior to development of hypertension (Iwado *et al.*, 2002) though it has been demonstrated that the presence of endothelial dysfunction does not predict development of hypertension (Shimbo *et al.*,

2010). Endothelial dysfunction is an imbalance of the factors controlled by the endothelium, which were discussed in 1.1.1.2, and manifests as increased inflammation, oxidative stress as well as impaired vasodilation among other aspects. It is likely that the disruption of the NO pathway is a key event in the development of endothelial dysfunction (Marchesi *et al.*, 2009). NO is known to be involved in several of the endothelium mediated functions, in addition to vasorelaxation, such as inhibition of platelet aggregation (Campelo *et al.*, 2012;May *et al.*, 1991;Radomski *et al.*, 1987), inflammation (Kubes *et al.*, 1991;May *et al.*, 1991) and oxidative stress (Ali *et al.*, 2009;Marchesi *et al.*, 2009).

Reduction in NO bioavailability can occur through increased NO degradation, reduced guanylyl cyclase sensitivity or prevention of NO synthesis (Tang & Vanhoutte, 2010). Prevention of NO synthesis can occur via reduced eNOS levels or activity, though eNOS mRNA and protein expression can be increased in endothelial dysfunction (Munzel *et al.*, 2005). Uncoupling of eNOS prevents NO synthesis (Li *et al.*, 2011;Munzel *et al.*, 2005) without altering eNOS activity as superoxide anion (O_2^-) is produced in these conditions (Forstermann, 2010). O_2^- is an oxidant promoting oxidative stress and thus further impairment of endothelial function (Munzel *et al.*, 2005). O_2^- can reduce NO bioavailability by reacting with NO forming peroxynitrate (Gao & Mann, 2009). Peroxynitrate can uncouple eNOS thus preventing NO formation and instead promoting O_2^- production (Brandes, 2010;Gao & Mann, 2009;Landmesser *et al.*, 2003). There is also evidence that oxidative stress can increase release of endothelial constrictor agents thus contributing to impairment of endothelial homeostasis (Priviero *et al.*, 2009). Inflammation can also contribute to a decrease in NO levels as CRP has been shown to reduce eNOS expression, phosphorylation and bioactivity (Schneeweis *et al.*, 2010;Venugopal *et al.*, 2002) and induce stiffening of the endothelium and reduce endothelial fluid permeability (Kusche-Vihrog *et al.*, 2011). Conversely to oxidative stress and inflammation altering endothelial function, endothelium is involved in oxidative stress and inflammation; both NO and prostacyclin can inhibit oxidative stress (Muzaffar *et al.*, 2008) and eNOS uncoupling has been shown to increase inflammation (Li *et al.*, 2011). The importance of NO synthase, and thus NO, in CVD has been shown in triple NOS knockout mice as when fed high fat diet they have increased cholesterol levels and oxidative stress and show signs of atherosclerosis (Yatera *et al.*, 2010). It should be noted that there appears to be some

compensatory mechanisms between the endothelial relaxation factors. In several disease states where the NO pathway is impaired the EDHF meditaed relaxation is upregulated (Triggle *et al.*, 2012) although impairment of the EDHF mediated relaxation can lead to disease as well (Feletou *et al.*, 2012;Triggle *et al.*, 2012).

The association of endothelial dysfunction with CVD has been discussed above as well as the interactions between endothelial dysfunction and inflammation and oxidative stress. The endothelium is important in maintaining the proper function of the vasculature and endothelial dysfunction has been shown early on in the development of the disease. Reduced NO bioavailability appears to be the key in endothelial dysfunction though involvement of other, less explored, endothelial factors are also implicated.

1.1.4 Cardiovascular disease risk factors

CVD risk factors can be divided into modifiable and non-modifiable risk factors (Table 1.1). Lifestyle factors are modifiable. High body mass index (BMI) is a risk factor for CVD (Lloyd-Jones *et al.*, 2006) and factors contributing to increased BMI, lack of exercise and high calorie diet, are also risk factors on their own (Mora *et al.*, 2007;Sattelmair *et al.*, 2010;Szostak & Laurant, 2011). This is partially due to cholesterol accumulation, a risk factor for CVD itself as well (Pencina *et al.*, 2009), though physical activity is a natural regulator of metabolism which appears to contribute to reduced CVD risk (Szostak & Laurant, 2011). Smoking increases the risk of CVD (Kondo *et al.*, 2011;Pencina *et al.*, 2009) and markers of inflammation, oxidative stress and platelet activation have been shown to be increased in smokers (Liu *et al.*, 2011).

The non-modifiable risk factors are mainly genetic. Men have a higher risk of CVD (Blankstein *et al.*, 2011;Pencina *et al.*, 2009), at least when compared to premenopausal women, and this appears to be at least partially due to protective effects of oestrogen in women (Perregaux *et al.*, 1999;Wildman *et al.*, 2008). Race is a risk factor for CVD, for example southern Asians have been shown to have increased risk as compared to whites (Bansal *et al.*, 2013;Quan *et al.*, 2013) probably reflecting the prevalence of certain alleles associated with CVD. Familial hypercholesterolemia is also well known risk factor for CVD (Orso *et al.*, 2009); several genes have been associated with familial hypercholesterolemia and a mutation in any of these can lead to abnormally high

cholesterol levels. The risk of CVD also increases with age (Blankstein *et al.*, 2011). The increase in risk could be related to reduction in NO bioavailability which can be increased with exercise (Nyberg *et al.*, 2012). Also *in utero* and childhood environment can be important for the development of CVD in later life as aspects associated with the disease can already be observed in children (Berenson *et al.*, 1998;Clarkson *et al.*, 1997;Huang *et al.*, 2009;Osiniri *et al.*, 2012) and these have been shown to be associated with impaired vascular function in adulthood (Aatola *et al.*, 2010). Other diseases can also increase the risk of CVD. Diabetes is one of these though type 2 diabetes actually shares several lifestyle risk factors with CVD.

Non-modifiable risk factors	**Modifiable risk factors**
Gender	Obesity
Ethnicity	Unhealthy diet
Genetics	Physical inactivity
Age	Cholesterol
Early life environment	Smoking
	Hypertension
	Diabetes

Table 1.1 Table of modifiable and non-modifiable CVD risk factors.

1.1.4.1 Cholesterol

Increased total cholesterol and LDL and reduced high-density lipoprotein (HDL) levels are well known risk factors for CVD(Criqui *et al.*, 1987;Davies, 2000;Klag *et al.*, 1993;Nichols *et al.*, 2013;Poulter, 2003;Ridker *et al.*, 1997) and many pharmacological therapies for CVD have been developed to target cholesterol. LDL has a central role in the development of atherosclerosis and increased LDL levels are associated with more prevalent atherosclerosis (Berenson *et al.*, 1998). LDL accumulates in the arterial wall and it can be oxidised contributing to atherosclerotic plaque formation. Cholesterol is essential for normal cell structure and function (Goedeke & Fernandez-Hernando, 2012) but increased levels and alterations in vessel wall properties lead to disease.

1.1.4.2 Hypertension

Hypertension is defined as increase in blood pressure due to increase in vascular resistance without change in cardiac output. High blood pressure can lead to end-organ damage but it is also associated with alterations in other aspects of vascular function,

such as endothelial dysfunction, and is a CVD risk factor (Blankstein *et al.*, 2011;Masley *et al.*, 2006;Nichols *et al.*, 2013;Ridker *et al.*, 1997) though it can also be the driving factor behind CVD. In the vast majority of hypertension, called primary or essential hypertension, there is no clear definable mechanism behind the increase in blood pressure. In secondary hypertension the alterations in physiological mechanisms are known and are related to other disease. Reduction of high blood pressure is known to reduce major cardiovascular events (Amery *et al.*, 1985;Czernichow *et al.*, 2010).

1.1.5 Treatment of cardiovascular disease

CVD can be treated through lifestyle measures and medication. Increased activity, dietary changes and stopping smoking reduce the risk of CVD events by lowering LDL and blood pressure (Brown & Hu, 2001;Kondo *et al.*, 2011;Libby & Theroux, 2005;Mora *et al.*, 2007;Paraskevas *et al.*, 2008) as well as modulating the mechanisms behind CVD (Szostak & Laurant, 2011). Weight loss in obese individuals has also been shown to reduce arterial stiffness (Dengo *et al.*, 2010). Effective pharmacological methods used in treatment of CVD include angiotensin-converting enzyme inhibitors, calcium-channel blockers and 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) (Beckman *et al.*, 2004;Brown & Hu, 2001;Libby & Theroux, 2005). Angiotensin- converting enzyme inhibitors inhibit constriction and improve vasodilation by inhibiting formation of angiotensin II and calcium-channel blockers reduce contractility in smooth and cardiac muscles by inhibiting voltage gated calcium channels (Brown & Hu, 2001). Statins have lipid lowering effects but their effectiveness is also suggestive of other, pleiotropic, properties (Brown & Hu, 2001;Ridker *et al.*, 2008a;Shepherd *et al.*, 1995;Torrens *et al.*, 2009b). Statin properties are further discussed below.

1.2 Statins

3’-hydroxy-3’-methylglutaryl-coenzyme A (HMG-Co A) reductase inhibitors, also known as statins, have been proven to be effective in their main objective of secondary prevention of cardiovascular events and mortality (Furberg *et al.*, 1994;Law *et al.*, 2003;McKinney & Kostis, 2012;Nissen *et al.*, 2006). Accumulating evidence indicates primary treatment with statins can also effectively reduce cardiovascular event rates and mortality (Ridker *et al.*, 2001;Ridker *et al.*, 2008b;Taylor *et al.*, 2011;Torrens *et al.*,

2009b)(Downs et al 1995). Statins are prescribed mainly for their lipid lowering properties but there is also increasing evidence for statins' pleiotropic effects which could contribute considerably to statins' effectiveness in disease prevention (Kinlay, 2005).

1.2.1 Development of statins

1.2.1.1 Mevalonate pathway

Statins were specifically developed to target HMG-Co A reductase which had been identified as being part of the rate limiting step in cholesterol synthesis. Although different statins vary in efficacy they all work by binding HMG-Co A reductase at lower concentrations than its natural substrate HMG-Co A and thus inhibit HMG-Co A reductase (Liao, 2002;Wang *et al.*, 2008). This inhibits the synthesis of mevalonate which has numerous sterol and non-sterol downstream targets involved in several different pathways (Figure 1.4). Mevalonate contributes to the formation of different isoprenoids, one of which is required for the cholesterol synthesis (Liao, 2002;Takemoto & Liao, 2001). HMG-CoA reductase activity is regulated on mRNA, protein and activity levels through a negative feedback loop from sterol and non-sterol products of the mevalonate pathway (Arebalo *et al.*, 1981;Auwerx *et al.*, 1989;Goldstein & Brown, 1990;Nakanishi *et al.*, 1988). HMG-Co A reductase is essential for normal cell function; cholesterol is required in cell membrane lipid layers and other HMG-Co A reductase downstream targets are involved in processes such as regulation of cell growth (Goldstein & Brown, 1990). Cholesterol is especially important during development and in early life *de novo* cholesterol synthesis, requiring HMG-CoA reductase, is increased (Dietschy *et al.*, 1993) with highest levels in rats having been shown immediately post weaning at three weeks (Smith *et al.*, 1995). Liver specific knockout of HMG-CoA reductase in mice has been shown to be highly lethal within six weeks of birth (Nagashima *et al.*, 2012).

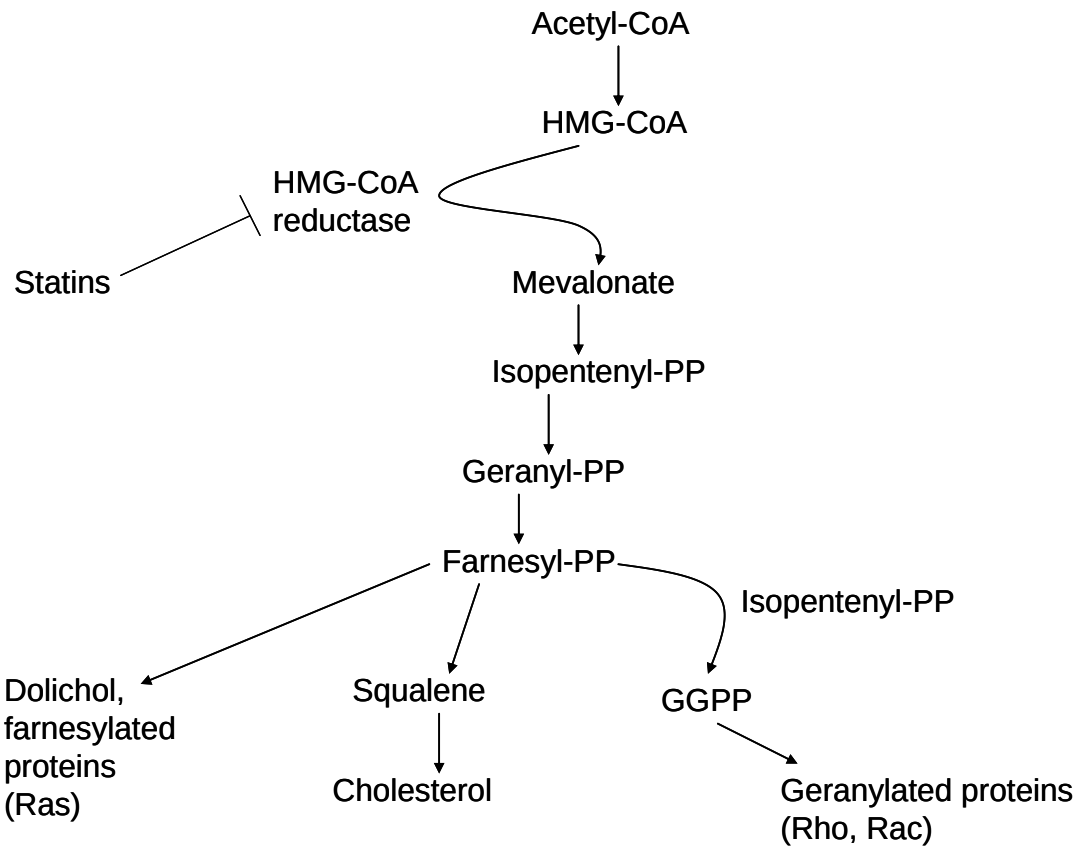


Figure 1.4 The mevalonate pathway and the effect of statins.

As mentioned, statins' ability to reduce cholesterol remains the main reason for their use to this day but new evidence indicates that their other properties could be as important in their effectiveness in preventing cardiovascular events and mortality. These pleiotropic effects are mainly thought to be mediated through the inhibition of isoprenylation. Isoprenylation is a post-transcriptional modification of proteins and involves addition of lipid attachments which is required for the membrane translocation of these proteins, such as members of protein families Rac and Rho GTPases (Takemoto & Liao, 2001). Several of these mevalonate pathway end-products are involved in regulation of cell growth (Beg *et al.*, 1978;Goldstein & Brown, 1990;Nakanishi *et al.*, 1988). The importance of inhibition of range of mevalonate pathway end-products is well demonstrated by studies comparing statins and other cholesterol lowering medication. Statins and ezetimibe, a cholesterol absorption inhibitor, have been shown to have similar cholesterol lowering capacities but unlike statins, ezetimibe does not improve endothelial function (Fichtlscherer *et al.*, 2006;Gounari *et al.*, 2010).

1.2.1.2 Discovery

Mevastatin, the first statin, was discovered in 1971. It was extracted from the fermentation broth of *Penicillium citrinum* as a part of experiments trying to identify agents for HMG-CoA inhibition (Endo, 1992) Animal studies and clinical trials were carried out during the decade until long-term high-dose trials on dogs were found to have toxic effects (Endo, 1992) Some years after the discovery of mevastatin, lovastatin was isolated as a fermentation product of *Aspergillus terreus* leading to its development for clinical use (Endo, 1992) Mevastatin and lovastatin are the only naturally occurring statins, with simvastatin and pravastatin being lovastatin derivatives, and atorvastatin, rosuvastatin, fluvastatin, pitavastatin and cerivastatin are fully synthetic.

1.2.2 Pharmacological properties of statins

All statins have a HMG-CoA reductase binding component but there are differences in their structure elsewhere (Istvan 2003). These differences are likely to account for the differences in overall efficacy and individual tolerance of different statins.

The natural and synthetic statins differ in their structure in addition to in their origins. The main difference in structure between these two groups involves the HMG-Co A binding moiety as the synthetic statins display more binding actions with HMG-Co A thus allowing tighter binding of the molecule (Istvan, 2003). Another important aspect in defining statin function is their lipophilicity and this determines which tissues statins can have a direct effect on. Lipophilic statins are able to pass through membranes by passive transport (Hamelin & Turgeon 1998) and can thus enter any cell. Hydrophilic statins, on the other hand, are hepatospecific as they require active transport to enter cells and these transporters are mainly located in the liver. The liver is the major organ for cholesterol synthesis, at least under normal cholesterol intake levels, and it is also the major organ controlling the overall cholesterol levels (Dietschy *et al.*, 1993) and thus hepatospecificity could allow better targeting of the cholesterol synthesis. Hepatospecificity could also be a protection against statins side effects. But as statins pleiotropic effects appear to be very important for statins ability to reduce CVD event rates and mortality it is possible that ability to enter other tissues, such as endothelial and smooth muscle cells, directly could be a benefit. Most of the statins are lipophilic,

by varying degrees, with only pravastatin and rosuvastatin being fully hydrophilic (Chong *et al.*, 2001;Hamelin & Turgeon, 1998;Sakamoto *et al.*, 2007).

Of the nine statins developed so far seven are on the market. As mentioned the first statin, mevastatin, never made it on the market due to severe adverse effects during testing and cerivastatin was withdrawn from the market after adverse side effects due to cerivastatin's ability to inhibit SMC proliferation were identified. Atorvastatin and rosuvastatin appear the most effective at least in lipid lowering followed by simvastatin and lovastatin (Chong *et al.*, 2001;Law *et al.*, 2003). Atorvastatin has the longest half life of 14 hours whereas the half lives of the other statins are up to 3 hours (Chong *et al.*, 2001). There is some variability in response to statins between individuals, for example genetics can affect susceptibility to statins (Chasman *et al.*, 2004).

1.2.3 Susceptibility to primary prevention

Statins have been shown to be effective in primary prevention (Ridker *et al.*, 2008b;Taylor *et al.*, 2011) but there is evidence that not everyone would benefit from primary treatment. Treatment of low risk groups might not incur benefits as statins have been shown to reduce coronary events only if either LDL or CRP was increased (Ridker *et al.*, 2001). Also in a systematic review it was indicated that in observational studies statin treatment at the time of stroke onset was associated with better outcome than without statin treatment but this association was not seen in clinical trials (Ni *et al.*, 2013). It is likely that the patients on statins in the observational studies had a higher risk of CVD prior to stroke onset than the patients in the clinical trials. This indicates that though primary prevention with statins is effective in high risk groups this protective effect is not evident in low risk groups. There is some evidence though that statin treatment can improve vascular parameters in apparently healthy subjects (Lunder *et al.*, 2011) though endothelial dysfunction was identified in the majority of the participants. These studies indicate that primary treatment with statins might not incur benefit in low risk groups.

1.2.4 Pleiotropic properties

The mechanisms behind statins' effectiveness in CVD prevention, both secondary and primary, have been investigated in clinical trials and translational studies. Changes have

been shown in different aspects of CVD on structural and functional level. These changes appear to be due to alterations in molecular levels as statins alter mRNA and protein levels as well as activity of molecules in a range of pathways.

1.2.4.1 Clinical trials

Both primary and secondary prevention of CVD by statins have been well demonstrated and have been associated with reduction in cholesterol (Ni Chroinin et al 2011)(1998;Downs *et al.*, 1998;Shepherd *et al.*, 1995). Statins have also been demonstrated to have beneficial effects on non-lipid related conditions such as Alzheimer's disease, rheumatoid arthritis and sepsis indicating pleiotropic properties. All the above mentioned conditions and CVD involve inflammation (Brookes *et al.*, 2009). There is also some evidence for statins effectiveness in cancer treatment (Kodach *et al.*, 2011;Liu *et al.*, 2012b). Statins' pleiotropic effects have also been shown in context of CVD in clinical trials.

Reduction in CVD event rates has shown to be greater when the reduction in not just cholesterol but also CRP levels is greater (Ridker et al 2005). In JUPITER study subjects were chosen based on increased CRP levels without elevated cholesterol and statin treatment lead to a reduction in CRP levels accompanied by hazard reduction (Ridker *et al.*, 2008b) though later post-hoc analysis found that in groups where at least one post-trial blood test showed low LDL levels protection against CVD events was greater (Hsia *et al.*, 2011;Ridker *et al.*, 2008b). Fluvastatin has been shown to reduce both LDL and inflammatory marker levels, though not CRP, in heart failure patients (Gurgun *et al.*, 2008). Rapid antioxidant effects via Rac1 and NADPH oxidase reduction, independently of cholesterol lowering and anti-inflammatory effects, have been demonstrated in patients undergoing coronary artery bypass graft (Antoniades *et al.*, 2010). Statins can also improve endothelial function as they have been shown to improve endothelium dependent vasodilation in smokers (Beckman *et al.*, 2004) and reduce platelet-collagen interactions (Molins *et al.*, 2010). Statin treatment has also been shown to slow down the development of atherosclerosis (Furberg *et al.*, 1994) and reduce plaques with vulnerable plaque characteristics (Kwee *et al.*, 2010). Although reduction in cholesterol levels would be expected to alter plaque characteristics there is evidence that the statin-related improvements in plaque composition could be at least partially mediated by a decrease in macrophage numbers and an increase in plaque

SMC content (Puato *et al.*, 2010). The above studies show that statins exert their pleiotropic properties in clinical setting. These studies have not conclusively confirmed the importance of these properties in event prevention though the JUPITER trial suggests that at least statins anti-inflammatory effects have some clinical relevance (Ridker *et al.*, 2008b).

1.2.4.2 Translational studies

The pleiotropic properties of statins have also been demonstrated in animal studies which have been further backed up by data from *in vitro* experiments allowing a better characterisation of the mechanisms behind statin actions. Several studies have demonstrated statins ability to improve vascular function, mainly endothelium-dependent relaxation. Statins have been shown to have similar effects on morphology and function in animals as in humans. Statin treatment has been shown to inhibit plaque formation in $ApoE^{-/-}$ mice (Bot *et al.*, 2010) and improve endothelium-dependent vasodilation in several animals models (Lee *et al.*, 2013;Suh *et al.*, 2010;Tian *et al.*, 2011). The improvement in endothelial function has been shown to be cholesterol independent (Kansui *et al.*, 2004;Torrens *et al.*, 2009b). Also incubation of arterial segments with statins improves endothelial function (Ghaffari *et al.*, 2011). Although the above studies have indicated improvement in endothelial function and especially increased NO bioavailability as the statin mechanisms, other endothelial aspects are indicated as well. Statin induced relaxation has been shown to be reduced by inhibition of prostanoid as well as NO production (Alvarez de *et al.*, 2000;Skogastierna *et al.*, 2011). Statins ability to increase NO bioavailability has also been indicated at molecular level. Statins have been shown to upregulate eNOS mRNA levels (Aoki *et al.*, 2012;Laufs *et al.*, 1998;Lee *et al.*, 2013;Skogastierna *et al.*, 2011), eNOS protein levels (Laufs & Liao, 1998;Wenzel *et al.*, 2008) and eNOS phosphorylation and activity (Aoki *et al.*, 2012;Lee *et al.*, 2013;Ohkawara *et al.*, 2010;Rossoni *et al.*, 2011;Suh *et al.*, 2010) Statin treatment also appears to increase BH4 (Meda *et al.*, 2010) which contributes towards eNOS recoupling (Wenzel *et al.*, 2008). Inhibition of RhoA could be one of the mechanisms increasing NO bioavailability as statins can inhibit RhoA activation (Gertz *et al.*, 2003) and RhoA has been shown to inhibit eNOS in endothelial cells (Laufs & Liao, 1998). Statins could also promote relaxation via inhibition of constriction agents

as statins have been shown to reduce ET-1 expression in cell culture (Hisada *et al.*, 2012).

Statin effects on other factors involved in CVD have also been shown in translational studies. Inhibition of transcription factor activation by statins has been shown to inhibit inflammatory and proliferative gene expression in cell culture (Dichtl *et al.*, 2003). Several studies have demonstrated statins antioxidant properties (Ali *et al.*, 2007;Bao *et al.*, 2009;Briones *et al.*, 2009;Moreno *et al.*, 2009;Wagner *et al.*, 2000;Yagi *et al.*, 2010) and statin induced upregulation of antioxidant genes has been indicated as a mechanism behind the increase in NO bioavailability (Ali *et al.*, 2009). Statins have also been shown to inhibit platelet activation in mice both dependent and independent of eNOS (Ni *et al.*, 2012).

1.2.5 Effects of statins over time

As discussed above clinical trials have shown that long term statin treatment reduces CVD events long term but there is also evidence that statin effects, especially pleiotropic properties, can be detected shortly after initiation of treatment. Statin treatment has been shown to improve endothelium-dependent vasodilation and elasticity and reduce cholesterol within thirty days in humans (Beckman *et al.*, 2004;Ratchford *et al.*, 2011) and the improvement in vasodilation and cholesterol levels accompanied with reduced CRP has been shown as soon as four days of initiation of treatment (Laufs *et al.*, 2001). The rapid improvement in endothelium-dependent vasodilation has been indicated to be due to prevention of eNOS uncoupling (Antoniades *et al.*, 2011). Some effects have been shown within hours in humans. Statin treatment has been shown to reduce vasoconstriction within 24 hours (Wassmann *et al.*, 2003) and reduced oxidative stress and platelet activation has been detected two hours after administration (Pignatelli *et al.*, 2012). The rapid beneficial effects of statins have also been shown in animals; in spontaneously hypertensive rat pravastatin administration prevented increase in blood pressure from the first week of treatment (Kassan *et al.*, 2009). Reduction in oxidative stress was implicated as the mechanisms behind reduction in blood pressure.

Not only do statins protective effects appear quickly but there is also indication that statins can exert their protective effects long after treatment discontinuation. Follow up studies investigating statins showed reduced CVD event rates and mortality in the

groups originally on statins up to five years after the end of the trial period (Bulbulia *et al.*, 2011;Ford *et al.*, 2007). These long term benefits of prior statin use could indicate that starting statin treatment before overt disease is established could provide protection even if the treatment is discontinued. There is also evidence that statin effects on function are not permanent, at least after shorter term treatment; improvement in forearm blood flow, cholesterol and CRP were shown to start to return to pre-treatment levels three days after 30 day treatment period (Laufs *et al.*, 2001).

Statins have been shown to be very effective in secondary and primary prevention of CVD. This is attributable to their cholesterol lowering properties though increasing evidence supports the importance of statins' pleiotropic effects. Primary prevention of CVD with statin has been shown to have protective effects beyond the treatment period although it is implied that only in those with increased risk of CVD. Statins exert their effects, especially the pleiotropic properties, rapidly although the degree to which statins pleiotropic properties contribute to disease prevention has not been fully determined despite number of studies that have looked at this. Also considering statins effectiveness in primary prevention and the indication that they could have protective effects beyond long term treatment period whether short term statin intervention could provide long term benefit needs to be further explored.

1.3 Developmental origins of cardiovascular disease

The increase in the prevalence of cardiovascular disease cannot be explained by hereditary and lifestyle factors alone. Early life environment can contribute to adult health as poor developmental environment has been associated with increased risk of disease, including CVD, diabetes and metabolic disorder. The earliest evidence for an association between developmental environment and adult health comes from epidemiological studies conducted in the 1980's and this has since been supported by data from further epidemiological studies and animal models.

1.3.1 Epidemiological studies

The first epidemiological studies looked at the association of birth weight with disease factors and mortality. Geographical studies found a negative correlation between CVD mortality and birth weight (Barker *et al.*, 1989b) and a similar association was also

found between adult blood pressure and birth weight (Barker *et al.*, 1989a). The association between low birth weight and CVD mortality and events has since been shown in cohort studies as well (Leon *et al.*, 1998;Osmond *et al.*, 1993;Osmond *et al.*, 2007). Several studies have been conducted since and these have further demonstrated the association between CVD and fetal growth by looking at not only CVD mortality and events but also risk factors and their association with a range of fetal growth markers, such as ponderal index (birth weight/birth length^3) and head circumference, in addition to birth weight. Ponderal index in men and shortness at birth in men and women has been associated with increased CVD mortality (Kajantie *et al.*, 2005). Shortness, thinness and small placental size have been associated with increased blood pressure in later life (Eriksson *et al.*, 2000). (Eriksson *et al.*, 2001;Huxley *et al.*, 2007) The association between blood pressure and birth weight has also been shown in later studies (Barker *et al.*, 1989a;Eriksson *et al.*, 2000) though some studies have not found this (Leeson *et al.*, 2001). The findings of these studies have been criticised as meta-analysis found the association between birth weight and later life blood pressure to be smaller than large studies indicated, but despite the criticism the association remained (Huxley *et al.*, 2002). Low birth weight has been shown to be associated with endothelial dysfunction and increased carotid intima-media thickness, an association which was also shown to be separately true for small for gestational-age preterm infants (Skilton *et al.*, 2011). Low birth weight has also been shown to be inversely associated with CRP levels in adulthood (Tzoulaki *et al.*, 2008). The effect of birth weight on CVD risk has been shown to be of same magnitude as current smoking when the overall risk factor burden is low (Leeson *et al.*, 2001). It has also been shown that the relationship between fetal growth and risk of CVD is not necessarily linear but rather inverse J shaped so that high birth weight is also associated with increased risk of CVD (Osmond *et al.*, 1993).

Though the above studies have demonstrated the association between CVD and fetal growth markers at birth it does not follow that the increase in risk is due to reduced fetal growth *per se*. It is possible that reduced nutrient availability alters both growth and tissue and organ development leading to altered function and increased risk in later life. Also it is not just the quantity but also the composition of maternal diet during pregnancy that can have an effect on vascular function of the offspring in adulthood. Famine exposure during fetal life has been shown to be associated with increased blood

pressure, endothelial dysfunction and adiposity (Stanner *et al.*, 1997;Stein *et al.*, 2006;Stein *et al.*, 2007) but there is evidence that the composition of this reduced nutrient supply can further alter the risk of impairment in cardiovascular function (Roseboom *et al.*, 2001).

Though the increase in risk and mortality has been demonstrated in adults there is also evidence of altered vascular function at younger age. These changes appear well before the development of overt disease. Endothelial dysfunction has been observed in young adults with low birth weight (Leeson *et al.*, 2001) which was also shown together with arterial stiffness already in 11 year children with low birth weight (Martin *et al.*, 2000). Increased blood pressure has also been shown in 5-11 year old children who were small for their gestational age at birth (Wolfenstetter *et al.*, 2012).

1.3.1.1 Hypotheses

The association between poor fetal growth and CVD could be due to compromised tissue and organ development though hypotheses have been put forward to explain this in evolutionary context. Thrifty phenotype hypothesis suggests that poor fetal environment increases the risk of disease in later life only if the postnatal environment is more abundant than the prenatal environment but would actually be beneficial in the same deprived environment experienced during fetal life (Hales & Barker, 1992). Evidence from epidemiological studies supports this as shift from poor nutritional status to a better one, especially during growth, increases the risk of diabetes in later life (Bhargava *et al.*, 2004;Schooling *et al.*, 2009). Predictive adaptive response hypothesis expands on the thrifty phenotype hypothesis. It suggests that a mismatch between the prenatal and postnatal environments brings out the disadvantageous effects of the prenatal adaptations; the greater the mismatch the greater the risk of disease (Hanson & Gluckman, 2005;Mcmillen & Robinson, 2005). This is indicated in humans as faster weight gain after birth increases the risk of CVD even further in children born small (Eriksson *et al.*, 2001;Oren *et al.*, 2004) implicating greater mismatch between the intrauterine and postnatal environments. Also impaired endothelial function has been shown to be associated with increased growth in early post natal life even in normal birth weight children (Touwslager *et al.*, 2012).

1.3.2 Animal models for developmental origins of cardiovascular disease

Several animal models have been developed to induce a disease phenotype developmentally; these include placental insufficiency, maternal dietary modulation and uterine ligation mainly in sheep and rodents. These models have been reviewed in (Vuguin, 2007) and (Mcmillen & Robinson, 2005). Despite the differences in the insult, timing, duration and severity, a similar disease phenotype is seen in the offspring. These models have been shown to exhibit a range of alterations in cardiovascular function, growth, glucose handling and behaviour among others (Samuelsson *et al.*, 2008;Torrens *et al.*, 2009a;Vickers *et al.*, 2005;Watkins *et al.*, 2008). Some of the rodent models of developmentally induced CVD will be discussed below.

1.3.2.1 Manipulation of fetal nutrient availability

In accordance with the association between low birth weight and CVD, disease models of intra-uterine growth restriction (IUGR) have been induced. These models include uterine vessel ligation as well as maternal undernutrition during pregnancy. The offspring phenotype includes reduced fetal growth, as implied by IUGR, and impaired vascular function in the adult offspring. Although the birth weights of pups born to undernourished rat dams are reduced, increased weight gain has been observed postnatally (Brennan *et al.*, 2008) and this is also true for offspring of uterine vessel ligation (Mazzuca *et al.*, 2012;Wlodek *et al.*, 2008). Uterine vessel ligation has also been shown to increase blood pressure in the offspring (Mazzuca *et al.*, 2012;Wlodek *et al.*, 2008). Increased constriction and endothelial dysfunction has been shown in the offspring of undernourished rat dams (Holemans *et al.*, 1999;Torrens *et al.*, 2009a) and they also display obesity and hyperinsulemia (Vickers *et al.*, 2005;Vickers *et al.*, 2008).

Cardiovascular function can also be impaired by alterations in availability of certain nutrients. Maternal protein restriction (PR) during and around pregnancy is one of these models. Maternal protein restriction has been shown to affect several aspects of vascular function in the offspring. NO induced vasodilation has been shown to be impaired both in the aorta and mesenteric arteries (Brawley *et al.*, 2003;Rodford *et al.*, 2008;Torrens *et al.*, 2009b). Some studies have demonstrated this to be completely endothelium dependent (Torrens *et al.*, 2009b) whereas others have also indicated alterations in the cGMP levels (Brawley *et al.*, 2003). In the brain microvessels the impaired relaxation

has been shown to be due to reduced cGMP availability (Lamireau *et al.*, 2002). Increase in blood pressure has also been shown in this model (Brawley *et al.*, 2003;Langley-Evans & Jackson, 1995;Mizuno *et al.*, 2013). This increase has been shown already in four-week old offspring in some studies (Sahajpal & Ashton, 2003;Sherman & Langley-Evans, 1998) though others have only shown this in the female offspring (Jackson *et al.*, 2002). Maternal PR solely during oocyte maturation or pre-implantation period, brief periods before and in the beginning of pregnancy, can also impair endothelium dependent relaxation and increase blood pressure in mouse offspring (Watkins *et al.*, 2008;Watkins *et al.*, 2010).

As demonstrated above maternal protein restriction impairs vascular function due to impaired fetal environment but it does not necessarily alter rate of growth during fetal life or long term (Sherman & Langley-Evans, 1998;Torrens *et al.*, 2003;Torrens *et al.*, 2009b). Some studies do show reduced weight at birth though this appears to manifest when the difference in protein content between the maternal diets is somewhat greater (Desai *et al.*, 1997;Guan *et al.*, 2005;Qasem *et al.*, 2010;Woods *et al.*, 2001). Increase in weight postnatally has also been reported (Rodford *et al.*, 2008;Sherman & Langley-Evans, 2000). These data indicate that even small differences in protein content can have great effects on offspring phenotype. It is also implied that although weight and other anthropometric measurements at birth can be used as markers of fetal growth they are not necessarily representative of the development of individual tissues and organs.

Not only does nutrient deprivation during development induce disease in later life but also excess can be detrimental to later life health as has been demonstrated in maternal high fat model. High fat diet models have been developed to reflect dietary habits in the Western world. Rats can adjust their food intake based on the calorie content and thus high fat diet does not necessarily lead to increase in weight and highly palatable diets high in sugars have been developed to induce obesity (Samuelsson *et al.*, 2008).Maternal high fat during pregnancy or suckling has been shown to lead to endothelial dysfunction and increased blood pressure (Kelsall *et al.*, 2012;Khan *et al.*, 2005;Torrens *et al.*, 2012).

Some animal studies have supported the predictive adaptive response hypothesis as postnatal high fat feeding has been shown to prevent some of the vascular impairment induced by maternal high fat feeding during pregnancy and lactation (Khan *et al.*,

2004). Also postnatal high fat diet has been shown to aggravate the effects of severe maternal protein restriction as both postnatal high fat and maternal PR increased blood pressure and had an additive effect when the animals were exposed to both (Souza-Mello *et al.*, 2007). Postnatal high protein diet has also been shown to increase blood pressure in the PR offspring even further (Chen *et al.*, 2010).

1.3.3 Epigenetics

Long term changes induced by the developmental environment could arise following changes in morphology but there is evidence to indicate that long term changes in heritable elements could be involved in developmentally induced disease. Changes in heritable elements are indicated as the effects of maternal PR and undernutrition have been shown in the F_2 generation (Ponzio *et al.*, 2012;Torrens *et al.*, 2008). Changes in heritable elements are also implicated in humans as father's early life nutrition has been shown to have an effect on offspring mortality (Pembrey *et al.*, 2006). Epigenetic mechanisms are known to have long term effects on gene expression. These mechanisms alter the binding of the transcription factors onto the promoter regions. Methylation affects gene promoter regions directly; methylation of the promoter region has an effect on the sensitivity of the promoter region and thus can alter the rate of gene expression. Maternal protein restriction has been shown to alter methylation in rat offspring (Lillycrop *et al.*, 2008) and these changes with altered gene expression have also been shown in the F_2 generation (Burdge *et al.*, 2007). Also in humans periconceptional famine exposure is known to alter methylation (Heijmans *et al.*, 2008). Histone acetylation can alter DNA packaging and thus alter the availability of the promoter regions. Maternal high fat diet during pregnancy has been shown to lead to alterations in histone acetylation in rat neonates (Strakovsky *et al.*, 2011).

1.3.4 Intervention in developmentally induced cardiovascular disease models

Both epidemiological and animal studies have demonstrated that poor fetal environment impairs vascular function and increases the risk of disease in later life. Animal studies have looked at interventions in mature animals and as changes in function have been seen already in adolescents, in humans and animals, the possibilities of early age interventions have also been investigated.

Chronic intervention and short term intervention in mature animals have been shown to be effective in the PR model. Chronic statin treatment from weaning onwards has been shown to ameliorate endothelial dysfunction in twenty week old PR offspring and this was shown to be cholesterol independent (Torrens *et al.*, 2009b). Renin-angiotensin system targeting drug captropil has been shown to reduce blood pressure after 18 day treatment in 23 week old female PR offspring (Langley-Evans & Jackson, 1995). This reduction was maintained for some weeks after the withdrawal of the treatment. Although the blood pressure started to increase again it did not reach pretreatment levels at least for thirteen weeks after the withdrawal.

As short term treatment can have long term effects in the PR model acute intervention at an earlier time point could allow more effective prevention of disease progression. Treatment of the mothers during pregnancy has been shown to have a protective effect on the vascular function of the offspring. Pravastatin treatment of high fat fed mice during pregnancy has been shown to reduce blood pressure and cholesterol levels in the offspring (Elahi *et al.*, 2008). It is likely though that the improvement in vascular parameters in the offspring was not due to direct effects of statin on the offspring but rather due to the treatment effect on the mothers. Thus the improvement in maternal parameters could have prevented some of the programming effects of maternal high fat diet from occurring in the first place, although a possibility remains that pravastatin might be able to cross the placenta following modification in the liver. Early age acute treatment of the offspring with captropil and losartan, another renin-angiotensin system targeting drug, has been shown to improve blood pressure long term in the PR offspring. Administration of losartan and captropil from two to four weeks, prior to weaning, reduced blood pressure immediately after treatment and at twelve weeks in the PR offspring (Sherman & Langley-Evans, 1998;Sherman & Langley-Evans, 2000).

Acute early age treatment has also been shown to be beneficial in undernourished rat offspring. Leptin has several roles including regulation of energy homeostasis and metabolism (Khan *et al.*, 2012) and acute early age leptin treatment has been shown to alter metabolic response to postnatal high fat diet in undernourished rat offspring. Leptin was administered by injection for 10 days from 3 days of age. There were differences between males and females but in general leptin protected the offspring of

undernourished rat dams against obesity at 100 days of age (Vickers *et al.*, 2005;Vickers *et al.*, 2008).

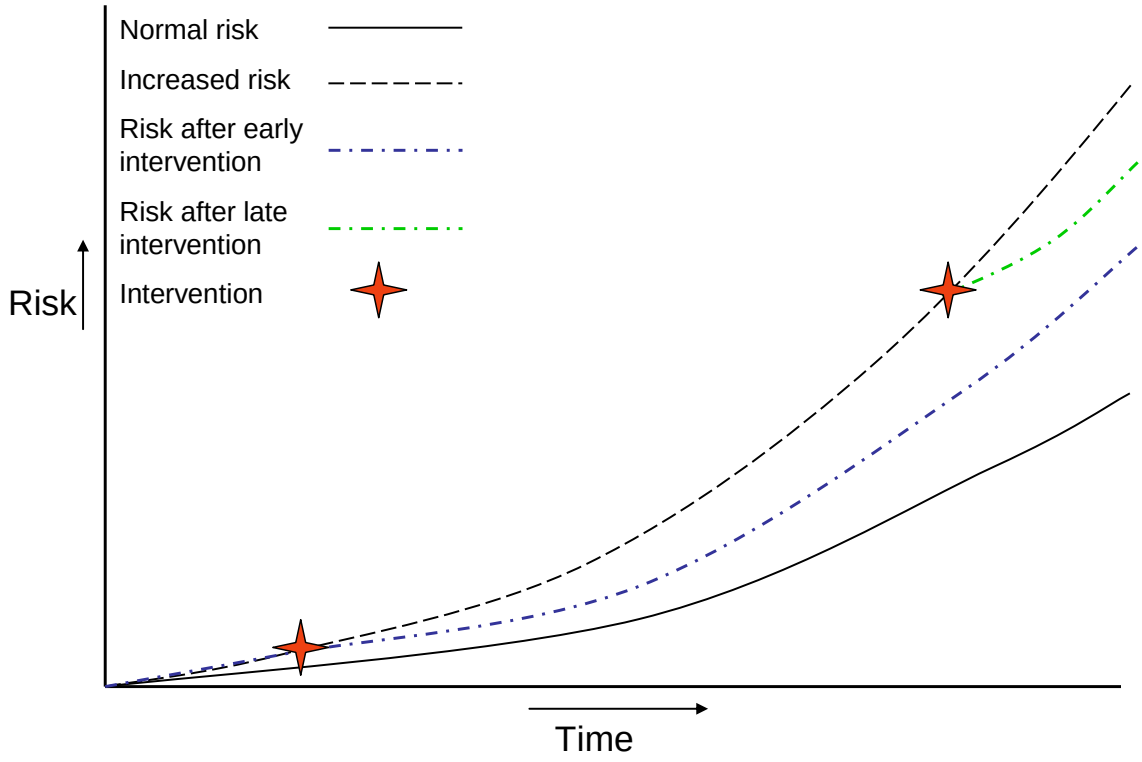


Figure 1.5 The risk of CVD in individuals with impaired fetal growth and the effect of treatment at different time points. Impairment of fetal growth increases risk of disease from childhood onwards which is somewhat reduced by late life intervention when the risk of disease is already high. Early life intervention on the other hand could allow lifelong reduction in risk. Adapted from (Hanson & Gluckman, 2011).

It has demonstrated above that poor fetal environment increases the risk of cardiovascular disease in later life. Animal models have demonstrated that intervention in mature rodents improves vascular function as does early life intervention, both acute and chronic (Figure 1.5). The possible mechanisms behind the long term effects of early age acute treatment with losartan and captropil have not been reported. Also it is not known if early age acute treatment with statins would improve vascular function in the PR model as chronic statin treatment does. This might be expected due to statins effectiveness in primary prevention as was discussed in 1.2. Also the mechanism of the statin effects in the PR model have not been extensively explored.

1.4 Rationale and aims

Maternal PR in rats has been shown to result in vascular dysfunction in the form of endothelial dysfunction and increased blood pressure in the offspring (Brawley *et al.*,

2003). Although raised blood pressure has been reported in the PR offspring already at four weeks of age (Jackson *et al.*, 2002) it is not known whether other aspects of vascular function are impaired in adolescent PR offspring. Endothelial dysfunction could be expected in the adolescent offspring as it has been observed in children and young adults with low birth weight in humans (Leeson, 2007;Martin *et al.*, 2000) As early age acute treatment with renin-angiotensin system targeting drugs has been shown to reduce blood pressure short and long term in the PR model (Sherman & Langley-Evans, 1998;Sherman & Langley-Evans, 2000) and chronic statin treatment has been shown to ameliorate the endothelial dysfunction in the adult PR offspring (Torrens *et al.*, 2009b) early age acute statin treatment could also improve vascular function short and long term. Chronic statin treatment in the PR model does not alter cholesterol levels (Torrens *et al.*, 2009b) and statins' pleiotropic effects could have an important role in this model.

It is hypothesised that early age acute statin treatment will provide immediate benefit for vascular function in a model of developmentally induced cardiovascular disease by altering endothelial and vascular smooth muscle signalling pathways. It is further hypothesised that this protective effect of the early age acute statin treatment is carried through to the mature offspring.

The main objectives of the current study were:

- to establish whether vascular function is impaired in the five week old maternally protein restricted offspring
- determine whether acute early age statin treatment improves vascular function (i) immediately after treatment in the programmed adolescent offspring and (ii) long term in the mature programmed offspring.
- to investigate the signalling pathways involved in statin-induced alterations in vascular function.

Chapter 2 Methods

2.1 Animals

All the animal procedures were carried out in accordance with the British Home Office Animals (Scientific Procedures) Act 1986 and approved by local ethics committee.

2.1.1 Pregnancy and lactation

The animals were maintained at 22˚C with 12:12 h light-dark cycle throughout the study. Animals were acquired from Harlan. Virgin female Wistar rats were mated with proven stud males. Pregnancies were confirmed by the presence of vaginal plug on the cage floor, which was checked for daily during mating, and this was taken as day 0 of pregnancy. On confirmation of pregnancy, females were housed individually and randomly assigned to either a control diet of 18 % protein (from casein, n=7) or a protein restricted diet of 9 % protein (from casein, n=6). The details of the diet compositions can be found in Appendix 1. Food intake in both groups was recorded throughout the pregnancy. Dams were weighed at the confirmation of pregnancy and again at day 20 of pregnancy. At term (22 days) dams were allowed to deliver naturally and were returned to standard laboratory chow after delivery was confirmed. To avoid maternal rejection pups were sexed and weighed and the litter sizes standardised minimum of 24 hours postpartum. To ensure comparable nutrition during lactation, litters were standardised to 8 with equal numbers of both sexes where possible.

2.1.2 Offspring

At three weeks of age, pups were weaned onto standard chow and male and female offspring separated. Half of the male and female pups from each maternal group received atorvastatin (10 mg/kg) dissolved in carboxymethylcellulose in their drinking water for 2 weeks. This gave four experimental groups for both males and females: C (18% diet, no statin, n=13/11), CS (18% diet, statin, n=13), PR (9% diet, no statin, n=11/10) and PRS (9% diet, statin, n=11/12). Atorvastatin was chosen as it is one of the most effective statins (Chong *et al.*, 2001;Law *et al.*, 2003) and has been shown to provide benefit in this model previously (Torrens *et al.*, 2009b). The offspring were weighed at weaning and weekly thereafter. Cardiovascular function was assessed by measuring blood pressure and vascular reactivity by wire myography at five and sixteen weeks. A schematic of the model can be seen in Figure 2.1.

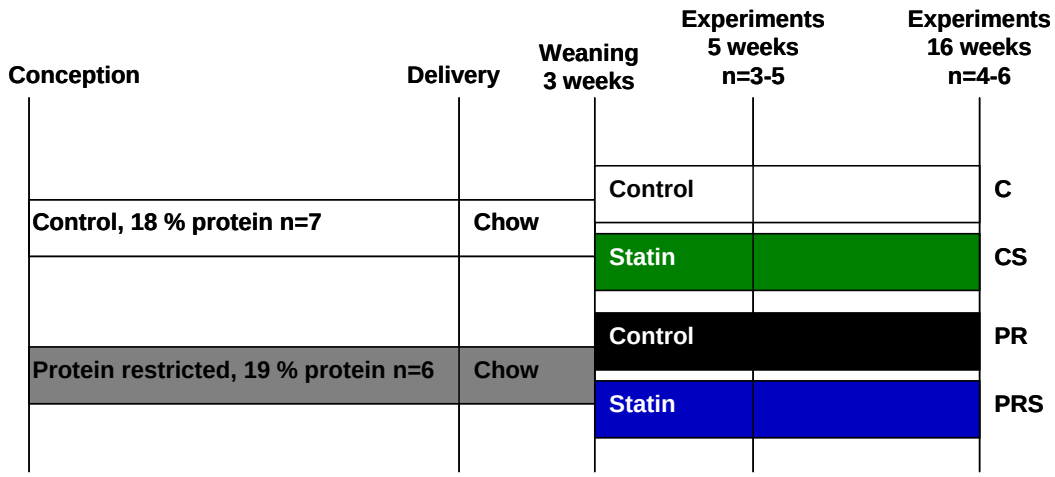


Figure 2.1 A schematic of the study design. C, control; CS, control with statin; PR, protein restricted, PRS; protein restricted with statin.

2.1.3 Post mortem

Prior to vascular reactivity experiments the animals were euthanised with CO_2 and cervical dislocation. Blood samples were taken, of which plasma was separated and snap frozen in liquid nitrogen. Liver, right kidney, heart and lungs were harvested, weighed and snap frozen. The thoracic aorta (five and sixteen week old offspring) and mesentery (sixteen week old offspring) were removed and placed in cold (4° C) physiological salt solution (PSS, composition in 2.7). Sections of aorta and mesenteric arteries were dissected out of connective tissue, some for myography and other were snap frozen for later molecular analysis.

2.2 Non-invasive tail-cuff plethysmography

Blood pressure assessment was carried out at five (n=4-5 in each group of males and females) and sixteen (n=4-6 in each group of males and females) weeks using non-invasive tail-cuff plethysmography (Columbus Instruments, USA). Two cuffs were placed around the tail to obtain readings: an occlusion cuff at the base of the tail and the sensory cuff next to it. Both of the cuffs were pressurised during the experiment. The sensory cuff detects the blood flow as the pressure asserted against it whereas the occlusion cuff inflates and deflates to induce changes in the blood flow. The blood pressure is determined based on the changes in the pressure detected by the sensory cuff.

The pressure exerted by the occlusion cuff and the pressure detected by the sensory cuff are both presented in trace form as demonstrated in Figure 2.2. Briefly after starting each measurement the occlusion cuff inflates to a preset level blocking the blood flow reflected by a flat sensory trace. After reaching the maximum pressure the occlusion cuff starts to deflate and the systolic blood pressure (SBP) can be detected when occlusion cuff pressure drops below this as the sensory trace starts to increase. The stabilisation of the sensory trace indicates diastolic blood pressure (DBP). Heart rate is indicated by the sensory trace during normal blood flow periods.

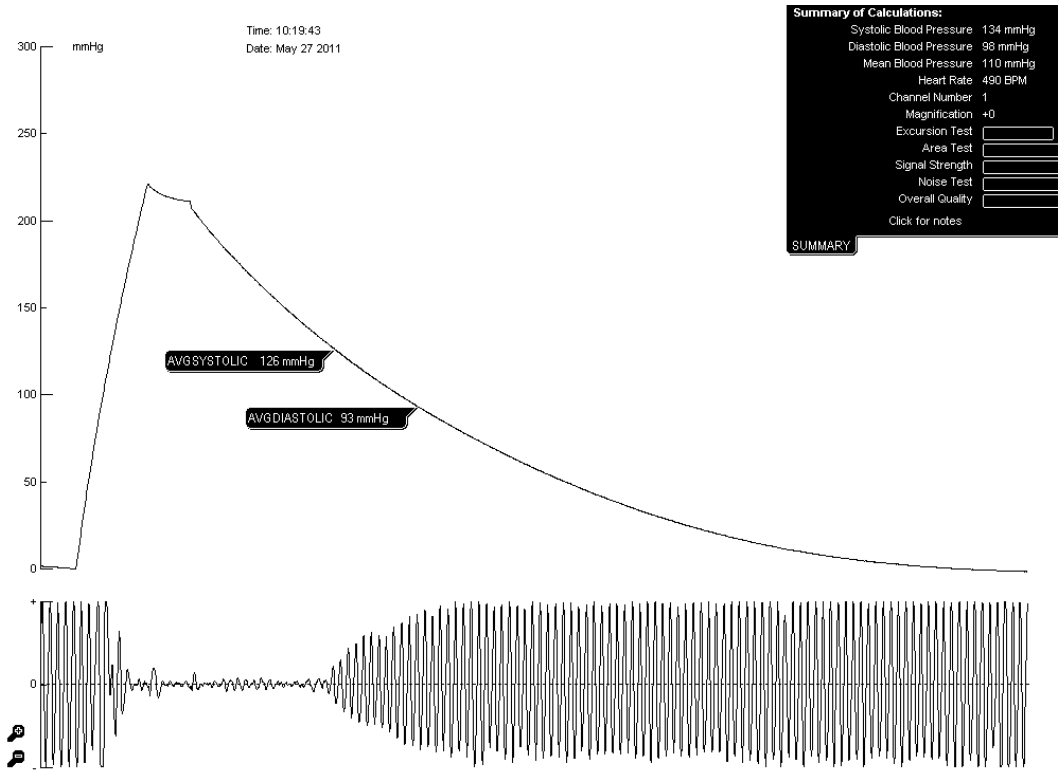


Figure 2.2 Blood pressure trace. The trace above indicates occlusion cuff pressure and the trace below shows the pressure detected by the sensory cuff.

The occlusion cuff pressure was set at 200 mmHg and the sensory cuff pressure was set at 60 mmHg to start with and altered when needed. The room was heated to at least 26º C and heat lamp was used to warm the animal in the restrainers if required to allow sufficient blood flow for the measurements from the tail. The animals were placed in the warmed room to acclimatise at least half an hour before the experiments. The animals were restrained in a tube during the measurements. This was to prevent the animal from removing the cuffs from the tail and limiting movement as this can cause anomalies in the trace. Five sets of measurements (SBP, DBP, MAP and HR for each set) of similar

SBP range were taken of which the sets with highest and lowest SBP readings were discarded.

2.3 Myography

Aortic reactivity at five (n=3-5 in each group of males and females) and sixteen (n=5-6 in each group of males and females) weeks and mesenteric artery reactivity at 16 weeks were tested using a myograph. The reactivity of the mesenteric arteries at 5 weeks was not tested as the success rate was estimated to be very low in the current study.

A myograph has two jaws, one of which is movable to adjust the distance between the two jaws and the other one connected to a force transducer that can detect changes in force. A segment of a vessel is mounted on two wires (diameter 40 μM each) connected to the jaws. The transducer detects contraction in the mounted artery as increased tension and relaxation as decreased tension. The jaw set up is presented in Figure 2.3.

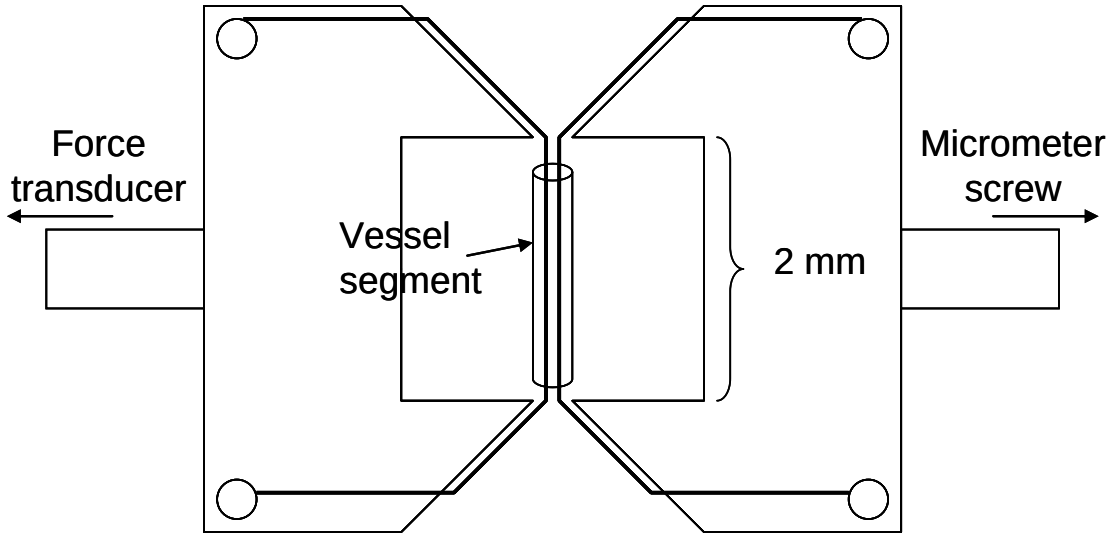


Figure 2.3 A schematic of the myograph jaws.

2.3.1 Thoracic aorta

The myograph was calibrated daily after which the aorta segments were mounted and stretched. The aorta was dissected clean of connective tissue and mounted on two pins, bathed in PSS heated to 37° C and gassed continuously with 95% O_2 and 5% CO_2. Before commencing experiments, the aortic segments were placed under a resting tension of 1 g. The segments were left to rest before the functionality was tested by

confirming constrictor response to 125 mM KPSS (PSS with equimolar substitution of KCl for NaCl, composition in 2.7).

2.3.2 Mesenteric arteries

Mesenteric arteries dissected free of connective tissue were mounted on two wires and bathed in the same conditions as the aortae. For normalisation of the mesenteric arteries internal circumference under transmural pressure of 100 mmHg (IC_{100}) was determined by stepwise increases in tension until the transmural pressure exceeded 100 mmHg (13.3 kPa). This allowed the calculation of IC_{100} and the internal circumference was then set to 90% of the IC_{100} (Mulvany & Halpern, 1977). The arteries were left to rest before functionality was tested. The functionality of SMC was confirmed by administering KPSS (125 mM) twice, noradrenalin (10 µM) once and KPSS with noradrenalin once. The arteries were deemed functional if the pressure equal to or greater than 13.3 kPa, which corresponds to 100 mmHg (the blood pressure in the mesenteric arteries *in vivo*), was achieved in response to the constrictors.

2.3.3 Assessment of vasoreactivity

Workflow diagrams for vascular reactivity studies can be seen in Appendix 2.

2.3.3.1 Assessment of vasoconstriction

Cumulative concentration-response curves were constructed to the α_1-adrenoceptor agonist phenylephrine (PE) and in the mesenteric arteries also to endothelin (ET). Examples of constriction trace and graph generated from the data can be seen in Figure 2.4.

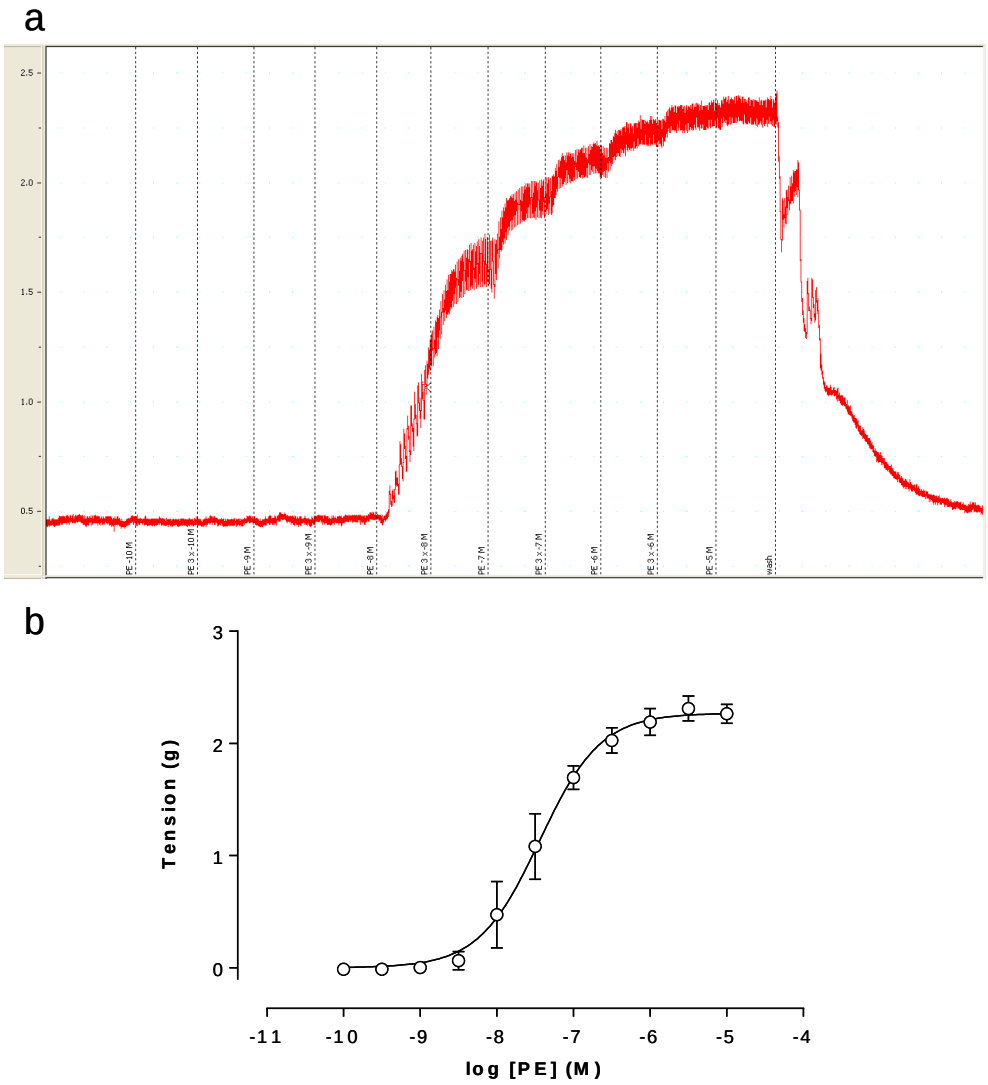


Figure 2.4 Representatives of the constriction curves in aorta. (a) Cumulative constriction response to phenylephrine in an arterial segment. (b) Sigmoidal dose response curve to phenylephrine for one of the experimental groups combined from the results for each animal in the group.

2.3.3.2 Assessment of vasodilatation

Prior to any relaxation the segments were pre-constricted with PE concentration required to produce 80% of the maximal constrictor response (EC_{80}). Acetylcholine (ACh) was used to assess endothelium-dependent relaxation. ACh leads to release of nitric oxide (NO) in both arterial beds tested and also other vasodilators in the mesenteric arteries. In the aorta ACh relaxation was also assessed in the presence of an eNOS inhibitor L-NAME to determine the NO component in the ACh induced relaxation. 100 μM L-NAME was used to fully block the relaxation and confirm the ACh induced relaxation was fully eNOS dependent. Submaximal dose of 100 nM L-NAME was used to assess possible differences in eNOS levels and NO sensitivity. In the mesenteric arteries ACh relaxation was also tested in presence of L-NAME,

indomethacin (INDO), a COX inhibitor, and 25 mM KPSS, to prevent EDHF response. Endothelium-independent relaxation was tested with sodium nitroprusside (SNP). SNP is a NO donor and thus allows assessment of relaxation independently of the endothelium. Examples of relaxation trace and graph generated from the data can be seen in Figure 2.5.

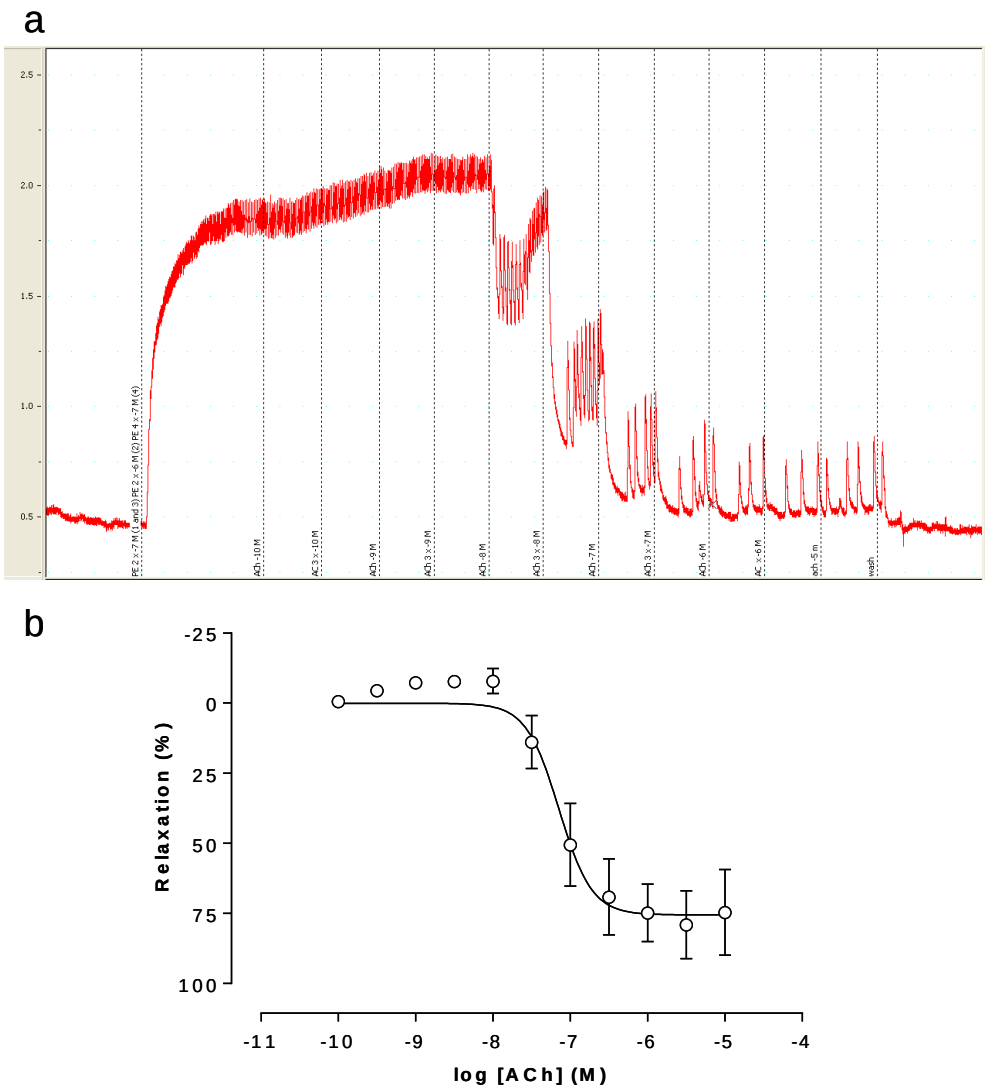


Figure 2.5 Representatives of the relaxation curves. (a) Sigmoidal dose response curve to acetylcholine combined of the data for each animal of one of the experimental groups. (b) Cumulative relaxation response to acetylcholine in an arterial segment.

2.4 mRNA levels in the liver and mesenteric arteries

The mRNA levels of RhoA, ROCK 1, ROCK 2, eNOS and HMG-CoA reductase were examined to assess gene expression in the liver and mesenteric arteries (see Table 2.1.)

HMG-CoA reductase	Forward primer	GCAGAGAAAGG-TGCGAAGTT
	Reverse primer	CGTCTCCATGAG-GGTTTCCA
	Probe	TCAATGCTAAGCACATCCCAGCCTACAAA
RhoA	Forward primer	TGAAGCAGGAGCCGGTAAAA
	Reverse primer	CCCAAAAGCGCCAATCC
	Probe	AGAAGGCAGAGATATGGCAA
ROCK1	Forward primer	TAGCCGACTTTGGTACTTGTATGAA
	Reverse primer	CACCGCCTTGTGATTTTAATACTTC
	Probe	ATGAATAAGGAAGGTATGGTACG
ROCK2	Forward primer	GCTGCTGACTGAGCGAACAC
	Reverse primer	TACCACGCTTGACAGGTTCTTTT
	Probe	CAAGACTCAAGCTGTGAATA
β-actin	Forward primer	CGTGAAAAGATGACCCAGATCA
	Reverse primer	CACAGCCTGGATGGCTACGT
	Probe	TTTGAGACCTTCAACACCCCAGCCAT

Table 2.1 Primer and probe sequencies for the real time PCR

2.4.1 Phenol-chloroform RNA extraction

RNA extraction from tissues was carried out using phenol-chloroform extraction technique. This relies in the use of different chemicals and centrifugations allowing the separation of RNA, DNA and protein into different layers.

Liver and sections of mesenteric arteries were mechanically homogenised in Trizol and incubated in room temperature for 5 minutes after which it was centrifuged to remove debris. Supernatant was taken up and chloroform added before another centrifugation step allowing the formation of three phases: a lower organic phase with DNA and protein, interphase and a top organic phase with RNA. The RNA phase was taken up and isopropanol was added. The samples were incubated at -20° C overnight for RNA precipitation. The samples were then centrifuged to allow RNA pellet formation. Supernatant was removed and the pellet was washed with ethanol and centrifuged. The ethanol was removed and the pellet left to air dry. The pellet was then dissolved in RNAse free water and stored in -80˚ C.

2.4.2 Reverse transcription

The extracted RNA was reverse transcribed into DNA with the help of viral reverse transcriptase. The samples were heated in the presence of random primers to allow the secondary structure of the RNA to melt and thus leave the template accessible for the

primers and reverse transcription reagents. M-MLV reverse transcriptase, dNTPs and RNAse inhibitors were added after this. The sample was heated to allow the annealing of the primers and then the temperature was increased further to allow the elongation of cDNA. The cDNA was stored at -20˚ C.

2.4.3 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (rtPCR) is a method used to determine relative levels of DNA. The method is based on the amplification of the DNA with measurement of the product accumulation over time.

With this method the amount of double stranded DNA is measured at the end of each replication cycle. This is done by using TaqMan double dye system. In this system forward and reverse primers, as well as double labelled probe, are required for each gene of interest. The probe is labelled with fluorescent dye FAM and quencher dye TAMRA. When TAMRA is attached to the probe it inhibits the fluorescence of FAM but when elongation occurs across the probe, TAMRA is released and FAM fluoresces thus allowing the detection of double stranded DNA. The amount of double stranded DNA and thus fluorescence increases exponentially from cycle to cycle. For each plate a threshold is set where the sample amplification is exponential, above baseline but before the amplification starts to plateau. Threshold cycle (Ct) for each sample is identified as the point when the fluorescence from that sample crosses the threshold. A standard curve is constructed on each plate with samples of known relative amounts of cDNA. These relative values of the standards are plotted against their Ct values creating a standard curve which is used for relative quantification of the unknown samples.

Primers and probes were designed using Primer Express 3.0 program. The mRNA sequences were obtained from NCBI nucleotide database and a BLAST search was then performed to identify exon locations. The nucleotide sequences and exon junctions were fed to Primer Express and this then identified several primer pairs and probes of which one set was chosen. One of the primers or the probe ran across an exon junction to prevent amplification of genomic DNA. These sequencies were run through BLAST to confirm their specificity to the gene of interest.

Each sample for each gene was run in duplicate. For each well forward and reverse primers were added with the probe. Universal mix containing required reagents for the synthesis was also added. The reactions were run on Applied Biosystems ABI 7500 thermal cycler and the fluorescence was measured at the end of each cycle. Example of fluorescence curves can be seen in Figure 2.6.

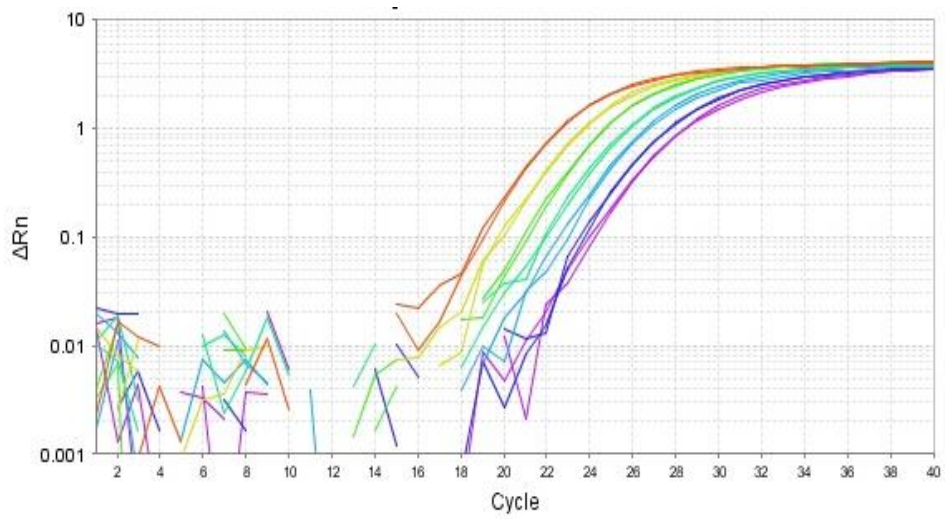


Figure 2.6 Quantitative real-time PCR curves for standards of known relative concentration

To allow for differences in the amount of starting material the results were expressed against a control gene. β-actin was chosen as a stable housekeeping gene after geNorm and Normfinder analysis.

2.4.4 Identification of stable housekeeping gene

To identify stable housekeeping gene (HKG) for relative quantification geNorm and NormFinder analyses were performed. rtPCR reaction was run on 11 commonly used HKGs. Primers were added to the reaction mix with detection agent SYBR green. SYBR green binds to double stranded DNA and fluoresces green thus allowing detection of amplification of the area of interest. The expression values for each gene were transformed using delta Ct method so that the expression in each sample was relative to the least expressed sample. The geNorm analysis is based on several rounds of pairwise comparisons with the elimination of the least stable gene at the end of each round until two genes are left (Andersen *et al.*, 2004). NormFinder is a model based tool that uses algorithms to calculate both intergroup and intragroup variation to determine

HKG stability (Vandesompele *et al.*, 2002). The results for the analyses from both programmes can be found in Appendix 4.

2.5 Analysis of protein expression

2.5.1 Protein extraction and subcellular fractination

Protein extraction is based on mechanical homogenisation of the tissue in extraction buffer to release proteins from the cells. Detergents are used to help break down the cellular structures and to allow the release of proteins from their subcellular compartments such as nucleus and membranes. Use of different buffers and centrifugation steps during the extraction procedure allows the separation of the proteins based on their subcellular location. Workflow diagram for subcellular fractination can be seen in Appendix 3.

The aortae were pooled for each experimental group due to the small amount of tissue. The combined samples were crushed in liquid nitrogen and then lysed in isotonic buffer. After this they were passed through a needle to mechanically disrupt the tissue but maintaining the subcellular structures. Part of the lysate was taken up as a whole cell fraction and detergent added to break the subcellular compartments. The whole cell lysate was then centrifuged at 10000 G after incubation on ice, to remove debris. The remaining lysate was centrifuged at 180000 G for 1 hour. This allowed the collection of cellular membranes, and thus proteins bound to the membrane, as pellet at the bottom of the tube. The supernatant was taken up as the cytosolic fraction and this was centrifuged through a concentrator column. The pellet was resuspended in buffer with a strong detergent (0.01% SDS) and passed through a needle to help the mechanical breakdown of the cellular structures.

2.5.2 Bradford analysis

After extraction protein in each sample was quantified. This was done using the Bradford method. Bradford reagent was added to each sample and BSA standard with known BSA concentrations. Each sample and standard was done in duplicate. The plate was then read at 600 nm. The BSA standards were used to generate a standard curve against which the samples were then quantified.

2.5.3 Western blotting

Western blotting is a technique that allows the detection and quantification of extracted proteins. First, sodium dodecyl sufate poly agrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins based on their size. The protein samples were prepared with Laemmli buffer and DTT reducer and then heated at 70° C for ten minutes to denature the proteins. The samples were loaded onto a polyacrylamide gel and the separation was carried out in running buffer within an electric current allowing protein separation vertically based on their molecular weight. After the separation of the proteins they were transferred horizontally onto a PVDF membrane again within an electric current.

After the transfer the membrane was prepared for protein detection. First the membrane was blocked with 5 % BSA to prevent unspecific binding of antibodies used for protein detection. The membrane was then probed with an antibody against the protein of interest. If the primary antibody was not HRP-conjugated, which was used for visualisation, incubation with a secondary HRP-conjugated antibody was performed.. After this incubation the membrane was prepared for imaging by exciting the HRP to produce a chemiluminescent signal. This was visualised with BioRad VersaDoc.

The separation of cytosolic and membrane fractions was confirmed by probing against a plasma membrane marker protein alpha 1 sodium potassium ATPase and β-actin was used as a loading control.

2.5.4 Quantification

The protein quantification was carried out using ImageJ programme which allowed the measurement of signal intensity of each band. To control against the amount of sample loaded into a well the samples were expressed against β-actin. Whole cell extracts were compared against each other. Cytosolic and membrane fractions for each sample group were run on the same gel and the protein was quantified as amount of protein in membrane fraction against protein in cytosolic fraction and these were then compared.

2.6 Statistics

Results are expressed as mean ± SEM unless otherwise stated. Significance was accepted at $p \leq 0.05$. Statistical calculations were performed using Prism 6.0, GraphPAD software Inc., San Diego, CA, U.S.A or IBM SPSS Statistics 19, SPSS Inc., Armonk, NY, USA. As sample sizes were small, normality could not be tested and parametric tests were used to ensure higher power in testing.

2.6.1 Maternal data and offspring data before statin treatment

The differences in weight before and after pregnancy in the two dam groups were assessed with two-way repeated measure ANOVA to account for the two time points at which the measurements were taken. Differences in food intake between the two maternal groups and litter data at birth were assessed using Student's t-test.

2.6.2 Blood pressure

Set of five readings (each including SBP, DBP, MAP and HR) with similar SBP were obtained of which sets with highest and lowest SBP were discarded. Means were calculated for each animal from the remaining three sets of readings. In cases where more than one offspring from the same litter were included in the same group, the readings were meaned and the results treated as n=1. Differences between the groups were assessed using two-way ANOVA with Tukey's post hoc correction as all the groups were compared to each other (six comparisons). Raw p-values reported with a comment on the results of the post hoc test.

2.6.3 Weekly and post mortem weights

For weekly weights on occasions where more than one offspring from the same litter were included in the same group, the readings were meaned and the results treated as n=1. The differences in weight gain from 3 to 16 weeks between the groups were assessed with three factor repeated measures ANOVA. The differences in post mortem body and organ weights were assessed using a two-way ANOVA with Tukey's post hoc correction as all the groups were compared to each other (six comparisons). Raw p-values reported with a comment on the results of the post hoc test.

2.6.4 Vascular reactivity

Two arterial segments were mounted from each animal where possible and data from these were meaned and treated as n=1 when response tested in both segments (see 1Appendix 2). The differences in mesenteric artery diameter were assessed with two-way ANOVA. Constrictor responses were measured as change in tension and relaxant responses as % reversal of PE-induced constriction. Cumulative concentration response curves to agonists were analysed by fitting to a four-parameter logistic equation using non-linear regression to obtain the pEC_{50} (effective concentration equal to 50% of maximum) and a maximal response, which were compared with two-way ANOVA with Tukey's post hoc correction as all the groups were compared to each other (six comparisons). Raw p-values reported with a comment on the results of the post hoc test.

2.6.5 rtPCR

Each of the duplicates was quantified from the standard curve run on the same plate. These values were then meaned and these means were standardised against similarly treated β-actin data for each sample. These data were then analysed for differences between the groups with two-way ANOVA with Tukey's post hoc correction as all the groups were compared to each other (six comparisons). Raw p-values reported with a comment on the results of the post hoc test.

2.6.6 Protein expression

No statistical analysis was performed as only one value was obtained per group due to pooling of samples.

2.7 Compositions, drugs and chemicals

Animals

Atorvastatin – Pfizer Ltd., Tadworth, Surrey, UK

Carboxymethylcellulose – Sigma-Aldrich, Gillingham, UK

Composition of statin drinking solution

carboxymethylcellulose 5 g/l

atorvastatin 100 mg/l

Vascular reactivity

Acetylcholine chloride, $CaCl_2$, endothelin, ethylenediaminetetraacetic acid, glucose, indomethacin, KCl, KH_2PO_4, $MgSO_4$, NaCl, $NaHCO_3$, Nω-nitro-L-arginine methyl ester, phenylephrine, sodium nitroprusside – Sigma-Aldrich, Gillingham, UK

Compositions for PSS and KPSS.

	PSS (mM)	KPSS (mM)
NaCl	119.00	-
KCl	4.70	124.00
MgSO4	1.17	1.17
NaHCO3	25.00	25.00
KH2PO4	1.18	1.18
D-glucose	5.50	5.50
EDTA	0.027	0.027
CaCl2	2.50	2.50

Acetylcholine, L-NAME, sodium nitroprusside, phenylephrine and endothelin 10^{-2} M stocks were prepared in water. Indomethacin 10^{-3} stock solution was prepared in phosphate buffer. All stocks were kept in -20°C and required dilutions were prepared in PSS at the time of the experiments.

mRNA quantification

Trizol Reagent – Life Technologies, Paisley, UK

Glycogen – Roche Diagnostics Ltd, Burgess Hill, UK

Ethanol – Fisher Scientific, Loughborough, UK

Chloroform, Propan-2-ol (iso-propyl alcohol) – VWR International Ltd, Lutterworth, UK

Random primers (500 µg / ml), M-MLV 5X Reaction Buffer, M-MLV Reverse Transcriptase (200 U/µl), PCR Nucleotide Mix (10 mM), Recombinant RNAsin Ribonuclease Inhibitor (40 U / µl) – Promega, Southampton, UK

Precision Mastermix, low rox, geNorm SYBR green kit (sequences not known), Precision Mastermix premixed with SYBR green – PrimerDesign, Southampton, UK

Primers and probes – Eurogentec, Seraing, Belgium

All primers were provided in 100 μM solution and were kept in -20°C. 10 μM substocks were generated by diluting in ultrapure water when needed. Probes were provided dry and diluted to 100 μM stock concentration with water according to manufacturer's instructions. Substocks of 5 μM were diluted with ultrapure water when needed.

Reverse transcription reaction

	Volume (μl) per reaction
Random primers	0.8
Water	12.2
RNA	2
Rt buffer	5
dNTPs	1.25
RNAsin	0.625
M-MLV transcriptase	1
Water	2.125

Real time PCR reaction

	Volume (μl) per sample
Probe (5 μM)	2
Forward primer (10 μM)	2
Reverse primer (10 μM)	2
Mastermix	33
H_2O	24
cDNA	3

2*30 μl reactions for each sample

Protein expression

Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, DL-Dithiothreitol solution, Glycerol, Methanol, KCl, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), MgCl2, ethylenediaminetetraacetic acid, PBS tablets, BSA, mouse monoclonal anti-β-actin−peroxidase antibody – Sigma-Aldrich, Gillingham, UK

Protease Inhibitor cocktail tablets – Roche Diagnostics Ltd, Burgess Hill, UK

Sucrose – Acros Organics (Fisher Scientific, Loughborough, UK)

Sodium dodecyl sulphate – VWR International Ltd, Lutterworth, UK

Bradford Ultra – Expedeon, Harston, UK

Tween 20 – Fisher Scientific, Loughborough, UK

4-15 % pre-cast gels, Laemmli buffer, TGS buffer (10X), TG buffer (10X) – BioRad, Hemel Hempstead, UK

Rabbit polyclonal anti-RhoA, mouse monoclonal anti-alpha 1 Sodium Potassium ATPase antibody, goat polyclonal secondary antibody to rabbit IgG (HRP) – abcam, Cambridge, UK

Antibodies were kept as undiluted aliquots at -20°C and diluted to required concentration in 5 % BSA at the time of use

Hypotonic buffer for protein extraction

	50 ml solution in H_2O	Final concentration (mM)
Sucrose	4.28 g	250
HEPES (1M)	1 ml	20
KCl	0.0373 g	10
$MgCl_2$ (1M)	75 µl	1.5
EDTA (0.5M)	100 µl	1
EGTA (0.5M)	100 µl	1

Detergent buffer made with 10 % glycerol and 0.01% SDS in the above.
1 mM DTT and protease inhibitors added to both at the time of use.

Stock solutions (1 M: HEPES, $MgCl_2$; 0.5 M: EDTA, EGTA) were made in 10 ml of H_2O and kept in 4°C.

Protease inhibitor tablets dissolved in H_2O to make a 25 X stock solution.

Running buffer and transfer buffer were prepared fresh for each run. PBS 0.1% Tween was prepared fresh every week and kept in 4°C. Blocker was prepared fresh daily.

Running buffer		Transfer buffer	
TGS buffer (10X)	70ml	TG buffer (10X)	100ml
H2O	630ml.	Methanol*	200ml
		H2O	700m

PBS 0.1 % Tween		5 % BSA Blocker	
H2O	1l	PBS Tween	10ml
PBS tablets	10	BSA	0.5
Tween	1ml		

Chapter 3 Pregnancy and cardiovascular function in the adolescent offspring

3.1 Introduction

Poor fetal growth is associated with increased risk of CVD in adult life (Barker *et al.*, 1989a;Eriksson *et al.*, 2001;Huxley *et al.*, 2007;Leon *et al.*, 1998) and impairment in vascular function can be seen already in young adults and children with low birth weight (Leeson *et al.*, 2001;Martin *et al.*, 2000). Similarly maternal protein restriction (PR) in rats has been shown to induce endothelial dysfunction and high BP in the offspring (Brawley *et al.*, 2003;Langley-Evans & Jackson, 1995;Torrens *et al.*, 2009b) and the increase in BP has been shown already at four weeks (Jackson *et al.*, 2002;Sherman & Langley-Evans, 1998). Short term early age treatment, from two to four weeks, with losartan and captropil can reduce BP in the PR model immediately after the treatment (Sherman & Langley-Evans, 1998;Sherman & Langley-Evans, 2000).

Statins are effective in secondary and primary prevention of CVD in humans (Law *et al.*, 2003;McKinney & Kostis, 2012;Ridker *et al.*, 2008b) and chronic statin treatment in the maternal PR model has been shown to ameliorate the endothelial dysfunction present in the adult offspring (Torrens *et al.*, 2009b). Statins have also been demonstrated to exert their effects on vascular function and properties rapidly in both animal models and humans (Kassan *et al.*, 2009;Pignatelli *et al.*, 2012;Wassmann *et al.*, 2003).

Although increase in blood pressure has been shown in the PR model early on it is not documented whether changes in other vascular parameters can be seen as early. Thus the aim of this part of the study was to investigate if endothelial dysfunction could be observed in 5 week old PR offspring. Considering that statins can exert some of their effects rapidly and that short term early age treatment with other drugs have been shown to have immediate benefits on vascular function this part of the study also aimed to identify if statins would alter vascular function immediately after early age, short term treatment in control and PR offspring. It was hypothesised that vascular function would be impaired in young PR offspring and that this would be improved by short term statin treatment.

3.2 Methods

3.2.1 The model

The model was set up as described in chapter 2. Briefly, on confirmation of pregnancy female Wistar rats were fed either PR or control diet. Food intake and the dams' weight gain were recorded. From delivery onwards the animals were fed standard chow. Litters were standardised to eight and weighed 1-2 days *post partum*. At 3 weeks litters were weaned and half of the offspring received atorvastatin (10 mg/kg) in their drinking water giving four experimental groups; control (C), control with statin (CS), protein restricted (PR) and protein restricted with statin (PRS). The average weight of the male offspring at post mortem was 136 g and females 119 g.

3.2.2 Blood pressure

Blood pressure of the 34-37 day old animals was measured using tail cuff plethysmography as described in Chapter 2. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) measurements were obtained. The occlusion cuff pressure was set at 200-235 mmHg and the sensory cuff pressure at 50-80 mmHg.

3.2.3 Post mortem

Animals were euthanised with CO_2 and cervical dislocation at 37-43 days. Liver, right kidney, heart and lungs were dissected, weighed and snap frozen. The aortae were collected and placed into cold PSS (4°C) for vascular reactivity experiments. Segments of aortae were also snap frozen after they were dissected out from fat and connective tissue.

3.2.4 Aortic reactivity

Aortic reactivity was investigated with myography, as described previously Chapter 2. Aortae were dissected clean of surrounding connective tissue and fat, cut into 2 mm long segments and mounted on a myograph under a resting tension of 1 g. Functionality was tested with KPSS. Cumulative concentration response curves were constructed to

the α1-adrenoceptor agonist PE (100 pM – 10 μM). Following pre-constriction to EC_{80} dose of PE, cumulative concentration-response curves were constructed to the endothelium-dependent vasodilator ACh (100 pM – 10 μM). To test the contribution on NO to ACh-induced dilatation, responses to ACh were repeated in the presence of two different concentrations of the eNOS inhibitor L-NAME (100 nM and 100 μM). Endothelium independent relaxation to the NO donor SNP (100 pM – 10 μM) was also assessed after pre-constriction with a submaximal dose of PE. Workflow diagram can be found in Appendix 2.

3.2.5 Statistics

The results were analysed with Student's t-test or two-way ANOVA, repeated measures when appropriate, as indicated in Chapter 2. All results are presented as mean ± SEM and significance was accepted at $p \leq 0.05$.

3.3 Results

3.3.1 Maternal weight gain and food intake during pregnancy

There were no differences in weight between the two groups of rat dams at the start of the pregnancy ($p>0.05$). A significant weight gain was observed during pregnancy in both groups ($p<0.0001$) which did not differ between the groups ($p>0,05$; Table 3.1). Food intake was increased in the PR dams during the whole of the pregnancy (total food intake: C 398.3±19.4 g, n=7; PR 464.9±21.6 g, n=6; $p<0.05$; Figure 3.1). When food intake was assessed in terms of calorie content no significant difference was observed (C 1517.9±73.9 kcal, n=7; PR 1608.5±74.6, n=6; $p>0.05$).

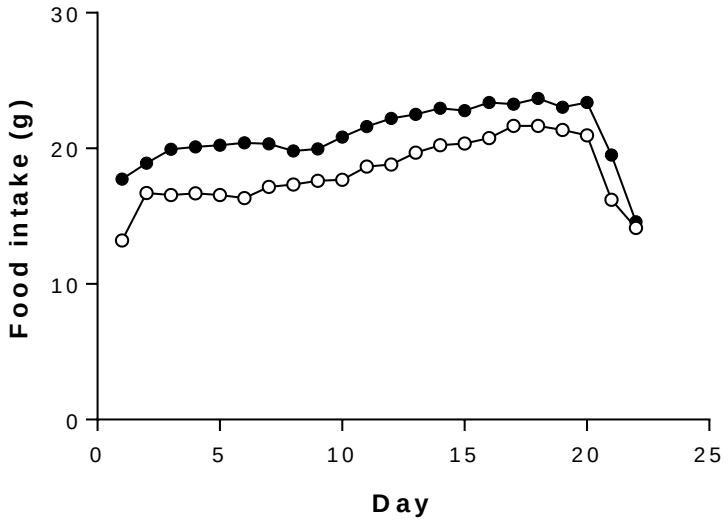


Figure 3.1 Food intake throughout pregnancy. ○ Control dams (n=7) ● PR dams (n=6)

3.3.2 Litter sizes and weight at birth

There were no differences in the litter sizes between the two maternal groups ($p>0.05$) nor in weights at birth either ($p>0.05$; Table 3.1).

	C (n = 7)	**PR** (n = 6)
Weight at the start of pregnancy (g)	238.1 ± 5.4	235.7 ± 4.2
Weight at the end of pregnancy (g)	341.1 ± 6.2	338.0 ± 7.5
Number of pups	9.7 ± 1.0	10.0 ± 1.3
Male pup birth weight (g)	7.60 ± 0.16	7.74 ± 0.40
Female pup birth weight (g)	7.41 ± 0.19	7.46 ± 0.34

Table 3.1 Dam and litter characteristics

3.3.3 Post mortem weights

There were no differences in body weights between groups in males or females at the time of euthanisation ($p>0.05$ for all factors). No differences were seen in weights of liver, heart, right kidney and lungs between groups in males or females and this was true whether expressed as absolute values in grams or as percentage of body weight ($p>0.05$; Table 3.2).

Males		C (n=5)	CS (n=5)	PR (n=4)	PRS (n=4)
Body	weight (g)	140.2 ± 6.0	129.8 ± 8.3	144.3 ± 8.8	128.1 ± 10.9
Liver	weight (g)	7.11 ± 0.43	6.26 ± 0.56	7.66 ± 0.46	6.52 ± 0.69
	% of body weight	5.06 ± 0.16	4.80 ± 0.15	5.31 ± 0.05	5.08 ± 0.23
Kidney	weight (g)	0.57 ± 0.03	0.57 ± 0.04	0.59 ± 0.02	0.54 ± 0.02
	% of body weight	0.41 ± 0.01	0.44 ± 0.01	0.41 ± 0.03	0.43 ± 0.02
Heart	weight (g)	0.65 ± 0.04	0.65 ± 0.07	0.67 ± 0.02	0.60 ± 0.01
	% of body weight	0.46 ± 0.01	0.50 ± 0.03	0.47 ± 0.03	0.48 ± 0.04
Lungs	weight (g)	1.20 ± 0.04	1.17 ± 0.02	1.24 ± 0.08	1.01 ± 0.09
	% of body weight	0.86 ± 0.06	0.91 ± 0.05	0.86 ± 0.05	0.79 ± 0.03
Females		**C (n=4)**	**CS (n=5)**	**PR (n=4)**	**PRS (n=4)**
Body	weight (g)	122.9 ± 6.6	117.8 ± 6.3	117.2 ± 1.6	117.5 ± 6.3
Liver	weight (g)	6.14 ± 0.41	5.81 ± 0.31	5.64 ± 0.13	5.92 ± 0.33
	% of body weight	4.99 ± 0.16	4.94 ± 0.18	4.82 ± 0.16	5.04 ± 0.1
Kidney	weight (g)	0.56 ± 0.02	0.53 ± 0.03	0.52 ± 0.03	0.53 ± 0.03
	% of body weight	0.46 ± 0.02	0.45 ± 0.01	0.44 ± 0.02	0.45 ± 0.03
Heart	weight (g)	0.58 ± 0.06	0.54 ± 0.03	0.66 ± 0.13	0.58 ± 0.05
	% of body weight	0.47 ± 0.04	0.46 ± 0.03	0.56 ± 0.11	0.49 ± 0.05
Lungs	weight (g)	1.12 ± 0.13	1.07 ± 0.08	1.00 ± 0.08	1.08 ± 0.11
	% of body weight	0.90 ± 0.06	0.91 ± 0.07	0.86 ± 0.08	0.91 ± 0.06

Table 3.2 Post mortem body and organ weights.

3.3.4 Blood pressure

No differences were seen in SBP or DBP in males or females as assessed by two-way ANOVA ($p>0.05$ for all factors; Figure 3.2). No differences were seen in MAP or HR either ($p>0.05$ for all factors; Table 3.3).

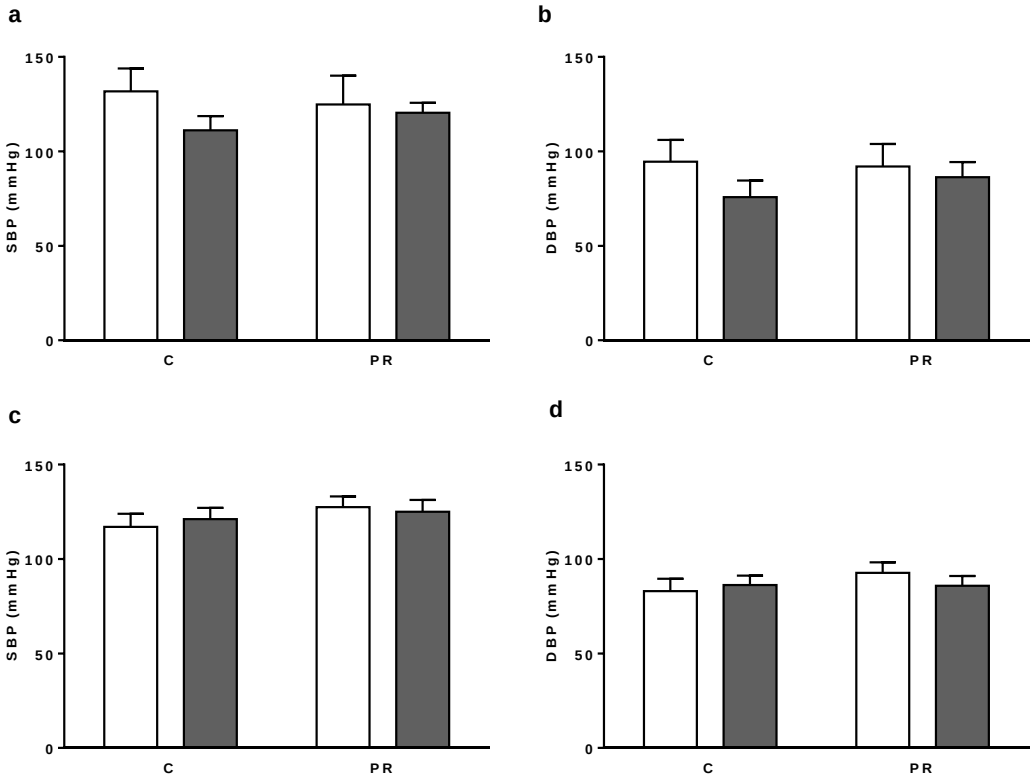


Figure 3.2 Systolic blood pressure (SBP) and diastolic blood pressure (DBP) of 5 week old male (a and b; n= 5 for C and CS; n=4 for PR and PRS) and female (c and d; n=4 for each group) offspring. □ Non treated offspring, ■ statin treated offspring

Males	C (n=5)	CS (n=5)	PR (n=4)	PRS (n=4)
SBP (mmHg)	132±12	111±8	125±15	120±5
DBP (mmHg)	95±11	76±9	92±12	86±8
MAP (mmHg)	107±12	87±8	103±13	98±7
HR (bpm)	487±7	480±17	482±15	465±21
Females	**C (n=4)**	**CS (n=4)**	**PR (n=4)**	**PRS (n=4)**
SBP (mmHg)	117±14	121±12	127±11	125±12
DBP (mmHg)	83±13	86±10	93±11	86±10
MAP (mmHg)	94±13	98±11	104±11	99±11
HR (bpm)	471±11	483±8	463±12	489±12

Table 3.3 Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) readings in the male and female offspring.

3.3.5 Vasomotor responses

3.3.5.1 Constrictor responses to phenylephrine

Phenylephrine caused a concentration-dependent constriction in all studied arteries. Maximal constriction to phenylephrine was significantly reduced in the male statin treated offspring irrespective of maternal diet (g: C, 2.3±0.1, n=5; CS 1.7±0.2, n=5; PR 1.9±0.1, n=4; PRS 1.6±0.2, n=4; $p<0.01$ for statin effect, significance maintained CS and PRS versus C following *post hoc* correction; $p>0.05$ for maternal diet effect and interaction; Figure 3.3 a) though there was a trend ($p<0.08$) for reduced maximal constriction in the protein restricted offspring. Reduced sensitivity was observed in the PR offspring irrespective of the statin treatment (pEC_{50}: C, 7.4±0.1, n=5; CS, 7.4±0.2, n=5; PR, 7.1±0.1, n=4; PRS, 7.1±0.2, n=4; $p<0.05$ for maternal diet effect, significance not maintained following *post hoc* correction; $p>0.05$ for statin effect and interaction).

In female offspring maternal protein restriction lead to a decrease in maximal constriction irrespective of statin treatment (g: C, 2.1±0.2, n=4; CS, 2.2±0.2, n=5; PR, 1.6±0.2, n=4; PRS, 1.6±0.2, n=4; $p<0.01$ for maternal diet effect, significance not maintained following *post hoc* correction; $p>0.05$ for statin effect and interaction; Figure 3.3 b). There were no significant differences in sensitivity though there was a trend ($p<0.06$) towards a reduced sensitivity in the PR offspring irrespective of statin treatment (pEC_{50}: C, 7.7±0.3, n=4; CS, 7.7±0.2, n=5; PR, 7.3±0.2, n=4; PRS, 7.3±0.2, n=4; $p>0.05$ for all factors).

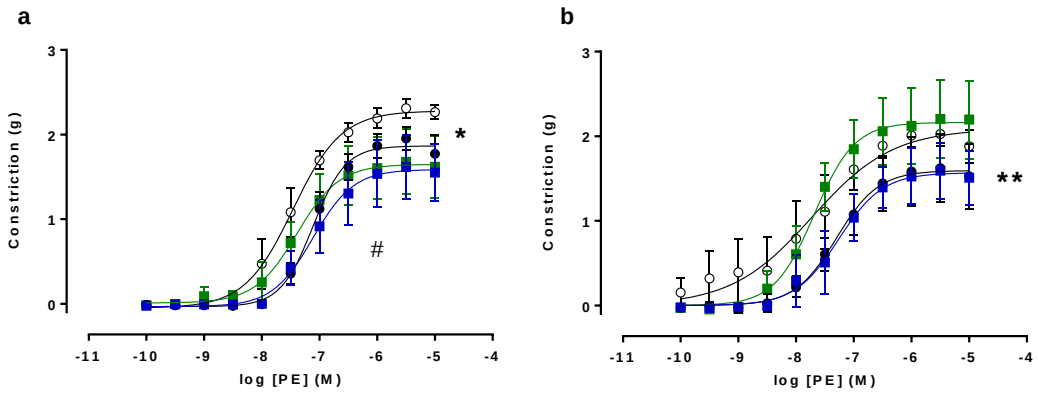


Figure 3.3 Phenylephrine (PE) induced constriction in the aorta of ○ C (n=5/4), ■ CS (n=5/5), ● PR (n=4/4) and ■ PRS (n=4/4) in male (a) and female (b) offspring. * p<0.05 maximal constriction

statin treated versus non-treated. # p<0.05 pEC_{50} PR groups versus C groups. ** p<0.01 maximal constriction PR offspring versus C offspring.

3.3.5.2 Relaxation responses to acetylcholine

Acetylcholine caused a concentration-dependent vasodilation in all the arteries. No differences were seen in maximal relaxation in the male offspring (% maximal response: C, 75.5±5.0, n=5; CS, 59.1±5.3, n=5; PR, 71.1±9.9, n=4; PRS, 82.4±8.3, n=4; p>0.05 for all factors; Figure 3.4 a). No differences were seen in sensitivity to ACh either (pEC_{50}: C, 7.1±0.1, n=5; CS 7.3±0.2, n=5; PR 7.3±0.3, n=4; PRS 7.4±0.2, n=4; p>0.05 for all factors).

No differences were seen in the maximal relaxation in the female offspring (% maximal response: C, 80.0±3.9, n=4; CS, 74.8±4.5, n=5; PR, 79.2±6.6, n=4; PRS, 86.1±8.7, n=4; p>0.05 for all factors; Figure 3.4 b). Maternal PR lead to increased sensitivity irrespective of statin treatment (pEC_{50}: C, 7.1±0.1, n=4; CS, 7.1±0.1, n=5; PR 7.4±0.2, n=4; PRS 7.5±0.1, n=4; p<0.05 for maternal die, significance not maintained following *post hoc* correction t; p>0.05 for statin effect and interaction).

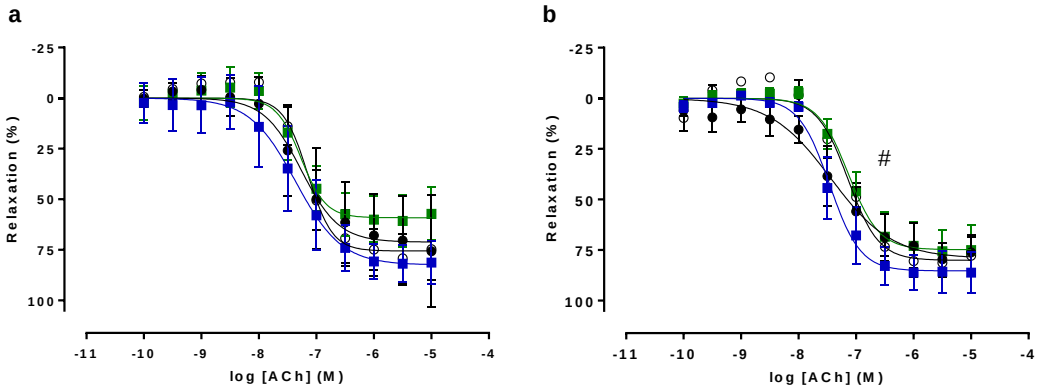


Figure 3.4 Acetylcholine (ACh) induced relaxation in the aorta of ○ C(n=5/4), ■ CS (n=5/5), ● PR (n=4/4) and ■ PRS (n=4/4) in male (a) and female (b) offspring. # p<0.05 pEC_{50} PR groups versus C groups.

Relaxation response to ACh was completely abolished by 100 μM L-NAME in all male aortae (% response following addition of final dose: C, -11.1±11.1, n=4; CS, -28.9±2.5, n=3; PR -7.7±6.2, n=4; PRS -24.3±2.9, n=3). In the presence of 100 nM L-NAME full relaxation was observed in CS group and partial in all the other groups (% maximal response: C, 19.9±16.7, n=5; CS, 46.1±25.3, n=4; PR, 34.2±16.9, n=4; PRS, 14.7±7.2, n=3; p<0.05 as compared to maximal naïve ACh relaxation for C, PR and PRS; p>0.05

as compared to naïve ACh relaxation for CS; Figure 3.5). No differences were seen in maximal relaxation between groups in presence of 100 nM L-NAME ($p>0.05$ for all factors).

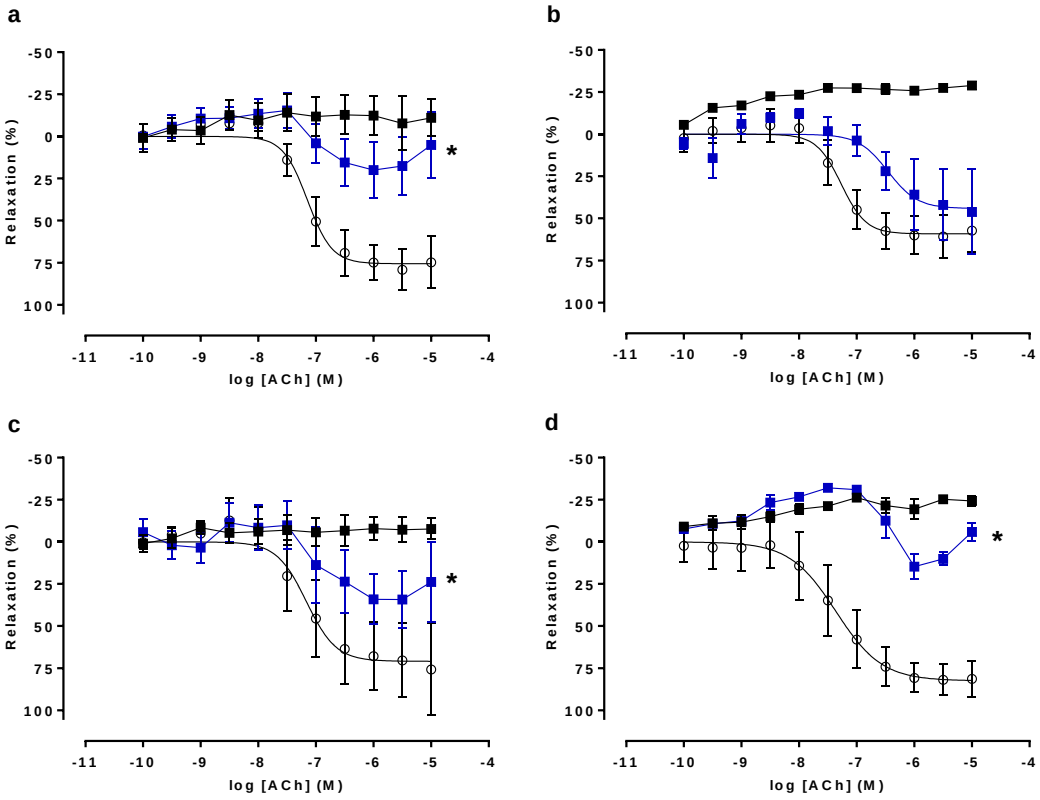


Figure 3.5 Acetylcholine (ACh) induced relaxation in the aorta of C (a; n=4-5), CS (b; n=3-5), PR (c; n=4) and PRS (d; n=3-4) male offspring; naïve ○, L-NAME 100 nM ■ and L-NAME 100 µM ■. * p<0.05 maximal relaxation naïve versus 100 nM L-NAME.

Relaxation response to ACh was completely abolished by 100 µM L-NAME in the aortae form all the female offspring (% response following addition of final dose: C -24.6±9.4, n=4; CS -18.4±8.7, n=4; PR -29.4±13.0; n=4; PRS, 7.3±19.5, n=3). In the presence of 100 nM L-NAME full relaxation was observed in the PRS group and partial in others (% maximal response: C, 36.1±7.4, n=4; CS, 48.1±7.8, n=5; PR, 36.3±3.9, n=4; PRS, 87.9±3.7, n=3; $p<0.05$ as compared to maximal naïve ACh relaxation for C, CS and PR ; $p>0.05$ as compared to naïve ACh relaxation for PRS; Figure 3.6). When the maximal ACh relaxation in the presence of 100 nM L-NAME was compared between the groups, interaction, maternal diet and statin treatment were indicated in having a significant effect ($p<0.05$ for interaction and maternal diet effect; $p<0.01$ for statin effect). When the maximal ACh relaxation in the presence of 100 nM L-NAME

was compared between the groups, interaction, maternal diet and statin treatment were indicated in having a significant effect ($p<0.05$ for interaction and maternal diet effect; $p<0.01$ for statin effect; significance not maintained following *post hoc* correction for any of the effects).

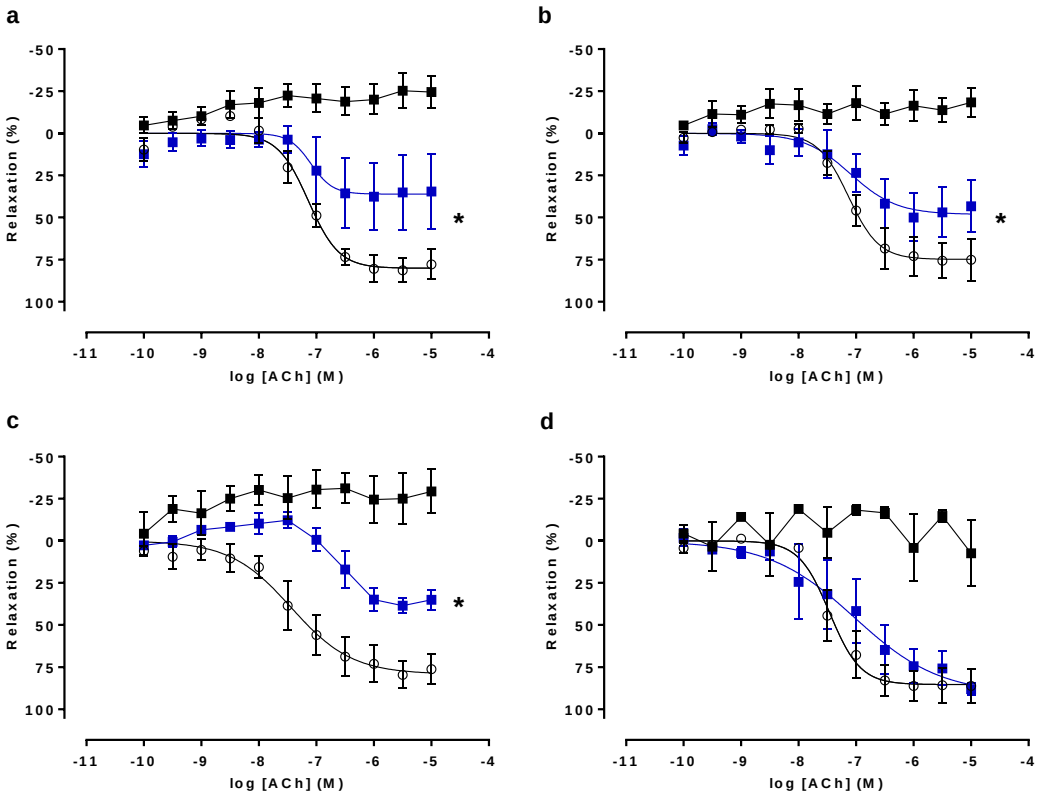


Figure 3.6 Acetylcholine (ACh) induced relaxation in the aorta of C (a; n=4), CS (b; n=4-5), PR (c; n=4) and PRS (d; n=3-4) female offspring; naïve ○, L-NAME 100 nM ■ and L-NAME 100 µM ■. * p<0.05 maximal relaxation naïve versus 100 nM L-NAME.

3.3.5.3 Relaxation responses to sodium nitroprusside

The NO donor SNP produced a concentration-dependent relaxation in all the arteries. No differences were seen in maximal relaxation between the groups in the male offspring (% maximal response: C, 106.8±6.6, n=4; CS 102.5±3.4, n=4; PR, 104.±5.2, n=3; PRS 101.±5.3, n=3; $p>0.05$ for all factors; Figure 3.7 a). No differences were seen in sensitivity to SNP (pEC_{50}: C, -8.9±0.1, n=4; CS -8.7±0.1, n=4; PR, -9.0±0.1, n=3; PRS, -8.6±0.1, n=3; $p>0.05$ for all factors).

No differences were seen in maximal relaxation between groups in female offspring (% maximal relaxation: C, 101.6±3.9, n=2; CS, 106.3±3.8, n=3; PR, 107.9±6.0, n=3; PRS,

102.7±3.2, n=3; p>0.05 for all factors; Figure 3.7 b). Maternal diet effect and interaction was observed in sensitivity to SNP (pEC_{50}: C, 9.1±0.1, n=2; CS 8.7±0.1, n=3; PR 8.4±0.1, n=3; PRS 8.9±0.1, n=3; p<0.05 for maternal diet effect; p<0.01 for interaction; p>0.05 for statin effect; significance maintained with reduced sensitivity in PR versus C and PRS groups following *post hoc* correction).

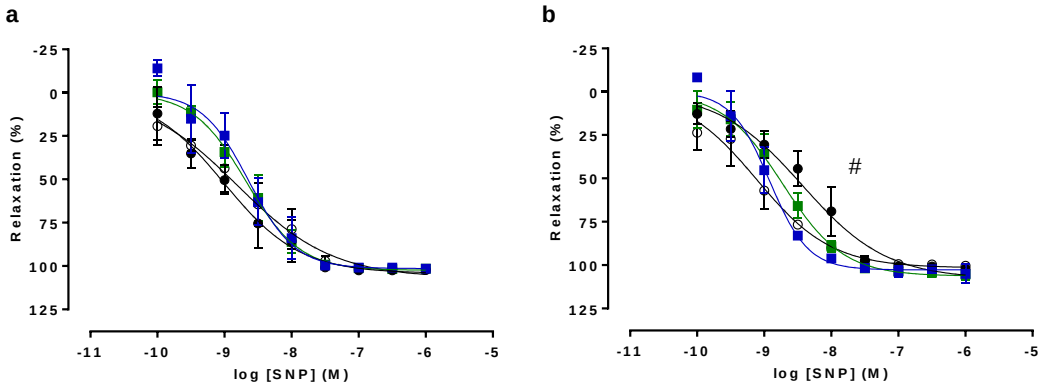


Figure 3.7 Sodium nitroprusside (SNP) induced relaxation in the aorta of ○ C, ■ CS, ● PR and ■ PRS in male (a) and female (b) offspring. # p<0.05 pEC_{50} PR offspring versus C offspring.

3.4 Discussion

Fetal environment has been shown to alter cardiovascular function and increase the risk of CVD in later life (Barker *et al.*, 1989b;Leon *et al.*, 1998) maternal protein restriction has been used to model this (Brawley *et al.*, 2003;Torrens *et al.*, 2009a). Early age, short term intervention with renin-angiotensin system targeting drugs has been shown to improve cardiovascular function in adolescent offspring of PR rat dams (Sherman & Langley-Evans, 1998;Sherman & Langley-Evans, 2000). The current study shows that short term statin treatment at an early age could have beneficial effects on vascular function immediately after the treatment. Maternal PR did not impair vascular function in the adolescent offspring in the current study. The data are summarised in Table 3.4.

		5 week aorta	
		Males	Females
PE max	PR	▼	▼
	S	▼	
PE EC50	PR	▼	▼
	S		
ACh max	PR		
	S		

ACh EC50	PRS	▲
SNP max	PRS	
SNP EC50	PRS	▼*

Table 3.4 Summary of aortic vasoreactivity results at five weeks. ▲ increased maximal constriction or sensitivity, ▼ reduced maximal constriction or sensitivity, ▲ / ▼ p<0.05, ▼ p<0.1, * interaction; PR versus C

There were differences in vasomotor function in both male and female statin-treated offspring. In the males reduced maximal constriction was observed in the statin treated offspring indicating possible beneficial effects of statins. Reduced constriction to several vasoconstrictors, including PE and ET, has been shown before in statin incubated rat mesenteric arteries (Ghaffari *et al.*, 2011;Rossoni *et al.*, 2011), in aorta and renal arteries of statin treated diabetic mice (Tian *et al.*, 2011) and aortae of statin treated $ApoE^{-/-}$ high fat fed mice (Maguire *et al.*, 2006). All these studies showed the reduction to be NO dependent as NOS inhibition abolished the differences. Thus increased basal NO levels could be responsible for the reduced constriction as statins have been shown to increase NO bioavailability through several pathways (Feron *et al.*, 2001;Hernandez-Perera *et al.*, 1998;Laufs & Liao, 1998;Ohkawara *et al.*, 2010;Ota *et al.*, 2010;Suh *et al.*, 2010). Alterations in NO bioavailability would be expected to alter relaxation responses to ACh and in the current study there were no differences in naïve ACh relaxation between the groups. In contrast full relaxation was observed with submaximal dose of L-NAME in the CS in the male offspring, and also PRS groups in females, which could indicate statin induced increase in NO bioavailability. Though the current study did not measure PE constriction in the presence of L-NAME, preconstriction to EC_{80} PE was induced with and without L-NAME and these measurements did not show any differences between the groups, suggesting that increased NO levels were not responsible for the reduced constriction in this study. It is possible that the changes induced by statin in response to PE were not endothelium-dependent but occurred in the SMC. Pitavastatin has been shown to reduce SMC constriction *in vivo* through RhoA pathway independent of the endothelium (Kuzuya *et al.*, 2004). Such changes might be expected to have similar functional effects as an increase in NO bioavailability.

Maternal PR also had an effect on to PE induced constriction in both males and females: both maximal relaxation and sensitivity were reduced, though the former was a trend in males and the latter in females. These changes show that maternal PR can alter vascular function in the offspring at a young age though these changes were not as expected as earlier studies in mature animals indicate impaired vascular, especially endothelial, function (Brawley *et al.*, 2003;Torrens *et al.*, 2009b). As the changes induced by PR are mainly apparent in constriction alterations in SMC are indicated in this study. No changes were seen in blood pressure measurements between any of the groups and this in contrast to earlier studies that have shown an increase in PR offspring blood pressure at 4 weeks (Jackson *et al.*, 2002;Sherman & Langley-Evans, 1998). This lack of increase in blood pressure in the PR offspring could be due to the measurement method as non-invasive plethysmography can be stressful for the animals and stress is known to alter blood pressure.

In the female offspring maternal PR increased sensitivity to ACh and reduced SNP sensitivity though the latter only in the non-statin treated group. Reduced sensitivity to SNP in the PR group indicates reduced NO sensitivity in the SMC which has previously been linked to reduced soluble guanylyl cyclase expression and activity (Lamireau *et al.*, 2002) and reduced cGMP response (Brawley *et al.*, 2003) and this appears to have been improved by statin treatment. As ACh sensitivity was increased in the current study, increased NO production is indicated which would have overcome the reduced NO sensitivity in the SMC. Upregulation of NO production could have been induced by reduced SMC sensitivity. As these changes were only seen in the female offspring it is possible that they are due to hormonal, puberty related changes as the stage of the oestrus cycle in rats can alter vascular function (Tare *et al.*, 2011). Female rats go through puberty around five weeks and maternal dietary manipulation has been shown to alter the timing. IUGR and undernourishment during lactation in rats have both been shown to delay the onset of menarche and first cycle (Engelbregt *et al.*, 2000;Engelbregt *et al.*, 2002) and protein restriction during lactation has been shown to delay vaginal opening (da Silva *et al.*, 2004). Thus it is possible that the changes seen in relaxation in the female offspring are related to hormonal differences due to developmentally induced changes in timing of puberty and oestrogen levels as oestrogen is known to affect NO levels (Mendelsohn, 2009).

There were no differences in dam weights or litter parameters reflecting data from previous studies (Rodford *et al.*, 2008;Torrens *et al.*, 2009b). The only difference observed between the two dam diet groups was an increased food intake in the dams on the protein restricted diet. There was no indication of this in the dam weights or litter characteristics so it is possible that this is an anomaly of the measuring method though increase in food intake has been shown in protein restricted rat dams before with no difference in weight gain (Mizuno et al 2013). Calorie content was somewhat higher though in the control diet than in the PR diet and when the food intake was converted to calories there was no significant difference between the two groups and thus the increase in food intake in weight could be explained by rodents ability to adjust food intake to the calorie content. Thus it is possible that the increase was real and this would have then increased protein intake above the 9% that was intended for the PR dams. The PR dams ate an average of 66.5g more during pregnancy which is 16.7% more than the control dams. The amount of protein would equate to 10.5% in same weight of food the control dams ate so still a significantly reduced amount of protein as compared to the control diet but this could be enough to ameliorate the increase in blood pressure observed previously in the PR offspring.

Maternal protein restriction did not induce vascular dysfunction as measured by BP and arterial reactivity in the five week old offspring in this study. This is in contrast to earlier studies as increased blood pressure has been shown in this same model at four weeks of age (Jackson *et al.*, 2002;Sherman & Langley-Evans, 1998). This is the first study to look at the presence of endothelial dysfunction at young age in the PR model and based on the results endothelial dysfunction is not present in five week old PR offspring though it is known to be apparent in adult offspring (Brawley *et al.*, 2003;Rodford *et al.*, 2008). In spontaneously hypertensive rat hypertension develops early and is clearly detectable by 5 weeks but endothelial dysfunction is not evident until at 12 weeks (Chamiot-Clerc *et al.*, 2001) thus, even though early elevation in blood pressure has been observed in this model before, this does not mean there is a detectable impairment in endothelial function at an early age. Possible alterations in SMC constriction in both sexes and timing of puberty in females were indicated in the PR offspring. But, as vascular dysfunction was not evident at five weeks in this study, improvement by early age short term statin treatment could not be observed though

possible beneficial effects were shown in the statin treated C and PR males as maximal constriction to PE was reduced.

Chapter 4 Cardiovascular function in the adult offspring

4.1 Introduction

Impaired fetal growth has been shown to impair vascular function in adult life leading to increased risk of cardiovascular disease (Eriksson *et al.*, 2001;Huxley *et al.*, 2007;Leon *et al.*, 1998) and this has been further demonstrated in animal models of developmentally induced CVD (Brawley *et al.*, 2003;Langley-Evans & Jackson, 1995;Rodford *et al.*, 2008;Torrens *et al.*, 2009a). Impaired endothelial function has been implicated in both humans (Leeson *et al.*, 2001;Martin *et al.*, 2000) and animal models (Brawley *et al.*, 2003;Rodford *et al.*, 2008). Early age acute treatment with losartan, captropil and leptin have been shown to improve cardiovascular function and metabolic parameters long term in models of developmentally induced CVD (Sherman & Langley-Evans, 1998;Sherman & Langley-Evans, 2000;Vickers *et al.*, 2005;Vickers *et al.*, 2008).

Treatment with statins has been shown to be very effective in primary and secondary intervention in reducing cardiovascular event rates and mortality in humans (Law *et al.*, 2003;Ridker *et al.*, 2001;Ridker *et al.*, 2008b) and clinical trials have indicated that statin treatment can have long term protective effects even after the treatment is withdrawn (Bulbulia *et al.*, 2011;Ford *et al.*, 2007). Statins are also known to be effective in the PR model as chronic statin treatment ameliorates the endothelial dysfunction observed in the adult offspring (Torrens *et al.*, 2009b).

Though chronic statin treatment has been shown to improve vascular function in the PR offspring the effects of early age acute statin treatment have not been investigated thus the purpose of this part of the study was to investigate long term effects of early age acute statin treatment in the PR model. Based on earlier research on statins and early age acute treatments in the developmentally induced CVD models it was hypothesised that early age acute treatment would provide long term protective effects on vascular function in the PR offspring.

4.2 Methods

4.2.1 The model

The model was set up as described in Chapter 2. Briefly, on confirmation of pregnancy female Wistar rats were fed either PR or control diet. From delivery onwards the animals were fed standard chow. Litters were standardised to eight and weighed 1-2 days *post partum*. At 3 weeks litters were weaned and half of the offspring were received atorvastatin (10 mg/kg) in their drinking water giving four experimental groups; control (C), control with statin (CS), protein restricted (PR) and protein restricted with statin (PRS). Statin was withdrawn after two weeks and the offspring were maintained on water and standard laboratory chow until 16 weeks. The animals were weighed weekly from weaning. The average post mortem weight of the male offspring was 390 g for and of the female offspring 229 g.

4.2.2 Blood pressure

Blood pressure of the 111-114 day old animals was measured using tail cuff plethysmography. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) measurements were obtained as described in Chapter 2. The occlusion cuff pressure was set as 200-255 mmHg and the sensory cuff pressure was set as 50-90 mmHg.

4.2.3 Post mortem

Animals were euthanised with CO_2 and cervical dislocation at 115-120 days. Liver, right kidney, heart and lungs were weighed and snap frozen. The aortae and gut were collected and placed into cold PSS (4°C) for vascular reactivity experiments. Segments of aorta and mesenteric arteries were also snap frozen in liquid nitrogen after they were dissected out of fat and connective tissue.

4.2.4 Aortic reactivity

Aortic reactivity was investigated using myography, as described in Chapter 2. Aortae were dissected clean of surrounding fat and connective tissue, cut into 2 mm long

segments and mounted on a myograph under a resting tension of 1 g. Functionality was tested with KPSS. Cumulative concentration response curves were constructed to the α1-adrenoceptor agonist PE (100 pM – 10 µM). Following pre-constriction to EC_{80} dose of PE, cumulative concentration-response curves were constructed to endothelium-dependent vasodilator ACh (100 pM – 10 µM). To test the contribution of NO to ACh-induced dilatation, responses to ACh were repeated in the presence of the eNOS inhibitor L-NAME (100 nM & 100 µM). Endothelium-independent relaxation to NO donor SNP (100 pM – 10 µM) was also assessed after pre-constriction to PE EC_{80}. Workflow diagram can be found in Appendix 2.

4.2.5 Mesenteric artery reactivity

Mesenteric artery reactivity was also investigated using myography, as described in Chapter 2. Segments of mesenteric arteries were dissected out of surrounding fat and 2 mm segment were mounted on a wire myograph. The data from vessel normalisation was used to estimate the diameter of the vessels. The functionality was tested with KPSS and noradrenaline. Cumulative concentration response curves were constructed to the α1-adrenoceptor agonist PE (10 nM – 100 µM). Constriction was also assessed to endothelin (1 pM – 100 nM). Following pre-constriction to PE EC_{80} cumulative concentration-response curves were constructed to the endothelium-dependent vasodilator ACh (100 pM – 10 µM) to observe endothelium-dependent relaxation. ACh relaxation was also assessed in the presence of 100 µM L-NAME, 10 µM indomethacin and 25 mM KPSS. Endothelium independent relaxation to the NO donor SNP (1 pM – 10 µM) was assessed after pre-constriction to PE EC_{80}. Workflow diagram can be found in Appendix 2.

4.2.6 Statistics

The results were analysed with Student's t-test or two-way ANOVA, with repeated measures when appropriate, as indicated in Chapter 2. All results are presented as mean ± SEM and significance was accepted at $p \leq 0.05$.

4.3 Results

4.3.1 Growth

There were no differences in weight gain from three to sixteen weeks in the male offspring ($p>0.05$; Figure 4.1 a). In the females the weight gain was reduced in the PR offspring irrespective of maternal diet ($p<0.05$ for maternal diet effect; $p>0.05$ for statin effect and interaction; Figure 4.1 b).

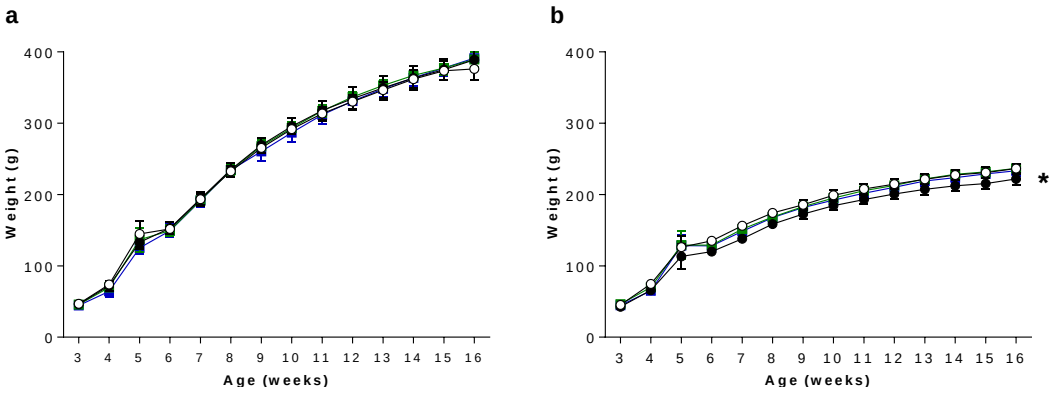


Figure 4.1 Growth curves of ○ C (n=6/5), ■ CS (n=6), ● PR (n=5) and ■ PRS (n=5/6) from three to sixteen weeks in the male (a) and female (b) offspring. * p<0.05 PR offspring versus C offspring.

4.3.2 Blood Pressure

No differences were seen in SBP or DBP in males or females as assessed by two-way ANOVA ($p>0.05$ for all factors; Figure 4.2). No differences were seen in MAP or HR either ($p>0.05$ for all factors; Table 4.1).

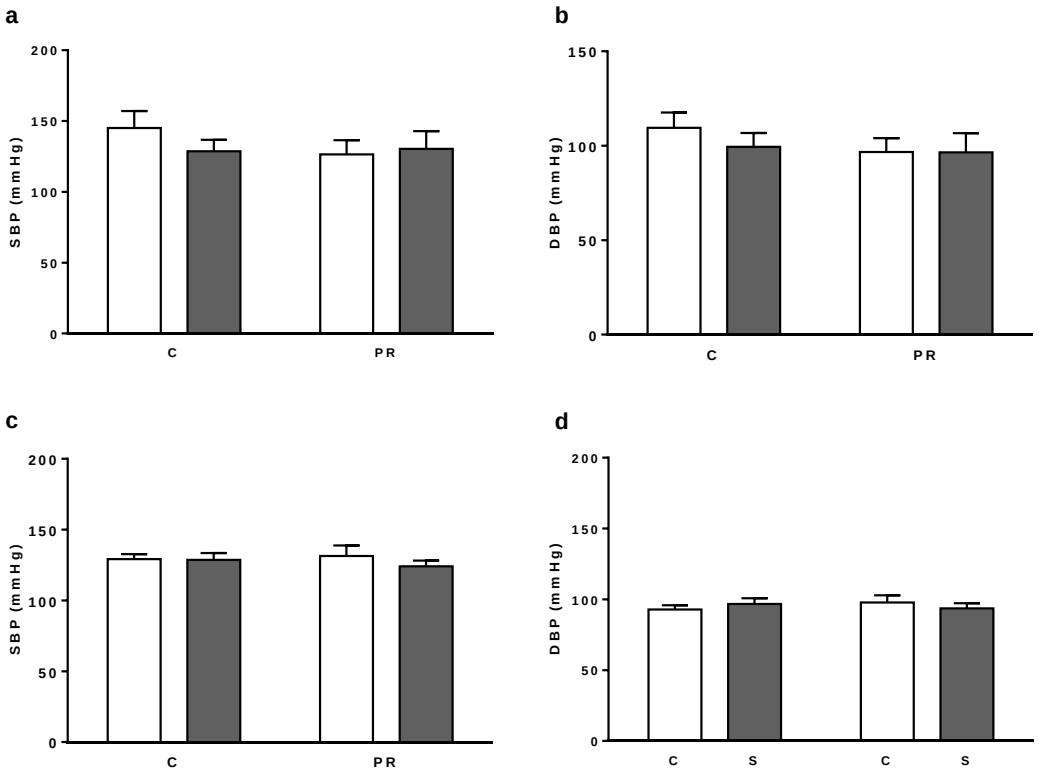


Figure 4.2 Systolic blood pressure (SBP) and diastolic blood pressure (DBP) of 16 week old male (a and b; n=4 for C and PR; n=6 for CS; n=5 for PRS) and female (c and d; n=4 for C and PR; n=5 for CS; n=6 for PRS) offspring. □ Non treated offspring, ■ statin treated offspring.

Males	C (n=4)	CS (n=6)	PR (n=4)	PRS (n=5)
SBP (mmHg)	145±12	129±8	127±10	130±13
DBP (mmHg)	109±8	99±7	97±7	96±10
MAP (mmHg)	121±9	109±8	107±8	108±11
HR (bpm)	447±15	449±7	445±14	419±11
Females	**C (n=4)**	**CS (n=5)**	**PR (n=4)**	**PRS (n=6)**
SBP (mmHg)	129±7	129±11	131±15	124±10
DBP (mmHg)	93±6	97±9	98±10	94±9
MAP (mmHg)	105±6	107±9	109±12	104±9
HR (bpm)	438±26	427±8	476±17	437±15

Table 4.1 Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) readings in the 16 week old male and female offspring

4.3.3 Organ weights

There were no differences in body weights between the groups at the time of euthanisation ($p>0.05$ for all factors). There were no differences in most of the organ sizes when measured either in absolute weight or as percentage of body weight ($p>0.05$

for all factors; Table 4.2) between the groups in males and females. Maternal protein restriction independent of statin treatment reduced percentage lung weight ($p<0.05$ for maternal diet effect, significance not maintained following *post hoc* correction; $p>0.05$ for statin effect and interaction) in males. In females the right kidney size was reduced in the PR offspring independent of statin treatment both in absolute weight ($p<0.01$ for maternal diet effect, significance maintained CS versus PR following *post hoc* correction; $p>0.05$ for statin effect and interaction) and percentage weight ($p<0.05$ for maternal diet effect, significance not maintained following *post hoc* correction; $p>0.05$ for statin effect and interaction).

Males		C (n=6/5)	CS (n=6)	PR (n=5/4)	PRS (n=5)
Body	weight (g)	390.9 ± 12.9	389.3 ± 10.2	391.9 ± 20.4	389.1 ± 11.6
Liver	weight (g)	13.84 ± 0.35	13.91 ± 0.63	13.65 ± 0.38	14.13 ± 0.70
	% of body weight	3.58 ± 0.04	3.57 ± 0.09	3.51 ± 0.08	3.63 ± 0.10
Kidney	weight (g)	1.05 ± 0.04	1.05 ± 0.03	1.02 ± 0.04	1.03 ± 0.04
	% of body weight	0.27 ± 0.01	0.27 ± 0.002	0.26 ± 0.02	0.26 ± 0.01
Heart	weight (g)	1.00 ± 0.03	1.03 ± 0.04	1.06 ± 0.02	0.99 ± 0.04
	% of body weight	0.26 ± 0.002	0.26 ± 0.01	0.28 ± 0.02	0.25 ± 0.003
Lungs	weight (g)	1.93 ± 0.06	1.80 ± 0.08	1.75 ± 0.06	1.74 ± 0.09
	% of body weight	0.50 ± 0.01	0.46 ± 0.02	0.44 ± 0.02*	0.45 ± 0.01*
Females		**C (n=5)**	**CS (n=6)**	**PR (n=5)**	**PRS (n=6)**
Body	weight (g)	234.2 ± 8.9	235.9 ± 3.8	217.9 ± 9.6	227.8 ± 6.3
Liver	weight (g)	8.42 ± 0.19	8.88 ± 0.31	8.09 ± 0.47	8.32 ± 0.39
	% of body weight	3.61 ± 0.08	3.77 ± 0.15	3.71 ± 0.06	3.65 ± 0.14
Kidney	weight (g)	0.72 ± 0.02	0.76 ± 0.01	0.62 ± 0.03#	0.68 ± 0.04#
	% of body weight	0.31 ± 0.01	0.32 ± 0.01	0.29 ± 0.01*	0.30 ± 0.01*
Heart	weight (g)	0.71 ± 0.03	0.72 ± 0.02	0.7 ± 0.03	0.74 ± 0.03
	% of body weight	0.30 ± 0.01	0.30 ± 0.01	0.32 ± 0.02	0.32 ± 0.01
Lungs	weight (g)	1.35 ± 0.07	1.35 ± 0.07	1.28 ± 0.06	1.33 ± 0.06
	% of body weight	0.58 ± 0.03	0.57 ± 0.03	0.59 ± 0.03	0.58 ± 0.02

Table 4.2 Post mortem body and organ weights of sixteen week old offspring. * p<0.05 PR groups versus C groups, # p<0.01 PR groups versus C groups.

4.3.4 Vasomotor responses in the aorta

4.3.4.1 Constriction to phenylephrine

Phenylephrine produced a concentration dependent constriction in all aortae. Maternal diet, statin treatment and interaction were all indicated as having a significant effect on maximal constriction to PE (C, 2.81±0.11 g, n=6; CS, 3.52±0.20 g, n=6; PR, 2.49±0.13

g, n=5; PRS, 2.42±0.05 g, n=5; $p<0.05$ for statin effect and interaction; $p<0.0001$ for maternal diet effect, significance maintained in CS versus C, PR and PRS following *post hoc* correction; Figure 4.3 a). Sensitivity to PE was reduced in the PR offspring irrespective of statin treatment (pEC_{50}: C 7.1±0.1, n=6; CS 6.9±0.1; PR 6 6.8±0.1, n=5; PRS 6.7±0.04, n=5; $p<0.05$ for maternal diet effect, significance not maintained following *post hoc* correction; $p>0.05$ for statin effect and interaction).

In the females maximal constriction to PE was reduced in the PR offspring irrespective of statin treatment (C, 2.18±0.35 g, n=5; CS, 2.17±0.20 g, n=6; PR, 2.03±0.15 g, n=5; PRS, 1.26±0.13 g, n=6; $p<0.05$ for maternal diet effect, significance maintained PRS versus C and CS following *post hoc* correction; $p>0.05$ for statin effect and interaction; Figure 4.3 b).Neither maternal diet nor statin altered sensitivity to PE (pEC_{50}: C 7.1±0.3, n=5; CS 6.8±0.2, n=6; PR 6.9±0.1, n=5; PRS 6.7±0.2, n=6; $p>0.05$ for all factors).

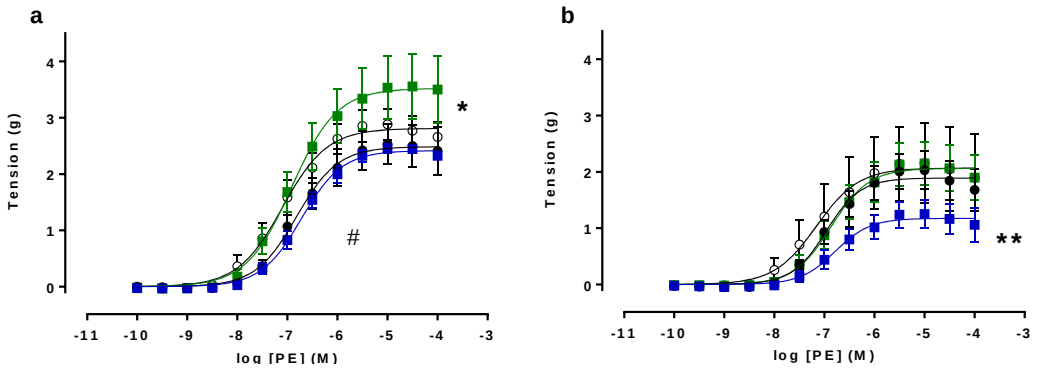


Figure 4.3 Phenylephrine (PE) induced constriction in the aorta of ○ C (n=6/5), ■ CS (n=6), ● PR (n=5) and ■ PRS (n=5/6) in male (a) and female (b) offspring. * $p<0.05$ maximal constriction CS versus C, PR and PRS. # $p<0.05$ pEC_{50} PR groups versus C groups. ** $p<0.05$ maximal constriction PR groups versus C groups.

4.3.4.2 Relaxation to acetylcholine

Acetylcholine induced a concentration-dependent relaxation in all aortae. In the male offspring interaction was detected (% maximal response: C, 68.4±4.1, n=6; CS, 58.3±3.8, n=6; PR, 65.4±5.2, n=5; PRS, 73.2±3.0, n=5; $p<0.05$ for interaction, significance not maintained following *post hoc* correction; $p>0.05$ for maternal diet and statin effect; Figure 4.4 a) No differences were seen in sensitivity between the groups (pEC_{50}: C 7.2±0.1, n=6; CS 7.3±0.1, n=6; PR 7.4±0.2, n=5; PRS 7.4±0.1, n=5; $p>0.05$ for all factors).

In the females interaction was observed in the maximal relaxation to ACh (% maximal response: C, 80.1±7.5, n=5; CS, 79.2±3.1, n=6; PR, 68.4±6.0, n=5; PRS, 105.3±8.2, n=6; $p<0.01$ for interaction; $p<0.05$ for statin effect; $p>0.05$ for maternal diet effect; significance maintained PRS versus CS and PR following *post hoc* correction; Figure 4.4 b). No differences were seen in sensitivity between the groups (pEC_{50}: C 7.8±0.3, n=5; CS 7.6±0.1, n=6; PR 7.6±0.2, n=5; PRS 7.5±0.2, n=6; $p>0.05$ for all factors).

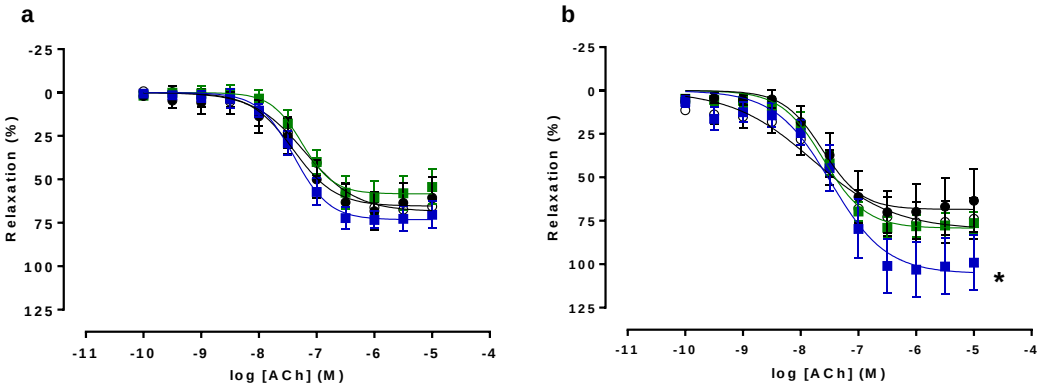


Figure 4.4 Acetylcholine (ACh) induced relaxation in the aorta of ○ C (n=6/5), ■ CS, (n=6) ● PR (n=5) and ■ PRS (n=5/6) in male (a) and female (b) offspring. * p<0.05 maximal relaxation PRS versus CS and PR.

ACh-induced relaxation was completely blocked by L-NAME 100 µM in all aortae tested in males (% response following addition of final dose: C, -19.9±12.7, n=6; CS, -30.7±7.6, n=6; PR, -12.4±17.7, n=5; PRS, -38.8±3.9, n=5). Maternal protein restriction increased maximal relaxation to ACh in the presence of 100 nM L-NAME irrespective of statin treatment in the male offspring (% maximal response: C, 6.2±20.8, n=6; CS, -0.2±18.2, n=6; PR, 75.6±11.7, n=5; PRS, 34.2±21.1, n=5; $p<0.05$ for maternal diet effect, significance maintained C versus PR following *post hoc* correction; $p>0.05$ for statin effect and interaction; Figure 4.5). Similar results were obtained when comparisons of maximal ACh relaxation with and without L-NAME were made within each groups (following p-values after *post hoc* correction); in C and CS groups maximal relaxation did not significantly differ from100 µM L-NAME ($p>0.05$) but was significantly reduced compared to naïve ACh ($p<0.01$) and in the PR and PRS groups 100 nM did not differ form naive ($p>0.05$) but did differ from 100 µM L-NAME though only in PR group ($p<0.001$).

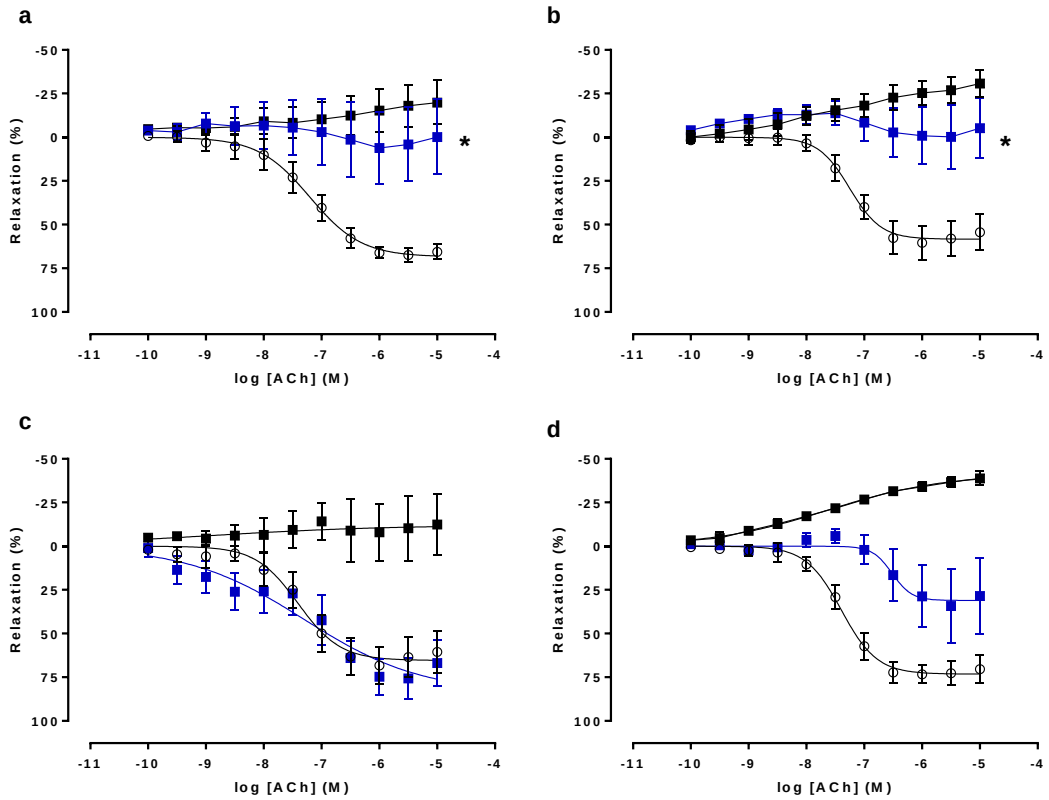


Figure 4.5 Acetylcholine (ACh) induced relaxation in the aorta of C (a; n=6), CS (b; n=6), PR (c; n;5) and PRS (d; n=5) male offspring; naïve ○, L-NAME 100 nM ■ and L-NAME 100 µM ■. * p<0.05 maximal relaxation naïve versus 100 nM L-NAME.

Relaxation response to ACh was abolished by 100 µM L-NAME in the aortae in females (% response following addition of final dose: C, 5.8±13.2, n=5; CS -36.5±9.7, n=6; PR, 4.9±21.0, n=5; PRS -16.9±9.1, n=6). As in the males maternal protein restriction increased maximal relaxation to ACh in the presence of 100 nM L-NAME irrespective of statin treatment (% maximal response: C, 27.3±8.7, n=5; CS, 24.9±6.9, n=6; PR, 75.3±5.2, n=5; PRS, 88.7±21.9, n=6; $p<0.001$ for maternal diet effect, significance maintained PRS versus C and CS; $p>0.05$ for statin effect and interaction; Figure 4.6). Again, similar results were obtained when comparisons of maximal ACh relaxation with and without L-NAME were made within each groups (following p-values after *post hoc* correction); in C and CS groups maximal relaxation did not significantly differ from100 µM L-NAME ($p>0.05$) but was significantly reduced compared to naïve ACh ($p<0.01$) and in the PR and PRS groups 100 nM did not differ form naive ($p>0.01$) but did differ from 100 µM L-NAME though only in PR group ($p<0.001$).

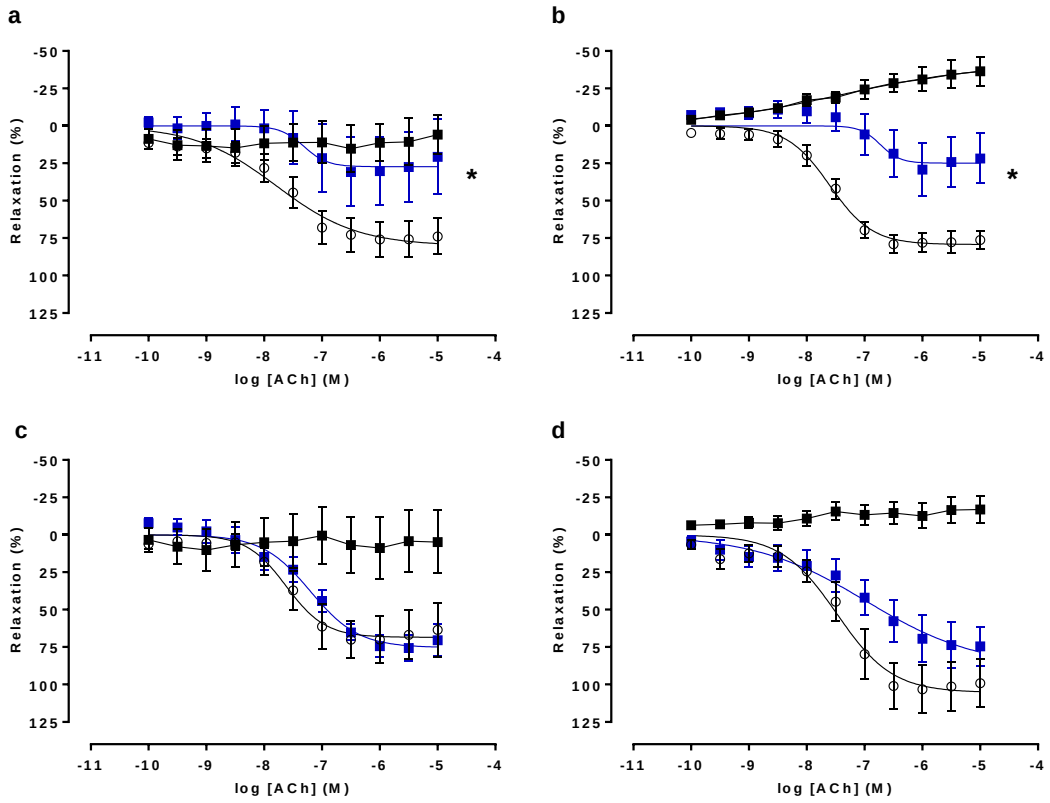


Figure 4.6 Acetylcholine (ACh) induced relaxation in the aorta of C (a; n=5), CS (b; n=6), PR (c; n=5) and PRS (d; n=6) female offspring; naïve ○, L-NAME 100 nM ■ and L-NAME 100 µM ■. * p<0.01 maximal relaxation naïve versus 100 nM L-NAME.

4.3.4.3 Relaxation to sodium nitroprusside

The NO donor SNP produced a concentration dependent relaxation in all aortae. No differences were seen in maximal relaxation to SNP in males (% maximal response: C, 103.9±3.0, n=6; CS, 105.4±3.8, n=6; PR, 101.9±3.0, n=5; PRS, 101.7±3.5, n=5; $p>0.05$ for all factors; Figure 4.7 a). Maternal PR increased sensitivity and statin treatment reduced sensitivity to SNP (pEC_{50}: C, 9.3±0.1, n=6; CS, 8.9±0.10, n=6; PR, 9.5±0.1, n=5; PRS, 9.3±0.1, n=5; $p<0.01$ for maternal diet and statin effect, significance maintained CS versus C, PR and PRS following *post hoc* correction; $p>0.05$ for interaction).

No differences were seen in maximal relaxation to SNP in females (% maximal response: C, 110.0±3.2, n=5; CS, 104.9±4.6, n=6; PR, 106.6±2.4, n=5; PRS, 110.6±3.9, n=6; $p>0.05$ for all factors; Figure 4.7 b). No difference was seen in sensitivity between the groups either (pEC_{50}: C 9.4±0.1, n=5; CS 9.2±0.1, n=6; PR 9.3±0.1, n=5; PRS 9.1±0.1, n=6; $p>0.05$ for all factors).

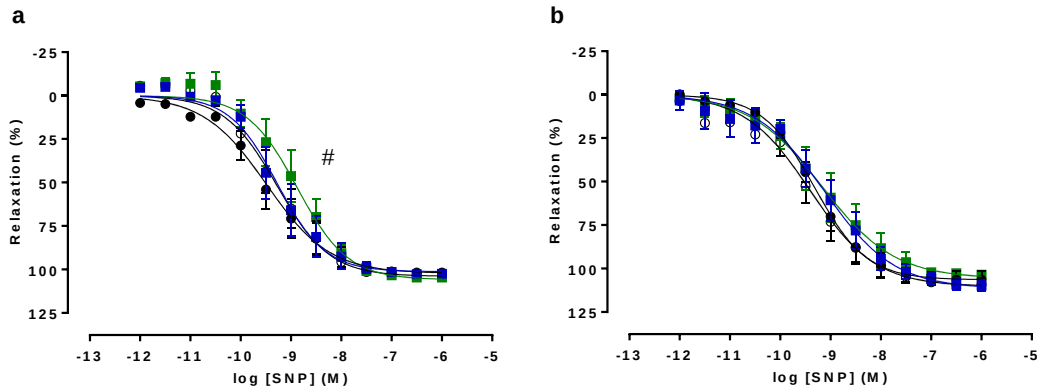


Figure 4.7 Sodium nitroprusside (SNP) induced relaxation in the aorta of ○ C (n=6/5), ■ CS (n=6), ● PR (n=5) and ■ PRS (n=5/6) in male (a) and female (b) offspring. # p<0.01 pEC_{50} PR groups versus C groups.

4.3.5 Vasomotor responses in the mesenteric arteries

Of the 44 segments of mesenteric arteries mounted from the male offspring 1 failed to achieve 100 mmHg active tension and was excluded from experiments. No differences were seen in the estimated mesenteric artery diameters of the functional segments between the groups (C, 345.21±8.83 µm, n=6; CS, 326.35±16.61 µm, n=6; PR, 326.46±11.70 µm, n=5; PRS, 317.47±22.22 µm, n=5; p>0.05 for all factors; Figure 4.8 a). Of the 44 segments of mesenteric arteries mounted from the female offspring 4 failed to achieve 100 mmHg active tension and were excluded from experiments. No differences were seen in the mesenteric artery diameters of the functional segments between the groups (C, 316.16±20.16 µm, n=5; CS, 339.07±11.00 µm, n=6; PR, 289.69±21.64 µm, n=5; PRS, 309.18±18.88 µm, n=6; p>0.05 for all factors; Figure 4.8 b).

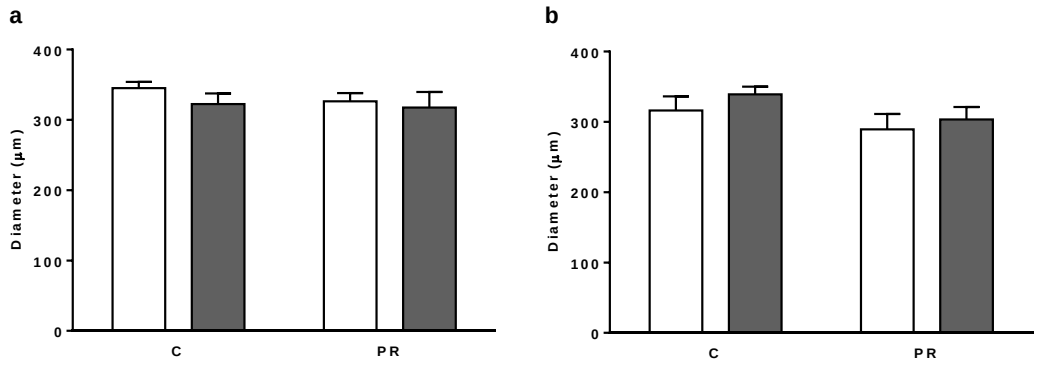


Figure 4.8 Mesenteric artery diameter of the 16 week old male (a; C, n=6, CS, n=6; PR, n=5; PRS, n=5) and female (b; C, n=5, CS, n=6; PR, n=5; PRS, n=6) offspring. □ non treated offspring, ■ statin treated offspring.

4.3.5.1 Constriction to phenylephrine

Phenylephrine produced a concentration dependent constriction in all tested mesenteric arteries. Both maternal protein restriction as well as statin treatment increased maximal constriction to PE in mesenteric arteries of male offspring (mN/mm^2: C 4.12±0.16, n=6; CS, 4.57±0.16, n=6; PR, 4.66±0.29, n=5; PRS, 5.27±0.21, n=5; $p<0.01$ for maternal diet effect; $p<0.05$ for statin effect; $p>0.05$ for interaction; significance maintained PRS versus C following *post hoc* correction; Figure 4.9 a). No differences were seen in sensitivity between the groups (pEC_{50}: C 8.5±0.1, n=6; CS 8.7±0.1, n=6; PR 8.5±0.1, n=5; PRS 8.6±0.1, n=5; $p>0.05$ for all factors).

In females statin treatment also increased constriction and there was a trend ($p<0.06$) for increased constriction in response to maternal diet (mN/mm^2: C 3.22±0.26, n=5; CS, 4.17±0.23, n=6; PR, 3.82±0.40, n=5; PRS, 4.71±0.24, n=6; $p<0.01$ for statin effect, significance maintained PRS verus C following *post hoc* correction; $p>0.05$ for maternal diet effect and interaction; Figure 4.9 b). No differences were seen in sensitivity between the groups (pEC_{50}: C 6.4±0.2, n=5; CS 6.6±0.1, n=6; PR 6.4±0.2, n=5; PRS 6.4±0.1, n=6; $p>0.05$ for all factors).

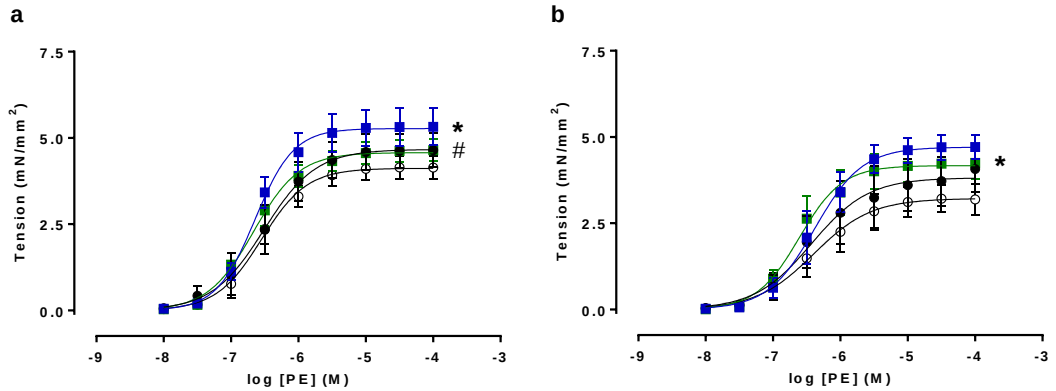


Figure 4.9 Phenylephrine (PE) induced constriction in the mesenteric arteries of ○ C (n=6/5), ■ CS (n=6), ● PR (n=5) and ■ PRS (n=5/6) in male (a) and female (b) offspring. * $p<0.05$ maximal constriction statin treated versus non-treated. # $p<0.05$ maximal constriction PR groups versus C groups.

4.3.5.2 Constriction to endothelin

Endothelin induced a concentration-dependent constriction in all tested mesenteric arteries. No significant differences were seen in maximal constriction to endothelin in male offspring (mN/mm^2: C 4.21±0.46, n=6; CS 5.19±0.516, n=5; PR 4.72±0.52, n=5;

PRS 5.83±0.71, n=5; p>0.05 for all factors; Figure 4.10 a) though there was a trend towards increased constriction in the statin treated offspring (p<0.08). No differences were seen in sensitivity between the groups (pEC_{50}: C 8.6±0.2, n=6; CS 8.9±0.2, n=6; PR 8.7±0.2, n=5; PRS 8.5±0.2, n=5; p>0.05 for all factors).

In the female offspring increased maximal constriction was seen in statin treated animals irrespective of maternal diet (mN/mm^2: C 3.27±0.56, n=4; CS 4.43±0.36, n=5; PR 3.44±0.62, n=4; PRS 6.08±0.64, n=5; p p<0.01 for statin effect, significance maintained PRS versus C and PR following *post hoc* correction; p>0.05 for maternal diet effect and interaction; Figure 4.10 a). No differences were seen in sensitivity between the groups (pEC_{50}: C 8.9±0.3, n=4; CS 9.0±0.1, n=5; PR 9.0±0.3, n=4; PRS 8.4±0.2, n=5; p>0.05 for all factors).

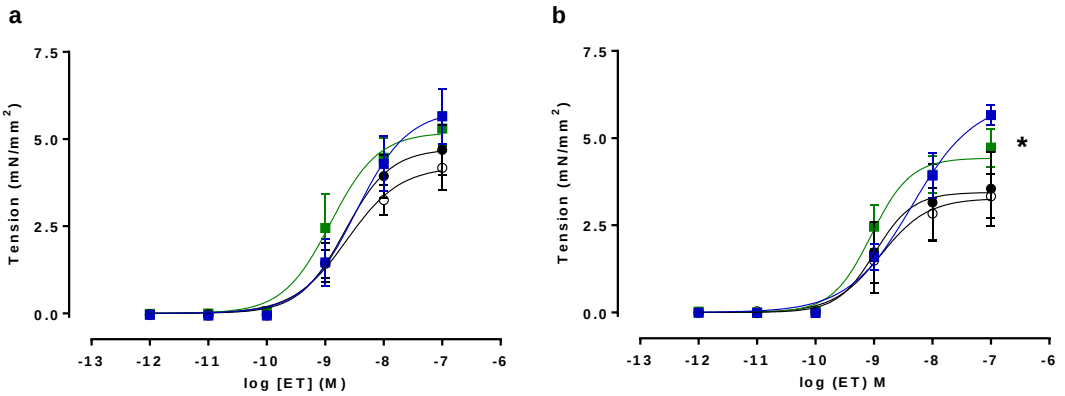


Figure 4.10 Endothelin (ET) induced constriction in the mesenteric arteries of ○ C (n=6/4), ■ CS (n=5), ● PR (n=5/4) and ■ PRS (n=5) in male (a) and female (b) offspring. * p<0.01 maximal constriction statin treated versus non-treated.

4.3.5.3 Relaxation to acetylcholine

No changes were seen in acetylcholine induced maximal constriction between the groups in the male (% maximal response: C 37.5±4.05, n=4; CS 31.43±2.24, n=5; PR 41.65±2.05, n=4; PRS 39.24±2.81, n=2; p>0.05 for all factors; Figure 4.11 a) or the female (% maximal response: C 35.00±8.40, n=3; CS 37.33±11.36, n=3; PR 56.55±8.05, n=2; PRS 32.19±5.83, n=4; p>0.05 for all factors; Figure 4.11 b) offspring.

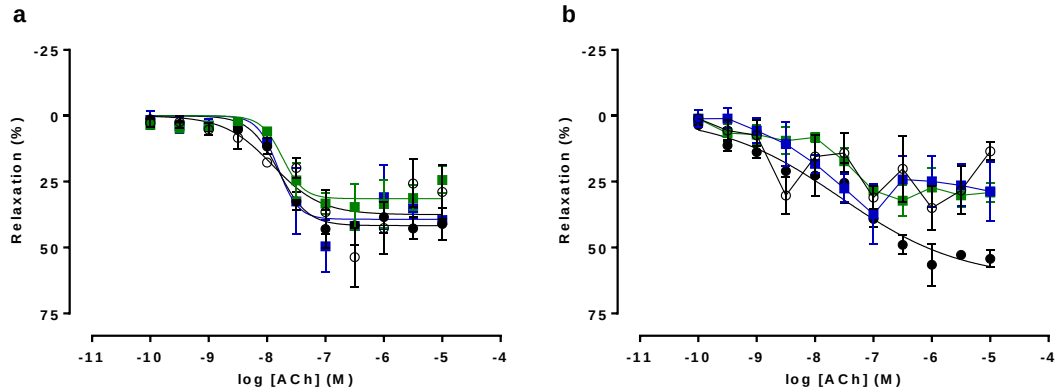


Figure 4.11 Acetylcholine (ACh) induced relaxation in the mesenteric arteries of ○ C (n=4/3), ■ CS (n=5/3), ● PR (n=4/2)and ■ PRS (n=2/4) in male (a) and female (b) offspring.

Incubation with 100µM eNOS inhibtor L-NAME, 10 µM COX-1 inhibitor indomethacin and 25 mM KPSS completely blocked the ACh induced relaxation in all the groups in males (% maximal response: C -7.00±3.87, n=6; CS -7.88±4.96, n=5; PR -0.08±2.51, n=5; PRS -3.80±4.82, n=5; Figure 4.12) and females (% maximal response: C -5.33±4.72, n=4; CS -6.70±3.54, n=5; PR -2.70±2.29, n=4; PRS -12.45±3.49, n=4; Figure 4.13).

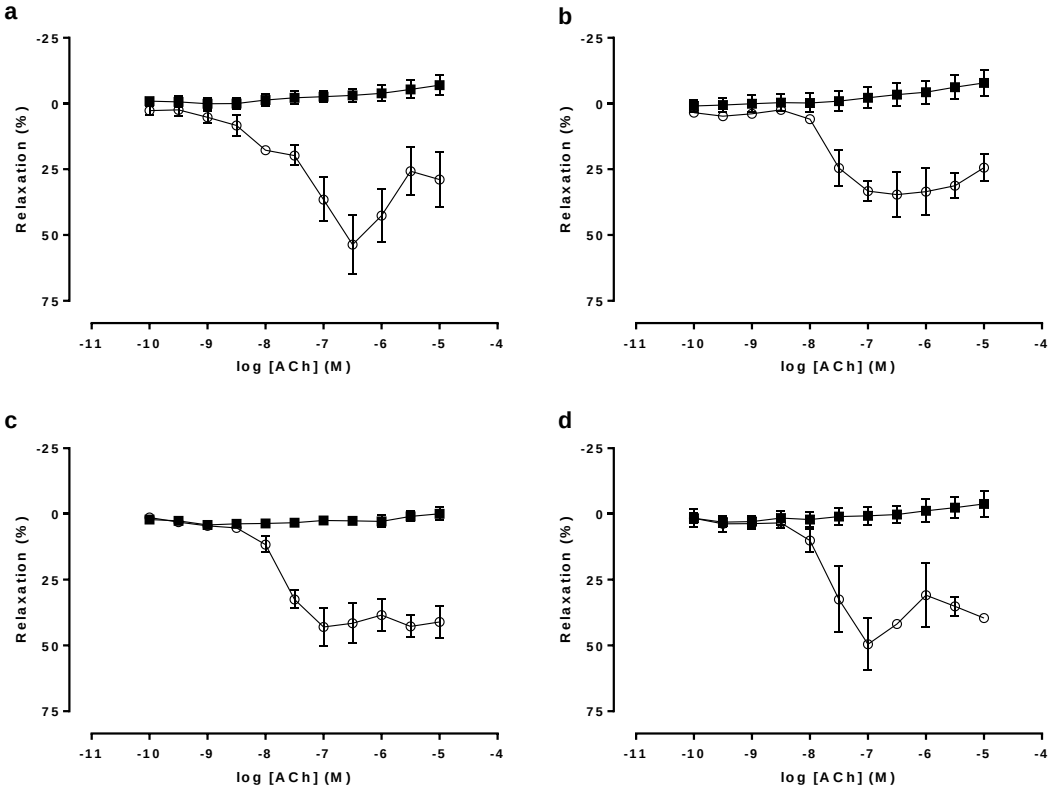


Figure 4.12 Acetylcholine (ACh) induced relaxation in the mesenteric arteries of C (a; n=6), CS (b; n=5,), PR (c; n=5) and PRS (d; n=5) male offspring; naïve ○ and 100 µL-NAME, 10 µM indomethacin and 25 mM KPSS.

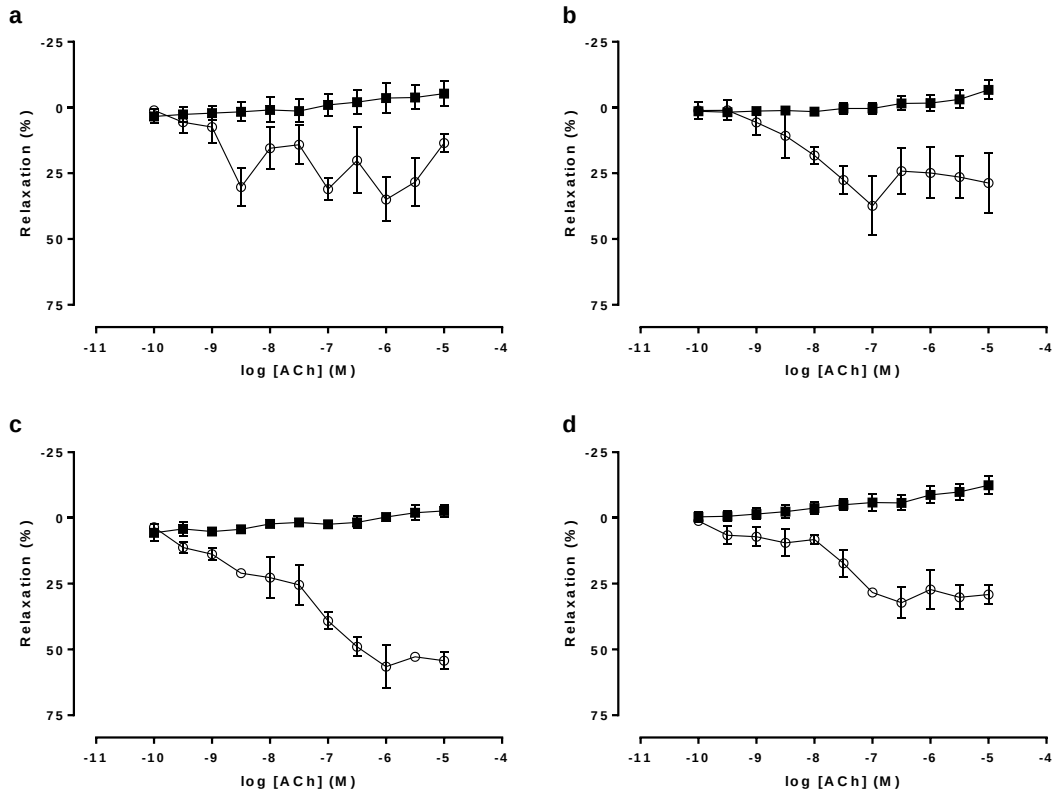


Figure 4.13 Acetylcholine (ACh) induced relaxation in the mesenteric arteries of C (a; n=4), CS (b; n=5,), PR (c; n=4) and PRS (d; n=4) female offspring; naïve ○ and 100 µL-NAME, 10 µM indomethacin and 25 mM KPSS.

4.3.5.4 Relaxation to sodium nitroprusside

Sodium nitroprusside induced a concentration dependent relaxation in all tested arteries. Statin treatment increased maximal relaxation in the male offspring independent of maternal diet (% maximal response: C 69.40±5.07, n=6; CS 78.07±2.63, n=6; PR 65.97±5.98, n=5; PRS 88.95±3.16, n=5; $p<0.01$ for statin effect significance maintained PRS versus C and PR following *post hoc* correction; $p>0.05$ for maternal diet and interaction; Figure 4.14 a). Interaction was observed in the SNP sensitivity (pEC_{50}: C 8.8±0.2, n=6; CS 8.6±0.1, n=6; PR 8.1±0.2, n=5; PRS 8.8±0.1, n=5; $p<0.05$ for interaction, significance maintained PRS versus PR following *post hoc* correction; $p>0.05$ for maternal diet and statin effect).

No differences were seen in maximal relaxation to SNP in the female offspring (% maximal response: C 80.63±7.59, n=5; CS 89.78±10.08, n=5; PR 77.84±4.64, n=5; PRS 75.46±4.51, n=5, $p>0.05$ for all factors; Figure 4.14 b). No differences were seen in sensitivity between the groups either (C 9.3±0.7, n=5; CS 9.8±0.3, n=5; PR 10.1±0.5, n=5; PRS 10.6±0.2, n=5; $p>0.05$ for all factors).

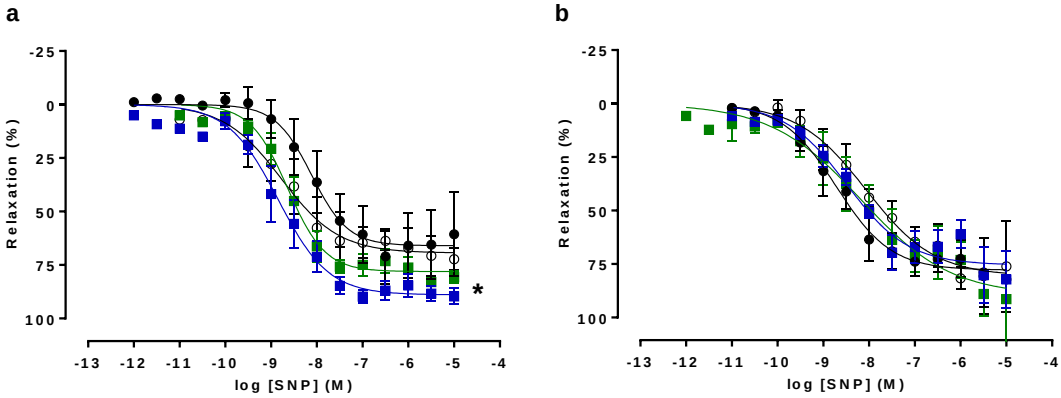


Figure 4.14 Sodium nitroprusside (SNP) induced relaxation in the mesenteric arteries of ○ C (n=6/5), ■ CS (n=6/5), ● PR (n=5) and ■ PRS (n=5) in male (a) and female (b) offspring. * p<0.01 maximal relaxation statin treated versus non-statin treated.

4.4 Discussion

Maternal protein restriction has been shown to impair vascular function in rat offspring (Brawley *et al.*, 2003;Rodford *et al.*, 2008) and early age acute treatment with renin-angiotensin system targeting drugs has been shown to improve this (Sherman & Langley-Evans, 1998;Sherman & Langley-Evans, 2000). As chronic statin treatment has been shown to improve vascular function in this model as well (Torrens *et al.*, 2009b) this study set out to investigate the long term effects of early age acute statin treatment in the PR model. This study shows that early age statin treatment is not protective long term but leads to increased constriction. The functional data for five and sixteen weeks old animals are summarised in Table 4.3.

		5 week aorta		16 week aorta		16 week mesenterics	
		Males	Females	Males	Females	Males	Females
PE max	PR	▼	▼		▼	▲	▲
	S	▼		▲**		▲	▲
PE EC50	PR	▼	▼	▼			
	S						
ET max	PR						
	S					▲	▲
ET EC50	PR						
	S						
ACh max	PR		▲	#			
	S			#	▲***		
ACh EC50	PR						
	S						
SNP max	PR		▼*				
	S					▲	
SNP EC50	PR			▲		#	
	S			▼		#	

Table 4.3 Summary of aortic and mesenteric vasoreactivity results at five and sixteen weeks. ▲ increased maximal constriction or sensitivity, ▼ reduced maximal constriction or sensitivity, ▲ / ▼ p<0.05, ▲ p<0.1, # interaction but no differences between groups detected by Bonferonni's multiple comparisons test, ** interaction: CS versus C, PR and PRS, * interaction: PRS versus CS and PRS.**

Increased constriction was observed in the aorta and mesenteric arteries of the statin treated animals in both males and females. In the aorta the only statin effect on constriction was observed in the CS group in males where the constriction was increased. In the mesenteric arteries increased constriction was seen in all statin treated offspring to PE and.to ET in the statin treated female offspring. The long term results are contradictory to those observed in the five week old animals as reduced maximal constriction was observed in the male offspring aorta and unlike the beneficial effects at five weeks these results at sixteen weeks show adverse effects. Interestingly, other animal studies have shown impairment of vascular and metabolic parameters after withdrawal of statin treatment (Gertz *et al.*, 2003;Laufs *et al.*, 2000;Otani *et al.*, 2010;Vecchione & Brandes, 2002) which could explain the results observed in the current study. Some of the withdrawal studies have looked at the effects of withdrawal after short term statin treatment (10-14 days) in mature mice and have shown that the impairment of vascular parameters is transient and returns to control levels some days after the withdrawal (Gertz *et al.*, 2003;Laufs *et al.*, 2000;Vecchione & Brandes, 2002).

Long term effects have also been shown in mature rats after a longer treatment period (16 weeks) where impairment was shown in glucose handling and pancreatic parameters fifty weeks after statin withdrawal (Otani *et al.*, 2010). Though the previous studies looking at the effects of short term treatment prior to withdrawal have shown transient effects, the young age at the time of the treatment and withdrawal in the current study could account for the long term effects observed. These studies have implicated different mechanisms behind the effects of statin withdrawal and the applicability of these mechanisms to the current study are discussed below.

As increased constriction in the mesenteric arteries was shown in response to both ET and PE the statin effects would appear to have either occurred in mechanisms promoting constriction in general or in the parts of the pathways shared by PE and ET. Reduction in relaxation agents could allow the increase in constriction to all agents as has been demonstrated by increased constriction after inhibition of NO synthesis (Dresner *et al.*, 1997;Vo *et al.*, 1992). As statins are known to upregulate NO (Aoki *et al.*, 2012;Feron *et al.*, 2001;Kansui *et al.*, 2004;Wenzel *et al.*, 2008) this appears a possibility for increased constriction especially as statin withdrawal induced reduction in ACh mediated relaxation has been shown to be NO-dependent (Vecchione & Brandes, 2002). As already discussed in Chapter 3, alterations in NO levels might be expected to alter ACh relaxation and no indication of this was seen in the mesenteric arteries. In the aorta the only indication of statin effect on ACh relaxation was seen in the female offspring, where no increase in constriction was observed, as ACh induced relaxation was increased in the PRS offspring. Thus the ACh relaxation data does not support reduced NO bioavailability as mechanism behind the increase in constriction.

PE elicits constriction through α_1-adrenoceptor and ET through ET_A receptor and the pathway downstream of these GPCRs is shared as they induce constriction via phospholipase C induced Ca^{2+} release leading to MLCK activation and MLC phosphorylation. There is some evidence that statins can upregulate phospholipase C activity (Mutoh *et al.*, 1999) and if the upregulation was maintained after statin withdrawal this could account for the increase in constriction. This appears unlikely though as at five weeks the constriction was reduced in the aortae. A likelier option appears the promotion of constriction via RhoA activation. Statins are well known to reduce RhoA activity by inhibiting its isoprenylation, which could explain reduced

constriction at five weeks, and it has been shown that statin withdrawal can lead to upregulation of RhoA activity (Laufs *et al.*, 2000). Inhibition of RhoA activity by statins leads to upregulation of RhoA mRNA levels and cytosolic protein via a negative feedback loop which leads to a transient increase in RhoA activity after statin withdrawal (Laufs *et al.*, 2000). Sustained upregulation of RhoA gene expression could lead to increase in constriction observed in this study. Also statin treatment has been shown to increase HMG-Co A reductase mRNA levels (Ness *et al.*, 1998;Torrens *et al.*, 2009b) and, if converted to activity levels, sustained upregulation of HMG-Co A reductase mRNA levels could lead to upregulation of RhoA activity thus promoting constriction.

These findings could well have clinical implications as there is evidence from clinical trials that statin withdrawal can lead to a rebound effect. Follow ups of up to a year after statin withdrawal have shown an increase in event rates and mortality as compared to continued statin treatment and never on statins (Daskalopoulou *et al.*, 2008;Heeschen *et al.*, 2002). Endothelial function seems to deteriorate after statin withdrawal (Laufs *et al.*, 2001;Taneva *et al.*, 2006) and increase in inflammation has also been shown (Lai *et al.*, 2005), at least briefly after withdrawal. On the other hand follow ups of trials up to five years after trial period have shown that event rates and mortality are reduced in the groups that were on statins during the trials as compared to the placebo group (Bulbulia *et al.*, 2011;Ford *et al.*, 2007). In these studies though statin treatment in the post-trial period was not taken into account in the follow up analysis and it was shown that a higher proportion of individuals on statins during the study period were on statins at the time of the follow up than those who were originally in the placebo group. Thus it is possible that the higher prevalence of statin use during post-trial period masked possible statin withdrawal effects.

Maternal protein restriction was also shown to have an effect on the vascular function in the sixteen week old offspring. In the mesenteric arteries PE increased constriction in the PR offspring though this did not quite reach significance in the female offspring and no changes were seen in ET induced constriction. This increase in constriction could be an indication of PR induced vascular dysfunction though no impairment was observed in the endothelial function which is usually observed in this model. In the aorta though, maximal constriction to PE was reduced in PR female offspring as was sensitivity in the

male offspring. Also increased relaxation in the aorta was observed in both sexes of the PR offspring in response to ACh in the presence of 100 nM L-NAME. Similar results have been shown in other studies where maximal relaxation to ACh in presence of L-NAME and indomethacin was not reduced in mesenteric arteries of PR offspring as compared to naïve relaxation whereas partial relaxation was observed in the control offspring (Torrens *et al.*, 2009b) indicating impaired NO and prostaglandin contribution to ACh induced relaxation in the PR offspring and compensatory EDHF response. In the current study the differences in relaxation in the presence of L-NAME were seen in the aorta at 16 weeks but no impairment was seen to naïve ACh. The relaxation in the aorta was fully NO dependent, as shown by abolished relaxation in the presence of 100 μM L-NAME, thus the full relaxation in presence of submaximal L-NAME indicates either increased NO biosynthesis or greater sensitivity to NO. In the male offspring though increased sensitivity was shown to SNP which indicates increased NO sensitivity. Increased NO biosynthesis could be explained by increased eNOS levels and activity though this is not expected in this model. Increased NO biosynthesis would not only explain the alterations in relaxation but also the reduced sensitivity to PE observed in the male PR offspring. No differences were seen in naïve ACh pEC_{50} though thus altered NO biosynthesis does not appear likely. In the male offspring though increased sensitivity was shown to SNP which indicates increased NO sensitivity. This could be an indication of changes in the smooth muscle component of NO mediated relaxation, namely increased cGMP or sGC levels.

Even though changes were observed between the two maternal groups as discussed above these were by no means the same as observed in previous studies. Most notably no impairment of endothelium-dependent vasodilation was observed. No changes were shown in the PR offspring blood pressure either even though this has been reported before (Brawley *et al.*, 2003;Langley-Evans & Jackson, 1995;Mizuno *et al.*, 2013) This is not the first study not to show differences in vascular function in the PR offspring; endothelial dysfunction has not been always detected (Watkins *et al.*, 2010) and neither has an increase in blood pressure (Brennan *et al.*, 2008;Rodford *et al.*, 2008). As already discussed in Chapter 3 it is possible that this was due to new diet used in this laboratory or increased food intake in the PR dams. The maternal diet effect on growth was different in males and females though both effects have been reported previously. Maternal diet did not affect growth in male offspring which fits in with previous studies

(Torrens *et al.*, 2009b) as does the reduced growth observed in the female PR offspring (Sherman & Langley-Evans, 2000).

Interestingly some differences were also seen between the two sexes. The constrictor response to PE both in the aorta and mesenteric arteries was reduced in the females as compared to the males. Also there was a trend towards reduced constriction to ET ($p<0.07$) in the females (univariate general linear model, SPSS). Reduced constriction in females has been observed before and it has been indicated that this could be due to oestrogen altering endothelium dependent relaxation (Raffetto *et al.*, 2010) and Ca^{2+} entry from the extracellular space (Murphy & Khalil, 2000;Crews *et al.*, 1999). Increased relaxation was not observed in the female offspring in the current study and thus it would appear more likely that altered Ca^{2+} entry into the SMC might explain the differences.

This study has shown that early age acute statin treatment and withdrawal leads to long term increase in constriction in the mesenteric arteries of rats. Based on the evidence from previous studies the likeliest mechanism for this appears to be upregulation of the RhoA pathway. Maternal PR induced impairment of vascular function was not observed in the current study.

Chapter 5 Molecular changes in vascular tissues

5.1 Introduction

Statins work by inhibiting HMG-CoA reductase and thus mevalonate synthesis. Downstream products of the mevalonate pathway are involved in post-translational modifications of proteins and thus can alter their activity (Laufs *et al.*, 2000;Vecchione & Brandes, 2002). In addition to alterations in posttranslational modifications, statin treatment can alter gene and protein expression levels (Ali *et al.*, 2007;Ali *et al.*, 2009;Aoki *et al.*, 2012;Dichtl *et al.*, 2003;Torrens *et al.*, 2009b). These changes have been shown as part of the mechanisms behind statins' pleiotropic properties. Transient changes in mRNA and protein levels have been shown in response to statin withdrawal in adult mice (Gertz *et al.*, 2003;Laufs *et al.*, 2000).

Statins inhibit isoprenylation of RhoA (Figure 1.4) and thus its membrane translocation required for its full activation (Laufs *et al.*, 2000;Laufs & Liao, 1998;Ohkawara *et al.*, 2005;Ohkawara *et al.*, 2010). Statin treatment has also been shown to upregulate RhoA mRNA and protein expression through a negative feedback loop leading to an increase in membrane bound RhoA, at least transiently, after statin withdrawal (Laufs *et al.*, 2000) Membrane bound RhoA activates ROCK 1 and 2 which mediate constriction promoting effects in the SMC by inhibiting MLCP (Khalil, 2010). Statins have also been shown to increase eNOS mRNA levels (Aoki *et al.*, 2012;Laufs *et al.*, 1998) and RhoA has been shown to reduce eNOS mRNA levels(Laufs & Liao, 1998). Alterations in NO bioavailability have been shown to be involved in statin-mediated reduction in constrictor responses (Ghaffari *et al.*, 2011;Rossoni *et al.*, 2011;Tian *et al.*, 2011) and impairment of vascular function after statin withdrawal (Laufs *et al.*, 2000;Vecchione & Brandes, 2002).

Statin treatment has also been shown to lead to upregulation of HMG-CoA reductase mRNA in cell culture (Wu *et al.*, 2010) and in the liver in rodent models (Ness *et al.*, 1998) including the PR model (Torrens *et al.*, 2009b). Whether the expression remains elevated after statin withdrawal is not known but if it does this could lead to increase in HMG-CoA reductase activity and thus upregulation of the mevalonate pathway (Liao, 2002;Takemoto & Liao, 2001;Wang *et al.*, 2008) including upregulation of RhoA activity.

The previous chapters have shown that early age acute statin treatment can alter vascular function both short and long term with the long term changes appearing to be due to statin withdrawal. It was hypothesised that statin treatment reduced RhoA translocation whereas statin withdrawal increased RhoA translocation through upregulation of HMG-CoA reductase. To confirm this protein expression of RhoA was investigated as well as the mRNA levels of HMG-CoA reductase, eNOS and genes associated with the RhoA pathway.

5.2 Methods

5.2.1 RNA extraction, reverse transcription and realtime PCR

RNA was extracted from liver (30-50 mg) and mesenteric arteries (2 arcades) using phenolchloroform extraction as described in Chapter 2. Glycogen was added to mesenteric artery samples with isopropanol to aid RNA recovery from the small amount of tissue. Mesenteric artery samples were heated at the end of the protocol to help to dissolve the RNA extracted.

Reverse transcription into cDNA was carried out as described in chapter 2.

Real time PCR was carried out using primers and probes and FAM/TAMRA detection system as described in Chapter 2. The primer and probe sequences for each gene can be found in Table 2.1. The standards used for generation of the standard curve were generated from a pooled sample of cDNA extracted from the liver of the control animals. The standards were 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 % dilutions of the pooled sample.

5.2.2 Protein extraction, subcellular fractionation and quantification

Half an aorta (20-50 mg) form each animal was used for protein extraction and these samples were pooled for analysis. Protein was extracted using subcellular fractination protocol as described in Chapter 2 to generate whole cell, cytosolic and membrane/nuclear fractions. After extraction the samples were quantified using the Bradford method as described in Chapter 2. All samples and BSA standards were analysed in duplicate. 20 µl of each standard was loaded per well and samples were diluted 1:1 in 10 µl of water and 300 µl of Bradford reagent was added.

5.2.3 Western blotting

SDS-PAGE electrophoresis, transfer and Western blotting were run as described in Chapter 2. Volume equivalent of 10 μg of protein was loaded into each well. The gel was run at 200 V for 30 minutes. The transfer was run at 100 V for 60 minutes. The membranes were blocked in 5% BSA. Membrane marker NaKATPase primary antibody was diluted 1:5000 and RhoA primary antibody 1:1000.The primary antibody incubations were carried out at 4°C overnight. Anti-mouse secondary for NaKATPase was diluted 1:12 000 and anti-rabbit for RhoA 1:100 000 and incubated at room temperature for 90 minutes. HRP-conjugated β-actin was diluted 1:50 000 and incubated for 60 minutes at room temperature. The band intensity was quantified using ImageJ as described in Chapter 2.

5.2.4 Statistics

All real-time PCR results are expressed as mean ± SEM. The qPCR results for each gene were normalised against β-actin that was selected as stable housekeeping gene after geNorm and Normfinder analysis (Appendix 4). The differences between the groups were analysed using two-way ANOVA. The differences for males and females were analysed separately.

No statistical analysis could be performed on the Western blotting data as the samples were pooled.

5.3 Results

5.3.1 mRNA expression

5.3.1.1 Mesenteric arteries

No differences were seen in HMG-CoA reductase mRNA expression in the 16 week old males between the experimental groups ($p>0.05$ for all factors; Figure 5.1 a). In the female mesenteric arteries an increase in HMG-CoA reductase mRNA expression was seen in the statin treated animals irrespective of maternal diet ($p<0.05$ for statin effect, significance not maintained following *post hoc* correction; $p>0.05$ for maternal diet effect and interaction; Figure 5.1 b).

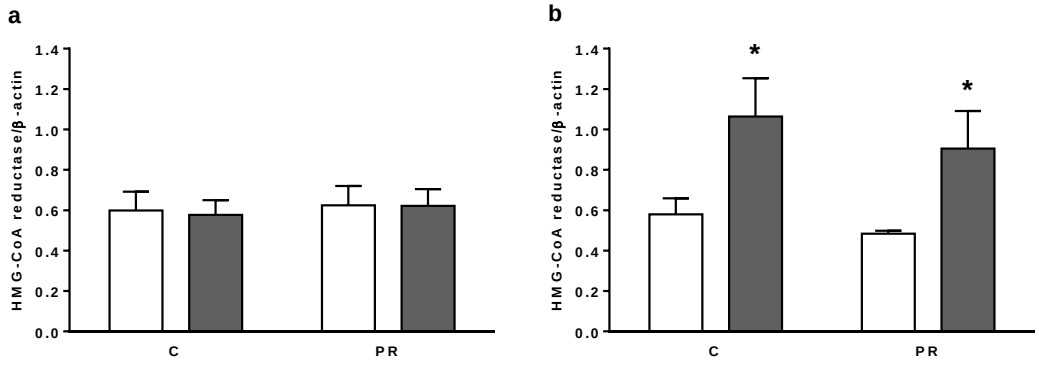


Figure 5.1 Relative HMG-CoA reductase mRNA expression in the mesenteric arteries of male (a; C n=6; CS n=6, PR n=5, PRS n=5) and female (b; C n=5; CS n=6, PR n=4, PRS n=5) offspring at 16 weeks; □ non treated offspring, ■ statin treated offspring. * p<0.05 statin treated versus non-treated.

Statin treatment increased eNOS mRNA expression in the 16 week old males irrespective of maternal diet ($p<0.05$ for statin effect, significance not maintained following *post hoc* correction; $p>0.05$ for maternal diet effect and interaction; Figure 5.2 a). No differences were seen between the groups in the 16 week old females ($p>0.05$ for all factors; Figure 5.2 b).

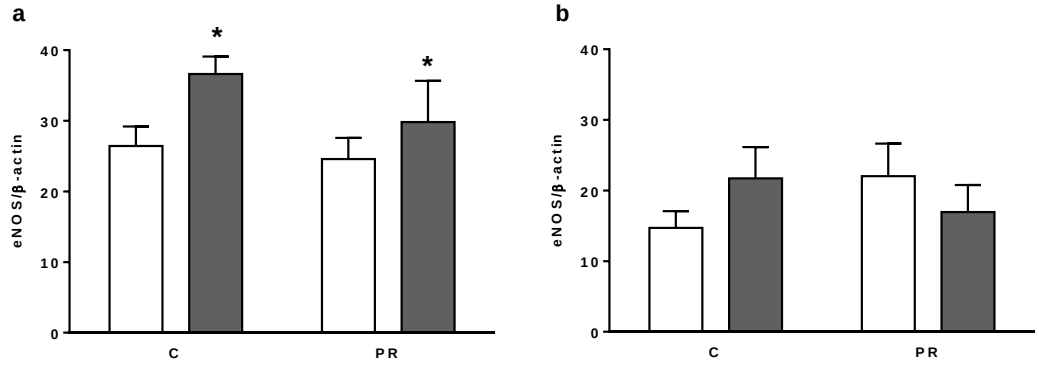


Figure 5.2 Relative eNOS mRNA expression in the mesenteric arteries of male (a; C n=6; CS n=6, PR n=5, PRS n=5) and female (b; C n=5; CS n=5, PR n=4, PRS n=5) offspring at 16 weeks; □ non treated offspring, ■ statin treated offspring. * p<0.05 statin treated versus non-treated.

No differences were seen in RhoA mRNA expression in the 16 week old males between the experimental groups ($p>0.05$ for all factors; Figure 5.3 a). In the female mesenteric arteries an increase was seen in RhoA mRNA expression in the statin treated animals irrespective of maternal diet ($p<0.05$ for statin effect, significance not maintained following *post hoc* correction; $p>0.05$ for maternal diet effect and interaction; Figure 5.3 b).

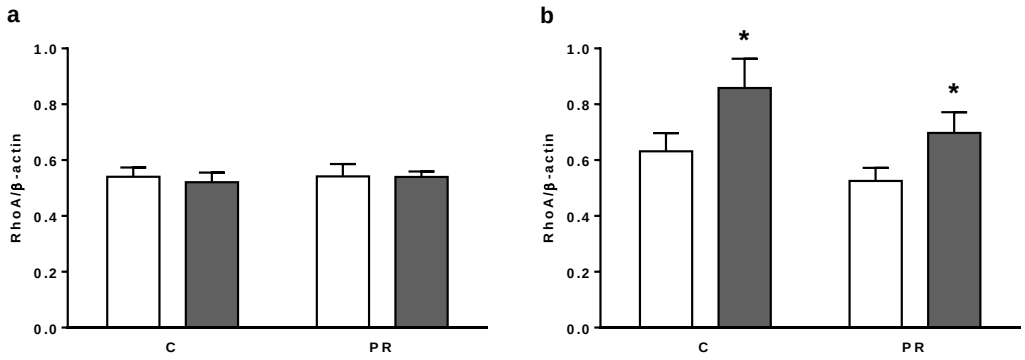


Figure 5.3 Relative RhoA mRNA expression in the mesenteric arteries of male (a; C n=6; CS n=6, PR n=5, PRS n=5) and female (b; C n=5; CS n=6, PR n=4, PRS n=5) offspring at 16 weeks; □ non treated offspring, ■ statin treated offspring. * p<0.05 statin treated versus non-treated.

Statin treatment increased ROCK 1 mRNA expression in the 16 week old males irrespective of maternal diet ($p<0.05$ for statin effect, significance not maintained following *post hoc* correction; $p>0.05$ for maternal diet effect and interaction; Figure 5.4 a). No significant differences were seen between the groups in the 16 week old females ($p>0.05$ for all factors; Figure 5.4 b).

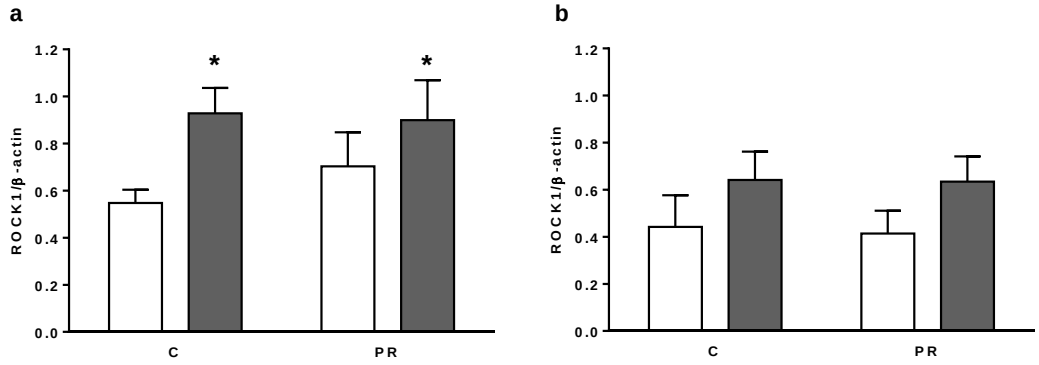


Figure 5.4 Relative ROCK 1 mRNA expression in the mesenteric arteries of male (a; C n=6; CS n=6, PR n=5, PRS n=5) and female (b; C n=5; CS n=6, PR n=4, PRS n=5) offspring at 16 weeks; □ non treated offspring, ■ statin treated offspring. * p<0.05 statin treated versus non-treated.

No significant differences were seen between the experimental groups in ROCK 2 mRNA expression in either males or females ($p>0.05$ for all factors; Figure 5.5) though there was a trend towards interaction in males ($p<0.07$) and increased expression in the statin treated females ($p<0.08$).

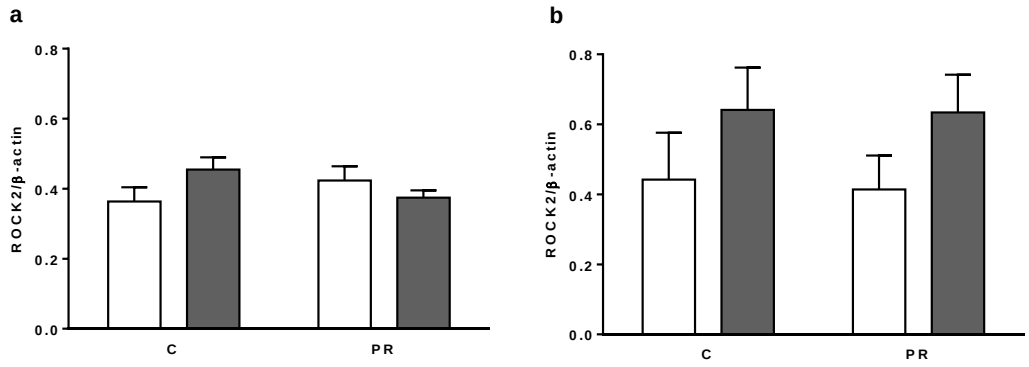


Figure 5.5 Relative ROCK 2 mRNA expression in the mesenteric arteries of male (a; C n=6; CS n=6, PR n=5, PRS n=5) and female (b; C n=5; CS n=6, PR n=4, PRS n=5) offspring at 16 weeks; □ non treated offspring, ■ statin treated offspring.

The relative expression values for all the tested genes in the mesenteric arteries can be found in Table 5.1.

Males	C	CS	PR	PRS
HMG-CoA reductase	0.60±0.09	0.58±0.07	0.62±0.10	0.62±0.08
eNOS	26.45±2.75	36.62±2.48*	24.58±3.00	29.82±5.84*
RhoA	0.54±0.03	0.52±0.03	0.54±0.04	0.54±0.02
ROCK 1	0.55±0.06	0.93±0.11*	0.70±0.14	0.90±0.17*
ROCK 2	0.36±0.04	0.45±0.03	0.42±0.04	0.37±0.02
Females				
HMG-CoA reductase	0.58±0.08	1.06±0.19*	0.48±0.01	0.91±0.19*
eNOS	14.72±2.37	21.72±4.43	22.04±4.61	16.96±3.81
RhoA	0.63±0.06	0.86±0.10*	0.53±0.05	0.70±0.07*
ROCK 1	0.44±0.13	0.64±0.12	0.41±0.10	0.63±0.11
ROCK 2	0.44±0.13	0.64±0.12	0.41±0.10	0.63±0.11

Table 5.1 Relative expression values for all the tested genes in the mesenteric arteries of male and female offspring.

5.3.1.2 Liver

No differences were seen in HMG-CoA reductase mRNA expression in males or females at either time point ($p>0.05$ for all factors; Figure 5.6).

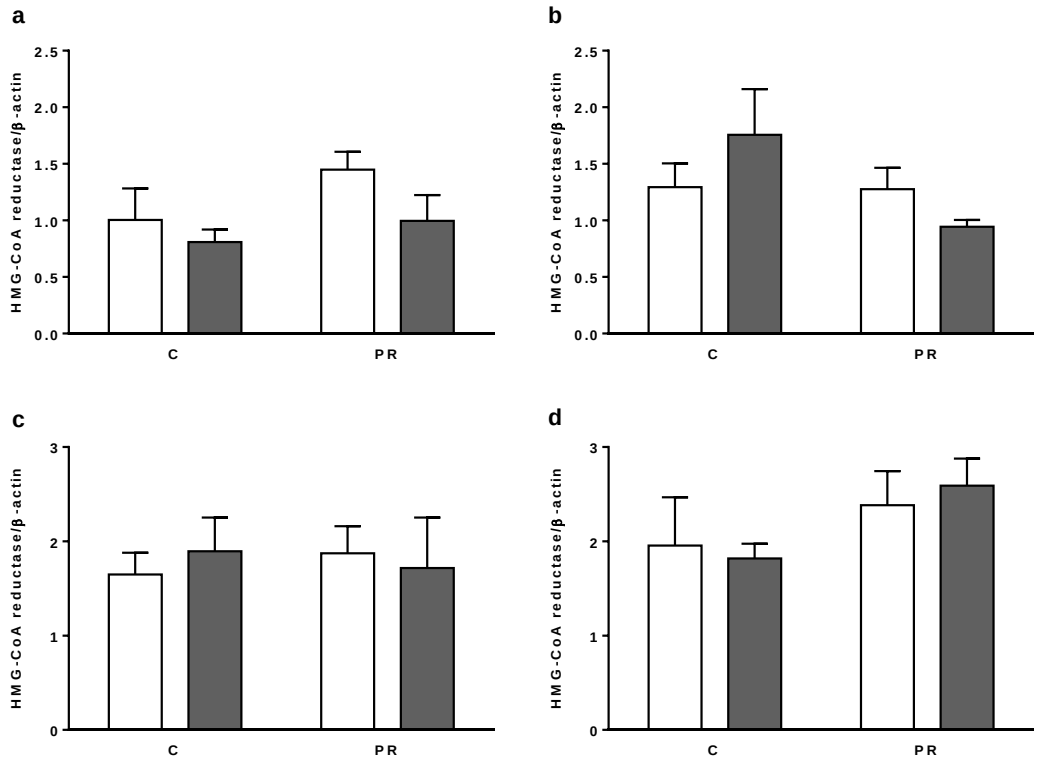


Figure 5.6 Relative HMG-CoA reductase mRNA expression in the liver of 5 week old male (a; C n=4; CS n=5; PR n=4; PRS n=4) and female (b; C n=4; CS n=5; PR n=4; PRS n=4) and 16 week old male (c; C n=6; CS n=6; PR n=5; PRS n=5) and female (d; C n=5; CS n=6; PR n=5; PRS n=6) offspring; □ non treated offspring, ■ statin treated offspring.

For the males at 5 weeks there was interaction in eNOS mRNA levels ($p<0.05$ for interaction, significance not maintained following *post hoc* correction; $p>0.05$ for maternal diet and statin effect; Figure 5.8 a). No differences were seen in eNOS mRNA expression in males at 16 weeks or females at either time point ($p>0.05$ for all factors; Figure 5.7).

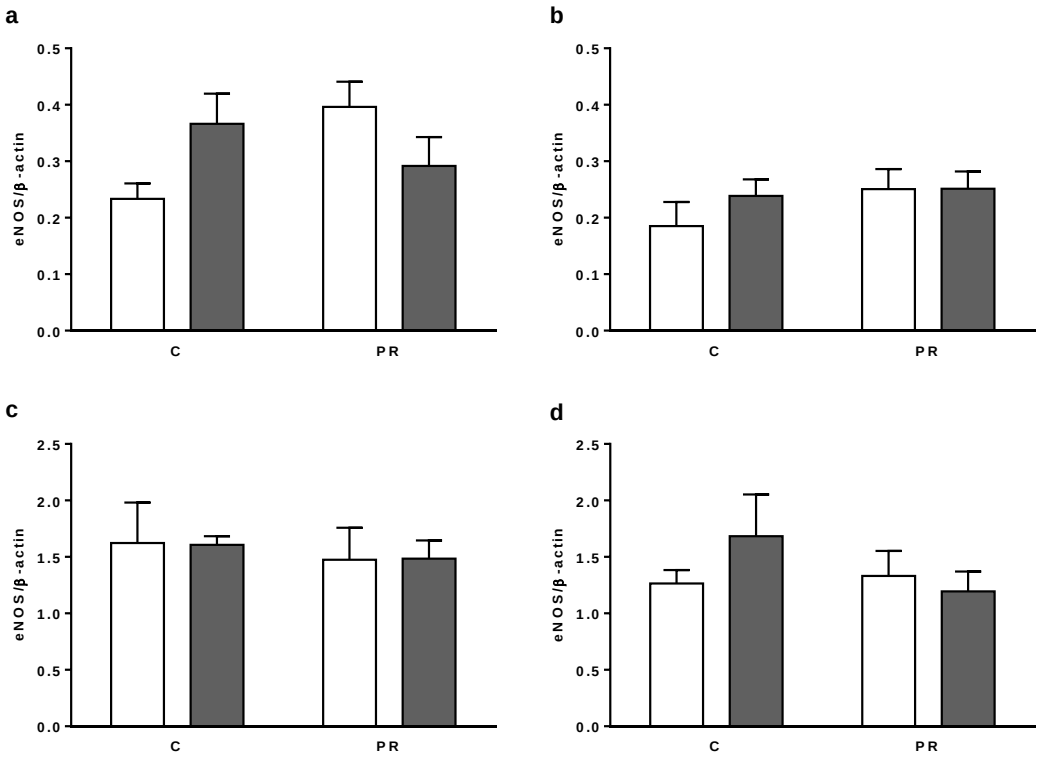


Figure 5.7 Relative eNOS mRNA expression in the liver of 5 week old male (a; C n=5; CS n=5; PR n=4; PRS n=4) and female (b; C n=4; CS n=5; PR n=4; PRS n=4) and 16 week old male (c; C n=6; CS n=6; PR n=5; PRS n=5) and female (d; C n=5; CS n=6; PR n=5; PRS n=6) offspring; □ non treated offspring, ■ statin treated offspring.

No significant differences were seen in RhoA mRNA expression in males or females at either time point ($p>0.05$ for all factors; Figure 5.8) though there was a trend towards increased expression in statin treated 5 week old males ($p<0.08$).

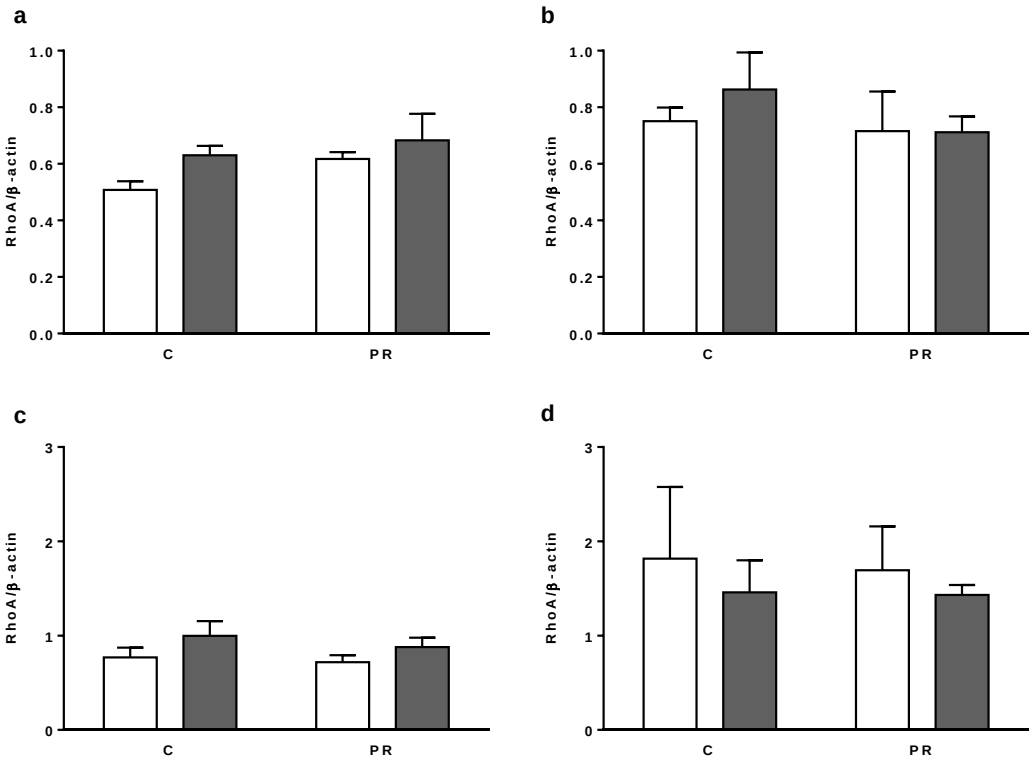


Figure 5.8 Relative RhoA mRNA expression in the liver of 5 week old male (; C n=5; CS n=5; PR n=4; PRS n=4) and female (b; C n=4; CS n=5; PR n=4; PRS n=4) and 16 week old male (c; C n=6; CS n=6; PR n=5; PRS n=5) and female (d; C n=5; CS n=6; PR n=5; PRS n=6) offspring; □ non treated offspring, ■ statin treated offspring.

No differences were seen in ROCK 1 mRNA expression in males or females at either time point ($p>0.05$ for all factors; Figure 5.9).

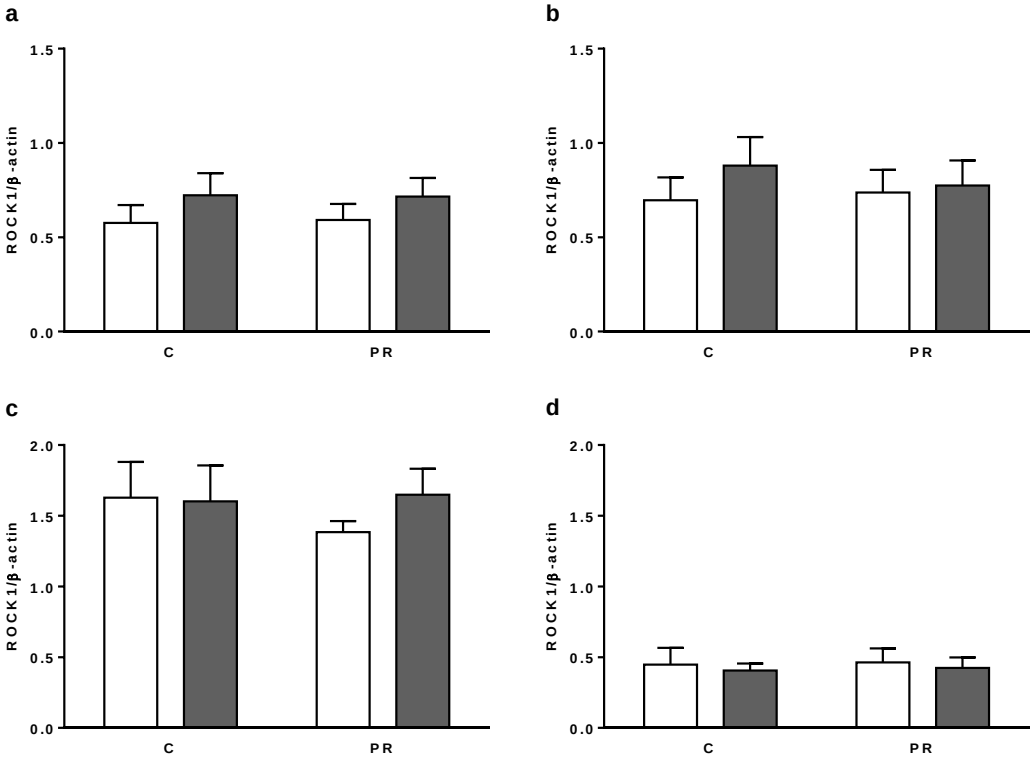


Figure 5.9 Relative ROCK 1 mRNA expression in the liver of 5 week old male (a; C n=5; CS n=5; PR n=4; PRS n=4) and female (b; C n=4; CS n=5; PR n=4; PRS n=4) and 16 week old male (c; C n=6; CS n=6; PR n=5; PRS n=5) and female (d; C n=5; CS n=6; PR n=5; PRS n=6) offspring; □ non treated offspring, ■ statin treated offspring.

No differences were seen in ROCK 2 mRNA expression in males or females at either time point ($p>0.05$ for all factors; Figure 5.10).

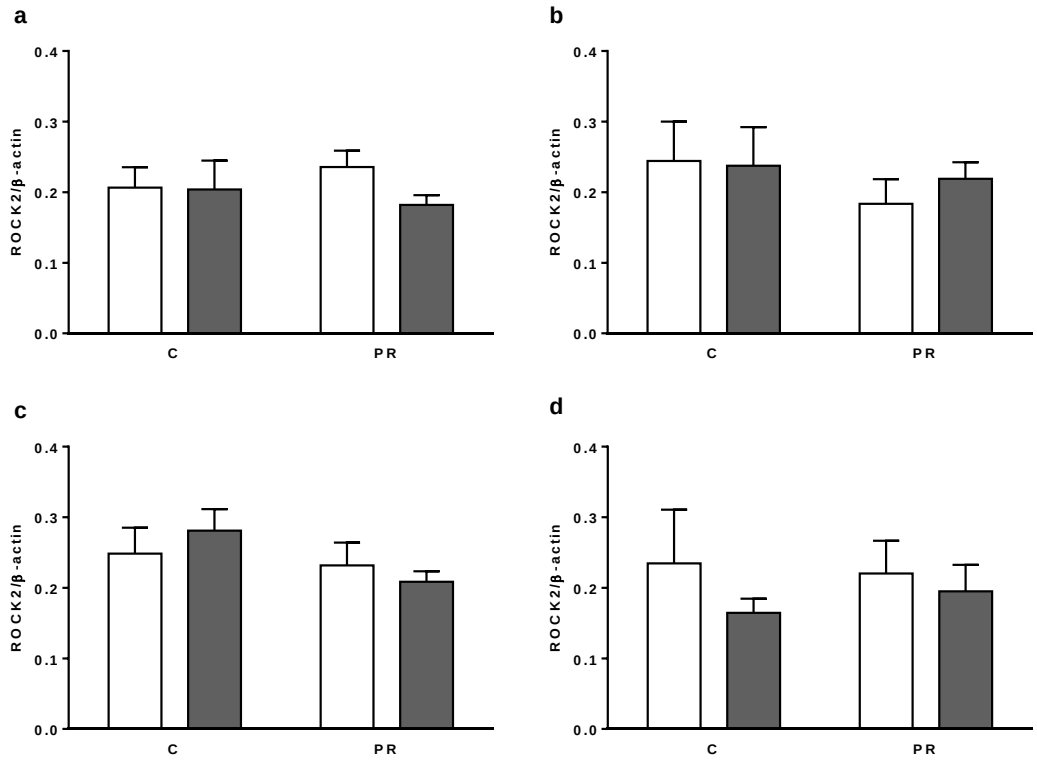


Figure 5.10 Relative ROCK 2 mRNA expression in the liver of 5 week old male (; C n=5; CS n=5; PR n=4; PRS n=4) and female (b; C n=4; CS n=5; PR n=4; PRS n=4) and 16 week old male (c; C n=6; CS n=6; PR n=5; PRS n=5) and female (d; C n=5; CS n=6; PR n=5; PRS n=6) offspring; □ non treated offspring, ■ statin treated offspring.

The relative expression values for all the tested genes in the liver can be found in Table 5.2 and Table 5.3.

Males	C (n=4/5)	CS (n=5)	PR (n=4)	PRS (n=4)
HMG-CoA reductase	1.00±0.28	0.81±0.11	1.45±0.16	1.00±0.23
eNOS	0.23±0.03	0.37±0.05	0.40±0.04	0.29±0.05
RhoA	0.51±0.03	0.63±0.03	0.62±0.02	0.68±0.09
ROCK 1	0.58±0.09	0.72±0.12	0.59±0.08	0.72±0.10
ROCK 2	0.21±0.03	0.20±0.04	0.24±0.02	0.18±0.01
Females	**C (n=4)**	**CS (n=5)**	**PR (n=4)**	**PRS (n=4)**
HMG-CoA reductase	1.29±0.21	1.76±0.40	1.28±0.19	0.94±0.06
eNOS	0.19±0.04	0.24±0.03	0.25±0.04	0.25±0.03
RhoA	0.75±0.05	0.86±0.13	0.72±0.14	0.71±0.06
ROCK 1	0.70±0.12	0.88±0.15	0.74±0.12	0.77±0.13
ROCK 2	0.24±0.06	0.24±0.05	0.18±0.03	0.22±0.02

Table 5.2 Relative expression values for all the tested genes in the liver of 5 week old male and female offspring.

Males	C (n=6)	CS (n=6)	PR (n=5)	PRS (n=5)
HMG-CoA reductase	1.65±0.23	1.89±0.36	1.87±0.29	1.72±0.54
eNOS	1.62±0.36	1.61±0.08	1.47±0.28	1.48±0.16
RhoA	0.77±0.10	1.00±0.16	0.72±0.07	0.88±0.10
ROCK 1	1.63±0.25	1.60±0.25	1.38±0.08	1.65±0.18
ROCK 2	0.25±0.04	0.28±0.03	0.23±0.03	0.21±0.01
Females	C (n=5)	CS (n=6)	PR (n=5)	PRS (n=6)
HMG-CoA reductase	1.96±0.51	1.82±0.16	2.38±0.36	2.59±0.29
eNOS	1.27±0.12	1.68±0.37	1.33±0.22	1.19±0.18
RhoA	1.82±0.76	1.46±0.34	1.69±0.46	1.43±0.11
ROCK 1	0.45±0.12	0.41±0.05	0.46±0.10	0.42±0.07
ROCK 2	0.23±0.08	0.16±0.02	0.22±0.05	0.19±0.04

Table 5.3 Relative expression values for all the tested genes in the liver of 16 week old male and female offspring.

5.3.2 RhoA protein expression in the aorta

For the protein analysis tissue from all the animals in each group was pooled and processed as one. The number of animals that tissue came from in each groups was as follows: five week old males C n=5, CS n=5, PR n=4, PRS n=4; five week old females C n=4, CS n=5, PR n=4, PRS n=5; sixteen week old males C n=6, CS n=6, PR n=5, PRS n=5; sixteen week old females C n=5, CS n=6, PR n=5, PRS n=6.

5.3.2.1 Whole cell expression

The levels of RhoA protein as expressed against β-actin in the pooled samples of the 5 week old males was similar across the groups (RhoA/β-actin: C 0.28; CS 0.34; PR 0.31; PRS 0.32; Figure 5.11 a). In the 5 week old female pooled samples considerably higher levels were detected in the C offspring, though this was at least partially due to low β-actin signal. Levels in the PRS group appeared somewhat elevated against CS and PR groups (RhoA/β-actin: C 1.14; CS 0.30; PR 0.27; PRS 0.38; Figure 5.11 a).

In the 16 week old males the expression in the pooled samples did not vary much between groups though maternal protein restriction and statin appeared to increase expression somewhat (RhoA/β-actin: C 0.25; CS 0.31; PR 0.36; PRS 0.40; Figure 5.11 b). In the 16 week old females again expression in the pooled samples was similar though statin appeared to reduce expression slightly (RhoA/β-actin: C 0.27; CS 0.18; PR 0.25; PRS 0.22; Figure 5.11 b).

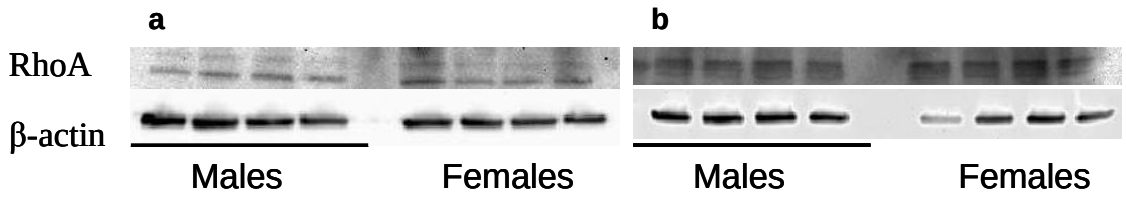


Figure 5.11 RhoA protein expression in the whole cell aortic lysates in the males and females at 5 (a) and 16 weeks (b).

5.3.2.2 Subcellular fractionation

Membrane fractionation of the pooled samples was successful as assessed by the expression of the membrane marker NaKATPase. Strong bands were detected around the expected 112 kDa in the membrane fractions of males and females at 5 and 16 weeks (Figure 5.12). In the 16 week samples a band was also detected in the cytosolic fraction though these were somewhat higher molecular weight and considerably fainter. β-actin was detectable in all samples.

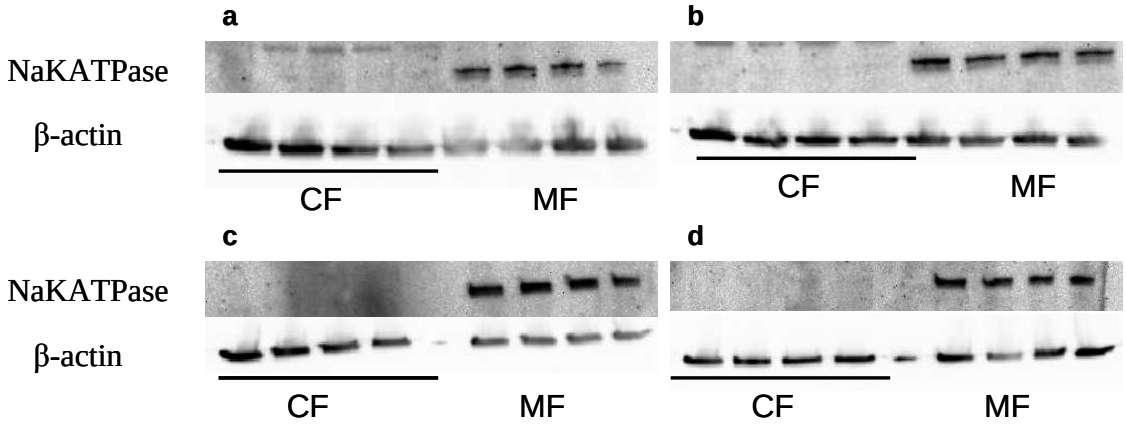


Figure 5.12 Sodium potassium ATPase protein expression in the cytosolic (CF) and membrane fractions (MF) in the 5 week old male (a) and female (b) and 16 week old male (c) and female (d) offspring

5.3.2.3 Membrane localisation of RhoA

In the 5 week old males cytosolic RhoA expression in the pooled samples was reduced in the PRS group with the other groups showing a fairly uniform expression (RhoA/β-actin: C 0.46; CS 0.47; PR 0.48; PRS 0.23; Figure 5.13 a) whereas the membrane bound RhoA appeared increased in the PRS group (RhoA/β-actin: C 0.07; CS 0.08; PR 0.06; PRS 0.15; Figure 5.13 a).

In the 5 week old females cytosolic RhoA expression in the pooled samples was reduced in both of the PR offspring groups and more so in the statin treated ones

(RhoA/β-actin: C 0.77; CS 0.81; PR 0.60; PRS 0.41; Figure 5.13 b). In the membrane fraction expression was again decreased in the PR groups but this time PRS showed slightly higher expression than PR (RhoA/β-actin: C 0.22; CS 0.20; PR 0.14; PRS 0.17; Figure 5.13 b).

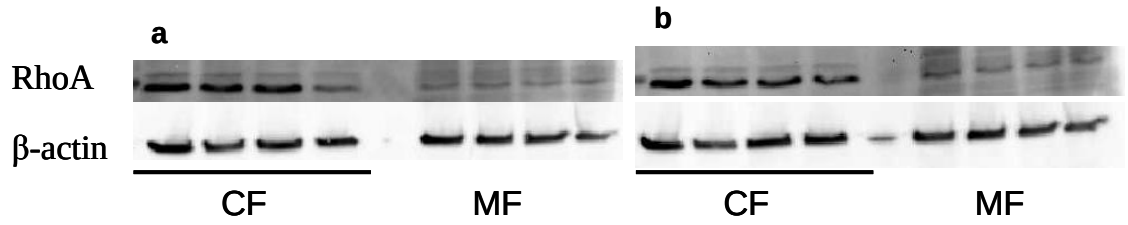


Figure 5.13 RhoA protein expression in the cytosolic (CF) and membrane fractions (MF) in the 5 week old male (a) and female (b) offspring

In the 16 week old males both of the protein restricted groups the cytosolic RhoA protein expression in the pooled samples appeared slightly increased as compared to the control groups (RhoA/β-actin: C 0.77; CS 0.71; PR 0.92; PRS 0.90; Figure 5.14 a). Again in the membrane fraction expression appeared higher in the protein restricted groups and also the statin treated groups showed a reduction in expression in each maternal diet group (RhoA/β-actin: C 0.67; CS 0.59; PR 0.85; PRS 0.71; Figure 5.14 a).

In the 16 week old females cytosolic expression of the pooled samples was reduced in the PR group with the other groups showing fairly uniform expression (RhoA/β-actin: C 0.23; CS 0.24; PR 0.15; PRS 0.22; Figure 5.14 b). No comparisons could be made on membrane fraction expression as no signal could be detected (Figure 5.14 b).

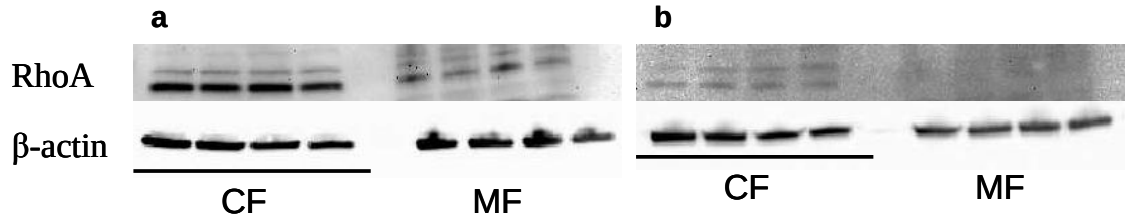


Figure 5.14 RhoA protein expression in the cytosolic (CF) and membrane fractions (MF) in the 16 week old male (a) and female (b) offspring.

5.4 Discussion

Statin treatment and its withdrawal have both been shown to lead to alterations in mRNA and protein levels. Possible molecular changes behind the altered function at five and sixteen weeks were investigated. This part of the study showed alterations in mRNA expression of several different genes in statin treated animals, especially in the

mesenteric arteries at 16 weeks. Protein expression and membrane translocation of RhoA was investigated as well, though no clear differences could be detected. Summary of the mRNA data is presented in Table 5.4.

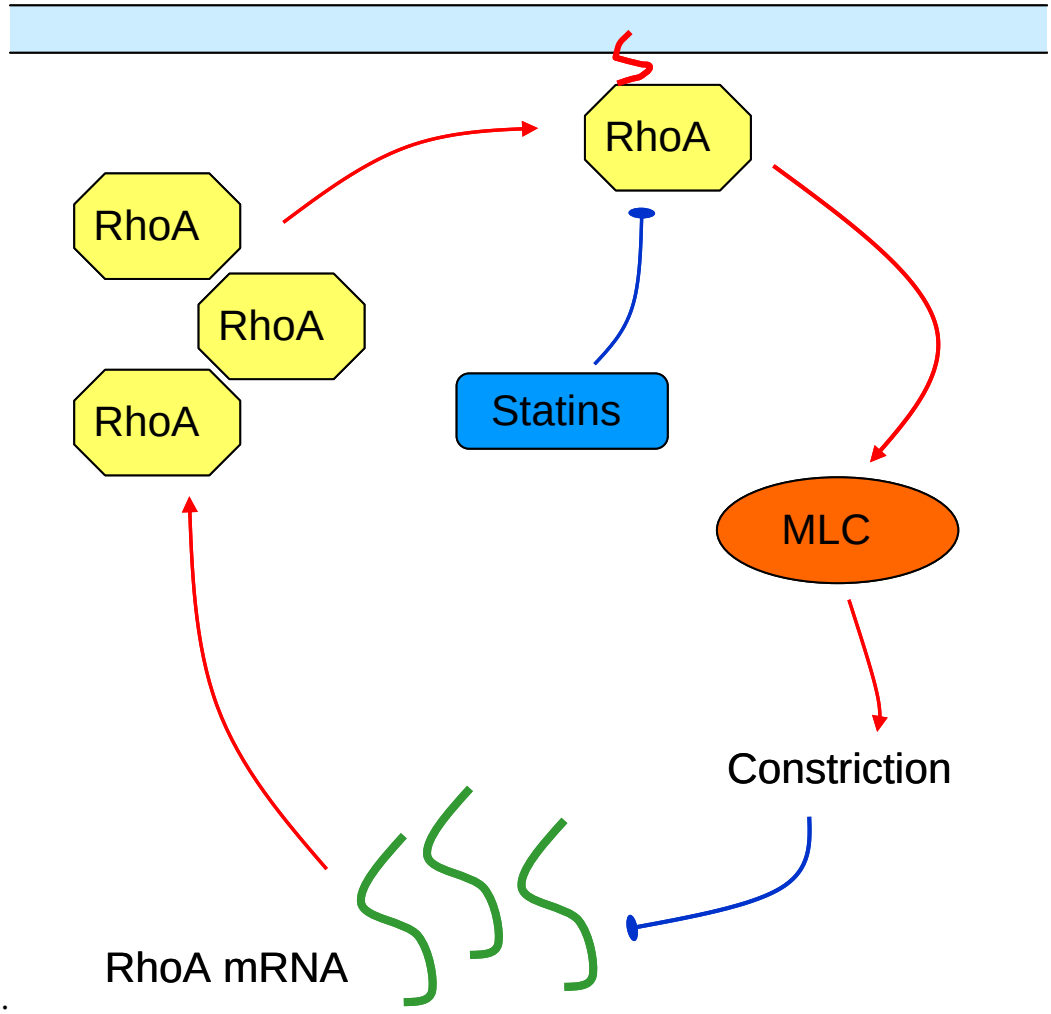
		5 week liver		16 week liver		16 week mesenterics	
		Males	Females	Males	Females	Males	Females
HMG-CoA reductase	PR						
	S						▲
eNOS	PR	#					
	S	#				▲	
RhoA	PR						
	S	△					▲
ROCK 1	PR						
	S					▲	
ROCK 2	PR						
	S						△

Table 5.4 Summary of mRNA levels in the liver and mesenteric arteries. ▲ increased mRNA levels, ▲ $p<0.05$, △ $p<0.1$, # interaction but no differences between groups detected by Bonferonni's multiple comparisons test.

In the mesenteric arteries of the 16 week old females HMG-CoA reductase mRNA levels were increased in the statin treated C and PR offspring though no changes were seen in the male arteries. This indicates sustained upregulation of HMG-CoA reductase expression in the mesenteric arteries several weeks after withdrawal of statin treatment. This could explain the increased constriction observed in the adult offspring as upregulation of HMG-CoA reductase at mRNA level could lead to increased mevalonate synthesis. Upregulation of the mevalonate synthesis end products would allow increased RhoA membrane localisation among other effects (Takemoto & Liao, 2001;Wang *et al.*, 2008). No changes were seen in the mRNA expression of HMG-CoA reductase in the liver at either time point. This is in contrast to previous studies as hepatic HMG-Co A reductase mRNA levels have been shown to be increased in mature rats after chronic treatment at twenty weeks (Torrens *et al.*, 2009b) and after three day treatment in adolescent rats (Ness *et al.*, 1998) likely through the negative feedback loop from mevalonate downstream targets. The current findings suggest that two week statin treatment in young rats is not enough to lead to upregulation of HMG-CoA reductase mRNA levels in the liver either short or long term. As HMG-CoA reductase gene expression is regulated by the downstream products of the mevalonate pathway (Goldstein & Brown, 1990) this could be an indication that statin treatment or withdrawal did not induce great alterations in the mevalonate pathway end products in

the liver. The 10 mg / kg of atorvastatin a day has been shown to be a sufficient dose to alter HMG-Co A reductase mRNA levels (Torrens *et al.*, 2009b) but as *de novo* cholesterol synthesis is increased during early life (Dietschy *et al.*, 1993), with highest levels immediately post weaning (Smith *et al.*, 1995), this dose might not have been high enough in the adolescent rats to allow the feedback loop mediated increase in HMG-CoA reductase mRNA. The differential effects seen in the liver and mesenteric arteries in the 16 week old female offspring could be explained by atorvastatin lipophilicity as this would allow localised effects.

Increased expression of mRNA levels of the RhoA pathway genes were observed in the mesenteric arteries at 16 weeks though the genes affected varied between the sexes. In the female offspring RhoA mRNA levels were increased in the statin treated offspring and in the male offspring same was seen with ROCK 1 mRNA levels. Also in the female offspring trend towards increased levels were seen in ROCK 2 mRNA levels. No changes were seen in the mRNA expression of RhoA, ROCK 1 or ROCK 2 in the liver at either time point indicating statins localised effects again though a trend towards increased RhoA mRNA levels were observed in the liver of the five week old males possibly indicating mild short term upregulation of RhoA mRNA levels due to statin treatment. These results indicate sustained upregulation of the RhoA pathway genes after withdrawal of early age acute statin treatment in mesenteric arteries. Also for the first time, in addition to increase in RhoA mRNA expression, statin related increase has been shown in mRNA levels of the RhoA target protein levels, ROCK 1 and 2. The upregulation of RhoA and the ROCK mRNA levels could account for the increased constriction observed in the mesenteric arteries at sixteen weeks if translated into protein and activity levels. Inhibition of RhoA activity by statins is known to upregulate RhoA mRNA and cytosolic protein levels through a negative feedback loop (Figure 5.15 (Laufs *et al.*, 2000) and in the current study statin inhibition of RhoA isoprenylation during statin treatment could have lead to sustained upregulation of mRNA levels of RhoA pathway genes observed



.

Figure 5.15 RhoA regulation and statins. RhoA maintains smooth muscle constriction by inhibiting myosin light chain (MLC) phosphorylation. This inhibits RhoA activity through a negative feedback loop by inhibiting RhoA mRNA levels. Statin treatment inhibits RhoA activity and thus leads to accumulation of RhoA mRNA and cytosolic protein.

RhoA protein levels and membrane translocation were investigated to confirm whether statin treatment and withdrawal could alter RhoA activity levels in the aorta. Because of the small amount of tissue available the samples were pooled for each experimental group and thus no statistical testing could be carried out. In the five week old offspring indication of increased membrane translocation was seen in PRS group in both males and females. In the males the expression was reduced in the cytosol and increased in the membrane fraction as compared to other groups. In the females the increase in membrane fraction was not as clear when compared to the other groups but there was a relative increase when membrane was compared to cytosol. This does not correspond to the functional results as no changes were seen in females and in males reduced constriction was observed. In the sixteen week old males no clear differences were apparent. In the females no RhoA protein was observed in the membrane and only the

amount of RhoA in the cytosol could be assessed. The whole cell RhoA levels did not reflect the mRNA expression and expression was fairly equal across groups. Due to pooling of the protein samples no statistical testing could be performed in the current study and the data does not show clear differences in protein levels between the groups. It might be possible to analyse the data by using an n-number as the number from which the samples came from. As the statcistical analysis would also require SEM this would have to be artificially determined thus reducing the reliability of the results. Thus the current study cannot confirm whether early age statin treatment and its withdrawal can alter RhoA protein expression short or long term in the aorta.

Early age acute statin treatment increased eNOS mRNA expression in the mesenteric arteries of the male offspring at sixteen weeks. Increase in NO availability was not indicated in the mesenteric artery function though impaired endothelial function has also been shown to lead to upregulation of eNOS mRNA levels (Munzel *et al.*, 2005) and in a similar manner increase in constriction in the current study could have led to upregulation of the eNOS mRNA levels. This evidence indicates that any changes in constriction in response to early age acute statin treatment are unlikely to be mediated through alterations in eNOS mRNA levels, either short or long term, and supporting the lack of changes in endothelium dependent relaxation in response to statin treatment at either time point. Further investigation of eNOS protein and cGMP and sGC levels, especially as changes in cGMP and sGC levels could explain differences seen in SNP responses, would have given a more comprehensive understanding of the factors controlling vasorelaxation but mainly due to time limitations this was not done as part of the current study.

These data show alterations in gene expression after early age acute statin treatment, several weeks after discontinuation of the treatment. As mesenteric artery function at five weeks was not investigated no samples were collected for molecular analysis either, thus it cannot be confirmed whether the upregulation of mRNA levels occurred during statin treatment leading to sustained upregulation of these genes or whether the upregulation occurred as a result of the withdrawal. The former appears the likelier option as statins are known to upregulate HMG-CoA reductase mRNA levels (Torrens *et al.*, 2009b) as well as RhoA mRNA levels which have also been shown to remain elevated at least transiently in adult rodents following statin withdrawal (Laufs *et al.*,

2000). The upregulation of the genes shown in the current study would not alter function during statin treatment as statins inhibit the activity of the proteins, but after statin withdrawal the increase in gene expression could lead to an increase in the activity and thus increase in constriction. The n-numbers were small in the current study and it is possible that the many trends observed in the mRNA levels could have been brought out to be significant should the n-numbers have been higher.

It has shown that early age acute statin treatment and withdrawal can induce long term changes in mRNA levels of genes whose activity at protein level can be altered by statins. The upregulation of these genes is more prominent in the mesenteric arteries than in the liver. Pooled Western blotting data was not able to confirm whether statins altered RhoA protein expression or membrane translocation in the aorta in the current study. The data from the mRNA expression experiments indicate that upregulation of the RhoA pathway could underlie the increased maximal constriction observed in the sixteen week old animals.

Chapter 6 Discussion

The current study shows that early age acute statin treatment can have both short and long term effects on cardiovascular function and molecular markers in rats. Early age acute statin treatment reduced vasoconstriction in the adolescent offspring whereas increased vasoconstriction was observed in the adult offspring. It is indicated that the long term effects have arisen as a consequence of the withdrawal rather than being a direct effect of the statin treatment. These data add to the emerging evidence of rebound effect after statin withdrawal demonstrated by clinical trials and animal studies. This study indicates that upregulation of the RhoA pathway is part of the mechanisms behind the effects of statin withdrawal.

Maternal PR did not impair vascular function in the current study though this has been demonstrated several times previously. No impairment of vascular function was detected in the form of increase in blood pressure or endothelial dysfunction. The likelihood is that the new diet and the possibly increased food intake in the PR dams ameliorated the differences usually apparent in this model. The possible reasons behind the lack of these changes were discussed in the respective chapters. As impairment of vascular function was not detected in the current study the effect of treatment and withdrawal could not be confirmed in a disease model.

This discussion will concentrate on the possible mechanisms behind the effects of early age acute statin treatment and its withdrawal. As the main findings of the current study concerning the statin effects were broadly similar in the two sexes this discussion will not differentiate between males and females with exception of some key differences. The clinical implications of the results of the current study will also be discussed.

6.1 Effects of early age acute statin treatment and withdrawal

Animal studies and clinical trials have demonstrated that both secondary and primary prevention of CVD with statins is due to their cholesterol lowering and pleiotropic properties (Law *et al.*, 2003;Ridker *et al.*, 2001;Ridker *et al.*, 2008b;Shepherd *et al.*, 1995;Torrens *et al.*, 2009b). Although the current study has demonstrated beneficial effects after short term statin treatment at an early age, supporting previous data, it has also indicated that withdrawal of the statin treatment at a young age can be detrimental to the vascular function by leading to increased vasoconstriction later in life.

Studies both in humans and rodents have demonstrated that withdrawal of statin treatment can have detrimental effects on vascular function. Studies in humans have shown an increase in event rates and mortality up to six months after statin withdrawal (Dowlatshahi *et al.*, 2012;Heeschen *et al.*, 2002) and an increase in all cause mortality was shown in a study based on records of up to a year after withdrawal (Daskalopoulou *et al.*, 2008). Withdrawal of statin after a four week treatment period has also been shown to reduce flow mediated dilation (Chen *et al.*, 2009). The effect of statin withdrawal on cholesterol has been investigated but increase has not been shown above pretreatment levels though increase has been shown compared to during treatment levels. Pretreatment data has not been available for all studies (Puccetti *et al.*, 2003) though a steady rise in cholesterol and platelet aggregation was shown for sixty days following statin withdrawal. It is also possible that the follow up period has not been long enough to detect changes, the cholesterol levels having been investigated one and six weeks after withdrawal (Chen *et al.*, 2009;Pappu *et al.*, 2003). Data from animal studies show that withdrawal of statin treatment can impair vascular function, decrease NO bioavailability, increase oxidative stress and platelet activation and accelerate progression of diabetes and pancreatic fibrosis (Gertz *et al.*, 2003;Laufs *et al.*, 2000;Otani *et al.*, 2010;Vecchione & Brandes, 2002). The current study in rodents supports previous reports that statin withdrawal can lead to a rebound effect and impair vascular function.

6.1.1 Timing of treatment and withdrawal

The clinical trials investigating statin withdrawal have looked at the effects up to a year after withdrawal thus far providing no evidence of the permanency of the effect. Contradictory to the results from the withdrawal studies, follow ups of statin trials have shown long term protective effects as a reduced mortality and event rates up to five years after the end of trial period (Bulbulia *et al.*, 2011;Ford *et al.*, 2007). These follow up studies were not designed to look at statin withdrawal and in analysis took into account only the in-trial statin use without correcting for after trial statin use. As a higher proportion of subjects from the statin-treated group were on statins than of the placebo group at the time of the follow up, the results of these studies may reflect a protective effect of longer term statin treatment rather than sustained protection after withdrawal.

The data from animal studies are conflicting concerning the permanency of the rebound effect. Some of the studies suggest that the effects of statin withdrawal are transient (Gertz *et al.*, 2003;Laufs *et al.*, 2000;Vecchione & Brandes, 2002) while others show long term effects (Otani *et al.*, 2010). The transient effects were shown on a range of vascular parameters whereas the long term effects were shown in metabolic parameters relating to diabetes or development of pancreatic fibrosis. It is a possibility that the effect of statin withdrawal could vary between different physiological aspects although long term changes were seen in vascular parameters in the current study. It is more likely though that the differences in the length of the treatment period explain the differences in the longevity of the withdrawal effect. The length of the treatment was only two weeks in the studies with the transient effects whereas the long term effects were seen after a treatment period of 16 weeks. In contrast in the current study long term effects were shown after short term (two weeks) treatment. The current study differs from the others in the timing of the treatment in that the treatment and withdrawal occurred in adolescent animals. In all the other animal studies statin treatment and withdrawal was studied in mature animals. Early age withdrawal was investigated in the current study as developmentally induced disease models have shown that alterations in environmental parameters during development can have long reaching implications on vascular function. In a similar manner to alterations in maternal diet short term statin treatment and withdrawal during development was shown to have long term effects in the current study in contrast to the transient withdrawal effects following short term treatment in mature animals. This could have implications on statin prescription for children and on maternal statin treatment during pregnancy and lactation. Treatment of high fat fed dams with statins during pregnancy, though, has been shown to have beneficial effects on the offspring vascular function (Elahi *et al.*, 2008). As hydrophilic pravastatin was used the treatment should not have had direct effects on the offspring but the maternal parameters were improved considerably. Thus it is likely that the improvement in the offspring parameters was due improved cardiovascular parameters in the dams. The current study adds to the understanding of the importance of timing of the withdrawal and the longevity of the effect.

6.1.2 Involvement of nitric oxide

Ability to increase NO bioavailability appears to be one of statins most important pleiotropic properties (Aoki *et al.*, 2012;Feron *et al.*, 2001;Kansui *et al.*, 2004;Wenzel *et al.*, 2008) and inhibition of NO synthesis can increase constriction (Dresner *et al.*, 1997;Vo *et al.*, 1992), suggesting that statins can reduce constriction via increased NO bioavailability. Increased NO bioavailability has been implicated in studies showing reduced constrictor response in aortae and renal arteries of statin treated animals (Tian *et al.*, 2011) and mesenteric arteries incubated with statins (Ghaffari *et al.*, 2011;Rossoni *et al.*, 2011). Thus the reduction in constriction observed at five weeks in the current study could have been due to increased NO bioavailability (Figure 6.1 a). As reduced NO bioavailability has been shown after statin withdrawal (Vecchione & Brandes, 2002) this could have explained the increase in constriction in the mature animals (Figure 6.1 b). In the current study no apparent changes were seen in endothelium-dependent relaxation in statin treated animals neither at the end of the treatment nor eleven weeks after withdrawal and this does not support an increase in NO bioavailability. These results are in contrast to previous data where an increase in endothelium-mediated relaxation has been shown in statin treated animals (Kansui *et al.*, 2004;Torrens *et al.*, 2009b) and impairment has been shown after withdrawal (Vecchione & Brandes, 2002). The molecular data in the current study does not support alterations NO bioavailability either as no changes were seen in eNOS mRNA levels. The mRNA levels are not necessarily indicative of NO levels as eNOS can be regulated at protein and activity levels although reduced NO bioavailability in oxidative stress has been shown to upregulate eNOS mRNA levels even when eNOS activity is increased (Munzel *et al.*, 2005). The molecular and functional data available in the current study do not support alterations in NO bioavailability.

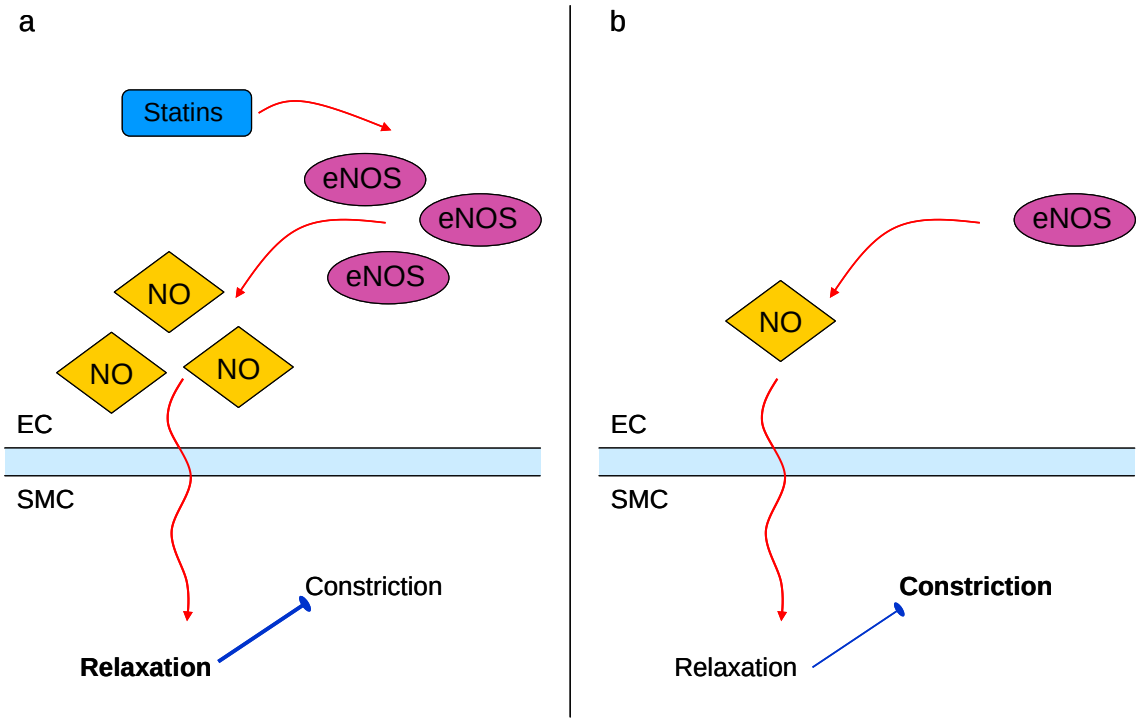


Figure 6.1 Nitric oxide (NO) as the mechanism mediating the effects of the early age acute statin treatment and withdrawal. a) Statin treatment increases NO bioavailability via upregulation of endothelial nitric oxide synthase (eNOS) in the endothelial cells (EC). NO induces relaxation in the smooth muscle which can counteract vasoconstriction. b) After statin withdrawal NO bioavailability and relaxation is reduced allowing increased constriction.

6.1.3 Involvement of the smooth muscle

Statin treatment and withdrawal have also been shown to have an effect on smooth muscle function and these changes could explain the altered function observed in the current study. Increased vasoconstriction was shown in response to both PE and ET in the mesenteric arteries and these both induce constriction via GPCR leading to Ca^{2+} release from the intracellular stores. Interestingly, there is some evidence that statins can increase intracellular Ca^{2+} levels, at least in cell culture (Cheng *et al.*, 2003;Mutoh *et al.*, 1999). The increase in Ca^{2+} by statins has been shown to occur via increase in phospholipase C (PLC) phosphorylation (Mutoh *et al.*, 1999) which PE and ET receptors activate to induce constriction (Figure 1.2). It is therefore a possibility that statin treatment leads to an increase in PLC phosphorylation which is maintained after statin withdrawal (Figure 6.2). This mechanism could explain the increase in vasoconstriction observed at sixteen weeks but if so, constriction at five weeks would also have been expected to be increased. In contrast to these studies showing statin induced increase in Ca^{2+} statin have also been shown to reduce Ca^{2+} release. Fluvastatin

has been shown to reduce Ca^{2+} release from the sarcoplasmic reticulum in myofibres (Tanaka *et al.*, 2010) and atorvastatin reduces plasma Ca^{2+} levels in a rat model of vascular calcification (Li *et al.*, 2010). Considering that majority of data from other studies indicates that statins reduce intracellular Ca^{2+} and that vasoconstriction was reduced in the five week old offspring statin induced increase in Ca^{2+} via PLC phosphorylation appears an unlikely mechanisms behind the increased vasoconstriction in the current study.

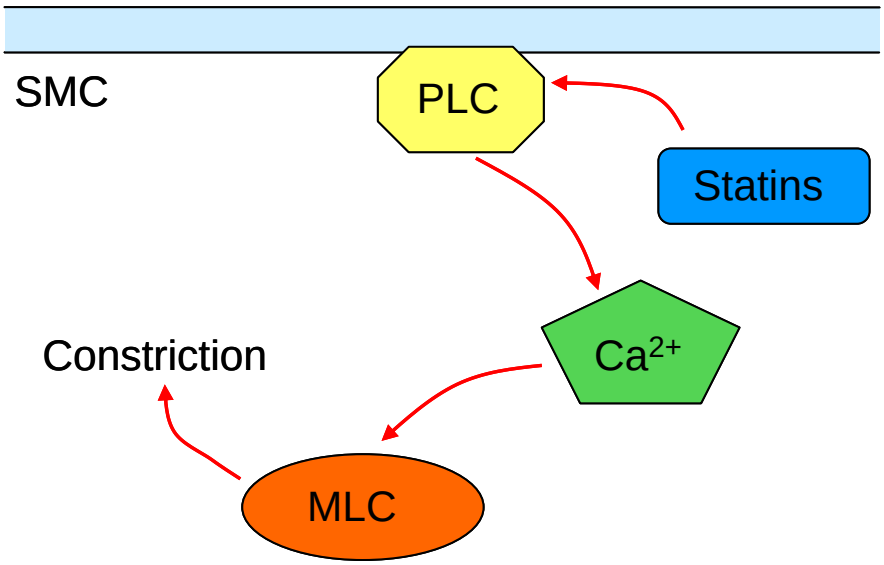


Figure 6.2 Phospholipase C (PLC) as the mechanism mediating the early age acute statin treatment and withdrawal effects. Statins induce PLC phosphorylation in vascular smooth muscle cells (SMC) which leads to an increase in intracellular Ca^{2+}. This activates myosin light chain (MLC) and constriction. If the increase in PLC phosphorylation was sustained following statin withdrawal this could explain the increase in vasoconstriction.

Changes in the vasoconstriction could also be explained by changes in the Ca^{2+}-independent constriction pathway. Both PE and ET maintain vasoconstriction via the RhoA pathway. The mevalonate pathway is important in the posttranscriptional modification of RhoA that allows it to become membrane bound and be activated by GPCRs (Figure 1.2) (Zhou & Liao, 2010). Several studies have shown reduction in RhoA activity during statin treatment (Laufs *et al.*, 2000;Ohkawara *et al.*, 2010;Yokoyama *et al.*, 2002). Statins have also been shown to reduce constriction independent of the endothelium while implicating the inhibition of posttranscriptional modification of RhoA and its activity (Kuzuya *et al.*, 2004). Statins have also been demonstrated to have effects on RhoA downstream targets, ROCKs, by reducing their activity (Liu *et al.*, 2012a) probably because of reduction in their activation due to inhibition of RhoA. Also statin treatment has been shown to increase RhoA protein

expression with reduction in RhoA membrane translocation whereas after statin withdrawal membrane translocation of RhoA has been shown to be elevated (Gertz *et al.*, 2003;Laufs *et al.*, 2000). Thus alterations in the RhoA pathway could explain the changes in vasoconstriction observed in the current study. Unlike in the previous studies no changes were seen in membrane the translocation of RhoA at either time point in the aorta though the pooling of the samples could have masked any differences. Also though no changes were observed in RhoA protein levels in the aortae it does not mean that no changes occurred in the mesenteric arteries where the increase in constriction following statin withdrawal was more prominent. The mRNA levels of the RhoA pathway genes in the mesenteric arteries also support the upregulation of this pathway as the mechanism behind the increase in constriction in this vascular bed. Previous studies have shown upregulation of RhoA at mRNA levels contributing to increased protein levels following statin treatment (Laufs *et al.*, 2000).(Laufs *et al.*, 2000) This is the first study to show changes in mRNA levels of RhoA target genes, ROCKs, following statin treatment and withdrawal. This indicates that statin treatment and withdrawal can induce molecular changes on pathways downstream of the mevalonate pathway end products. The current study indicates that alterations in the RhoA and its targets' mRNA levels in the mesenteric arteries contribute to the increased vasoconstriction following early age acute statin treatment and withdrawal.

Also there is some evidence that direct inhibition of ROCK can protect aspects of EDHF response as did statin incubation of these arteries (McNeish *et al.*, 2012). Thus increased RhoA pathway activity could also account for increased constriction in the mesenteric arteries due to inhibition of the EDHF mediated relaxation. The relative contribution of the EDHF was not investigated in the current study but it would be expected to have an effect on the overall relaxation and no differences were seen in this in the current study. Also other studies have indicated that statins do not alter the EDHF response (Kansui *et al.*, 2004;Brandes *et al.*, 1999) alterations in the EDHF response are not indicated in the current study.

6.1.4 Upregulation of the RhoA pathway

There are some possibilities as the mechanism behind the upregulation of the RhoA pathway. Most of the aspects of these possible mechanisms were not investigated in the current study but some inferences can be made based on data from other studies and

aspects examined. Statins inhibit RhoA geranylation via inhibition of HMG-CoA reductase (Zhou & Liao, 2010) and statin treatment has been shown to upregulate HMG-CoA reductase at least at mRNA level (Torrens *et al.*, 2009b), likely through a negative feedback loop by mevalonate pathway endproducts (Figure 6.3 a). Upregulation of HMG-CoA reductase following statin withdrawal could lead to upregulation of RhoA activity (Figure 6.3 b) and provided that the upregulation of HMG-CoA reductase was sustained this could explain the increased constriction at sixteen weeks. No upregulation of HMG-CoA reductase mRNA was detected in the liver of the acutely treated adolescent or mature animals in the current study in contrast to previous reports (Ness *et al.*, 1998;Torrens *et al.*, 2009b). Upregulation was shown in the female offspring mesenteric arteries. Thus there is some evidence that HMG-CoA reductase activity could be increased following statin withdrawal. Considering statins pleiotropic effects and the numerous downstream targets of mevalonate, increase in HMG-CoA reductase could have contributed to the increase in constriction via pathways other than RhoA and could have had effects on other aspects than just increase in constriction. Increase in cholesterol might have been expected in such a situation, at least if HMG-CoA reductase upregulation occurred in the liver. Previous studies in this model though do not support alterations in cholesterol levels at least during statin treatment (Torrens *et al.*, 2009b). Processes such as inflammation and oxidative stress could have been increased and these could have been expected to alter NO bioavailability. As discussed above, alterations in NO levels do not appear likely thus not indicating changes in inflammation or oxidative stress. As the involvement of inflammation and oxidative stress were not directly investigated in the current study alterations in these processed cannot be conclusively ruled out. Though HMG-Co A reductase can alter RhoA activity by allowing its membrane translocation, upregulation of HMG-Co A reductase cannot explain the increased mRNA levels of the RhoA pathway genes.

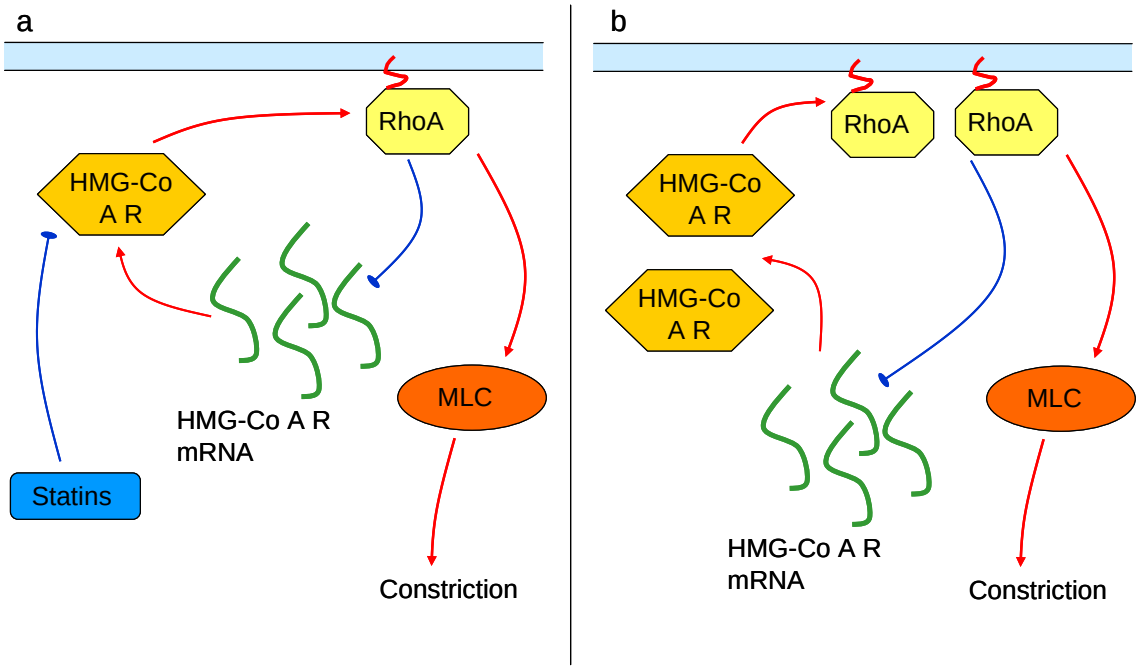


Figure 6.3 HMG-Co A reductase (HMG-Co A R) as the mechanism mediating the early age acute statin treatment and withdrawal effects. a) HMG-CoA reductase contributes to smooth muscle cell constriction by allowing isoprenylation and membrane translocation of RhoA. Statins inhibit HMG-CoA reductase activity and thus RhoA membrane translocation and can reduce constriction. Inhibition of HMG-Co A reductase activity leads to upregulation of its mRNA levels through a negative feedback loop by the mevalonate pathway end products. b) After statin withdrawal increased activity of HMG-Co A reductase could allow increased RhoA membrane translocation and thus constriction.

Increased mRNA levels could be explained by altered histone deacetylation. Statins have been shown to alter histone deacetylase (HDAC) activity in cell culture though the data is contradictory as to which way. Some studies indicate that statins can increase HDAC activity (Dje *et al.*, 2009;Schmeck *et al.*, 2008) whereas others indicate statins reduce this (Ishikawa *et al.*, 2010;Lin *et al.*, 2008). Data from functional studies and clinical trials indicates that HDAC inhibition has beneficial effects on vascular function. HDAC inhibition has been shown to reduce blood pressure and vasoconstriction in the sponataneously hypertensive rat (Usui *et al.*, 2012) and reduce bronchoconstriction in human tissue (Banerjee *et al.*, 2012). Thus HDAC inhibition appears to be beneficial for cardiovascular function in disease and thus inhibition of HDAC *in vivo* by statins appears more likely. Inhibition of HDAC has been shown to alter RhoA pathway mRNA and protein levels in cell culture thus statin-mediated changes in HDAC activity could explain the changes in mRNA levels of the RhoA pathway genes. HDAC inhibition has been shown to upregulate MLC phosphorylation and upregulation of ROCK 1 mRNA and protein levels appeared to contribute to this (Kim *et al.*, 2005) but HDAC inhibition has also been shown to reduce RhoA activity (Shieh *et al.*, 2012).

Again considering the *in vivo* data on HDAC inhibition, HDAC activation of the RhoA pathway appears more likely. Even though these data are contradictory it shows that HDAC can have an effect on RhoA itself as well as its target genes. Based on these data inhibition of HDAC activity could account for the reduced constriction at five weeks whereas upregulation of HDAC levels and activity could explain the increased constriction at sixteen weeks (Figure 6.4). This would require sustained upregulation of HDAC activity. The effect of statin withdrawal on HDAC levels or activity has not been explored yet. Data concerning statins and HDAC activity are contradictory and thus alterations in HDAC activity as the mechanism behind the changes observed in the current study should be approached with caution.

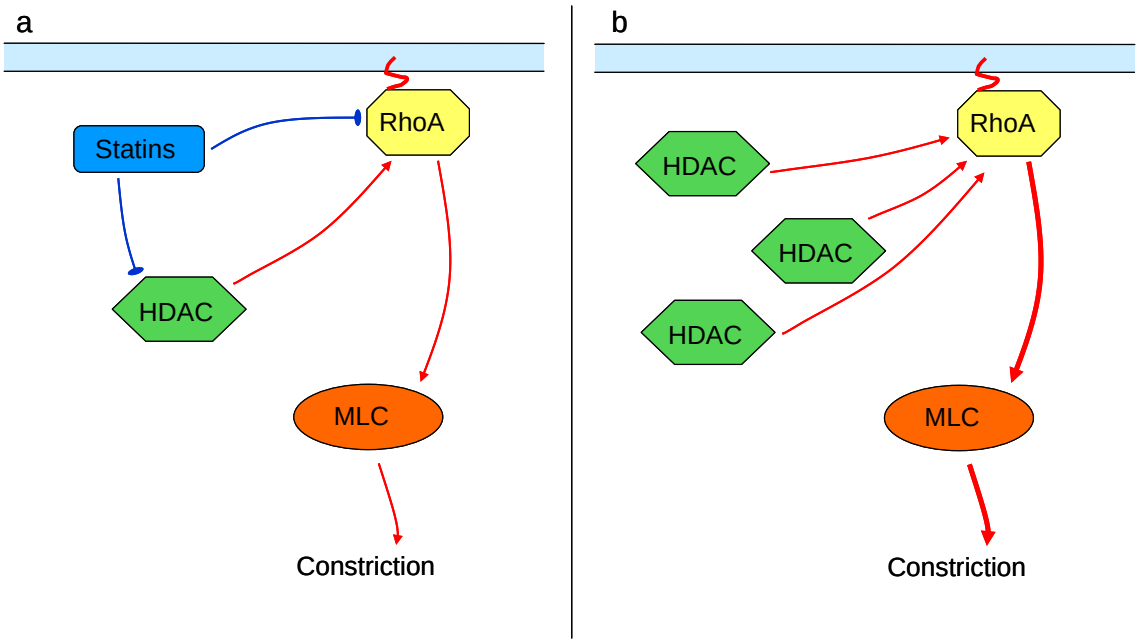


Figure 6.4 Histone deacetylase (HDAC) as the mechanism mediating the early age acute statin treatment and withdrawal effects. a) Statin-mediated inhibition of HDAC activity and thus indirect inhibition of RhoA in addition to direct inhibition of RhoA by statins could account for the reduced constriction observed at five weeks in the current study. b) Sustained increase in HDAC levels and activity following statins withdrawal could increase RhoA pathway activity and thus constriction.

It is also possible that changes in the RhoA pathway gene mRNA levels were induced following statin-induced changes in vascular function. RhoA has been shown to be upregulated at several levels by the actin cytoskeleton (Laufs *et al.*, 2000).(Laufs *et al.*, 2000) Thus reduced constriction at five weeks, possibly due reduced RhoA activity, could have induced an increase in RhoA mRNA and protein expression, which had no effect due to statin-mediated inhibition of RhoA function at the time (Figure 6.5 a). After statin withdrawal though the increase in RhoA mRNA and protein levels could have led to increased RhoA membrane translocation and thus constriction as has been

shown before (Figure 6.5 b) (Laufs *et al.*, 2000). The current study shows increased mRNA expression of RhoA targets, as well as RhoA itself, and it is possible that these could be regulated by the same negative feedback loop that has been shown to upregulate RhoA (Laufs *et al.*, 2000). Thus sustained upregulation of the RhoA pathway genes following reduced constriction at five weeks could have explained the increased constriction at sixteen weeks.

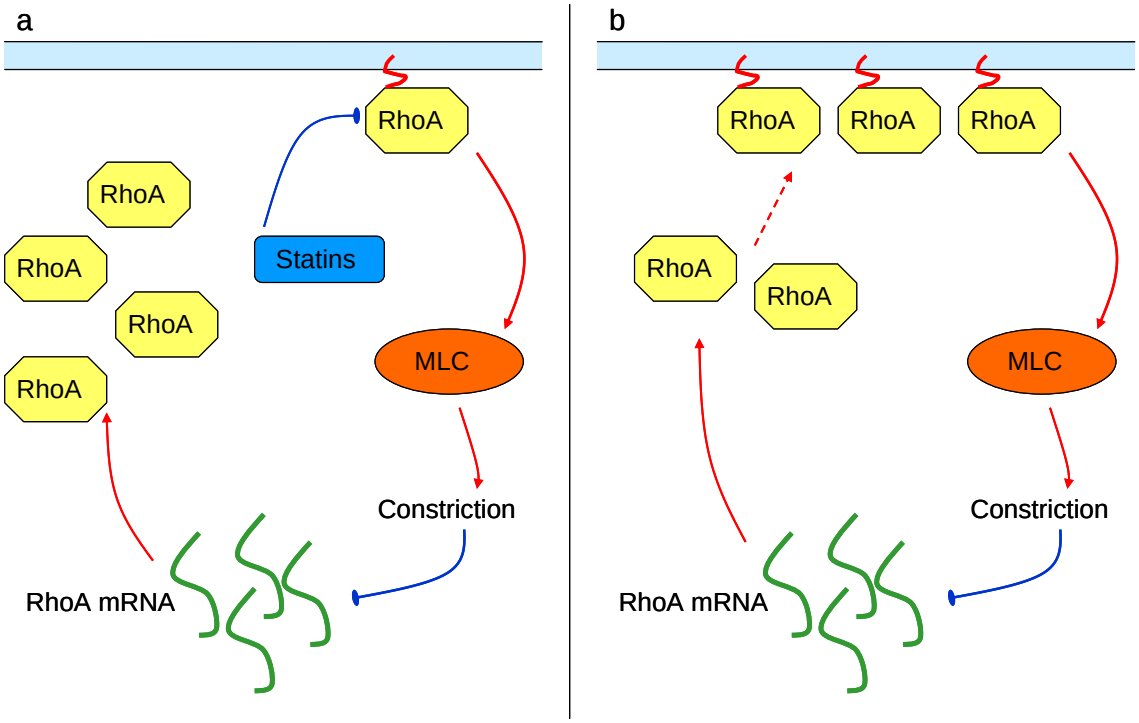


Figure 6.5 RhoA as the mechanism mediating the early age acute statin treatment and withdrawal effects. a) RhoA maintains phosphorylation of myosin light chain (MLC) and thus promotes constriction. Increased constriction, on the other hand, maintains a negative feedback loop to RhoA by inhibiting RhoA mRNA expression. In the presence of statins RhoA prenylation, and thus activity, is inhibited leading to upregulation of RhoA mRNA and accumulation of cytosolic, inactive, RhoA. b) After statin withdrawal RhoA can be prenylated again and due to the accumulation of the protein in the cytosol this leads to increase in constriction.

The different possible mechanism mediating upregulation of the RhoA pathway have been discussed above. Of these mechanisms the upregulation of HMG-Co A reductase appears the least likely as it does not explain the changes observed in RhoA and ROCK mRNA levels. The direct upregulation of the RhoA via negative feedback loop from the actin cytoskeleton appears the most likely as it is best supported by data from other studies. All the mechanisms though require sustained upregulation of the genes in question.

6.1.5 Epigenetic mechanisms

Sustained long term upregulation of gene expression could be induced by epigenetic mechanism such as methylation. Prenatal environment can induce methylation changes as has been shown in famine exposed humans (Heijmans et al., 2008) and the PR offspring (Lillycrop et al., 2008). Also postnatal environment has been shown to be able to induce changes in methylation; maternal grooming behaviour has been shown to alter stress response in 90 day old offspring with changes in gene methylation (Bagot et al., 2012;Weaver et al., 2004;Weaver et al., 2005) and this was also associated with alterations in histone acetylation. Infusion of HDAC inhibitor or methionine in mature animals can reverse some of the effects on gene expression and behaviour (Weaver et al., 2004;Weaver et al., 2005;Weaver et al., 2006) although the longevity of this effect has not been investigated beyond two weeks. Methylation changes induced by IUGR have been shown to be permanently reversed by postnatal treatment with a GLP-1 analogue, used for treatment of diabetes, from day one to six and again changes were seen in histone acetylation as well (Pinney et al., 2011). A high fat diet from weaning has also been shown to alter gene methylation in adult animals (Vucetic et al., 2011). These studies indicate that long term epigenetic changes can be induced in early postnatal life and possibly even in fully mature animals. There is indication that statins can alter methylation in cell culture as lovastatin has been shown to increase demethylation (Kodach et al., 2011) and as methylation can be altered postnatally the changes in function and mRNA levels could have been induce by changes in methylation by the statin treatment and withdrawal. Whether methylation of CpG sites of the RhoA or ROCKs promoter regions are involved in regulation of their mRNA levels has not been investigated. HMG-Co A reductase promoter region is known to contain CpG methylation sites but altered methylation was not associated with altered gene expression in tumour cells (Hentosh et al., 2001). Sustained upregulation of mRNA levels could thus be explained by alterations in gene methylation though this has not been confirmed in the current study.

6.1.6 Effect on structure

Structural changes could have also explained the changes in function observed in the current study. For example alterations in SMC mass or number could account for the changes observed and there is infact some evidence that statins can affect SMC apoptosis and proliferation. Cell culture studies have shown that statin incubation can induce apoptosis in the SMC (Xu *et al.*, 2007;Blanco-Colio *et al.*, 2002) although this might not occur in vivo (Doyon *et al.*, 2011). Statins have also been shown ihibit proliferations (Zhang *et al.*, 2013;Kaneda *et al.*, 2010) and interestingly the RhoA pathway has been shown to be involved in this (Ma *et al.*, 2012). Thus reduction in SMC number at 5 weeks could have lead to reduction in constriction and in response to statin withdrawal there could have been an increase in SMC proliferation via the RhoA pathway leading to long term increase in constriction. The current study did not investigate SMC numbers or mass and thus the effect of statin treatment and withdrawal on this cannot be confirmed.

There is also some evidence that statins can have an effect on cytoskeleton organisation. Statins have been shown to affect endothelial cell shape by altering the cytoskeleton organisation (Dick *et al.*, 2013). This adverse effect could be induced by statin treatment and maintained following the withdrawal this could explain the increased constriction observed in the adult offspring. But if the effect was induced in the young animals the function could be expected to be impaired in the young animals which was not observed in the current study.

6.1.7 Effect on cholesterol

It is interesting to consider why statins did not have an effect on the hepatic parameters measured in this study especially during statin treatment. In the current study no alterations were seen in hepatic HMG-CoA reductase mRNA expression although changes have been shown previously (Torrens *et al.*, 2009b). This could be an indication that alterations observed in the current study were tissue specific effects rather than mediated through the statins effect in the liver. This is possible as atorvastatin is lipophilic and thus can enter cells via passive transport. The tissue specific effects are not only indicated by the lack of differences in mRNA levels in the liver but also by the functional differences in the two vascular beds. As mentioned

before the effect of the statin treatment on cholesterol levels is not known in the current study but considering that HMG-CoA reductase gene expression was not altered in the liver this indicates that it is more likely that plasma cholesterol levels were not altered as they are mainly regulated by the liver. Liver specific deletion of HMG-CoA reductase has been shown to be lethal with the median survival age around 5 weeks (Nagashima *et al.*, 2012). This indicates that statin treatment did not completely block the mevalonate pathway in this study and that the remaining cholesterol synthesis may have been sufficient to maintain adequate cholesterol levels. However the possibility remains that cholesterol levels were reduced during statin treatment and that this depletion could have had long term effects. Six week statin treatment in adult rats has been shown to alter brain lipid compositions (Vecka *et al.*, 2004) and alterations in membrane fatty acids have been shown as well (Nyalala *et al.*, 2008). Alterations in membrane properties have been shown to affect membrane permeability and signal transduction pathways (de Jonge *et al.*, 1996;Miersch *et al.*, 2008) and thus changes in membrane properties in the vasculature could have altered the response to receptor mediated constriction. As *de novo* cholesterol synthesis, and thus HMG-Co A reductase activity, are naturally increased in young rats immediately post weaning (Dietschy *et al.*, 1993;Smith *et al.*, 1995) this increase might have been adequate to prevent statin-mediated changes from occurring in the liver in the current study. Previous studies indicate that statin effects are cholesterol independent in the PR model (Torrens *et al.*, 2009b) and as no changes were seen in the liver parameters, especially in hepatic HMG-Co A reductase mRNA levels, in the current study changes in cholesterol levels are not indicated.

In summary this study shows that early age acute statin treatment is detrimental to vascular function long term even though the immediate effects appear mildly beneficial. These data do not support the original hypothesis about long term protective effect of early age acute statin treatment on vascular function (Figure 6.6). These findings support rebound effect after statin withdrawal leading to increased risk of CVD as compared to without treatment in later life. Molecular data indicates that there is a tissue specific involvement of the RhoA pathway. Investigating the role of other mechanisms known to be affected by statins, such as inflammation and oxidative stress, after statin withdrawal could allow better understanding of the rebound effect.

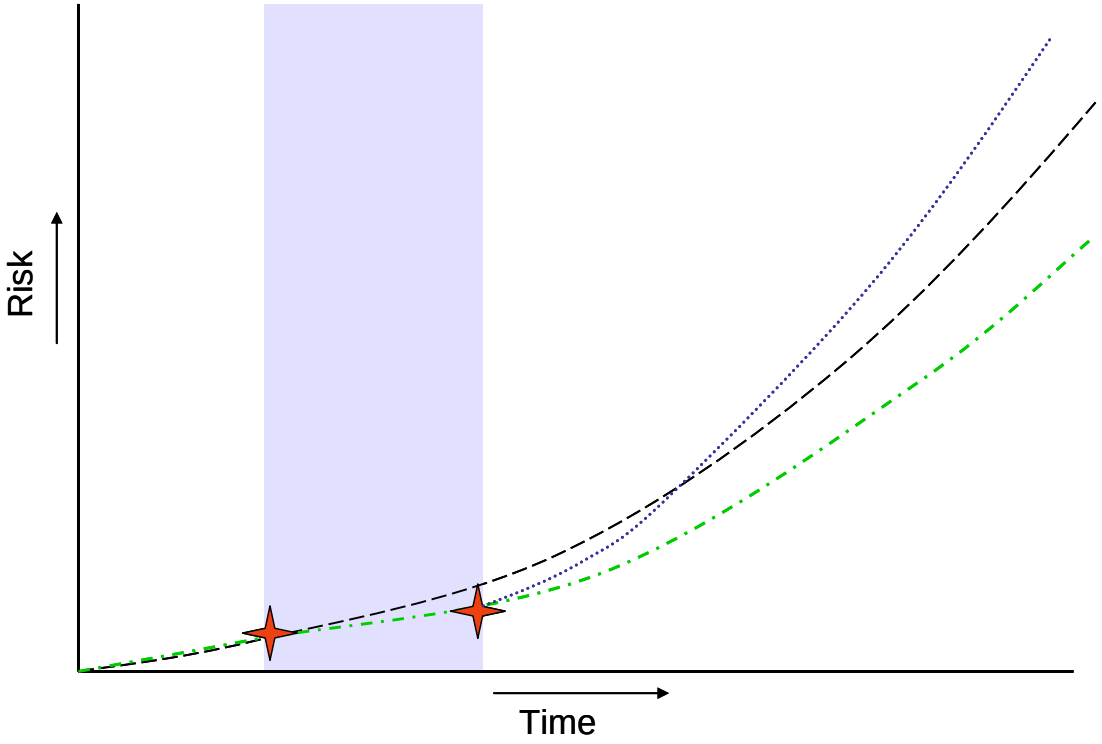


Figure 6.6 The predicted and indicated risk of CVD following early age acute statin treatment and withdrawal. This study indicates that unlike predicted protective effects of statin treatment are not maintained after statin withdrawal but this leads to a rebound effect where the risk becomes greater than without treatment. Baseline risk ---; predicted risk -·-·-·-; observed risk ····· ; intervention.

6.2 Translational implications

The current study shows that statin withdrawal can impair vascular function long term at least in an animal model and, if applicable to humans, indicates that the risk of CVD increases after withdrawal of short term statin treatment at an early age. Although there are clinical studies on the rebound effect of statins, the studies conducted so far have not been able to confirm the mechanisms behind the phenomenon, who is susceptible and if the increase in risk and impairment in function are transient as has been implied in animal studies (Gertz *et al.*, 2003;Laufs *et al.*, 2000;Vecchione & Brandes, 2002). The conflicting data from the clinical trial follow ups and statin withdrawal trials have not been satisfactorily addressed either (Bulbulia *et al.*, 2011;Daskalopoulou *et al.*, 2008;Ford *et al.*, 2007;Heeschen *et al.*, 2002). Double blind, placebo controlled, sufficiently powered clinical trials take a long time and due to ethical reasons can be difficult or even impossible to conduct thus animal models need to be utilised to investigate the mechanisms and the characteristics of the rebound effect in more detail.

The first issue is whether the effects observed in the clinical trials do represent a rebound effect. In majority of the clinical withdrawal studies the withdrawal was not conducted under controlled conditions and this raises the possibility of risk treatment mismatch. Statin withdrawal on hospitalisation leads to worse outcome (Sposito *et al.*, 2009) but it has been indicated that statins were more likely to be withdrawn in cases were the prognosis was worse on admittance (Spencer *et al.*, 2004) indicating a possibility of risk treatment mismatch. Risk treatment mismatch is not an issue in animal studies and thus results from the current study as well as other *in vivo* as well as *in vitro* studies support the occurrence of a rebound effect following statin withdrawal (Gertz *et al.*, 2003;Laufs *et al.*, 2000;Otani *et al.*, 2010;Vecchione & Brandes, 2002). Also shorter term studies in humans, looking at parameters such as CRP and cholesterol, indicate that there is a withdrawal effect (Chen *et al.*, 2009;Puccetti *et al.*, 2003;Taneva *et al.*, 2006) and impairment in function has been shown in healthy individuals as well after statin withdrawal (Chen *et al.*, 2009;Laufs *et al.*, 2001).

Another point the clinical trials so far have not clearly addressed is the longevity of the rebound effect and whether certain groups are more susceptible to it. There is a contradiction between the trials that have looked at statin withdrawal and the follow ups of original statin trials. It is possible that rebound effect was not detected in the follow up trials as they did not take into account statin use following the study period (Bulbulia *et al.*, 2011;Ford *et al.*, 2007). The observations from both groups of studies could be real if short term withdrawal effect is detrimental but there is a protective long term effect. The rebound effect could be transient, as shown in some animal studies (Laufs *et al.*, 2000;Vecchione & Brandes, 2002), with impairment in function after withdrawal due to a sudden shift in balance with the restabilisation occurring on the side of benefit. The evidence indicates though that if the effect is transient the stabilisation occurs at the pretreatment levels (Chen *et al.*, 2009;Gertz *et al.*, 2003;Lai *et al.*, 2005;Laufs *et al.*, 2000;Laufs *et al.*, 2001;Vecchione & Brandes, 2002). Also there is no evidence of detrimental short term effect in the follow up studies, as when the cardiovascular events are plotted against time no spike can be detected shortly after withdrawal (Bulbulia *et al.*, 2011;Ford *et al.*, 2007). The discrepancy between the follow up and withdrawal studies could also be explained by population subgroups with different rebound risk. The severity of the disease could be the factor defining the risk of rebound effect; in the follow up trials the original population sample consisted of people in increased risk of

CVD but before the onset of the overt disease (Bulbulia *et al.*, 2011;Ford *et al.*, 2007) whereas in the majority of the withdrawal studies the sample population consisted of people with manifest disease (Chen *et al.*, 2009;Daskalopoulou *et al.*, 2008;Heeschen *et al.*, 2002) (Dowlatshahi *et al.*, 2012;Spencer *et al.*, 2004;Sposito *et al.*, 2009). The increased risk of rebound effect in those with manifest disease is further backed up by a study showing that statin withdrawal reduces FMD which returns back to baseline a week after withdrawal in healthy subjects but not in patients with coronary artery disease (Chen *et al.*, 2009). Defining the rebound risk could be even more complicated though as data from the current study combined with other animal studies (Laufs *et al.*, 2000;Otani *et al.*, 2010;Vecchione & Brandes, 2002) indicate that other factors, such as length and timing of treatment, can alter the longevity and possibly severity of the rebound effect.

The clinical trials conducted so far have not confirmed the mechanism behind the withdrawal effect though both endothelial dysfunction and inflammation appear to be involved (Chen *et al.*, 2009;Lai *et al.*, 2005;Laufs *et al.*, 2001;Taneva *et al.*, 2006). In the current study alterations in NO bioavailability were not indicated but alterations were seen in the RhoA pathway. The mRNA levels of RhoA pathway genes were upregulated in the mesenteric arteries. Considering that statin treatment confers its benefits through several pathways it seems likely that this could be the same for the rebound effect. This study indicates that one of the pathways involved is the RhoA pathway which is known to be altered by statin treatment and withdrawal.

This study supports the idea of rebound effect following statin withdrawal and that withdrawal of short term treatment can have long lasting effects. Taken together with other animal studies it indicates that the nature and severity can be altered by several factors such as timing and length of treatment and withdrawal. Also it is indicated that, as statin effects, the withdrawal effects may be meditated through several pathways. The evidence from animal studies cannot directly be applied to humans though they can help to determine the direction of clinical trials. Further clinical trials, as well as animal studies, are needed to confirm the effect in humans and identify the mechanisms behind the rebound effect. Understanding the risk of rebound effect would also allow more accurate assessment to be made on whether to start statin therapy or not on individual patients.

6.3 Conclusions

This study has demonstrated both short and long term effects of acute early age statin treatment in rats. Acute early age statin treatment reduced maximal constriction in the adolescent offspring but following statin withdrawal maximal constriction was increased. In the mature animals the increase in constriction was associated with upregulation of the RhoA vasoconstrictor pathway mRNA levels. These results are indicative of a rebound effect which is supported by data from other studies in rodents as well as clinical trials. The current study is the first one to demonstrate that withdrawal of short term statin treatment can have long term effects. Maternal protein restriction did not alter vascular reactivity in the current study. The current study does not support early age acute statin treatment in primary prevention of CVD as the withdrawal of the treatment leads to impairment of vascular function long term.

Appendices

Appendix 1 Diet compositions

AIN-76A w/ 18% Protein — 5SZ3

DESCRIPTION

Modification of TestDiet® AIN-76A Semi-Purified Diet 58B0 with 18% Protein.

Storage conditions are particularly critical to TestDiet® products. To provide maximum protection against possible changes during storage, purified diets, high fat diets (>10% fat) and diets containing temperature sensitive experimental compounds should be stored under refrigeration (2° C) or frozen (-20° C) upon receipt. All other diets can be stored in a dry, cool location. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*

*Other Forms Available On Re

INGREDIENTS (%)

Sucrose	49.9990
Casein - Vitamin Free	20.0158
Corn Starch	15.0000
Corn Oil	5.0000
Powdered Cellulose	4.9842
AIN-76 Mineral Mix	3.5000
AIN-76A Vitamin Mix	1.0000
DL-Methionine	0.3000
Choline Bitartrate	0.2000
Ethoxyquin (a preservative)	0.0010

NUTRITIONAL PROFILE [1]

Protein, %	***18.0***
Arginine, %	0.70
Histidine, %	0.52
Isoleucine, %	0.96
Leucine, %	1.73
Lysine, %	1.45
Methionine, %	0.82
Cystine, %	0.07
Phenylalanine, %	0.96
Tyrosine, %	1.01
Threonine, %	0.77
Tryptophan, %	0.22
Valine, %	1.14
Alanine, %	0.55
Aspartic Acid, %	1.29
Glutamic Acid, %	4.09
Glycine, %	0.39
Proline, %	2.36
Serine, %	1.10
Taurine, %	0.00
Fat, %	***5.0***
Cholesterol, ppm	0
Linoleic Acid, %	2.86
Linolenic Acid, %	0.05
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.05
Total Saturated Fatty A	0.64
Total Monounsaturated Fatty Acids, %	1.21
Polyunsaturated Fatty Acids, %	2.90
Fiber (max), %	***5.0***
Carbohydrates, %	***66.4***
Energy (kcal/g) [2]	***3.81***

From:	kcal	%
Protein	0.720	18.8
Fat (ether extract)	0.453	11.8
Carbohydrates	2.656	69.4

Minerals	
Calcium, %	0.53
Phosphorus, %	0.56
Phosphorus (available), %	0.56
Potassium, %	0.36
Magnesium, %	0.05
Sodium, %	0.12
Chloride, %	0.21
Fluorine, ppm	0.0
Iron, ppm	35
Zinc, ppm	36
Manganese, ppm	59
Copper, ppm	6.0
Cobalt, ppm	0.0
Iodine, ppm	0.21
Chromium, ppm	2.0
Molybdenum, ppm	0.00
Selenium, ppm	0.17
Vitamins	
Vitamin A, IU/g	4.0
Vitamin D-3 (added), IU/g	1.0
Vitamin E, IU/kg	50.0
Vitamin K (as menadione), ppm	0.50
Thiamin Hydrochloride, ppm	6.1
Riboflavin, ppm	6.7
Niacin, ppm	30
Pantothenic Acid, ppm	16
Folic Acid, ppm	2.1
Pyridoxine, ppm	5.8
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	14
Choline Chloride, ppm	1,000
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only; NOT for human consumption.

10/19/2009

ISO 9001:2000 CERTIFIED

TestDiet
www.testdiet.com

AIN-76A Purified Diet w/ 9.0% Protein — 5SZT

DESCRIPTION

Modification of TestDiet® AIN-76A Semi-Purified Diet 58B0 with 9.0% Protein.

Storage conditions are particularly critical to TestDiet® products. To provide maximum protection against possible changes during storage, purified diets, high fat diets (>10% fat) and diets containing temperature sensitive experimental compounds should be stored under refrigeration (2° C) or frozen (-20° C) upon receipt. All other diets can be stored in a dry, cool location. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1814008

*Other Forms Available On Re

INGREDIENTS (%)

Sucrose	49.9990
Powdered Cellulose	15.1768
Corn Starch	15.0000
Casein - Vitamin Free	9.8232
Corn Oil	5.0000
AIN-76 Mineral Mix	3.5000
AIN-76A Vitamin Mix	1.0000
DL-Methionine	0.3000
Choline Bitartrate	0.2000
Ethoxyquin (a preservative)	0.0010

NUTRITIONAL PROFILE [1]

Protein, %	***9.0***
Arginine, %	0.34
Histidine, %	0.25
Isoleucine, %	0.47
Leucine, %	0.85
Lysine, %	0.71
Methionine, %	0.55
Cystine, %	0.04
Phenylalanine, %	0.47
Tyrosine, %	0.50
Threonine, %	0.38
Tryptophan, %	0.11
Valine, %	0.56
Alanine, %	0.27
Aspartic Acid, %	0.63
Glutamic Acid, %	2.01
Glycine, %	0.19
Proline, %	1.15
Serine, %	0.54
Taurine, %	0.00
Fat, %	***5.0***
Cholesterol, ppm	0
Linoleic Acid, %	2.86
Linolenic Acid, %	0.05
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.05
Total Saturated Fatty A	0.64
Total Monounsaturated Fatty Acids, %	1.21
Polyunsaturated Fatty Acids, %	2.90
Fiber (max), %	***15.2***
Carbohydrates, %	***66.4***
Energy (kcal/g) [2]	***3.46***

From:	kcal	%
Protein	0.360	10.4
Fat (ether extract)	0.453	13.1
Carbohydrates	2.656	76.6

Minerals	
Calcium, %	0.53
Phosphorus, %	0.48
Phosphorus (available), %	0.48
Potassium, %	0.36
Magnesium, %	0.05
Sodium, %	0.12
Chloride, %	0.21
Fluorine, ppm	0.0
Iron, ppm	35
Zinc, ppm	32
Manganese, ppm	59
Copper, ppm	6.0
Cobalt, ppm	0.0
Iodine, ppm	0.21
Chromium, ppm	2.0
Molybdenum, ppm	0.00
Selenium, ppm	0.14
Vitamins	
Vitamin A, IU/g	4.0
Vitamin D-3 (added), IU/g	1.0
Vitamin E, IU/kg	50.0
Vitamin K (as menadione), ppm	0.50
Thiamin Hydrochloride, ppm	6.0
Riboflavin, ppm	6.3
Niacin, ppm	30
Pantothenic Acid, ppm	15
Folic Acid, ppm	2.1
Pyridoxine, ppm	5.8
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	12
Choline Chloride, ppm	1,000
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only; NOT for human consumption.

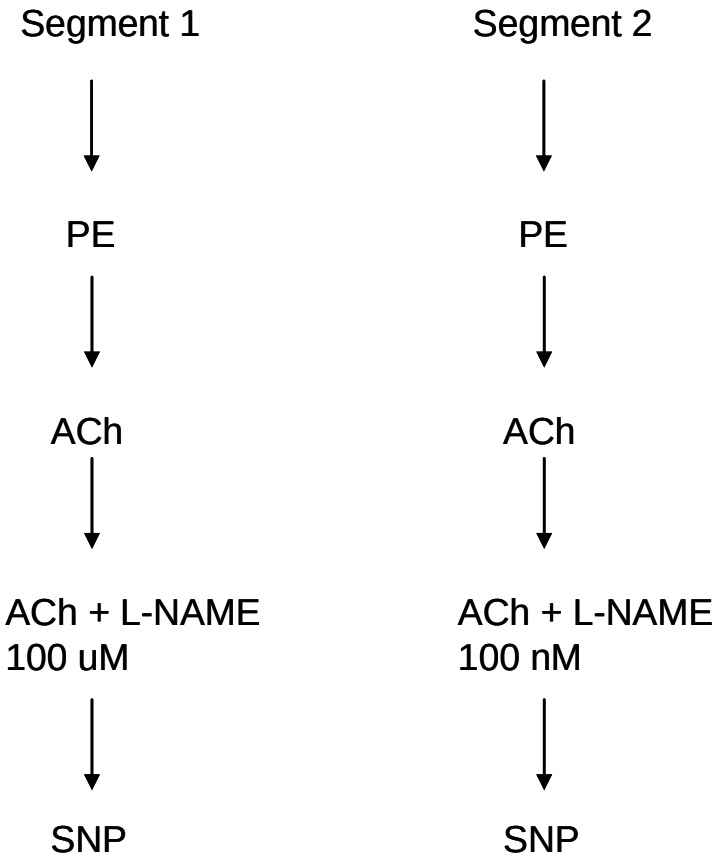
10/29/2009

ISO 9001:2000

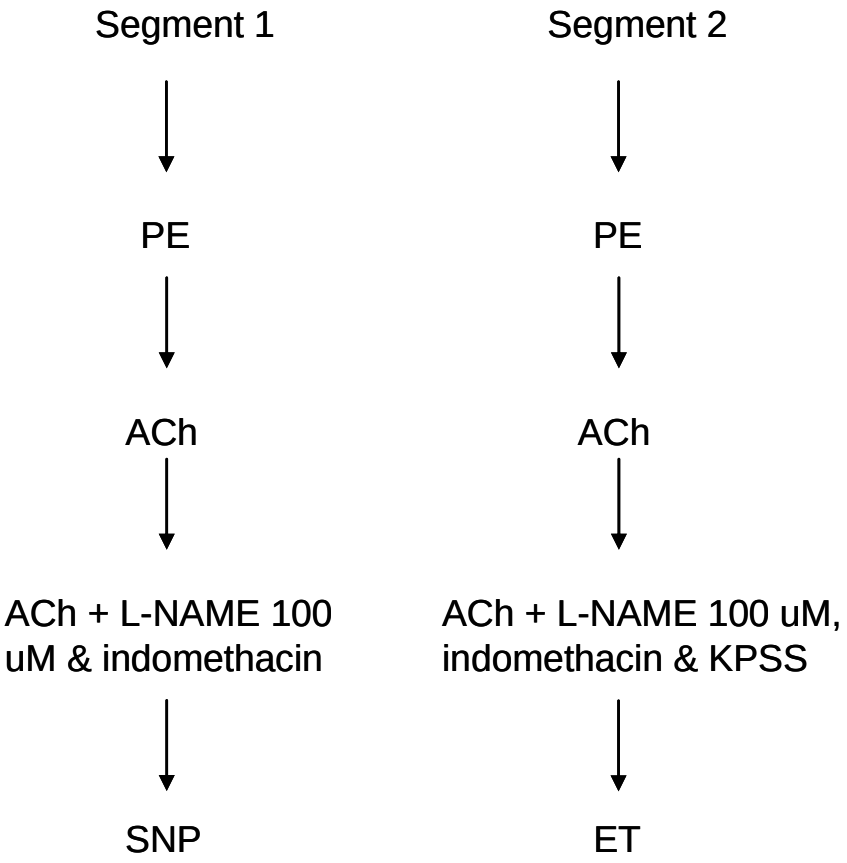
TestDiet
www.testdiet.com

Appendix 2 Myography protocols

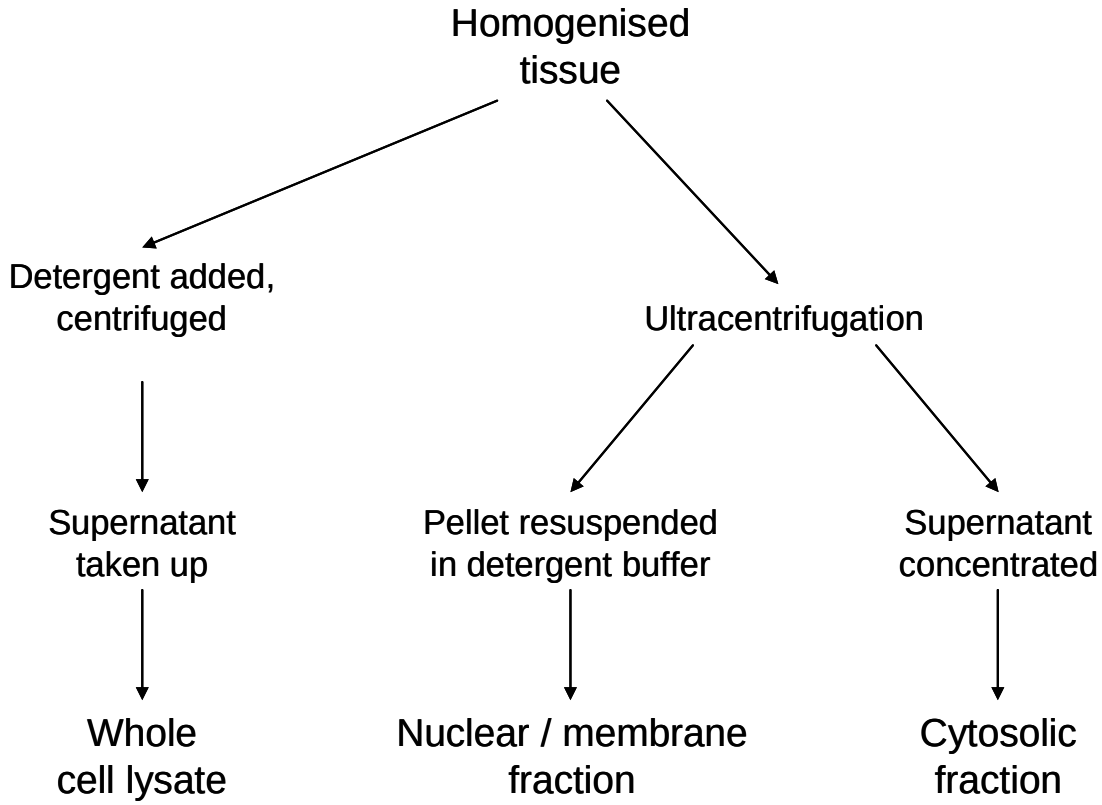
Aorta



Mesenteric arteries



Appendix 3 Subcellular fractination protocol



Appendix 4 Housekeeping gene stability determination

GeNorm

Individual stability values

Gene name	Stability value
B2M	1.646
ACTB	1.737
CYC1	1.743
RPL13A	1.789
UBC	1.802
CANX	1.835
YWHAZ	2.087
ATP5B	2.142
GAPDH	2.211
MDH1	2.231
TOP1	2.534

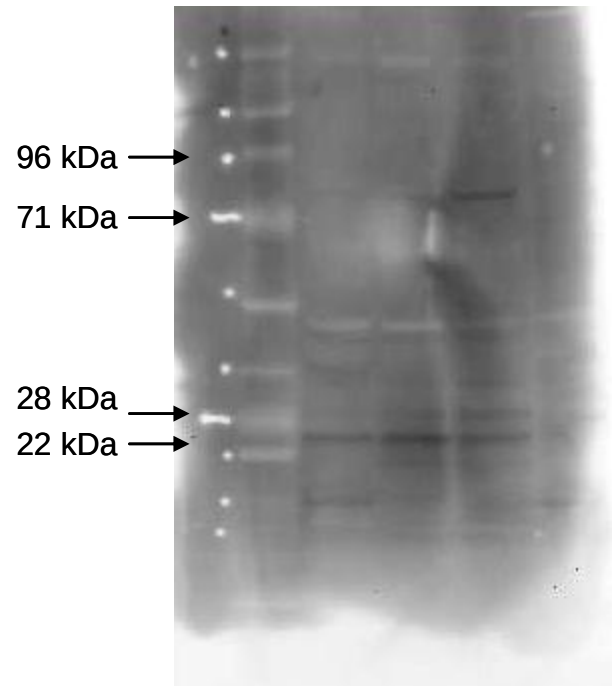
Pair-wise comparisons

Normfinder

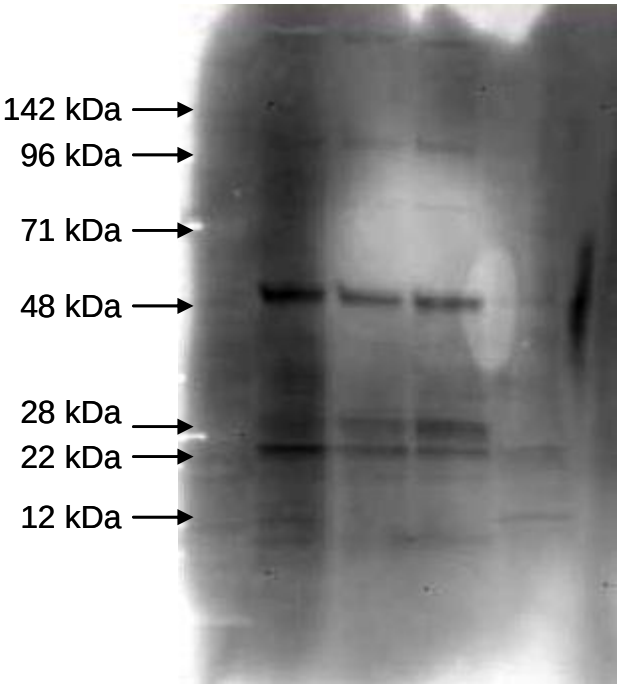
Gene name	Stability value
ACTB	11.823
CYC1	11.987
UBC	12.513
RPL13A	12.591
B2M	12.896
CANX	13.897
YWHAZ	14.953
ATP5B	18.550
MDH1	23.171
TOP1	45.402
GAPDH	60.343

Appendix 5 Western blotting secondary antibody controls

Anti-rabbit antibody



Anti-mouse antibody



Appendix 6 Published abstracts

Physiological Society Main Meeting, University of Oxford, UK, 11.-14. July 2011

C7

Short term statin treatment does not affect blood pressure in 5 week old rats

P.H. Keskivali[1], N.P. Curzen[1,2], G.F. Clough[1] and C. Torrens[1]

[1]*Vascular Biology Group, Faculty of Medicine, University of Southampton, Southampton, UK and* [2]*Wessex Cardiothoracic Unit, University of Southampton, Southampton, UK*

Statins (HMG Co-A reductase inhibitors) have been proven to be effective in secondary prevention of cardiovascular disease (CVD) with increasing evidence for their effectiveness in primary prevention (Ridker et al., 2008). In rats, maternal protein restriction leads to raised blood pressure and endothelial dysfunction in adult male offspring, which can be improved with long term chronic statin treatment (Torrens et al., 2009). The aim of the current study was to determine if statin treatment over an early two week period had any effect on blood pressure.

Female Wistar rats were fed either a control (C, 18 % casein) or protein restricted (PR, 9% casein) diet throughout gestation from conception to term. On delivery dams and pups were returned to standard chow. At 3 weeks of age pups were weaned and further divided into two subgroups of controls and those receiving statin. This gave the four experimental groups; control (C), control + statin (CS), protein restricted (PR) and PR + statin (PRS). At 5 weeks blood pressure was recorded using a non-invasive blood pressure monitor. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured and mean arterial pressure (MAP) was calculated. To minimise variations five stable readings were obtained, the highest and lowest of which were then discarded and the three remaining readings were used to calculate the mean. Results are expressed as mean ± SEM. Differences were assessed by one-way ANOVA. Significance was assumed at $p<0.05$.

Maternal weight gain, litter size and birth weight were similar between the groups. Neither systolic pressure (mmHg: C, 130.5±9.5, n=9; CS, 113.1±6.4, n=9; PR, 121.4±11.8, n=7; PRS, 119.3±6.6, n=7) nor diastolic pressure (mmHg: C, 93.3±9.0, n=9; CS, 78.6±6.0, n=9; PR, 89.3±9.2, n=7; PRS, 85.0±8.6, n=7) were different in male offspring. This was also true in female offspring for both systolic (mmHg: C, 117.2±11.3, n=7; CS, 121.2±9.7, n=8; PR, 126.4±9.7, n=7; PRS, 125.0±9.8, n=8) and diastolic pressure (mmHg: C, 82.8±10.5, n=7; CS, 86.2±8.5, n=8; PR, 91.7±9.7, n=7; PRS, 85.8±4.4, n=8)

The current data suggest that the blood pressure rises previously reported in this model are not present at five weeks of age. In addition, they show that even though long-term statin treatment has been shown to have positive effects in this model, they do not appear to be effective after a shorter exposure.

Ridker et al. (2008) NEJM 359:2195-2207

Torrens et al. (2009) Hypertension 2009; 53:661-667

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

7th World congress on Developmental Origins of Health and Disease, Portland, Oregon, USA, 18-21 September 2011

Young adults born preterm at VLBW are more insulin resistant than their term-born peers with a similar body mass index. This is in part compensated by their higher insulin secretion.

PII-241

Preterm Birth at Very Low Birth Weight and Nutrient Intake in Adult Life. Nina Kaseva[1,2], Karoliina Wehkalmpi[1,2], Katri Hemiö[1], Petteri Hovi[1,2], Anna-Liisa Järvenpää[2], Sture Andersson[2], Johan G. Eriksson[1,2,3], Jaana Lindström[1], Eero Kajantie[1]. *[1]National Institute for Health and Welfare, Finland; [2]Helsinki University Central Hospital, Finland; [3]University of Helsinki, Finland.*

The period after severely preterm birth is characterized by immaturity-associated illness and inadequate nutrition and thus constitutes a model of early nutritional deprivation. We studied dietary intake in healthy young adults born preterm at very low birth weight (VLBW, <1500g).

151 unimpaired young adults, aged 19-27 years, born at VLBW and 156 age-, sex-, and birth hospital-matched term-born controls completed a 3-day food record and used a picture booklet with food portions to estimate portion sizes. A nutritionist interviewed the participants after completion of the food record. Nutrient intakes were calculated with a dietary analysis program based on the national FINELI® database. A subset of participants underwent dual-energy x-ray absorptiometry for body composition measurement. Data were analyzed by multiple linear regression.

As compared with controls, the VLBW subjects reported 194.1 kcal lower daily energy intake (95% CI; -311.9 to -76.4 adjusted for age, sex, socio-economic status, daily smoking and perinatal factors), which was due to their smaller body size; (mean difference adjusted in addition for height and BMI -82.6 kcal/d, 95% CI; -206.7 to 41.5). There was no difference between groups in the proportion of energy from carbohydrates [-1.0% (95% CI; -2.8 to 0.9), fat [1.2% (95% CI;-0.5 to 3.0)] or protein [-0.6% (95% CI; -1.5 to 0.4)]. The mean values for the proportions of daily energy intake in VLBW and controls from carbohydrate were as follows: 46.3% (SD 7.4%) and 47.2% (8.4%), for protein 16.1% (3.8%) and 16.2% (3.9%) and for fat 34.7% (7.0%) and 33.4% (7.1%). Compared with controls, VLBW participants had lower intake of calcium -219.8 mg (95% CI; -328.7 to -110.9) and cholesterol -29.9 mg (95% CI; -49.3 to -10.6). Vitamin D intake was 3.7 µg/d (SD 2.6) in VLBW and 4.4 µg/d (3.6) in control subjects [mean difference -0.8 (95% CI -1.6 to -0.03)].

We found no evidence for altered macronutrient intake in young adults born preterm at VLBW. Further analysis of micronutrient intake is ongoing. Calcium intake was lower in the VLBW group; this may be of importance as increased risk for osteoporosis has been linked to preterm birth at VLBW.

PII-242

Young Adults Born Preterm at Very Low Birth Weight Undertake Less Physical Activity Than Their Peers Born at Term. Nina Kaseva[1,2], Karoliina Wehkalampi[1,2], Sonja Strang-Karlsson[1,2], Minna Salonen[1], Anu-Katriina Pesonen[3], Katri Räikkönen[3], Tuija Tammelin[4], Petteri Hovi[1,2], Jari Lahti[3], Kati Heinonen[3], Anna-Liisa Järvenpää[2], Sture Andersson[2], Johan G. Eriksson[1,2,3,5], Eero Kajantie[1,2]. *[1]National Institute for Health and Welfare, Finland; [2]Helsinki University Central Hospital, Finland; [3]University of Helsinki, Finland; [4]LIKES Research Center for Sport and Health Sciences, Finland; [5]Folkhälsan Research Centre, Finland.*

A recent meta-analysis (Andersen *et al.* PLoS One 2009;4:e8192) showed that the association between birth weight and undertaking leisure-time physical activity (LTPA) is very weak within the normal birth weight range, but both low and high birth weights may be associated with low rates of self-reported LTPA. We assessed LTPA and its components in healthy young adults born preterm at very low birth weight (VLBW, <1500g) compared with term-born controls.

We studied 94 unimpaired young adults, aged 21-29 years, born at VLBW and 101 age-, sex-, and birth hospital-matched term-born controls. The participants completed a validated 30-item 12-month physical activity questionnaire and the NEO-Personality Inventory measuring the "big five" personality traits. Yearly frequency, total time, total volume and energy expenditure of conditioning and non-conditioning LTPA and commuting physical activity were compared between VLBW and term-born subjects. A subset of participants underwent dual-energy x-ray absorptiometry for body composition measurement. Data were analyzed by multiple linear regression.

Compared with controls, VLBW participants had lower frequency [-38.5% (95% CI; -58.9, -7.7)], total time [-47.4% (95% CI; -71.2, -4.1)], total volume [-44.3% (95% CI; -65.8, -9.2)] and energy expenditure [-55.9% (95% CI; -78.6, -9.4)] of conditioning LTPA when adjusted for age, sex, body mass index, smoking, parental education and personality traits. Adjusting for lean body mass instead of body mass index attenuated the difference. There were no differences in non-conditioning LTPA or commuting physical activity.

Compared with term-born controls, unimpaired VLBW adults undertake less frequent LTPA with lower total time and volume of exercise resulting in lower energy expenditure. This finding reinforces previous suggestions that physical activity is programmed early in life. It also underlies the importance of early promotion of physical activity in the prevention of chronic non-communicable diseases in VLBW individuals.

PII-243

Does Preterm Birth Affect Vascular Health Status in Young Adulthood? Gerthe F. Kerkhof, Petra E. Breukhoven, Ralph W.J. Leunissen, Ruben H. Willemsen, Anita C.S. Hokken-Koelega. *Pediatrics, subdivision of Endocrinology, ErasmusMC/Sophia Children's Hospital, Netherlands.*

Both preterm birth and small birth size for gestational age (SGA) have been associated with an increased risk for developing cardiovascular diseases (CVD), but controversies still exist. We investigated the effect of preterm birth on several parameters of vascular health status. We hypothesized that preterm birth is associated with increased risk for CVD in young adulthood, independent of size at birth.

In 406 young adults of the PROGRAM/PREMS study, aged 18-24 yr, the effect of preterm birth (gestational age <36 weeks) on systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure, blood pressure variability, heart rate, Pulse Wave Velocity (PWV), and carotid Intima Media Thickness (cIMT) was analyzed. To study the differential effect of preterm and SGA birth on vascular health, these parameters were also analyzed in subgroups: young adults born small for gestational age with short stature (SGA-S) or normal stature (SGA-CU), born either preterm or term, and young adults born appropriate for gestational age with normal stature (AGA), born either preterm or term.

Subjects born preterm had a higher SBP, pulse pressure, blood pressure variability, heart rate, and a lower DBP than subjects born at term. In the total group, the continuous variable gestational age was inversely associated with SBP via an increased heart rate, inversely associated with pulse pressure and blood pressure variability, and positively associated with DBP, also after adjustment for confounders. There was no effect of gestational age on PWV and cIMT. Of all vascular health parameters, higher pulse pressure affected cIMT the most. Subgroup analyses showed that all preterm subgroups had a significantly higher pulse pressure and DBP variability than the reference group (AGA subjects born at term), but lower DBP. SGA-CU and AGA subjects born preterm had a higher heart rate than the reference group. There were no differences in vascular health parameters between the SGA and AGA groups born at term.

Our results show that young adults born preterm have a less favorable vascular health status than those born at term, independent of birth size. Subgroup comparisons showed that the effect of preterm birth on vascular health is likely not to be due to SGA birth or catch up growth.

PII-244

Effects of Maternal Protein Restriction and Short Term Statin Treatment on the Vascular Function of the Offspring. P. H.M. Keskivali[1], N. P. Curzen[1,2], G. F. Clough[1], C. Torrens[1]. *[1]Faculty of Medicine, University of Southampton, United Kingdom; [2]Wessex Cardiothoracic Unit, University of Southampton, United Kingdom.*

In rats maternal protein restriction during pregnancy leads to cardiovascular dysfunction in the offspring. Administration of statins (HMG Co-A reductase inhibitors) from weaning onwards can improve this (Torrens *et al.*, 2009). The aim of this study was to determine if early age short term statin treatment improves cardiovascular function in offspring of protein restricted dams.

Wistar rat dams were fed isocaloric control (C, 18% casein) or protein restricted (PR, 9% casein) diet during pregnancy and standard chow during

suckling. At weaning (3 weeks) offspring either received atorvastatin in drinking water (10mg/kg per day) or acted as control giving four offspring groups; control (C), control+statin (CS), protein restricted (PR) and PR+statin (PRS). At five weeks of age offspring blood pressure (BP) was recorded by tail cuff plethysmography and aorta reactivity was assessed post mortem using wire myography. Cumulative concentration response curves were constructed to phenyleprine (PE), acetylcholine (ACh) and sodium nitroprusside (SNP). Results are presented as mean±SEM. Differences were assessed by one-way ANOVA, significance assumed at p£0.05.

No differences were seen in BP across the groups. Statin treatment reduced the maximal constrictor response to PE in CS and PRS as compared to controls in male (g: C 2.27±0.09, n=5; CS 1.65±0.15, n=5; PR 1.86±0.07, n=4; PRS 1.58±0.15, n=4, p<0.01) but not female offspring. No differences were seen in maximal relaxation response to ACh in male (% of constriction: C 75.2±6.4, n=5; CS; 59.1±5.3, n=5; PR 70.8±9.5, n=4; PRS 82.4±8.3, n=4) or female (% of constriction; C 76.4±5.9, n=4; CS 71.4±5.3, n=5; PR 76.2±6.4, n=4; PRS 83.9±4.9, n=4) offspring at five weeks. Smooth muscle function as assessed by relaxation to SNP was similar in all groups.

These results show that short term statin treatment reduces maximal constriction to PE regardless of pre-natal diet. At five weeks endothelial function was unaffected by either maternal diet or short term statin treatment, suggesting a later onset of endothelial dysfunction and beneficial effects of statins.

Torrens *et al.*. (2009) *Hypertension* 2009; **53**:661-667.

PII-245

Ouabain Decreases the Risk of Rat Hypertension and Renal Disease by Rescuing Kidney Development during Intrauterin Growth Restriction.

Juan Li[1], Jing Yue[2], Pinhao Xie[1], Qingxing Zhai[1], Yali Hu[2]. *[1]Department of Hematology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu Province, China; [2]Department of Obstetrics and Gynecology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu Province, China.*

Low birth weight due to intrauterine growth restriction (IUGR) is associated with an increased risk of hypertension and end stage renal disease in later life. Overwhelming evidence suggests that this is because of IUGR endangers kidney development and results in an irreversible loss of nephrons. Yet, no drug available that would alleviate the effects of IUGR on nephron formation. In our previous work in vitro study, using explanted rat embryonic kidneys, we found that ouabain, the Na,K-ATPase ligand, by triggering a calcium–nuclear factor- κB signal, protected embryonic kidney development from IUGR. Here we report that ouabain rescues embryonic kidney development in vivo study and decreases the risk of hypertension and end stage renal disease in later life.

Protocol The Animal Welfare Board at Nanjing University approved the protocol for this investigation. Pregnant SD rats were given either a low (9%) or a normal (18%) protein diet immediately after mating. Under Isofluran anaesthesia, osmotic pumps (Alzet Osmotic Pumps), delivering either ouabain dissolved in PBS (15 μ g ouabain per kg body weight per day) or vehicle, were implanted subcutaneously on the second day of pregnancy. **Glomeruli counting** Half of the embryos were killed at birth and their kidneys were processed for stereological examination of glomerular number. Stereological analysis was performed using a modified form of the disector method. **Blood Pressure (BP)** Half of the embryos were followed 18 monthes, SBP was measured each month by tail plethysmography (Softron,Japan). **Renal function** Serum urea nitrogen and creatinine were measured using the VetACE clinical chemistry system(Hitachi 7600, Japan).

In ouabain treated rats, the embryos' nephrons were increased compare to vehicle treated rats. We followed up the left embryos for 18 months, measuring the blood pressure, serum urea nitrogen and serum creatinine. We found that in ouabain treated group, the risk of hypertension and end stage renal disease is decreased compare to vehicle treated group.

Thus we have identified a novel medicine by which kidney development can be protected under IUGR.

PII-246

Effects of a Direct Fetal Amino Acid Infusion on Oxygenation and Acid-Base Balance in Sheep.

Anne M. Maliszewski, Monika Gadhia, Meghan O'Meara, Stephanie Thorn, William Hay, Paul J. Rozance, Laura D. Brown. *Pediatrics, University of Colorado, CO, USA.*

Maternal amino acid infusion in pregnant sheep results in competitive inhibition of amino acid transport across the placenta, increased fetal oxygen (O_2) consumption, fetal hypoxia and acidosis. A direct fetal amino acid infusion, bypassing competitive inhibition of amino acid transport across the placenta, will increase fetal O_2 consumption but preserve acid-base balance.

Late gestation singleton fetal sheep were intravenously infused with a complete amino acid mixture (AA, n=8) or saline (C, n=10) for an average of 12 days. The effects of treatment and day of infusion on fetal arterial plasma branched chain amino acid (BCAA) concentrations; pH; blood gasses; hematocrit; plasma lactate, glucose, and insulin concentrations, and glucose/O_2 quotient. On final day of infusion, umbilical blood flow, fetal O_2 consumption, and net rates of fetal glucose, lactate and amino acid uptakes from the placenta were measured.

Fetal [BCAA] were increased by 50% in AA vs. C (P<0.005). Glucose decreased in AA (22.6±1.5 baseline vs. 18.0±1.2 mg/dl final infusion day, P<0.0005). Fetal pH, pCO_2, hematocrit, hemoglobin-O_2 saturation, and blood O_2 content did not change. Fetal arterial blood pO_2 decreased in AA from baseline (18.9±0.7 mmHg) on days 5 (16.5±1.4 mmHg, P<0.05) and 8 (16.3±1.3 mmHg, P<0.05) then returned to baseline. Fetal arterial plasma lactate concentrations increased in the AA group initially (2.05±0.13 baseline vs 4.41±1.3 mmol/L on day 7, P<.005) then returned to baseline. The fetal glucose/O_2 quotient decreased in AA (P<0.05) but not C.

On final day of infusion, fetal arterial essential amino acid concentrations increased by 32%; concentrations of non-essential amino acids did not change.Net fetal uptake of most amino acids were not inhibited by amino acid infusion. Net fetal glucose uptake was lower in AA vs. C (2.52±0.36 vs 3.86±0.11 mg/kg/min, P<0.05). Umbilical blood flow, net fetal lactate uptake, and O_2 consumption rates did not change in AA.

Prolonged infusion of amino acids directly into fetal sheep increased essential fetal arterial amino acid concentrations but did not inhibit net amino acid uptake. Fetal O_2 consumption was not increased and acid-base balance was preserved. We speculate that decreased net fetal glucose uptake and fetal glucose/O_2 quotient in the AA group is due to increased fetal amino acid oxidation substituting for glucose oxidation.

PII-247

Impaired Insulin Receptor Tyrosine Phosphorylation in Pancreatic Islets from MSG-Obese Mice Is Improved by Early Swim Training.

Rosiane A. Miranda[1], Renato C.S. Branco[1], Luiz F. Barella[1], Ana E. Andreazzi[2], Júlio C. de Oliveira[1], Maria C. Picinato[3], Paulo C.F. Mathias[1]. *[1]Department of Cell Biology and Genetics, State University of Maringá, Paraná, Brazil; [2]Department of Biology, Federal University of Juiz de Fora, Minas Gerais, Brazil; [3]Department of Biologic Sciences, Foundation of Higher Teaching of Passos- State University of Minas Gerais, Minas gerais, Brazil.*

Obesity produced by hypothalamic injury, such as that obtained by neonatal treatment with monosodium L-glutamate (MSG), is characterized by hyperinsulinaemia without normal glucose uptake, which is due to severe tissue insulin resistance from dysfunction of the insulin receptor (IR) and its downstream signalling pathways. Insulin receptor substrate-1 (IRS-1) plays a key role in transmitting signals from the insulin receptor in several tissues including pancreatic beta cells. In both humans and experimental animals, deregulation of glucose homeostasis and insulin sensitivity/secretion were improved by exercise training. Nevertheless, the mechanisms underlying the changes in insulin signalling pathways are currently unknown.

Swim training of 90-day-old MSG-mice was used to evaluate whether signalling pathways of the IR and IRS-1 in islets are involved with the insulin resistance and glucose intolerance observed in this obese animal model.

IR tyrosine phosphorylation (pIR) was reduced by 42% in MSG-obese mice (MSG, 6.7±0.2 arbitrary units (au); control, 11.5±0.4au); on the other hand, exercise training increased pIR by 76% in MSG-mice without affecting control mice (MSG, 11.8±0.3; control, 12.8±0.2au). Although the treatment

traction so that it was indistinguishable from EC-denuded vessels, P>0.05). In aorta from males, EC-dependent depression of PE (max contraction in EC-containing rings was 1.0±0.3g vs. 2.0±0.2g in EC-denuded preparations; P<0.001) was also shown to be eNOS and sGC dependent (contraction in L-NAME and ODQ-treated tissue was 1.9±0.1g and 2.1±0.2g, respectively; neither statistically different from EC-denuded rings, P>0.05). However, in powerful contrast to females, inactivation of NO with hydroxocobalamin only partly impaired the influence of EC (1.6±0.2g; P<0.01 compared to EC-denuded rings) suggesting the presence of a non-NO mediator. Use of the established HNO scavenger L-cysteine (3mM) produced a partial inhibition of the vasodepressor influence of EC (1.5±0.1g; P<0.05 compared to EC-containing rings) but in combination with hydroxocobalamin produced an effect greater than when either was used independently (1.9±0.2g; indistinguishable from EC-denuded rings, P>0.05).
In conclusion, tonically active eNOS in aortic EC from male, but not female, rats generates both NO and HNO but the HNO contribution is lost when the EC are stimulated by ACh. These data identify a gender-specific role for HNO.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC176

Withdrawal of statin treatment leads to increased constriction in the mesenteric arteries of adult rats

P.H. Keskivali[1], N.P. Curzen[2], G.F. Clough[1] and C. Torrens[1]

[1]Human Development and Health, University of Southampton, Southampton, UK and [2]Wessex Cardiothoracic Unit, University of Southampton, Southampton, UK

HMG-CoA reductase inhibitors (statins) are widely used for secondary prevention of cardiovascular disease and increasing evidence indicates benefits from primary prevention in risk groups. Chronic statin treatment from weaning has been shown to attenuate endothelial dysfunction in adult rats in a model of cardiovascular disease (Torrens et al. 2009) and evidence suggests that early targeted interventions can have long term benefits in this same model (Sherman and Langley-Evans 2000;Vickers et al. 2005). It was hypothesised that early age acute statin treatment would also improve the cardiovascular function in later life in a rat model of developmentally induced cardiovascular disease.
Pregnant Wistar rat dams were fed either 18% protein, control (C) or 9% protein restricted (PR) diet throughout pregnancy and were returned to standard chow on delivery. The pups were divided into controls and those receiving atorvastatin, 10mg/kg, (S) for two weeks from weaning at 3 weeks. This gave four experimental groups: control (C), control + statin (CS), protein restricted (PR) and protein restricted + statin (PRS). At 16 weeks blood pressure was assessed with non-invasive tail-cuff plethysmography and vascular reactivity of the mesenteric arteries (MA) was measured *ex vivo* by wire myography. The differences were assessed with one-way ANOVA and results are expressed as mean±SEM. Significance was assumed at p≤0.05.
Increased constriction to PE was observed in PRS groups in both males (C, 4.12±0.16 mN/mm, n=6; CS, 4.57±0.16 mN/mm, n=6; PR, 4.66±0.29 mN/mm, n=5; PRS, 5.27±0.21 mN/mm, n=5; p<0.01) and females (C, 3.22±0.26 mN/mm, n=5; CS, 4.17±0.23 mN/mm, n=6; PR, 3.82±0.40 mN/mm, n=5; PRS, 4.71±0.24 mN/mm, n=6; p<0.01). In female MA increased constriction to endothelin was also shown in the PRS group (C, 3.27±0.56 mN/mm, n=4; CS, 4.43±0.36 mN/mm, n=5; PR, 3.44±0.62 mN/mm, n=4; PRS, 6.20±0.89 mN/mm, n=5; p<0.01). No differences were seen in systolic or diastolic blood pressure or heart rate between the groups.
These data show increased constriction in the statin treated PR offspring in adulthood. As data during statin treatment at 5 weeks indicated reduced constriction, the detrimental effects are attributed to statin withdrawal and subsequent rebound. The PR offspring appear to be more susceptible to this as no increase in constriction was observed in the control MA. Investigation into the mechanisms is ongoing.

Sherman, R.C. & Langley-Evans, S.C. 2000. Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat. Clin.Sci.(Lond), 98, (3) 269-275 available from: PM:10677384

Torrens, C., Kelsall, C.J., Hopkins, L.A., Anthony, F.W., Curzen, N.P., & Hanson, M.A. 2009. Atorvastatin restores endothelial function in offspring of protein-restricted rats in a cholesterol-independent manner. Hypertension, 53, (4) 661-667

Vickers, M.H., Gluckman, P.D., Coveny, A.H., Hofman, P.L., Cutfield, W.S., Gertler, A., Breier, B.H., & Harris, M. 2005. Neonatal leptin treatment reverses developmental programming. Endocrinology, 146, (10) 4211-4216 available from: PM:16020474

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC177

Modulation of aortic reactivity to angiotensin-(1-7) with intermittent hyperbaric oxygenation in diabetic rats

A. Kibel[1], A. Cavka[1], S. Novak[1], M. Mihalj[1], Z. Mihaljevic[1], A. Cosic[1], J.R. Falck[2] and I. Drenjancevic[1]

[1]Department of Physiology and Immunology, Faculty of Medicine Osijek, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia and [2]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA

INTRODUCTION: We previously reported a facilitating effect of hyperbaric oxygen therapy (HBOT) on responses of rat aortic rings to angiotensin-(1-7) (ANG-(1-7)) in healthy rats ('Physiology 2011')[1]. It was suggested that this influence was mediated by changes in production or sensitivity to epoxyeicosatrienoic acids (EETs). The aim was to assess whether HBOT exerts similar effects in diabetic rats and to further explore the role of cytochrome P450 (CYP) enzymes in changes induced by HBOT.
METHODS: Diabetes was induced by streptozocin in 6 weeks old male Sprague-Dawley rats. After 8 weeks, they were divided into control and HBOT group (exposed to 100% O2(2 bars) 2 hours/day for 4 consecutive days). Before decapitation, rats were anesthetized with 75 mg/kg ketamine with 2.5 mg/kg midazolam. Thoracic aortic rings were used to test responses to 1 μM ANG-(1-7) after precontraction with noradrenaline for 5 minutes (tension was read 3 minutes after ANG-(1-7) addition and the response expressed as percentage of precontraction decrease). Intactness of endothelium was previously tested. A separate series of HBOT rats was used to test ANG-(1-7) responses after the epoxygenases inhibitor MS-PPOH (10 μM) – inhibitor of the CYP4A2 AND CYP4A3 epoxygenation reactions – was added for 15 minutes to the tissue bath. CYP 4A1, 4A2, 4A3 and 2J3 mRNA expression was determined with Quantitative real time PCR and was normalized to the expres-

synchronous in the whole cortex, are readily recordable with relatively high amplitude with any electrode pair inside the head or on the surface of the head, including lower face.

F09

Withdrawal of statin treatment leads to increased constriction in the mesenteric arteries of adult rats

KESKIVÄLI PHM, CURZEN NP, CLOUGH GF, TORRENS C
Human Development and Health, IDS Building, Tremona Road, Southampton General Hospital, SO166YD Southampton, UK

HMG-CoA reductase inhibitors (statins) are used in primary and secondary prevention of cardiovascular disease (CVD). In a rat model of maternally-induced CVD, chronic statin treatment restored endothelial function in adult rats. In a similar model, early targeted, acute interventions have long term benefits on cardiovascular function so it was hypothesised that early age acute statin treatment would improve cardiovascular function in later life in this model. Wistar rat dams were fed 18% protein (control, C) or 9% protein restricted (PR) diet throughout pregnancy and returned to standard chow on delivery. At weaning (3 weeks) pups were divided into a statin group (S) receiving atorvastatin (10mg/kg; Pfizer, UK) for 2 weeks or controls. This gave four groups: control (C), control + statin (CS), protein restricted (PR) and protein restricted + statin (PRS). At 16 weeks vascular reactivity of mesenteric arteries (MA) was measured by wire myography. Results are expressed as mean ± SEM and differences were assessed by one-way ANOVA. Significance was assumed at p0.05. Enhanced constriction to phenylephrine was observed in PRS groups in both males (mN/mm: C, 4.120.16, n=6; CS, 4.570.16, n=6; PR, 4.660.29, n=5; PRS, 5.270.21, n=5; $p<0.01$) and females (mN/mm: C, 3.220.26, n=5; CS, 4.170.23, n=6; PR, 3.820.40, n=5; PRS, 4.710.24, n=6; $p<0.01$). In female MA increased constriction to endothelin was also shown in the PRS group (mN/mm: C, 3.270.56, n=4; CS, 4.430.36, n=5; PR, 3.440.62, n=4; PRS, 6.200.89, n=5; $p<0.01$). These data show enhanced constriction in PR offspring, but not in controls, following statin withdrawal. The PR offspring appear to be more susceptible to statin withdrawal and subsequent rebound than controls.

F10

The Na^+,HCO_3^- cotransporter NBCn1 is upregulated in human breast tumors and by ErbB2: Mechanisms and implications

PEDERSEN SF, BOEDTKJER E, GORBATENKO A, LAURITZEN G, OLESEN CW, MOREIRA J, AALKJAER C
Department of Biology, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark

Cancer cells in solid tumors exhibit greatly increased dependence on acid extrusion compared to normal cells, making acid extruding transporters potential treatment targets. We previously demonstrated strong upregulation of both acid extrusion and Na^+,HCO_3^- cotransporter NBCn1 (SLC4A7) expression in MCF-7 human breast cancer cells by a constitutively active, truncated ErbB2/HER2 receptor, ΔNErbB2[Lauritzen et al. 2010]. Here, we address the mechanisms of NBCn1 regulation by ErbB receptors, and the *in vivo* relevance of NBCn1 in human breast cancer. NBCn1 upregulation in ΔNErbB2-expressing MCF-7 cells was dependent on ERK and Src, and PI3K-Akt regulated NBCn1 expression in both ΔNErbB2- and vector cells. NBCn1 expression was also increased by stimulation of full-length ErbB1 or ErbB3-4. Promoter deletions and luciferase reporter assays identified a ΔNErbB2 responsive region upstream of SLC4A7 and several relevant transcription factor (TF) binding sites. Of these TFs, Sp1 and Sp3 activity was increased by ΔNErbB2, and overexpression of their dominant-negative forms decreased NBCn1 expression. Using matched sets of patient tissue, we showed that NBCn1 is upregulated in primary breast carcinomas

List of References

(1998). Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med* **339**, 1349-1357.

Aatola H, Hutri-Kahonen N, Juonala M, Viikari JS, Hulkkonen J, Laitinen T, Taittonen L, Lehtimaki T, Raitakari OT, & Kahonen M (2010). Lifetime risk factors and arterial pulse wave velocity in adulthood: the cardiovascular risk in young Finns study. *Hypertension* **55**, 806-811.

Ali F, Hamdulay SS, Kinderlerer AR, Boyle JJ, Lidington EA, Yamaguchi T, Soares MP, Haskard DO, Randi AM, & Mason JC (2007). Statin-mediated cytoprotection of human vascular endothelial cells: a role for Kruppel-like factor 2-dependent induction of heme oxygenase-1. *J Thromb Haemost* **5**, 2537-2546.

Ali F, Zakkar M, Karu K, Lidington EA, Hamdulay SS, Boyle JJ, Zloh M, Bauer A, Haskard DO, Evans PC, & Mason JC (2009). Induction of the cytoprotective enzyme heme oxygenase-1 by statins is enhanced in vascular endothelium exposed to laminar shear stress and impaired by disturbed flow. *J Biol Chem* **284**, 18882-18892.

Alvarez de SM, Herrera MD, Marhuenda E, & Andriantsitohaina R (2000). Characterization of endothelial factors involved in the vasodilatory effect of simvastatin in aorta and small mesenteric artery of the rat. *Br J Pharmacol* **131**, 1179-1187.

Amery A, Birkenhager W, Brixko P, Bulpitt C, Clement D, Deruyttere M, De SA, Dollery C, Fagard R, Forette F, & . (1985). Mortality and morbidity results from the European Working Party on High Blood Pressure in the Elderly trial. *Lancet* **1**, 1349-1354.

Andersen CL, Jensen JL, & Orntoft TF (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* **64**, 5245-5250.

Antoniades C, Bakogiannis C, Leeson P, Guzik TJ, Zhang MH, Tousoulis D, Antonopoulos AS, Demosthenous M, Marinou K, Hale A, Paschalis A, Psarros C, Triantafyllou C, Bendall J, Casadei B, Stefanadis C, & Channon KM (2011). Rapid, direct effects of statin treatment on arterial redox state and nitric oxide bioavailability in human atherosclerosis via tetrahydrobiopterin-mediated endothelial nitric oxide synthase coupling. *Circulation* **124**, 335-345.

List of references

Antoniades C, Bakogiannis C, Tousoulis D, Reilly S, Zhang MH, Paschalis A, Antonopoulos AS, Demosthenous M, Miliou A, Psarros C, Marinou K, Sfyras N, Economopoulos G, Casadei B, Channon KM, & Stefanadis C (2010). Preoperative atorvastatin treatment in CABG patients rapidly improves vein graft redox state by inhibition of Rac1 and NADPH-oxidase activity. *Circulation* **122**, S66-S73.

Aoki C, Nakano A, Tanaka S, Yanagi K, Ohta S, Jojima T, Kasai K, Takekawa H, Hirata K, & Hattori Y (2012). Fluvastatin upregulates endothelial nitric oxide synthase activity via enhancement of its phosphorylation and expression and via an increase in tetrahydrobiopterin in vascular endothelial cells. *Int J Cardiol* **156**, 55-61.

Arebalo RE, Hardgrave JE, & Scallen TJ (1981). The in vivo regulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. Phosphorylation of the enzyme as an early regulatory response following cholesterol feeding. *J Biol Chem* **256**, 571-574.

Arnal JF, Dinh-Xuan AT, Pueyo M, Darblade B, & Rami J (1999). Endothelium-derived nitric oxide and vascular physiology and pathology. *Cell Mol Life Sci* **55**, 1078-1087.

Auwerx JH, Chait A, & Deeb SS (1989). Regulation of the low density lipoprotein receptor and hydroxymethylglutaryl coenzyme A reductase genes by protein kinase C and a putative negative regulatory protein. *Proc Natl Acad Sci U S A* **86**, 1133-1137.

Bagot RC, Zhang TY, Wen X, Nguyen TT, Nguyen HB, Diorio J, Wong TP, & Meaney MJ (2012). Variations in postnatal maternal care and the epigenetic regulation of metabotropic glutamate receptor 1 expression and hippocampal function in the rat. *Proc Natl Acad Sci U S A* **109 Suppl 2**, 17200-17207.

Banerjee A, Trivedi CM, Damera G, Jiang M, Jester W, Hoshi T, Epstein JA, & Panettieri RA, Jr. (2012). Trichostatin A abrogates airway constriction, but not inflammation, in murine and human asthma models. *Am J Respir Cell Mol Biol* **46**, 132-138.

Bansal N, Fischbacher CM, Bhopal RS, Brown H, Steiner MF, & Capewell S (2013). Myocardial infarction incidence and survival by ethnic group: Scottish Health and Ethnicity Linkage retrospective cohort study. *BMJ Open* **3**, e003415.

Bao XM, Wu CF, & Lu GP (2009). Atorvastatin attenuates homocysteine-induced apoptosis in human umbilical vein endothelial cells via inhibiting NADPH oxidase-related oxidative stress-triggered p38MAPK signaling. *Acta Pharmacol Sin* **30**, 1392-1398.

Barker DJ, Osmond C, Golding J, Kuh D, & Wadsworth ME (1989a). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* **298**, 564-567.

Barker DJ, Osmond C, & Law CM (1989b). The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. *J Epidemiol Community Health* **43**, 237-240.

Beckman JA, Liao JK, Hurley S, Garrett LA, Chui D, Mitra D, & Creager MA (2004). Atorvastatin restores endothelial function in normocholesterolemic smokers independent of changes in low-density lipoprotein. *Circ Res* **95**, 217-223.

Beg ZH, Stonik JA, & Brewer HB, Jr. (1978). 3-Hydroxy-3-methylglutaryl coenzyme A reductase: regulation of enzymatic activity by phosphorylation and dephosphorylation. *Proc Natl Acad Sci U S A* **75**, 3678-3682.

Bellien J, Favre J, Iacob M, Gao J, Thuillez C, Richard V, & Joannides R (2010). Arterial stiffness is regulated by nitric oxide and endothelium-derived hyperpolarizing factor during changes in blood flow in humans. *Hypertension* **55**, 674-680.

Berenson GS, Srinivasan SR, Bao W, Newman WP, III, Tracy RE, & Wattigney WA (1998). Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. *N Engl J Med* **338**, 1650-1656.

Bhargava SK, Sachdev HS, Fall CH, Osmond C, Lakshmy R, Barker DJ, Biswas SK, Ramji S, Prabhakaran D, & Reddy KS (2004). Relation of serial changes in childhood body-mass index to impaired glucose tolerance in young adulthood. *N Engl J Med* **350**, 865-875.

Blankstein R, Budoff MJ, Shaw LJ, Goff DC, Jr., Polak JF, Lima J, Blumenthal RS, & Nasir K (2011). Predictors of coronary heart disease events among asymptomatic persons with low low-density lipoprotein cholesterol MESA (Multi-Ethnic Study of Atherosclerosis). *J Am Coll Cardiol* **58**, 364-374.

Bot I, Jukema JW, Lankhuizen IM, van Berkel TJ, & Biessen EA (2010). Atorvastatin inhibits plaque development and adventitial neovascularization in ApoE deficient mice independent of plasma cholesterol levels. *Atherosclerosis*.

Brandes RP (2010). Vascular functions of NADPH oxidases. *Hypertension* **56**, 17-21.

Brawley L, Itoh S, Torrens C, Barker A, Bertram C, Poston L, & Hanson M (2003). Dietary protein restriction in pregnancy induces hypertension and vascular defects in rat male offspring. *Pediatr Res* **54**, 83-90.

List of references

Brennan KA, Kaufman S, Reynolds SW, McCook BT, Kan G, Christiaens I, Symonds ME, & Olson DM (2008). Differential effects of maternal nutrient restriction through pregnancy on kidney development and later blood pressure control in the resulting offspring. *Am J Physiol Regul Integr Comp Physiol* **295**, R197-R205.

Briones AM, Rodriguez-Criado N, Hernanz R, Garcia-Redondo AB, Rodrigues-Diez RR, Alonso MJ, Egido J, Ruiz-Ortega M, & Salaices M (2009). Atorvastatin prevents angiotensin II-induced vascular remodeling and oxidative stress. *Hypertension* **54**, 142-149.

Brookes ZL, McGown CC, & Reilly CS (2009). Statins for all: the new premed? *Br J Anaesth* **103**, 99-107.

Brown AA & Hu FB (2001). Dietary modulation of endothelial function: implications for cardiovascular disease. *American Journal of Clinical Nutrition* **73**, 673-686.

Bulbulia R, Bowman L, Wallendszus K, Parish S, Armitage J, Peto R, & Collins R (2011). Effects on 11-year mortality and morbidity of lowering LDL cholesterol with simvastatin for about 5 years in 20,536 high-risk individuals: a randomised controlled trial. *Lancet* **378**, 2013-2020.

Burdge GC, Slater-Jefferies J, Torrens C, Phillips ES, Hanson MA, & Lillycrop KA (2007). Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. *Br J Nutr* **97**, 435-439.

Caballero AE (2005). Metabolic and vascular abnormalities in subjects at risk for type 2 diabetes: the early start of a dangerous situation. *Archives of Medical Research* **36**, 241-249.

Campelo AE, Cutini PH, & Massheimer VL (2012). Testosterone modulates platelet aggregation and endothelial cell growth through nitric oxide pathway. *J Endocrinol* **213**, 77-87.

Celermajer DS, Sorensen KE, Bull C, Robinson J, & Deanfield JE (1994). Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol* **24**, 1468-1474.

Chamiot-Clerc P, Renaud JF, & Safar ME (2001). Pulse pressure, aortic reactivity, and endothelium dysfunction in old hypertensive rats. *Hypertension* **37**, 313-321.

Chasman DI, Posada D, Subrahmanyan L, Cook NR, Stanton VP, Jr., & Ridker PM (2004). Pharmacogenetic study of statin therapy and cholesterol reduction. *JAMA* **291**, 2821-2827.

Chen H, Ren JY, Xing Y, Zhang WL, Liu X, Wu P, Wang RJ, & Luo Y (2009). Short-term withdrawal of simvastatin induces endothelial dysfunction in patients with coronary artery disease: a dose-response effect dependent on endothelial nitric oxide synthase. *Int J Cardiol* **131**, 313-320.

Chen J, Xu H, Shen Q, Guo W, & Sun L (2010). Effect of postnatal high-protein diet on kidney function of rats exposed to intrauterine protein restriction. *Pediatr Res* **68**, 100-104.

Chen Y, Ruan XZ, Li Q, Huang A, Moorhead JF, Powis SH, & Varghese Z (2007). Inflammatory cytokines disrupt LDL-receptor feedback regulation and cause statin resistance: a comparative study in human hepatic cells and mesangial cells. *Am J Physiol Renal Physiol* **293**, F680-F687.

Cheng G, Shan J, Xu G, Huang J, Ma J, Ying S, & Zhu L (2003). Apoptosis induced by simvastatin in rat vascular smooth muscle cell through Ca2+-calpain and caspase-3 dependent pathway. *Pharmacol Res* **48**, 571-578.

Chong PH, Seeger JD, & Franklin C (2001). Clinically relevant differences between the statins: implications for therapeutic selection. *Am J Med* **111**, 390-400.

Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM, & Deanfield JE (1997). Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. *Circulation* **96**, 3378-3383.

Codoner-Franch P, Tavarez-Alonso S, Murria-Estal R, Megias-Vericat J, Tortajada-Girbes M, & Alonso-Iglesias E (2011). Nitric oxide production is increased in severely obese children and related to markers of oxidative stress and inflammation. *Atherosclerosis* **215**, 475-480.

Criqui MH, Cowan LD, Tyroler HA, Bangdiwala S, Heiss G, Wallace RB, & Cohn R (1987). Lipoproteins as mediators for the effects of alcohol consumption and cigarette smoking on cardiovascular mortality: results form the Lipid Research Clinics Follow-up Study. *Am J Epidemiol* **126**, 629-637.

Czernichow S, Ninomiya T, Huxley R, Kengne AP, Batty GD, Grobbee DE, Woodward M, Neal B, & Chalmers J (2010). Impact of blood pressure lowering on cardiovascular outcomes in normal weight, overweight, and obese individuals: the Perindopril Protection Against Recurrent Stroke Study trial. *Hypertension* **55**, 1193-1198.

List of references

da Silva FT, da Fonte RC, & Sampaio FJ (2004). Puberty onset in the female offspring of rats submitted to protein or energy restricted diet during lactation. *J Nutr Biochem* **15**, 123-127.

Daskalopoulou SS, Delaney JA, Filion KB, Brophy JM, Mayo NE, & Suissa S (2008). Discontinuation of statin therapy following an acute myocardial infarction: a population-based study. *Eur Heart J* **29**, 2083-2091.

Davies MJ (2000). The pathophysiology of acute coronary syndromes. [Review] [20 refs]. *Heart* **83**, 361-366.

de Jonge HW, Dekkers DH, & Lamers JM (1996). Polyunsaturated fatty acids and signalling via phospholipase C-beta and A2 in myocardium. *Mol Cell Biochem* **157**, 199-210.

Dengo AL, Dennis EA, Orr JS, Marinik EL, Ehrlich E, Davy BM, & Davy KP (2010). Arterial destiffening with weight loss in overweight and obese middle-aged and older adults. *Hypertension* **55**, 855-861.

Desai M, Byrne CD, Meeran K, Martenz ND, Bloom SR, & Hales CN (1997). Regulation of hepatic enzymes and insulin levels in offspring of rat dams fed a reduced-protein diet. *Am J Physiol* **273**, G899-G904.

Dichtl W, Dulak J, Frick M, Alber HF, Schwarzacher SP, Ares MP, Nilsson J, Pachinger O, & Weidinger F (2003). HMG-CoA reductase inhibitors regulate inflammatory transcription factors in human endothelial and vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **23**, 58-63.

Dietschy JM, Turley SD, & Spady DK (1993). Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res* **34**, 1637-1659.

Dje NP, Riediger F, Vardarova K, Scharf S, Eitel J, Opitz B, Slevogt H, Weichert W, Hocke AC, Schmeck B, Suttorp N, & Hippenstiel S (2009). Statins control oxidized LDL-mediated histone modifications and gene expression in cultured human endothelial cells. *Arterioscler Thromb Vasc Biol* **29**, 380-386.

Dowlatshahi D, Demchuk AM, Fang J, Kapral MK, Sharma M, & Smith EE (2012). Association of statins and statin discontinuation with poor outcome and survival after intracerebral hemorrhage. *Stroke* **43**, 1518-1523.

Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, Langendorfer A, Stein EA, Kruyer W, & Gotto AM, Jr. (1998). Primary prevention of acute coronary

events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *JAMA* **279**, 1615-1622.

Dresner LS, Wang SP, West MW, Ponomarenko IN, Mueller CM, & Wait RB (1997). Nitric oxide inhibition simulates the enhancement of alpha 1 agonist-induced vasoconstriction in diabetes. *J Surg Res* **70**, 119-123.

Duran WN, Breslin JW, & Sanchez FA (2010). The NO cascade, eNOS location, and microvascular permeability. *Cardiovasc Res* **87**, 254-261.

Earley S & Nelson MT (2006). Central role of Ca2+-dependent regulation of vascular tone in vivo. *J Appl Physiol* **101**, 10-11.

Elahi MM, Cagampang FR, Anthony FW, Curzen N, Ohri SK, & Hanson MA (2008). Statin treatment in hypercholesterolemic pregnant mice reduces cardiovascular risk factors in their offspring. *Hypertension* **51**, 939-944.

Endo A (1992). The discovery and development of HMG-CoA reductase inhibitors. *J Lipid Res* **33**, 1569-1582.

Engelbregt MJ, Houdijk ME, Popp-Snijders C, & Delemarre-van de Waal HA (2000). The effects of intra-uterine growth retardation and postnatal undernutrition on onset of puberty in male and female rats. *Pediatr Res* **48**, 803-807.

Engelbregt MJ, van Weissenbruch MM, Popp-Snijders C, & Delemarre-van de Waal HA (2002). Delayed first cycle in intrauterine growth-retarded and postnatally undernourished female rats: follicular growth and ovulation after stimulation with pregnant mare serum gonadotropin at first cycle. *J Endocrinol* **173**, 297-304.

Eriksson J, Forsen T, Tuomilehto J, Osmond C, & Barker D (2000). Fetal and childhood growth and hypertension in adult life. *Hypertension* **36**, 790-794.

Eriksson JG, Forsen T, Tuomilehto J, Osmond C, & Barker DJ (2001). Early growth and coronary heart disease in later life: longitudinal study. *BMJ* **322**, 949-953.

Feletou M, Kohler R, & Vanhoutte PM (2012). Nitric oxide: orchestrator of endothelium-dependent responses. *Ann Med* **44**, 694-716.

Feletou M & Vanhoutte PM (2009). EDHF: an update. *Clin Sci (Lond)* **117**, 139-155.

List of references

Feng J, Damrauer SM, Lee M, Sellke FW, Ferran C, & Abid MR (2010). Endothelium-dependent coronary vasodilatation requires NADPH oxidase-derived reactive oxygen species. *Arterioscler Thromb Vasc Biol* **30**, 1703-1710.

Feron O, Dessy C, Desager JP, & Balligand JL (2001). Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. *Circulation* **103**, 113-118.

Fichtlscherer S, Schmidt-Lucke C, Bojunga S, Rossig L, Heeschen C, Dimmeler S, & Zeiher AM (2006). Differential effects of short-term lipid lowering with ezetimibe and statins on endothelial function in patients with CAD: clinical evidence for 'pleiotropic' functions of statin therapy. *Eur Heart J* **27**, 1182-1190.

Ford I, Murray H, Packard CJ, Shepherd J, Macfarlane PW, Cobbe SM, & West of Scotland Coronary Prevention Study Group (2007). Long-term follow-up of the West of Scotland Coronary Prevention Study. *New Eng J Med* **357**, 1477-1486.

Forstermann U (2010). Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch* **459**, 923-939.

Furberg CD, Adams HP, Jr., Applegate WB, Byington RP, Espeland MA, Hartwell T, Hunninghake DB, Lefkowitz DS, Probstfield J, Riley WA, & . (1994). Effect of lovastatin on early carotid atherosclerosis and cardiovascular events. Asymptomatic Carotid Artery Progression Study (ACAPS) Research Group. *Circulation* **90**, 1679-1687.

Furchgott RF & Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376.

Gao L & Mann GE (2009). Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling. *Cardiovasc Res* **82**, 9-20.

Gertz K, Laufs U, Lindauer U, Nickenig G, Bohm M, Dirnagl U, & Endres M (2003). Withdrawal of statin treatment abrogates stroke protection in mice. *Stroke* **34**, 551-557.

Ghaffari N, Ball C, Kennedy JA, Stafford I, & Beltrame JF (2011). Acute modulation of vasoconstrictor responses by pravastatin in small vessels. *Circ J* **75**, 1506-1514.

Giannotti G, Doerries C, Mocharla PS, Mueller MF, Bahlmann FH, Horvath T, Jiang H, Sorrentino SA, Steenken N, Manes C, Marzilli M, Rudolph KL, Luscher TF, Drexler H, & Landmesser U (2010). Impaired endothelial repair capacity of early endothelial progenitor cells in prehypertension: relation to endothelial dysfunction. *Hypertension* **55**, 1389-1397.

Goedeke L & Fernandez-Hernando C (2012). Regulation of cholesterol homeostasis. *Cell Mol Life Sci* **69**, 915-930.

Goldstein JL & Brown MS (1990). Regulation of the mevalonate pathway. *Nature* **343**, 425-430.

Gounari P, Tousoulis D, Antoniades C, Kampoli AM, Stougiannos P, Papageorgiou N, Roulia G, Stefanadi E, Siasos G, Tsioufis C, & Stefanadis C (2010). Rosuvastatin but not ezetimibe improves endothelial function in patients with heart failure, by mechanisms independent of lipid lowering. *Int J Cardiol* **142**, 87-91.

Guan H, Arany E, van Beek JP, Chamson-Reig A, Thyssen S, Hill DJ, & Yang K (2005). Adipose tissue gene expression profiling reveals distinct molecular pathways that define visceral adiposity in offspring of maternal protein-restricted rats. *Am J Physiol Endocrinol Metab* **288**, E663-E673.

Gurgun C, Ildizli M, Yavuzgil O, Sin A, Apaydin A, Cinar C, & Kultursay H (2008). The effects of short term statin treatment on left ventricular function and inflammatory markers in patients with chronic heart failure. *Int J Cardiol* **123**, 102-107.

Hales CN & Barker DJ (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* **35**, 595-601.

Hamelin BA & Turgeon J (1998). Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors. *Trends Pharmacol Sci* **19**, 26-37.

Hanson M & Gluckman P (2005). Endothelial dysfunction and cardiovascular disease: the role of predictive adaptive responses. *Heart* **91**, 864-866.

Hanson M & Gluckman P (2011). Developmental origins of noncommunicable disease: population and public health implications. *Am J Clin Nutr* **94**, 1754S-1758S.

Hashiba Y, Tosaka M, Saito N, Imai H, Shimizu T, & Sasaki T (2007). Vasorelaxing effect of the Rho-kinase inhibitor, Y-27632, in isolated canine basilar arteries. *Neurol Res* **29**, 485-489.

Heeschen C, Hamm CW, Laufs U, Snapinn S, Bohm M, & White HD (2002). Withdrawal of statins increases event rates in patients with acute coronary syndromes. *Circulation* **105**, 1446-1452.

Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, & Lumey LH (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* **105**, 17046-17049.

Hentosh P, Yuh SH, Elson CE, & Peffley DM (2001). Sterol-independent regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in tumor cells. *Mol Carcinog* **32**, 154-166.

Hernandez-Perera O, Perez-Sala D, Navarro-Antolin J, Sanchez-Pascuala R, Hernandez G, Diaz C, & Lamas S (1998). Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J Clin Invest* **101**, 2711-2719.

Hisada T, Ayaori M, Ohrui N, Nakashima H, Nakaya K, Uto-Kondo H, Yakushiji E, Takiguchi S, Terao Y, Miyamoto Y, Adachi T, Nakamura H, Ohsuzu F, Ikewaki K, & Sakurai Y (2012). Statin inhibits hypoxia-induced endothelin-1 via accelerated degradation of HIF-1alpha in vascular smooth muscle cells. *Cardiovasc Res* **95**, 251-259.

Holemans K, Gerber R, Meurrens K, De CF, Poston L, & Van Assche FA (1999). Maternal food restriction in the second half of pregnancy affects vascular function but not blood pressure of rat female offspring. *Br J Nutr* **81**, 73-79.

Hsia J, MacFadyen JG, Monyak J, & Ridker PM (2011). Cardiovascular event reduction and adverse events among subjects attaining low-density lipoprotein cholesterol <50 mg/dl with rosuvastatin. The JUPITER trial (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin). *J Am Coll Cardiol* **57**, 1666-1675.

Huang RC, Mori TA, Burke V, Newnham J, Stanley FJ, Landau LI, Kendall GE, Oddy WH, & Beilin LJ (2009). Synergy between adiposity, insulin resistance, metabolic risk factors, and inflammation in adolescents. *Diabetes Care* **32**, 695-701.

Huxley R, Neil A, & Collins R (2002). Unravelling the fetal origins hypothesis: is there really an inverse association between birthweight and subsequent blood pressure? *Lancet* **360**, 659-665.

Huxley R, Owen CG, Whincup PH, Cook DG, Rich-Edwards J, Smith GD, & Collins R (2007). Is birth weight a risk factor for ischemic heart disease in later life? *Am J Clin Nutr* **85**, 1244-1250.

Ishikawa S, Nagai Y, Masuda T, Koga Y, Nakamura T, Imamura Y, Takamori H, Hirota M, Funakosi A, Fukushima M, & Baba H (2010). The role of oxysterol binding protein-related protein 5 in pancreatic cancer. *Cancer Sci* **101**, 898-905.

Istvan E (2003). Statin inhibition of HMG-CoA reductase: a 3-dimensional view. *Atheroscler Suppl* **4**, 3-8.

Iwado Y, Yoshinaga K, Furuyama H, Ito Y, Noriyasu K, Katoh C, Kuge Y, Tsukamoto E, & Tamaki N (2002). Decreased endothelium-dependent coronary vasomotion in healthy young smokers. *Eur J Nucl Med Mol Imaging* **29**, 984-990.

Jackson AA, Dunn RL, Marchand MC, & Langley-Evans SC (2002). Increased systolic blood pressure in rats induced by a maternal low-protein diet is reversed by dietary supplementation with glycine. *Clin Sci (Lond)* **103**, 633-639.

Kajantie E, Osmond C, Barker DJ, Forsen T, Phillips DI, & Eriksson JG (2005). Size at birth as a predictor of mortality in adulthood: a follow-up of 350 000 person-years. *Int J Epidemiol* **34**, 655-663.

Kansui Y, Fujii K, Goto K, Abe I, & Iida M (2004). Effects of fluvastatin on endothelium-derived hyperpolarizing factor- and nitric oxide-mediated relaxations in arteries of hypertensive rats. *Clin Exp Pharmacol Physiol* **31**, 354-359.

Kassan M, Montero MJ, & Sevilla MA (2009). Chronic treatment with pravastatin prevents early cardiovascular changes in spontaneously hypertensive rats. *Br J Pharmacol* **158**, 541-547.

Kauffenstein G, Laher I, Matrougui K, Guerineau NC, & Henrion D (2012). Emerging role of G protein-coupled receptors in microvascular myogenic tone. *Cardiovasc Res* **95**, 223-232.

Kelsall CJ, Hoile SP, Irvine NA, Masoodi M, Torrens C, Lillycrop KA, Calder PC, Clough GF, Hanson MA, & Burdge GC (2012). Vascular dysfunction induced in offspring by maternal dietary fat involves altered arterial polyunsaturated fatty acid biosynthesis. *PLoS One* **7**, e34492.

Khalil RA (2010). *Regulation of Vascular Smooth Muscle Function*.

Khan I, Dekou V, Hanson M, Poston L, & Taylor P (2004). Predictive adaptive responses to maternal high-fat diet prevent endothelial dysfunction but not hypertension in adult rat offspring. *Circulation* **110**, 1097-1102.

List of references

Khan IY, Dekou V, Douglas G, Jensen R, Hanson MA, Poston L, & Taylor PD (2005). A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *Am J Physiol Regul Integr Comp Physiol* **288**, R127-R133.

Khan SM, Hamnvik OP, Brinkoetter M, & Mantzoros CS (2012). Leptin as a modulator of neuroendocrine function in humans. *Yonsei Med J* **53**, 671-679.

Kim YB, Yu J, Lee SY, Lee MS, Ko SG, Ye SK, Jong HS, Kim TY, Bang YJ, & Lee JW (2005). Cell adhesion status-dependent histone acetylation is regulated through intracellular contractility-related signaling activities. *J Biol Chem* **280**, 28357-28364.

Kinlay S (2005). Potential vascular benefits of statins. *Am J Med* **118 Suppl 12A**, 62-67.

Klag MJ, Ford DE, Mead LA, He J, Whelton PK, Liang KY, & Levine DM (1993). Serum cholesterol in young men and subsequent cardiovascular disease. *N Engl J Med* **328**, 313-318.

Kodach LL, Jacobs RJ, Voorneveld PW, Wildenberg ME, Verspaget HW, van WT, Morreau H, Hommes DW, Peppelenbosch MP, van den Brink GR, & Hardwick JC (2011). Statins augment the chemosensitivity of colorectal cancer cells inducing epigenetic reprogramming and reducing colorectal cancer cell 'stemness' via the bone morphogenetic protein pathway. *Gut* **60**, 1544-1553.

Kondo T, Osugi S, Shimokata K, Honjo H, Morita Y, Maeda K, Yamashita K, Muramatsu T, Shintani S, Matsushita K, & Murohara T (2011). Smoking and smoking cessation in relation to all-cause mortality and cardiovascular events in 25,464 healthy male Japanese workers. *Circ J* **75**, 2885-2892.

Kubes P, Suzuki M, & Granger DN (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* **88**, 4651-4655.

Kusche-Vihrog K, Urbanova K, Blanque A, Wilhelmi M, Schillers H, Kliche K, Pavenstadt H, Brand E, & Oberleithner H (2011). C-reactive protein makes human endothelium stiff and tight. *Hypertension* **57**, 231-237.

Kuzuya M, Cheng XW, Sasaki T, Tamaya-Mori N, & Iguchi A (2004). Pitavastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, blocks vascular smooth muscle cell populated-collagen lattice contraction. *J Cardiovasc Pharmacol* **43**, 808-814.

Kwee RM, van Oostenbrugge RJ, Prins MH, Ter Berg JW, Franke CL, Korten AG, Meems BJ, van Engelshoven JM, Wildberger JE, Mess WH, & Kooi ME (2010).

Symptomatic patients with mild and moderate carotid stenosis: plaque features at MRI and association with cardiovascular risk factors and statin use. *Stroke* **41**, 1389-1393.

Lai WT, Lee KT, Chu CS, Voon WC, Yen HW, Tsai LY, & Sheu SH (2005). Influence of withdrawal of statin treatment on proinflammatory response and fibrinolytic activity in humans: an effect independent on cholesterol elevation. *Int J Cardiol* **98**, 459-464.

Lamireau D, Nuyt AM, Hou X, Bernier S, Beauchamp M, Gobeil F, Jr., Lahaie I, Varma DR, & Chemtob S (2002). Altered vascular function in fetal programming of hypertension. *Stroke* **33**, 2992-2998.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, & Harrison DG (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *Journal of Clinical Investigation* **111**, 1201-1209.

Langley-Evans SC & Jackson AA (1995). Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. *Comp Biochem Physiol A Physiol* **110**, 223-228.

Laufs U, Endres M, Custodis F, Gertz K, Nickenig G, Liao JK, & Bohm M (2000). Suppression of endothelial nitric oxide production after withdrawal of statin treatment is mediated by negative feedback regulation of rho GTPase gene transcription. *Circulation* **102**, 3104-3110.

Laufs U, La F, V, Plutzky J, & Liao JK (1998). Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* **97**, 1129-1135.

Laufs U & Liao JK (1998). Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* **273**, 24266-24271.

Laufs U, Wassmann S, Hilgers S, Ribaudo N, Bohm M, & Nickenig G (2001). Rapid effects on vascular function after initiation and withdrawal of atorvastatin in healthy, normocholesterolemic men. *Am J Cardiol* **88**, 1306-1307.

Law MR, Wald NJ, & Rudnicka AR (2003). Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. *BMJ* **326**, 1423.

Leduc L, Levy E, Bouity-Voubou M, & Delvin E (2010). Fetal programming of atherosclerosis: Possible role of the mitochondria. *Eur J Obstet Gynecol Reprod Biol*.

List of references

Lee HY, Youn SW, Cho HJ, Kwon YW, Lee SW, Kim SJ, Park YB, Oh BH, & Kim HS (2013). FOXO1 impairs whereas statin protects endothelial function in diabetes through reciprocal regulation of Kruppel-like factor 2. *Cardiovasc Res* **97**, 143-152.

Leeson CP, Kattenhorn M, Morley R, Lucas A, & Deanfield JE (2001). Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life. *Circulation* **103**, 1264-1268.

Leeson P (2007). Pediatric prevention of atherosclerosis: targeting early variation in vascular biology. *Pediatrics* **119**, 1204-1206.

Leon DA, Lithell HO, Vagero D, Koupilova I, Mohsen R, Berglund L, Lithell UB, & McKeigue PM (1998). Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29. *BMJ* **317**, 241-245.

Li H, Tao HR, Hu T, Fan YH, Zhang RQ, Jia G, & Wang HC (2010). Atorvastatin reduces calcification in rat arteries and vascular smooth muscle cells. *Basic Clin Pharmacol Toxicol* **107**, 798-802.

Li L, Chen W, Rezvan A, Jo H, & Harrison DG (2011). Tetrahydrobiopterin deficiency and nitric oxide synthase uncoupling contribute to atherosclerosis induced by disturbed flow. *Arterioscler Thromb Vasc Biol* **31**, 1547-1554.

Liao JK (2002). Isoprenoids as mediators of the biological effects of statins. *J Clin Invest* **110**, 285-288.

Libby P & Theroux P (2005). Pathophysiology of coronary artery disease. [Review] [58 refs]. *Circulation* **111**, 3481-3488.

Lillycrop KA, Phillips ES, Torrens C, Hanson MA, Jackson AA, & Burdge GC (2008). Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPAR alpha promoter of the offspring. *Br J Nutr* **100**, 278-282.

Lin YC, Lin JH, Chou CW, Chang YF, Yeh SH, & Chen CC (2008). Statins increase p21 through inhibition of histone deacetylase activity and release of promoter-associated HDAC1/2. *Cancer Res* **68**, 2375-2383.

Lind L, Berglund L, Larsson A, & Sundstrom J (2011). Endothelial function in resistance and conduit arteries and 5-year risk of cardiovascular disease. *Circulation* **123**, 1545-1551.

Liu B, Zhang JY, Cao HM, Wang Q, & Wang HB (2012a). Effect of rosuvastatin on ROCK activity, endothelial function, and inflammation in Asian patients with atherosclerosis. *Intern Med* **51**, 1177-1182.

Liu J, Liang Q, Frost-Pineda K, Muhammad-Kah R, Rimmer L, Roethig H, Mendes P, & Sarkar M (2011). Relationship between biomarkers of cigarette smoke exposure and biomarkers of inflammation, oxidative stress, and platelet activation in adult cigarette smokers. *Cancer Epidemiol Biomarkers Prev* **20**, 1760-1769.

Liu W, Choueiri TK, & Cho E (2012b). Statin use and the risk of renal cell carcinoma in 2 prospective US cohorts. *Cancer* **118**, 797-803.

Lloyd-Jones DM, Leip EP, Larson MG, D'Agostino RB, Beiser A, Wilson PW, Wolf PA, & Levy D (2006). Prediction of lifetime risk for cardiovascular disease by risk factor burden at 50 years of age. *Circulation* **113**, 791-798.

Lunder M, Janic M, Habjan S, & Sabovic M (2011). Subtherapeutic, low-dose fluvastatin improves functional and morphological arterial wall properties in apparently healthy, middle-aged males a pilot study. *Atherosclerosis*.

Maguire JJ, Wiley KE, Kuc RE, Stoneman VE, Bennett MR, & Davenport AP (2006). Endothelin-mediated vasoconstriction in early atherosclerosis is markedly increased in ApoE-/- mouse but prevented by atorvastatin. *Exp Biol Med (Maywood)* **231**, 806-812.

Marchesi C, Ebrahimian T, Angulo O, Paradis P, & Schiffrin EL (2009). Endothelial nitric oxide synthase uncoupling and perivascular adipose oxidative stress and inflammation contribute to vascular dysfunction in a rodent model of metabolic syndrome. *Hypertension* **54**, 1384-1392.

Martin H, Hu J, Gennser G, & Norman M (2000). Impaired endothelial function and increased carotid stiffness in 9-year-old children with low birthweight. *Circulation* **102**, 2739-2744.

Masley SC, Phillips SE, & Schocken DD (2006). Blood pressure as a predictor of cardiovascular events in the elderly: the William Hale Research Program. *J Hum Hypertens* **20**, 392-397.

May GR, Crook P, Moore PK, & Page CP (1991). The role of nitric oxide as an endogenous regulator of platelet and neutrophil activation within the pulmonary circulation of the rabbit. *Br J Pharmacol* **102**, 759-763.

Mazzuca MQ, Tare M, Parkington HC, Dragomir NM, Parry LJ, & Wlodek ME (2012). Uteroplacental insufficiency programmes vascular dysfunction in non-pregnant rats: compensatory adaptations in pregnancy. *J Physiol* **590**, 3375-3388.

McDaniel NL, Rembold CM, Richard HM, & Murphy RA (1991). Cyclic AMP relaxes swine arterial smooth muscle predominantly by decreasing cell Ca2+ concentration. *J Physiol* **439**, 147-160.

McKinney JS & Kostis WJ (2012). Statin therapy and the risk of intracerebral hemorrhage: a meta-analysis of 31 randomized controlled trials. *Stroke* **43**, 2149-2156.

Mcmillen IC & Robinson JS (2005). Developmental Origins of the Metabolic Syndrome: Prediction, Plasticity, and Programming. *Physiol Rev* **85**, 571-633.

Meda C, Plank C, Mykhaylyk O, Schmidt K, & Mayer B (2010). Effects of statins on nitric oxide/cGMP signaling in human umbilical vein endothelial cells. *Pharmacol Rep* **62**, 100-112.

Mendelsohn ME (2009). Estrogen actions in the cardiovascular system. *Climacteric* **12 Suppl 1**, 18-21.

Miersch S, Espey MG, Chaube R, Akarca A, Tweten R, Ananvoranich S, & Mutus B (2008). Plasma membrane cholesterol content affects nitric oxide diffusion dynamics and signaling. *J Biol Chem* **283**, 18513-18521.

Mizuno M, Siddique K, Baum M, & Smith SA (2013). Prenatal programming of hypertension induces sympathetic overactivity in response to physical stress. *Hypertension* **61**, 180-186.

Molins B, Pena E, Padro T, Casani L, Mendieta C, & Badimon L (2010). Glucose-Regulated Protein and Platelet Deposition. Effect of Rosuvastatin. *Arterioscler Thromb Vasc Biol*.

Mora S, Cook N, Buring JE, Ridker PM, & Lee IM (2007). Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms. *Circulation* **116**, 2110-2118.

Moreno M, Ramalho LN, Sancho-Bru P, Ruiz-Ortega M, Ramalho F, Abraldes JG, Colmenero J, Dominguez M, Egido J, Arroyo V, Gines P, & Bataller R (2009). Atorvastatin attenuates angiotensin II-induced inflammatory actions in the liver. *Am J Physiol - Gastr L Physiology* **296**, G147-G156.

Mulvany MJ & Halpern W (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* **41**, 19-26.

Munzel T, Daiber A, Ullrich V, & Mulsch A (2005). Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. *Arterioscler Thromb Vasc Biol* **25**, 1551-1557.

Mutoh T, Kumano T, Nakagawa H, & Kuriyama M (1999). Role of tyrosine phosphorylation of phospholipase C gamma1 in the signaling pathway of HMG-CoA reductase inhibitor-induced cell death of L6 myoblasts. *FEBS Lett* **446**, 91-94.

Muzaffar S, Shukla N, Bond M, Newby AC, Angelini GD, Sparatore A, Del Soldato P, & Jeremy JY (2008). Exogenous Hydrogen Sulfide Inhibits Superoxide Formation, NOX-1 Expression and Rac 1 Activity in Human Vascular Smooth Muscle Cells. *J Vasc Res* **45**, 521-528.

Nagashima S, Yagyu H, Ohashi K, Tazoe F, Takahashi M, Ohshiro T, Bayasgalan T, Okada K, Sekiya M, Osuga J, & Ishibashi S (2012). Liver-specific deletion of 3-hydroxy-3-methylglutaryl coenzyme A reductase causes hepatic steatosis and death. *Arterioscler Thromb Vasc Biol* **32**, 1824-1831.

Nakanishi M, Goldstein JL, & Brown MS (1988). Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. *J Biol Chem* **263**, 8929-8937.

Ness GC, Chambers CM, & Lopez D (1998). Atorvastatin action involves diminished recovery of hepatic HMG-CoA reductase activity. *J Lipid Res* **39**, 75-84.

Ni CD, Asplund K, Asberg S, Callaly E, Cuadrado-Godia E, Diez-Tejedor E, Di NM, Engelter ST, Furie KL, Giannopoulos S, Gotto AM, Jr., Hannon N, Jonsson F, Kapral MK, Marti-Fabregas J, Martinez-Sanchez P, Milionis HJ, Montaner J, Muscari A, Pikija S, Probstfield J, Rost NS, Thrift AG, Vemmos K, & Kelly PJ (2013). Statin therapy and outcome after ischemic stroke: systematic review and meta-analysis of observational studies and randomized trials. *Stroke* **44**, 448-456.

Ni R, Peleg T, & Gross PL (2012). Atorvastatin Delays Murine Platelet Activation In Vivo Even in the Absence of Endothelial NO Synthase. *Arterioscler Thromb Vasc Biol*.

Nichols GA, Joshua-Gotlib S, & Parasuraman S (2013). Independent Contribution of A1C, Systolic Blood Pressure, and LDL Cholesterol Control to Risk of Cardiovascular Disease Hospitalizations in Type 2 Diabetes: An Observational Cohort Study. *J Gen Intern Med*.

Nissen SE, Nicholls SJ, Sipahi I, Libby P, Raichlen JS, Ballantyne CM, Davignon J, Erbel R, Fruchart JC, Tardif JC, Schoenhagen P, Crowe T, Cain V, Wolski K, Goormastic M, & Tuzcu EM (2006). Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. *JAMA* **295**, 1556-1565.

Nuyt AM (2008). Mechanisms underlying developmental programming of elevated blood pressure and vascular dysfunction: evidence from human studies and experimental animal models. *Clin Sci* **114**, 1-17.

Nyalala JO, Wang J, Dang A, Faas FH, & Smith WG (2008). Hypertriglyceridemia and hypercholesterolemia: effects of drug treatment on fatty acid composition of plasma lipids and membranes. *Prostaglandins Leukot Essent Fatty Acids* **78**, 271-280.

Nyberg M, Blackwell JR, Damsgaard R, Jones AM, Hellsten Y, & Mortensen SP (2012). Lifelong physical activity prevents an age-related reduction in arterial and skeletal muscle nitric oxide bioavailability in humans. *J Physiol* **590**, 5361-5370.

Ohkawara H, Ishibashi T, Saitoh SI, Inoue N, Sugimoto K, Kamioka M, Uekita H, Kaneshiro T, Ando K, Takuwa Y, Maruyama Y, & Takeishi Y (2010). Preventive effects of pravastatin on thrombin-triggered vascular responses via Akt/eNOS and RhoA/Rac1 pathways in vivo. *Cardiovasc Res*.

Ohkawara H, Ishibashi T, Sakamoto T, Sugimoto K, Nagata K, Yokoyama K, Sakamoto N, Kamioka M, Matsuoka I, Fukuhara S, Sugimoto N, Takuwa Y, & Maruyama Y (2005). Thrombin-induced rapid geranylgeranylation of RhoA as an essential process for RhoA activation in endothelial cells. *J Biol Chem* **280**, 10182-10188.

Oren A, Vos LE, Uiterwaal CS, Gorissen WH, Grobbee DE, & Bots ML (2004). Birth weight and carotid intima-media thickness: new perspectives from the atherosclerosis risk in young adults (ARYA) study. *Ann Epidemiol* **14**, 8-16.

Orso E, Ahrens N, Kilalic D, & Schmitz G (2009). Familial hypercholesterolemia and lipoprotein(a) hyperlipidemia as independent and combined cardiovascular risk factors. *Atheroscler Suppl* **10**, 74-78.

Osiniri I, Sitjar C, Soriano-Rodriguez P, Prats-Puig A, Casas-Satre C, Mayol L, de ZF, Ibanez L, Bassols J, & Lopez-Bermejo A (2012). Carotid intima-media thickness at 7 years of age: relationship to C-reactive protein rather than adiposity. *J Pediatr* **160**, 276-280.

Osmond C, Barker DJ, Winter PD, Fall CH, & Simmonds SJ (1993). Early growth and death from cardiovascular disease in women. *BMJ* **307**, 1519-1524.

Osmond C, Kajantie E, Forsen TJ, Eriksson JG, & Barker DJ (2007). Infant growth and stroke in adult life: the Helsinki birth cohort study. *Stroke* **38**, 264-270.

Ota H, Eto M, Kano MR, Kahyo T, Setou M, Ogawa S, Iijima K, Akishita M, & Ouchi Y (2010). Induction of endothelial nitric oxide synthase, SIRT1, and catalase by statins inhibits endothelial senescence through the Akt pathway. *Arterioscler Thromb Vasc Biol* **30**, 2205-2211.

Otani M, Yamamoto M, Harada M, & Otsuki M (2010). Effect of long- and short-term treatments with pravastatin on diabetes mellitus and pancreatic fibrosis in the Otsuka-Long-Evans-Tokushima fatty rat. *Br J Pharmacol* **159**, 462-473.

Panza JA, Quyyumi AA, Brush JE, Jr., & Epstein SE (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* **323**, 22-27.

Pappu AS, Bacon SP, & Illingworth DR (2003). Residual effects of lovastatin and simvastatin on urinary mevalonate excretions in patients with familial hypercholesterolemia. *J Lab Clin Med* **141**, 250-256.

Paraskevas KI, Bessias N, Papas TT, Gekas CD, Andrikopoulos V, & Mikhailidis DP (2008). Do different vascular risk factors affect all arteries equally? *Angiology* **59**, 397-401.

Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, & Golding J (2006). Sex-specific, male-line transgenerational responses in humans. *Eur J Hum Genet* **14**, 159-166.

Pencina MJ, D'Agostino RB, Sr., Larson MG, Massaro JM, & Vasan RS (2009). Predicting the 30-year risk of cardiovascular disease: the framingham heart study. *Circulation* **119**, 3078-3084.

Perregaux D, Chaudhuri A, Mohanty P, Bukhari L, Wilson MF, Sung BH, & Dandona P (1999). Effect of gender differences and estrogen replacement therapy on vascular reactivity. *Metabolism* **48**, 227-232.

Pignatelli P, Carnevale R, Pastori D, Cangemi R, Napoleone L, Bartimoccia S, Nocella C, Basili S, & Violi F (2012). Immediate antioxidant and antiplatelet effect of atorvastatin via inhibition of Nox2. *Circulation* **126**, 92-103.

Pinney SE, Jaeckle Santos LJ, Han Y, Stoffers DA, & Simmons RA (2011). Exendin-4 increases histone acetylase activity and reverses epigenetic modifications that silence Pdx1 in the intrauterine growth retarded rat. *Diabetologia* **54**, 2606-2614.

Ponzio BF, Carvalho MH, Fortes ZB, & do Carmo FM (2012). Implications of maternal nutrient restriction in transgenerational programming of hypertension and endothelial dysfunction across F1-F3 offspring. *Life Sci* **90**, 571-577.

Poulter N (2003). Global risk of cardiovascular disease. *Heart* **89 Suppl 2**, ii2-ii5.

Priviero FBM, Zemse SM, Teixeira CE, & Webb RC (2009). Oxidative Stress Impairs Vasorelaxation Induced by the Soluble Guanylyl Cyclase Activator BAY 41-2272 in Spontaneously Hypertensive Rats. *Am J Hypertens* **22**, 493-499.

Puato M, Faggin E, Rattazzi M, Zambon A, Cipollone F, Grego F, Ganassin L, Plebani M, Mezzetti A, & Pauletto P (2010). Atorvastatin Reduces Macrophage Accumulation in Atherosclerotic Plaques. A Comparison of a Nonstatin-Based Regimen in Patients Undergoing Carotid Endarterectomy. *Stroke*.

Puccetti L, Pasqui AL, Pastorelli M, Bova G, Di RM, Leo A, Cercignani M, Palazzuoli A, Auteri A, & Bruni F (2003). Platelet hyperactivity after statin treatment discontinuation. *Thromb Haemost* **90**, 476-482.

Qasem RJ, Cherala G, & D'mello AP (2010). Maternal protein restriction during pregnancy and lactation in rats imprints long-term reduction in hepatic lipid content selectively in the male offspring. *Nutr Res* **30**, 410-417.

Quan H, Chen G, Walker RL, Wielgosz A, Dai S, Tu K, Campbell NR, Hemmelgarn BR, Hill MD, Johansen H, McAlister FA, & Khan N (2013). Incidence, cardiovascular complications and mortality of hypertension by sex and ethnicity. *Heart*.

Radomski MW, Palmer RM, & Moncada S (1987). The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol* **92**, 639-646.

Ratchford EV, Gutierrez J, Lorenzo D, McClendon MS, Della-Morte D, DeRosa JT, Elkind MS, Sacco RL, & Rundek T (2011). Short-term effect of atorvastatin on carotid artery elasticity: a pilot study. *Stroke* **42**, 3460-3464.

Raymenants E, Yang B, Nicolini F, Behrens P, Lawson D, & Mehta JL (1993). Verapamil and aspirin modulate platelet-mediated vasomotion in arterial segments with intact or disrupted endothelium. *J Am Coll Cardiol* **22**, 684-689.

Ridker PM, Cushman M, Stampfer MJ, Tracy RP, & Hennekens CH (1997). Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* **336**, 973-979.

Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, & Glynn RJ (2008a). Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med* **359**, 2195-2207.

Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ, & JUPITER Study Group (2008b). Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein.[see comment]. *N Eng J Med* **359**, 2195-2207.

Ridker PM, Hennekens CH, Buring JE, & Rifai N (2000a). C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med* **342**, 836-843.

Ridker PM, Rifai N, Clearfield M, Downs JR, Weis SE, Miles JS, & Gotto AM, Jr. (2001). Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events. *N Engl J Med* **344**, 1959-1965.

Ridker PM, Rifai N, Stampfer MJ, & Hennekens CH (2000b). Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* **101**, 1767-1772.

Rizzoni D, Porteri E, Castellano M, Bettoni G, Muiesan ML, Tiberio G, Giulini SM, Rossi G, Bernini G, & Agabiti-Rosei E (1998). Endothelial dysfunction in hypertension is independent from the etiology and from vascular structure. *Hypertension* **31**, 335-341.

Rodford JL, Torrens C, Siow RC, Mann GE, Hanson MA, & Clough GF (2008). Endothelial dysfunction and reduced antioxidant protection in an animal model of the developmental origins of cardiovascular disease.[see comment]. *J Physiol* **586**, 19-20.

Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJ, & Bleker OP (2001). Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Twin Res* **4**, 293-298.

Ross R (1999). Atherosclerosis--an inflammatory disease. *N Eng J Med* **340**, 115-126.

Rossoni LV, Wareing M, Wenceslau C, Al-Abri M, Cobb C, & Austin CE (2011). Acute simvastatin increases endothelial nitric oxide synthase phosphorylation via AMP-activated protein kinase and reduces contractility of isolated rat mesenteric resistance arteries. *Clin Sci (Lond)*.

Sahajpal V & Ashton N (2003). Renal function and angiotensin AT1 receptor expression in young rats following intrauterine exposure to a maternal low-protein diet. *Clin Sci (Lond)* **104**, 607-614.

Sakamoto T, Kojima S, Ogawa H, Shimomura H, Kimura K, Ogata Y, Sakaino N, & Kitagawa A (2007). Usefulness of hydrophilic vs lipophilic statins after acute myocardial infarction: subanalysis of MUSASHI-AMI. *Circ J* **71**, 1348-1353.

Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L, & Taylor PD (2008). Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. *Hypertension* **51**, 383-392.

Sardo MA, Campo S, Mandraffino G, Saitta C, Bonaiuto A, Castaldo M, Cinquegrani M, Pizzimenti G, & Saitta A (2008). Tissue factor and monocyte chemoattractant protein-1 expression in hypertensive individuals with normal or increased carotid intima-media wall thickness. *Clin Chem* **54**, 814-823.

Sattelmair JR, Kurth T, Buring JE, & Lee IM (2010). Physical activity and risk of stroke in women. *Stroke* **41**, 1243-1250.

Scarborough P, Bhatnagar P, Wickramasinghe K, Smolina K, Mitchell C, & Rayner M (2011). *Coronary heart disease statistics 2010 edition* British Heart Foundation, London.

Schmeck B, Beermann W, N'Guessan PD, Hocke AC, Opitz B, Eitel J, Dinh QT, Witzenrath M, Krull M, Suttorp N, & Hippenstiel S (2008). Simvastatin reduces Chlamydophila pneumoniae-mediated histone modifications and gene expression in cultured human endothelial cells. *Circ Res* **102**, 888-895.

Schneeweis C, Grafe M, Bungenstock A, Spencer-Hansch C, Fleck E, & Goetze S (2010). Chronic CRP-exposure inhibits VEGF-induced endothelial cell migration. *J Atheroscler Thromb* **17**, 203-212.

Schooling CM, Lam TH, Janus ED, Cowling BJ, & Leung GM (2009). A socio-historical hypothesis for the diabetes epidemic in Chinese--preliminary observations from Hong Kong as a natural experiment. *Am J Hum Biol* **21**, 346-353.

Setoguchi M, Ohya Y, Abe I, & Fujishima M (1997). Stretch-activated whole-cell currents in smooth muscle cells from mesenteric resistance artery of guinea-pig. *J Physiol* **501 (Pt 2)**, 343-353.

Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, Macfarlane PW, McKillop JH, & Packard CJ (1995). Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* **333**, 1301-1307.

Sherman RC & Langley-Evans SC (1998). Early administration of angiotensin-converting enzyme inhibitor captopril, prevents the development of hypertension programmed by intrauterine exposure to a maternal low-protein diet in the rat. *Clin Sci (Lond)* **94**, 373-381.

Sherman RC & Langley-Evans SC (2000). Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat. *Clin Sci (Lond)* **98**, 269-275.

Shieh JM, Wei TT, Tang YA, Huang SM, Wen WL, Chen MY, Cheng HC, Salunke SB, Chen CS, Lin P, Chen CT, & Wang YC (2012). Mitochondrial apoptosis and FAK signaling disruption by a novel histone deacetylase inhibitor, HTPB, in antitumor and antimetastatic mouse models. *PLoS One* **7**, e30240.

Shimbo D, Muntner P, Mann D, Viera AJ, Homma S, Polak JF, Barr RG, Herrington D, & Shea S (2010). Endothelial dysfunction and the risk of hypertension: the multi-ethnic study of atherosclerosis. *Hypertension* **55**, 1210-1216.

Shimokawa H, Aarhus LL, & Vanhoutte PM (1987). Porcine coronary arteries with regenerated endothelium have a reduced endothelium-dependent responsiveness to aggregating platelets and serotonin. *Circ Res* **61**, 256-270.

Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cordiano C, Catapano A, Tramontana S, Perticone F, Naccarato P, Camici P, Picano E, Cortigiani L, Bevilacqua M, Milazzo L, Cusi D, Barlassina C, Sarzi-Puttini P, & Turiel M (2010). From endothelial dysfunction to atherosclerosis. *Autoimmun Rev* **9**, 830-834.

Skilton MR, Viikari JS, Juonala M, Laitinen T, Lehtimaki T, Taittonen L, Kahonen M, Celermajer DS, & Raitakari OT (2011). Fetal growth and preterm birth influence cardiovascular risk factors and arterial health in young adults: the Cardiovascular Risk in Young Finns Study. *Arterioscler Thromb Vasc Biol* **31**, 2975-2981.

Skogastierna C, Luksha L, Kublickiene K, Eliasson E, Rane A, & Ekstrom L (2011). Beneficial vasoactive endothelial effects of fluvastatin: focus on prostacyclin and nitric oxide. *Heart Vessels* **26**, 628-636.

Smith JL, Lear SR, & Erickson SK (1995). Developmental expression of elements of hepatic cholesterol metabolism in the rat. *J Lipid Res* **36**, 641-652.

Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ, & Deanfield JE (1994). Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *J Clin Invest* **93**, 50-55.

Souza-Mello V, Mandarim-de-Lacerda CA, & Aguila MB (2007). Hepatic structural alteration in adult programmed offspring (severe maternal protein restriction) is aggravated by post-weaning high-fat diet. *Br J Nutr* **98**, 1159-1169.

Spencer FA, Fonarow GC, Frederick PD, Wright RS, Every N, Goldberg RJ, Gore JM, Dong W, Becker RC, & French W (2004). Early withdrawal of statin therapy in patients with non-ST-segment elevation myocardial infarction: national registry of myocardial infarction. *Arch Intern Med* **164**, 2162-2168.

Sposito AC, Carvalho LS, Cintra RM, Araujo AL, Ono AH, Andrade JM, Coelho OR, & Quinaglia e Silva JC (2009). Rebound inflammatory response during the acute phase of myocardial infarction after simvastatin withdrawal. *Atherosclerosis* **207**, 191-194.

Stanner SA, Bulmer K, Andres C, Lantseva OE, Borodina V, Poteen VV, & Yudkin JS (1997). Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study. *BMJ* **315**, 1342-1348.

Stein AD, Kahn HS, Rundle A, Zybert PA, van der Pal-de Bruin, & Lumey LH (2007). Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine. *Am J Clin Nutr* **85**, 869-876.

Stein AD, Zybert PA, van der Pal-de Bruin, & Lumey LH (2006). Exposure to famine during gestation, size at birth, and blood pressure at age 59 y: evidence from the Dutch Famine. *Eur J Epidemiol* **21**, 759-765.

Strakovsky RS, Zhang X, Zhou D, & Pan YX (2011). Gestational high fat diet programs hepatic phosphoenolpyruvate carboxykinase gene expression and histone modification in neonatal offspring rats. *J Physiol* **589**, 2707-2717.

Suh JW, Choi DJ, Chang HJ, Cho YS, Youn TJ, Chae IH, Kim KI, Kim CH, Kim HS, Oh BH, & Park YB (2010). HMG-CoA reductase inhibitor improves endothelial dysfunction in spontaneous hypertensive rats via down-regulation of caveolin-1 and activation of endothelial nitric oxide synthase. *J Korean Med Sci* **25**, 16-23.

Sussan TE, Jun J, Thimmulappa R, Bedja D, Antero M, Gabrielson KL, Polotsky VY, & Biswal S (2008). Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice. *PLoS One* **3**, e3791.

Swirski FK & Nahrendorf M (2013). Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure. *Science* **339**, 161-166.

Szostak J & Laurant P (2011). The forgotten face of regular physical exercise: a 'natural' anti-atherogenic activity. *Clin Sci (Lond)* **121**, 91-106.

Takemoto M & Liao JK (2001). Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol* **21**, 1712-1719.

Tanaka S, Sakamoto K, Yamamoto M, Mizuno A, Ono T, Waguri S, & Kimura J (2010). Mechanism of statin-induced contractile dysfunction in rat cultured skeletal myofibers. *J Pharmacol Sci* **114**, 454-463.

Taneva E, Borucki K, Wiens L, Makarova R, Schmidt-Lucke C, Luley C, & Westphal S (2006). Early effects on endothelial function of atorvastatin 40 mg twice daily and its withdrawal. *Am J Cardiol* **97**, 1002-1006.

Tang EH & Vanhoutte PM (2010). Endothelial dysfunction: a strategic target in the treatment of hypertension? *Pflugers Arch* **459**, 995-1004.

Tare M, Emmett SJ, Coleman HA, Skordilis C, Eyles DW, Morley R, & Parkington HC (2011). Vitamin D insufficiency is associated with impaired vascular endothelial and smooth muscle function and hypertension in young rats. *J Physiol* **589**, 4777-4786.

Taylor F, Ward K, Moore TH, Burke M, Davey SG, Casas JP, & Ebrahim S (2011). Statins for the primary prevention of cardiovascular disease. *Cochrane Database Syst Rev* **1**, CD004816.

Tian XY, Wong WT, Xu A, Chen ZY, Lu Y, Liu LM, Lee VW, Lau CW, Yao X, & Huang Y (2011). Rosuvastatin Improves Endothelial Function of db/db Mice: Role of Angiotensin II Type 1 Receptors and Oxidative Stress. *Br J Pharmacol*.

Tong X, Hou X, Jourd'heuil D, Weisbrod RM, & Cohen RA (2010). Upregulation of Nox4 by TGF{beta}1 Oxidizes SERCA and Inhibits NO in Arterial Smooth Muscle of the Prediabetic Zucker Rat. *Circ Res*.

Torrens C, Brawley L, Barker AC, Itoh S, Poston L, & Hanson MA (2003). Maternal protein restriction in the rat impairs resistance artery but not conduit artery function in pregnant offspring. *J Physiol* **547**, 77-84.

Torrens C, Ethirajan P, Bruce KD, Cagampang FR, Siow RC, Hanson MA, Byrne CD, Mann GE, & Clough GF (2012). Interaction between maternal and offspring diet to

impair vascular function and oxidative balance in high fat fed male mice. *PLoS One* **7**, e50671.

Torrens C, Hanson MA, Gluckman PD, & Vickers MH (2009a). Maternal undernutrition leads to endothelial dysfunction in adult male rat offspring independent of postnatal diet. *Brit J Nutr* **101**, 27-33.

Torrens C, Kelsall CJ, Hopkins LA, Anthony FW, Curzen NP, & Hanson MA (2009b). Atorvastatin restores endothelial function in offspring of protein-restricted rats in a cholesterol-independent manner. *Hypertension* **53**, 661-667.

Torrens C, Poston L, & Hanson MA (2008). Transmission of raised blood pressure and endothelial dysfunction to the F2 generation induced by maternal protein restriction in the F0, in the absence of dietary challenge in the F1 generation. *Brit J Nutr* **100**, 760-766.

Touwslager RN, Gerver WJ, Tan FE, Gielen M, Zeegers MP, Zimmermann LJ, Houben AJ, Blanco CE, Stehouwer CD, & Mulder AL (2012). Influence of growth during infancy on endothelium-dependent vasodilatation at the age of 6 months. *Hypertension* **60**, 1294-1300.

Triggle CR, Samuel SM, Ravishankar S, Marei I, Arunachalam G, & Ding H (2012). The endothelium: influencing vascular smooth muscle in many ways. *Can J Physiol Pharmacol* **90**, 713-738.

Tzoulaki I, Jarvelin MR, Hartikainen AL, Leinonen M, Pouta A, Paldanius M, Ruokonen A, Canoy D, Sovio U, Saikku P, & Elliott P (2008). Size at birth, weight gain over the life course, and low-grade inflammation in young adulthood: northern Finland 1966 Birth Cohort study. *Eur Heart J* **29**, 1049-1056.

Usui T, Okada M, Mizuno W, Oda M, Ide N, Morita T, Hara Y, & Yamawaki H (2012). HDAC4 mediates development of hypertension via vascular inflammation in spontaneous hypertensive rats. *Am J Physiol Heart Circ Physiol* **302**, H1894-H1904.

Vandesompele J, De PK, Pattyn F, Poppe B, Van RN, De PA, & Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034.

Vanhoutte PM (1989). Endothelium and control of vascular function. State of the Art lecture. *Hypertension* **13**, 658-667.

Vanhoutte PM & Mombouli JV (1996). Vascular endothelium: vasoactive mediators. *Prog Cardiovasc Dis* **39**, 229-238.

Vanhoutte PM, Shimokawa H, Tang EH, & Feletou M (2009). Endothelial dysfunction and vascular disease. *Acta Physiol (Oxf)* **196**, 193-222.

Vecchione C & Brandes RP (2002). Withdrawal of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors elicits oxidative stress and induces endothelial dysfunction in mice. *Circ Res* **91**, 173-179.

Vecka M, Tvrzicka E, Stankova B, Novak F, Novakova O, & Zak A (2004). Hypolipidemic drugs can change the composition of rat brain lipids. *Tohoku J Exp Med* **204**, 299-308.

Venugopal SK, Devaraj S, Yuhanna I, Shaul P, & Jialal I (2002). Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation* **106**, 1439-1441.

Vickers MH, Gluckman PD, Coveny AH, Hofman PL, Cutfield WS, Gertler A, Breier BH, & Harris M (2005). Neonatal leptin treatment reverses developmental programming. *Endocrinology* **146**, 4211-4216.

Vickers MH, Gluckman PD, Coveny AH, Hofman PL, Cutfield WS, Gertler A, Breier BH, & Harris M (2008). The effect of neonatal leptin treatment on postnatal weight gain in male rats is dependent on maternal nutritional status during pregnancy. *Endocrinology* **149**, 1906-1913.

Vo PA, Reid JJ, & Rand MJ (1992). Attenuation of vasoconstriction by endogenous nitric oxide in rat caudal artery. *Br J Pharmacol* **107**, 1121-1128.

Vucetic Z, Kimmel J, & Reyes TM (2011). Chronic high-fat diet drives postnatal epigenetic regulation of mu-opioid receptor in the brain. *Neuropsychopharmacol* **36**, 1199-1206.

Vuguin PM (2007). Animal models for small for gestational age and fetal programming of adult disease. *Horm Res* **68**, 113-123.

Wagner AH, Kohler T, Ruckschloss U, Just I, & Hecker M (2000). Improvement of nitric oxide-dependent vasodilatation by HMG-CoA reductase inhibitors through attenuation of endothelial superoxide anion formation. *Arterioscler Thromb Vasc Biol* **20**, 61-69.

Wallace SM, Yasmin, McEniery CM, Maki-Petaja KM, Booth AD, Cockcroft JR, & Wilkinson IB (2007). Isolated systolic hypertension is characterized by increased aortic stiffness and endothelial dysfunction. *Hypertension* **50**, 228-233.

Wang CY, Liu PY, & Liao JK (2008). Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. *Trends Mol Med* **14**, 37-44.

Wang Y, Zheng XR, Riddick N, Bryden M, Baur W, Zhang X, & Surks HK (2009). ROCK isoform regulation of myosin phosphatase and contractility in vascular smooth muscle cells. *Circ Res* **104**, 531-540.

Wassmann S, Faul A, Hennen B, Scheller B, Bohm M, & Nickenig G (2003). Rapid effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition on coronary endothelial function. *Circ Res* **93**, e98-103.

Watkins AJ, Lucas ES, Torrens C, Cleal JK, Green L, Osmond C, Eckert JJ, Gray WP, Hanson MA, & Fleming TP (2010). Maternal low-protein diet during mouse pre-implantation development induces vascular dysfunction and altered renin-angiotensin-system homeostasis in the offspring. *Br J Nutr* **103**, 1762-1770.

Watkins AJ, Wilkins A, Cunningham C, Perry VH, Seet MJ, Osmond C, Eckert JJ, Torrens C, Cagampang FR, Cleal J, Gray WP, Hanson MA, & Fleming TP (2008). Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. *J Physiol* **586**, 2231-2244.

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, & Meaney MJ (2004). Epigenetic programming by maternal behavior. *Nat Neurosci* **7**, 847-854.

Weaver IC, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ, & Szyf M (2005). Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. *J Neurosci* **25**, 11045-11054.

Weaver IC, Meaney MJ, & Szyf M (2006). Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. *Proc Natl Acad Sci U S A* **103**, 3480-3485.

Wenzel P, Daiber A, Oelze M, Brandt M, Closs E, Xu J, Thum T, Bauersachs J, Ertl G, Zou MH, Forstermann U, & Munzel T (2008). Mechanisms underlying recoupling of eNOS by HMG-CoA reductase inhibition in a rat model of streptozotocin-induced diabetes mellitus. *Atherosclerosis* **198**, 65-76.

Wildman RP, Colvin AB, Powell LH, Matthews KA, Everson-Rose SA, Hollenberg S, Johnston JM, & Sutton-Tyrrell K (2008). Associations of endogenous sex hormones with the vasculature in menopausal women: the Study of Women's Health Across the Nation (SWAN). *Menopause* **15**, 414-421.

Wlodek ME, Westcott K, Siebel AL, Owens JA, & Moritz KM (2008). Growth restriction before or after birth reduces nephron number and increases blood pressure in male rats. *Kidney Int* **74**, 187-195.

Wolfenstetter A, Simonetti GD, Poschl J, Schaefer F, & Wuhl E (2012). Altered cardiovascular rhythmicity in children born small for gestational age. *Hypertension* **60**, 865-870.

Woods LL, Ingelfinger JR, Nyengaard JR, & Rasch R (2001). Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatr Res* **49**, 460-467.

World Health Organization. World Health Report. 2004. Geneva, World Health Organization.
Ref Type: Report

Wu T, Fujihara M, Tian J, Jovanovic M, Grayson C, Cano M, Gehlbach P, Margaron P, & Handa JT (2010). Apolipoprotein B100 secretion by cultured ARPE-19 cells is modulated by alteration of cholesterol levels. *J Neurochem* **114**, 1734-1744.

Yagi S, Akaike M, Aihara K, Ishikawa K, Iwase T, Ikeda Y, Soeki T, Yoshida S, Sumitomo-Ueda Y, Matsumoto T, & Sata M (2010). Endothelial nitric oxide synthase-independent protective action of statin against angiotensin II-induced atrial remodeling via reduced oxidant injury. *Hypertension* **55**, 918-923.

Yatera Y, Shibata K, Furuno Y, Sabanai K, Morisada N, Nakata S, Morishita T, Toyohira Y, Wang KY, Tanimoto A, Sasaguri Y, Tasaki H, Nakashima Y, Shimokawa H, Yanagihara N, Otsuji Y, & Tsutsui M (2010). Severe dyslipidaemia, atherosclerosis, and sudden cardiac death in mice lacking all NO synthases fed a high-fat diet. *Cardiovasc Res* **87**, 675-682.

Yokoyama K, Ishibashi T, Ohkawara H, Kimura J, Matsuoka I, Sakamoto T, Nagata K, Sugimoto K, Sakurada S, & Maruyama Y (2002). HMG-CoA reductase inhibitors suppress intracellular calcium mobilization and membrane current induced by lysophosphatidylcholine in endothelial cells. *Circulation* **105**, 962-967.

Zebrack JS, Anderson JL, Maycock CA, Horne BD, Bair TL, & Muhlestein JB (2002a). Usefulness of high-sensitivity C-reactive protein in predicting long-term risk of death or acute myocardial infarction in patients with unstable or stable angina pectoris or acute myocardial infarction. *Am J Cardiol* **89**, 145-149.

Zebrack JS, Muhlestein JB, Horne BD, & Anderson JL (2002b). C-reactive protein and angiographic coronary artery disease: independent and additive predictors of risk in subjects with angina. *J Am Coll Cardiol* **39**, 632-637.

Zhou Q & Liao JK (2009). Rho kinase: an important mediator of atherosclerosis and vascular disease. *Curr Pharm Des* **15**, 3108-3115.

Zhou Q & Liao JK (2010). Pleiotropic effects of statins. - Basic research and clinical perspectives -. *Circ J* **74**, 818-826.