Women's Health and Perinatology Research Group
Department of Clinical Medicine
Faculty of Health Sciences

Cardiovascular adaptation in pregnancy

Effects of angiotensin II, transverse aorta constriction and high-intensity interval training on pregnant rats

-

Nils Thomas Songstad

A dissertation for the degree of Philosophiae Doctor - September 2014

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UiT – The Arctic University of Norway
-
Department of Pediatrics
Division of Child and Adolescent Health
University Hospital of Northern Norway
Northern Norway Regional Health Authority

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List of papers

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B. Songstad NT, Johansen D, How O-J, Kaaresen PI, Ytrehus K and Acharya G. Effect of transverse aortic constriction on cardiac structure, function and gene expression in pregnant rats. *PLoS ONE 2014, 9(2): e89559. doi:10.1371/journal.pone.0089559*

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Abbreviations

ACE	Angiotensin converting enzyme
AngII	Angiotensin II
AT_1	Angiotensin II-receptor type 1
AT_1-AA	AT_1 -agonistic autoantibody
ANOVA	Analysis of variance
ANKRD1	Ankyrin repeat domain-containing protein 1
ANP	Atrial natriuretic peptide
BNP	B-type natriuretic peptide
BP	Blood pressure
BW	Body weight
CAT	Catalase
CO	Cardiac output
CFR	Coronary flow reserve
CFR_{peak}	Coronary flow reserve, ratio between peak coronary flow velocities
CFR_{VTI}	Coronary flow reserve, ratio between velocity-time integrals
COL1A1	Collagen type I-α1
COL3A1	Collagen type III-α1
CRL	Crown-rump length (fetal)
CTH	Cystathionase
dP/dt_{max}	Maximal rate of left ventricle pressure rise in early systole
dP/dt_{min}	Maximal rate of left ventricle pressure drop in late systole
E/A-ratio	The ratio of the early (E) to late (A) left ventricle filling velocities
Ees	Left vetricular end-systolic elastance
eNOS	Endothelial nitric oxide synthase
ET	Ejection time
FMD	Flow mediated dilatation
FN1	Fibronectin 1
GD	Gestational day
GPx	Glutathione peroxidase
HK2	Hexokinase II
HIIT	High-intensity interval training
HW	Heart weight

iNOS	Inducible nitric oxide synthase
ICT	Isovolumetric contraction time
IRT	Isovolumetric relaxation time
IV	Intravenous
LMCA	Left main coronary artery
LV	Left ventricle
MAP	Mean arterial pressure
mRNA	Messenger ribonucleic acid
MHC	Myosin heavy chain
NaCl	Sodium chloride
NP	Non-pregnant
TP53	Tumor protein p53
PKC	Protein kinase C
PV	Pressure-volume
RT-PCR	Reverse transcription polymerase chain reaction
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
SV	Stroke volume
TAC	Transverse aortic constriction
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metallopeptidase
TNFα	Tumor necrosis factor-α
TPR	Total peripheral resistance
VEGF	Vascular endothelial growth factor
$\dot{V}O_2$	Oxygen consumption
$\dot{V}O_{2max}$	Maximal oxygen consumption
VTI	Velocity-time integrals

Summary of the thesis

Objectives: To investigate how cardiac function, remodeling and gene expression are affected in pregnancy in response to cardiovascular stress and to detect possible adverse effects of chronic AngII infusion, transverse aortic constriction (TAC) or high-intensity interval training (HIIT) on the fetus. Additionally we wanted to evaluate if coronary endothelial function is influenced by pregnancy and increased cardiac afterload.

Materials and methods: Pregnant and non-pregnant rats were subjected to chronic AngII-infusion, TAC or HIIT in three separate studies. Echocardiography was used to evaluate maternal heart function and fetal hemodynamics. Blood flow velocities in the left main coronary artery were measured using Doppler echocardiography, and coronary flow reserve (CFR) was assessed using 3.5% inhaled isoflurane as a vasodilating agent. A conductance catheter placed via the right carotid artery was used for invasive measurements of aortic blood pressure and left ventricle (LV) pressure-and volume. Histological sections of the maternal LV were used to determine collagen content (Sirius Red staining), vessel density (β-actin immunolabelling) and myocyte size (Toluidine Blue). RT-PCR was used to quantify the gene expression in maternal myocardium, placenta, fetal heart and fetal liver. Total antioxidant capacity and oxidative stress (peroxidase and superoxide dismutase activity and malondialdehyde content) was measured in the placentas, fetal hearts and livers in the HIIT-study.

Results: Chronic AngII infusion resulted in an increase in myocardial collagen content, and pregnancy reduced this effect. Vessel density in LV was decreased in AngII infused compared to sham non-pregnant rats, but not significantly in pregnant rats. Fetal hemodynamics was not affected by chronic AngII-infusion. Calculated stroke work in pregnant TAC rats was double compared to pregnant shams, whereas it was only 35% higher (not significant) in non-pregnant TAC rats compared to non-pregnant shams. The ratio of β-MHC to α-MHC expression was higher in pregnant TAC compared to non-pregnant TAC. Myocyte transverse circumference was increased by pregnancy, but not by TAC. HIIT did not alter maternal cardiac structure or function, fetal growth or oxidative stress and total antioxidant capacity in the placenta, fetal heart and fetal liver. However, the expression of some genes related to oxidative stress or cardiac remodeling was changed in fetal heart and liver. CFR could be calculated in 60 of 75 (80%) rats. There were no differences in CFR between rats with

increased afterload (AngII or TAC) and sham controls. CFR was lower in pregnant sham compared to non-pregnant sham rats.

Main conclusions: AngII infusion caused cardiac hypertrophy in pregnant rats. However, pregnancy was protective against fibrosis and preserved angiogenesis in AngII infused rats. On the other hand, the differences in cardiac structure, function and gene expression between pregnant and non-pregnant rats following TAC indicated that increased afterload may be less tolerated in pregnancy. Pregnancy does not lead to significant heart hypertrophy in rats, but induces changes in the expression of a wide range of genes involved in cardiac remodeling independent of afterload. HIIT is feasible and well tolerated by pregnant rats. CFR is reduced in late pregnancy, but not influenced by increased afterload caused by TAC or chronic AngII infusion. HIIT does not induce significant changes in oxidative stress in the fetus, but altered the expression of some genes in fetal liver and heart indicating that adaptive mechanisms are activated.

1. Introduction

1.1 Cardiovascular adaptations to pregnancy

This thesis will focus on how pregnancy influences heart function, structure and gene expression in health and disease. Women who become pregnant undergo profound alterations of the cardiovascular system. In this section the pregnancy induced adaptive changes of the heart and cardiovascular system are summarized.

1.1.1 Heart rate and cardiac output

The first hemodynamic adaptation to take place is an increase in resting heart rate which starts between 2 and 5 weeks gestation and continues throughout pregnancy [1, 2]. Maternal heart rate is also increased during exercise, but less evident at higher intensities. The increase in heart rate is primarily a result of reduced parasympathetic modulation [3]. LV stroke volume starts to increase early in pregnancy, reaching a plateau at approximately 24 weeks of gestation [2]. Thus the rise in total cardiac output, the product of stroke volume and hear rate, is most pronounced in the first trimester with more than 50% of the change in cardiac output taking place before 8 weeks gestation [4]. In the third trimester, the gravid uterus may obstruct the inferior vena cava when lying in the supine position, leading to reduced venous return to the heart and a subsequent decrease in cardiac output.

1.1.2 Myocardial contractility

Data on how pregnancy will influence myocardial function is conflicting. Some researchers have reported an increase in LV contractility during pregnancy [5-7], while others have found no change [8] or decrease in contractile function of the maternal heart in health pregnancies [9]. *In vivo* measurements of cardiac function in pregnancy are highly dependent on the loading conditions of the heart and these are influenced by a variety of factors in pregnancy [10, 11].

1.1.3 Systemic vascular resistance and blood pressure

During early pregnancy active vasodilatation through the action of local mediators as nitric oxide and prostacyclin as well as increased blood flow in the uteroplacental circulation leads to a decrease in systemic vascular resistance [5, 8, 9, 11-13]. Systemic BP falls early in

gestation and diastolic BP will average 10 mmHg bellow non-pregnant values in the second trimester before gradually increasing towards basal values at term [11, 13].

1.1.4 Blood volume

In normal pregnancies blood volume will start to increase between 10 and 20 weeks of pregnancy averaging a total blood volume 40-45% above non-pregnant levels [14-18]. Two thirds of the rise consists of increased plasma volume where as the rest is attributed to an increase in red blood cell volume, leading to a relative hemodilution and lower levels of hemoglobin. Similarly, colloid osmotic pressure decreases during pregnancy, lowest at 30-34 weeks of gestation [19]. The increase in plasma volume is likely to be triggered by the fall in systemic vascular tone in pregnancy [12].

1.1.5 Aerobic capacity

Resting or submaximal $\dot{V}O_2$ during weight-bearing exercise increases proportional to maternal weight gain during pregnancy whereas $\dot{V}O_{2max}$ during pregnancy is poorly explored due to the perceived risk of inducing fetal stress during testing including reports of fetal bradycardia [3, 20]. However there is evidence that $\dot{V}O_{2max}$ is conserved during pregnancy, and athletes and physically fit women may even increase their $\dot{V}O_{2max}$ following pregnancy indicating that pregnancy may have an added effect in well trained women [17, 21].

1.1.6 Adaptation of the endocrine system

Pregnancy influences a wide range of hormones. Progesterone is produced by the corpus luteum in the luteal phase of the menstrual cycle and early pregnancy. After eight weeks of pregnancy, placental trophoblasts become the main source of progesterone. Progesterone and estrogens increases during pregnancy, and both sex steroid hormones can influence cardiac growth, cardiac output and blood volume in animals. Progesterone can induce hypertrophy whereas estradiol have anti-hypertrophic properties and increases cardiac output [22]. Relaxin produced by the corpus luteum contribute to the decrease in vascular resistance and increased cardiac output in pregnancy [23]. How the levels of circulating catecholamines are affected by pregnancy is debated [22], but noradrenalin infusion leads to less vasoconstriction in healthy pregnant women, compared to both non-pregnant women and women with pregnancy-induced hypertension [24]. In pregnant women an increase in estrogens leads to an upregulation the renin-angiotensin system (RAS) and the serum AngII levels are increased [25]. However, in

healthy pregnancies RAS activity remains low as AngII sensitivity is decreased [26], due to specific changes in the AngII-receptors sensitivity [25, 27, 28].

1.2 Differences between physiological and pathological heart hypertrophy

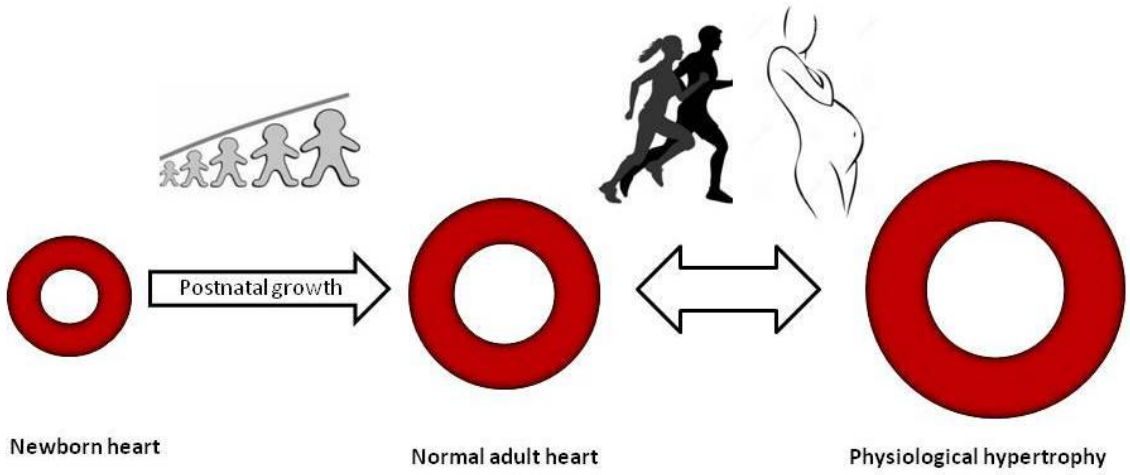

Figure 1 Physiological heart hypertrophy

Schematic representation of the left ventricle in short axis showing physiological postnatal heart growth. In the human heart endurance training or pregnancy leads to heart hypertrophy characterized by a proportional increase in wall thickness and chamber enlargement. The hypertrophy is reversible, i.e. the heart will go back to normal size postpartum or if the training ceases. Figure modified from Bernardo et al [29] .

Increased ventricular mass as a response to chronically increased afterload or volume overload on the heart is referred to as pathological hypertrophy. Cardiac hypertrophy is considered a poor prognostic sign and may represent the first stage in development of heart failure. However, the heart hypertrophy observed in postnatal growth [30], in response to exercise training [29, 31, 32] and in pregnancy [7, 22, 33, 34] differs from pathological hypertrophy both at the structural, functional and molecular levels and may be referred to as physiological hypertrophy [29, 31, 35] (Figure 1). The growth of human hearts is most rapid during the first postnatal months. The neonatal period is characterized by myocyte hyperplasia whereas in the heart growth of childhood the total number of myocytes remains relatively constant while myocyte volumes and deposition of collagen and the number of connective tissue cells increases [30].

Different stimuli induce different forms of ventricular hypertrophy. A pathologically increased afterload, such as in hypertension or in obstruction of the outflow tracts of the

ventricles, increases systolic wall stress and typically lead to a concentric hypertrophy with thickening of the walls of the heart, a small ventricular chamber and increased myocyte diameter whereas a pathologically increased volume load, as can be seen in valve disease, such as in aortic regurgitation, produces increased diastolic wall stress and may lead to an eccentric hypertrophy with an increased luminal diameter, a thinner ventricular wall and increased myocyte length [22, 29, 35] (Table 1 and Figure 2).

Table 1 Characteristics of physiological and pathological heart hypertrophy

	Physiological	**Concentric**	**Eccentric/dilated**
LV wall and chamber growth	LV wall = LV chamber	LV wall > LV chamber	LV wall << LV chamber
Myocyte growth	Proportional	Length < Width	Length >> Width
Cardiac dysfunction	No	Maybe	Advanced
Fibrosis	No	Yes	Extensive
Myocyte damage	No	Necrosis and apoptosis	Myocyte cell death

Table based on Bernardo et al [29], Chung et al [22] and Heineke et al [35].

Physiological stimuli may also induce concentric and eccentric hypertrophy. Both endurance training and pregnancy will increase venous return to the heart leading to increased volume load and an hypertrophy characterized by a proportional increase in wall thickness *and* chamber enlargement, in contrast to the thinning of the ventricular walls seen in pathological eccentric hypertrophy following volume overload or the concentric hypertrophy following pressure overload [29].

Despite some similarities at the macroscopic level, there are distinct differences between physiological and pathological heart hypertrophy at the structural, functional, metabolic and molecular level (Table 1). Pathological hypertrophy causes cell death via apoptosis and necrosis leaving room for extracellular accumulation of collagen causing fibrosis. The increased stiffness of the ventricles and impaired electrical signal conduction lead to impaired mechanical function of the ventricles. Reduced capillary density and interstitial accumulation of fibrotic tissue lead to reduced tissue oxygen tension, myocardial ischemia, and further cell death. These sequences of structural and functional changes may eventually lead to advanced cardiac dysfunction and heart failure [29, 35]. The heart is capable of utilizing fatty acids,

glucose and lactate as substrates for ATP-production, with fatty acid oxidation responsible for 2/3rds of the ATP synthesis in the normal heart [36]. In pressure induced heart hypertrophy there is an early impairment of fatty acid oxidation followed by a progressive decrease in glucose oxidation and overall ATP-production before development of heart failure [37]. Thus the failing heart could be referred to as *an engine out of fuel* [38]. In heart hypertrophy following high intensity aerobic exercise, there is improved mitochondrial function and an increase in glucose oxidation allowing more effective energy production as glucose oxidation will produce more ATP per molecule of oxygen consumed compared to oxidation of fatty acids [39].

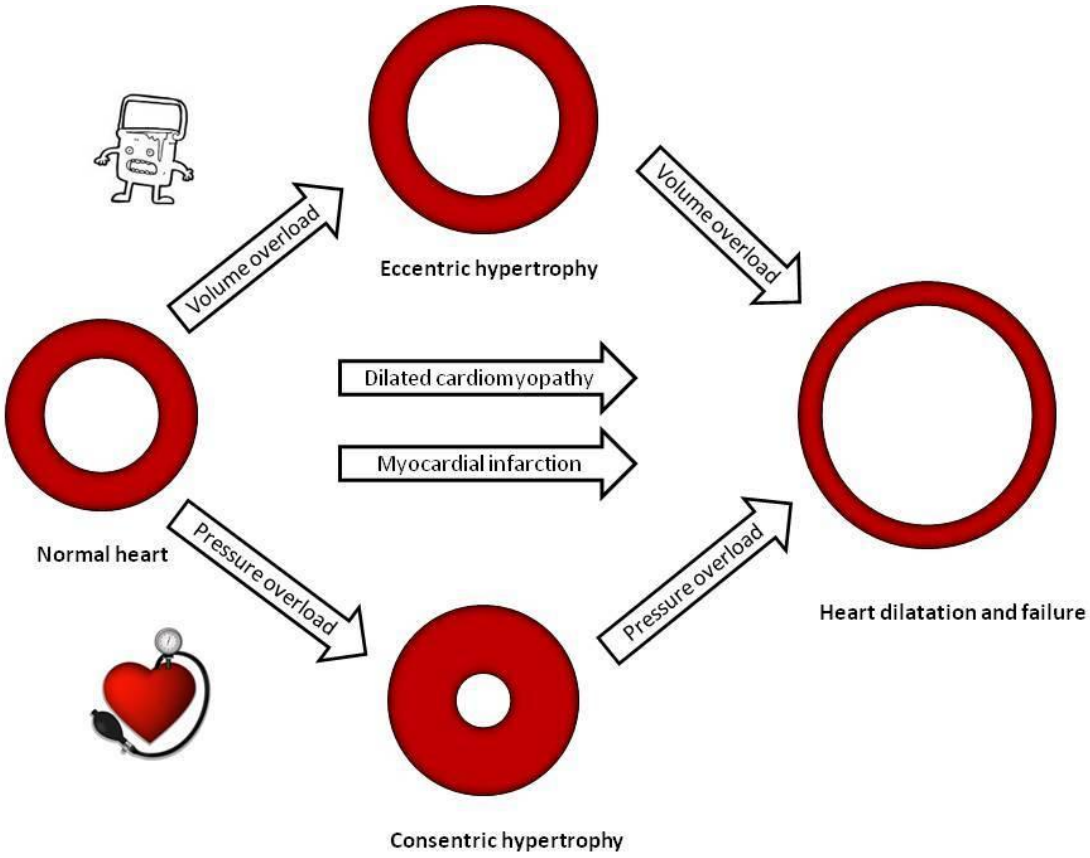

Figure 2 Pathological heart hypertrophy

Schematic representation of the left ventricle in short axis. Volume overload, as seen in valve regurgitation, leading to eccentric hypertrophy. Pressure overload, as in systemic hypertension or obstruction of LV outflow tract, leads to concentric hypertrophy. Heart dilatation and failure represent the end stage of these processes. A sick or damaged myocardium, as seen in dilated cardiomyopathy or after massive myocardial infarction, may lead to dilated heart failure without hypertrophy. Figure adapted from Heineke et al [35] and Bernardo et al [29] .

Table 2 Differences between physiological (caused by pregnancy and exercise) and pathological cardiac hypertrophy

	Pregnancy	Exercise	Pathological
Cardiac function	-	-/↑	↓↓
Reversibility	+	+	-
Fetal gene induction	-	-	↑
Fibrosis	-	-	↑
Angiogenesis	-	-/↑	↓
Signaling pathways	PI3K/Akt ERK1/2 Calcineurin	PI3K/Akt	Gaq MAPKs Calcineurin

Table adapted from Chung et al [22].

Physiological heart hypertrophy following endurance training and in pregnancy are often regarded as similar phenomena following different stimuli. However, Chang et al have pointed out several characteristics that distinguish heart hypertrophy in pregnancy from hypertrophy following exercise training [22] (Table 2), and describe evidence of specific cardiac transcriptional profiles defining pregnancy and exercise [40].

1.3 Increased afterload on the heart in pregnancy

The cardiovascular and hemodynamic changes that take place in pregnancy has a potential to make the heart more vulnerable to stress, and manifestations of heart conditions well compensated for before conception can unmask during pregnancy. The pregnant woman may be at risk for complication during pregnancy, delivery and in the postpartum period [11].

1.3.1 Pregnancy in women with congenital heart disease

Advances in cardiac surgery and improved care for children with congenital heart defects has lead to an improved survival, and there is a growing population of women of childbearing age with congenital heart disease [11, 41, 42]. Many of these will have residual impairment of their heart function that increases their risk of cardiovascular complications during pregnancy [11, 43, 44]. Significant LV outflow tract obstruction, as can be seen in aortic stenosis, coarctation of the aorta, interrupted aortic arch and in some complex cardiac defects, will

increase afterload on the ventricles. Severe symptomatic LV outflow tract obstruction is a contraindication for pregnancy and should be treated before pregnancy, or the woman should be counseled against pregnancy [11]. However, some women with significantly increased afterload on the heart will get pregnant, and knowledge of how pregnancy influences cardiac structure and function is crucial for providing optimal care for these women and their fetuses.

1.3.2 Hypertensive disorders of pregnancy

Systemic hypertension is often the cause of increased cardiac afterload in pregnancy. Hypertension may be preexisting, as in essential hypertension or as a part of metabolic syndrome, or it can be secondary to other medical conditions, such as renal disease or endocrine disorders. Failure of the cardiovascular system to adapt to physiological changes of pregnancy can lead to hypertensive disorders of pregnancy, frequently associated with adverse outcomes for mother and offspring [11, 45]. Approximately 3-10 % of pregnancies are complicated by preeclampsia, characterized by hypertension developing together with proteinuria after 20 weeks of gestation. Although preeclampsia is a heterogenous condition and several mechanisms may be involved, abnormal placentation, with poor penetration of cytotrophoblasts into the maternal deciduas and adjacent spiral arteries, appears to be central in its pathophysiology [46]. Early onset disease is more severe and associated with a higher rate of premature birth, small for gestational age neonates as well as a higher rate of recurrence. As we are currently not able to effectively predict and prevent the development of preeclampsia, early recognition is required to assure adequate antenatal care and management. Premature delivery may be necessary to prevent eclampsia, the end stage of the disease characterized by generalized seizures, or to rescue fetuses with severely compromised circulation and growth restriction due to placental insufficiency.

In contrast to healthy pregnancies, where decreased systemic vascular tone leads to an increase in circulating blood volume, relative hemodilution and decrease in colloid osmotic pressure, preeclampsia is characterized by high vascular resistance due to peripheral vasoconstriction and decreased arterial compliance [47]. Thus the increase in blood volume is small, or absent, leading to a relative hemoconcentration compared to in healthy pregnancies [16]. Endothelial dysfunction may play a central role in the pathogenesis of preeclampsia. There is firm evidence that several circulating factors that are released by the injured or

activated placental endothelium in preeclamptic women are capable of inducing endothelial dysfunction in the maternal circulation [47].

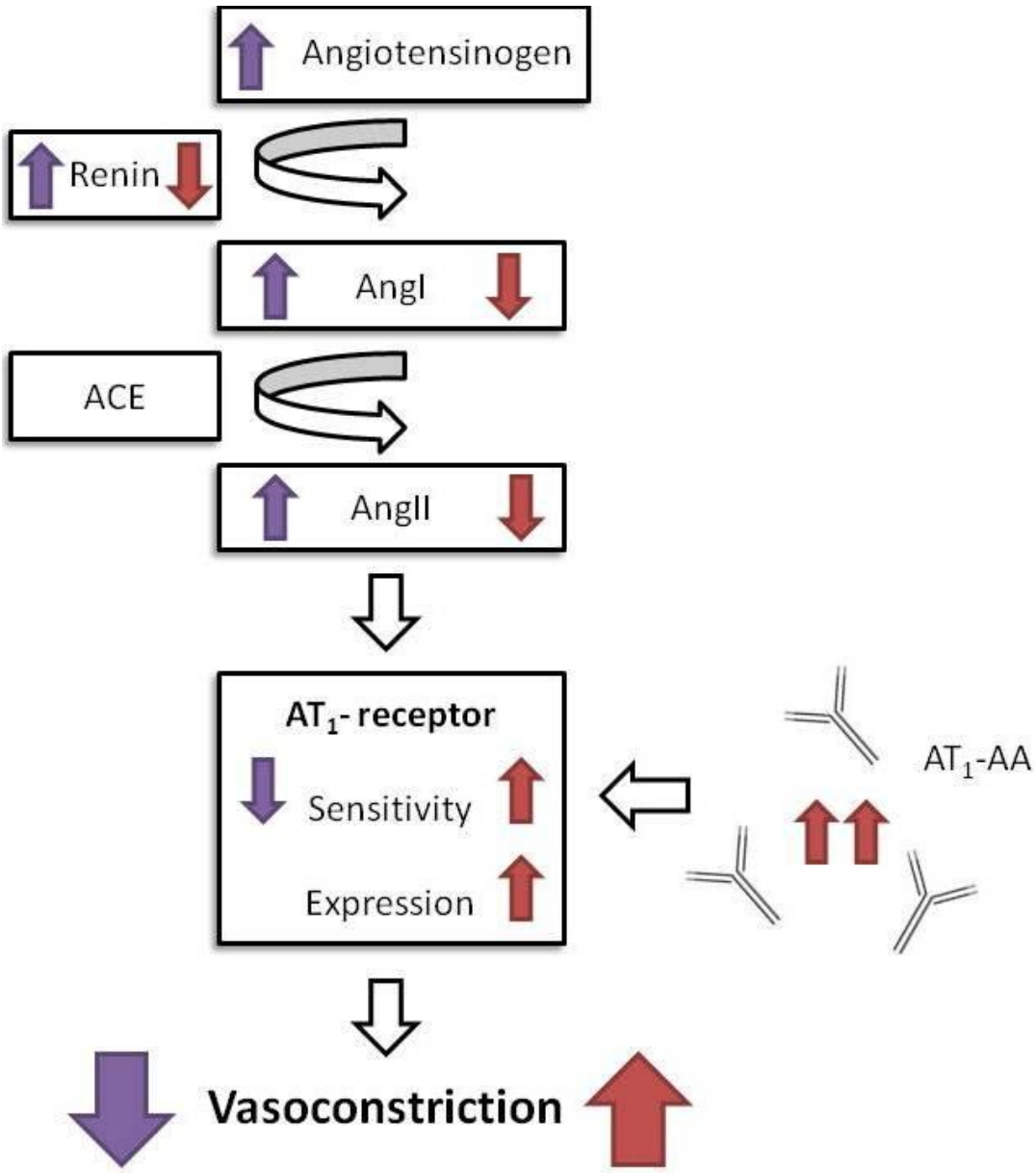

Figure 3 The renin-angiotensin system (RAS) in pregnancy.

RAS components and AT_1-receptors in healthy pregnancy (purple arrows) and preeclampsia (red arrows).

The regulation of the RAS in preeclampsia differs from that in healthy pregnancy. AngII levels are high but the pressor response to AngII is reduced in normal pregnancy. Preeclamptic women have lower circulating levels of RAS components, but increased AT_1-receptor activation [25, 27, 28] (Figure 3). Renin synthesis is suppressed in preeclampsia, possibly due to negative-feedback as stimulation of the AT_1-receptor suppress renin release

[25]. The discovery of AT_1-receptor agonistic autoantibodies (AT_1-AA) in preeclamptic women may explain this feature [48-52]. In rats AT_1-AA reduces trophoblast invasion [51] and induces renal changes and atherosis-like lesions in the spiral arteries of the placenta similar to in what is seen in women with preeclampsia [52]. Furthermore, AT_1-AA can activate the inflammatory response similar to what is seen in preeclampsia through stimulation of ROS production [27, 51, 53], and AT_1-AA from preeclamptic women can induce preeclampsia-like changes if infused into pregnant mice [54].

1.4 Endurance training and exercise in pregnancy

Cardiovascular training, in terms of repeated episodes of physical activity performed over a longer period causes adaptations in the cardiovascular system that enables the trained person to increase physical performance i.e. exercise at a higher intensity or at the same intensity for a longer period of time. Regular endurance training increases LV cavity volume, stroke volume and thus maximal CO leading to increased $\dot{V}O_{2max}$ reflecting an improved overall aerobic capacity and a higher level of cardiovascular fitness [17].

1.4.1 Training in normal pregnancies

Clinical guidelines encourage moderate exercise in pregnancy due to its multiple beneficial effects for both the mother and her offspring [17, 55-57]. In the long-term, women who continue to exercise during pregnancy appear to exercise at a higher intensity, deposit less fat, improve fitness, have a lower cardiovascular risk profile, a more favorable metabolic profile with less gestational diabetes, lower incidence of low back pain and urinary incontinence and reduced symptoms of depression compared to women who cease to exercise during pregnancy [17, 58, 59]. In a study by *Clapp et al* women who continue to engage in vigorous training during pregnancy had babies that weigh slightly less than among the regular training women who quit exercise in pregnancy. There were no negative effects on physical growth or neurodevelopmental outcome at five years, exercise offsprings performed slightly better on Wechsler scales and tests for oral language skills, and they weighed less and had less subcutaneous fat deposits [60]. Well-trained athletes tolerate high volumes of training during uncomplicated pregnancies and aerobic training during pregnancy may even have an additive effect on physical fitness post pregnancy compared to before pregnancy [21, 61]. However, there remains some concern regarding high intensity training because of the possible adverse effect on the placental blood flow and episodes of fetal bradycardia observed in pregnant elite

athletes training at high intensity above 90% of maximal heart rate examined in the second trimester [20]. Thus, a recent comparison of guidelines for physical activity during pregnancy identified conflicting recommendations in between countries regarding vigorous-intensity activity in pregnancy [57].

1.4.2 Training in complicated pregnancies

Although there is good evidence that training during healthy pregnancy is beneficial, there are concerns about how to advice women with complicated pregnancies.

1.4.2.1 Preeclampsia

A systematic review of evidence indicates a trend towards a protective effect of physical activity in the prevention of preeclampsia [62]. Exercise may protect against preeclampsia by reducing oxidative stress, improving placentation and preventing endothelial dysfunction. Training during pregnancy might mitigate the effects of placental insufficiency or the angiogenic imbalance associated with preeclampsia [62, 63]. However, a large (>85000 pregnant women) prospective cohort study from Denmark indicated that physical activity for more than 270 minutes per week may *increase* risk of severe preeclampsia.

1.4.2.2 Heart disease

Evidence based recommendations of how to balance the possible risk related to strenuous exercise against the advantages of physical fitness in pregnancies complicated by heart disease are lacking. In the *European Society of Cardiology (ESC) Guidelines on the management of cardiovascular disease during pregnancy* exercise testing is recommended to assess disease severity and predict outcome but do not advise for or against physical activity in pregnancy in different settings of heart disease in pregnant women [11].

1.4.2.3 Adiposity and diabetes

There is some evidence that physical training in pregnancy reduces risk of gestational diabetes and restricts weight gain in healthy pregnant women [56, 59, 64, 65]. However, the information available in the literature is limited with regard to the role of physical activity for pregnant women with established diabetes mellitus or severe obesity. According to the American College of Obstetricians and Gynecologist committee opinion for obesity in

pregnancy "*...all overweight or obese women, ... should be encouraged to follow an exercise program*." However, what such an exercise program should entail is not specified [66].

1.5 Clinical evaluation of cardiovascular function in pregnancy

As summarized earlier, pregnancy induces a variety of hemodynamic changes which may alter the balance in compensated cardiovascular diseases and put the pregnant woman or fetus at risk. All women in reproductive age with significant cardiovascular disease should be counseled before planning to get pregnant. In pregnant women with cardiovascular disease a throughout cardiovascular assessment is required to detect women at risk of adverse outcome and to customize an adequate cardiovascular follow-up throughout pregnancy, labor and in the postnatal period [11]. In addition, previously undiagnosed heart conditions may become symptomatic due to the hemodynamic alterations in pregnancy and pregnancy associated cardiovascular diseases like peripartum cardiomyopathy. More commonly, hypertensive disorders like preeclampsia will debut during pregnancy.

In all pregnancies, a general history should be taken and a clinical examination including auscultation of the heart and measurement of blood pressure should be performed at regular intervals, and if heart disease is suspected further examinations should be done, and followed up accordingly [11].

Procedures involving radiation exposure should be minimized, and if possible, deferred to after 12 weeks of gestation to reduce the risk of radiation induced congenital malformations. Computer tomography is not recommended. Magnetic resonance imaging may be used to diagnose complex heart defects or aorta disease, but gadolinium should be avoided in pregnancy.

Electrocardiography (ECG) and echocardiography are non-invasive procedures that can be performed safely, and repeated if necessary, in pregnancy. In late pregnancy the heart is rotated towards the left and there is a 15-20 left axis deviation on ECG. Changes in ST segments, Q wave and T wave as the heart changes position can mimic LV hypertrophy. Echocardiography is indicated when dyspnoea occurs during pregnancy or a new pathological murmur is heard. In some cases, such as in women with complex congenital heart disease, transeosophagal echocardiography may be a useful tool [11].

Exercise testing is useful in assessing functional capacity in pregnant women with congenital heart disease or asymptomatic valve disease. According to ESC Guidelines submaximal exercise test to 80% of predicted maximal heart rate should be used in asymptomatic pregnant women with suspected cardiovascular disease [11]. Dobutamine stress test should be avoided. Stress echocardiography can be used to detect ischemia or prior to conception to assess myocardial reserve in patients with cardiomyopathy, valvular disease or congenital heart defects and reduced LV function.

Impedance cardiography (ICG) is a highly accessible, non-invasive, operator-independent and easy to perform non-invasive test to measure CO in pregnancy, and the new generation of ICG machines have been validated and shown to be accurate, reproducible, reliable and useful also in pregnant population [67, 68]

Endothelium-dependent vasodilatation can be examined non-invasively in humans measuring flow mediated dilatation (FMD) of the brachial artery [69, 70], and several studies have evaluated FMD in pregnancy [71-77]. However, it is debated whether endothelial function in the peripheral vessels correlates with endothelial function in the coronary vascular bed [70, 78-80]. We are not aware of any published studies evaluating endothelial function in the coronary circulation during pregnancy, and to our knowledge the effect of increased afterload on coronary flow reserve (CFR) during pregnancy has not been reported.

2. Aims of the thesis

The aims of this thesis were to investigate how cardiac function, remodeling and gene expression are affected in pregnancy in response to cardiovascular stress and to detect possible adverse effects of chronic AngII infusion, TAC or HIIT on the fetus. Additionally we wanted to evaluate if coronary endothelial function is influenced by pregnancy and increased cardiac afterload.

The *specific objectives* were to:

- Investigate the effects of chronic AngII infusion on the hearts of pregnant rats and to test the hypothesis that pregnancy protects against presumed detrimental changes caused by AngII.
- Investigate the effect of isolated chronic pressure load induced by TAC on the hearts of pregnant rats and to test the hypothesis that pregnancy is protective against the negative effects of increased afterload on the heart.
- Evaluate whether an established model of HIIT in rats is applicable in pregnancy and to investigate the effect of HIIT on the maternal heart.
- Determine if HIIT has any adverse effects on the fetus.
- Evaluate a non-invasive method of assessing CFR in rats using high concentration of inhaled isoflurane for coronary vasodilation.
- Investigate the differences in CFR between pregnant and non-pregnant rats and study how CFR is affected by increased afterload in pregnant and non-pregnant rats.

3. Methods and methodological considerations

We used animal experiments to approach clinical problems related to cardiac remodeling in pregnancy. Ideally experiments should have been performed on humans. However, to test the effects of increased afterload on the heart, we had to do invasive procedures that could not be performed in pregnant women for obvious ethical reasons. Despite species differences, animal studies have some advantage compared to studies in human as they can be performed under strictly controlled laboratory conditions. Using animals of the same strain, age and size results in considerably less individual variability, and thus a lower number of subjects are necessary to find relevant differences between groups.

Rats are often used as animal models in pregnancy research because they have a similar type of placenta to human [81] and the duration of rat pregnancy is relatively short (~21-22 days). Furthermore, rats are regarded as robust when it comes to tolerating surgical interventions and there is an abundance of experiments performed investigating circulatory physiology in non-pregnant rats. Compared to mice, larger size of rats makes it is easier to investigate their heart function using echocardiography and intracardiac conductance catheter.

3.1 Animal models

All animal experiments were performed at Unit of Comparative Medicine, Faculty of Health Sciences, University of Tromsø and experiments conformed to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes [82]. All procedures were approved by the Norwegian Committee on Ethics in Animal Experimentation with project ID 907 (Paper A and D), ID 2177 (Paper B and D) and ID 2853 (Paper C). In Paper A, B and D Wistar rats where used. In Paper C Sprague-Dawley rats were used based on experience from previous studies on a HIIT-protocol similar to ours in non-pregnant rats. The CFR studies presented in Paper D were performed on the same rats presented in Papers A and B.

3.1.1 Power analysis

In order to comply with good ethical standards in animal research [83], we strived towards using as few animals as possible needed to give reliable results by doing power analyses before starting experiments. Assuming that following interventions eight out of ten rats will develop cardiac hypertrophy compared to less than one out of ten in the control group, we

calculated that nine animals are required in each experimental group to detect the effect of intervention at a significance level (alpha) of 0.05 with a power (beta) of 80%. In Paper C fewer rats than expected became pregnant after mating and the number of pregnant animals is smaller than predicted when designing the study (five and seven, respectively). .

3.1.2 General considerations on animal models in pregnancy

In all our studies we used young female rats aged 9-12 weeks at the start of experiments. Thus the rats were not fully grown and non-pregnant as well as pregnant rats continued to gain weight during the observation period. The animals were housed in cages in pairs under controlled conditions of temperature and humidity and light-dark periods of 12 h, and with free access to water and food. All rats (pregnant and non-pregnant) were fed a pellet diet especially produced for breeding rodents (Rat and Mouse NO.3 Breeding, Special Diet Services, Witham, Essex, U.K.) *ad libitum* and had free access to tap water.

3.1.3 Mating and length of gestation

In Papers A and B pregnant rats were obtained by mating with a male rat housed together with two female overnight for 12-18 hours. The rats where constrained in a cylindrical tube, an otoscope was inserted in the rat vagina and the presence of a vaginal sperm plug confirmed that copulation had taken place. The day of the vaginal plug was considered gestational day (GD) 0.5. Rats are naturally nocturnal animals, and since the training sessions for practical reasons had to take place in daytime, the circadian rhythm of the rats in Paper C was changed by reversing light/dark (12/12 hour) cycle. The rats were housed together with a male rat and mated at daytime for 6-7 hours, and the day of the mating was considered GD 0. In Paper C we experienced that fewer animals conceived after mating than in Papers A and B, both in HIIT and sedate rats. Thus the decreased pregnancy rate was not caused by decreased fertility in training rats, but probably related to a shorter time of pairing together with a male. As the estrous cycle in rats is strongly influenced by light periodicity the reversed light/dark cycle applied in Paper C may also have affected the pregnancy rate negatively.

The normal gestational length in the rat is 21-22 days. In Paper A the terminal experiments were performed at GD 18.5-20.5 in pregnant animals. However, fetal weight gain in the last few days of pregnancy is significant [84], and the mean body weight of the fetuses in each dam more than doubled from GD 18.5 (1.36±0.03 gram) to GD 20.5 (3.08±0.65 gram). Thus in Paper B and Paper C we strived towards doing all experiments at the same length of

gestation. In Paper B 20 out of 22 pregnant rats was examined at GD 20.5, one at GD 19.5 and one at 21.5. Two rats delivered between GD 20.5 and 21.5 and were excluded from further analyzes. In Paper C all pregnant rats were examined at GD 20.

3.1.4 Chronic angiotensin II infusion

Increase in circulating AngII is a key component in the mechanisms behind pathological remodeling of the heart and thus inhibition of the response to AngII is essential in treatment of cardiovascular diseases [85-87]. AngII may induce heart remodeling through its direct effects on the heart and via increased afterload due to vasoconstriction in the systemic circulation [87-89]. In pregnancy the renin-angiotensin system (RAS) is upregulated leading to an increase in plasma concentrations of AngII [25, 90]. Even if the exact mechanism causing preeclampsia is not known, angiotensin receptor activation appears to drive the vascular maladaptation seen in pregnancy [91], pregnant women who subsequently develop preeclampsia appear to be more sensitive to infused AngII [25, 92] and there is some evidence of AT_1-AA playing an important part in the development of preeclampsia [48-54]. Thus the heart's response to AngII in pregnancy is of particular interest.

In Paper A heart hypertrophy was induced by implanting mini osmotic pumps (Alzet® Model 2002, Cupertino, CA, USA) releasing AngII subcutaneously. Before including pregnant animals, four different concentrations of AngII infusion (0, 150, 300 and 400 ng/kg/min) were tested in a total of 16 animals. The lowest rate of AngII infusion (150 ng/kg/min) that led to heart hypertrophy was chosen as a low concentration was considered closest to a physiological challenge, and higher concentrations of chronic AngII infusions may lead to cachexia without cardiac hypertrophy [89]. The pumps were implanted 9-10 days before terminal experiments, corresponding to GD 8.5-9.5 in pregnant dams. Mini osmotic pumps releasing saline were used in control animals (sham). Thus four groups were studied; non-pregnant and pregnant sham, and non-pregnant AngII and pregnant AngII.

3.1.5 Transverse aortic constriction

Whereas Ang II may induce heart remodeling through direct effects on the heart as well as increased afterload, inducing LV outflow obstruction by mechanical constriction of the aorta will mimic the effects of purely increased afterload, as seen in aortic stenosis, co-arctation of the aorta or an interrupted aortic arch.

In Paper B the surgical procedure was performed on intubated and ventilated rats under general anesthesia with inhaled isoflurane (2.5% in 100% oxygen for maintenance). The rats were put in a closed chamber filled with 4% isoflurane in 100% oxygen (Vevo Compact Anesthesia System, VisualSonics, Toronto, Canada). The spontaneously breathing rats were fixated on a semi upright worktop hanging by the incisors on a tight string. Isoflurane was provided by a mask held over the snout to maintain anesthesia. The tongue was carefully pulled out by a pair of blunt tweezers and the vocal cords were visualized by pointing a bright light source at the external larynx. A 16G peripheral venous catheter with a shortened and blunted stylet used as mandrin was inserted in the rat trachea, the stylet was removed and the catheter was connected to a ventilator (New England Medical Instruments Inc., Medway, MA, USA) delivering tidal volumes of 2-3 ml at a frequency of 60 per minute. 2.5% isoflurane in 100% oxygen was used to maintain the anesthesia. The rats were placed supine on a warm electric pad and the temperature was kept stable at approximately 38°C. The heart rate and the rectal temperature were monitored continuously. A heating lamp was used when required. Hair was removed with a mechanical shaver and application of depilatory cream (Vichy Laboratories, Paris, France). Surgery was performed under sterile conditions. Analgesia was provided with subcutaneous buprenorphine (Temgesic, Reckitt Benckiser, UK) 0.05 mg/kg and local bupivacaine (Marcain, AstraZeneca, Sweden). Pain reflexes were checked before surgery and concentration of inhaled isoflurane was increased if appropriate. After skin incision, the upper half of the sternum was divided in the midline using blunt scissors. Care was taken to remove the thymus in one piece, as in our experience this would reduce the risk of extensive bleeding. In most cases satisfactory hemostasis could be obtained by applying gentle pressure with a sterile swab. Then the aortic arch was carefully dissected free of the surrounding tissues. TAC was performed by tying a blunted and bended stylet from a 16G or 18G IV catheter (Optiva, Smith Medical International Ltd., Rossendale, UK) tightly to the aortic arch between the brachiocephalic trunk and the left common carotid artery. When the stylet was removed, a partial constriction of the transverse aorta was created. In sham animals, the exact same procedure was performed; the aortic arch was lifted, but not tied. The sternotomy and the skin incision were closed with 5-0 sutures (Polysorb, Synture, Mansfield, MA, USA). The rats were extubated and put in an incubator (Vetario S10 Intensive Care Unit, Brinsea Products Ltd, N. Somerset, UK) at 28-30° C for the recovery period. Postoperatively they were kept in separate cages with free access to water and food. Analgesia with buprenorphine 0.05 mg/kg subcutaneously was provided every 12 hour for 48 hours.

Surgery was performed in a total of 57 rats. Four animals died during surgery. In three animals we were unable to control extensive bleeding from the vascular bed of the thymus or the internal thoracic arteries. In one rat the transverse aorta was tied completely without having the stylet in place and the animal developed acute heart failure and died of respiratory failure within minutes. Six animals died or were euthanized after surgery; three developed symptoms of acute heart failure (dyspnoea, cyanosis and hemoptysis), one respiratory distress due to pneumothorax, one stopped breathing shortly after extubation, probably related to drug overdose, and one was found dead in the cage. In the 47 surviving rats, there was no statistically significant difference in weight gain after surgery in TAC animals compared to sham animals.

In the initial experiments we tested two sizes of TAC, using stylets from 16 and 18 G catheters. A total of nine rats had a tighter TAC using a stylet from an 18G IV catheter. Three of them developed symptoms of acute heart failure postoperatively and were euthanized, while this did not happen to any rats where the bigger stylet was used. Thus the less pronounced constriction was performed in the majority of animals. Two rats with a tighter aorta constriction were excluded due to inappropriate banding time, and two rats are included in each TAC group (pregnant and non-pregnant).

We performed the surgery 14 (range, 13-17) days before terminal experiments, corresponding to GD 5.5-8.5 in pregnant rats, thus imitating hypertensive disorders of pregnancy. A longer period following TAC could have lead to more pronounced LV hypertrophy and overt heart failure [93], but the short duration of pregnancy in rats did not allow this.

3.1.6 High intensity interval training

Rats are used as an animal model in exercise in pregnancy for several reasons. The rat placenta is quite similar to the human placenta, rats adapt to training similar as humans do and, most importantly, rats appear to enjoy running [81]. Sprague-Dawley rats were subjected to high intensity interval training (HIIT) modified from a protocol originally described by Wisløff et al [94] and previously used in mice at our facility [39, 95].

3.1.6.1 High intensity interval training in pregnant rats

Five days/week one group of rats was subjected to exercise sessions of 10 bouts of 4 min high intensity treadmill running at 25° inclination separated by 2 min of treadmill running at low intensity (50-60% of the speed required to achieve estimated $\dot{V}O_{2max}$). Three or four rats ran

in parallel tracks on the same treadmill. Stimulation such as gentle physical handling, an airbrush or low current shock grids were used to secure high intensity. Usually the use of the shock grids was not necessary and painful stimulus was kept to a minimum. Contrary to described by Wisløff et al [94], rewarding the rats with chocolate was not effective in stimulating the rats to maximal effort. Speed was set in bouts of 85 to 90% of the speed required to achieve estimated $\dot{V}O_{2max}$ of rats at baseline, slowest in the first bouts of each session. Typically rats tolerated and achieved highest speed in the last bouts of each session. All rats were continuously monitored during the training sessions. Throughout the training period treadmill speed was increased gradually as long as the rats did not show signs of exhaustion.

After 11-12 sessions of training, training was stopped for one day and one male rat was put in a cage with two female rats for 6-7 hours. The second day after mating, the rats resumed HIIT again. In pregnant rats, the day of mating was considered GD 0. HIIT was stopped corresponding to GD17-18 in pregnant rats out of concern for how the highly pregnant rat would perform on the tread mill and with regards to animal welfare as advised by the responsible veterinarian. The rats in the HIIT groups completed a total of 24 one hour sessions of HIIT with an average total running distance of 83±4 km per rat. Rats of the same age and size was kept as sedentary controls, pregnant and non-pregnant.

3.1.6.2 Measuring maximal oxygen consumption in the rat

Maximal oxygen consumption ($\dot{V}O_{2max}$) was measured in seven rats during the first days of training. The rats ran on a treadmill at 25° inclination in a metabolic chamber (Modular treadmill with Oxymax open circuit calorimeter, Columbus Instruments, OH, USA). Ambient air was pumped into the chamber at a fixed rate and samples of extracted air form the chamber were continuously analyzed for oxygen and carbon dioxide. The speed was gradually increased until oxygen consumption leveled off despite increased running speed, and $\dot{V}O_{2max}$ was defined as $\dot{V}O_2$ measured at this speed divided by rat weight [39, 94]. The treadmill velocity at $\dot{V}O_{2max}$ was used to set the speed of the treadmill for HIIT. $\dot{V}O_{2max}$ was measured again in the same rats after 11-12 training sessions, before mating.

When running on a treadmill in a closed chamber, the rat is not accessible to physical stimulation except low current shock grids. We found it difficult to assure that the rats actually ran at their highest performance, and sometimes the rats would sit down on the low

current grid instead of running, even if V̇O2-measurements indicated that they was not running at their $\dot{V}O_{2max}$. The estimated $\dot{V}O_{2max}$ varied considerably. Before training, mean $\dot{V}O_{2max}$ was 78 (range 70-90) ml/kg/min, corresponding to a maximal running speed of mean 19 (range 17-21) m/min. When $\dot{V}O_{2max}$ was measured again after 11-12 training sessions mean measured $\dot{V}O_{2max}$ was not increased, 75 (range 65-93) ml/kg/min, while average speed at $\dot{V}O_{2max}$ had increased by 16% to 22 (range 19-27) m/min, indicating that that the $\dot{V}O_{2max}$ measured did not detect an actual increase in physical performance. Thus $\dot{V}O_{2max}$-measurements were stopped after the first group of seven animals, and running speed in each session was set by the best judgment of the operator in the rest of experiments.

3.2 Small animal echocardiography

Echocardiography was performed using a high resolution ultrasound imaging system equipped with a RMV-710B transducer with a frequency of 25 MHz and a fixed focal length of 15 mm mounted on an integrated rail system (Vevo 770, Visualsonics, Toronto, Canada). Prewarmed ultrasound gel was used. The rats were anesthetized with isoflurane as previously described and silk tape (Leukosilk® 1.25cm, Smith&Nephew, London, UK) was used to fix the paws to the electrodes integrated in the plate provided with the ultrasound equipment. Conductance cream was used to secure good ECG signals. In Paper A the rats were spontaneously breathing 1.5-3.5% isoflurane in 100% oxygen provide by a mask over the snout. In Paper B and C the rats were intubated.

In Paper A the studies were performed by two investigators, M.C.S. and N.T.S., and all analyzes were performed offline by M.C.S. N.T.S. did only studies on non-pregnant (Ang II and sham) animals in Paper A. In Paper B and C all studies were performed and analyzed by N.T.S. In Paper D, only CFR calculations performed and analyzed by M.C.S. were included from the Ang II-study and N.T.S. performed and analyzed all studies from the TAC-study.

3.2.1 Motion-mode echocardiography

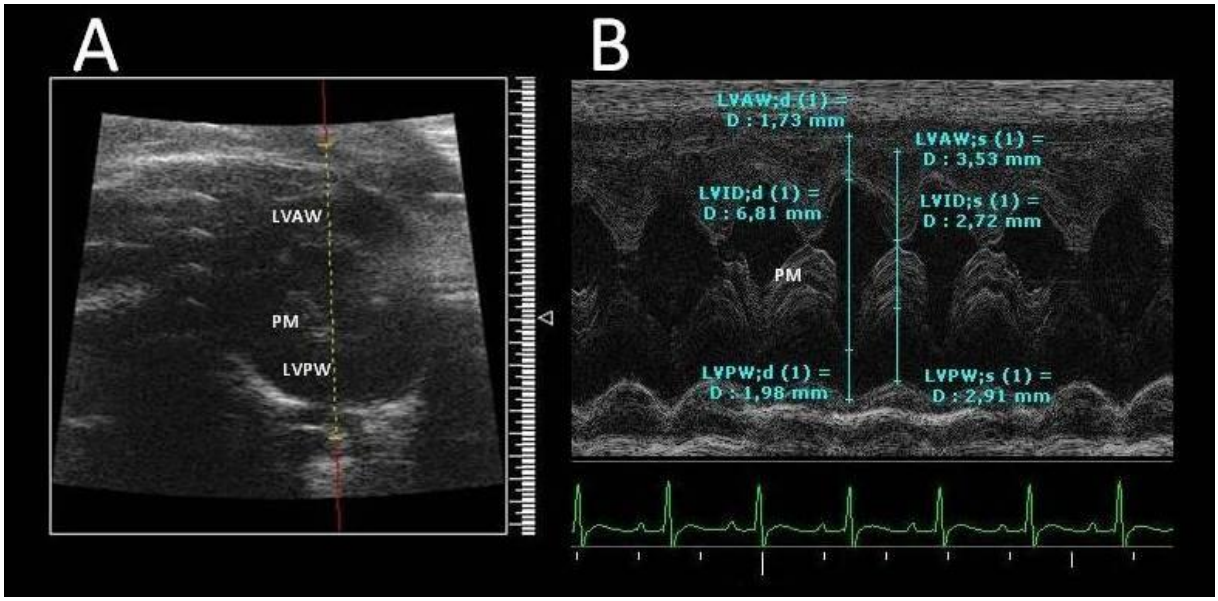

Figure 4 M-mode echocardiography from maternal left ventricle (LV)
A. Parasternal short axis view through the LV anterior wall (AW), papillary muscle (PM) and LV posterior wall (PW). B. Measurement of LVAW, LV inner diameter (ID) and LVPW in diastole (d) and systole (s).

Motion (M-) mode echocardiography recordings were obtained from the parasternal short- or long-axis views. All ultrasound based measurements were performed off-line without the knowledge of the animals' identity (Figure 4). Care was taken to select three consecutive cycles with good quality signals. The internal dimensions of the LV cavity and thickness of the anterior and posterior LV walls were measured. The heart rate (HR) was obtained from the electro-cardiogram signals. LV fractional shortening % was calculated as 100 x ((LVIDd-LVIDs)/LVIDd) where, LVIDd = LV internal diameter in diastole and LVIDs = LV internal diameter in systole. Stroke volume (SV) was calculated as LV EDV – ESV where, EDV = end diastolic volume and ESV = end systolic volume. The LV EDV was calculated as 7.0/(2.4 + LVIDd)x$LVIDd^3$ and the LV ESV was calculated as 7.0/(2.4 + LVIDs)x$LVIDs^3$ [96]. Cardiac output per minute (CO) was calculated as SV x HR. Relative wall thickness (RWT) was calculated using the formula: RWT = (LVPWd + LVAWd)/LVIDd where, LVPWd= LV posterior wall thickness and LVAWd = LV anterior wall thickness. LV mass was calculated using the formula: LV mass =1.04x$(LVIDd + LVPWd + LVAWd)^3$ - $LVIDd^3$ [97].

3.2.2 Coronary flow reserve

Coronary flow reserve (CFR) is used as a measure of coronary endothelial function. In the AngII- and TAC-studies, Doppler echocardiography was used to measure velocities in coronary artery and thus calculating CFR. This method is described in detail and evaluated in Paper D. In short, high concentrations (3.5%) of inhaled isoflurane were used as a vasodilating agent and were compared to the standard dose (1.5%) of isoflurane used to maintain anesthesia in rats not exposed to painful stimuli. CFR was calculated as the ratio between the peak coronary flow velocities (CFR_{peak}) and the velocity-time integrals (CFR_{VTI}) recorded at hyperemia (3.5%) and at baseline (1.5%).

3.2.3 Doppler echocardiography

Doppler echocardiography was performed in the AngII-study. Ejection time (ET), isovolumetric relaxation time (IRT), isovolumetric contraction time (ICT) and Tei-index was calculated from pulsed-wave Doppler signals from the LV inflow and outflow recorded simultaneously as following: ET, ejection time (total time of LV outflow). IRT, time from the end of LV outflow signal to start of the mitral flow signal. ICT, time from the end of mitral flow signal to the start LV outflow signal. Tei-index = (ICT+IRT)/ET [98]. Mitral valve E/A-ratio is the ratio of the early (E) to late (A) LV peak filling velocities. Results are presented in Appendix 1.

In the TAC-study we planned to evaluate the size of the banding and estimate the pressure gradient across the banding by B-mode and Doppler echocardiography. However, in most animals we were not able to visualize the banding site satisfactory, possibly due post-operative scaring. Color Doppler mode may have been useful in locating the constriction and thus facilitating obtaining useful measurement, but this was not available in the ultrasound machine used (Vevo 770) when these studies were performed.

3.2.4 Fetal echocardiography

In the AngII-study fetal echocardiography was performed in 126 fetuses in 29 dams. Data from two AngII-treated rats was excluded due to fetal bradycardia indicating that the rats were hemodynamically unstable during the experiment. Thus ultrasound measurements from a total 119 fetuses from 27 animals (12 AngII and 15 sham) were included. E/A-ratio, ICT, IRT and Tei-index [98] were calculated from fetal mitral flow signals. Doppler flow velocity

waveforms were obtained from the umbilical artery, ductus venosus and ductus arteriosus and the pulsatility index (the difference between peak systolic and minimum diastolic velocities divided by time-averaged maximum velocity) was calculated for all vessels. Results are presented in Appendix 2.

3.3 Intravascular blood pressure measurements and pressure-volume analysis of the rat heart

In the AngII- and TAC studies a 2F microtip pressure-volume (PV) catheter (SPR-838; Millar Instruments Inc, Houston, TX, USA) was inserted via the right carotid artery into the ascending aorta and through the aortic valve into the LV. The procedure was performed immediately after echocardiographic examinations (Figure 5A). Pain reflexes were checked and isoflurane concentrations increased as appropriate. Sharp scissors were used to cut through the skin and platysma muscles and trachea was identified (Figure 5B). The right carotid artery and vagus nerve was carefully dissected free, lifted up and split apart (Figure 5C). A micro vascular clamp was fixed to the proximal artery (Figure 5D) and the distal part of the artery was tied by sutures an externally fixated. A bend needle of an insulin syringe (Figure 5E, Omnican®, B. Braun Medical AG, Melsungen, Germany) was used to perforate artery and keep the perforation open as the catheter was introduced. Two sutures were tied tightly around the artery and the catheter to prevent bleeding as the clamp was removed and the catheter advanced into the carotid artery and ascending aorta (Figure 5D). Aortic BP was measured before the catheter was introduced into the LV. The PV-signal was recorded by a PowerLab using a LabChart 7 acquisition system (AD Instruments) and was used to verify proper position of the catheter in the LV (Figure 5F). The animal was allowed to stabilize before the baseline PV-loops were recorded. In Paper B PVAN 3.6 software (Millar Instruments Inc) was used to analyze PV-loop data.

Raw signals from volume measurements were calibrated with SV calculated from M-mode echocardiography. Mean arterial pressure (MAP) was calculated as 2/3 x diastolic BP + 1/3 x systolic BP, total peripheral resistance (TPR) as MAP divided by CO, and LV stroke work was calculated as the difference between maximum and minimum LV pressure multiplied with SV. Effective arterial elastance (Ea) was calculated as the ratio of LV end-systolic pressure to SV. dP/dt_{max}, dP/dt_{min} and isovolumetric relaxation constant (Tau) were calculated

by the software. LV end-systolic elastance (Ees) was calculated as the ratio of end systolic pressure to ESV.

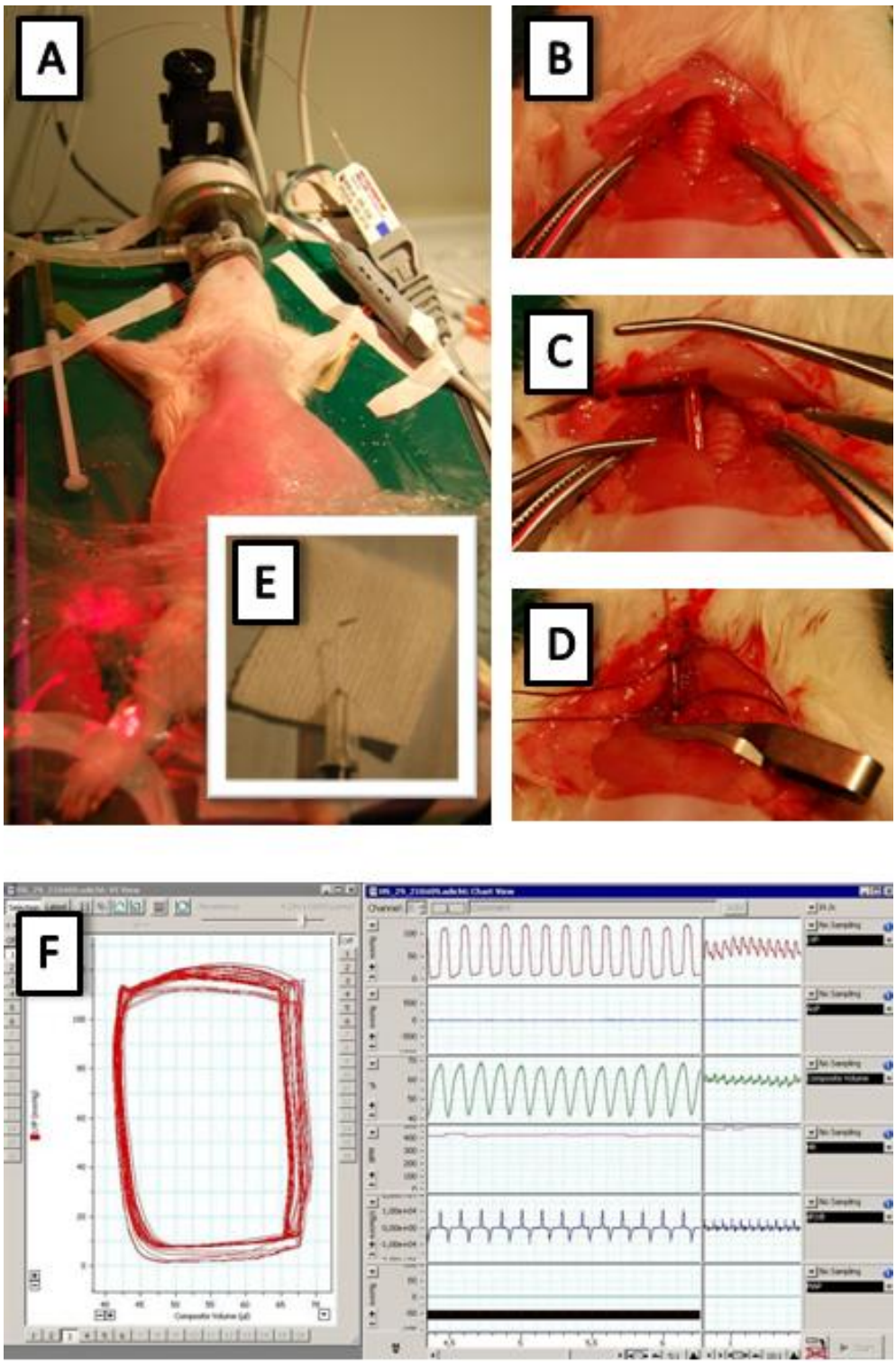

Figure 5 Pressure-Volume loop recording from the left ventricle of a rat

Insertion of conductance catheter via right carotid artery (A-E) and screen capture of PV-loop recordings from LV (F).

In Paper B the right carotid artery was cannulated in three rats to measure the pressure gradient across the aortic constriction. However, these rats became clinically unstable (drop in BP, cyanosis, bradycardia) shortly after catheter placement, and thus we refrained from bilateral catheter placement in the majority of animals. The cardiovascular deterioration associated with bilateral catheter placement may be related to cerebral ischemia or direct effects on vascular baroreceptors.

3.4 Morphometry

Rats were weighed and body weight (BW) determined before terminal experiments were performed, immediately before anesthesia. In the end of terminal experiments, the rats were euthanized with sodium pentobarbital 100 mg/kg administered intravenously or intraperitoneally. The hearts were excised immediately, washed in phosphate-buffered saline (PBS) or 0.9% NaCl and dried rapidly on a piece of gauze before it was weighed (HW). In the TAC- and HIIT-studies the LV was dissected free from the right ventricle, the atria and the mitral and tricuspid valves, and weighed (LV weight) and the right tibia was dissected free and measured with calipers. Which parameter best describes cardiac hypertrophy in pregnancy remains controversial. Conventionally used parameters include HW, LV weight and HW/BW-ratio. As maternal BW increases with advancing gestation, use of HW/tibia ratio [99] may be better than HW/BW ratio in pregnancy and was therefore used in Papers B and C. The fetoplacental units were removed from the uterus and put in a petri dish filled with PBS or 0.9% NaCl. Placentas and fetuses were dissected free, gently dried on gauze and weighed and crown-rump length (CRL) was measured without actively extending the fetus. The fetus was put back into the petri dish and the thoracic cavity cut open and the fetal heart was removed using a pair of bended sharp tweezers. A magnifying glass was used to ascertain that the tissue collected was from the heart as liver or lung tissue can easily be mistaken for heart tissue in small fetuses. Fetal hearts were put on individually preweighed eppendorf tubes filled with tissue storage reagent (RNAlater®, Qiagen, Hilden, Germany) and fetal heart weight was calculated as the difference of weight of the tubes before and after.

3.5 Histology

Myocardial tissue samples were taken from the LV and fixed in formalin. Slides stained with Toluidine Blue were used for the measurement of cardiomyocyte size, Sirius Red staining was

used for quantification of collagen content and β-actin immunolabeling for vessel density calculation.

3.5.1 Cardiomyocyte size

In the AngII-study pictures were taken using a Leitz Aristoplan microscope with a Leica DFC320 digital camera (Leica Microsystems Digital Imaging, Cambridge, United Kingdom). Minimum myocyte diameter at the level of the nucleus was measured using computer based morphometry (Leica CTR 600 & Leica Qwin V3, Leica Microsystems, Wetzlar, Germany) on a minimum of thirty cells in each heart. Only cells with visible nucleus were used for quantification. In the TAC-study a Leica DM2000 microscope was used for viewing slides, and sixteen photographs from each slide were taken with a Leica DFC 425 digital camera. 97±7 cardiomyocytes cut in short axis and containing a nucleus were identified from each heart. Cell circumference was outlined digitally using Image J (National Institutes of Health, Bethesda, MD, USA) and the myocyte transverse circumference was expressed in arbitrary units (Paper B, Figure 3). In the HIIT-study we refrained from doing microscopic studies of tissues as there were no significant changes in gross measurement, cardiac function as measured by ultrasound or expression of any mRNAs related to heart hypertrophy.

3.5.2 Fibrosis and collagen content in heart tissue

Collagen content was analyzed in the AngII- and TAC-studies. Formalin-fixed sections of the left ventricle were paraffin embedded and sliced, and Sirius Red staining of collagen fibers (Direct Red 80, Sigma-Aldrich, Germany) was performed. In the AngII-study transverse ventricular sections were examined under microscope using conventional and polarized light at magnification 50x and 200x. The level of staining as well as tissue changes and injury was evaluated and scored by an experienced pathologist (dr. Samer Al-Saad), who was blinded to information about AngII or pregnancy status. Each heart was finally assigned to one category with respect to collagen content and also scored according to presence of necrosis or not. The results are presented in Paper A, Table 4. In Papers A and B myocardial collagen content was measured in a minimum of 20 (Paper A) or 16 (Paper B) sampled images (200x) from each heart. Image J was used to analyze % tissue area stained by Sirius Red as described online by the developer [100]. Perivascular areas (containing higher concentrations of extracellular collagen) were outlined using Image J and excluded from quantification. As we did not expect

pathological accumulation of collagen in HIIT, we did not quantify collagen content in this study.

3.5.3 Vessel density in left ventricular myocardium

In Paper A angiogenesis was evaluated by estimating vessel density in hearts by immunohistochemistry using non-muscular β-actin to identify endothelial cells [101, 102]. Staining was performed using primary antibody against non-muscular β-actin protein (mouse anti rat, Beta-Actin; clone AC-74, Sigma). The slides were counterstained with haematoxylin to visualize the nuclei. Sections were examined under light microscope at 200x and twelve pictures of each heart section were taken from the left ventricle from four different areas. Using Image J software, a grid of 80 points (area per point was 3.22 cm^2) was applied on each picture and relative vessel density was expressed as the number of points crossing a blood vessel.

3.6 Gene expression in tissues

Gene expression analysis was performed to study changes induced by pregnancy or experimental interventions (AngII infusion, TAC or HIIT). Real time polymerase chain reaction (RT-PCR) was used to quantify expression of genes in maternal myocardium, placenta and fetal heart and liver. In all studies presented, relative quantification was used. Tissue was stored in RNA later (Qiagen, Hilden, Germany) and the samples were homogenized and lysed and total RNA was isolated according to the RNeasyFibrous Tissue protocol (Qiagen). RNA concentration was measured by spectroscopy (NanoDrop, Witec, Switzerland), and stored at - 80 °C before use. Reverse transcription of RNA was carried out according to High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The qualitative RT-PCR was performed in an ABI PRISM 7900 HT Fast real-time thermal cycler using the SYBR green or TaqMan Fast Universal PCR master mix (Applied Biosystems). The relative expression ratio of the target gene was calculated based on its real-time efficiency and threshold cycle differences between groups. The expression of the target genes were normalized to the stably expressed reference gene based on testing by Norm-Finder (Aarhus University Hospital, Aarhus, Denmark) of possible reference genes [103]. The presented data is normalized to controls. The reference genes and primers used are presented in Appendix 5. In fetal heart, the expression of Ddx3y (DEAD box polypeptide 3,

Y-linked) and Eif2s3y (eukaryotic translation initiation factor 2, subunit 3, Y-linked) was used to different male from female fetuses.

3.7 Oxidative stress and total antioxidant capacity

In Paper C level of oxidative stress and total antioxidant capacity was measured in placentas and fetal hearts and livers. Decreased total antioxidant capacity and increased level of malondialdehyde (MDA) together with the decreased superoxide dismutase (SOD) and peroxidase activities are indicators of oxidative stress.

Frozen tissue samples from 2-3 feto-placental units from each dam was thawed, weighed and homogenized for 2-3 min with a mechanical homogenizer in the buffer supplied in the assay kits to prepare 50 mg/ml suspensions. The homogenized suspension was checked with light microscope and centrifuged to 14000 x g for 15 min at 4° C. The supernatant was separated and stored in working aliquots at -70° C. All analyses were performed on the supernatant according to the instructions provided by the manufacturers of the assay kits.

3.7.1 Malondialdehyde level

MDA content was quantified by using OxiSelectTM TBARS Assay kit (Cell Biolabs, Inc., San Diego, CA, USA). All samples and standards were assayed in duplicate. The thiobarbituric acid reaction was completed in a microcentrifuge tube (1.5 mL) at 95° C for 1 hour. 200 µL of the reaction product was transferred into a 96 well microplate and the color was read with a spectrophotometric plate reader at 532 nm using blank as control.

3.7.2 Superoxide dismutase activity

SOD activity was carried out by using the procedure as provided with the SOD activity assay kit (Abnova GmbH EMBLEM, Heidelberg, Germany). This assay kit measures total (both cytosolic and mitochondrial) SOD activity and the results are reported as inhibition rate %. All reactions were carried out in a 96 well microplate in triplicate, and the absorbance was read at 450 nm using a microplate reader.

3.7.3 Peroxidase activity

Peroxidase activity reactions (Sigma-Aldrich, St. Louis, MO, USA) were carried out in a 96 well microplate in quadruplet, and the absorbance was read at 570 nm.

3.7.4 Total antioxidant capacity

Cumulative antioxidant capacity was quantified using an antioxidant assay kit (Sigma-Aldrich, St. Louis, MO, USA). All reactions were carried out in 96 well microplate in duplicate. The absorbance was read at 405 nm using a microplate reader and the results are presented as the inverse of optical density readings.

3.8 Data analysis and statistical methods

All measurements and analyzes were performed without the knowledge of pregnancy status and intervention. For obvious reasons the operator could not be blinded for pregnancy status when performing echocardiography, inserting PV-catheters and collecting tissue samples. However, echocardiograhy and PV-data were analyzed off-line with the investigator blinded to the allocation of experimental groups.

All data are reported as mean±standard error of the mean (SEM). A p-value <0.05 was considered statistically significant. Logarithmic transformation was used to achieve normal distribution of continuous variables when appropriate. As fetoplacental units from the same mother should not be handled as independent units, mean values for each mother was used when analyzing effects of interventions on pregnancy outcome (biometry, fetal echocardiography, oxidative stress and RT-PCR). Correlation between parametric variables was checked using Pearson Correlation coefficient. Categorical data comparison between groups was performed using chi-square test. Independent-Samples T-test was used to compare two groups. One way analysis of variance (ANOVA) was used to compare the 4 groups of rats. Two way ANOVA was used to investigate the influence and interaction between pregnancy status and interventions (AngII, TAC or HIIT) and the influence and interaction between fetal sex and interventions on gene expression in fetal tissues. Only differences in influence without significant interaction between pregnancy and TAC are presented for two way ANOVA. The Holm–Sidak method was used as post-hoc test. Sigma Plot 12.0 (Systat Software Inc, San Jose, CA, USA) software was used for two way ANOVA and PASW Statistics 18.0.3 (SPSS Inc., Chigaco, IL, USA) software was used for all other statistical analyses.

4. Results

In this section the results from Papers A-D are summarized and some supplementary data are presented.

Paper A: Pregnancy protects against antiangiogenic and fibrogenic effects of angiotensin II in rat hearts

4.1 Summary of the results

The effects of chronic AngII infusion on pregnant and non-pregnant Wistar rats were compared to infusion of 0.9% NaCl (sham). AngII infusion for 9-10 days led to an increase in diastolic and systolic BP compared to sham, while pregnancy status did not significantly influence BP. Heart weight and LV relative wall thickness were higher in pregnant AngII infused rats compared to AngII infused non-pregnant rats, pregnant sham and non-pregnant sham. Myocyte diameter was not affected by AngII or pregnancy. Ejection fraction was higher in AngII infused pregnant rats than in non-pregnant and pregnant sham. AngII infusion resulted in an increase in collagen content, and pregnancy ameliorated this effect. Vessel density in LV was decreased in AngII infused compared to sham non-pregnant rats, but not significantly decreased in pregnancy. AngII infusion led to significant changes in LV expression of genes related to function, fibrosis and apoptosis, with an increase in ANF, BNP, ANKRD1, PKCα, PKCδ and TP53. Pregnancy itself did not induce increased expression of known markers of pathological remodeling, but reduced the expression of α-MHC, TNFα, TP53, eNOS and iNOS. Fetal size, placenta weight or litter sizes were not influenced by AngII infusion.

We concluded that pregnancy and chronic exposure to a moderate dose of AngII has contrasting effects on fibrosis and angiogenesis in the heart. Pregnancy seemed to counteract the detrimental effects of AngII on fibrosis and angiogenesis in heart despite synergistic effects with respect to heart hypertrophy. Pregnancy and AngII lead to partly opposite changes in the expression of some of the genes related to heart function and remodeling.

4.1.1 Supplementary data

Data presented in Paper A are from six randomly selected rats from each group; Sham, Pregnant, AngII and Pregnant Ang II. Supplementary echocardiographic measurements from

all rats are presented in Appendix 1. Calculations derived from B-mode echocardiography were not significantly affected by pregnancy or AngII infusion.

Fetal echocardiography was performed on a total of 126 fetuses in 29 dams. Results are presented in Appendix 2. There were no significant differences between any of the measured variables.

Expression of genes in fetal hearts from pregnant rats infused with AngII and sham rats is presented in Appendix 3. Expression of genes is normalized to fetuses from sham rats and mean values of fetal hearts (n=2-3) from each dam is calculated. Fetal sex did not significantly affect gene expression in fetal heart (data not presented).

Paper B: Effect of transverse aortic constriction on cardiac structure, function and gene expression in pregnant rats

4.2 Summary of the results

The effect of increased afterload on the heart was studied ~2 weeks after TAC in non-pregnant and pregnant Wistar rats. Aortic systolic, but not diastolic BP was increased by TAC. Diastolic BP was decreased in pregnant compared to non-pregnant sham operated rats. TAC for ~2 weeks did not lead to overt heart failure. Heart weight and LV weight was increased by TAC, but not by pregnancy. Ejection fraction or cardiac output was not influenced by pregnancy or TAC. Stroke work in pregnant TAC rats was double compared to pregnant shams, whereas it was only 35% higher (not significant) in non-pregnant TAC rats compared to non-pregnant shams. Myocyte transverse circumference was increased by pregnancy, but not by TAC. Nine out of the 19 genes examined related to cardiac remodeling were affected by pregnancy independent of TAC. The increase in expression of β-MHC was higher in pregnant (5-fold) compared to non-pregnant rats (2-fold) after TAC, and the ratio of β-MHC to α-MHC expression was higher in pregnant TAC compared to non-pregnant TAC. Myocardial tissue collagen was not influenced by pregnancy or TAC, but expression of fibrosis related genes (COL3A1, COL1A1, FN1 and TIMP1) was up-regulated by TAC. Fetal size, placental weight and litter sizes were not influenced by TAC.

In conclusion this study demonstrated that pregnancy *per se* does not lead to significant cardiac hypertrophy in Wistar rats and does not protect against the negative effects of increased afterload caused by TAC. The differences in cardiac structure, function and gene

expression between pregnant and non-pregnant rats following TAC indicated that increased afterload may be less tolerated in pregnancy.

4.2.1 Supplementary data

Gene expression in fetal hearts from TAC and sham operated pregnant rats is presented in Appendix 4. Expression of genes is normalized to female sham fetuses and mean values of two fetal hearts from each dam is calculated. Fetal sex did not significantly affect gene expression in fetal heart (data not presented).

Paper C: High intensity interval training in pregnant rats alters gene expression in fetal heart and liver without inducing oxidative stress

4.3 Summary of the results

We studied the effects of six weeks of HIIT on the heart, placenta and fetuses of Sprague-Dawley rats. HIIT was well tolerated by pregnant rats. HIIT for six weeks or pregnancy did not lead to significant heart hypertrophy. Myocardial expression of 22 genes related to cardiac remodeling was not influenced by HIIT, but the expression of 11 of these genes was decreased in the myocardium of pregnant compared to non-pregnant rats. Fetal and placental growth or litter size was not affected by HIIT. Oxidative stress (peroxidase and superoxide dismutase activity and malondialdhyde level) and total antioxidant capacity in placenta, fetal liver and fetal heart was not influenced by HIIT, and HIIT did not alter the expression of genes related to oxidative stress in the placenta. However, HIIT reduced the expression of eNOS, HIF1A and GPx4.2 in the fetal liver and increased the expression of VEGF-β, SOD1 and TIMP3 in the fetal heart. Total antioxidant capacity was higher in fetal liver than in fetal heart and placenta independent of HIIT, suggesting that the placenta and developing heart are more vulnerable to oxidative stress compared to liver.

We concluded that HIIT is feasible in pregnant rats, but HIIT for six weeks did not lead to cardiac remodeling. There were no obvious adverse effects of HIIT in pregnancy on the mother. Fetal and placental growth as well as the level oxidative stress and total antioxidant capacity in placenta, fetal heart and liver were not affected by HIIT. However, some genes related to oxidative stress were altered in the fetal heart and liver indicating that adaptive mechanisms are activated.

Paper D: Coronary flow reserve in pregnant rats with increased left ventricular afterload

4.4 Summary of the results

We evaluated a Doppler echocardiographic method to non-invasively measure CFR in rats using different concentrations of inhaled isoflurane. High concentration (3.5%) of inhaled isoflurane was used as a coronary vasodilator. CFR was measured in the same pregnant and non-pregnant rats that were used in AngII and TAC studies. We found that CFR can be measured non-invasively in rats using this technique. We were able to calculate CFR using Doppler flow velocity waveforms of LMCA in most animals. CFR was reduced in pregnant rats compared to non-pregnant. CFR was not significantly affected by AngII infusion for ~10 days or TAC for ~2 weeks.

5. Discussion

5.1 Study design

In Papers A, B and C we used a similar design where an intervention (AngII, TAC or HIIT) was applied to pregnant and non-pregnant animals. Thus we had four groups of rats of same sex, similar age and size for studying the effects of both the pregnancy and the intervention. This simple study design reduces confounding to a minimum as all rats are as equal as possible except for the intervention and pregnancy status. We are aware of only a few other experimental studies in pregnancy that has used a similar design [104-107]. In most previous studies non-pregnant animals has been compared to pregnant animals [6, 7, 33, 108-114], pregnant control animals have been compared to pregnant animals subjected to an intervention [54, 63, 92, 115, 116] or both [117]. In some studies pregnant animals have been compared to non-pregnant animals subjected to an intervention [33, 40, 63, 118]. To illustrate, in one frequently cited study of physiological hypertrophy, pregnant mice were compared to non-pregnant mice and to *male* mice subjected to TAC [33], thus not taking possible effects of gender and differences in size into account. In addition to minimizing confounding, our study design enabled us to determine if changes in structure, function or gene expression can be attributed to pregnancy, intervention, or both, allowing us to get reliable data using a relative small sample size. We found this design particularly advantageous when analyzing gene-expression data using two-way ANOVA as a statistical method for comparing groups. Reducing the unnecessary use of animals is important when performing research on animals for animal welfare reasons [83], as well as out of obvious economical and practical considerations.

5.2 Physiological effects of pregnancy on the heart in the rat

In all three studies we included groups of non-pregnant and pregnant control animals. Thus we could evaluate the effects of pregnancy *per se* on the heart, as well as the effects of the interventions applied (AngII infusion, TAC or HIIT).

5.2.1 Pregnancy does not lead to significant heart hypertrophy in rats

It is widely believed that pregnancy causes physiological cardiac hypertrophy characterized by chamber enlargement without any increase in LV wall thickness/chamber diameter ratio [2, 22, 33, 40, 119, 120] (Figure 1). Pregnancy leads to significant heart hypertrophy in

humans [2, 9, 119, 120] and mice [7, 33, 40], and some authors have previously reported increased cardiac mass in pregnant rats [108-110, 118]. Thus, when planning and designing the first (AngII) study we expected to find evidence of physiological heart hypertrophy in pregnant as well as AngII infused rats. Interestingly, we did not find any evidence of significant heart hypertrophy in pregnant compared to non-pregnant control animals in any of the presented studies.

Total HW, LV weight and HW/BW-ratio are conventionally used parameters of heart hypertrophy. As BW increases with advancing gestation, use of HW/tibia ratio [99, 118] may be better than HW/BW ratio. However, none of the parameters measured supported the evidence of significant cardiac hypertrophy in pregnant Wistar (Paper A and Paper B) or Sprague Dawley rats (Paper C). These findings are in contrast to some other studies performed in rats. Jankowski et al. found a 28% increase in both HW and LV weight in pregnant Sprague-Dawley rats at GD21 (n=8 in each group, age not reported) [109], and Rimbaud et al. found an increase in both HW and HW/tibia-ratio of 10% in nine pregnant 15-weeks old Wistar rats at GD18-19 compared to in ten non-pregnant rats [118]. Virgen-Oritz et al. report an *increase* in HW/BW-ratio from 3.69 in non-pregnant (n=8) to 4.34 in pregnant (n=8) Sprague-Dawley rats at GD18-21 without providing actual weight [110]. This is in contrast to ours as well as others [109, 118] findings of decreased HW/BW-ratio in pregnant rats. Gonzalez et al. [108] report an increase of 70 % in LV mass calculated using echocardiography in pregnant compared to non-pregnant Sprague-Dawley rats. However, uncertainty in morphological data presented for five rats in each group make their conclusions questionable as the HW (89 – 95 g) and LV weights (78 – 93 mg) presented appear way out of range for adult, female rats. The TAC-study had an 80% statistical power to detect an increase in HW of 13%. When all pregnant (n=24) and non-pregnant (n=18) sham animals from the AngII- and TAC-studies were pooled together there was still no difference in HW between groups (730±20 vs 700±14 mg, p=0.24). We are not aware of studies in rats that report increased cardiac mass in pregnancy with equally high number of pregnant animals included. In support of our findings, Buttrick et al did not find increased heart weight in pregnant compared to non-pregnant 12-14 weeks old Wistar rats (eight rats in each group) [6], and Bassien-Capsa et al even report a significant *decrease* in wet heart weight in 13-14 weeks old pregnant Sprague-Dawley rats (n=17) compared to non-pregnant (n=19). Dry heart weights were similar in pregnant and non-pregnant animals. Both Buttrick and Bassien-Capsa refrain

from discussing the lack of hypertrophy in pregnancy further in their papers [6, 117]. Interestingly LV wall thickness measured with M-mode echocardiography was increased in pregnancy in the same rats [117]. In both studies reporting increased LV wall thickness in pregnant rats using ultrasound, the authors do not report if the investigators performing echocardiography or performing offline measurements were blinded to rats pregnancy status [108, 117]. Thus one may suspect overestimation of the LV mass in pregnant rats (error of anticipation) using echocardiography.

In humans, pregnancy is associated with an increase in blood volume leading to an increase in preload and the LV end-diastolic volume [14, 15, 17, 18]. Increased filling of the heart leads to increased SV and CO. However, in the TAC-study LV internal diameter in diastole and LV end-diastolic pressure were not increased in pregnant sham rats indicating that preload is not increased in pregnancy in rats. Slangen et al used electromagnetic flow probes around ascending aorta to show that pregnancy increased CO in pregnant Wistar rats. Heart weights, or any other measures of hypertrophy were not reported [121]. Studies in mice indicates that there is a good correlation between measurements obtained by flow probes and echocardiography [122]. However, our studies did not detect differences in CO between pregnant and non-pregnant control animals using M-mode echocardiography. Lack of an increase in CO in pregnancy may explain why the heart size was not increased by pregnancy in our studies on rats. High estrogen levels in pregnant animals may also be responsible for this, as in a recent study by Pedram et al [123] estrogen is shown to prevent cardiac hypertrophy in cultured neonatal rat cardiomyocytes via the estrogen receptor-β.

In general, concentric cardiac hypertrophy due to increased afterload on the LV is associated with a greater increase in cardiac myocyte width compared to length, whereas eccentric cardiac hypertrophy is associated with an increase in myocyte length [29, 35] (Table 1). Thus increased myocyte size in pregnancy has been attributed to increased myocyte length [33, 110]. In the AngII-study there was no difference in cardiomyocyte diameter and in the TAC-study there were no significant difference in cardiomyocyte transverse circumference between pregnant and non-pregnant sham rats. However, in the TAC-study the myocyte circumference was increased in pregnancy when the analysis was performed on all rats independent of TAC-status (two way ANOVA, $p=0.01$). Reliable measurements of myocyte length are not possible to obtain using histological sections of LV myocardium. To explore this aspect of heart hypertrophy at the cellular level, single myocytes should be examined [110, 117]. As

pregnancy *per se* did not lead to cardiac hypertrophy in rats in our studies, and there was no echocardiographic evidence of LV dilatation (similar LV inner diameter in diastole in all studies) studies on single myocytes were not done. Bassien-Capsa et al report a small (113.4 vs 117.5 µm) but significant difference in myocyte length between the hearts of pregnant and nonpregnant rats. However, as the statistical analysis was performed on the single cell level, and not on individual animal level this result remains questionable. In our opinion, statistics should be performed on mean myocyte length from each rat as individual single cells from the same heart should not be regarded as independent.

The lack of significantly increased heart mass in pregnant rats was surprising, but was reproduced in all three studies. Echocardiographic measurements have several limitations, but the accuracy of the HW and LV weight should be indisputable. Thus we conclude that pregnancy does not lead to a significant increase in heart mass in rats, and we would advise against using pregnant rat as an experimental model of physiological heart hypertrophy.

5.2.2 Heart function and pregnancy

In all studies echocardiographic evaluation of heart function was performed measuring LV fractional shortening and ejection fraction. SV and CO output were calculated from M-mode echocardiographic measurements. In AngII-study, the evaluation of heart function was supplemented with indices of mitral valve blood flow velocity signals (Appendix 1). In Paper B we presented pressure-volume data obtained from pregnant and non-pregnant sham operated animals. The only significant difference in any measure of cardiac function between pregnant and non-pregnant control rats was a decreased dP/dt_{max} in pregnant (7.7±1.1 mmHg/sec*10^3) compared to non-pregnant (10.9±0.7 mmHg/sec*10^3) animals in the TAC-study (Paper B, Table1). dP/dt_{max} is used as a measure of LV systolic/contractile function, and is highly dependent on both preload and afterload [124]. Thus, a significantly lower dP/dt_{max} may also be explained by a lower BP in pregnancy (Paper B, Figure 2) rather than reduced contractile function.

The data on how pregnancy influences heart contractility is conflicting. In a longitudinal study of healthy, pregnant women Gilson et al used M-mode echocardiography to demonstrate enhanced myocardial performance in late gestation pregnancy compared to early pregnancy and post-partum [5]. In contrast, Clark et al did not find any significant difference in LV function when central hemodynamic measurements (pulmonary artery catheterization

and arterial line placement) were performed at 36-38 weeks gestation and 11-13 weeks post partum [8], and Geva et al found a transient *decrease* in contractility in the second trimester using serial echocardiographic measurements during and after pregnancy [9]. As described in the introduction heart function in pregnancy is vastly influenced by the variability in the loading conditions in pregnancy [9-11]. In clinical studies as well as in *in vivo* animal studies, it is not possible to correct for all parameters influencing pre- and afterload in pregnancy. However, in the isolated working heart both pre- and afterload can be tightly controlled and neuroendocrine influences neutralized [6, 125]. Buttrick et al found an increased contractile performance in isolated rat hearts of third trimester pregnant rats, compared to controls, whereas cardiac contractility was not significantly improved in the heart of first trimester or postpartum rats [6]. Data from our studies could not underpin these findings. More studies on the isolated hearts of pregnant animals might prove to be valuable when exploring the effects of pregnancy on cardiac function and metabolism. Since we did not detect cardiac hypertrophy in pregnant rats, mouse models may be more appropriate [126].

5.2.3 The coronary circulation and pregnancy

Coronary flow reserve (CFR) calculated from Doppler measurements of LMCA flow velocities at low and high concentrations of inhaled isoflurane in the AngII and TAC-studies are presented in Paper D. In the TAC-study CFR_{peak} was significantly reduced in pregnant compared to non-pregnant sham operated rats (Paper D, Figure 2C). The same trend was observed for CFR_{VTI} in the TAC-study (Paper D, Figure 2D) and in sham treated rats in the AngII-study. Combining data on sham animals from two studies, we found that both CFR_{peak} and CFR_{VTI} were significantly lower in pregnant compared to non-pregnant rats (Paper D, Figure 3).

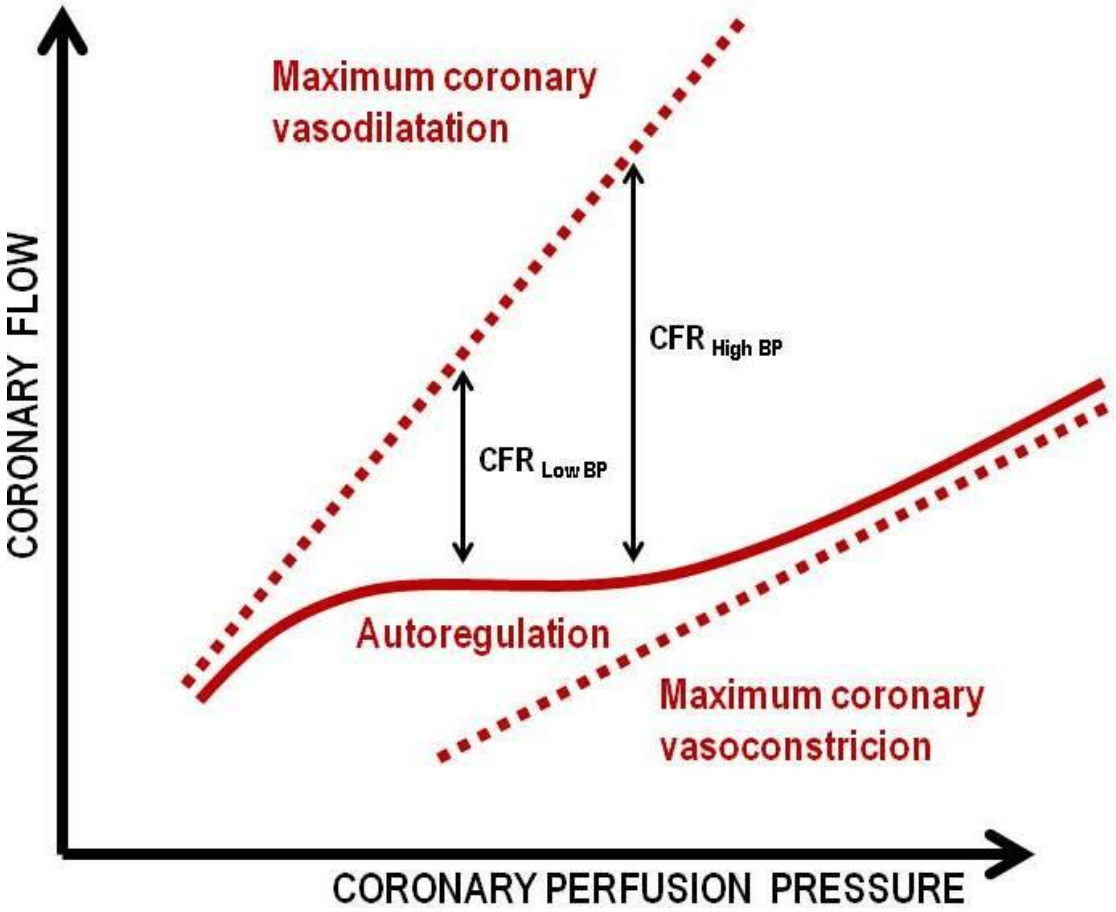

Figure 6 Coronary autoregulation, blood pressure and coronary flow reserve
Coronary flow is determined by coronary perfusion pressure. It is regulated by coronary vascular resistance and will remain relatively constant over a wide range of perfusion pressures. When high doses of inhaled isoflurane are administered, the vessels of the coronary bed are maximally dilated (corresponding to the upper dotted line) and the autoregulation is revoked. Thus the calculated CFR is directly dependent on the coronary perfusion pressure. Adapted from Baumgart et al [127].

Flow mediated vasodilatation (FMD) is believed to reflect the endothelial function in the peripheral resistance vessels but may not correlate with that of the coronary vascular bed [70, 78, 79, 128]. Studies in pregnant women have shown that FMD decreases towards term [72, 73, 76] which is in line with our findings. The coronary circulation is subjected to autoregulation and coronary blood flow is constant over a wide range of pressures [80, 129] (Figure 6). When the vascular bed of the heart is maximally vasodilated (e.g. by adenosine or isoflurane) the coronary autoregulation is revoked and coronary blood flow exhibits a linear relationship with the myocardial perfusion pressure [80, 127]. As coronary flow is predominantly diastolic in the rat LMCA (Paper D, Figure 1BC), an increase in diastolic BP may increase calculated CFR. Therefore, the reduced CFR in late pregnancy may be a result

of reduced diastolic BP rather than impaired coronary endothelial function in healthy pregnancy (Figure 6). Examining CFR and BP at mid-term could have added valuable information, but we refrained from anaesthetising the animals twice during pregnancy to avoid adverse effects on mother and fetuses.

A possible explanation for the reduction in CFR at late term in rats could be vasodilatation of the coronary vascular bed due to metabolic or endocrine changes in pregnancy. Hirata et al. have shown an *increase* in CFR associated with increasing levels of 17β-estrogen in the follicular phase of the menstrual cycle in young, healthy women and after administration of conjugated estrogen in postmenopausal women [130]. In third trimester pregnancy estrogen levels are high. However, profound changes in several other hormones that occur during pregnancy could be responsible for reduced CFR that we observed in rats close to term.

5.3 Three models of heart hypertrophy in the rat

Three different models of cardiac hypertrophy were applied to pregnant and non-pregnant rats to explore how pregnancy affects cardiovascular adaptation to pathological and physiological stress.

5.3.1 Angiotensin II infusion

As elaborated in the Introduction RAS is activated in preeclampsia, and the presence of autoantibodies to the AngII-receptor type 1 (AT_1-AA) may play a key role in the development of preeclampsia [50-52]. Thus a model of chronic AngII infusion may be of particular interest when exploring the possible mechanism behind heart remodeling in preeclampsia. Interestingly, infusion of AngII for 9-10 days led to hypertrophy in terms of a significant increased heart weight and LV wall thickness only in pregnant rats (Paper A, Table 3). Pregnant AngII infused rats also had increased ejection fraction compared to non-pregnant AngII infused rats. Although there were no significant differences in BPs measured in the ascending aorta, there was a trend towards lower systolic and diastolic BP in the pregnant AngII infused rats compared to non-pregnant AngII infused rats. Thus a difference in BP cannot explain increased heart mass. When looking at components related to interstitial remodeling such as collagen content, qualitative grading of fibrosis and vessel density, pregnant rats were less affected by AngII infusion than non-pregnant rats. As the increase in heart mass caused by AngII infusion in pregnancy did not appear to be related to increased deposits of extracellular components one would expect an increase in total myocyte mass.

However, there was no significant difference in myocyte diameter between any groups. The increase in heart mass in pregnant AngII infused rats could be related to an increase in myocyte length, which was not measured.

5.3.2 Transverse aortic constriction

TAC is used as a model of chronic pressure overload and non-pregnant rats typically develop progressive concentric LV hypertrophy and ultimately LV dilatation and heart failure [93] (Figure 2). However, in our TAC-study there were no differences in filling pressure, heart rate, SV, CO and weight gain between TAC and sham operated animals irrespective of whether they were pregnant or not. This indicates that moderate constriction of the transverse aorta for ~2 weeks is well compensated with LV hypertrophy and increased stroke work (Paper B, Table 1, Figures 2 and 4).

Women with hypertensive disorders of pregnancy are susceptible to pulmonary edema [131]. The more pronounced increase in cardiac stroke work after TAC observed in pregnant rats compared to in non-pregnant rats indicate that he hearts of pregnant TAC rats may had entered a hypercontractile state with increased myocardial metabolic demand representing the stage before decompensated heart failure [132] and thus indicating that pregnancy renders the heart more vulnerable to additional stress. A longer time following TAC could lead to overt heart failure [93], but the short duration of pregnancy in rats (about 21-22 days) did not allow this. Performing TAC before mating, or banding the ascending aorta on juvenile rats [133], could be options that may be more relevant to simulate clinical scenarios of pregnancy in women with congenital heart defects or acquired heart diseases with LV outflow obstruction, and these options should be considered for future studies. However, the effect of aortic constriction on fertility in rats is not known.

5.3.3 High intensity interval training

We were surprised to find that HIIT for six weeks did not lead to significant heart hypertrophy and that HIIT did not significantly change the expression of any of the genes related to cardiac hypertrophy we studied. This lack of cardiac hypertrophy stands in contrast to Wisløff et al [94] who reported a significant increase in LV mass of ~10% in female Sprague-Dawley rats after four weeks of HIIT and a ~35% increase after 13 weeks. In the study by Wisløff et al, the rats exercised for a longer time period (2 h/day) at intervals of 8 min duration at 85-90% of $\dot{V}O_{2max}$[94]. Longer training sessions or longer intervals might

have increased the LV mass in animals in the HIIT-study. We felt reluctant to use such an aggressive model of HIIT on pregnant rats, and chose a protocol based on what has previously been done in mice at our facilities [39] and similar to the more recent studies on rats from Wisløffs group [134, 135]. As pregnant rats were able to run ten intervals of four minutes per day, and both longer lasting bouts of high intensity running or longer training sessions may be tolerated by pregnant rats, and this could be explored in future studies. A longer total period of HIIT could have lead to significant increase in LV mass, but due to the short duration of pregnancy in rats (~21 days) this was not possible. As the main goal of the study was to investigate the effect of HIIT in pregnancy and vice versa we regarded a longer training period before mating as not relevant.

It is a paradox that six week of hard interval training including 24 one hour sessions of HIIT with an average total running distance of 83±4 km pr rat did not lead to significant change in heart mass or function and that none of the 22 examined genes related to cardiac function and remodeling was significantly affected. HIIT rats were able to increase their maximal running speed at intervals by 42% during the training period, indicating that their physical fitness was improved. Out of animal welfare reasons, HIIT was not performed in the last days of gestation, when the rats were heavily pregnant, and thus the rats rested for 2 or 3 days before terminal experiments. We chose to do the terminal experiments close to term, at 20 GD, as we wanted to investigate the effects of HIIT on the fetus and placenta, and half of the increase in fetal and placental weight will take place during the last few days of pregnancy [84]. We would not expect cardiac hypertrophy to regress following a couple of days rest towards the end of pregnancy. However, the lack of change in expression of genes could be attributed to the time lag between cessation of HIIT and collection of samples. The average half-life of mRNA (2.6-7 hours) is much shorter than the half-life of proteins (46 hours) in mammalian cells [136]. Thus we believe that a long duration from cessation of stimulus (training) to sampling of specimens may be the most important explanation for not finding any significant changes in expression of mRNAs. In contrast, AngII infusions were continued and the increased afterload due to TAC was present until tissues were sampled.

We used young (9-11 weeks at inclusion) female rats that continued to grow during the training period. The hearts of maturing young female rats may respond to the physiologic stimuli of pregnancy and/or exercise differently than the heart of mature adult rats. In future studies where the primary aim is to investigate effects of HIIT on the heart, one could

consider doing terminal experiments and tissue sampling at an earlier stage in pregnancy, closer to cessation of HIIT. One could consider using fully grown female rats not needing to take into account the natural physical growth of the body and heart in the young, maturing rat. Using ultrasound to evaluate cardiac function and to calculate LV mass longitudinally may also be possible, but the effects of applying repeated sessions of isoflurane anesthesia in pregnancy is not known and repeated procedures could interfere with the intensive training regime.

5.3.4 Comparing models

Whereas AngII infusion could mimic some of the patho-physiologic changes observed in preeclampsia (increased RAS activation), TAC imitates the situation of mechanical obstruction of the LV outflow tract as seen in some congenital heart defects (coarctation of the aorta, interrupted aortic arch and aortic stenosis). Both interventions lead to an increase in afterload, however TAC is in principle a mechanical intervention predominantly leading to an increase in systolic BP, whereas by AngII infusion, both diastolic and systolic BP is increased. Probably the increased diastolic pressure generated by TAC in the proximal aorta is absorbed by increased vascular elastance in the unaffected vascular bed supplied by the right brachial artery. AngII leads to increased afterload via its vasoconstricting properties on the peripheral circulation. Furthermore there is evidence that AngII stimulate heart hypertrophy through direct effects on myocytes as ACE-inhibitors prevents hypertrophy in aortic banded rats even if afterload is not reduced [137] , and AngII is able to induces cell growth and increases protein synthesis in isolated cardiac myocytes [138].

The increase in heart weight following AngII infusion was significantly ($p=0.006$) increased in pregnant compared to non-pregnant rats, whereas pregnancy did not significantly affect heart weight, LV weight or HW/tibia-ratio following TAC. The increase in mass was not related to a higher content of collagen and there was no increase in myocyte diameter. Increased myocyte length in pregnancy may account for increased mass; however, there were no signs of cardiac dilatation on echocardiography. Water content is increased in heart muscle after excessive stretch in dogs [139] and there are reports of increased water content in myocytes of pregnant rats [117]. Increased water content may explain an increase in heart weight. To our knowledge AngII does not alter water content in cardiac tissue. This could easily be addressed in future studies by comparing wet and dry heart weights [117].

5.4 Influence of pregnancy on heart remodeling

Even if pregnancy and HIIT did not lead to significant cardiac hypertrophy, pregnancy *per se* altered expression of a wide range of genes whereas HIIT did not. These data may support Chung et al's assertion that the mechanisms driving the physiologic remodeling following exercise are different from those in pregnancy [22, 40] (Table 1).

5.4.1 Effect of pregnancy on cardiac function in models of increased afterload

In Paper B we presented pressure-volume data showing that TAC doubled stroke work in pregnant rats, whereas the increase in stroke work attributed to TAC in non-pregnant rats was only 35% (Paper B, Figure 4D). TAC is used as a model of chronic pressure overload and non-pregnant rats generally develop progressive concentric LV hypertrophy and ultimately LV dilatation and heart failure [37, 93]. Doenst et al studied the effects of TAC applied in juvenile (3 weeks old) Sprague-Dawley rats 2, 6, 10 and 20 weeks later. The pressure overload led to progressive heart hypertrophy with preserved systolic cardiac function (EF) at 2, 6 and 10 weeks and dilatation and impaired function at 20 weeks. However, impairment in fatty acid oxidation and reduced cardiac power was demonstrated ex vivo as early as 2 weeks after TAC [37]. In the TAC-study there were no differences in filling pressure, heart rate, EF, CO and weight gain between TAC and sham operated animals irrespective of whether they were pregnant or not, indicating that a moderate constriction of the transverse aorta for ~2 weeks is well compensated with LV hypertrophy and increased stroke work. As expected TAC increased afterload, but the increase in afterload attributed to TAC was not affected by pregnancy. In contrast to chronic pressure load caused by AngII, aortic diastolic BP was not affected by TAC. The increase in systolic afterload caused by TAC was compensated by an increase in stroke work and this increase was more pronounced in pregnant animals. This may be related to the fact that pregnancy in combination with TAC led to an increase in myocyte circumference. Additionally, cardiac remodeling following TAC was associated with a more than three-fold increase in the ratio of β-MHC/α-MHC-gene expression in pregnant compared to non-pregnant rats.

5.4.2 Influence of pregnancy on cardiomyocytes

As discussed earlier (5.2.1) pregnancy per se did not lead to significant increase in myocyte diameter or circumference, and heart hypertrophy following AngII for ~10 days or TAC for ~2 weeks did not increase the myocyte diameter/circumference significantly. However, the

myocyte transverse circumference was significantly higher in pregnant TAC rats compared to non-pregnant sham operated rats. An increased β-MHC expression and decrease in α-MHC expression has been considered as hallmarks of cardiac hypertrophy [140, 141]. As expected, expression of β-MHC was increased and expression of α-MHC was decreased following TAC. Interestingly this shift in MHC-expression towards the β- isoform was more pronounced in pregnant rats. A recent study in mice has shown that β-MHC protein is induced by pressure overload only in a minor subpopulation of myocytes that are smaller than myocytes containing α-MHC only [142]. This may explain why TAC for ~2 weeks did not lead to an increase in average myocyte circumference even if the ratio of β-MHC to α-MHC expression and contractility was increased.

5.4.3 Myocardial fibrosis in pregnancy

In Paper A we showed that AngII infusion for ~10 days lead to a marked deposition of extracellular collagen and that pregnancy opposed this effect. There was a significant increase in the expression of COL3A1 in AngII and a trend towards less expression of fibrosis related genes COL1A1, COL3A1 and FN1 in AngII treated pregnant rats compared to AngII treated non-pregnant rats (Paper A, Figure 1 and Table 5). In Paper B the up-regulation of COL1A1, COL3A1, FN1 and TIMP1 genes was less pronounced following TAC for ~14 days compared to following AngII infusion and there was no increase in collagen deposition. Pregnancy increased the expression of TIMP1 independent of TAC ($p=0.02$). There was a non-significant trend towards less expression of COL1A1 and COL3A1 in pregnant compared to non-pregnant TAC (Paper B, Figure 3B and Tables 2 and 3). In Paper C, the expression of COL1A1, COL3A1 and TIMP1 genes was reduced by pregnancy, but not affected by HIIT. As HIIT did not lead to significant change in cardiac mass or changes in gene expression and fibrosis is not induced by exercise training, we did not analyze collagen content in tissue in the HIIT study.

The difference in myocardial response to increased afterload caused by AngII compared to the effects of obstruction of LV outflow tract is probably related to the direct effect AngII on the heart independent of increase in BP [137, 138, 143]. TGF-β acts as a regulator of cardiac remodeling through its direct actions on the cardiomyocyte, the fibroblast and the extracellular matrix, and is induced by hemodynamic overload [144]. TGF-β1 acts downstream to AngII in inducing myocardial fibrosis [144]. The expression TGF-β genes was

not examined in Paper A, but in Papers B and C the expression of TGF-β1, TGF-β2 and TGF-β3 in LV myocardium was lower in pregnant compared to non-pregnant rats, both in intervention groups and in controls. Thus downregulation of TGF-β signaling pathways may explain why pregnancy counteracts the development of fibrotic remodeling of the LV in AngII infused rats presented in Paper A. In addition, some AngII-receptors are less sensitive to AngII during pregnancy as they are switched from a heterodimeric to a monomeric state that is inactivated by reactive oxygen species [28]. Furthermore, the high estrogen levels of late pregnancy may inhibit fibrosis via direct effects on the estrogen receptor-β in cardiac fibroblast and cardiomyocytes [145].

Heart hypertrophy following TAC for ~14 days did not lead to significant myocardial fibrosis, but fibrosis is inevitable if the heart is exposed to increased afterload for a longer period of time [93]. However, due to the short gestation (~21 days), rat models with banding of the aorta may not be suitable to determine if pregnancy protects against fibrosis following increased LV outflow obstruction as seen in pregnant women with congenital heart defects.

5.4.4 Effects of pregnancy on the coronary circulation in models of increased afterload

Effects of pregnancy and increased afterload on the coronary circulation were evaluated in several ways; through measurement of expression of genes related to angiogenesis, by evaluating vessel density and by using CFR measurements to evaluate endothelial function.

5.4.4.1 Microcirculation and vessel density

Gene expression of VEGF-α and VEGF-β was decreased in pregnant compared to non-pregnant rats in all studies (Paper A; Table 5, Paper B; Tables 2 and 3, Paper C; Table 2). None of the examined interventions (AngII, TAC or HIIT) influenced the expression of VEGF-α or VEGF-β. In the AngII-study, vessel density tended to increase in pregnant compared to non-pregnant in both sham and AngII infused rats, and decrease in both pregnant and non-pregnant AngII infused rats (Paper A, Figure 2). Thus we concluded that pregnancy may counteract the detrimental effects of AngII on angiogenesis in the heart. VEGF-α is an angiogenic factor activated by hypoxia and is essential for angiogenesis and remodeling in several organs, including the heart [146]. Expression of VEGF-β is critical for the survivel of vascular endothelial cells [147]. Unlike in other studies [101], AngII alone did not increase the expression of VEGF in this study, and the decrease in vessel density in AngII infused rats

was not accompanied with significant changes in VEGF expression. Furthermore, the increase in vessel density in pregnancy was accompanied with lower expression of VEGF. Thus vessel density in the heart in AngII treated rats might be regulated through other mechanisms than the VEGFs or the change in of VEGF gene expression is not reflected at the protein level.

5.4.4.2 Coronary flow reserve

In contrast to previous findings in dogs [148] and mice [149] with increased afterload , in our study CFR was not reduced by AngII infusion or TAC in rats. In addition to species differences, there could be several other reasons for this discrepancy. Hittinger et al. found that CFR was preserved in dogs with compensated heart hypertrophy, but was exhausted when the dogs developed decompensated pressure overload left ventricular hypertrophy [148]. In our studies the animals did not have decompensated heart failure when CFR was measured .

The course and outcome of pregnancy may be affected by maternal heart disease [150], and death from heart disease is the leading cause of indirect maternal death in the UK [151]. A recent study showed that the utero-placental blood flow is impaired in pregnant women with congenital heart diseases [152]. As the effects of pregnancy on CFR have not been studied before, and it is not known if reduced CFR can predict an increased risk of adverse cardiovascular events in pregnancy, there is a need for non-invasive tests to asses risk and predict outcome in pregnant women at risk. Thus, studies of CFR in healthy human pregnancies and in pregnant women with hypertension or heart diseases using non-invasive methods and a safe vasodilating agent are warranted. Adenosine is probably safe, but is associated with unpleasant side effects such as dyspnea, chest discomfort and transient asystole. Dipyridamole, a FDA category B drug, could be a safe alternative [127].

Inhalation of isoflurane is not an alternative for pregnant women, but the findings in Paper D indicates that estimating CFR using different concentrations of isoflurane is an easy, quick and non-invasive method of evaluating CFR in research animals. This is of particular importance when several procedures are scheduled as increased time in anesthesia increases the risk of destabilizing the animal and thus failure in getting valuable results from the study.

5.5 High intensity interval training during pregnancy

HIIT was well tolerated by pregnant rats. However, six weeks of HIIT did not lead to significant changes in cardiac structure, function, and gene expression irrespective of whether the rats became pregnant or not. As high-intensity training in humans leads to heart hypertrophy, and others have demonstrated hypertrophy in rats subjected to HIIT [94], one could argue that the rats were not exposed to adequate doses of training. However, as the rats ran ten bouts of four minutes at 85-90% at $\dot{V}O_{2max}$ five days/week for six weeks, this is not likely. Looking at the translational aspect of our experiments it is not likely that even the most vigorously training pregnant women would train at this level during pregnancy.

HIIT did not affect body weight significantly in non-pregnant or pregnant rats and all rats gained weight during the course of experiments. This suggests that HIIT is not effective in reducing weight in young female non-obese rats, independent of pregnancy status. Maternal obesity and excessive weight gain in pregnancy are known risk factors for adverse pregnancy outcomes [66, 153]. As the prevalence of obesity in pregnancy is increasing [66], from a translational perspective it would be of interest to examine the effects of HIIT in pregnant obese animals. It is possible that the ratio of skeletal muscle to adipose tissue mass is affected by training. Measures of body composition and other markers of metabolic profile could be addressed in future studies on HIIT in pregnancy.

The present study was performed in young, healthy rats. In the developed world the average maternal age is rising [154] and in future studies one could consider replacing young, adolescent rats with fully mature rats to add translational relevance.

5.6 Rat models of heart hypertrophy and the fetus

None of the interventions tested, i.e. HIIT carried out from 3 weeks before pregnancy and continued until 2-3 days before term gestation, chronic AngII infusion or TAC in the last half of pregnancy, led to significant changes in fetal or placental size or the litter size. In the AngII-study the fetal hemodynamics was not affected (Appendix 2). There were subtle changes in gene expression in fetal heart in all three studies, but no consistent differences in findings were observed (Paper C, Table 5 and Appendix 3 and 4). Based on our findings, we can conclude that these interventions applied to pregnant rats do not seriously affect the fetuses.

One of the objectives of our study was to evaluate the safety of HIIT in pregnancy. In HIIT fetuses there were some minor changes in gene expression in liver tissue indicative of oxidative stress, but we did not find any significant changes in ROS-activity in placenta, fetal liver or heart (Paper C, Figures 3 and 4). A more extreme model of HIIT (longer training sessions, longer intervals, HIIT continued until term) might compromise fetal wellbeing, but the translational relevance of such protocol is questionable and there would be obvious animal welfare concerns. The changes in gene expressions were small and the translational significance of these findings is uncertain. However, they may indicate that protective mechanisms are activated. Thus, before extensive training at the anaerobic threshold can be ruled in as safe in pregnancy, our findings should be tested by other researchers and preferably in other animal species. Based on the combined experience, clinical studies with meticulous monitoring of the fetus may then be performed.

6. Main conclusions

- Cardiac remodeling caused by chronic infusion of moderate doses of AngII in pregnant rats is associated with heart hypertrophy. However, pregnancy protected against fibrosis and preserved angiogenesis in AngII infused rats.
- Some differences in cardiac structure, function and gene expression observed between pregnant and non-pregnant rats following TAC indicate that afterload increase is less tolerated in pregnancy.
- Pregnancy induces changes in the expression of a wide range of genes involved in cardiac remodeling independent of afterload.
- Pregnancy *per se* does not lead to heart hypertrophy in rats. Therefore, rat models may not be suitable for studying physiological heart hypertrophy of pregnancy.
- It is feasible to measure CFR in rats non-invasively using Doppler echocardiography and high concentration of inhaled isoflurane as the vasodilating agent.
- CFR is reduced in late pregnancy in rats. CFR is not affected by increased LV afterload caused by chronic infusion of moderate doses of Ang II or TAC, regardless of pregnancy status.
- HIIT is feasible and well tolerated by pregnant rats. It does not appear to alter maternal cardiac structure or function, and oxidative stress and total antioxidant capacity in the placenta, fetal heart and fetal liver.
- Litter size, placental weight and fetal size are not affected by AngII infusion, TAC or HIIT. Some genes related to cardiac function and oxidative stress are altered in the fetal heart and liver in HIIT, indicating that adaptive mechanisms are activated.

7. Translational relevance and future perspectives

Management of pregnant women with cardiac disease or hypertension remains a challenge. Experimental studies on cardiac remodeling in pregnancy, especially those relevant to heart diseases and pregnancy complications are sparse. Studies presented in this thesis try to explore basic mechanisms involved in cardiovascular adaptation to pregnancy under physiological and pathological circumstances, and therefore have some translational value. However, further studies are needed to better understand physiological and pathological cardiac remodeling in pregnancy at basic and clinical levels that may lead to new therapeutic approaches when dealing with hypertension and cardiac diseases in pregnancy.

8. Erratum

Paper C, page 20.

Table 2 Maternal myocardial expression of genes related to cardiac remodeling in pregnant and non-pregnant HIIT compared to pregnant and non-pregnant sedentary rats and influence of pregnancy on gene expression

Influence of pregnancy (sixth column); ns (not significant) , corrected to na (non-applicable) due to significant interaction between pregnancy and HIIT for α-MHC, BNP and FN1.

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Appendices

Appendix 1

Echocardiography performed on pregnant and non-pregnant rats infused with saline or angiontensin II

	Sham	Pregnant	AngII	Pregnant AngII
M-mode echocardiography (n)	7	14	8	14
Fraction of shortening (%)	44±4	46±2	54±5	61±4*#
Ejection fraction (%)	72±4	76±2	82±5	88±3*#
Stroke volume (µL)	149±13	158±10	141±10	132±11
Cardiac output (mL/min)	59±4	68±4	61±4	55±5
LV inner diameter in diastole (mm)	1.6±0.1	1.6±0.1	1.8±0.1	2.0±0.1
LV relative wall thickness (%)	51±4	45±3	57±2	76±7*#
LV mass (g)	0.64±0.02	0.58±0.04	0.61±0.04	0.72±0.06
Doppler echocardiography (n)	6	15	8	14
Mitral valve E/A-ratio	0.99±0.04	1.13±0.03	1.04±0.04	1.01±0.04
Isovolumetric contraction time (ms)	15±1	15±1	13±1	14±1
Isovolumetric relaxation time (ms)	18±2	19±2	20±3	21±2
Tei-index	0.59±0.05	0.64±0.05	0.63±0.09	0.70±0.06

Data were analyzed using one-way ANOVA and Holm-Sidak post hoc test and is presented as mean±SEM, $p<0.05$ compared to sham (*) and pregnant (#). LV, left ventricle, AngII, angiotensin II.

Appendix 2

Fetal echocardiography performed on pregnant rats infused with saline (sham) and angiotensin II

	Sham (n=15)	AngII (n=12)	p-value
Fetal heart rate (min^{-1})	254±5 (69)	242±5 (50)	0.09
E/A-ratio	0.37±0.04 (37)	0.33±0.01 (37)	0.3
Isovolumetric contraction time (ms)	24±1 (69)	24±1 (50)	0.8
Isovolumetric relaxation time (ms)	43±2 (69)	45±2 (50)	0.5
Tei-index	0.65±0.03 (69)	0.66±0.02 (50)	0.9
Umbilical artery pulsatility index	1.70±0.02 (69)	1.72±0.03 (50)	0.5
Ductus venosus pulsatility index	0.87±0.03 (67)	0.94±0.03 (47)	0.1
Ductus arteriosus pulsatility index	1.75±0.02 (68)	1.75±0.03 (49)	0.9

Data is presented as mean±SEM. AngII, angiotenin II, (n), number of fetuses

Appendix 3

Gene expression in fetal hearts from pregnant rats infused with angiotensin II or sham

Gene expression	Sham (n=8)	AngII (n=6)	p-value
Protein kinase C α	1.00±0.05	0.99±0.05	0.9
Protein kinase C δ	1.00±0.03	1.00±0.05	1.0
Protein kinase C ε	1.00±0.05	1.03±0.02	0.6
α-Myosin heavy chain	1.00±0.09	0.97±0.14	0.8
β-Myosin heavy chain	1.00±0.07	1.03±0.05	0.8
Atrial natriuretic peptide	1.00±0.05	1.06±0.11	0.6
Brain natriuretic peptide	1.00±0.08	1.31±0.11	0.031
Vascular endothelial growth factor-α	1.00±0.07	1.03±0.06	0.7
Vascular endothelial growth factor-β	1.00±0.05	1.00±0.05	1.0
Superoxide dismutase 1	1.00±0.04	0.99±0.05	0.9
Superoxide dismutase 2	1.00±0.04	1.01±0.01	0.9
Inducible nitric oxide synthase	1.00±0.04	0.84±0.06	0.025
Tissue inhibitor of metallopeptidase 1	1.00±0.11	0.89±0.10	0.5
Tissue inhibitor of metallopeptidase 3	1.00±0.04	1.11±0.05	0.08
Tissue inhibitor of metallopeptidase 4	1.00±0.06	0.90±0.05	0.3
Hypoxia-inducible factor 1α	1.00±0.3	0.99±0.04	0.8

Relative expression of genes in fetal heart tissue normalized to mean values in fetuses of sham rats. Data is presented as mean±SEM. (*), $p<0.05$

Appendix 4

Gene expression of fetal hearts of rats subjected to transverse aorta constriction compared to sham surgery

Gene expression	Sham (n=9)	TAC (n=7)	p-value
Protein kinase C α	1.00±0.07	1.10±0.10	0.4
Protein kinase C δ	1.01±0.02	1.06±0.02	0.1
Protein kinase C ε	1.03±0.06	1.10±0.11	0.6
α-Myosin heavy chain	0.98±0.08	1.12±0.15	0.4
β-Myosin heavy chain	0.99±0.13	1.05±0.09	0.8
Atrial natriuretic peptide	1.14±0.14	1.02±0.29	0.7
Brain natriuretic peptide	0.92±0.07	0.96±0.11	0.7
Vascular endothelial growth factor-α	0.98±.011	1.01±0.12	0.9
Vascular endothelial growth factor-β	1.02±0.02	1.20±0.09	0.06
Superoxide dismutase 1	0.96±0.08	0.92±0.07	0.8
Superoxide dismutase 2	0.99±0.07	0.86±0.09	0.3
Inducible nitric oxide synthase	0.92±0.08	1.20±0.09	0.6
Tissue inhibitor of metallopeptidase 1	0.93±0.12	0.97±0.18	0.9
Tissue inhibitor of metallopeptidase 3	0.97±0.09	0.89±0.09	0.6
Tissue inhibitor of metallopeptidase 4	1.03±0.10	1.20±0.20	0.4
Hypoxia-inducible factor 1α	0.90±0.08	0.87±0.10	0.8

Relative expression of genes in fetal heart tissue normalized to mean values in female fetuses of sedentary rats. Data is presented as mean of the mean value of fetuses in each pregnant rat ±SEM. TAC, transverse aorta constriction

Appendix 5

Primers used for real-time polymerase chain reaction analysis. Primer sequence (5'-3')

Rat mRNA

Forward primer

Reverse primer

Reference genes:

GADPH: Glyceraldehyde-3phosphate dehydrogenase

CTG-CAC-CAC-CAA-CTG-CTT-AC

CAG-AGG-TGC-CAT-CCA-GAG-TT

HPRT: Hypoxanthine phosphoribosyltransferase

GAC-CGG-TTC-TGT-CAT-GTC-G

ACC-TGG-TTC-ATC-ATC-ACT-AAT-CAC

Cyclo: Cyclophilin

CTG-ATG-GCG-AGC-CCT-TG

TCT-GCT-GTC-TTT-GGA-ACT-TTG-TC

B2M: β-2 microglobulin

TGC-CAT-TCA-GAA-AAC-TCC-CC

GAG-GAA-GTT-GGG-CTT-CCC-ATT

LDHA: Lactate dehydrogenase

GAT-CTC-GCG-CAC-GCT-ACT

CAC-AAT-CAG-CTG-GTC-CTT-GAG

SDHA: Succinate dehydrogenase complex, subunit A

CCC-TGA-GCA-TTG-CAG-AAT-C

CAT-TTG-CCT-TAA-TCG-GAG-GA

RPL13a: 60S ribosomal protein L13a

GAT-CTC-GCG-CAC-GCT-ACT

GGT-ACT-TCC-ACC-CGA-CCT-C

Studied genes:

PKC-α: Protein kinase C-α

CAA-GCA-GTG-CGT-GAT-CAA-TGT

GGT-GAC-GTG-CAG-CTT-TTC-ATC

PKC-δ: Protein kinase C-δ

TCA-AGA-ACC-ACG-AGT-TCA-TCG

GCA-TTG-CCT-GCA-TTT-GTA-GC

PKC-ε: Protein kinase C-ε

CGT-CAC-TGA-TGT-GTG-CAA-TG

TCG-AAC-TGG-ATG-GTG-CAG-TTG

α-MHC: α-Myosin heavy chain

CAA-GGC-AAA-CCT-GGA-GAA-AG

GGG-TAT-AGG-AGA-GCT-TGC-CC

β-MHC: β- Myosin heavy chain

GAG-GAG-AGG-GCG-GAC-ATT

ACT-CTT-CAT-TCA-GGC-CCT-TG

ANP: Atrial natriuretic peptide

CAA-CAC-AGA-TCT-GAT-GGA-TTT-CA

CGC-TTC-ATC-GGT-CTG-CTC

BNP: B-type natriuretic peptide

GTC-AGT-CGC-TTG-GGC-TGT

CAG-AGC-TGG-GGA-AAG-AAG-AG

Studied genes cont.:

ANKRD1: Ankyrin repeat domain-containing protein 1

GCTGGAGCCCAGATTGAA

CTCCACGACATGCCCAGT

TNF-α: Tumor necrosis factor-α

GCC-CAG-ACC-CTC-ACA-CTC

CCA-CTC-CAG-CTG-CTC-CTC-T

TGF-β1: Transforming growth factor β1

AAG-AAG-TCA-CCC-GCG-TGC-TA

TGT-GTG-ATG-TCT-TTG-GTT-TTG-TCA

TGF-β2: Transforming growth factor β2

ATC-GAT-GGC-ACC-TCC-ACA-TAT-G

GCG-AAG-GCA-GCA-ATT-ATC-CTG

TGF-β3: Transforming growth factor β3

CCC-GAT-GGC-GAA-AGG-CCG-AG

TAG-GGT-AGC-CGG-AGG-CCC-CT

Cth: Cystathinase

TGG-GAC-CAG-AGC-CGG-AGC-AA

AAG-GCC-CCG-AGC-GAA-GGT-CA

VEGF-α: Vascular endothelial growth factor-α

CAA-GCC-AAG-GCG-GTG-AGC-CA

TCT-GCC-GGA-GTC-TCG-CCC-TC

VEGF-β: Vascular endothelial growth factor-β

ACC-AGA-AGA-AAG-TGG-TGT-CAT-G

TGA-GGA-TCT-GCA-TTC-GGA-CTT-G

PGF: Placental growth factor

GTG-TGG-GGC-CGC-AGC-TAC-TG

AGC-GCC-ACA-CAG-TGC-AGA-CC

SOD1: Superoxide dismutase 1

TTC-GTT-TCC-TGC-GGC-GGC-TT

TTC-AGC-ACG-CAC-ACG-GCC-TT

SOD2: Superoxide dismutase 2

ATT-AAC-GCG-CAG-ATC-ATG-CA

CCT-CGG-TGA-CGT-TCA-GAT-TGT

eNOS: Endothelial nitric oxide synthase

TGA-CCC-TCA-CCG-ATA-CAA-CA

CGG-GTG-TCT-AGA-TCC-ATG-C

iNOS: Inducible nitric oxide synthase

ACCATGGAGCATCCCAAGTA

CAGCGCATACCACTTCAGC

TP53: Tumour suppressor gene(TP53)

GTT-AGG-GGG-TAC-CTG-GCA-TC

CGA-CTG-TGA-ATC-CTC-CAT-GA

Casp-3: Apoptosis related cysteine protease; caspase 3

CCG-ACT-TCC-TGT-ATG-CTT-ACT-CTA

CAT-GAC-CCG-TCC-CTT-GAA

Bcl-2: B-cell leukaemia/lymphoma 2

GTA-CCT-GAA-CCG-GCA-TCT-G

GGG-GCC-ATA-TAG-TTC-CAC-AA

COL1A1: Collagen type I-α1

CAT-GTT-CAG-CTT-TGT-GGA-CCT

GCA-GCT-GAC-TTC-AGG-GAT-GT

COL3A1: Collagen type III-α1

TCC-CCT-GGA-ATC-TGT-GAA-TC

TGA-GTC-GAA-TTG-GGG-AGA-AT

FN1: Fibronectin 1

CAG-CCC-CTG-ATT-GGA-GTC

TGG-GTG-ACA-CCT-GAG-TGA-AC

TIMP1: Tissue inhibitor of metallopeptidase 1

CAG-CAA-AAG-GCC-TTC-GTA-AA

TGG-CTG-AAC-AGG-GAA-ACA-CT

TIMP 3: Tissue inhibitor of metallopeptidase 3

GAA-CGG-AAG-CGT-GCA-CAT-G

CAG-CTT-CTT-TCC-CAC-CAC-TTT-G

TIMP 4: Tissue inhibitor of metallopeptidase 4

AGG-GAG-AGC-CTG-AAT-CAT-CA

GCA-CTG-CAT-AGC-AAG-TGG-TG

HIF1A: Hypoxia-inducible factor 1α

TGC-TTG-GTG-CTG-ATT-TGT-GA

GGT-CAG-ATG-ATC-AGA-GTC-CA

CAT: Catalase

TTT-TCA-CCG-ACG-AGA-TGG-CA

CCC-ACA-AGG-TCC-CAG-TTA-CC

HK2: Hexokinase II

TCG-CAT-ATG-ATC-GCC-TGC-TT

GCC-ATT-GTC-CGT-CAC-CCT-TA

GPx1: Glutathione peroxidase 1

AGT-TCG-GAC-ATC-AGG-AGA-A

AGG-GCT-TCT-ATA-TCG-GGT-TC

GPx2: Glutathione peroxidase 2

GCC-TAG-TGG-TTC-TCG-GCT-TCC

AGG-GTA-GGG-CAG-CTT-GTC-TTT-C

GPx4.1: Glutathione peroxidase 4 transcript variant 1

GCC-GCT-TAT-TGA-AGC-CAG-C

GTG-GGC-ATC-GTC-CCC-ATT-TA

GPx4.2: Glutathione peroxidase 4 transcript variant 2

CCC-ATT-CCC-GAG-CCT-TTC-AA

TAT-CGG-GCA-TGC-AGA-TCG-AC

Ddx3y: DEAD box polypeptide 3, Y-linked

ACG-GTG-GCT-TGC-TCC-GTG-AA

GCC-AAC-CGT-ATT-TTC-CGC-CGC

Eif2s3y: eukaryotic translation initiation factor 2, subunit 3, Y-linked

GGT-TGG-GCA-GGT-CCT-TGG-TGC

CGC-CAG-TGC-TTT-TCA-ACT-CGT-CG

Paper A

Aljabri MB, Songstad NT, Lund T, Serrano MC, Andreasen TV, Al-Saad S, Lindal S, Sitras V, Acharya G and Ytrehus K.

Pregnancy protects against antiangiogenic and fibrogenic effects of angiotensin II in rat hearts.

Acta Physiol 2011, 201: 445-456.

Paper B

Songstad NT, Johansen D, How O-J, Kaaresen PI, Ytrehus K and Acharya G.

Effect of transverse aortic constriction on cardiac structure, function and gene expression in pregnant rats.

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Paper C

Songstad NT, Kaspersen K-H, Hafstad A, Basnet P, Ytrehus K and Acharya G.

High intensity interval training in pregnant rats alters gene expression in fetal heart and liver without inducing oxidative stress.

Submitted manuscript

Paper D

Songstad NT, Serrano MC, Sitras V, Johansen D, Ytrehus K and Acharya G.

Coronary flow reserve in pregnant rats with increased left ventricular afterload.

Accepted for publication in PLoS ONE