“The most exciting phrase to hear in science,
the one that heralds the most discoveries,
is not Eureka! I found it!
but Hummm… that's funny"
~Isaac Asimov

University of Alberta

Long-Term Cardiovascular and Metabolic Effects of Hypoxia-Induced Intrauterine Growth Restriction

by

Christian Federico Rueda-Clausen

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

Spring 2011
Edmonton, Alberta

DEDICATION

All the hard work and sacrifices that this thesis represents are dedicated with love and respect to my family.

ABSTRACT

Cardiovascular and metabolic diseases are still the primary cause of death and disability in modern society. Although genetic factors play a fundamental role in the development of these chronic conditions, the remarkable variability in an individual's susceptibility to develop these pathologies cannot be completely explained by genetics. The early programming of adult diseases theory became established in the 1980's, and is now supported by a growing body of evidence demonstrating that exposure to suboptimal environmental conditions during crucial periods of time can predispose an individual to the development of chronic conditions (including cardiovascular and metabolic diseases) later in life.

Among the multitude of factors that can cause early programming, we have focused on the study of pregnancy complications leading to fetal hypoxia and causing intrauterine growth restriction (IUGR). To this end, we have used an animal model in which pregnant Sprague Dawley rats were exposed to either normal (~21% O_2) or hypoxic (~11.5% O_2) conditions during the last third of pregnancy. We then followed and studied the cardiovascular and metabolic characteristics of the offspring later in life.

The studies presented in this thesis demonstrate that hypoxic prenatal insults have long-term consequences on cardiac structure, function and susceptibility to ischemia. We also demonstrated that programmed susceptibility to myocardial ischemia was associated with changes in cardiac energy metabolism and increased levels of myocardial oxidative stress.

Moreover, we described the interaction between prenatal hypoxic insults, aging and sex differences in the later development of cardiovascular conditions.

Additional studies presented in this thesis demonstrate that offspring born IUGR are more susceptible to develop most components of the metabolic syndrome when exposed to a high-fat (HF) diet. Furthermore, we also demonstrated that the exacerbated deleterious response to a HF diet described in offspring born IUGR can be prevented by postnatal administration of Resveratrol, which is a natural compound with anti-oxidant and anti-aging properties.

In conclusion, the results presented in this thesis are an important contribution to the understanding of the long-term cardiovascular and metabolic effects of prenatal hypoxic insults causing IUGR and provide evidence regarding possible mechanisms and treatment alternatives that could be considered.

ACKNOWLEDGEMENTS

So many people to acknowledge and so few words to describe my immense gratitude.

I would like to start by thanking Sandy, whom I met when she became my supervisor a few years ago and who has since become my mentor, model and friend. No matter how busy the days were or how far her academic endeavors took her, she was always there, ready to provide the right advice and the encouraging words that I needed so often. I would also like to acknowledge my committee members, Dr. Gary Lopaschuk and Dr. Zam Kassiri, who have accompanied me on this journey with their thoughtful advice and constructive criticism.

The thesis that sits below this page wouldn't have been possible without the participation and collaboration of a wonderful team of people including trainees, technicians and researchers from the Heritage Medical Research Centre (HMRC) and the Cardiovascular Research Center (CVRC) at the University of Alberta. They were always ready to share their knowledge or discuss my results, and these people made this journey a fantastic learning experience and all of them have my eternal gratitude for that. From all of these wonderful people I would like to specially mention Dr. Jude Morton, who I worked with shoulder to shoulder and from whom I learned a lot of lessons relevant to language, science and life (Thanks Mort!!). I would also like to thank Dr. Joanna Stanley who participated in the style revision of this thesis, as well as Dr. Yanyan Jiang and Mrs. Sheila McManus who provided invaluable technical and administrative support during these years. In the same way I would like to thank all of the people with whom I had the good fortune to collaborate with, including Dr. Vernon Dolinsky, Dr. Jason R.B. Dyck, Dr. Spencer Proctor, Dr. Ferrante Gragasin, Dr. Gavin Oudit, Dr. Geoff Ball, Dr. Andrea Haqq, and Dr. Valerie Luyckx. I would also like to thank all of the staff and faculty from the department of Physiology who always supported me and made me feel like I was amongst family.

I am honored to have been part of the TORCH program and I want to thank all of the mentors, alumni and administrative personnel involved in this program; it helped me break paradigms and opened new doors to wonderful opportunities.

I would also like to express my immense gratitude to the following funding agencies; the Canadian Institutes of Health Research (CIHR), Pfizer Canada [protocol NRA2580076], the Women and Children's Health Research Institute (WCHRI), the Heart and Stroke Foundation of Canada (HSFC) and Alberta Innovates Health Solutions (AIHS), previously known as Alberta Heritage Foundation for Medical Research (AHFMR). Without their support this work wouldn't have been possible.

Finally, I want to especially thank my family for always supporting and encouraging me to pursue my dreams. Cris, this journey would not have been possible without you. I have no words to thank you for being by my side and sharing with me the best years of my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

The following abbreviations, definitions and units have been used throughout this thesis.

$^{14}CO_2$	Radiolabeled carbon dioxide
2D	Bi-dimensional
3D	Tri-dimensional
$^{3}H_2O$	Tritium
Acetyl-CoA	Acetyl coenzyme A
AHA	American Heart Association
AHFMR	Alberta Heritage Foundation for Medical Research
AIHS	Alberta Innovates Health Solutions
Akt	Protein kinase B
AMPK	5' AMP-activated protein kinase
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
BMI	Body mass index
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CE	Cholesterol esters
CF	Coronary flow
Ci	Curies
CIHR	Canadian Institutes of Health Research
CO	Cardiac output
CO_2	Carbon dioxide
CP	Chamber pressure
CT scan	Computed axial tomography scan
CV	Caloric value
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of adulthood heath and disease

dP/dt_{max}	First positive derivative of pressure over time
dP/dt_{min}	First negative derivative of pressure over time
ECM	Extracellular matrix
EDP	End-diastolic perfusion pressure
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
ET	Ejection time
Fe	Iron
Fe^{2}	Ferrous iron
Fe^{3}	Ferric iron
FFA	Free fatty acids
FITC-MAb1	Fluorescein-isothiocyanate-conjugated IgG-1 mouse monoclonal antibody
FPLC	Fast protein liquid chromatography
FPN	Ferroportin
G	Gestational day
GnRH	Gonadotropin releasing hormone
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GTP	Guanosine triphosphate
GTT	Glucose tolerance test
H^{+}	Protons
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDL-C	High density lipoprotein cholesterol
HF	High-fat
HIF-1α	Hypoxia-inducible factor 1 alpha
HOMA index	Homeostatic model assessment index
HR	Heart rate
HSE	Hugo Sachs Electronik
HSFC	Heart and Stroke Foundation of Canada
I/R	Ischemia reperfusion
IL-1ß	Interleukin 1 beta

IL-6	Interleukin 6
IP	Intraperitoneal
IRE	Iron response elements
IRS-1	Insulin receptor substrate 1
ITT	Insulin tolerance test
IUGR	Intrauterine growth restriction
IVCT	Isovolumetric contraction time
IVRT	Isovolumetric relaxation time
IVS	Interventricular septum thickness
LF	Low-fat
LV	Left ventricle
MetS	Metabolic syndrome
mmHg	Millimeters of mercury
MMP	Matrix metalloproteinase
MPA	Metaphosphoric acid
MPP	Mean perfusion pressure
Na	Sodium
NaCl	Sodium chloride
NaF	Sodium fluoride
NCX	Na/Ca exchangers
NHE-1	Na/H exchanger type 1
$O_2^{\bullet-}$	Superoxide anion
O_2^{2-}	Peroxide ion
OH^-	Hydroxyl radical
P/O	Phosphate/oxygen
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PKCε	Protein kinase C epsilon
PKCθ	Protein kinase C theta
pO_2	Partial oxygen pressure
PPARγ	Peroxisome proliferator-activated receptor gamma
PT	Perfusate temperature
RAS	Renin-angiotensin system

RER	Respiratory exchange ratio
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SGA	Small for gestational age
SIRT1	NAD^{+}-dependent protein deacetylase
β/αMHC	Beta/alpha myosin heavy chain ratio
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TD-NMR	Time-domain nuclear magnetic resonance
Tei index	Myocardial performance index
Tf	Transferrin
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TG	Triglycerides
TIMP	Matrix metalloproteinase tissue inhibitor
TNFα	Tumor necrosis factor alpha
UBM	High-resolution ultrasound biomicroscopy
UCP-1	Uncoupling protein1
VCO_2	Carbon dioxide production
V_iCO_{2i}	Carbon dioxide input
V_iO_{2i}	Input oxygen flow
VO_2	Oxygen consumption
V_oCO_{2o}	Carbon dioxide output
V_oO_{2o}	Output oxygen flow
WCHRI	Women and Children's Health Research Institute
WHO	World Health Organization

Mathematical prefixes

K……	kilo	(10^{3})
d……	deci	(10^{-1})
c……	centi	(10^{-2})
m……	milli	(10^{-3})
μ……	micro	(10^{-6})
n……	nano	(10^{-9})
ρ……	pico	(10^{-12})

Symbols

α……	alpha
β……	beta
γ……	gamma
δ……	delta
ε……	epsilon
θ……	theta

Time units

Mo……	month
d……	day
h……	hour
min……	minute
s……	second

CHAPTER 1 GENERAL INTRODUCTION*

1.1 Overview

The classic pathophysiological conception of many chronic diseases considers the interaction of two key elements: genetic predisposition and environmental factors.[1] Interestingly, a growing body of evidence has proven that among subjects with similar or identical genetic background, substantial differences can be observed in their susceptibility to develop adverse health outcomes such as cardiovascular diseases (CVDs) when exposed to similar environmental conditions.[2-7] These results suggest that there could be other elements influencing the development of health conditions.

In the late 1980's, a number of epidemiological observations linking low birth-weight and increased risk of developing chronic diseases later in life popularized the hypothesis that today is known as the "developmental origins of adulthood heath and disease (DOHaD)" or "the early programming theory".[8-12] This theory constitutes an interesting scientific proposal, not only because it could explain why some subjects are more susceptible to develop certain pathological conditions (regardless of their genetics), but also because it opens the door to a completely new approach for prophylactic and therapeutic alternatives aiming to reduce the prevalence and severity of chronic diseases.

---

* *Components of this chapter have previously been published in (or submitted to) peer-review journals including:*

- *"**Rueda-Clausen CF**, Morton JS and Davidge ST. The Early Origins of Cardiovascular Health and Disease: Who, When and How. Submitted to: Seminars in Reproductive Medicine in-press 2011"(Review).*
- *"Davidge ST, Morton JS and **Rueda-Clausen CF**. Oxygen and perinatal origins of adulthood diseases: is oxidative stress the unifying element? Hypertension. 2008;52:808-810. (Editorial comment)*

***Contribution:** Rueda-Clausen CF. wrote the first draft of the articles (including all the figures) and coordinated with the other authors in compiling the final version of the manuscript.*

In the last couple of decades, a growing body of basic fundamental, clinical and epidemiological evidence has linked the early programming theory to many chronic conditions including the most prevalent and relevant cardiovascular[13-16] and metabolic diseases.[17-22] However, limitations in the models used to study this phenomenon, as well as a lack of clear pathophysiological mechanisms, make the early programming theory a very polemic and controversial subject. Moreover, these gaps in knowledge constitute a fundamental barrier limiting the translation of this pathophysiological model to clinical scenarios. Therefore, understanding this phenomenon constitutes a promising area of research that could have many implications for the prevention and management of highly prevalent diseases.

1.2 The early programming theory

After Charles Darwin's Origin of the Species theory was published in 1854, most evolutive/adaptative responses were attributed to a process of natural selection in which random phenotypical variations occur sporadically in all species. According to this model, environmental conditions play a role by selecting and perpetuating the "strongest" phenotype. However, the environment itself was thought to have little or no direct influence on initially changing the phenotype of the species.[23]

Interestingly, by the time Darwin's hypothesis became popular, reports had been published describing a group of organisms (mainly insects) in which environmental conditions played a fundamental role in regulating their phenotypical characteristics.[24] However, these organisms were initially considered oddities and treated as exceptions to the evolutionary rule.[25] The further development of population genetics in the 1920's and 1930's demonstrated that Mendelian genetics were compatible with the natural selection and gradual evolution model initially proposed by Darwin, and

established foundations for the Modern Evolutionary Synthesis approach that is now widely accepted by the scientific community.[26]

More recent evidence suggests that contrary to the slow, permanent and random evolutive model, species evolution and adaptation is more likely to occur in a cyclic fashion in which periods with significant changes in the environment trigger the accelerated apparition of phenotypical variations.[27] Interestingly, and supporting this theory, some groups have published evidence that highlights the influence of environmental conditions during early development and suggests that the animal genome responds to certain environmental conditions by inducing specific phenotypical changes.[27] Consequently, the consensus of opinion has itself evolved to conclude that the interaction between genes and the environment during development is not as unusual as initially thought and appears to be the rule rather than the exception.[28]

1.2.1 The origins of the early programming theory

The recognition of species growth and development as a dynamic phenomenon that can be modulated by surrounding environmental conditions has now been around for more than a century.[29] In the previous decades, a number of authors have made interesting contributions to the understanding of this phenomena that has been identified by a variety of names such as early origins, developmental origins, *in utero* programming, the thrifty phenotype hypothesis, fetal programming and the Barker hypothesis, among others.[30] However, for the purposes of this thesis we will refer to it as the early programming phenomenon.

The primordial origins of this theory are spread throughout history and can be traced back for centuries depending on how previous publications and quotes are interpreted. Probably the earliest references to a programming phenomenon can be extracted from popular expressions imbedded in many ancient cultures, such as an ancient Greek quote, which

said "*healthy mother, healthy baby*". However, the first epidemiological evidence supporting the clinical relevance of this observation resulted from the work of Kermack *et al.* in 1934[31] who analyzed demographic information from England and Wales from 1845, Scotland from 1860 and Sweden from 1751, these being the earliest periods from which reliable demographic data was available. Based on their observations, the authors suggested that the death rates of adolescents and adults depended on the constitution acquired during the first 15 years or so of life.

Following the same line of thinking but thirty years later, Rose *et al.*[32] reported the association between rates of infant mortality and rates of ischemic heart disease some decades later in subjects with the same genetic background. A few years later, in 1976, Ravelli *et al.*[33] published a paper demonstrating an association between nutritional restriction during pregnancy and the later development of obesity. This was followed by a paper by Forsdahl[34] reporting that the incidence of atherosclerotic heart disease in a certain age group could be correlated with the infant mortality rate of the same population many years before.

Regardless of the implicit references to the programming phenomenon made in the past, it is well accepted that the early programming concept was popularized when David Barker and collaborators brought what they called "the developmental origins theory" to the fore in the mid 1980's.[12, 35]

Barker's initial and most popular studies of the early programming phenomenon focused on documenting the long-term health outcomes in a population from England and Wales; some of whom were *in utero* during starvation conditions in World War II and from whom extensive and accurate postnatal medical records were available.[8-11] Using this valuable information, Barker and collaborators demonstrated that birth weight, as well as other postnatal conditions such as breast feeding and certain anthropometric parameters at one year of age, were strongly associated with the later

development of diseases such as hypertension,[36] chronic bronchitis[10] and coronary heart disease[37] among others. Barker's work constitutes, without question, the most influential contribution and one of the cornerstones supporting the early programming phenomenon.[35]

1.2.2 Proposed pathophysiological models of early programming

The precise mechanisms by which environmental conditions can modulate the phenotypical manifestations associated with health or disease in humans are still elusive. However, multiple candidates have been proposed.[38]

1.2.2.1 The impaired genesis model

Some organs such as the heart, kidney and brain undergo an intense replication and differentiation process during fetal and early life stages while organogenesis occurs.[39] The impaired genesis model suggests that once this developmental period is over, the replication and differentiation potential of the cells from these organs is minimal. Therefore, the exposure of a developing organism to external stressors during these critical periods of rapid replication and differentiation could delay and reduce the amount and/or quality of these long-lasting cells and, in that manner, reduce tolerance to external stressors affecting these particular groups of cells later in life.

One observation that illustrates this particular model has been described in renal development and the effect of maternal protein restriction during the development of the kidney; which constitutes one of the foundations of the Brenner hypothesis.[40] Human nephron development starts around the 5th week of fetal development and, about 30 weeks later, nephrogenesis comes to a halt when the final number of nephrons an individual will have is set.[41] Throughout the normal aging process, the number and function of nephrons declines gradually and by the 8th decade of life, a normal healthy person has lost 40% of total kidney mass and close to

80% of renal function (in terms of creatinine clearance).[42] If external stressors impair nephrogenesis during high replication/differentiation periods, the final number of functional nephrons that a person will have (and consequently his/her renal reserve) will be reduced. Therefore, it is probable that these subjects may be more susceptible to develop premature renal failure as their renal function declines later in life.[43] A similar phenomenon has been described in the myocardium.[44, 45] During fetal development, heart growth occurs initially via hyperplasia of mononucleated cardiomyocytes.[46] Throughout pregnancy there is a transition from hyperplasic to hypertrophic growth as increasing numbers of cardiomyocytes become bi-nucleated and are terminally differentiated to mature cells.[47] At term, the fetal heart contains almost the full number of cardiomyocytes that it will have for the rest of its life and 90% of these cells are bi-nucleated. Given the relative maturity and limited ability for regeneration of the myocardium after birth,[48] environmental factors affecting the proliferation and differentiation of cardiomyocytes during gestation are likely to have long-lasting consequences for heart growth and function.[49] Similar conditions have been described in other cells with a low turnover during adulthood such as neurons.[50]

1.2.2.2 The neuroendocrine model

One of the best-recognized models of neuroendocrine programming in mammals is described in sexual differentiation. The reproductive neuroendocrine axis is sexually differentiated through exposure of the fetus/neonate to male steroid hormones.[51] This process seems not only to be mediated by the effects of hormones on the network of neurons that regulate Gonadotropin Releasing Hormone (GnRH) secretion from the hypothalamus,[52] but also to be susceptible to environmental conditions affecting the availability of hormonal stimuli during crucial periods of development. In lambs, for instance, this sexual differentiation process extends from approximately day 30–90 of gestation. Treatment of female lambs with testosterone during this critical period results in long-term

changes in the offspring including birth weight, infertility, insulin resistance, impairment of hypothalamic sensitivity to steroid negative feedback, pituitary sensitivity to GnRH and marked advancement of the timing of puberty later in life; probably due to long-term changes in the sensitivity of the neuroendocrine axis to the physiological feedback of sexual hormones.[53]

Another well-studied model of neuroendocrine programming is that resulting from chronic exposure to glucocorticoids and its effects on the hypothalamic-pituitary-adrenal (HPA) axis. Maternal corticoids levels can be increased physiologically as part of the normal response to stress,[54, 55] experimentally by direct administration to the mother,[56, 57] or pathologically as a result of other conditions affecting the corticoids production such as polycystic ovary diseases, pituitary and adrenal tumors.[58] Animal studies have demonstrated that an increase in the maternal circulating glucocorticoids can cross the placenta and affect the fetal HPA development, resulting in changes in function that persist throughout life. These changes appear to be modulated at the level of glucocorticoid and mineralocorticoid receptors in the brain and pituitary gland. [59-62] Interestingly, these early-programmed changes in neuroendocrine function are also influenced by sex and age.[63, 64] Moreover, these potential long-term changes in the HPA axis have also been proposed to be involved in the etiology of many long-term programming complications such as hypertension,[65, 66] obesity[67] and type 2 diabetes mellitus (T2DM).[68]

In humans, the most relevant example of neuroendocrine programming was initially described in pregnant women with classical 21-hydroxylase deficiencies in which fetuses are exposed to high concentrations of testosterone, leading to ambiguous genitalia, progressive virilization, menstrual irregularities, reduced fertility and aggressive behavior of the female offspring.[69] Interestingly, little is known about the potential effects of neuroendocrine programming on the development of CVDs. However, the

clearly described effects of hormones in the pathophysiology of CVDs[70] suggests that this mechanism could play a relevant role.

1.2.2.3 The environment-endocrine interaction model

The examples that better illustrate this programming model have been described in vertebrates such as reptiles[71] and some birds,[72] in which the environmental temperature can modulate the sex of the embryo by activating an aromatase capable of converting testosterone into estrogen and, thereby, modulate sexual differentiation.[73] The relevance of this particular model in mammals still needs to be studied. However, it has been described that in mammals, early exposure to specific environmental conditions such as nutritional restriction can modulate the expression (either increased or decreased) of hormonally active agents such as leptin, insulin and estrogens.[74-77]

1.2.2.4 The premature aging model

Aging is an unavoidable degenerative process that is inherent in all living organisms and is characterized by a gradual increase in oxidative stress, decreased cell turnover and function and accumulative deoxyribonucleic acid (DNA) damage,[78, 79] that can eventually progress to pathological states such as hypertension, T2DM, obesity, heart failure and dementia.[80-82] Interestingly, most of these pathological states that are characteristic in an aging population have also been associated with early programming. Moreover, a growing group of authors have proposed that early programming is mediated by the same mechanisms that cause premature aging.[83-85]

Besides the aforementioned mechanisms that could affect the expression of aging-related molecules,[86, 87] two other major potential pathways have been proposed to link early programming and premature aging. One is a reduction in the number, development, function and longevity of stem cells and consequent impairment of the mechanisms that mediate

cellular turnover and tissue regeneration in the adult offspring.[88] The second proposed mechanism, which may be more relevant for CVDs, suggests that early programming induces multiple mediators affecting the stability and regeneration of DNA or reducing the normal defense mechanisms used by the cell to protect the integrity of DNA exposed to normal external and internal stressors.[89] Studies in rodents conducted by our group, have shown that hypoxia-induced fetal growth restriction causes an increased susceptibility to myocardial ischemia/reperfusion (I/R) injury that emulates the effect of aging.[90] Additionally, studies in pregnant ewes have shown that a short cycle of dexamethaxone given at the end of pregnancy can produce a long-term increase in the myocardial production of reactive oxygen species (ROS) similar to that associated with aging. [91] Telomerase length, a cellular marker of DNA integrity that has been inversely associated with aging and the risk of developing CVDs[92] have shown to be reduced in babies born small relative to normal weight newborns.[93]

1.2.2.5 The transcriptional model

The potential capacity of environmental conditions to regulate DNA modifications constitutes a more recently described mechanism of early programming that may have more relevance in mammals.[76] The transcriptional model is based on epigenetics and implies that environmental agents can affect the transcriptional capabilities of genes either by altering their histone methylation or demethylation patterns[76] or by causing a direct methylation of promoter sites;[94] which result in a long-term phenotypic change in the offspring. Advances in the understanding of epigenetic influences during normal development have evolved substantially in the last decades and have been extensively reviewed by other authors.[95-97]

Despite the number of described mechanisms to explain the early programming phenomenon, there is still no consensus regarding which mechanisms are more relevant or better candidates for prophylactic or therapeutic interventions. Moreover, it is possible that different

organs/species have multiple and/or different mechanisms of early programming, or that some of these mechanisms have a certain degree of overlap and synergism in inducing long-term changes in phenotypical manifestations.

1.2.3 Models for the study of the early programming phenomenon

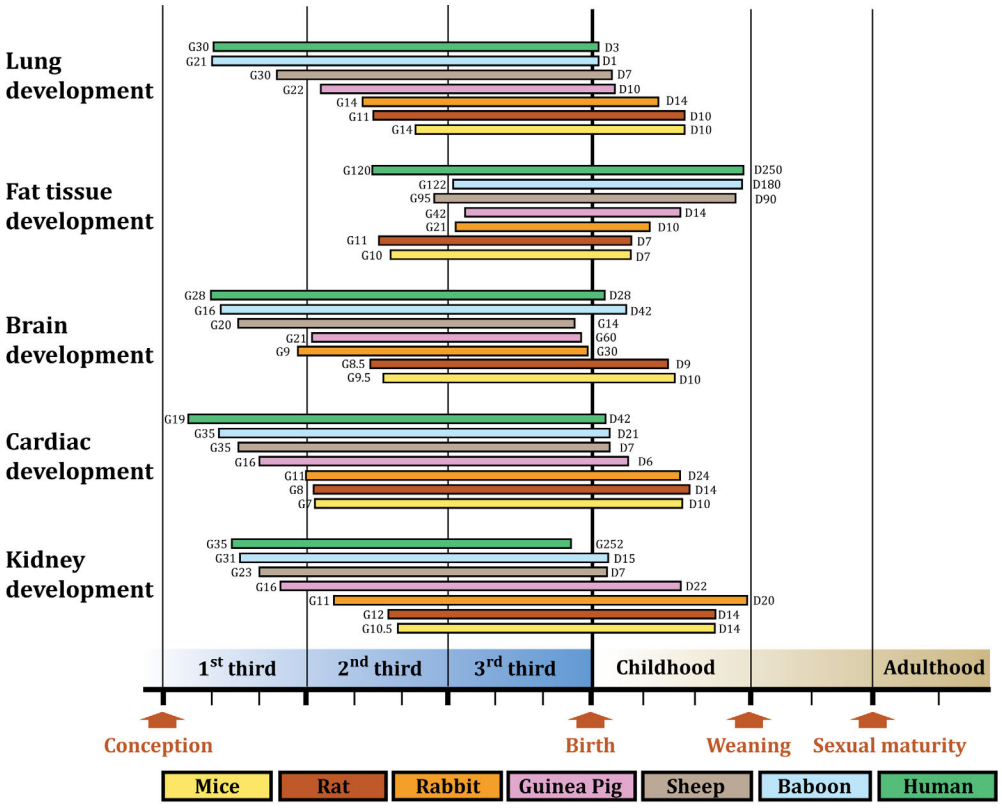
Throughout the years, several groups have used different models to study the pathophysiolgy of the early programming phenomenon. These models can, broadly speaking, be divided in two main groups: clinical and epidemiological models (involving human subjects) and experimental models (involving animals). Among the clinical and epidemiological approaches, the most popular ones are the historical cohort studies in populations with different birth weights and perinatal complications (such as those used by Barker *et al.* in the early 1980's).[12, 35] Other clinical models that have been used in the past include the study of fetal and early development using cadaveric specimens[98] and, more recently, prospective cohort studies in children born from pregnancies complicated by intrauterine growth restriction (IUGR)[99, 100] or twins with differential growth patterns.[7, 101, 102] The use of clinical and epidemiological approaches to the study of the programming phenomenon has several limitations; including high cost, ethical considerations, presence of confounding factors and long periods of time between exposure to the programming insult and development of strong clinical outcomes.[103-105]

Despite the fact that no animal model of pregnancy can fully mimic the human,[19, 106] methodological challenges inherent to clinical research in the early programming area has encouraged the development of several animal models.[100] Indeed, experimental models of early programming have been created using a wide variety of species including bird embryos[107, 108] small rodents (such as mice,[109] rats[110] and guinea pigs[111 112]), larger mammals (such as sheep[113] and llamas[114]) and, to a lesser extent, non-human primates

like the baboon.[115, 116] From these models, those developed in small rodents offer multiple benefits including short pregnancy length and life span of the offspring, convenient litter size, a relatively low cost and the possibility of using genetically modified animals. Experimental models, on the other hand, have their own problems such as species differences and the limited translation of results to clinical scenarios.[109] One of the limitations of using rodent models for studying early programming is that these are a polytocous species and, as a consequence, experimental approaches need to be adjusted for the effect of variation in litter sizes.

1.2.3.1 Timing of insults used to study early programming

During fetal and early childhood development, periods of increased replication and differentiation can differ from one organ or tissue to another. Therefore, there could be different and specific time windows in which every cell, organ or system is more or less susceptible to early programming. Consequently, the length and timing of the exposure to a programming-inducing insult can have substantial consequences on the programming outcome and need to be considered when interpreting the results obtained by each model used to study early programming. In addition, special considerations need to be made when making comparisons across multiple animal models of early programming given that different species have different lengths of gestation, levels of fetal maturity at birth and characteristics of postnatal development that could compromise the interpretation and extrapolation of the results. Figure 1-1 presents a summary of the estimated periods of rapid replication and differentiation of different organs across species relative to common milestones of development.



	Mice	Rat	Rabbit	Guinea Pig	Sheep	Baboon	Human
Length of gestation (days)	19±1	22±1	31±2	67±3	147±4	187±9	266±14
Implantantion (G)	4.5±0.5	5±0.5	7±1	6 ±1	6±1	7±1	7±1
Litter size (n)	10±5	14±4	8±4	3±2	1-2	1	1
Weaning (days)	21	21	28±5	35±7	100±35	185±60	270±70
Sexual maturity (months)	2	0.8	3±1	1±0.3	8±4	84±24	168±10
Life expectancy (years)	2±1	2.7±1.5	7±2	8±2	12±3	30±9	70±20

Figure 1-1 Summarized data showing periods of rapid replication and differentiation among species commonly used to study the early programming phenomenon

G: gestational age (days), D: postnatal age (days).

1.2.3.2 Common types of prenatal insults used to induce early programming in experimental models

In terms of the type of insult used to induce programming, approaches have changed with the identification of numerous elements as potential programming inducers.[117] From an experimental perspective, the insults that are more commonly used are characterized by conditions of deficiency, such as macro[118] and micronutrient restriction,[119] anemia,[120] hypoxia[121, 122] or feto/placental blood flow restriction.[123] This last can be achieved through a

number of mechanisms: including ligation of uterine and/or ovarian blood vessels,[123] injection of microspheres into the placental circulation[124] or manipulation of the materno/placental interface (carunculectomy).[125]

All of these deficiency models are attractive because they emulate either adverse environmental conditions during pregnancy (such as famine, high altitude pregnancy, maternal anemia, smoking or pulmonary disease) or pathological conditions affecting the normal development and function of the placenta (such as pregnancy induced hypertension, gestational diabetes, maternal anemia or placental insufficiency, among many others).

From this variety of programming-inducing insults, those causing a prenatal restriction of fetal oxygen availability (also known as fetal hypoxia) are particularly interesting and clinically relevant.[13, 126-128] Fetal hypoxia can result from either conditions restricting the maternal intake of oxygen (including heavy smoking, living at high altitude[129, 130] and severe pulmonary diseases[117, 131]), or conditions reducing the placental ability to allow gaseous interchange between the fetal and the maternal circulation. This second group of prenatal conditions constitute the most common causes of perinatal morbidity and mortality[132, 133] and include a broad spectrum of common obstetric conditions (such as gestational diabetes, placental insufficiency, preeclampsia and anemia) as well as environmental/social factors like cocaine use.[134]

1.2.4 Intrauterine growth restriction (IUGR) and early programming

One common characteristic in many clinically relevant insults that produce early programming, is that they cause some degree of intrauterine growth restriction (IUGR). This term refers to a pathological condition of pregnancy in which the developing fetus does not reach its growth potential for any given gestational age.[135] As correct and broad as this definition of IUGR is, it is also clinically imprecise since the specific growth potential for any given fetus at any given point during pregnancy is very difficult to

determine.[136] In practicality, clinicians and researchers have been using surrogate outcomes such as body weight below the 10th percentile.[117] Babies with body weights below this cutoff point are also known as babies born small for their gestational age (SGA) and, from a clinical perspective, are considered to have some degree of IUGR.[135]

1.2.4.1 Prevalence of IUGR

Despite having a clear and well accepted criteria to diagnose IUGR, the prevalence of this condition is still elusive and has tremendous variability (between 3.2 to 26%) from one study to another.[137] One recent study including all singleton births documented in the United States from 1995 to 2004 (excluding those born in California), reported that from 19,768,411 births occurring during this window of observation, 3,203,346 (16.18%) had birth weights that were below the 10th percentile (according to the normal growth charts that have been validated for that population).[138]

1.2.4.2 Hypoxia and IUGR

The etiology of IUGR is variable, however, in most instances fetal growth is ultimately constrained by a limitation of oxygen and nutrient delivery.[139] As mentioned in Section 1.2.3.2 of this thesis, prenatal insults limiting oxygen availability are highly relevant and strongly associated with both IUGR and early programming. Fetuses have several mechanisms to compensate for an acute nutritional restriction while they have very limited reserves to compensate for oxygen insufficiency.[140] Hence, even small variations in the availability of oxygen are unlikely to be compensated for by alternative metabolic pathways and are, therefore, very likely to affect fetal growth.[128] Pregnancies that occur at high altitude constitute an interesting condition that illustrates the fundamental effect of oxygen deprivation on fetal growth. Observational studies conducted in populations born at high altitude show that, independent of diet and genotype, the decrease in oxygen availability associated with high altitude is directly correlated with changes

in average birth weight,[136, 141-143] and inversely related to neonatal mortality and morbidity.[129, 144]

1.3 Early programming and the increased risk of developing chronic medical conditions

From the long list of chronic conditions that have been linked to the early programming phenomenon, cardiovascular and metabolic pathologies have received particular attention. This is due not only to their high prevalence, but also to the cost they represent to the health care system. Consequently, and for the purposes of this thesis, we have centered our attention on the potential effects of early programming in these areas. Specifically, we have focused on the long-term effects of hypoxia-induced IUGR on two specific areas: i) cardiac function, structure and response to ischemic insults (Section 1.3.1) and ii) susceptibility to develop components of the metabolic syndrome (MetS) such as obesity, dyslipidemia and insulin resistance (Section 1.3.2).

1.3.1 Early programming of cardiovascular diseases

1.3.1.1 The global burden of cardiovascular diseases

Despite a general public awareness regarding the magnitude of the CVD epidemic, the growing interest of the scientific community and the implementation of several public health strategies during recent decades, CVDs are still the leading cause of death and disability in the world.[145] According to a recent report published by the World Health Organization (WHO), an estimated 17.1 million people die from CVDs in the world each year. This represents 29% of all deaths and affects men and women almost equally.[146] In Canada, CVDs account for more deaths than any other disease: over 72,000 people die of CVDs every year, accounting for 31% of male deaths and 33% of female deaths and causing an estimated cost to the Canadian economy of about $18 billion over the same period of time.[147]

Even more dramatic than the mortality rates is the fact that a far larger proportion of individuals have asymptomatic disease and other organ damage secondary to the presence of undetected cardiovascular risk factors such as high blood pressure, hyperlipidemia, obesity and T2DM.[148] Current estimates show that the global burden of CVDs far exceeds that of its death rates, affecting an estimated 128 million people, or nearly eight times the number of casualties attributable to it.[149] In the United States alone, 71.3 million adults (around 30% of the population) have at least one type of CVD.[148] Thus, the greater burden of CVDs and the majority of costs to the health care system are not only due to mortality but also to non-fatal cardiovascular events, their respective long-term disability and complications such as post-ischemic cardiac arrhythmias and heart failure.[149]

A recent report made by the American Heart Association (AHA) showed that the mortality rates of a first cardiovascular event have decreased in the last 20 years, and consequently, the number of patients discharged from hospital with chronic CVDs and heart failure is increasing.[148] Moreover, this report also mentioned that these subjects are living longer and requiring more resources from the health care system.[148] Furthermore, populations from several developing countries are undergoing a period of epidemiological transition in which life expectancy is increasing and mortality causes are changing toward non communicable and chronic diseases (Figure 1-2).

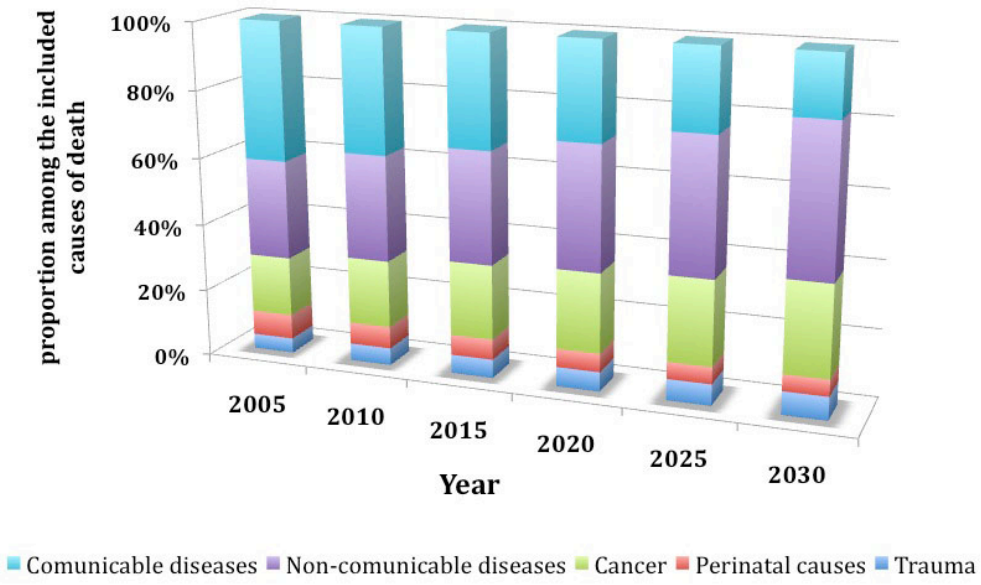


Figure 1-2 Worldwide actual and estimated shifts towards non-communicable and chronic diseases as causes of death

Extracted from "The world health report 2008: primary health care, now more than ever".[150]

As the world's population gets older and the lifestyles of developing societies emulate the nutritional models described in developed countries, the global prevalence of CVDs is expected to increase in the years to come.[145, 147] In fact, it has been estimated that if the current trends continue, the number of casualties attributable to CVDs by 2020 will be around 25 million per year.[148]

Since Barker and his group published their work linking birth weight and cardiovascular outcomes[12] (see Section 1.2.1 for details), several authors have focused on the early programming of cardiovascular pathologies such as cardiac hypertrophy,[151, 152] heart failure,[153] hypertension[15, 105, 118, 152, 154] and endothelial dysfunction[153, 155-157] among others. As a result, a substantial body of evidence has been collected to prove that early exposure to programming-inducing conditions increases the risk of presenting several classic cardiovascular risk factors such as dyslipidemia, hypertension and T2DM,[154] Interestingly, the increased risk of developing CVDs in subjects born IUGR cannot be fully explained by differences in the prevalence of other

cardiovascular risk factors; as shown by Stein and collaborators[158] who used an epidemiological approach to demonstrate that the long-term cardiovascular deleterious effects associated with being born IUGR are clearly independent of the presence of other cardiovascular risk factors. So far, the specific mechanisms leading to an increased risk of CVDs in subjects born IUGR are still elusive. However, special attention has been given to a number of cardiac pathophysiological mechanisms that could be involved.

1.3.1.2 Impaired myocardiogenesis

This pathophysiological mechanism follows the same principle of the impaired genesis model for early programming (Section 1.2.2.1). The mammalian heart is one of the first organs to form during embryogenesis.[159] During early embryogenesis, growth of this organ occurs initially via hyperplasia of mononucleated cardiomyocytes.[46] Throughout pregnancy, myocardial growth undergoes a progressive transition from hyperplasic to hypertrophic growth as increasing numbers of cardiomyocytes become bi-nucleated and are terminally differentiated to mature cells.[47] At term, the heart of a human fetus contains almost the complete number of cardiomyocytes that it will have for the rest of its life and 90% of its cells are bi-nucleated (similar levels of binucleated myocardial cells are reached in rodents by around five days of life).[160] Using a sheep model of IUGR induced by uterine caruncle resection, it has been demonstrated that offspring with IUGR due to a placental deficiency exhibit an increased proportion of mononucleated cardiomyocytes relative to controls[45], which suggests that prenatal hypoxic insults could have a direct effect on cardiac development and maturation. Given the limited ability for regeneration of the myocardium after birth,[48] environmental factors affecting the proliferation and differentiation of cardiomyocytes during gestation are likely to have long-lasting consequences for heart growth and function.[49] Therefore, the potential effect of different perinatal insults on cardiac genesis and maturation is likely to be involved in the cardiac programming

phenomenon.[14] Animal models of chronic fetal hypoxia or protein intake reduction, for instance, have shown that both newborn as well as adult offspring born under these conditions exhibit changes in their number of cardiomyocytes.[44, 161] This due to decreased cell replication and increased levels of apoptosis in the myocardium during early stages of life.[49, 161]

1.3.1.3 Fibrosis, cardiac remodeling and early programming

The extracellular matrix (ECM) is a very organized structure of collagen, proteoglycans, glycoproteins, and other bioactive molecules.[162] This component of the myocardium plays a fundamental role in integrating the individual sarcomeric contractile components, transferring the kinetic energy and determining the passive viscoelastic properties of the heart.[163] The ECM is also a very dynamic element of the myocardium and its turnover is tightly controlled by coordinated degradation and synthesis of its components.[164] Several pathological conditions of the myocardium (such as cardiac hypertrophy, heart failure and diastolic dysfunction) are characterized by a disorganized proliferation/degradation of some components of the ECM leading to changes in structural and viscoelastic properties of the myocardium and decreased mechanical efficiency of the heart.[165]

Studies performed in rodent models demonstrated that the heart of offspring born IUGR, as a result of a maternal nutritional restriction, exhibit a 15% increase in the amount of myocardial interstitial fibrosis compared to controls.[166] Additionally, studies performed by our group using a hypoxia-induced IUGR model, demonstrated that in rats, this kind of prenatal insult produced changes in the cardiac ECM composition during adulthood; characterized by increased deposition of type I and type III collagen fibers.[90]

Among the mechanisms that regulate ECM synthesis and degradation, a family of zinc-dependent matrix metalloproteinase (MMPs), together with their tissue inhibitors (TIMPs), are key regulators.[167] Interestingly, our

previous results also showed that adult offspring, which were prenatally exposed to hypoxia, exhibit decreased myocardial activity of MMP-2.[90] Altogether, these results suggest that pathways involved in the modulation of myocardial remodeling and ECM turnover could be involved in the development of the cardiac phenotype observed in adult rats exposed to prenatal hypoxia.

1.3.1.4 Early programming and increased myocardial susceptibility to ischemia/reperfusion injury

Ischemia refers to a condition in which tissue blood flow (and consequently nutrient and oxygen availability) is reduced to a level that is below physiological requirements. Reperfusion injury refers to the tissue damage that occurs when blood flow is restored after a sustained ischemic period.[168] The particularly deleterious effects of I/R injury, and the fact that I/R can be more deleterious than ischemia alone, were observations initially described by Jennings in the 1960's.[169] It took, however, 20 years of research before some of the molecular and cellular events leading to I/R injury could be elucidated.

The pathophysiological mechanisms involved in I/R injury are very complex and may be mediated by the interaction of several pathways, including ion accumulation,[170] mitochondrial dysfunction,[171] free radical production,[172] apoptosis and autophagy induction,[173] endothelial dysfunction,[174] platelet aggregation and immune activation among others.[175] Interestingly, the relevance of each one of these specific pathways in human disease and response to I/R injury are still controversial.

Although I/R injury cannot be used to evaluate the susceptibility to develop a cardiovascular event, it is very useful to evaluate the cardiac response to a prolonged ischemic period. Therefore, this model emulates the kind of heart injury that is observed after intraoperatory cardioplegia or emergency coronary revascularizations.[176]

Different rodent models have been used to study the effects of prenatal insults and early programming on myocardial susceptibility to I/R injury. Fetal exposure to cocaine, for instance, is known to have a deleterious effect on myocardial sensitivity to I/R injury in adult rat offspring.[177] This may be due to a decreased expression of cardioprotective factors such as protein kinase C epsilon (PKC-ε).[178] It is still, however, unclear whether the long-term effects generated by early exposure to cocaine are due to direct effects of the alkaloid on the adult myocardium, or if they are secondary to other common effects of this compound in pregnancy (such as fetal hypoxia or increased levels of sympathetic neurotransmitters).

Our laboratory[90] and others[122, 179] have shown that adult rat offspring (at four and seven months of age) born IUGR secondary to a prenatal hypoxic insult, have an increased predisposition to I/R injury. Interestingly, other authors did not observe this particular phenotype when studying animals exposed to similar prenatal insults at two months of age.[122] Although there were small differences in the protocols used by these authors, the results suggest that, in this particular model, the increased vulnerability to I/R injury is not a congenital condition associated with hypoxic prenatal insults but rather a phenotype that depends on an aging effect to manifest.

1.3.1.5 Cardiac energy metabolism and early programming

Among the constellation of factors that could be involved in the cardiac phenotype described in adult offspring born IUGR, potential changes in cardiac energy metabolism constitute an interesting and relevant area that requires to be studied in more detail. The heart is an organ with high energy requirements. Its intracellular content of adenosine triphosphate (ATP), however, is very limited. The heart, therefore, depends on its ability to continuously produce large amounts of ATP to guarantee its proper function and viability.[180]

Under aerobic conditions, the main pathways used by the heart to produce ATP include the oxidative phosphorylation of pyruvate, lactate and fatty acids, which are the sources of more that 95% of all the ATP produced by the heart. The remaining ATP production results from other metabolic pathways such as glycolysis or guanosine triphosphate (GTP) derived from the tricarboxylic acid cycle (TCA).[181] The substrate used to produce ATP through oxidative phosphorylation in the mitochondria, has important implications for cardiac energetic efficiency.[182] In the healthy myocardium, ~60% of the energy obtained by oxidative phosphorylation comes from free fatty acid (FFA) oxidation, while the remaining ~40% comes from the oxidation of carbohydrates.[183] Interestingly, the mechanisms that regulate the myocardial uptake of both carbohydrates and FFAs (and consequently determine the predominance of the substrate used to produce energy) are strongly related to each other.[183] In a well-perfused heart, for instance, an elevation of circulating and intra-myocardial levels of FFAs produces a reduction in the rate of glucose oxidation. Moreover, inhibition of fatty acid β-oxidation produces an increase in the rate of glucose oxidation.[184] This phenomenon is also known as the "glucose-fatty acid cycle" or "Randle cycle", in honor of Philip Randle who first described it in the 1960's.[185]

The relevance of the glucose-fatty acid cycle, with respect to myocardial susceptibility to I/R injury, relies on the fact that changes in the substrate used by the heart to produce energy can affect the "oxygen efficiency" of the myocardium (determined as the amount of work performed by the heart for a given amount of oxygen consumed). Studies performed in dogs, for instance, have shown that increasing the rate of myocardial FFA oxidation can affect the performance of the heart by reducing its oxygen efficiency by about 25%.[186]

One factor that can explain why an increase in the utilization of fat as an energetic substrate affects the oxygen efficiency of the heart is the decreased phosphate/oxygen (P/O) ratio of fat metabolism. The P/O ratio

represents the amount of ATP that can be produced by the mitochondria per unit of oxygen[187] and depends on the substrate used to produce ATP. Each mol of palmitate, which is a common FFA used by the myocardium to produce energy, requires 46 mols of oxygen to undergo complete mitochondrial oxidation and produces 105 mols of ATP. On the other hand, one mol of glucose requires 12 mols of oxygen to be catabolized by the mitochondria and in the process produces 31 mols of ATP. By adjusting the amount of ATP produced by the amount of oxygen required for the metabolism of each one of these substrates, it is evident that a myocardial cell using 100% fat as energetic substrate is ~12% less "oxygen efficient" than a cell using 100% glucose as substrate.

Palmitate oxygen efficiency relative to glucose oxygen efficiency:

=Palmitate (ATP prod/O_2 req)/Glucose (ATP prod/O_2 req)
=(105 /46)/(31/12)
=(2.28)/(2.58)
=(0.88)

prod: produced, req: required

The selection of the substrates utilized to produce energy is under very complex control and is regulated by a number of mechanisms including the availability of substrate, the energy demands of the heart, the availability of oxygen and intra-mitochondrial regulatory mechanisms.[188-191] Interestingly, all of these mechanisms that govern energy substrate selection in the myocardium are known to be key elements affecting glucose metabolism coupling, cardiac aerobic performance and cardiac tolerance to I/R injury.[192-194] In fact, it has been demonstrated that interventions modulating cardiac selection of energy substrates can have a beneficial impact by improving cardiac function in a failing heart[195, 196] and cardiac function recovery after ischemic challenges.[193, 197]

Additionally, sub-products derived from glucose metabolism uncoupling constitute another factor that could affect the myocardial susceptibility to ischemia. Under ischemic or hypoxic conditions, when myocardial oxygen availability is reduced, the oxidative phosphorylation capacity of the mitochondria is limited and anaerobic glycolysis takes over to maintain a modest production of ATP that partially compensates for the energy depleted myocardium.[198] A sustained increase in the glycolytic rate in the presence of a reduced pyruvate oxidation capacity (glucose metabolism uncoupling), however, results in the accumulation of other metabolites such as protons (H^+), lactate and sugar phosphates.[182] The production of these by-products can adversely affect cardiac energetic efficiency and may outweigh the benefits of ATP produced from anaerobic mechanisms.[199]

Despite the proven effects of cardiac energy metabolism modulators on cardiac function and susceptibility to ischemia,[200-203] the potential role of these metabolic pathways in mediating some of the long-term cardiac effects due to early programming are still unknown.

1.3.1.6 Early programming, oxidative stress and cardiac function

Oxidative stress refers to a state of imbalance between the production of ROS and antioxidant mechanisms,[204] leading to toxic effects through the production of peroxides and free radicals that damage the structure and function of several components of the cell; including proteins, lipids and DNA. Generally speaking, ROS are not one but a group of atoms or molecules which readily gain (reducing factors) or lose (oxidizing factors) electrons.[205] Due to their electrochemical properties these compounds are "highly reactive" with other molecules. Among ROS some of the most common include superoxide anions ($O_2^{\cdot -}$), peroxide ions (O_2^{2-}) and hydrogen peroxide (H_2O_2). The production of ROS can be mediated by enzymatic processes and constitutes a fundamental part of many physiological processes such as mitochondrial respiration (Figure 1-3), cell signaling and immune system function.

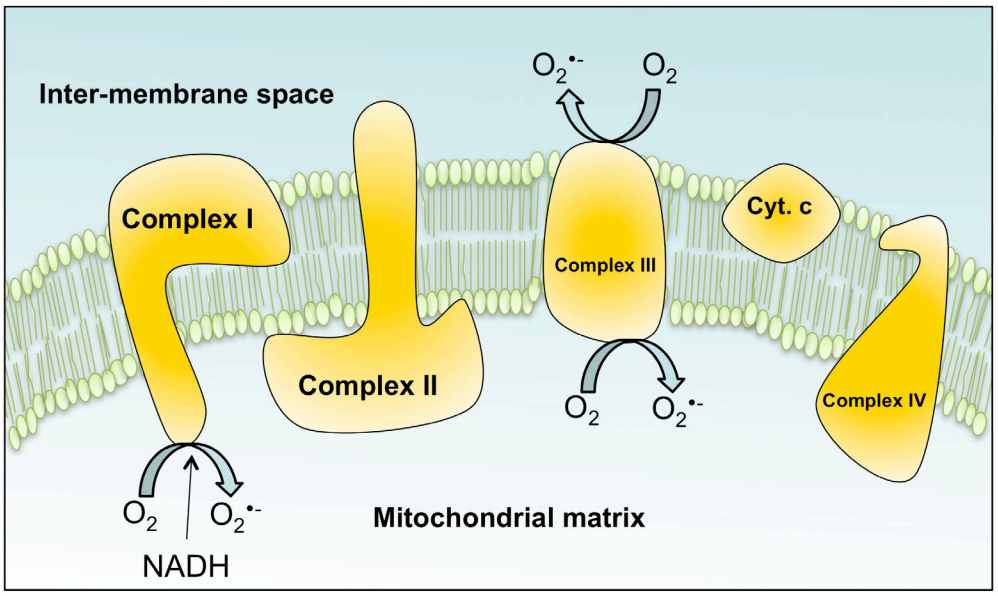


Figure 1-3 Schematic diagram of reactive oxigen species produced by the electron-transport chain during mitochondrial respiration

Schematic of the mitochondrial membrane, showing complexes I–IV of the electron transport chain. Arrows identify superoxide anions ($O_2^{\bullet-}$) produced from complexes I and III. Cyt.c: cytochrome c.

Since ROS are so prone to react and interact with other molecules, organisms have developed antioxidant defenses to limit the activity of these molecules; such as glutathione, vitamin C, and vitamin E and enzymes such as catalase, superoxide dismutase and various peroxidases.[206]

In humans, oxidative stress has been shown to be involved in many cardiovascular diseases including atherosclerosis,[205] heart failure[207] and myocardial infarction.[208] Moreover, several mechanisms involved in aging and the development of cardiac dysfunction are associated with an increased oxidative stress in the myocardium.[206, 209-212]

Some studies suggest that oxidative stress may play a role in the development of the cardiovascular phenotype observed in animals exposed to particular prenatal insults leading to early programming. Studies in sheep exposed to corticoids *in utero,* for instance, have identified that the production of ROS was increased in the coronary arteries of programmed

animals;[213] probably due to an increased mitochondrial production of H_2O_2 despite the presence of increased levels of antioxidants such as catalase.[91] A set of studies made by Elmes *et al*., demonstrated that postnatal enhancement of the antioxidant status (by administering N-acetylcysteine or diethylmaleate) can revert the increased susceptibility to I/R that is present in adult offspring born from dams exposed to protein restriction during pregnancy.[214, 215] However, the role of oxidative stress in the development of the cardiac phenotype described in animals exposed to hypoxia-induced early programming has not been explored.

1.3.1.7 Iron metabolism and its potential role in early cardiac programming

Iron (Fe) is an essential molecule involved in many physiological processes including oxygen transport (as a component of hemoglobin and myoglobin molecules), cellular respiration (as a component of cytochrome enzymes in the mitochondria) and cellular signaling (as a component of enzymes such as nitric oxide synthase, NOS).[216, 217] The total amount of iron in the body of a healthy person is 3-4 g, from which approximately two thirds are found as hemoglobin either in erythrocytes, the bone marrow or the reticulo-endothelial system. The remaining iron is distributed in tissue stores, mainly in the liver, spleen and skeletal muscle.[218]

Iron's fundamental role in several metabolic processes relies on its high reduction-oxidation potential, which also makes it potentially deleterious for the redox balance.[219] For instance, the addition of a single electron to H_2O_2 results in the generation of the hydroxyl radical (OH^-), one of the most deleterious oxidants. This process can occur in the presence of free iron in a chemical process known as the Haber-Weiss-Fenton reaction.[220]

Haber-Weiss-Fenton reaction:	
First step	$Fe^{3+} + {\bullet}O_2^- \rightarrow Fe^{2+} + O_2$
Second step (Fenton reaction):	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {\bullet}OH$
Net reaction:	${\bullet}O_2^- + H_2O_2 \rightarrow {\bullet}OH + OH^- + O_2$

Some pathological conditions characterized by exaggerated iron accumulation (such as primary hemochromatosis or secondary iron overload), are also characterized by a multisystem failure secondary to the deleterious effects of iron deposition in several organs including brain, pancreas, liver, kidney and heart.[221-224] Iron accumulation in the heart is also known as cardiac siderosis and constitutes the most common cause of death in people with chronic iron intoxication.[225] Other common conditions caused by iron overload include iron toxicosis,[226] iron-overload cardiomyopathy,[211] Friedreich-ataxia associated cardiomyopathy,[227] iron-overload endocrinopathies,[228] and increased susceptibility to myocardial I/R injury[229-231] among others.

Because of its toxic properties, iron uptake, transport and storage are highly regulated processes.[232] Iron absorption occurs principally in the duodenum through a coordinated series of protein interactions.[233] Once inside the enterocytes, it either binds to ferritin and turns into an iron storage molecule, or moves to the basolateral membrane to be transported into the circulation by the transporter ferroportin (FPN).[234] In the circulation, ferrous iron (Fe^{2+}) is oxidized to its trivalent state as ferric iron (Fe^{3+}) by a number of enzymes. Fe^{3+} is the only form of Fe that can bind to transferrin (Tf) for safe transport of iron in the circulation.[235]

Transferrin is an 80 kDa glycoprotein synthesized mainly by the liver,[220] that can be present in the circulation in its apo, monoferric or diferric forms depending on the plasma concentrations of iron.[236] Plasma levels of Tf are regulated at multiple levels by multiple transcription factors including iron response elements (IRE) and hypoxia-inducible factor-1 alpha (HIF-1α);

which may be important for iron mobilization during hypoxic conditions.[237] Typically, Tf remains 20-50% saturated with iron, providing a buffer range for acute increases in plasma iron levels. Therefore, Tf acts as both an iron donor and acceptor, depending on supply and demand.[233] Absorption of Tf-bound iron occurs via one of two Tf receptors that have been identified; TfR1 and TfR2.

Peripheral storage of iron depends on Ferritin, which is a water soluble protein that consists of 24 subunits and is capable of binding several thousand atoms of iron.[238] Ferritin synthesis is regulated by the presence of iron via IRE.[239] Despite being predominantly distributed in intracellular compartments, plasma levels of Ferritin are closely correlated with intracellular levels[240] and constitute the most commonly used parameter to evaluate iron metabolism in clinical scenarios.[236]

The acute effects of different environmental conditions on iron homeostasis in adult subjects have been well described using different animal models.[241-245] Both, hypoxic insults and acute infections, for instance, can enhance iron absorption by the intestinal mucosa and increase the mobilization of this element from hepatic stores into the circulation.[236, 244, 246] Moreover, deficiency of iron or other oligo-elements during pregnancy is known to cause early cardiovascular programming.[120, 247, 248] However, the potential effects of prenatal insults (such a hypoxia) leading to early programming in the later regulation of iron metabolism remain unexplored.

In addition to its direct association with several cardiovascular conditions (as previously mentioned), the early programming phenomenon has also been linked to an increased cardiovascular risk by inducing or favoring the development of pathological metabolic conditions. These conditions include obesity, insulin resistance and dyslipidemia; all of which are associated with an increased risk of developing CVDs and constitute relevant health outcomes.

1.3.2 Early programming and metabolic diseases

As previously mentioned, a strong association between cardiovascular pathologies and cardiometabolic risk factors has been clearly established.[249] In fact, it is estimated that up to 50% of all cardiovascular events could be prevented by reducing the prevalence and severity of a number of cardiovascular and metabolic risk factors.[249-251]

Interestingly, the early programming phenomenon has also been demonstrated to be involved in the etiology of many cardiovascular and metabolic risk factors.[248, 252-254] Given the close interaction and relevance of these three elements (early programming, cardiovascular and metabolic pathologies), we have developed a particular interest in the study of other cardiometabolic factors that could influence the future development of cardiovascular health outcomes and could be associated with the antecedent of being born from complicated pregnancies.

1.3.2.1 The metabolic syndrome

Since preventive strategies have proven to be the most cost-effective approach to reduce morbidity-mortality attributable to CVDs,[255, 256] much effort has been directed toward the identification, classification, understanding and prevention of different cardiovascular risk factors.[257-259] In this process, several terms have been coined to integrate the multifactorial nature of CVDs and facilitate cardiovascular risk assessment in clinical scenarios.[260-262] Of these terms, metabolic syndrome (MetS) is among the most commonly used.[263-265]

By definition, the MetS constitutes a cluster of the most relevant cardiovascular risk factors which coexist more frequently than would be expected by chance alone.[263] The specific clinical criteria that define MetS have been the subject of controversy in the last decades.[266-268] A recent joint interim statement, however, made by the most prominent organizations interested in the study of the MetS,[249] proposed that the presence of three

abnormal findings out of five (which include: T2DM or insulin resistance, abdominal obesity, hypertriglyceridemia, low plasma levels of high density lipoprotein cholesterol (HDL-C) and high blood pressure) are required to diagnose a person with this entity (Table 1-1).[249]

Table 1-1 Criteria for the clinical diagnosis of metabolic syndrome

Measure	Categorical Cutoff Points
Elevated waist circumference	Population-specific definitions. For United States and Canada ≥102 cm in males ≥88 cm in females
Elevated triglycerides (drug treatment for elevated triglycerides is an alternate indicator)*	≥150 mg/dL (1.7 mmol/L)
Reduced HDL-C (drug treatment for reduced HDL-C is an alternate indicator)*	<40 mg/dL (1.0 mmol/L) in males; <50 mg/dL (1.3 mmol/L) in females
Elevated blood pressure (antihypertensive drug treatment in a patient with a history of hypertension is an alternate indicator)	Systolic blood pressure ≥130 and/or Diastolic blood pressure ≥85 mmHg
Elevated fasting glucose (drug treatment for elevated glucose is an alternate indicator)	≥100 mg/dL

Extracted from Alberti *et al*. Circulation 2009.[249] HDL-C indicates high-density lipoprotein cholesterol. ***** The most commonly used drugs for elevated triglycerides and reduced HDL-C are fibrates and nicotinic acid. A patient taking any of these drugs can be presumed to have high triglycerides and low HDL-C.

Based on these recently introduced diagnostic criteria, it was determined that in 2004 the prevalence of MetS among subjects over 20 years old in the United States was around 40.1% and that this prevalence is very similar in men (41%) and women (39%).[249]

Even though the identification of this cluster of cardiovascular risk factors as a "syndrome" is questionable,[269] it provides a widely accepted tool to identify subjects at risk of developing other cardiovascular risk factors as well as CVDs.[266, 270] It has been estimated that people with MetS are twice as likely to die from CVDs and three times more likely to have a cardiovascular event than people without it.[148]

1.3.2.2 Obesity and metabolic syndrome

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have an adverse effect on health.[271] A recent Canadian study showed that ~50% of all cases of T2DM, ~25% of all cancers and ~22% of all CVDs, as well as some co-morbidities, are considered to be directly attributable to obesity.[271] For ease of diagnosis, simplified criteria such as body mass index (BMI), defined as weight (Kg)/second power of height (m^2), have been widely incorporated into clinical practice. The use of this specific parameter, however, to diagnose obesity is controversial since several epidemiological and clinical studies have shown that not all fat deposits are identical and that it is intra-abdominal fat (central adiposity) which is more strongly associated with cardiovascular risk factors and the development of adverse cardiometabolic outcomes.[249, 253, 272-274] In fact, many authors consider that central obesity is the most important element in the MetS since it is strongly associated and involved in the etiology and pathophysiology of all other components of this syndrome.[263, 269, 275]

Several pathways have been identified as potential mechanisms by which central obesity is associated with other cardiovascular and metabolic conditions. An increase in the intra-abdominal fat content has been associated with adipose tissue dysfunction,[276] which is characterized by a number of morphological and physiological changes. These changes include: increased adipocyte size, adipocyte hypoxia and increased plasma concentrations of inflammatory, atherogenic, and diabetogenic adipokines, increased circulating levels of triglycerides (TG) and FFA, ectopic fat accumulation and impaired peripheral insulin sensitivity.[276-278]

1.3.2.3 Early onset of obesity and metabolic syndrome

Relative to adults, there is a less-clear diagnostic criteria of obesity or MetS in the pediatric population.[279, 280] Moreover, the actual diagnostic criteria for pediatric obesity likely underestimate the prevalence of this

condition.[281] Despite these limitations, it is estimated that close to 20% of children between 2 and 18 years old are overweight or obese and close to 50% of these children have some other metabolic condition associated with obesity such as dyslipidemia, insulin resistance and non-alcoholic fatty liver.[282-284]

A sustained and dramatic rise in the prevalence of obesity and its metabolic complications among children has occurred during the last three decades and is considered to be one of the most alarming and challenging public health problems.[282, 285] In fact, it has been suggested that for the first time in two centuries, the current generation of children in America may have shorter life expectancies than their parents by as much as five years, mainly due to the increase in obesity and other obesity-associated co-morbidities.[286] Therefore, particular attention has been given to novel interventions leading to a reduction in the prevalence, delay in the onset and decrease in the severity of each one of the elements of this "syndrome", particularly during the early stages of life.[287]

1.3.2.4 Early programming of obesity and other components of the metabolic syndrome

The amount of data supporting the association between certain prenatal insults and the later susceptibility to develop obesity (using both clinical and animal models) is extensive.[252, 288-293] Most of these studies, however, focus on the long-term effects of macro and micronutrients (either deficit[289, 294, 295] or excess[296-298]) as program-inducing insults. Generally, prenatal insults characterized by nutritional deficit lead to a decrease in the maternal weight-gain during pregnancy and a decreased birth weight in the offspring.[289, 299] Additionally, most nutrient deficiency models are characterized by exaggerated postnatal weight gain of the offspring; this phenomenon is also known as catch-up growth.[300, 301] This particular postnatal growth pattern seems to be mediated by a postnatal up-regulation in appetite control signaling and has been recognized as a strong driving

force in the obesity early programming phenomenon.[100, 302] In fact, postnatal nutritional intervention, designed to decrease the exaggerated weight gain in offspring born small, has been shown to ameliorate the susceptibility to develop obesity and other components of the MetS later in life.[100, 303]

In the case of animal models using maternal obesity to examine the programming effects of this prenatal condition, offspring do not exhibit decreased birth weight or exaggerated catch-up after birth.[288] Adult offspring from most of these excess nutritional models, however, have a phenotype that closely resembles the MetS; including abnormal glucose homeostasis, increased blood pressure, abnormal serum lipid profiles, increased adiposity and endothelial dysfunction.[304, 305] Interestingly, this particular model of early programming also exhibits long-term changes in the hypothalamus and endocrine mechanisms related to appetite regulation (such as leptin).[22, 306]

Other animal models using non-nutritional insults (such as maternal stress or maternal infection) have also been employed to study the early programming of obesity. The information available from these models, however, is very limited.[288] Finally, there is one recently published paper showing the association of prenatal hypoxia and insulin intolerance later in life.[307] The results presented in this manuscript also suggest that prenatal hypoxic insults leading to IUGR cause a long-term impairment in the insulin signaling pathway of organs such as liver and muscle. Interestingly, there are no studies designed to identify the potential long-term effects of prenatal hypoxia on the later susceptibility to develop components of the MetS such as obesity, dyslipidemia and hypertension.

1.3.2.5 Resveratrol for the treatment of early programmed obesity and metabolic syndrome

Regardless of our understanding of the etiology and pathophysiology of components of the MetS, the management and prevention of these

cardiovascular risk factors still constitutes a clinical challenge. Since the MetS is mainly mediated by increased energy intake and decreased energy expenditure, agents that mimic caloric restriction may offer a new therapeutic approach to prevent the early onset of these conditions in susceptible populations.

Resveratrol (3, 4', 5 trihydroxystilbene) is a polyphenol that is naturally synthesized by plants in response to attacks by certain pathogens.[308] This molecule is known to be present in relatively high amounts (0.1-14.3 mg/L) in red wine[309] and has been associated with the cardioprotective effects of red wine consumption.[310] Interest in this particular molecule has increased substantially in the last decade due to its diverse beneficial effects demonstrated in a variety of animal models.[311, 312] Previous studies, for instance, have shown that administration of Resveratrol improves insulin sensitivity and vascular function,[313, 314] inhibits tumor formation,[315] and ameliorates the early mortality associated with a high-fat diet in mice.[316]

The exact mechanisms by which Resveratrol produces its beneficial effects are not fully understood. Many of the beneficial effects of this molecule, however, are attributed to its ability to upregulate the SIRT1 gene which decodes the protein Sirtuin1.[317] Sirtuin1 down-regulates p53 activity, increasing lifespan and cell survival; it also deacetylates peroxisome proliferator-activated receptor-gamma (PPARγ) and its coactivator 1alpha (PGC-1α), promoting fat mobilization, increasing mitochondrial size and number, and positively regulating insulin secretion.[317]

Caloric restriction is a non-pharmacological intervention that is capable of increasing lifespan and decreasing most elements of the MetS. Caloric restriction is also known to cause activation of Sirtuin1 and for that reason pharmacological agents capable of activating Sirtuin1 are known as nutrition restriction mimetics.[318] Since Resveratrol is a very powerful up-regulator of Sirtuin1 it is plausible that its administration may have

beneficial effects on the treatment or prevention of obesity and other co-morbidities of the MetS. Among the multiple effects of Resveratrol, the Akt-induced facilitation of nitric oxide production and activity and the protection from oxidative stress seem to be among the most important and could constitute a key pathway connecting the vascular and metabolic beneficial effects of this natural compound.[319]

1.4 Early programming and increased susceptibility to a "second insult"

One interesting characteristic that is shared by most models (both clinical and experimental) of early programming, is that offspring exposed to programming-inducing insults do not exhibit a well-established pathological phenotype during early stages in life; after recovering from the acute perinatal effects of being exposed to hypoxia in utero (i.e. IUGR, increase in relative cardiac weight, etc). In fact, most young offspring born from complicated pregnancies tend to be healthy during the initial stages of life.[19, 109] However, as they age or are exposed to new challenges such as stress or unhealthy diets, pathological phenotypes rapidly become evident and demonstrate an accelerated progression.[13, 16, 320, 321]

These observations raise the "second insult" concept; this postulates that programming-inducing insults affect individuals by decreasing their physiological reserve, (through any of the mechanisms described in Section 1.2.2) and decreasing their capacity to respond/adapt when exposed to a "second insult". A complicating factor is that each "second insult" could have different pathways to cause pathological conditions, and each programming-inducing insult could have different effects on multiple organs and homeostatic systems. For the purposes of this thesis we have, therefore, focused on two specific "second insults" that are highly prevalent and relevant; aging and diet-induced obesity.

1.4.1 Aging as a "second insult"

Aging is an unavoidable process characterized by progressive organ function loss, decreased physiological cellular replication, decreased metabolic rates, impaired immunological function and increased oxidative stress.[322, 323] The normal aging process is also characterized by an increased incidence and prevalence of several pathological conditions such as hypertension,[321, 324] renal failure,[98] T2DM,[320] obesity,[325] coronary heart disease,[153] and heart failure.[326] Interestingly, most of these aging-associated conditions are also known to appear earlier and/or with more severity in subjects born from pregnancies complicated by IUGR.[41, 98, 100, 153]

Since aging is an unavoidable consequence of life, and considering that the life expectancy in developed and developing societies is reaching record values,[327-329] the study of early programming and its effects on the aging process are extremely relevant.

1.4.2 Diet-induced obesity as a "second insult"

Reaching a minimal nutritional intake is a very basic and intuitive behavior for any organism. Exceeding the nutritional requirements of energy intake, however, doesn't necessarily imply a benefit for health or survival. In fact, nutritional caloric restriction is the only intervention conclusively shown to slow aging and maintain health and vitality in mammals.[330] At the other extreme, lifestyles and experimental interventions characterized by high-energy intake and decreased energy expenditure are associated with a higher risk of developing several chronic diseases.[331-334]

Although chronic cardiovascular and metabolic diseases have a complex pathophysiology, characterized by a strong influence of inherited factors[335], the strong association between diet, obesity and cardiovascular and metabolic diseases has been well documented.[336, 337] Moreover, the nutritional habits of populations from both developed and developing societies have suffered a dramatic change in recent years, moving toward

western models of food consumption.[250, 338] These nutritional behaviors are characterized by an increased caloric intake rich in saturated fat, sodium and refined sugars[339-341] and constitute one of the most relevant and prevalent modifiable risk factors for several cardiovascular and metabolic diseases.

Actual trends in the prevalence of obesity have caused a general awareness and concern regarding an imminent epidemic of obesity.[146, 274, 342, 343] This problem is of particular concern in young adult and pediatric populations in which the proportion of subjects who are overweight or obese has exhibited a 10 fold increase during the last twenty years.[284, 344-347] Among the number of elements that have been associated with an increased susceptibility to becoming obese,[284, 343, 345] prenatal factors appear to play a fundamental role.[103, 348-350] Therefore, understanding the interactions between early programming and susceptibility to diet-induced obesity (DIO) is highly relevant; especially when considering the social, economical and health consequences of the early onset of obesity.

1.5 Sex differences in early programming

Despite the notable amount of epidemiological evidence illustrating differences in the pathophysiology of chronic diseases between men and women, there is an enormous paucity of information regarding sex differences in the early programming phenomenon. Several experimental models have shown that, in addition to their effect on the differentiation of primary sexual characteristics, sex hormones also play a fundamental role in the regulation of fetal growth and development throughout pregnancy and early infancy.[351] Moreover, hormonal influences may also modulate the long-term effects of insults inducing early programming; this may result in different phenotypic manifestations in male and female offspring exposed to similar insults early in life.

Depending on the model used and the outcomes evaluated, different authors have described either sex-dependent programming protection, sex-

independent programming effects or a sexually dimorphic, deleterious long-term response to prenatal insults.

Studies performed in various animal models indicate that relative to females, male fetuses are more likely to exhibit long-term changes in vascular function[352] and cardiac susceptibility to ischemia[179] when exposed to hypoxia, nutritional restriction or glucocorticoids[353] *in utero*. Moreover, models using maternal stress to induce programming suggest that male offspring may also be more susceptible to maladaptive behavioral stress responses relative to female offspring.[354] Further studies designed to understand the influence of hormones on programming mechanisms have determined that programming of blood pressure regulation appears to be mediated, at least in part, by testosterone. In male rats in which fetal growth restriction was induced by a reduction of uterine perfusion during the last third of pregnancy, castration at 10 weeks of age abolished the increase in blood pressure that was normally observed in these animals.[355] Estrogens, on the other hand, may play a protective role against early programming of specific conditions. It has been described that in offspring born small, the induction of an estrogen deficit by ovariectomy can accelerate the development of hypertension; an effect that can be reversed by estrogen supplementation or pharmacological blockade of the renin-angiotensin system.[356]

Importantly, not all studies suggest that female offspring are protected against all kinds of programming insults. In fact, relative to their male counterparts, female offspring seem to be more susceptible to develop hypertension when exposed to a high-fat diet during pregnancy[357] and alterations in the hypothalamo-lactotroph axis when exposed to glucocorticoids *in utero.*[358] Moreover, not all kinds, intensities or timing of programming insults have differential effects depending on the sex of the offspring and most interactions seem to depend partly on the species involved.[359]

All together, these observations illustrate the complexity of the programming/aging/sex interactions and suggest that programming-inducing mechanisms associated with each of the described pathological outcomes could be different.

1.6 General objectives and hypotheses

1.6.1 General objectives

Given the high prevalence of perinatal insults that can induce early programming, and the potential influence of these conditions in the later development of clinically relevant health outcomes, it is crucial to pursue a better understanding of the mechanisms behind this fascinating phenomenon and the potential preventive and therapeutic alternatives for this segment of the population.

As discussed in Section 1.2.3.2 of this introductory chapter, many different perinatal conditions have been linked to the early programming of several chronic diseases. Interestingly, a significant body of evidence suggests that there could be substantial differences in the specific mechanisms that cause early programming depending on the nature of the insult affecting the developing organism.[19, 22, 104, 360-365]

The objective of this thesis, therefore, was to study the long-term effects of prenatal hypoxic insults, which constitutes one of the most prevalent causes of IUGR in developed countries like Canada.[366] Moreover, in consideration of both the actual perinatal epidemiological profile as well as the global burden of cardiovascular and metabolic diseases (Sections 1.3.1.1 and 1.3.2.1), this thesis focused on how hypoxic prenatal insults (causing IUGR) may increase the offspring's susceptibility to develop cardiovascular and metabolic outcomes in response to common "second insults" such as aging and DIO.

Despite well-described differences between males and females in the pathophysiology of chronic cardiovascular and metabolic diseases,[367, 368] and the availability of some evidence demonstrating the effect of sex-differences in the early programming of health and diseases (Section 1.5), most basic fundamental approaches to study the early programming phenomenon have been conducted exclusively in male animals. Therefore, potential differences and/or interactions between sex and programming-inducing conditions remain to be explored and have been incorporated as a secondary objective of this thesis.

1.6.2 General hypotheses

The specific cardiac and metabolic changes leading to the phenotype described in offspring born from pregnancies complicated by hypoxia-induced IUGR are not fully known. We, therefore, approached the following four general hypotheses:

1. In addition to the previously described increased susceptibility to cardiac I/R injury, adult offspring born IUGR would also exhibit changes in the *in vivo* cardiac function, and those phenotypical differences would be exacerbated by the effect of aging.
2. The increased susceptibility to cardiac I/R injury observed in offspring born IUGR would be associated with the presence of long-term changes in cardiac energy metabolism.
3. Prenatal hypoxic insults causing IUGR would produce long-term changes in the mechanisms that regulate iron homeostasis in adult offspring, leading to chronic myocardial siderosis and increased cardiac oxidative stress.
4. Offspring born IUGR due to prenatal hypoxic insults would exhibit an increased susceptibility to develop components of the MetS when exposed to a high-fat diet, and those

deleterious metabolic effects of prenatal hypoxia could be reversed by the administration of a caloric-restriction mimetic such as Resveratrol.

1.7 References

1. Andreassi MG. Metabolic syndrome, diabetes and atherosclerosis: influence of gene-environment interaction. *Mutation Research*. 2009;667:35-43.

2. Vaag A, Jensen CB, Poulsen P, Brons C, Pilgaard K, Grunnet L, *et al.* Metabolic aspects of insulin resistance in individuals born small for gestational age. *Horm Res*. 2006;65 Suppl 3:137-143.

3. Rumball CW, Harding JE, Oliver MH, Bloomfield FH. Effects of twin pregnancy and periconceptional undernutrition on maternal metabolism, fetal growth and glucose-insulin axis function in ovine pregnancy. *J Physiol*. 2008;586:1399-1411.

4. Rose RJ. Prenatal programming of behavior: a twin-study perspective. *Neuroscience and Biobehavioral Reviews*. 2005;29:321-327.

5. Frasch MG, Muller T, Wicher C, Weiss C, Lohle M, Schwab K, *et al.* Fetal body weight and the development of the control of the cardiovascular system in fetal sheep. *J Physiol*. 2007;579:893-907.

6. de Boo HA, Harding JE. The developmental origins of adult disease (Barker) hypothesis. *Aust N Z J Obstet Gynaecol*. 2006;46:4-14.

7. Bebbington M. Twin-to-twin transfusion syndrome: current understanding of pathophysiology, in-utero therapy and impact for future development. *Semin Fetal Neonatal Med*. 2010;15:15-20.

8. Barker DJ, Osmond C. Diet and coronary heart disease in England and Wales during and after the second world war. *J Epidemiol Community Health*. 1986;40:37-44.

9. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet*. 1986;1:1077-1081.

10. Barker DJ, Osmond C. Childhood respiratory infection and adult chronic bronchitis in England and Wales. *Br Med J (Clin Res Ed)*. 1986;293:1271-1275.

11. Barker DJ, Osmond C. Death rates from stroke in England and Wales predicted from past maternal mortality. *Br Med J (Clin Res Ed)*. 1987;295:83-86.

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

13. Thornburg KL. Hypoxia and cardiac programming. *J Soc Gynecol Investig*. 2003;10:251.

14. Meyer K, Lubo Z. Fetal programming of cardiac function and disease. *Reprod Sci*. 2007;14:209-216.

15. Ojeda NB, Grigore D, Alexander BT. Intrauterine growth restriction: fetal programming of hypertension and kidney disease. *Adv Chronic Kidney Dis*. 2008;15:101-106.

16. Barker DJ. In utero programming of cardiovascular disease. *Theriogenology*. 2000;53:555-574.

17. Godfrey KM, Barker DJ. Fetal programming and adult health. *Public Health Nutr*. 2001;4:611-624.

18. Padmanabhan V, Sarma HN, Savabieasfahani M, Steckler TL, Veiga-Lopez A. Developmental reprogramming of reproductive and metabolic dysfunction in sheep: native steroids vs. environmental steroid receptor modulators. *Int J Androl*.

19. Bertram CE, Hanson MA. Animal models and programming of the metabolic syndrome. *Br Med Bull*. 2001;60:103-121.

20. Ozanne SE, Hales CN. Pre- and early postnatal nongenetic determinants of type 2 diabetes. *Expert Rev Mol Med*. 2002;4:1-14.

21. Stocker CJ, Arch JR, Cawthorne MA. Fetal origins of insulin resistance and obesity. *Proc Nutr Soc*. 2005;64:143-151.

22. Vickers MH. Developmental programming and adult obesity: the role of leptin. *Curr Opin Endocrinol Diabetes Obes*. 2007;14:17-22.

23. Mayr E. Commemorating the 20th century Darwin: Ernst Mayr's words and thoughts, five years later. Interview by Rob J. Kulathinal. *Genome*. 2010;53:157-159.

24. Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden PJ, Monteiro A*, et al.* Development, plasticity and evolution of butterfly eyespot patterns. *Nature*. 1996;384:236-242.

25. Kutschera U. Charles Darwin's Origin of Species, directional selection, and the evolutionary sciences today. *Naturwissenschaften*. 2009;96:1247-1263.

26. "The scientific consensus around evolution is overwhelming". "Appendix: Frequently Asked Questions" (php). Science and Creationism: A View from the National Academy of Sciences Second ed. Washington, DC: The National Academy of Sciences.; 1999.

27. Gilbert SF. The genome in its ecological context: philosophical perspectives on interspecies epigenesis. *Ann N Y Acad Sci*. 2002;981:202-218.

28. McFall-Ngai MJ. Unseen forces: the influence of bacteria on animal development. *Dev Biol*. 2002;242:1-14.

29. Hertwig O. Zeit- und Streitfragen der Biologie I. Präfor- mation oder Epigenese? Grundzüge einer Entwicklungstheorie der Organismen. Translated as The bio- logical problem of to-day: Preformation or epigenesis? . In: Jena GF, editor.: (P C Mitchell transl.) (New York: Macmillan); 1894.

30. Barker DJ. The origins of the developmental origins theory. *J Intern Med*. 2007;261:412-417.

31. Kermack WO, Mckendrick AG, P.L. M. Death rates in Great Britain and Sweden; some general regularities and their significance. *Lancet*. 1934;i:698-703.

32. Rose G. FAMILIAL PATTERNS IN ISCHAEMIC HEART DISEASE. *Br J Prev Soc Med*. 1964;18:75-80.

33. Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med*. 1976;295:349-353.

34. Forsdahl A. Are poor living conditions in childhood and adolescence an important risk factor for arteriosclerotic heart disease? *Br J Prev Soc Med*. 1977;31:91-95.

35. Barker DJ. The fetal and infant origins of adult disease. *Bmj*. 1990;301:1111.

36. Barker DJ, Osmond C. Low birth weight and hypertension. *Bmj*. 1988;297:134-135.

37. Barker DJ. Fetal origins of coronary heart disease. *Br Heart J*. 1993;69:195-196.

38. Gilbert SF. Mechanisms for the environmental regulation of gene expression: ecological aspects of animal development. *J Biosci*. 2005;30:65-74.

39. Schreuder MF, Nauta J. Prenatal programming of nephron number and blood pressure. *Kidney International*. 2007;72:265-268.

40. Luyckx VA, Brenner BM. Low birth weight, nephron number, and kidney disease. *Kidney International Supplement*. 2005:S68-77.

41. Thomas R, Kaskel FJ. It's not over till the last glomerulus forms. *Kidney International*. 2009;76:361-363.

42. Lerma EV. Anatomic and physiologic changes of the aging kidney. *Clinics in Geriatric Medicine*. 2009;25:325-329.

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

44. Corstius HB, Zimanyi MA, Maka N, Herath T, Thomas W, van der Laarse A*, et al.* Effect of intrauterine growth restriction on the number of cardiomyocytes in rat hearts. *Pediatr Res*. 2005;57:796-800.

45. Morrison JL, Botting KJ, Dyer JL, Williams SJ, Thornburg KL, McMillen IC. Restriction of placental function alters heart development in the sheep fetus. *Am J Physiol Regul Integr Comp Physiol*. 2007;293:R306-313.

46. Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ. Cardiomyocyte DNA synthesis and binucleation during murine development. *Am J Physiol*. 1996;271:H2183-2189.

47. Clubb FJ, Jr., Bishop SP. Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. *Lab Invest*. 1984;50:571-577.

48. Woodcock EA, Matkovich SJ. Cardiomyocytes structure, function and associated pathologies. *Int J Biochem Cell Biol*. 2005;37:1746-1751.

49. Cheema KK, Dent MR, Saini HK, Aroutiounova N, Tappia PS. Prenatal exposure to maternal undernutrition induces adult cardiac dysfunction. *Br J Nutr*. 2005;93:471-477.

50. Allen ND. Temporal and epigenetic regulation of neurodevelopmental plasticity. *Philos Trans R Soc Lond B Biol Sci*. 2008;363:23-38.

51. Clarke IJ, Scaramuzzi RJ, Short RV. Effects of testosterone implants in pregnant ewes on their female offspring. *J Embryol Exp Morphol*. 1976;36:87-99.

52. Savabieasfahani M, Lee JS, Herkimer C, Sharma TP, Foster DL, Padmanabhan V. Fetal programming: testosterone exposure of the female sheep during midgestation disrupts the dynamics of its adult gonadotropin secretion during the periovulatory period. *Biol Reprod*. 2005;72:221-229.

53. Padmanabhan V, Manikkam M, Recabarren S, Foster D. Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female. *Mol Cell Endocrinol*. 2006;246:165-174.

54. Andersson IJ, Jiang YY, Davidge ST. Maternal stress and development of atherosclerosis in the adult apolipoprotein E-deficient mouse offspring. *Am J Physiol Regul Integr Comp Physiol*. 2009;296:R663-671.

55. Bernberg E, Andersson IJ, Gan LM, Naylor AS, Johansson ME, Bergstrom G. Effects of social isolation and environmental enrichment on atherosclerosis in ApoE-/- mice. *Stress*. 2008;11:381-389.

56. Bogdarina I, Haase A, Langley-Evans S, Clark AJ. Glucocorticoid effects on the programming of AT1b angiotensin receptor gene methylation and expression in the rat. *PLoS One*. 2010;5:e9237.

57. Dagan A, Habib S, Gattineni J, Dwarakanath V, Baum M. Prenatal programming of rat thick ascending limb chloride transport by low-protein diet and dexamethasone. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R93-99.

58. Vilar L, Freitas Mda C, Lima LH, Lyra R, Kater CE. Cushing's syndrome in pregnancy: an overview. *Arq Bras Endocrinol Metabol*. 2007;51:1293-1302.

59. Levitt NS, Lindsay RS, Holmes MC, Seckl JR. Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. *Neuroendocrinology*. 1996;64:412-418.

60. Meaney MJ, Diorio J, Francis D, Widdowson J, LaPlante P, Caldji C*, et al.* Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. *Dev Neurosci*. 1996;18:49-72.

61. Clark PM. Programming of the hypothalamo-pituitary-adrenal axis and the fetal origins of adult disease hypothesis. *Eur J Pediatr*. 1998;157 Suppl 1:S7-10.

62. Lingas R, Dean F, Matthews SG. Maternal nutrient restriction (48 h) modifies brain corticosteroid receptor expression and endocrine function in the fetal guinea pig. *Brain Res*. 1999;846:236-242.

63. Reznikov AG, Nosenko ND, Tarasenko LV, Sinitsyn PV, Lymareva AA. Prenatal dexamethasone prevents early and long-lasting neuroendocrine and behavioral effects of maternal stress on male offspring. *Fiziol Zh*. 2008;54:28-39.

64. Levay EA, Paolini AG, Govic A, Hazi A, Penman J, Kent S. HPA and sympathoadrenal activity of adult rats perinatally exposed to maternal mild calorie restriction. *Behav Brain Res*. 2010;208:202-208.

65. Bertram CE, Hanson MA. Prenatal programming of postnatal endocrine responses by glucocorticoids. *Reproduction*. 2002;124:459-467.

66. Braems G. Fetal hypoxemia on a molecular level: adaptive changes in the hypothalamic-pituitary-adrenal (HPA) axis and the lungs. *Eur J Obstet Gynecol Reprod Biol*. 2003;110 Suppl 1:S63-69.

67. Nieuwenhuizen AG, Rutters F. The hypothalamic-pituitary-adrenal-axis in the regulation of energy balance. *Physiol Behav*. 2008;94:169-177.

68. Reynolds RM. Corticosteroid-mediated programming and the pathogenesis of obesity and diabetes. *J Steroid Biochem Mol Biol*. 2010;122:3-9.

69. New MI, Carlson A, Obeid J, Marshall I, Cabrera MS, Goseco A*, et al.* Prenatal diagnosis for congenital adrenal hyperplasia in 532 pregnancies. *J Clin Endocrinol Metab*. 2001;86:5651-5657.

70. Bhupathy P, Haines CD, Leinwand LA. Influence of sex hormones and phytoestrogens on heart disease in men and women. *Womens Health (Lond Engl)*. 2010;6:77-95.

71. Bull JJ, Vogt RC. Temperature-dependent sex determination in turtles. *Science*. 1979;206:1186-1188.

72. Yang X, Zheng J, Na R, Li J, Xu G, Qu L*, et al.* Degree of sex differentiation of genetic female chicken treated with different doses of an aromatase inhibitor. *Sex Dev*. 2008;2:309-315.

73. Crews D, Bergeron JM. Role of reductase and aromatase in sex determination in the red-eared slider (Trachemys scripta), a turtle with temperature-dependent sex determination. *Journal of Endocrinology*. 1994;143:279-289.

74. Waterland RA, Lin JR, Smith CA, Jirtle RL. Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. *Human Molecular Genetics*. 2006;15:705-716.

75. Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition*. 2004;20:63-68.

76. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Molecular and Cellular Biology*. 2003;23:5293-5300.

77. Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect*. 2006;114:567-572.

78. Schumacher B, Garinis GA, Hoeijmakers JH. Age to survive: DNA damage and aging. *Trends in Genetics*. 2008;24:77-85.

79. Garinis GA, van der Horst GT, Vijg J, Hoeijmakers JH. DNA damage and ageing: new-age ideas for an age-old problem. *Nat Cell Biol*. 2008;10:1241-1247.

80. Hasty P, Campisi J, Hoeijmakers J, van Steeg H, Vijg J. Aging and genome maintenance: lessons from the mouse? *Science*. 2003;299:1355-1359.

81. Hasty P, Vijg J. Accelerating aging by mouse reverse genetics: a rational approach to understanding longevity. *Aging Cell*. 2004;3:55-65.

82. Schumacher B, Hoeijmakers JH, Garinis GA. Sealing the gap between nuclear DNA damage and longevity. *Molecular and Cellular Endocrinology*. 2009;299:112-117.

83. Fowden AL, Forhead AJ. Endocrine mechanisms of intrauterine programming. *Reproduction*. 2004;127:515-526.

84. Fernandez-Capetillo O. Intrauterine programming of ageing. *EMBO Rep*. 2010;11:32-36.

85. Sinclair DA, Oberdoerffer P. The ageing epigenome: damaged beyond repair? *Ageing Res Rev*. 2009;8:189-198.

86. Simons JW. Epigenetic hereditary transcription profiles II, aging revisited. *Biol Direct*. 2007;2:39.

87. Thompson RF, Einstein FH. Epigenetic Basis for Fetal Origins of Age-Related Disease. *J Womens Health (Larchmt)*. 2010.

88. Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. *Cell*. 2008;132:681-696.

89. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature*. 2007;447:725-729.

90. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

91. von Bergen NH, Koppenhafer SL, Spitz DR, Volk KA, Patel SS, Roghair RD*, et al.* Fetal programming alters reactive oxygen species production in sheep cardiac mitochondria. *Clin Sci (Lond)*. 2009;116:659-668.

92. Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol*. 2003;23:842-846.

93. Akkad A, Hastings R, Konje JC, Bell SC, Thurston H, Williams B. Telomere length in small-for-gestational-age babies. *BJOG*. 2006;113:318-323.

94. van Meer H, van Straten EM, Baller JF, van Dijk TH, Plosch T, Kuipers F*, et al.* The effects of intrauterine malnutrition on maternal-fetal cholesterol transport and fetal lipid synthesis in mice. *Pediatr Res*. 2010.

95. Burdge GC, Hanson MA, Slater-Jefferies JL, Lillycrop KA. Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *Br J Nutr*. 2007;97:1036-1046.

96. Delage B, Dashwood RH. Dietary manipulation of histone structure and function. *Annu Rev Nutr*. 2008;28:347-366.

97. El-Osta A, Wolffe AP. DNA methylation and histone deacetylation in the control of gene expression: basic biochemistry to human development and disease. *Gene Expr*. 2000;9:63-75.

98. Dotsch J, Plank C, Amann K, Ingelfinger J. The implications of fetal programming of glomerular number and renal function. *J Mol Med*. 2009;87:841-848.

99. Jaquet D, Leger J, Chevenne D, Czernichow P, Levy-Marchal C. Intrauterine growth retardation predisposes to insulin resistance but not to hyperandrogenism in young women. *J Clin Endocrinol Metab*. 1999;84:3945-3949.

100. Morrison JL, Duffield JA, Muhlhausler BS, Gentili S, McMillen IC. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol*. 2010;25:669-677.

101. Cheung YF, Taylor MJ, Fisk NM, Redington AN, Gardiner HM. Fetal origins of reduced arterial distensibility in the donor twin in twin-twin transfusion syndrome. *Lancet*. 2000;355:1157-1158.

102. Poulter NR, Chang CL, MacGregor AJ, Snieder H, Spector TD. Association between birth weight and adult blood pressure in twins: historical cohort study. *BMJ*. 1999;319:1330-1333.

103. Remacle C, Bieswal F, Reusens B. Programming of obesity and cardiovascular disease. *Int J Obes Relat Metab Disord*. 2004;28 Suppl 3:S46-53.

104. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev*. 2005;85:571-633.

105. Nuyt AM, Alexander BT. Developmental programming and hypertension. *Curr Opin Nephrol Hypertens*. 2009;18:144-152.

106. Vuguin P. Animal models for assessing the consequences of intrauterine growth restriction on subsequent glucose metabolism of the offspring: a review. *J Matern Fetal Neonatal Med*. 2002;11:254-257.

107. Liu C, Zhang LF, Song ML, Bao HG, Zhao CJ, Li N. Highly efficient dissociation of oxygen from hemoglobin in Tibetan chicken embryos compared with lowland chicken embryos incubated in hypoxia. *Poult Sci*. 2009;88:2689-2694.

108. Tintu A, Rouwet E, Verlohren S, Brinkmann J, Ahmad S, Crispi F*, et al.* Hypoxia induces dilated cardiomyopathy in the chick embryo: mechanism, intervention, and long-term consequences. *PLoS One*. 2009;4:e5155.

109. Vuguin PM. Animal models for small for gestational age and fetal programming of adult disease. *Horm Res*. 2007;68:113-123.

110. Barry JS, Rozance PJ, Anthony RV. An animal model of placental insufficiency-induced intrauterine growth restriction. *Semin Perinatol*. 2008;32:225-230.

111. Carter AM, Enders AC, Jones CJ, Mess A, Pfarrer C, Pijnenborg R*, et al.* Comparative placentation and animal models: patterns of trophoblast invasion -- a workshop report. *Placenta*. 2006;27 Suppl A:S30-33.

112. Mess A. The Guinea pig placenta: model of placental growth dynamics. *Placenta*. 2007;28:812-815.

113. Roselli CE, Stormshak F. Prenatal programming of sexual partner preference: the ram model. *J Neuroendocrinol*. 2009;21:359-364.

114. Giussani DA, Riquelme RA, Sanhueza EM, Hanson MA, Blanco CE, Llanos AJ. Adrenergic and vasopressinergic contributions to the cardiovascular response to acute hypoxaemia in the llama fetus. *J Physiol*. 1999;515 (Pt 1):233-241.

115. Farley D, Tejero ME, Comuzzie AG, Higgins PB, Cox L, Werner SL*, et al.* Feto-placental adaptations to maternal obesity in the baboon. *Placenta*. 2009;30:752-760.

116. Aida K, Wang XL, Wang J, Li C, McDonald TJ, Nathanielsz PW. Effect of betamethasone administration to the pregnant baboon at 0.75 gestation on placental eNOS distribution and activity. *Placenta*. 2004;25:780-787.

117. Rosenberg A. The IUGR newborn. *Semin Perinatol*. 2008;32:219-224.

118. Godfrey KM, Forrester T, Barker DJ, Jackson AA, Landman JP, Hall JS*, et al.* Maternal nutritional status in pregnancy and blood pressure in childhood. *Br J Obstet Gynaecol*. 1994;101:398-403.

119. Latini G, De Mitri B, Del Vecchio A, Chitano G, De Felice C, Zetterstrom R. Foetal growth of kidneys, liver and spleen in intrauterine growth restriction: "programming" causing "metabolic syndrome" in adult age. *Acta Paediatr*. 2004;93:1635-1639.

120. McArdle HJ, Andersen HS, Jones H, Gambling L. Fetal programming: causes and consequences as revealed by studies of dietary manipulation in rats -- a review. *Placenta*. 2006;27 Suppl A:S56-60.

121. Williams SJ, Hemmings DG, Mitchell JM, McMillen IC, Davidge ST. Effects of maternal hypoxia or nutrient restriction during pregnancy on endothelial function in adult male rat offspring. *J Physiol*. 2005;565:125-135.

122. Li G, Bae S, Zhang L. Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. *Am J Physiol Heart Circ Physiol*. 2004;286:H1712-1719.

123. Gilbert JS, Babcock SA, Granger JP. Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression. *Hypertension*. 2007;50:1142-1147.

124. Cheung CY, Bogic L, Gagnon R, Harding R, Brace RA. Morphologic alterations in ovine placenta and fetal liver following induced severe placental insufficiency. *J Soc Gynecol Investig*. 2004;11:521-528.

125. Giles WB, Trudinger BJ, Stevens D, Alexander G, Bradley L. Umbilical artery flow velocity waveform analysis in normal ovine pregnancy and after carunculectomy. *J Dev Physiol*. 1989;11:135-138.

126. Teitel D, Rudolph AM. Perinatal oxygen delivery and cardiac function. *Advances in pediatrics*. 1985;32:321-347.

127. Thornburg KL. Fetal response to intrauterine stress. *Ciba Foundation symposium*. 1991;156:17-29; discussion 29-37.

128. Jensen A, Berger R. Fetal circulatory responses to oxygen lack. *J Dev Physiol*. 1991;16:181-207.

129. McCullough RE, Reeves JT, Liljegren RL. Fetal growth retardation and increased infant mortility at high altitude. *Obstetrical and Gynecological Survey*. 1977;32:596-598.

130. Moore LG, Rounds SS, Jahnigen D, Grover RF, Reeves JT. Infant birth weight is related to maternal arterial oxygenation at high altitude. *J Appl Physiol*. 1982;52:695-699.

131. Tuder RM, Yun JH, Bhunia A, Fijalkowska I. Hypoxia and chronic lung disease. *J Mol Med*. 2007;85:1317-1324.

132. Cogswell ME, Yip R. The influence of fetal and maternal factors on the distribution of birthweight. *Semin Perinatol*. 1995;19:222-240.

133. Hickey RJ, Clelland RC, Bowers EJ. Maternal smoking, birth weight, infant death, and the self-selection problem. *Am J Obstet Gynecol*. 1978;131:805-811.

134. Zhang L. Prenatal hypoxia and cardiac programming. *J Soc Gynecol Investig*. 2005;12:2-13.

135. Rizzo G, Arduini D. Intrauterine growth restriction: diagnosis and management. A review. *Minerva Ginecol*. 2009;61:411-420.

136. Cogswell ME, Yip R. The influence of fetal and maternal factors on the distribution of birthweight. *Semin Perinatol*. 1995;19:222-240.

137. Verkauskiene R, Figueras F, Deghmoun S, Chevenne D, Gardosi J, Levy-Marchal M. Birth weight and long-term metabolic outcomes: does the definition of smallness matter? *Horm Res*. 2008;70:309-315.

138. Ananth CV, Vintzileos AM. Distinguishing pathological from constitutional small for gestational age births in population-based studies. *Early Hum Dev*. 2009;85:653-658.

139. Marsal K. Intrauterine growth restriction. *Curr Opin Obstet Gynecol*. 2002;14:127-135.

140. Jobgen WS, Ford SP, Jobgen SC, Feng CP, Hess BW, Nathanielsz PW*, et al.* Baggs ewes adapt to maternal undernutrition and maintain conceptus growth by maintaining fetal plasma concentrations of amino acids. *J Anim Sci*. 2008;86:820-826.

141. Zamudio S. High-altitude hypoxia and preeclampsia. *Front Biosci*. 2007;12:2967-2977.

142. Ali KZ, Burton GJ, Morad N, Ali ME. Does hypercapillarization influence the branching pattern of terminal villi in the human placenta at high altitude? *Placenta*. 1996;17:677-682.

143. Reshetnikova OS, Burton GJ, Milovanov AP. Effects of hypobaric hypoxia on the fetoplacental unit: the morphometric diffusing capacity of the villous membrane at high altitude. *Am J Obstet Gynecol*. 1994;171:1560-1565.

144. Moore LG, Armaza F, Villena M, Vargas E. Comparative aspects of high-altitude adaptation in human populations. *Adv Exp Med Biol*. 2000;475:45-62.

145. Dahlof B. Cardiovascular disease risk factors: epidemiology and risk assessment. *Am J Cardiol*. 2010;105:3A-9A.

146. Wyatt SB, Winters KP, Dubbert PM. Overweight and obesity: prevalence, consequences, and causes of a growing public health problem. *Am J Med Sci*. 2006;331:166-174.

147. Statistical fact sheet-Populations, Internationla cardiovascular diseases statistics from the American Heart Association. Cardiovascular diseases (CVD) 2009 [cited 2010 May 13]; Available from: http://www.americanheart.org/downloadable/heart/1236204012112INTL.pdf

148. Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K*, et al.* Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*. 2007;115:e69-171.

149. Ezzati M, Vander Hoorn S, Lawes CM, Leach R, James WP, Lopez AD*, et al.* Rethinking the "diseases of affluence" paradigm: global patterns of nutritional risks in relation to economic development. *PLoS Med*. 2005;2:e133.

150. WHO. The world health report 2008 : primary health care now more than ever.; 2008.

151. Porrello ER, Widdop RE, Delbridge LM. Early origins of cardiac hypertrophy: does cardiomyocyte attrition programme for pathological 'catch-up' growth of the heart? *Clin Exp Pharmacol Physiol*. 2008;35:1358-1364.

152. Soukhova-O'Hare GK, Ortines RV, Gu Y, Nozdrachev AD, Prabhu SD, Gozal D. Postnatal intermittent hypoxia and developmental programming of hypertension in spontaneously hypertensive rats: the role of reactive oxygen species and L-Ca2+ channels. *Hypertension*. 2008;52:156-162.

153. Louey S, Thornburg KL. The prenatal environment and later cardiovascular disease. *Early Hum Dev*. 2005;81:745-751.

154. Osmond C, Barker DJ. Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. *Environ Health Perspect*. 2000;108 Suppl 3:545-553.

155. Patterson AJ, Zhang L. Hypoxia and fetal heart development. *Curr Mol Med*. 2010;10:653-666.

156. Pladys P, Sennlaub F, Brault S, Checchin D, Lahaie I, Le NL*, et al.* Microvascular rarefaction and decreased angiogenesis in rats with fetal programming of hypertension associated with exposure to a low-protein diet in utero. *Am J Physiol Regul Integr Comp Physiol*. 2005;289:R1580-R1588.

157. Han HC, Austin KJ, Nathanielsz PW, Ford SP, Nijland MJ, Hansen TR. Maternal nutrient restriction alters gene expression in the ovine fetal heart. *J Physiol*. 2004;558:111-121.

158. Stein CE, Fall CH, Kumaran K, Osmond C, Cox V, Barker DJ. Fetal growth and coronary heart disease in south India. *Lancet*. 1996;348:1269-1273.

159. Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet*. 2005;6:826-835.

160. Li F, Wang X, Capasso JM, Gerdes AM. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J Mol Cell Cardiol*. 1996;28:1737-1746.

161. Bae S, Xiao Y, Li G, Casiano CA, Zhang L. Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart. *Am J Physiol Heart Circ Physiol*. 2003;285:H983-990.

162. Daniels A, van Bilsen M, Goldschmeding R, van der Vusse GJ, van Nieuwenhoven FA. Connective tissue growth factor and cardiac fibrosis. *Acta Physiol (Oxf)*. 2009;195:321-338.

163. Kania G, Blyszczuk P, Eriksson U. Mechanisms of cardiac fibrosis in inflammatory heart disease. *Trends Cardiovasc Med*. 2009;19:247-252.

164. Follonier Castella L, Gabbiani G, McCulloch CA, Hinz B. Regulation of myofibroblast activities: calcium pulls some strings behind the scene. *Exp Cell Res*. 2010;316:2390-2401.

165. Swynghedauw B. Molecular mechanisms of myocardial remodeling. *Physiol Rev*. 1999;79:215-262.

166. Lim K, Zimanyi MA, Black MJ. Effect of maternal protein restriction in rats on cardiac fibrosis and capillarization in adulthood. *Pediatr Res*. 2006;60:83-87.

167. D'Armiento J. Matrix metalloproteinase disruption of the extracellular matrix and cardiac dysfunction. *Trends Cardiovasc Med*. 2002;12:97-101.

168. Moens AL, Claeys MJ, Timmermans JP, Vrints CJ. Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process. *Int J Cardiol*. 2005;100:179-190.

169. Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch Pathol*. 1960;70:68-78.

170. Krause S, Hess ML. Characterization of cardiac sarcoplasmic reticulum dysfunction during short-term, normothermic, global ischemia. *Circ Res*. 1984;55:176-184.

171. Kim JS, Jin Y, Lemasters JJ. Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. *Am J Physiol Heart Circ Physiol*. 2006;290:H2024-2034.

172. Wang P, Zweier JL. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. *J Biol Chem*. 1996;271:29223-29230.

173. Takagi H, Matsui Y, Hirotani S, Sakoda H, Asano T, Sadoshima J. AMPK mediates autophagy during myocardial ischemia in vivo. *Autophagy*. 2007;3:405-407.

174. Loke KE, McConnell PI, Tuzman JM, Shesely EG, Smith CJ, Stackpole CJ*, et al.* Endogenous endothelial nitric oxide synthase-derived nitric oxide is a physiological regulator of myocardial oxygen consumption. *Circ Res*. 1999;84:840-845.

175. Turer AT, Hill JA. Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. *Am J Cardiol*. 2010;106:360-368.

176. Ikonomidis JS, Weisel RD, Mickle DA. Ischemic preconditioning: cardioprotection for cardiac surgery. *J Card Surg*. 1994;9:526-531.

177. Bae S, Zhang L. Prenatal cocaine exposure increases apoptosis of neonatal rat heart and heart susceptibility to ischemia-reperfusion injury in 1-month-old rat. *Br J Pharmacol*. 2005;144:900-907.

178. Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D*, et al.* Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc Natl Acad Sci U S A*. 2001;98:11114-11119.

179. Xue Q, Zhang L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: role of protein kinase C epsilon. *J Pharmacol Exp Ther*. 2009;330:624-632.

180. Ingwal J. ATP and the Heart. Massachuset: Kluwer Academic Publisher; 2002.

181. Lopaschuk GD, Belke DD, Gamble J, Itoi T, Schonekess BO. Regulation of fatty acid oxidation in the mammalian heart in health and disease. *Biochimica et Biophysica Acta*. 1994;1213:263-276.

182. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev*. 2010;90:207-258.

183. Stanley WC, Lopaschuk GD, Hall JL, McCormack JG. Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. *Cardiovasc Res*. 1997;33:243-257.

184. Schwartz GG, Greyson C, Wisneski JA, Garcia J. Inhibition of fatty acid metabolism alters myocardial high-energy phosphates in vivo. *Am J Physiol*. 1994;267:H224-231.

185. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet*. 1963;1:785-789.

186. Mjos OD. Free fatty acids and oxygen consumption in dogs. *Scand J Clin Lab Invest*. 1971;28:121-125.

187. Hinkle PC. P/O ratios of mitochondrial oxidative phosphorylation. *Biochim Biophys Acta*. 2005;1706:1-11.

188. Lopaschuk GD, Stanley WC. Malonyl-CoA decarboxylase inhibition as a novel approach to treat ischemic heart disease. *Cardiovascular Drugs and Therapy*. 2006;20:433-439.

189. Stanley WC, Morgan EE, Huang H, McElfresh TA, Sterk JP, Okere IC*, et al.* Malonyl-CoA decarboxylase inhibition suppresses fatty acid oxidation and reduces lactate production during demand-induced ischemia. *Am J Physiol Heart Circ Physiol*. 2005;289:H2304-2309.

190. Lopaschuk GD, Barr RL. Measurements of fatty acid and carbohydrate metabolism in the isolated working rat heart. *Molecular and Cellular Biochemistry*. 1997;172:137-147.

191. Lopaschuk GD, Russell JC. Myocardial function and energy substrate metabolism in the insulin-resistant JCR:LA corpulent rat. *J Appl Physiol*. 1991;71:1302-1308.

192. Lopaschuk GD. Optimizing cardiac energy metabolism: how can fatty acid and carbohydrate metabolism be manipulated? *Coronary Artery Disease*. 2001;12 Suppl 1:S8-11.

193. Lopaschuk GD. Targets for modulation of fatty acid oxidation in the heart. *Curr Opin Investig Drugs*. 2004;5:290-294.

194. Lopaschuk GD. AMP-activated protein kinase control of energy metabolism in the ischemic heart. *Int J Obes (Lond)*. 2008;32 Suppl 4:S29-35.

195. Bersin RM, Stacpoole PW. Dichloroacetate as metabolic therapy for myocardial ischemia and failure. *American Heart Journal*. 1997;134:841-855.

196. Bersin RM, Wolfe C, Kwasman M, Lau D, Klinski C, Tanaka K*, et al.* Improved hemodynamic function and mechanical efficiency in congestive heart failure with sodium dichloroacetate. *Journal of the American College of Cardiology*. 1994;23:1617-1624.

197. Lopaschuk G. Regulation of carbohydrate metabolism in ischemia and reperfusion. *American Heart Journal*. 2000;139:S115-119.

198. Lopaschuk GD, Stanley WC. Glucose metabolism in the ischemic heart. *Circulation*. 1997;95:313-315.

199. King LM, Opie LH. Glucose and glycogen utilisation in myocardial ischemia--changes in metabolism and consequences for the myocyte. *Molecular and Cellular Biochemistry*. 1998;180:3-26.

200. Dyck JR, Barr AJ, Barr RL, Kolattukudy PE, Lopaschuk GD. Characterization of cardiac malonyl-CoA decarboxylase and its putative role in regulating fatty acid oxidation. *Am J Physiol*. 1998;275:H2122-2129.

201. Lopaschuk GD. Malonyl CoA control of fatty acid oxidation in the diabetic rat heart. *Adv Exp Med Biol*. 2001;498:155-165.

202. Dyck JR, Lopaschuk GD. Malonyl CoA control of fatty acid oxidation in the ischemic heart. *J Mol Cell Cardiol*. 2002;34:1099-1109.

203. Dyck JR, Cheng JF, Stanley WC, Barr R, Chandler MP, Brown S*, et al.* Malonyl coenzyme a decarboxylase inhibition protects the ischemic heart by inhibiting fatty acid oxidation and stimulating glucose oxidation. *Circ Res*. 2004;94:e78-84.

204. Forstermann U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch*. 2010;459:923-939.

205. Uno K, Nicholls SJ. Biomarkers of inflammation and oxidative stress in atherosclerosis. *Biomark Med*. 2010;4:361-373.

206. Dai DF, Rabinovitch PS. Cardiac aging in mice and humans: the role of mitochondrial oxidative stress. *Trends Cardiovasc Med*. 2009;19:213-220.

207. Pacher P, Schulz R, Liaudet L, Szabo C. Nitrosative stress and pharmacological modulation of heart failure. *Trends in pharmacological sciences*. 2005;26:302-310.

208. Galili O, Versari D, Sattler KJ, Olson ML, Mannheim D, McConnell JP*, et al.* Early experimental obesity is associated with coronary endothelial dysfunction and oxidative stress. *Am J Physiol Heart Circ Physiol*. 2007;292:H904-911.

209. Csont T, Viappiani S, Sawicka J, Slee S, Altarejos JY, Batinic-Haberle I, *et al.* The involvement of superoxide and iNOS-derived NO in cardiac dysfunction induced by pro-inflammatory cytokines. *J Mol Cell Cardiol*. 2005;39:833-840.

210. Leon H, Bautista-Lopez N, Sawicka J, Schulz R. Hydrogen peroxide causes cardiac dysfunction independent from its effects on matrix metalloproteinase-2 activation. *Can J Physiol Pharmacol*. 2007;85:341-348.

211. Oudit GY, Sun H, Trivieri MG, Koch SE, Dawood F, Ackerley C, *et al.* L-type Ca2+ channels provide a major pathway for iron entry into cardiomyocytes in iron-overload cardiomyopathy. *Nat Med*. 2003;9:1187-1194.

212. Park SK, Prolla TA. Gene expression profiling studies of aging in cardiac and skeletal muscles. *Cardiovasc Res*. 2005;66:205-212.

213. Dodic M, May CN, Wintour EM, Coghlan JP. An early prenatal exposure to excess glucocorticoid leads to hypertensive offspring in sheep. *Clin Sci (Lond)*. 1998;94:149-155.

214. Elmes MJ, McMullen S, Gardner DS, Langley-Evans SC. Prenatal diet determines susceptibility to cardiac ischaemia-reperfusion injury following treatment with diethylmaleic acid and N-acetylcysteine. *Life Sci*. 2008;82:149-155.

215. Elmes MJ, Gardner DS, Langley-Evans SC. Fetal exposure to a maternal low-protein diet is associated with altered left ventricular pressure response to ischaemia-reperfusion injury. *Br J Nutr*. 2007;98:93-100.

216. Salim Ur R, Huma N, Tarar OM, Shah WH. Efficacy of non-heme iron fortified diets: a review. *Crit Rev Food Sci Nutr*. 2010;50:403-413.

217. Zhu A, Kaneshiro M, Kaunitz JD. Evaluation and treatment of iron deficiency anemia: a gastroenterological perspective. *Dig Dis Sci*. 2010;55:548-559.

218. Neilands JB. A brief history of iron metabolism. *Biol Met*. 1991;4:1-6.

219. Galey JB. Recent advances in the design of iron chelators against oxidative damage. *Mini Rev Med Chem*. 2001;1:233-242.

220. Hershko C. Mechanism of iron toxicity. *Food Nutr Bull*. 2007;28:S500-509.

221. Fine JS. Iron poisoning. *Curr Probl Pediatr*. 2000;30:71-90.

222. Madiwale T, Liebelt E. Iron: not a benign therapeutic drug. *Curr Opin Pediatr*. 2006;18:174-179.

223. Oudit GY, Trivieri MG, Khaper N, Liu PP, Backx PH. Role of L-type Ca2+ channels in iron transport and iron-overload cardiomyopathy. *J Mol Med*. 2006;84:349-364.

224. Tenenbein M. Hepatotoxicity in acute iron poisoning. *J Toxicol Clin Toxicol*. 2001;39:721-726.

225. Olivieri NF, Nathan DG, MacMillan JH, Wayne AS, Liu PP, McGee A*, et al.* Survival in medically treated patients with homozygous beta-thalassemia. *N Engl J Med*. 1994;331:574-578.

226. Vernon DD, Banner W, Jr., Dean JM. Hemodynamic effects of experimental iron poisoning. *Ann Emerg Med*. 1989;18:863-866.

227. Seznec H, Simon D, Monassier L, Criqui-Filipe P, Gansmuller A, Rustin P*, et al.* Idebenone delays the onset of cardiac functional alteration without correction of Fe-S enzymes deficit in a mouse model for Friedreich ataxia. *Hum Mol Genet*. 2004;13:1017-1024.

228. Schafer AI, Cheron RG, Dluhy R, Cooper B, Gleason RE, Soeldner JS*, et al.* Clinical consequences of acquired transfusional iron overload in adults. *N Engl J Med*. 1981;304:319-324.

229. Turoczi T, Jun L, Cordis G, Morris JE, Maulik N, Stevens RG, *et al.* HFE mutation and dietary iron content interact to increase ischemia/reperfusion injury of the heart in mice. *Circ Res*. 2003;92:1240-1246.

230. Bel A, Martinod E, Menasche P. Cardioprotective effect of desferrioxamine. *Acta Haematol*. 1996;95:63-65.

231. Polla AS, Polla LL, Polla BS. Iron as the malignant spirit in successful ageing. *Ageing Res Rev*. 2003;2:25-37.

232. Worwood M. An overview of iron metabolism at a molecular level. *J Intern Med*. 1989;226:381-391.

233. Pootrakul P, Huebers HA, Finch CA, Pippard MJ, Cazzola M. Iron metabolism in thalassemia. *Birth Defects Orig Artic Ser*. 1988;23:3-8.

234. Fontecave M, Pierre JL. Iron metabolism: the low-molecular-mass iron pool. *Biol Met*. 1991;4:133-135.

235. Finch CA, Huebers HA. Iron metabolism. *Clin Physiol Biochem*. 1986;4:5-10.

236. Anderson GJ, Frazer DM, McLaren GD. Iron absorption and metabolism. *Curr Opin Gastroenterol*. 2009;25:129-135.

237. Lok CN, Ponka P. Identification of a hypoxia response element in the transferrin receptor gene. *J Biol Chem*. 1999;274:24147-24152.

238. Aisen P. Iron metabolism in isolated liver cells. *Ann N Y Acad Sci*. 1988;526:93-100.

239. Dandekar T, Stripecke R, Gray NK, Goossen B, Constable A, Johansson HE*, et al.* Identification of a novel iron-responsive element in murine and human erythroid delta-aminolevulinic acid synthase mRNA. *EMBO J*. 1991;10:1903-1909.

240. Taher AT, Musallam KM, Inati A. Iron overload: consequences, assessment, and monitoring. *Hemoglobin*. 2009;33 Suppl 1:S46-57.

241. Batey R, Gallagher N. Effect of iron stores and hysterectomy on iron absorption and distribution in pregnant mice. *Am J Physiol*. 1977;232:E57-61.

242. Hahn PF, Carothers EL, et al. Iron uptake in 750 cases of human pregnancy using the radioactive isotope Fe59. *Fed Proc*. 1947;6:392.

243. Nathanson MH, Muir A, McLaren GD. Iron absorption in normal and iron-deficient beagle dogs: mucosal iron kinetics. *Am J Physiol*. 1985;249:G439-448.

244. O'Riordan DK, Debnam ES, Sharp PA, Simpson RJ, Taylor EM, Srai SK. Mechanisms involved in increased iron uptake across rat duodenal brush-border membrane during hypoxia. *J Physiol*. 1997;500 (Pt 2):379-384.

245. Raja KB, Bjarnason I, Simpson RJ, Peters TJ. In vitro measurement and adaptive response of Fe3+ uptake by mouse intestine. *Cell Biochem Funct*. 1987;5:69-76.

246. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I*, et al.* The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest*. 2002;110:1037-1044.

247. Andersen HS, Gambling L, Holtrop G, McArdle HJ. Maternal iron deficiency identifies critical windows for growth and cardiovascular development in the rat postimplantation embryo. *J Nutr*. 2006;136:1171-1177.

248. Ashworth CJ, Antipatis C. Micronutrient programming of development throughout gestation. *Reproduction*. 2001;122:527-535.

249. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA*, et al.* Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation*. 2009;120:1640-1645.

250. Bersamin A, Luick BR, King IB, Stern JS, Zidenberg-Cherr S. Westernizing diets influence fat intake, red blood cell fatty acid composition, and health in remote Alaskan Native communities in the center for Alaska Native health study. *J Am Diet Assoc*. 2008;108:266-273.

251. Ferdinandy P, Schulz R, Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev*. 2007;59:418-458.

252. Achard V, Boullu-Ciocca S, Desbriere R, Grino M. Perinatal programming of central obesity and the metabolic syndrome: role of glucocorticoids. *Metab Syndr Relat Disord*. 2006;4:129-137.

253. Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J Physiol*. 2004;561:355-377.

254. Barker DJ. In utero programming of chronic disease. *Clin Sci (Lond)*. 1998;95:115-128.

255. Kahn R, Alperin P, Eddy D, Borch-Johnsen K, Buse J, Feigelman J*, et al.* Age at initiation and frequency of screening to detect type 2 diabetes: a cost-effectiveness analysis. *Lancet*. 2010;375:1365-1374.

256. Paulweber B, Valensi P, Lindstrom J, Lalic NM, Greaves CJ, McKee M*, et al.* A European evidence-based guideline for the prevention of type 2 diabetes. *Hormone and Metabolic Research*. 2010;42 Suppl 1:S3-36.

257. Chamnan P, Simmons RK, Khaw KT, Wareham NJ, Griffin SJ. Estimating the population impact of screening strategies for identifying and treating people at high risk of cardiovascular disease: modelling study. *Bmj*. 2010;340:c1693.

258. Hackam DG, Khan NA, Hemmelgarn BR, Rabkin SW, Touyz RM, Campbell NR*, et al.* The 2010 Canadian Hypertension Education Program recommendations for the management of hypertension: part 2 - therapy. *Canadian Journal of Cardiology*. 2010;26:249-258.

259. Tolfrey K. American heart association guidelines for preventing heart disease in women: 2007 update. *Phys Sportsmed*. 2010;38:162-164.

260. Alberti KG. Impaired glucose tolerance: what are the clinical implications? *Diabetes Research and Clinical Practice*. 1998;40 Suppl:S3-8.

261. Fujimoto WY, Bergstrom RW, Boyko EJ, Chen KW, Kahn SE, Leonetti DL*, et al.* Preventing diabetes--applying pathophysiological and epidemiological evidence. *British Journal of Nutrition*. 2000;84 Suppl 2:S173-176.

262. Zimmet P, Thomas CR. Genotype, obesity and cardiovascular disease--has technical and social advancement outstripped evolution? *J Intern Med*. 2003;254:114-125.

263. Day C. Metabolic syndrome, or What you will: definitions and epidemiology. *Diab Vasc Dis Res*. 2007;4:32-38.

264. Mancia G, Bombelli M, Facchetti R, Casati A, Ronchi I, Quarti-Trevano F*, et al.* Impact of different definitions of the metabolic syndrome on the prevalence

of organ damage, cardiometabolic risk and cardiovascular events. *Journal of Hypertension*. 2010;28:999-1006.

265. Sakurai T, Iimuro S, Araki A, Umegaki H, Ohashi Y, Yokono K*, et al.* Age-associated increase in abdominal obesity and insulin resistance, and usefulness of AHA/NHLBI definition of metabolic syndrome for predicting cardiovascular disease in Japanese elderly with type 2 diabetes mellitus. *Gerontology*. 2010;56:141-149.

266. Eddy DM, Schlessinger L, Heikes K. The metabolic syndrome and cardiovascular risk: implications for clinical practice. *Int J Obes (Lond)*. 2008;32 Suppl 2:S5-10.

267. Kranz S, Mahood LJ, Wagstaff DA. Diagnostic criteria patterns of U.S. children with Metabolic Syndrome: NHANES 1999-2002. *Nutr J*. 2007;6:38.

268. Strazzullo P, Barbato A, Siani A, Cappuccio FP, Versiero M, Schiattarella P*, et al.* Diagnostic criteria for metabolic syndrome: a comparative analysis in an unselected sample of adult male population. *Metabolism*. 2008;57:355-361.

269. Duvnjak L, Duvnjak M. The metabolic syndrome - an ongoing story. *Journal of Physiology and Pharmacology*. 2009;60 Suppl 7:19-24.

270. Hui WS, Liu Z, Ho SC. Metabolic syndrome and all-cause mortality: a meta-analysis of prospective cohort studies. *Eur J Epidemiol*.

271. Anis AH, Zhang W, Bansback N, Guh DP, Amarsi Z, Birmingham CL. Obesity and overweight in Canada: an updated cost-of-illness study. *Obes Rev*. 2009.

272. Yusuf S, Hawken S, Ounpuu S, Bautista L, Franzosi MG, Commerford P*, et al.* Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. *Lancet*. 2005;366:1640-1649.

273. Chan JC, Cheung JC, Stehouwer CD, Emeis JJ, Tong PC, Ko GT*, et al.* The central roles of obesity-associated dyslipidaemia, endothelial activation and cytokines in the Metabolic Syndrome--an analysis by structural equation modelling. *Int J Obes Relat Metab Disord*. 2002;26:994-1008.

274. Chaput JP, Berube-Parent S, Tremblay A. Obesity and cardiovascular physiology: impact of some pharmacological agents. *Curr Vasc Pharmacol*. 2005;3:185-193.

275. Maiorana A, Del Bianco C, Cianfarani S. Adipose Tissue: A Metabolic Regulator. Potential Implications for the Metabolic Outcome of Subjects Born Small for Gestational Age (SGA). *Rev Diabet Stud*. 2007;4:134-146.

276. Bluher M. Adipose tissue dysfunction in obesity. *Exp Clin Endocrinol Diabetes*. 2009;117:241-250.

277. Bakhai A. Adipokines--targeting a root cause of cardiometabolic risk. *QJM*. 2008;101:767-776.

278. Gutierrez DA, Puglisi MJ, Hasty AH. Impact of increased adipose tissue mass on inflammation, insulin resistance, and dyslipidemia. *Curr Diab Rep*. 2009;9:26-32.

279. Brambilla P, Pietrobelli A. Behind and beyond the pediatric metabolic syndrome. *Ital J Pediatr*. 2009;35:41.

280. Thivel D, Malina RM, Isacco L, Aucouturier J, Meyer M, Duche P. Metabolic syndrome in obese children and adolescents: dichotomous or continuous? *Metab Syndr Relat Disord*. 2009;7:549-555.

281. Mancini MC. Metabolic syndrome in children and adolescents - criteria for diagnosis. *Diabetol Metab Syndr*. 2009;1:20.

282. Benson L, Baer HJ, Kaelber DC. Trends in the diagnosis of overweight and obesity in children and adolescents: 1999-2007. *Pediatrics*. 2009;123:e153-158.

283. Flodmark CE, Ohlsson T. Childhood obesity: from nutrition to behaviour. *Proc Nutr Soc*. 2008;67:356-362.

284. Hudson CE. Being overweight and obese: Black children ages 2-5 years. *ABNF J*. 2008;19:89-91.

285. Baker JL, Olsen LW, Sorensen TI. Childhood body-mass index and the risk of coronary heart disease in adulthood. *N Engl J Med*. 2007;357:2329-2337.

286. Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, *et al.* A potential decline in life expectancy in the United States in the 21st century. *N Engl J Med*. 2005;352:1138-1145.

287. Despres JP, Lemieux I, Bergeron J, Pibarot P, Mathieu P, Larose E*, et al.* Abdominal obesity and the metabolic syndrome: contribution to global cardiometabolic risk. *Arterioscler Thromb Vasc Biol*. 2008;28:1039-1049.

288. Armitage JA, Taylor PD, Poston L. Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. *J Physiol*. 2005;565:3-8.

289. Bellinger L, Langley-Evans SC. Fetal programming of appetite by exposure to a maternal low-protein diet in the rat. *Clin Sci (Lond)*. 2005;109:413-420.

290. Bol VV, Delattre AI, Reusens B, Raes M, Remacle C. Forced catch-up growth after fetal protein restriction alters the adipose tissue gene expression program leading to obesity in adult mice. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R291-299.

291. Boullu-Ciocca S, Dutour A, Guillaume V, Achard V, Oliver C, Grino M. Postnatal diet-induced obesity in rats upregulates systemic and adipose tissue glucocorticoid metabolism during development and in adulthood: its relationship with the metabolic syndrome. *Diabetes*. 2005;54:197-203.

292. Bouret SG. Leptin, nutrition, and the programming of hypothalamic feeding circuits. *Nestle Nutr Workshop Ser Pediatr Program*. 2010;65:25-35; discussion 35-29.

293. Bursztyn M, Ariel I. Maternal-fetal deprivation and the cardiometabolic syndrome. *J Cardiometab Syndr*. 2006;1:141-145.

294. Langley-Evans SC, Bellinger L, McMullen S. Animal models of programming: early life influences on appetite and feeding behaviour. *Matern Child Nutr*. 2005;1:142-148.

295. Vickers MH, Breier BH, Cutfield WS, Hofman PL, Gluckman PD. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab*. 2000;279:E83-87.

296. Cripps RL, Martin-Gronert MS, Ozanne SE. Fetal and perinatal programming of appetite. *Clin Sci (Lond)*. 2005;109:1-11.

297. Martin-Gronert MS, Ozanne SE. Programming of appetite and type 2 diabetes. *Early Hum Dev*. 2005;81:981-988.

298. Morris MJ, Chen H. Established maternal obesity in the rat reprograms hypothalamic appetite regulators and leptin signaling at birth. *Int J Obes (Lond)*. 2009;33:115-122.

299. Augustyniak RA, Singh K, Zeldes D, Singh M, Rossi NF. Maternal Protein Restriction Leads to Hyper-Responsiveness to Stress and Salt-Sensitive Hypertension in Male Offspring. *Am J Physiol Regul Integr Comp Physiol*. 2010.

300. Coupe B, Grit I, Darmaun D, Parnet P. The timing of "catch-up growth" affects metabolism and appetite regulation in male rats born with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R813-824.

301. Hales CN, Ozanne SE. The dangerous road of catch-up growth. *J Physiol*. 2003;547:5-10.

302. Jimenez-Chillaron JC, Patti ME. To catch up or not to catch up: is this the question? Lessons from animal models. *Curr Opin Endocrinol Diabetes Obes*. 2007;14:23-29.

303. Ross MG, Desai M. Gestational programming: population survival effects of drought and famine during pregnancy. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R25-33.

304. Caluwaerts S, Lambin S, van Bree R, Peeters H, Vergote I, Verhaeghe J. Diet-induced obesity in gravid rats engenders early hyperadiposity in the offspring. *Metabolism*. 2007;56:1431-1438.

305. Calvert JW, Lefer DJ, Gundewar S, Poston L, Coetzee WA. Developmental programming resulting from maternal obesity in mice: effects on myocardial ischaemia-reperfusion injury. *Exp Physiol*. 2009;94:805-814.

306. Gupta A, Srinivasan M, Thamadilok S, Patel MS. Hypothalamic alterations in fetuses of high fat diet-fed obese female rats. *J Endocrinol*. 2009;200:293-300.

307. Camm EJ, Martin-Gronert MS, Wright NL, Hansell JA, Ozanne SE, Giussani DA. Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring. *Faseb J*. 2011;25:420-427.

308. Pervaiz S. Resveratrol: from grapevines to mammalian biology. *Faseb J*. 2003;17:1975-1985.

309. Sadruddin S, Arora R. Resveratrol: biologic and therapeutic implications. *J Cardiometab Syndr*. 2009;4:102-106.

310. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov*. 2006;5:493-506.

311. Liu L, Wang Y, Lam KS, Xu A. Moderate wine consumption in the prevention of metabolic syndrome and its related medical complications. *Endocr Metab Immune Disord Drug Targets*. 2008;8:89-98.

312. Potenza MV, Mechanick JI. The metabolic syndrome: definition, global impact, and pathophysiology. *Nutr Clin Pract*. 2009;24:560-577.

313. Akar F, Pektas MB, Tufan C, Soylemez S, Sepici A, Ulus AT*, et al.* Resveratrol Shows Vasoprotective Effect Reducing Oxidative Stress Without Affecting Metabolic Disturbances in Insulin-dependent Diabetes of Rabbits. *Cardiovasc Drugs Ther*. 2010.

314. Fitzpatrick DF, Hirschfield SL, Coffey RG. Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am J Physiol*. 1993;265:H774-778.

315. Soleas GJ, Grass L, Josephy PD, Goldberg DM, Diamandis EP. A comparison of the anticarcinogenic properties of four red wine polyphenols. *Clin Biochem*. 2002;35:119-124.

316. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A*, et al.* Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*. 2006;444:337-342.

317. Alcain FJ, Villalba JM. Sirtuin activators. *Expert Opin Ther Pat*. 2009;19:403-414.

318. Chaudhary N, Pfluger PT. Metabolic benefits from Sirt1 and Sirt1 activators. *Curr Opin Clin Nutr Metab Care*. 2009;12:431-437.

319. Gresele P, Cerletti C, Guglielmini G, Pignatelli P, de Gaetano G, Violi F. Effects of resveratrol and other wine polyphenols on vascular function: an update. *J Nutr Biochem*. 2010.

320. Stocker CJ, Arch JR, Cawthorne MA. Fetal origins of insulin resistance and obesity. *Proc Nutr Soc*. 2005;64:143-151.

321. Schwartz J, Thornburg KL. The influence of various physiological challenges on permanent changes to the cardiovascular system. *Arch Physiol Biochem*. 2003;111:3-7.

322. Montuschi P, Barnes P, Roberts LJ, 2nd. Insights into oxidative stress: the isoprostanes. *Curr Med Chem*. 2007;14:703-717.

323. Tosato M, Zamboni V, Ferrini A, Cesari M. The aging process and potential interventions to extend life expectancy. *Clin Interv Aging*. 2007;2:401-412.

324. Ojeda NB, Grigore D, Alexander BT. Intrauterine growth restriction: fetal programming of hypertension and kidney disease. *Adv Chronic Kidney Dis*. 2008;15:101-106.

325. Taylor PD, Poston L. Developmental programming of obesity in mammals. *Exp Physiol*. 2007;92:287-298.

326. Godfrey KM, Barker DJ. Fetal programming and adult health. *Public Health Nutr*. 2001;4:611-624.

327. Canudas-Romo V. Three measures of longevity: time trends and record values. *Demography*. 2010;47:299-312.

328. Carter KN, Blakely T, Soeberg M. Trends in survival and life expectancy by ethnicity, income and smoking in New Zealand: 1980s to 2000s. *N Z Med J*. 2010;123:13-24.

329. Kmietowicz Z. Life expectancy in Scotland remains lower than in many EU countries. *BMJ*. 2010;341:c4337.

330. Roth GS, Ingram DK, Lane MA. Caloric restriction in primates and relevance to humans. *Ann N Y Acad Sci*. 2001;928:305-315.

331. Acheson KJ. Carbohydrate for weight and metabolic control: where do we stand? *Nutrition*. 2010;26:141-145.

332. Dandona P, Ghanim H, Chaudhuri A, Dhindsa S, Kim SS. Macronutrient intake induces oxidative and inflammatory stress: potential relevance to atherosclerosis and insulin resistance. *Exp Mol Med*. 2010;42:245-253.

333. Hackam DG, Khan NA, Hemmelgarn BR, Rabkin SW, Touyz RM, Campbell NR*, et al.* The 2010 Canadian Hypertension Education Program recommendations for the management of hypertension: part 2 - therapy. *Can J Cardiol*. 2010;26:249-258.

334. Janiszewski PM, Ross R. The utility of physical activity in the management of global cardiometabolic risk. *Obesity (Silver Spring)*. 2009;17 Suppl 3:S3-S14.

335. Wardle J, Carnell S, Haworth CM, Plomin R. Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment. *Am J Clin Nutr*. 2008;87:398-404.

336. Hawkins SS, Cole TJ, Law C, Millennium Cohort Study Child Health G. An ecological systems approach to examining risk factors for early childhood overweight: findings from the UK Millennium Cohort Study. *J Epidemiol Community Health*. 2009;63:147-155.

337. He Y, Ma G, Zhai F, Li Y, Hu Y, Feskens EJ*, et al.* Dietary patterns and glucose tolerance abnormalities in Chinese adults. *Diabetes Care*. 2009;32:1972-1976.

338. Frassetto L, Morris RC, Jr., Sellmeyer DE, Todd K, Sebastian A. Diet, evolution and aging--the pathophysiologic effects of the post-agricultural inversion of the potassium-to-sodium and base-to-chloride ratios in the human diet. *Eur J Nutr*. 2001;40:200-213.

339. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA*, et al.* Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr*. 2005;81:341-354.

340. Jew S, AbuMweis SS, Jones PJ. Evolution of the human diet: linking our ancestral diet to modern functional foods as a means of chronic disease prevention. *J Med Food*. 2009;12:925-934.

341. Larsen CS. Animal source foods and human health during evolution. *J Nutr*. 2003;133:3893S-3897S.

342. Formiguera X, Canton A. Obesity: epidemiology and clinical aspects. *Best Pract Res Clin Gastroenterol*. 2004;18:1125-1146.

343. Pan WH, Lee MS, Chuang SY, Lin YC, Fu ML. Obesity pandemic, correlated factors and guidelines to define, screen and manage obesity in Taiwan. *Obes Rev*. 2008;9 Suppl 1:22-31.

344. Friedman RR, Schwartz MB. Public policy to prevent childhood obesity, and the role of pediatric endocrinologists. *J Pediatr Endocrinol Metab*. 2008;21:717-725.

345. Han JC, Lawlor DA, Kimm SY. Childhood obesity. *Lancet*. 2010;375:1737-1748.

346. Sinha A, Kling S. A review of adolescent obesity: prevalence, etiology, and treatment. *Obes Surg*. 2009;19:113-120.

347. Tzotzas T, Krassas GE. Prevalence and trends of obesity in children and adults of South Europe. *Pediatr Endocrinol Rev*. 2004;1 Suppl 3:448-454.

348. Shankar K, Kang P, Harrell A, Zhong Y, Marecki JC, Ronis MJ, *et al.* Maternal Overweight Programs Insulin and Adiponectin Signaling in the Offspring. *Endocrinology*. 2010;151:2577-2589.

349. Simmons R. Perinatal programming of obesity. *Semin Perinatol*. 2008;32:371-374.

350. Cripps RL, Archer ZA, Mercer JG, Ozanne SE. Early life programming of energy balance. *Biochem Soc Trans*. 2007;35:1203-1204.

351. Lampl M, Jeanty P. Timing is everything: a reconsideration of fetal growth velocity patterns identifies the importance of individual and sex differences. *Am J Hum Biol*. 2003;15:667-680.

352. Hemmings DG, Williams SJ, Davidge ST. Increased myogenic tone in 7-month-old adult male but not female offspring from rat dams exposed to

hypoxia during pregnancy. *Am J Physiol Heart Circ Physiol*. 2005;289:H674-682.

353. O'Regan D, Kenyon CJ, Seckl JR, Holmes MC. Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology. *Am J Physiol Endocrinol Metab*. 2004;287:E863-870.

354. Mueller BR, Bale TL. Sex-specific programming of offspring emotionality after stress early in pregnancy. *J Neurosci*. 2008;28:9055-9065.

355. Ojeda NB, Grigore D, Yanes LL, Iliescu R, Robertson EB, Zhang H*, et al.* Testosterone contributes to marked elevations in mean arterial pressure in adult male intrauterine growth restricted offspring. *Am J Physiol Regul Integr Comp Physiol*. 2007;292:R758-763.

356. Ojeda NB, Grigore D, Robertson EB, Alexander BT. Estrogen protects against increased blood pressure in postpubertal female growth restricted offspring. *Hypertension*. 2007;50:679-685.

357. Khan IY, Taylor PD, Dekou V, Seed PT, Lakasing L, Graham D*, et al.* Gender-linked hypertension in offspring of lard-fed pregnant rats. *Hypertension*. 2003;41:168-175.

358. McArthur S, Siddique ZL, Christian HC, Capone G, Theogaraj E, John CD*, et al.* Perinatal glucocorticoid treatment disrupts the hypothalamo-lactotroph axis in adult female, but not male, rats. *Endocrinology*. 2006;147:1904-1915.

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

360. Vickers MH, Ikenasio BA, Breier BH. Adult growth hormone treatment reduces hypertension and obesity induced by an adverse prenatal environment. *J Endocrinol*. 2002;175:615-623.

361. Loos RJ, Beunen G, Fagard R, Derom C, Vlietinck R. Birth weight and body composition in young adult men--a prospective twin study. *Int J Obes Relat Metab Disord*. 2001;25:1537-1545.

362. Barker DJ, Gluckman PD, Robinson JS. Conference report: fetal origins of adult disease--report of the First International Study Group, Sydney, 29-30 October 1994. *Placenta*. 1995;16:317-320.

363. Reynolds RM. Corticosteroid-mediated programming and the pathogenesis of obesity and diabetes. *J Steroid Biochem Mol Biol*. 2010:[Epub ahead of print].

364. Simmons RA. Developmental origins of diabetes: the role of epigenetic mechanisms. *Curr Opin Endocrinol Diabetes Obes*. 2007;14:13-16.

365. Young JB. Developmental origins of obesity: a sympathoadrenal perspective. *Int J Obes (Lond)*. 2006;30 Suppl 4:S41-49.

366. Garite TJ, Clark R, Thorp JA. Intrauterine growth restriction increases morbidity and mortality among premature neonates. *Am J Obstet Gynecol*. 2004;191:481-487.

367. Miller AA, De Silva TM, Jackman KA, Sobey CG. Effect of gender and sex hormones on vascular oxidative stress. *Clin Exp Pharmacol Physiol*. 2007;34:1037-1043.

368. Ordovas JM. Gender, a significant factor in the cross talk between genes, environment, and health. *Gend Med*. 2007;4 Suppl B:S111-122.

CHAPTER 2 ANIMAL MODELS AND EXPERIMENTAL METHODS†

All procedures used in this thesis were approved by the University of Alberta Animal Welfare Committee (Protocol number 301/11/07/D), and are in accordance with the guidelines of the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.[1]

2.1 Animal experimental models

2.1.1 Hypoxia-induced IUGR model

Female Sprague Dawley rats were obtained at three months of age (body weight 260±30 g) from Charles River, Quebec, Canada; acclimatized for one week at the animal facilities of the University of Alberta and then mated with a young male rat by leaving them together in a standard rat cage for a 12-hour period during the dark cycle. A vaginal smear obtained the following morning was examined under light microscopy to determine the presence of sperm, which signified day 0 of pregnancy (term ≈ 21 days). Throughout pregnancy, rats were housed in standard rat cages (one animal per cage)

† All methods described in this chapter have been published in (or submitted to) one or more peer-review journals including:

- *"**Rueda-Clausen CF**, Morton JS and Davidge ST. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. Cardiovascular Research 2009;81:713-722.*
- *"**Rueda-Clausen CF**, Morton JS, Lopaschuk GD and Davidge ST. Long-term effects of intrauterine growth restriction on cardiac metabolism and susceptibility to ischemia reperfusion. In press, Cardiovascular Research 2010."*
- *"**Rueda-Clausen CF**, Dolinsky VW, Morton JS, Dyck JRB and Davidge ST. Intrauterine growth restriction increases the susceptibility of rats to high-fat diet-induced metabolic syndrome. in press, Diabetes 2011"*
- *"Dolinsky VW, **Rueda-Clausen CF**, Morton JS, Dyck JRB and Davidge ST. Resveratrol reverts the susceptibility of high-fat diet-induced metabolic syndrome in rats born growth restricted. In preparation for: Diabetes 2011"*

***Contribution:** Glucose metabolism studies, lipid determinations and all western blots were performed by Dolinsky VW. Unless otherwise stated, all other methods described in this chapter were performed by Rueda-Clausen CF.*

with *ad libitum* access to water and standard laboratory rat chow LabDiet® Ref 5001 (see Table 2-1 for details on diet composition).

Table 2-1 Detailed composition of standard laboratory rat chow

Caloric content	**(4.07 Kcal/g)**	
Composition	**g %**	**Kcal %**
Fat	4.5	13.4
Soybean Oil	4.5	6.5
Lard	0	-
Carbohydrate	62	57.9
Corn starch	31.9	33
Maltodextrin 10	-	-
Sucrose	3.7	3.5
Cellulose	5.1	0
Protein	23	28.5
Casein		
L-Cysteine	0.31	0.36
Mineral mix	0.97	-

On gestational day (G) 15, rats were randomly allocated to either standard housing with an oxygen concentration of 21% (control) or maternal hypoxia with an oxygen concentration of ~11.5% (IUGR). Rats assigned to the maternal hypoxia group were placed inside a sealed acrylic chamber (Animal Chamber for Disease Modeling type A, Biospherix. Lacona, NY U.S.A.) controlled by an oxygen sensor (ProOx Oxygen Controller, Biospherix. Lacona, NY U.S.A.). This system performed a continuous evaluation of the gas composition inside the chamber and maintained an internal concentration of oxygen at 11.5±0.3% by regulating the flow of nitrogen infused into the chamber. In the lower level of the chamber a plastic tray was placed with 250 g of soda lime (J.T. Baker, Phillipsburg, NJ U.S.A. catalog #3448-05) to scavenge excess CO_2 inside the chamber. After three days inside the chamber (G18) rats were given clean cages and fresh food and water during a rapid cage change. [2-4]

After replacing cages, the oxygen concentration in the chamber returned to hypoxia levels within five minutes. Throughout experiments, the

oxygen level in the chamber was continuously monitored and recorded every 10 minutes by a computer connected to the ProOx Oxygen Controller. Previous studies have shown that exposure to re-oxygenation following hypoxia can itself induce deleterious effects on both placental and fetal tissues, probably due to induction of oxidative stress.[2-4] Although the exposure to room air during cage changing was very short relative to the full length of the hypoxic insult, it is plausible that this short period of re-oxygenation may have an effect on the fetal response to hypoxia and should be considered among the limitations of the study. At day 21 of pregnancy, 6 to 12 hours before birth, all dams were placed in clean cages with fresh food and water and dams randomized to the IUGR group were returned to normal housing conditions (21% oxygen).

Postnatal protocols for the management and assessment of control and IUGR offspring varied depending on the objectives of the particular study. Further details are provided in the following sections:

2.1.1.1 Fetal hypoxia and fetal growth assessment protocol

A specific set of experiments was performed to evaluate the effect of maternal hypoxia on fetal oxygen availability and determine the effect of this prenatal intervention on fetal biometry and placental weight just before birth. On G20.5 of pregnancy, dams allocated to hypoxia or control groups received an intraperitoneal (IP) injection of either 60 mg/Kg of Pimonidazole previously conjugated with fluorescein-isothiocyanate-conjugated IgG1 mouse monoclonal antibody (Hypoxyprobe 1 Plus, HPI Inc. Burlington, MA U.S.A.), or an equivalent volume of vehicle. After injection, Pimonidazole distributes to all tissues and adducts to thiol containing proteins only in those cells that have an oxygen concentration less than 14 mmol/L (equivalent to a partial oxygen pressure (pO_2) =10 mmHg at 37°C).[5, 6] When injected IP, the mean life of Pimonidazole is four hours.

To avoid post-mortem binding of Pimonidazole to fetal tissues, dams were euthanized six hours after Pimonidazole injection and before the beginning of parturition. Dams were anesthetized using inhaled isoflourane and euthanized by exsanguination laparotomy and hysterectomy was rapidly performed and all fetuses were counted and sexed. Both fetuses and placentas were gently dried with gauze and weighed. A minimum of three male offspring were randomly selected from each litter and euthanized with sodium thiopental (0.05 mL IP). Heart, liver and placenta from these fetuses were rapidly excised, embedded in medium for frozen tissue specimens (Tissue-tek, Sakura. Torrance, CA U.S.A.) and snap frozen. In at least six offspring from each litter (three males and three females) the right tibia was dissected and measured.

Remaining fetal tissues from male fetuses were frozen or fixed in buffered neutral formalin 10% (VWR, West Chester, PA U.S.A.) for 24 hours, dehydrated with increasing concentrations of methanol and embedded in paraffin. Histological preparations of samples were performed by the Histology Core of the Alberta Diabetes Institute (Edmonton, AB Canada). Tissue levels of Pimonidazole were determined in placenta, liver and heart tissue by immunostaining and following the protocol provided by the manufacturer.

Additional experiments were performed in a different set of animals using similar procedures but a different hypoxia marker. At G19.5 of pregnancy both control and dams exposed to hypoxia were injected with 60 mg/Kg IP of Hypoxyprobe F6 (Hypoxyprobe TM-F6/CCI-103F, HPI Inc. Burlington, MA U.S.A.). After injection, Hypoxiprobe F6 behaves similar to Pimonidazole but has a longer mean life (12-17 hours). Dams were euthanized 24 hours after injection and before the beginning of parturition. Maternal and fetal tissues were collected and preserved as previously mentioned. Levels of Hypoxyprobe F6 in the offspring's liver were determined by immunostaining and following the protocol provided by the manufacturer.

In the same set of animals, liver levels of HIF-1α were measured by immunostaining to determine whether maternal hypoxia was causing fetal activation of hypoxia-induced signaling. For that end a rabbit polyclonal antibody (1:500, catalog # NB100-134, Novus Biologicals. Littleton, CO U.S.A.) was used.

2.1.1.2 Hypoxia-induced IUGR/aging interaction model

In a different set of animals and immediately after parturition, stillborn were removed, counted and sexed. Viable offspring were then counted, sexed, weighed and the litter was reduced to eight viable pups (four males and four females) in order to control the postnatal environment and competition for milk. Immediately after litter reduction, excess offspring were sexed, weighed and euthanized by exsanguination. The hearts from these pups were rapidly extracted, gently dried and weighed.

During lactation, pups and dams were weighed and cages were changed bi-weekly. All offspring were weaned at three weeks of age and placed in standard rat cages (four rats of the same sex per cage from week three to week six and thereafter two rats of the same sex per cage). Throughout experiments, all rats were housed at the animal facilities of the University of Alberta in a room with 60% humidity, a 12 hour light: 12 hour darkness light cycle and *ad libitum* access to water and standard rat chow (LabDiet Ref 5001 distributed by Canadian Lab Diets, Inc. Leduc, AB Canada; see Table 2-1 for details on diet composition). If individual body weight exceeded 800 g, or signs of violence/stress were detected, animals were separated and singly housed until the experimental day. To evaluate the interaction between hypoxia induced-IUGR and the normal aging process, experiments were performed at 4 and 12 months of age, time points when rats were considered young adults and aging adults respectively (Figure 2-1).

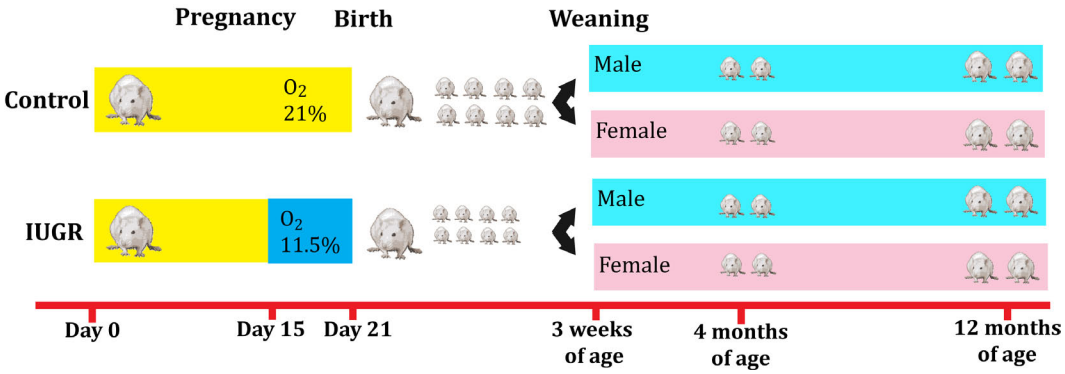


Figure 2-1 Schematic of the hypoxia-induced IUGR/aging interaction model

2.1.1.3 Hypoxia-induced IUGR/diet interaction model

In a separate set of animals and following parturition, stillborn pups were removed, counted and sexed. Viable offspring were then counted, sexed, weighed and the litter was reduced to eight viable male pups in order to control the postnatal environment and competition for milk. In cases where litters contained less than eight viable male pups, the groups were reduced to eight offspring with as many males as possible. At weaning, however, female offspring were euthanized and only male offspring were used for experimental purposes. At the same time point (three weeks of age), animals from each experimental group (IUGR and control) were randomly allocated to receive either a purified standardized low-fat diet (LF; 10% fat, ResDiets D12450B distributed by Canadian Lab Diets, Inc. Leduc, AB Canada) or a purified standardized high-fat diet (HF; 45% fat, ResDiets D12451, Research Diets, Inc. New Brunswick, NJ U.S.A). Detailed composition of these diets is presented in Table 2-2.

Table 2-2 Detailed characteristics of special diets

	LF diet ResDiets D12450B		HF diet ResDiets D12451	
Caloric content	(3.85 Kcal/g)		(4.73 Kcal/g)	
	gr%	Kcal%	Gr%	Kcal%
Fat	4.3	10	24	45
Soybean Oil	2.3	5.55	2.91	5.55
Lard	1.9	4.44	20.7	39.4
Carbohydrate	67.3	68	41	35
Corn starch	29.8	31	8.4	7.1
Maltodextrin 10	3.3	3.4	11.6	9.8
Sucrose	33.2	34.5	20.1	17
Cellulose	4.74	0	5.83	0
Protein	19.2	20	24	20
Casein	18.9	19.7	23.3	19.7
L-Cysteine	0.28	0.3	0.35	0.3
Mineral mix	0.95	-	1.17	-

During nutritional intervention periods, rats were given *ad libitum* access to water and either diet for four or nine weeks; following which experimental protocols were performed (Figure 2-2).

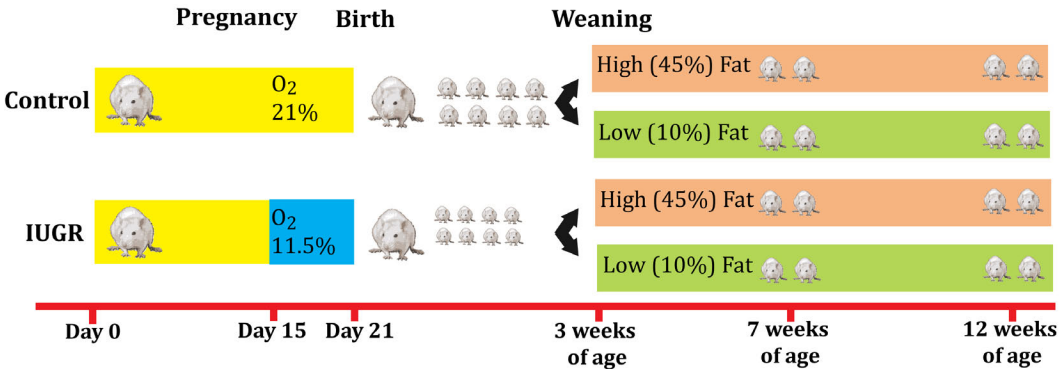


Figure 2-2 Schematic of the hypoxia-induced IUGR/diet interaction model

2.1.1.4 Hypoxia-induced IUGR plus high-fat diet /Resveratrol interaction model

Additional experiments were conducted in a different group of animals to evaluate the potential usefulness of Resveratrol (3,5,4'-trihydroxy-trans-stilbene) in the prevention of long-term deleterious effects of being born from pregnancies complicated by hypoxia-induced IUGR.

In order to approach this research question, a variation of the hypoxia-induced IUGR/diet interaction model presented in Section 2.1.1.3 was used. In this case, both experimental groups (control and IUGR) were randomized to receive either a HF diet alone (HF; 45% fat, ResDiets D12451, Research Diets, Inc. New Brunswick, NJ U.S.A) or HF diet supplemented with Resveratrol 4 g/Kg (Figure 2-3). The dose of Resveratrol added to the diet was chosen based on previous studies showing beneficial effects of nutritional supplementation with Resveratrol.[7] The bioavailability of Resveratrol administered in the diet is actually quite low, mainly due to intestinal breakdown, absorption and a high level of first pass clearance in phase one metabolism by the liver. Therefore, the final plasma concentration of Resveratrol achieved with this dose was within the expected therapeutic values (in the μg/L range).[8]

In this particular set of experiments, offspring were evaluated only at 12 weeks of age based on previous results showing no significant differences between experimental groups at seven weeks of age.

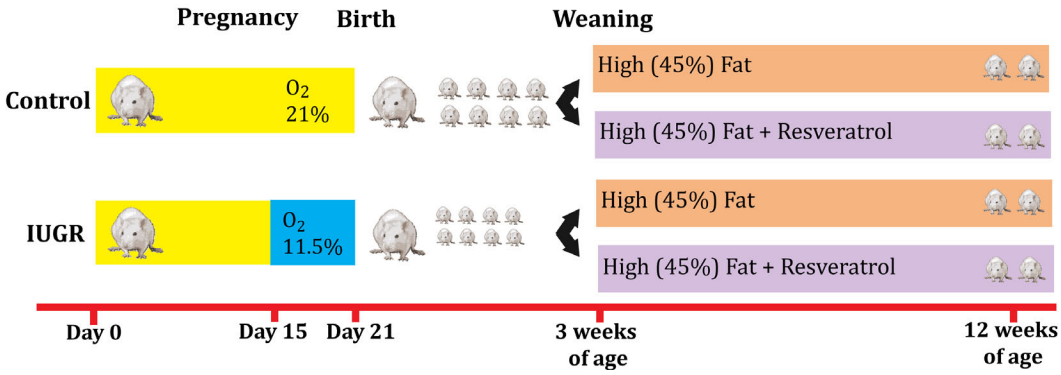


Figure 2-3 Schematic of the hypoxia-induced IUGR plus high-fat diet/Resveratrol interaction model

2.2 Experimental methods for the evaluation of cardiovascular parameters

2.2.1 In vivo *cardiac function and structure evaluation using ultrasound biomicroscopy*

To evaluate *in vivo* cardiac structure and function, adult offspring were anesthetized using sodium thiopental 60 mg/Kg IP and placed in a supine position on a temperature controlled heating pad. The chest and abdomen were shaved and the extremities were gently fixed to electrodes on the pad surface using tape and a highly conductive electrode gel. A single channel electrocardiogram signal and respiratory rate were continuously recorded on the imaging system and body temperature was monitored using a rectal probe. Echocardiographic evaluations were performed using a high-resolution ultrasound biomicroscopy (UBM) system Vevo-770 (by Visualsonics Inc. Toronto, ON Canada) with a real-time micro visualization scan head of 17.5 Mhz and following the guidelines of the American Society of Echocardiography.[9]

Images and videos were recorded and analyzed in triplicate and data was extracted using a form designed for such purposes (Appendix 10.1). M-

mode bi-dimensional (2D) echocardiography images were obtained in the parasternal long- and short-axes (Figure 2-4).

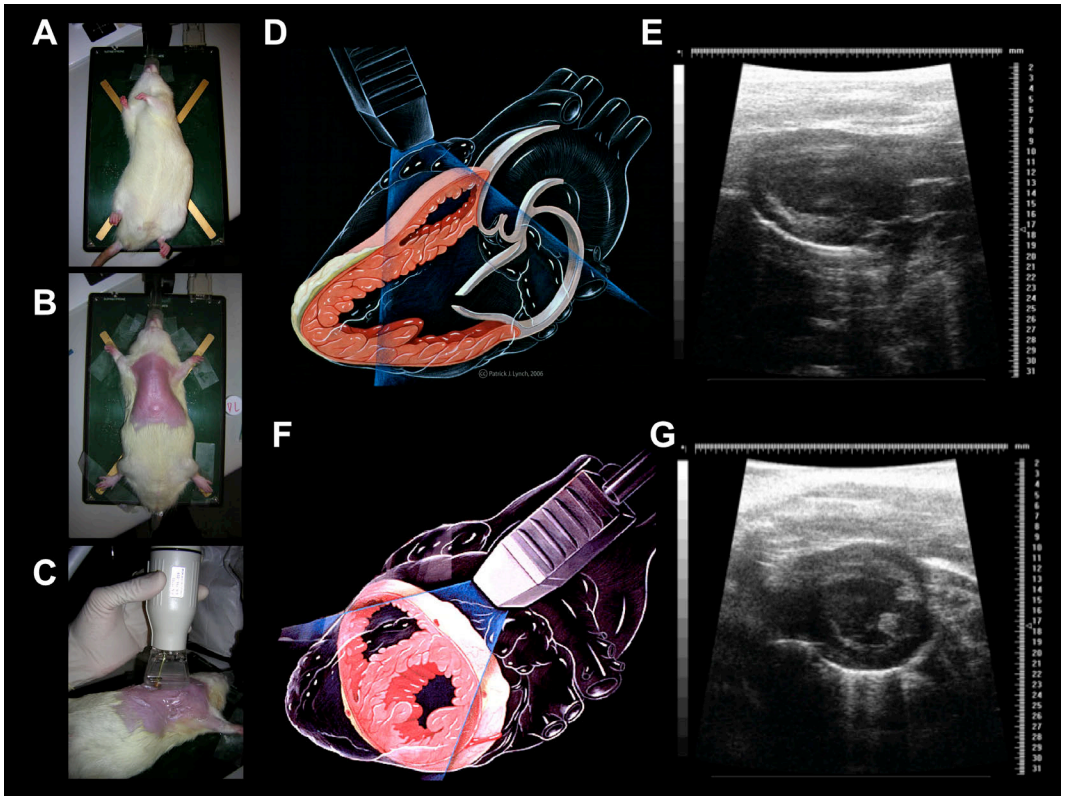


Figure 2-4 Standard ultrasound biomicroscopy set-up for adult rats and illustration of long- and short-axis views in B-mode

Anatomical position of the rat during UBM studies before (**A**) and after (**B**) hair removal and position of the scan-probe (**C**). Panels **D** and **F** illustrate the perspectives of long and short cardiac axes respectively. Panels **E** and **G** present actual images obtained using B-mode UBM in the long and short cardiac axes respectively.

The dimensions of the left ventricle (LV) diameter and wall thickness were measured using a left parasternal long-axis view of the heart with the M-mode beam positioned just beyond the mitral valve tips; perpendicular to the long-axis of the ventricle and centered in the short-axis. The LV posterior wall endocardium was identified in the M-mode image as the most intense continuous line with the steepest systolic motion. The posterior wall epicardium was identified as the echo reflection immediately anterior to the posterior pericardium. The septal endocardium was identified as the steepest

motion in systole with a continuous reflection throughout the cycle (Figure 2-5).

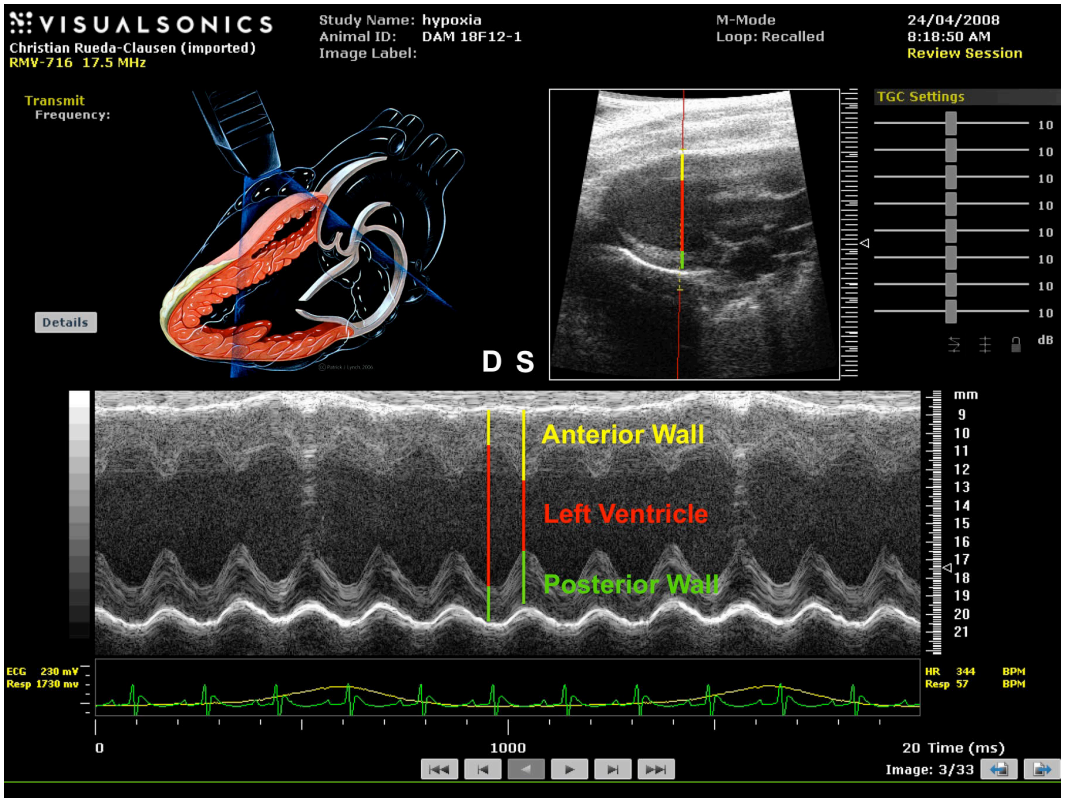


Figure 2-5 Representation of the cardiac long-axis view in B-mode (mono-ventricular view) and its respective projection in M-mode obtained by ultrasound biomicroscopy

Colored lines represent the different structures of the heart in both B-mode (top) and M-mode (bottom). Including left ventricle anterior wall (yellow), left ventricle internal diameter (red) and left ventricle posterior wall (green). S: systole D: diastole.

The LV volumes were estimated using the 'cubed' formula, which assumes that the long-axis of the LV is equal to twice the short-axis diameter. The volume of the LV at any given moment can then be approximated from a single, short-axis measurement as $Volume=Diameter^3$. Cardiac images were obtained using a variation of the short-axis view in which the scan head was located on the right thoracic wall pointed almost horizontal toward the contra-lateral chest wall. This view is also known as bi-ventricular short-axis view (Figure 2-6).

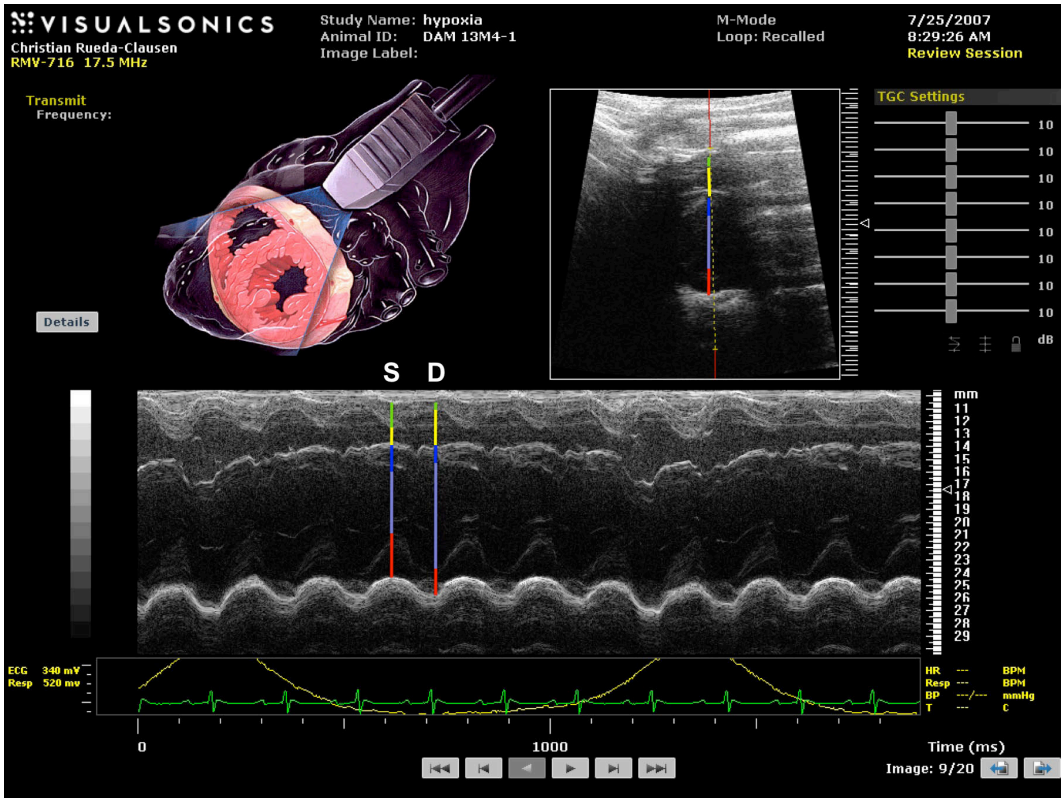


Figure 2-6 Representation of the cardiac short-axis (bi-ventricular view) in B-mode and its respective projection in M-mode obtained by ultrasound biomicroscopy

Colored lines represent the different structures of the heart in both the B-mode (top) and the M-mode (bottom). Including right ventricle free wall (green), right ventricle internal diameter (yellow), inter-ventricular septum (blue), left ventricle internal diameter (purple) and left ventricle posterior wall (red). S: systole D: diastole

To estimate ventricular mass, the following formula was applied using images obtained in the bi-ventricular view and using M-mode UBM.

$$LVmass = 1.04 * ((LVID_{dias}+PW+IVS)^3-LVIDd^3)*0.8$$

Were LVmass = LV mass (g), $LVID_{dias}$ = LV internal diameter in diastole (mm), IVS= interventricular septum thickness (mm) and PW = posterior wall thickness (mm).

Aortic outflow velocity/time integral (mmHg), aortic peak ejection velocity (cm/s) and aortic ejection time (ms) were determined using a Doppler signal of the ascending aorta that was recorded in a variation of the

long-axis view (aortic outlet view) in which the scan head was pointed 30 degrees cephalocaudal (Figure 2-7).

Figure 2-7 Representative images of the cardiac modified long-axis (aortic outlet view) in B-mode and the respective Doppler signal of the aortic flow obtained by ultrasound biomicroscopy

The blue arrow indicates the flow direction of the ascending aorta recorded in B-mode (top right). Colored lines represent the different parameters determined by Doppler imaging (top left and bottom) of the aortic flow including: aortic outflow velocity/time integral (pink), aortic peak ejection velocity (green) and aortic ejection time (red).

Aortic root and left atrium diameters were determined by M-mode imaging using a modified long-axis view (transversal aortic view) with the scan head on the long-axis and the beam parallel to the axis of the aortic valve (Figure 2-8).

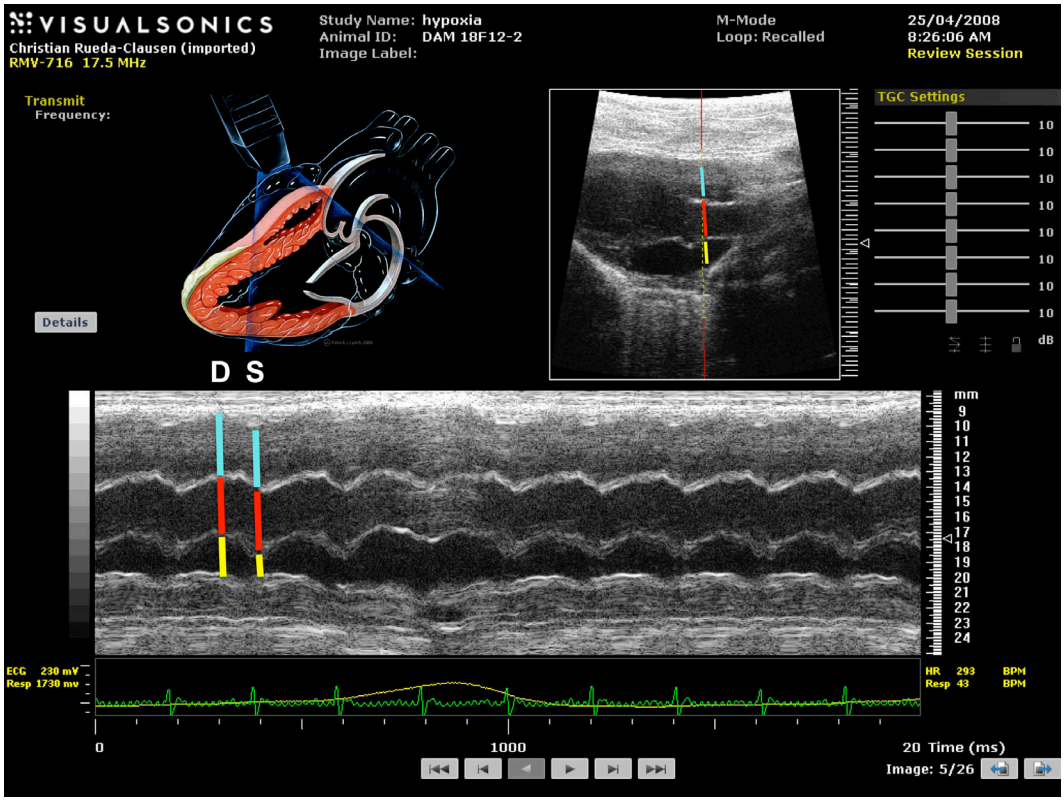


Figure 2-8 Representation of the cardiac modified long-axis (transversal aortic view) and images obtained by ultrasound biomicroscopy in B- and M-mode visualizing the aortic root and right atrium

Colored lines represent the different structures that can be identified in both the B-mode (top right) and M-mode projections (Bottom) including: right ventricle internal diameter (blue), aortic internal diameter (red) and right atrium internal diameters (yellow). S: systole, D: diastole.

LV function was evaluated by estimating ejection fraction, shortening fraction and cardiac output from images obtained in M-mode of the LV long-axis.

Ventricular diastolic function was assessed using both the transmitral Doppler and mitral tissue Doppler signals. E and A wave velocities and gradients were measured in a modified parasternal long-axis (mitral view) with the beam placed on the tip of the mitral leaflet. E/A index, mitral deceleration time and myocardial performance index (Tei index)[10] were calculated using the following formula:

$$E/A = E_{max}/ A_{max}$$

$$Tei\ index = (IVRT+IVCT)/ET$$

Were E= passive left ventricle filling velocity, A= active left ventricle filling velocity (atrial kick), IVRT= left ventricle isovolumetric relaxation time, IVCT= left ventricle isovolumetric contraction time, ET= left ventricle ejection time (Figure 2-9).

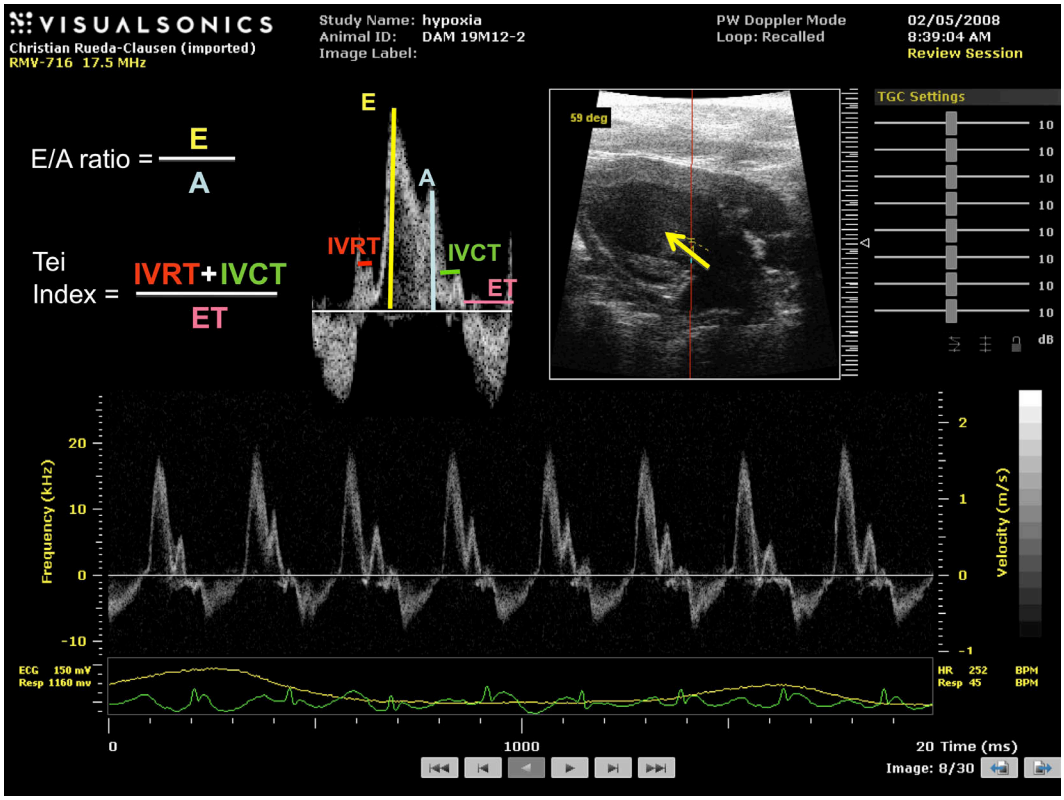


Figure 2-9 Representative images obtained in a cardiac modified long-axis (mitral view) in B-mode and the respective Doppler signal of the mitral outlet obtained by ultrasound biomicroscopy

Yellow arrow represents the direction of the trans-mitral flow in a B-mode image (top right). Colored lines represent the different parameters that can be determined from the Doppler signal of the mitral outlet (bottom) including: E= passive left ventricle filling velocity (yellow), A= active (atrial kick) left ventricle filling velocity (blue), IVRT= left ventricle isovolumetric relaxation time (red), IVCT= left ventricle isovolumetric contraction time (green), ET: left ventricle ejection time (pink).

Pulmonary artery Doppler was measured in parasternal long-axis orientation after B-mode identification of the pulmonary artery lateral to the aortic root (pulmonary artery view). Pulmonary vascular resistance was determined using the following formula:

Pulmonary vascular resistance = PAAT/RVET

Were PAAT = pulmonary artery acceleration time and RVET = right ventricle ejection time (Figure 2-10).

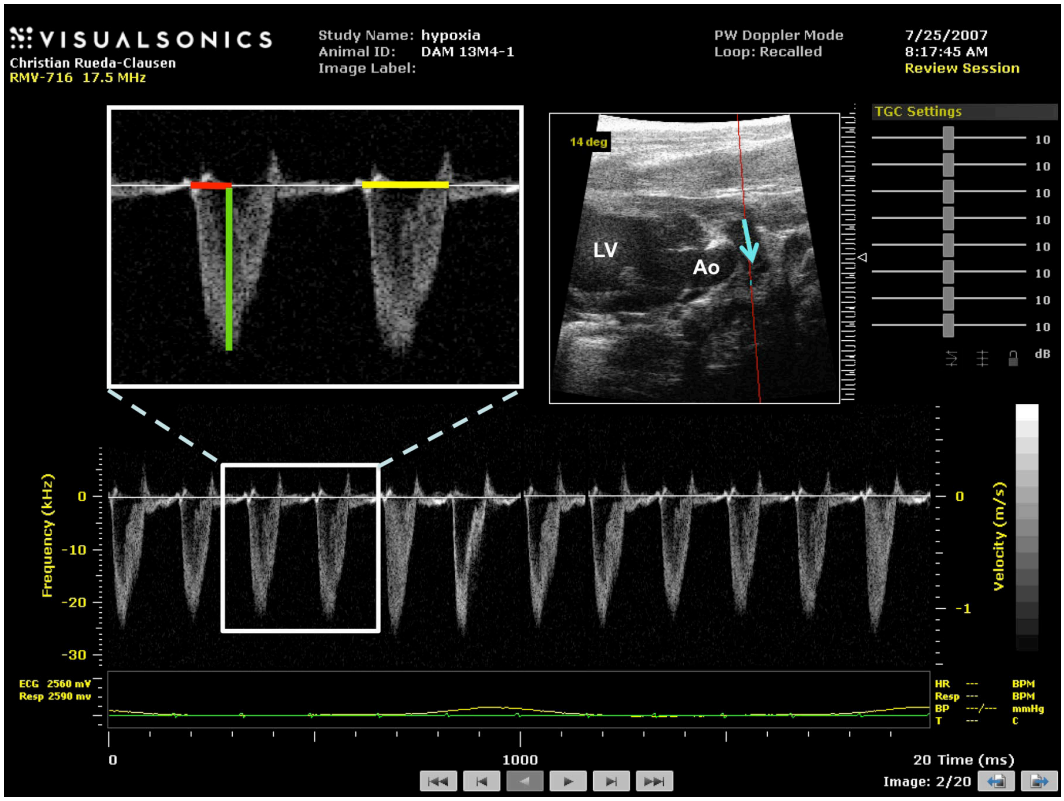


Figure 2-10 Representation of the cardiac modified long-axis (pulmonary artery view) in B-mode and the respective Doppler signal of the pulmonary artery atrium obtained by ultrasound biomicroscopy

Blue arrow indicates the blood flow direction in the pulmonary artery viewed in B-mode (top right), Ao: aortic outlet, LV: left ventricle. Colored lines represent the different parameters that can be determined using Doppler (bottom), including: PAAT: pulmonary artery acceleration time (red), RVET: right ventricle ejection time (yellow), pulmonary artery peak ejection velocity (green).

All measurements were made in triplicate and analyzed using the cardiac imaging analysis software (Vevo Cardiac Measurements Ver.1.3.2.5) provided by the UBM machine manufacturer.

2.2.2 Lung histology and vascular morphometry

A separate set of animals was euthanized at 12 months of age with a lethal injection of sodium pentobarbital (100 mg/Kg IP). The trachea was exposed in the neck and a tracheostomy performed with a metal cannula. The lungs were then perfused using 10% formalin (12.5 cmH_2O for 10 minutes). After perfusion, the trachea was ligated and lungs were carefully extracted from the thorax (being careful to not pinch them in the process). Lungs were then preserved in formalin 10%. After a 72-hour fixation period, the right antero-inferior lobe was dissected and preserved in methanol 10%.[11, 12] Lung samples in methanol were then sent to the Alberta Diabetes Institute Histology Core (Edmonton, Canada) where histopathological preparations (by paraffin embedding) and staining with hematoxylin and eosin were performed. Using a standard light microscope at 40X magnification, small pulmonary arterioles (diameter 30-110 μm) were identified from five cross-sectional regions. Using a digital micrometer, internal and external diameters were determined in all of the identified pulmonary arterioles. Percent wall thickness from each identified artery was calculated using the following formula:

$$\textit{Wall thickness (\%)} = \frac{\textit{Cross sectional diameter} - \textit{luminal diameter}}{\textit{Cross sectional diameter}} * 100$$

The independent values from each sample were then averaged to determine the percent wall thickness from each animal.

2.2.3 Isolated working heart preparations

2.2.3.1 Basic working heart set up

On the experimental day, animals were anesthetized using sodium pentobarbital (60 mg/Kg IP). Once the animal reached an appropriate anesthetic plane, a complete thoracotomy was performed; the heart was rapidly excised using scissors and placed in a beaker with 100 mL of fresh, cold (4 °C) Krebs solution. Excess blood was rinsed from the heart and the aorta was identified, rapidly fixed to a cannula and perfused for ~10 minutes in retrograde Langendorff mode against a constant perfusion pressure of 60 mmHg. Hearts were perfused with a Krebs-Henseleit solution (37±0.5 °C) containing (in mmol/L) 120 NaCl, 25 $NaHCO_3$, 5.5 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$ and 2.5 $CaCl_2$ (pH 7.4 gassed with 95% O_2 and 5% CO_2) as previously reported.[13-15] The time between the induction of a pneumothorax and the initiation of retrograde perfusion never exceeded 30 seconds.

During this initial perfusion period, the heart was trimmed of excess tissue and the right atrium cannulated. After an equilibration period, hearts were then switched to anterograde working mode by clamping the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow line. The closed, recirculating system was filled with 120 ml of modified Krebs-Henseleit solution containing (in mmol/L) 2.5 $CaCl_2$, 5.5 glucose, 1.2 palmitate bound to bovine serum albumin (BSA) 3% (fraction V), 0.5 lactate and 100 mU/L insulin (Novolin-GE Toronto, ON)[15] and warmed by a water jacket set at 37±0.5 °C.

Perfusate entered the cannulated left atrium at 11.5±0.5 mmHg pressure and passed to the LV from which it was spontaneously ejected through the aortic cannula into a compliance chamber against a pressure of 80 mmHg. During the first 10 minutes of equilibration, the pressure in the compliance chamber was checked, the preload was adjusted and the system was purged. Then the tip of a silver electrode was placed immediately

posterior and a little inferior to the right atrium being careful not to restrict the left atrium distension or forcing the position of the heart in such way that the aortic flow was compromised. Hearts were then paced using a Stimulator P type 201 (by HSE; Hugo Sachs Electronik – Harvard Apparatus. Hugstetten, Germany) previously set with a monophasic (+) basic rhythm, a faradic stimulation mode with a fixed wavelength of 0.3 to 0.5 ms and an output of three to six Volts. Pacing rates were set at 300 beats/min (delay=200 ms) in 4 month old rats or 260 beats/min (delay =240ms) in 12 month old rats. Pacing rates were selected based on preliminary studies showing that hearts from 12 month old animals do not tolerate pacing at the same rates as hearts from younger rats.

Temperature of the perfusate in the reservoir was measured constantly by an immersed thermocouple. Preload pressure, aortic pressures and chamber pressure were recorded using HSE Isoheart® sensors (P20 or P100). Both cardiac output and aortic flow were measured using in-line flow sensors (Transonic Systems. Ithaca, NY U.S.A.). Signals from all sensors were acquired using an HSE interface and recorded by a PC using the IsoHeart software (Ver 2.0.1.1) for windows 2000 (by HSE). Parameters that were determined using this technique are presented in Table 2-3.[16, 17]

Table 2-3 Parameters determined using the working heart system

Parameter	Description	Description/calculation	Units
Chamber pressure (CP)	Differential pressure developed by the flow of gas through the closed system	Estimated by a pressure monitor sensing the pressure inside the system	mmHg
Pre-load pressure (PLP)	Pressure of the fluid coming from the oxygenator into the left atrium	End-diastolic inflow pressure estimated by a pressure monitor sensing the pressure in the in-flow line minus the end-diastolic chamber pressure	mmHg
After-load pressure (ALP)	Minimal resistance against which the heart has to pump fluid.	End-diastolic aortic pressure measured by a pressure monitor sensing the pressure in the out-flow line minus the end-diastolic CP	mmHg
Perfusate temperature (PT)	Temperature of the fluid in the reservoir	Determined by a thermocouple in the reservoir	°C
Heart rate (HR)	Cardiac beats per minute	Measured by Millar catheter or ECG electrodes	bpm
Peak systolic perfusion pressure (PSP)	Maximal aortic pressure at the end of systole	Maximum systolic aortic pressure measured by a pressure monitor sensing the pressure in the out-flow line minus the end-systolic CP	mmHg
End-diastolic perfusion pressure (EDP)	Minimal aortic pressure at the end of diastole	End-diastolic aortic pressure measured by a pressure monitor sensing the pressure in the out-flow line minus the end-diastolic CP	mmHg
Mean perfusion pressure (MPP)	Used to describe an average fluid pressure in the system over time	PSP*1/3+EDP*2/3	mmHg

Aortic pulse pressure (AoPP)	Change in aortic pressure during a cardiac cycle	PSP-EDP	mmHg
Cardiac output (CO)	Volume of fluid pumped by the heart in one minute	Equivalent to the average flow in the atrium cannula during one minute Also reported adjusted by cardiac dry weight	mL/min cm^3/min or mL/g dry wt cm^3/g dry wt
Stroke volume (SV)	Volume of fluid pumped during each heartbeat	CO/HR Also reported adjusted by cardiac dry weight	μL or μL/g dry wt
Aortic flow (AoF)	Volume of fluid pumped through the aorta in one minute	Mean aortic flow per minute Also reported adjusted by cardiac dry weight	mL/min or mL•min^{-1}•g dry wt^{-1}
Coronary flow (CF)	Volume of fluid that circulates through the coronary system in one minute	CO–AoF Also reported adjusted by cardiac dry weight	mL/min or mL•min^{-1}•g dry wt^{-1}
Cardiac work	Unit of cardiac function	((PSP-CP)*CO_{max})/dry wt)	mL/min •mmHg •g dry wt^{-1}
Cardiac power	Unit of cardiac function	((PSP-max preload)*CO *0,13)/dry wt	Joules•min^{-1} •g dry wt^{-1}

mmHg can be transformed to cmH_2O (multiply by 1.38) or kPa (multiply by 0.1333), dry wt: total cardiac dry weight.

2.2.3.2 Isolated working heart left ventricle function evaluation

In a separate set of experiments, animals were anesthetized using sodium pentobarbital (60 mg/Kg IP) and hearts were rapidly excised and set in a working heart apparatus as described in Section 2.2.3. While in anterograde working mode, LV pressure was continuously recorded using a 1.4 Fr micro manometer (Millar instruments. Houston, TX U.S.A.) inserted through the left atrium cannula. Heart rate, LV pressure, first derivates of the maximal (dP/dt_{max}) and minimal (dP/dt_{min}) pressures and acceleration in the

LV were recorded using HSE Isoheart software for windows 2000 (Harvard Apparatus Canada, Saint-Laurent, Quebec, Canada). Other hemodynamic parameters determined using this technique are presented in Table 2-4.

Table 2-4 Parameters determined using the working heart system with a Millar catheter in the left ventricle

Parameter	Description	Description/calculation	Units
LV pressure in systole (LVESP)	Pressure inside the LV at the end of systole	Peak SBP measured	mmHg
LV end diastole pressure (LVEDP)	Pressure inside the LV at the end of diastole	Measurement of the relaxation capacity of the ventricle	mmHg
LV pressure amplitude (LVPA) or LV developed pressure (LVDP)	Ventricular capacity to increase blood pressure	LVESP-LVEDP	mmHg
LV hydraulic power (LVHP)	The product of the CO and the pressure gradient generated by the LV	(CO/dry wt) *LVDP*0.1333	mW /g dry wt
LV dP/dt_{max}	Maximum acceleration in pressure delta inside the LV	First derivate of the acceleration/time curve in the LV, measured by the Millar catheter.	mmHg/s
LV dP/dt_{min}	Maximum deceleration in pressure delta inside the LV	First derivate of the deceleration/time curve in the LV, measured by the Millar catheter.	mmHg/s

mmHg can be transformed to cmH_2O (multiply by 1.38) or kPa (multiply by 0.1333).

2.2.3.3 Ischemia/reperfusion protocol

Measurements of cardiac function were carried out every 10 or 20 minutes during an 80-minute protocol that included a 30- or 40-minute period of stabilization under aerobic conditions (pre-ischemia), 10 minutes of no-flow ischemia and 40 minutes of reperfusion. The duration of the no-flow ischemia insult was based on pilot data from our laboratory indicating that in order to achieve recovery in aging, IUGR offspring, the ischemic insult could not be longer than 10 minutes. Hearts that showed non-reversible cardiac arrhythmia were excluded.

2.2.3.4 Cardiac energy metabolism

Myocardial production of acetyl coenzyme A (acetyl-CoA) and ATP were calculated using a previously described and validated technique.[18, 19] Glycolysis rates were determined by adding 0.1 μCi/ml of D-[5-^{3}H(N)] glucose to the buffer and measuring the change in levels of tritium ($^{3}H_2O$) released into the buffer.[19]

Rates of glucose oxidation were simultaneously determined by adding 0.1 μCi/ml of D-[U^{14}C] glucose to the buffer and measuring the amount of radiolabeled carbon dioxide ($^{14}CO_2$) that was either released as gas in the oxygenation chamber and contained in a hyamine hydroxide trap, or diluted in the buffer as bicarbonate and extracted from the samples as previously described.[13]

If one mol of glucose passes through glycolysis to lactate without subsequent oxidation by the TCA a net production of two mols of H^+ occurs.[20] Therefore, rates of H^+ production derived from incomplete metabolism of glucose were determined by calculating the difference in the rates of glycolysis and glucose oxidation at any given point using the equation:

H^+ production = 2 x (glycolysis rate – glucose oxidation rate)

and reported as μmol•gram^{-1}•min^{-1}.[21] In a different set of experiments, fatty acid and lactate oxidation rates were measured by adding both 0.1 μCi/ml of [9,10-^{3}H(N)] palmitic acid and 0.1 μCi/ml [^{14}C-U] lactic acid to the buffer and measuring the production of both $^{3}H_2O$ and $^{14}CO_2$ as described above.

To calculate the amount of energy produced by the myocardium, it was assumed that each mol of glucose undergoing glycolysis produces two mols of ATP, each mol of glucose undergoing glucose oxidation produces two mols of acetyl-CoA or 30.5 mols of ATP, each mol of palmitate undergoing oxidation produces eight mols of acetyl-CoA or 105 mols of ATP and each mol of lactate produces 1 acetyl-CoA or 15 mols of ATP.[21] Cardiac efficiency was determined by dividing the amount of developed cardiac work by the estimated amount of acetyl-CoA produced at any given period of time and reported as Joules/μmol of TCA derived acetyl-CoA•10^2.[21]

Data from cardiac perfusions and metabolism studies were recorded in a form designed for such purposes (Appendix 10.2).

2.2.3.5 Cardiac dry weight

Following perfusion, hearts were placed in dry gauze to remove excess water, rapidly weighed and frozen by immersion in liquid nitrogen and stored at -80 °C. Before immersion in liquid nitrogen a small sample of LV was collected, weighed and dehydrated by placement in a conventional oven at 50 °C for four days. Dehydrated samples were reweighed and the total dry weight (dry wt) of the heart was calculated.

Myocardial histology

Perfused hearts were preserved in 10% formalin for 48 hours and then transferred to 10% methanol for dehydration.

2.2.3.6 Hematoxylin-eosin and Masson's trichrome

Following dehydration on methanol, axial myocardial samples, including left and right ventricle, were sent to the Alberta Diabetes Institute

Histology Core (Edmonton, Canada) for histopathological preparation (by paraffin embedding) followed by slicing and staining (hematoxylin/eosin and Masson's trichrome). All procedures were performed following standardized protocols.

2.2.3.7 Prussian blue

Prussian blue staining is one of the most sensitive histochemical tests to detect iron deposits. Following this procedure, any Fe^{3+} present in the tissue combines with ferrocyanide and results in the formation of a bright blue pigment called Prussian blue, or ferric ferrocyanide.

Prussian blue staining for the detection of iron deposits in the myocardium was performed in slices of myocardial tissue previously fixed in 10% formalin and embedded in paraffin according to the following protocol:

1. Deparaffinize and hydrate sections with distilled water.

2. Mix equal parts of hydrochloric acid (HCl 20% in distilled water) and potassium ferrocyanide ($K_4Fe(Cn)6.3H_2O$, FW 422.4 [Sigma Cat# P3289] 10g in 100 ml of distilled water) immediately before use. Immerse slides in this solution for 20 minutes.

3. Wash in distilled water three times.

4. Counterstain with nuclear fast red for five minutes.

5. Rinse twice in distilled water.

6. Dehydrate by rinsing once with 95% alcohol and twice with 100% alcohol.

7. Clear using xylene, two rinses - three minutes each.

8. Coverslip with resinous mounting medium.

2.2.4 Myocardial iron content

Myocardial samples (100-200 mg) were obtained from the free wall of the LV of frozen myocardial specimens from both control and IUGR, 12 month old male offspring previously used for cardiac I/R protocols (Section 2.2.3.3.). Cardiac tissue samples were dried at 50 °C for four days, stored in eppendorf vials and sent to the Trace Elements Laboratory (London, ON Canada). Samples underwent nitric acid digestion followed by dilution and injection into a High Resolution Sector Field Inductively Coupled Plasma Mass Spectrometer (Element 2®, Thermo Scientific). The reported coefficient of variation of this technique is 3-14% and the minimal detectable concentration of iron is 0.5 μg/g.

2.2.5 Blood withdrawal and processing

Blood samples were obtained from anesthetized animals by either LV puncture or aortic transection. Blood samples were collected in vacuum tubes containing Ethylenediaminetetraacetic acid (EDTA), heparin or no anticoagulant and placed on ice for 10 minutes and then centrifuged (10 minutes at 4000 rpm, 4°C). Serum and plasma were then separated into aliquots and stored at -80°C. Lipemic or severely hemolytic samples were discarded.

2.2.6 Blood markers of systemic iron homeostasis

Blood samples were collected from anesthetized animals (sodium pentobarbital 60 mg/Kg IP) and preserved in vacutainer tubes with EDTA, placed on ice and immediately sent to the Clinical Laboratory of the University of Alberta Hospital for the determination of plasma free iron, total iron binding capacity and iron saturation index. Ferritin plasma levels were determined using a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring ferritin in biological samples (Immunology Consultants Laboratory Inc. Newberg, OR U.S.A.; Catalog number E-25F). The

reported detection range for this test is 12.5 to 400 ng/mL.

2.2.7 Myocardial levels of total, oxidized and reduced glutathione

Myocardial samples (80-100 mg) were obtained from the free wall of the LV of frozen myocardial specimens previously used for cardiac I/R protocols (Section 2.2.3.3.) and a 10% w/v homogenate was created by adding ice-cold 5% metaphosphoric acid (0.9 mL/100 mg tissue) and homogenizing. The homogenates were then centrifuge at 12,000 rpm for 15 minutes at 4°C and supernatants were collected. Myocardial levels of glutathione in its reduced (GSH) and oxidized (GSSG) states were determined using a commercially available kit (OxiSelect™ Total Glutathione [GSSG/GSH] Assay Kit Cat# STA-312, Cell Biolabs Inc. San Diego, CA U.S.A.) and following the manufacturer specifications.

2.2.8 Blood pressure measurements

Prior to blood pressure determinations, rats were trained by placing them in a plexiglass restrainer for five minutes per day on three non-consecutive days. Non-invasive blood pressure determinations were made on awake, pre-trained animals using a tail cuff Multi Channel Blood Pressure System (IITC Life Sciences, Woodland Hills, CA U.S.A.). Measurements of heart rate, systolic and diastolic blood pressure were performed five consecutive times under basal conditions and following an air puff stress test.

2.3 Experimental methods for the evaluation of glucose metabolism and other components of the metabolic syndrome

2.3.1 Body weight and energy intake

After weaning, body weight and energy intake were measured weekly. To maintain a consistent follow-up of each of the animals, one of the rats from each cage was marked on the tail using a permanent marker. Food intake was measure by determining changes in the amount of food in both

the cage top and food pellets pulled inside the cage and adjusted by total body weight of the rats in the same cage. Energy intake data was reported as calculated caloric intake per day per kilo of rat ($Kcal \bullet day^{-1} \bullet Kg^{-1}$).

Additional measurements of energy and water intake, and body weight were performed in individual animals every day at the same time in the morning (8:00 a.m.) during three consecutive days while performing metabolic cage studies (refer to Section 2.3.2 for details).

2.3.2 Indirect calorimetry

One day before metabolic cage studies, rats were separated and singly housed in standard rat cages in the animal facilities of the University of Alberta with 60% humidity, a 12h light: 12h darkness light cycle and *ad libitum* access to food and water. On the experimental day, rats were weighed and placed in metabolic cages (Oxymax/CLAMS; Columbus Instruments. Columbus, OH U.S.A.). After 24 hours of acclimatization in the metabolic cages, oxygen consumption (VO_2), and carbon dioxide production (VCO_2) were measured using the Comprehensive Lab Animal Monitoring System (Columbus Instruments. Columbus, OH U.S.A.). Measurements of VO_2 and VCO_2 were recorded during one-minute periods every 11 minutes in an observation window of 24 hours. For analyses purposes, averages of each parameter during light and dark cycles were calculated separately for each animal.

The overall oxygen consumption was calculated by measuring the difference between the input oxygen flow (V_iO_{2i}) and the output oxygen flow (V_oO_{2o}) and reported as ml of oxygen per Kilo of weight per hour ($ml \bullet Kg^{-1} \bullet h^{-1}$).

$$VO_2 = V_i\, O_{2i} - V_o\, O_{2o}$$

Similarly, overall carbon dioxide (CO_2) Production was calculated by measuring the difference between the CO_2 output (V_oCO_{2o}) and CO_2 input

(V_iCO_{2i}) carbon dioxide flows and reported as ml of carbon dioxide produced per Kilo of weight per hour ($ml \bullet Kg^{-1} \bullet h^{-1}$).

$$VCO_2 = V_oCO_{2o} - V_iCO_{2i}$$

The respiratory exchange ratio (RER) is the ratio between CO_2 production and oxygen consumption that is calculated before any unit conversion or weight correction. This value is a ratio and thus does not have a unit.

$$RER = VCO_2/VO_2$$

Metabolic cages provide valuable information regarding the amount of substrate used for energy production as well as the amount of physical activity performed by the experimental subjects during a determined period of time. Following stoichiometric rules, the complete oxidation of one mol of glucose requires 6 mols of oxygen and produces 6 mols of CO_2, 6 mols of water and 38 mols of ATP.

$$6\ O_2 + C_6H_{12}O_6 \rightarrow 6\ CO_2 + 6\ H_2O + 38\ ATP$$

Therefore, in the hypothetical condition of using only glucose as a substrate for oxidative phosphorylation, the RER would be = 1.

$$RER\ glucose\ oxidation = VCO_2/VO_2 = 6CO_2/6O_2 = 1$$

Alternatively, oxidation of one molecule of palmitate (a common fatty acid) has an RER of 0.7.

$$23\ O_2 + C_{16}H_{32}O_2 \rightarrow 16\ CO_2 + 16\ H_2O + 129\ ATP$$

$$RER\ palmitate\ oxidation = VCO_2/VO_2 = 16\ CO_2/23\ 0_2 = 0.7$$

Based on this concept, measuring oxygen consumption and carbon dioxide production during a defined period of time provides information not only on the amount of energy that is produced by oxidative mechanisms but also on the energetic substrates that are being used to produce this energy (Table 2-5).

Table 2-5 Changes in the respiratory exchange ratio and heat production per unit of oxygen according to metabolic substrate selection

	% of total O_2 consumed by the metabolism of		% of heat produced by the metabolism of		Heat produced per liter of O_2	
RER	Carbs	Fat	Carbs	Fat	KJouls	Kcal
0.707	0	100	0	100	19.62	4.68
0.75	14.7	85.3	15.6	84.4	19.84	4.73
0.8	31.7	68.3	33.4	66.6	20.1	4.8
0.85	48.8	51.2	50.7	49.3	20.35	4.86
0.9	65.9	34.1	67.5	32.5	20.61	4.92
0.95	82.9	17.1	84	16	20.87	4.98
1	100	0	100	0	21.13	5.04

Extracted from [22] , RER: respiratory exchange ratio, Carbs: Carbohydrates

This table demonstrates how the amount of energy (Kcal) that is produced using a determined amount of oxygen depends on the substrate used for oxidation (as also illustrated in Figure 2-11).

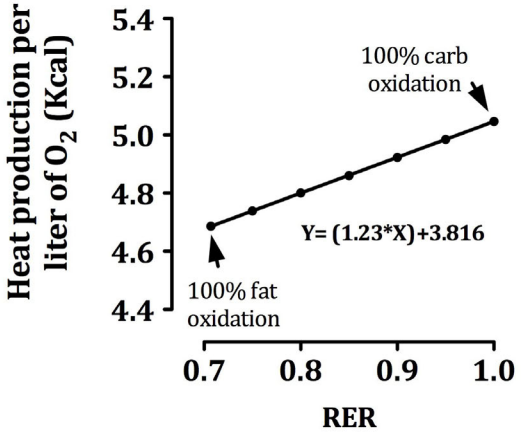


Figure 2-11 Heat production per liter of oxigen consumed according to the respiratory exchange ratio

Therefore, the amount of energy produced by an individual (heat) can be determined based on the caloric value (CV) of the substrate used to produce energy and the amount of oxygen consumed (VO_2).

$$Heat = CV \times VO_2$$

$$CV = 3.815 + 1.232 \times RER$$

$$Heat = (3.815 + 1.232 \times RER) \times VO_2$$

2.3.3 Physical activity assessment

Simultaneous to indirect calorimetry determinations (described in Section 2.3.2), physical activity was monitored by dual axis detection (X, Z) using infra-red photocell technology and reported as counts per minute. Total physical activity was calculated by adding Z-counts (rearing or jumping) to total counts associated with ambulatory movement and typical behavior (grooming, scratching etc.). Measurements of physical activity were recorded during one-minute periods every 11 minutes during an observation window of 24 hours. For analysis purposes, averages of each parameter during light and dark cycles were analyzed separately.

2.3.4 In vivo *body composition analyses*

A sub-group of non-fasted, conscious animals were carefully weighed and placed in a plexiglass restrainer. Body composition was determined using either a Bruker's minispec (Bruker LF 90II® Hamilton, ON Canada) or an EchoMRI 4-in-1/1000 (Echo Medical Systems. Houston, TX U.S.A.); a whole body composition analyzer based on time-domain nuclear magnetic resonance (TD-NMR) technology. This equipment acquires and analyzes TD-NMR signals from all protons in the entire sample volume and can provide three parameters of interest: body fat, body fluid (water) and body lean

tissue. The difference between the summary of these three components and the total body weight constitutes the weight of elements of the body with a very low water content such as bones and skin (referred to as 'other'). This technique provides a precise method for the *in vivo* measurement of body composition in rodents that does not require anesthesia. The precision of this equipment was tested using a repeated measures approach. In all experimental domains the test-retest correlation of this technique was >0.995 and the bias variability was <0.1% (Figure 2-12).

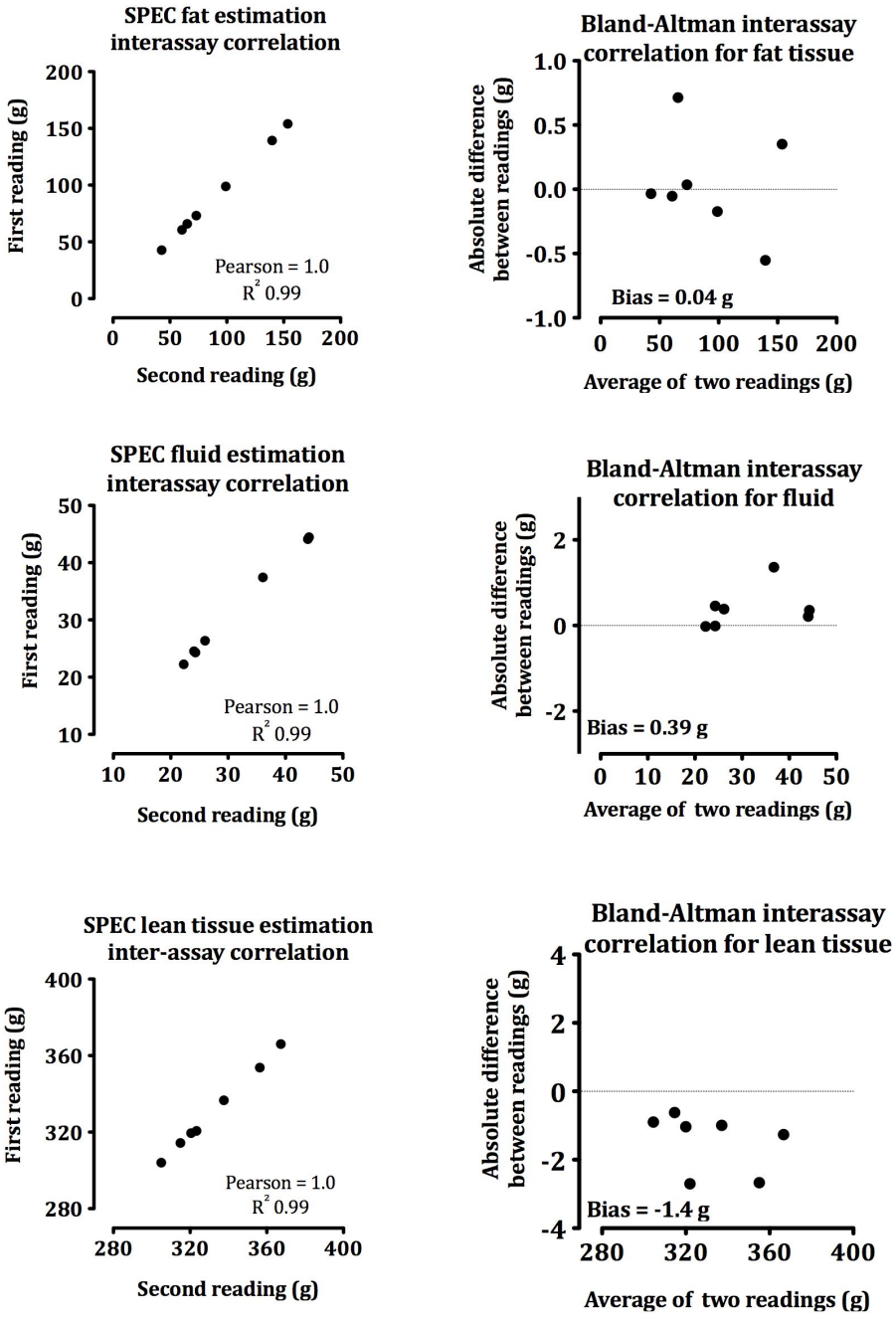


Figure 2-12 Test-retest inter-assay correlation and Bland-Altman analysis of body composition studies

Measurements performed on the Bruker's minispec (Bruker LF 90II® Hamilton. ON Canada) Whole Body Composition Analyzer.

2.3.5 Intra-abdominal high-resolution computer tomography scan

A micro-computed axial tomography (CT) scanner is a piece of equipment based on the same underlying physical principle as the CT scanners used in clinical practice but designed for higher resolution imaging. The micro-CT scanner produces tri-dimensional (3D) tomographic data at a microscopic resolution (discrimination limit; 100 μm^3) by recording 512 consecutive 2D projections, at four frames/projection, over a complete 360^o around the animal. The x-ray source produces a cone-shaped beam, which is projected through the specimen with the resultant radiographic density of the tissues projected onto a 2D detector. The multiple projections are combined using a reconstruction method that is generally based on a filtered back projection algorithm. The resultant micro-CT scan is a 3D matrix of points with values proportional to the X-ray penetration coefficient of the material within each point. Since the density of different tissues (bone, water, fat and lean tissue) and, therefore, X-ray penetrations are different, this technique can be used to discriminate and quantify intra-abdominal fat content with a very standardized technique that is not operator dependent.

To perform these measurements, non-fasted rats were anesthetized using inhaled isoflourane (~2% in compresses air) and placed into a micro CT scanner FLEX Pre-Clinical Platform X_O-X_{PET}-X_{SPET} instrument (Gamma Medica Ideas. Northridge, CA U.S.A.). The instrument gamma camera was programmed to scan 512 projections (sum of frames = 4, voltage 60 kDP and 390 uA). The observation window was set between the diaphragmatic membrane and the acetabulum for each animal (~91.78 mm) and the magnification factor on the gamma camera was set to 1.29.

To calculate the intra-abdominal fat content, 3D reconstructions of the intra-abdominal cavity were made based on axial, coronal and sagittal projections. The intra-abdominal fat volume was calculated using the software GMI - Amira 3.1.1. Threshold density was adjusted by internal

volume so that internal organs and large vessels were not counted as intra-abdominal fat tissue and were excluded from calculations.

2.3.6 Intra-abdominal organ weight and fat content evaluation by mechanical extraction

Animals were anesthetized using inhaled isoflourane and euthanized by exsanguination. Different intra-abdominal fat pads (mesenteric-epiploic, epididymal (male rats), peri-uterine (female rats), sub-diaphragmatic and retroperitoneal) were surgically extracted and weighed. Intra-abdominal organs including, liver, spleen, pancreas and kidneys were dissected and weighed. Finally, the right tibia bone was dissected and measured.

Intra-abdominal fat data are presented as either total fat weight or fat weight adjusted by body weight, lean weight or total body fat weight as determined by TD-NMR (Section 2.3.4). The Pearson correlation between the two methods used to determine the intra-abdominal fat content (mechanical extraction and micro CT scan) was =0.65, p=0.002. The Bland and Altman analysis demonstrated a bias between the techniques of -0.14 (Figure 2-13).

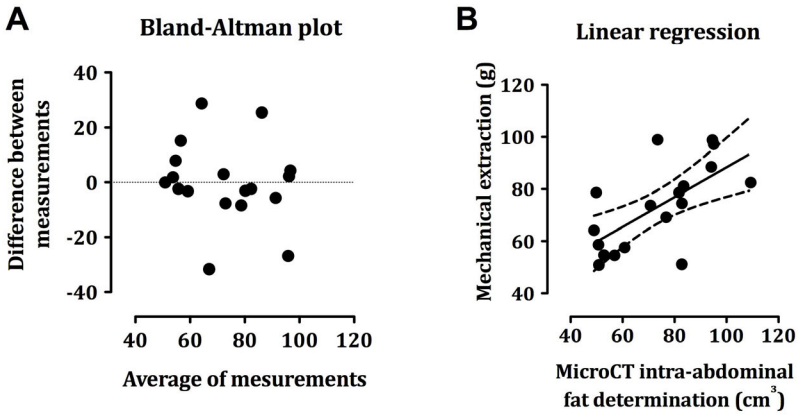


Figure 2-13 Correlation of two techniques (microCT scan vs. mechanical extraction) to determine intra-abdominal fat content

Correlation analyses performed using data from 24 rats after a nutritional intervention of nine weeks receiving HF diet. (**A**) The Bland-Altman analysis estimated bias between the techniques of -0.14. (**B**) Linear regression (solid line) with 95% CI (dash line) and scatter plot for both techniques. The Pearson correlation between the two methods used to determine the intra-abdominal fat content was =0.65, p=0.002.

2.3.7 Glucose and insulin tolerance tests

Glucose plasma concentrations were determined using an ACCU-CHEK Advantage® glucometer (Roche Diagnostics. NY U.S.A.) using blood collected from the tail. The reported mean±SD of the bias of this glucometer, relative to a reference laboratory, ranged from 5.3±3.7% to 31.5±7.5%. The reported intra-assay variability for this equipment is <8%, making it one of the most accurate pieces of equipment on the market.[23] To determine tolerance to a glycemic challenge (glucose tolerance test; GTT), a set of animals in which no other determinations or experiments were performed was used. Glucose plasma concentrations were determined after an initial five hour fasting period and at 15, 30, 60, 90 and 120 minutes following a glucose injection. Rats were injected intraperitoneally with a 50% glucose solution (2 g/Kg body weight).

Insulin tolerance tests (ITT) were performed one week later in the same set of animals in which GTT were performed. To do these experiments, human recombinant insulin (Novolin-GE. Toronto, ON Canada) was used to prepare an insulin-saline solution injected intraperitoneally (1 mU/Kg body weight) after a two hour fasting period. As described above, blood glucose from the tail was measured at baseline and following insulin injection (at 15, 30, 60, 90 and 120 minutes).

2.3.8 Insulin concentrations and homeostatic model assessment index

Following a fasting period of greater than three hours, rats were anesthetized using inhaled isoflourane and euthanized by exsanguination. Circulating concentrations of insulin were determined in EDTA-preserved plasma using either a radioimmunoassay kit for rodent insulin (Cat.# EZRMI-13K Linco St. Charles, MO U.S.A.) or an ELISA kit for rodent insulin determination (Cat. 80-INSRT-E01, ALPCO diagnostics, Salem, NH, U.S.A.). The lowest level of insulin that can be detected using any of these assays is 0.2 ng/ml (35 pM) when using a 10 µl sample. The specificity of these

analytical tests for rat insulin is 100%. The inter- and intra-assay variability reported for the test were <3.6 and <9.1% respectively. The insulin resistance was determined using the homeostatic model assessment (HOMA) index[24] was determined using the following formula:

HOMA = insulin (μU/mL) × [glucose (mmol/L)/22.5]

2.3.9 Pancreas insulin content

After euthanasia by exsanguination, the pancreas was surgically extracted, weighed, minced using fine scissors, placed in 1.0 ml of acidified ethanol (75% ethanol, 1.5% 12 mmol/L HCl) and incubated for 24 hours (4°C) to extract insulin from the tissue.[25] Samples were then centrifuged (8000 rpm/15 minutes) and supernatants were separated. Insulin levels were determined in using a radioimmunoassay kit for rodent insulin (Cat.# EZRMI-13K, Linco. St. Charles, MO U.S.A.). Refer to Section 2.3.8 for accuracy and precision information of this technique.

2.3.10 Tissue homogenization and immunoblotting for the study of insulin signaling

Rats were fasted overnight, injected with 1 mU/g body weight insulin and 15 minutes later euthanized by injecting a lethal dose of pentobarbital (100 mg/Kg). Samples of gastrocnemius muscle and liver were collected and frozen in liquid nitrogen. Subsequently, frozen tissue homogenates were prepared in ice-cold sucrose homogenation buffer (20 mmol/L Tris-HCl, pH 7.4 (4 °C), 50 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L Na pyrophosphate, 0.25 mmol/L sucrose).[26] Fresh protease and phosphatase inhibitors were added to the buffer mixture. Protein content on each homogenate was determined using a Bradford protein assay. Equal amounts of protein were loaded into a 10% sodium dodecyl sulfate polyacrylamide gel for electrophoresis (SDS-PAGE) and run at 100-120 V. for one hour. Protein was then transferred to a nitrocellulose membrane and probed with affinity-

purified primary antibodies for Akt (1:2000), activated Akt (p-AKT S473; 1:1000), Insulin Receptor Substrate-1 (IRS-1; 1:1000), inactive IRS-1 (p-IRS S1101; 1:500), Protein Kinase C theta (PKCθ; 1:500), 5' AMP-activated protein kinase (AMPK; 1:2000), phosphorylated AMPK (p-AMPK; 1:1000), Peroxisome Proliferator-Activated Receptor-Coactivator 1 alpha (PGC-1α; 1:1000) and tubulin (1:5000). Primary antibodies were then detected using goat anti-rabbit secondary antibody (1:1000-5000). Membranes were then visualized using the Amersham Pharmacia Enhanced Chemiluminescence Western Blotting Detection System (Little Chalfont, Buckinghamshire England). All the secondary antibodies and primary antibodies for PGC-1α were from Santa Cruz Biotechnology (Santa Cruz, CA U.S.A). All other primary antibodies utilized in this study were purchased from Cell Signaling Technology (Danvers, MA U.S.A.).

2.3.11 Adipokines plasma concentration

Plasma was collected and preserved with heparin as previously described in Section 2.2.5 and shipped to Millipore Biomarker Services to perform a multiple adipokine determination using the Rat Serum Adipokine Milliplex system / Panel 5-plex (Millipore. Billerica, MA U.S.A.) on a Luminex 100 machine (Millipore. Billerica, MA U.S.A.) The reported minimal detectable concentrations for this technique are leptin 9.7 pg/ml, adiponectin 6.1 pg/ml, Interleukin 1 beta (IL-1ß) 1.2 pg/mL, Interleukin-6 (IL-6) 8.8 pg/mL and Tumor Necrosis Factor alpha (TNFα) 3.2 pg/mL. The reported intra-assay variability for these determinations was <4%. All measurements were performed in duplicate in one single assay.

2.3.12 Lipid profile

To determine the lipid profile of experimental animals, blood samples were collected as described in section 2.2.5 in vacutainer tubes containing EDTA and immediately stored on ice to inhibit lipase activity without the use of chemical inhibition. Lipids were extracted from 200 μL of plasma by the

method of Folch.[27] Triacylglycerol (TG), cholesterol, cholesterol ester (CE) and FFA were separated and measured by fast protein liquid chromatography (FPLC) according to the method described by Christie *et al.*[28]

2.3.13 Determination of liver and muscle lipid levels

Following a four hours fasting period, rats were anesthetized with isoflourane and euthanized by exsanguination. Then, samples of liver and skeletal muscle (gastrocnemius) were extracted and homogenized. The phospholipids in the samples were digested with phospholipase C (2h, 30°C) and the amount of FFA, TG, cholesterol, CE were determined FPLC.[26]

2.3.14 Adipocyte histology and morphometry

After intra-abdominal fat extraction was completed and its weight was determined, samples of abdominal adipose tissue from the major omentum were collected and fixed in 10% formalin for 48 hours and then in 10% methanol. Histopathological preparations and hematoxylin/eosin staining were performed at the Alberta Diabetes Institute Histology Core (Edmonton, AB. Canada) following standardized protocols. Digital images of three representative fields were taken using a digital camera mounted on a light microscope at 40X magnification. All images were analyzed with the ImageJ software (Ver 1.43u, National Institute of Health, U.S.A.).

2.4 Statistical analyses

Data analyses were performed using the statistical software Prism 5.0c for Mac (GraphPad Software. La Jolla, CA USA) and Stata Statistical Software Release 10SE (StataCorp. College Station, TX U.S.A.). Data are reported as mean±standard error of the mean (SE), except where stated otherwise. Before analyses, data distribution was tested using a D'Agostino and Pearson omnibus normality test. When n values were <10, normal distribution was assumed. In all cases, the animals included in each experimental group

belong to different litters.

Comparisons of continuous parameters performed between two groups were tested using a t-test or Willcoxon rank-sum test according to the data distribution. Analyses of continuous variables measurements over time (such as body weight, food consumption, metabolic parameters, cardiac power, GTT, ITT, etc.) were analyzed using a two-way analysis of variance (ANOVA) with both time and group as sources of variation. Comparisons of single measurements performed among four groups (in a two by two factorial design) were analyzed using a non-matched two-way ANOVA with IUGR and the other factor of interest (either sex, age, diet or Resveratrol supplementation) as sources of variation. A Bonferroni post-hoc analysis was then used to compare means by group. For these analyses, overall effect of each one of the factors included in the ANOVA, their interaction, and Bonferroni post-hoc significance were reported only when statistical significance was reached (Figure 2-14).

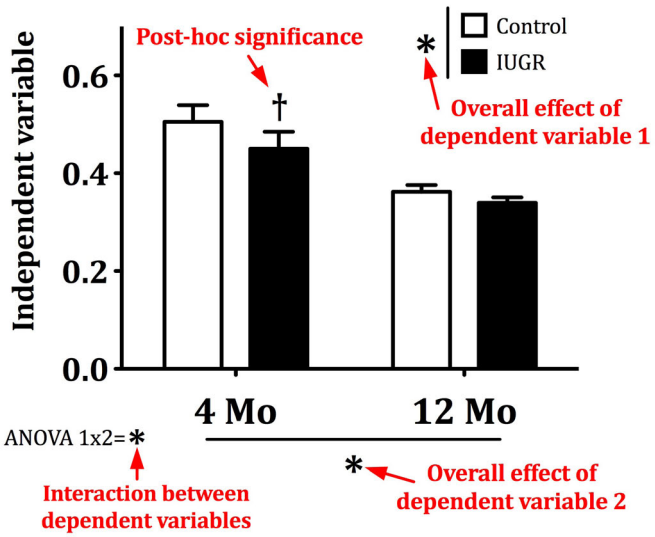


Figure 2-14 Representation of statistical analyses in figures presenting two-factorial analyses

Due to the marked phenotypical differences between sexes, data obtained from male and female adult offspring were analyzed separately. For the analyses of categorical variables, differences in proportions among groups were compared using a χ^2 test. Evaluations of correlation within and between different techniques were analyzed and reported using both Pearson correlation and linear regression as well as Bland-Altman plots.

For most statistical analyses a p-value <0.05 was considered statistically significant. In order to decrease the multiple comparisons effect in the data obtained by echocardiographic studies, a p value <0.01 was considered statistically significant when analyzing this kind of data.

2.5 References

1. Lopaschuk GD. Alterations in myocardial fatty acid metabolism contribute to ischemic injury in the diabetic. *Canadian Journal of Cardiology*. 1989;5:315-320.

2. Antonova OA, Loktionova SA, Romanov YA, Shustova ON, Khachikian MV, Mazurov AV. Activation and damage of endothelial cells upon hypoxia/reoxygenation. Effect of extracellular pH. *Biochemistry (Mosc)*. 2009;74:605-612.

3. Hung TH, Chen SF, Li MJ, Yeh YL, Hsieh TT. Differential effects of concomitant use of vitamins C and E on trophoblast apoptosis and autophagy between normoxia and hypoxia-reoxygenation. *PLoS One*. 2010;5:e12202.

4. Raddatz E, Thomas AC, Sarre A, Benathan M. Differential contribution of mitochondria, NADPH-oxidases and glycolysis to region-specific oxidant stress in the anoxic-reoxygenated embryonic heart. *Am J Physiol Heart Circ Physiol*. 2010.

5. Zampino M, Yuzhakova M, Hansen J, McKinney RD, Goldspink PH, Geenen DL*, et al.* Sex-related dimorphic response of HIF-1 alpha expression in myocardial ischemia. *Am J Physiol Heart Circ Physiol*. 2006;291:H957-964.

6. Dubois L, Landuyt W, Haustermans K, Dupont P, Bormans G, Vermaelen P*, et al.* Evaluation of hypoxia in an experimental rat tumour model by [(18)F]fluoromisonidazole PET and immunohistochemistry. *British Journal of Cancer*. 2004;91:1947-1954.

7. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F*, et al.* Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell*. 2006;127:1109-1122.

8. Dolinsky VW, Chan AY, Robillard Frayne I, Light PE, Des Rosiers C, Dyck JR. Resveratrol prevents the prohypertrophic effects of oxidative stress on LKB1. *Circulation*. 2009;119:1643-1652.

9. Dittoe N, Stultz D, Schwartz BP, Hahn HS. Quantitative left ventricular systolic function: from chamber to myocardium. *Crit Care Med*. 2007;35:S330-339.

10. Pellett AA, Tolar WG, Merwin DG, Kerut EK. The Tei index: methodology and disease state values. *Echocardiography*. 2004;21:669-672.

11. Rey-Parra GJ, Archer SL, Bland RD, Albertine KH, Carlton DP, Cho SC*, et al.* Blunted hypoxic pulmonary vasoconstriction in experimental neonatal chronic lung disease. *Am J Respir Crit Care Med*. 2008;178:399-406.

12. van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M*, et al.* Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. *Am J Respir Crit Care Med*. 2009;180:1131-1142.

13. Lopaschuk GD, Spafford MA, Davies NJ, Wall SR. Glucose and palmitate oxidation in isolated working rat hearts reperfused after a period of transient global ischemia. *Circ Res*. 1990;66:546-553.

14. Barr RL, Lopaschuk GD. Direct measurement of energy metabolism in the isolated working rat heart. *Journal of Pharmacological and Toxicological Methods*. 1997;38:11-17.

15. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

16. Xu Y, Clanachan AS, BI. J. Enhanced expression of angiotensin II type 2 receptor, inositol 1,4, 5-trisphosphate receptor, and protein kinase cepsilon during cardioprotection induced by angiotensin II type 2 receptor blockade. *Hypertension*. 2000;36:506-510.

17. Xu Y, Kumar D, Dyck JR, Ford WR, Clanachan AS, Lopaschuk GD*, et al.* AT(1) and AT(2) receptor expression and blockade after acute ischemia-reperfusion in isolated working rat hearts. *Am J Physiol Heart Circ Physiol*. 2002;282:H1206-1215.

18. Belke DD, Larsen TS, Lopaschuk GD, Severson DL. Glucose and fatty acid metabolism in the isolated working mouse heart. *Am J Physiol*. 1999;277:R1210-1217.

19. Barr RL, Lopaschuk GD. Methodology for measuring in vitro/ex vivo cardiac energy metabolism. *Journal of Pharmacological and Toxicological Methods*. 2000;43:141-152.

20. Dennis SC, Gevers W, Opie LH. Protons in ischemia: where do they come from; where do they go to? *J Mol Cell Cardiol*. 1991;23:1077-1086.

21. Liu B, el Alaoui-Talibi Z, Clanachan AS, Schulz R, Lopaschuk GD. Uncoupling of contractile function from mitochondrial TCA cycle activity and MVO2 during reperfusion of ischemic hearts. *Am J Physiol*. 1996;270:H72-80.

22. McLean, Tobin. "Animal and Human Calorimetry"; 1987.

23. Li HY, Hua CH, Lin YR, Lin MS, Wei JN. Effect of fluoride-containing tubes on accuracy of glucometers. *Diabetes Care*. 2008;31:e33.

24. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412-419.

25. Suarez-Pinzon WL, Power RF, Yan Y, Wasserfall C, Atkinson M, Rabinovitch A. Combination therapy with glucagon-like peptide-1 and gastrin restores normoglycemia in diabetic NOD mice. *Diabetes*. 2008;57:3281-3288.

26. Koonen DP, Jacobs RL, Febbraio M, Young ME, Soltys CL, Ong H*, et al.* Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity. *Diabetes*. 2007;56:2863-2871.

27. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957;226:497-509.

28. Christie WW. Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J Lipid Res*. 1985;26:507-512.

CHAPTER 3 PHENOTYPICAL CHARACTERISTICS OF THE HYPOXIA-INDUCED IUGR MODEL‡

3.1 Introduction

A complete and accurate description of the perinatal characteristics of animals exposed to hypoxia *in utero* is fundamental for a better understanding and comparison of this specific animal model and prenatal insult. The rat model of maternal hypoxia used for the studies included in this thesis (Section 2.1.1), has previously been used by our own group[1-5] as well as others[6, 7] to study the long-term effects of being born IUGR. To ensure consistency in reproducing the model between investigators, there are some phenotypical characteristics that can be readily compared (such as maternal weight gain during hypoxic insults, litter size and birth weight). Moreover, there are some additional parameters, such as measures of fetal hypoxia, sex distribution of the litters, fetal and placental weight that are difficult to measure accurately due to methodology, and remain to be clarified.

One of the limitations of the hypoxia-induced IUGR protocol used in this thesis and described in Section 2.1.1, is that obstetric and perinatal outcomes cannot be measured very accurately for a number of reasons: for instance, early manipulation of the offspring could stress the dam and affect the quality of nursing, therefore, interaction with the newborn litter must be reduced to a minimum in the first weeks of life. In addition, the timing of

---

‡ Most components of this chapter have been published in (or submitted to) one or more peer-review journals including:

- *"**Rueda-Clausen CF**, Morton JS and Davidge ST. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. Cardiovascular Research 2009;81:713-722.*
- *"**Rueda-Clausen CF**, Morton JS, Lopaschuk GD and Davidge ST. Long-term effects of intrauterine growth restriction on cardiac metabolism and susceptibility to ischemia reperfusion. In press, Cardiovascular Research 2010."*

***Contribution:** Rueda-Clausen CF. was the project coordinator, performed all the experiments and data analyses, wrote the first draft of the review article (including all the figures) and coordinated with the other authors in compiling the final version of the manuscript.*

nursing and lactation of the offspring is variable among different litters. In some cases, newborn feeding can start immediately after birth while the parturition process could last as long as 12 hours, therefore differences in the weight of the offspring (between the first and last born) at the end of parturition could be very large due to differences in the length of time of postnatal feeding. Dams also eat fetal membranes and placentas immediately after birth, therefore, the evaluation of these organs without handling the dam during parturition is not possible. Moreover, dams may also eat some offspring after parturition and variability in this behavior could account for differences in litter size and sex distribution.

Previous studies conducted in clinical models of high-altitude pregnancy suggest that a healthy placenta is capable of adapting when exposed to low oxygen concentrations and is able to ameliorate the effects on fetal development by increasing its functional capacity for gaseous exchange.[8] Therefore, another question that has been raised regarding this model of IUGR is whether a maternal hypoxic insult is in fact limiting oxygen availability to the fetuses.

3.2 Objectives

In order to deal with some of the previously described limitations and to reach a better understanding of the animal model of hypoxia-induced IUGR used in this thesis, an additional set of experiments were performed on a separate set of animals to study the effects of this prenatal insult (on factors such as fetal hypoxia, litter size, fetal weight and placenta weight) independent of the already mentioned postnatal confounding factors. The results presented in this chapter constitute a description of the phenotypical characteristics of this model and apply for all consequent experiments in which the hypoxia-induced IUGR model was used.

3.3 Methods

Some components of this chapter refer to "prenatal" characteristics of the hypoxia-induced IUGR model and present data obtained from fetuses studied immediately before parturition, see experimental protocol Section 2.1.1.1. Other components of this chapter refer to "postnatal" characteristics of the offspring and present data obtained from both newborn offspring (6 to 12 hours after birth) exposed to the prenatal hypoxia protocol (Section 2.1.1) and offspring culled immediately after reduction of litters (Section 2.1.1.2).

3.4 Results

3.4.1 Effect of prenatal hypoxia on maternal weight gain

There were no differences in age or body weight of dams randomized to hypoxia or control protocols either before mating or at the beginning of the hypoxic insult ($p>0.4$ for both parameters). Exposure to hypoxia reduced maternal weight gain (Figure 3-1). Post-partum, however, dams rapidly regain weight and after one week of lactation the body weight of dams previously exposed to hypoxia was comparable to controls (Figure 3-1).

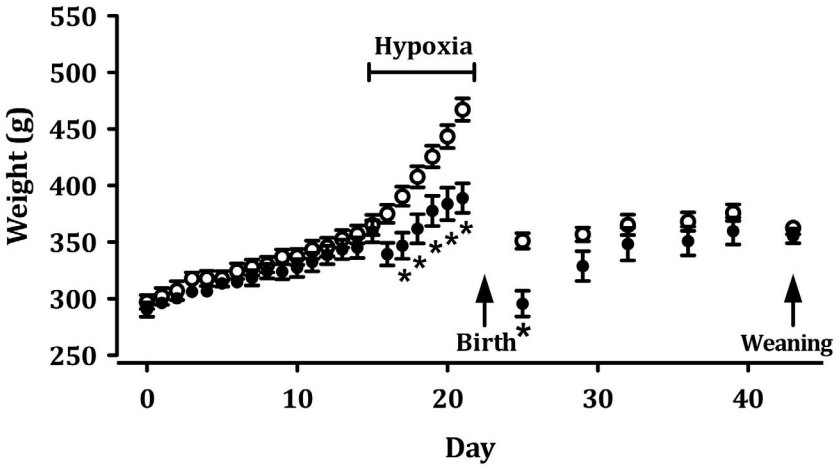


Figure 3-1 Effect of prenatal exposure to hypoxia on body weight change in the dams during pregnancy and lactation periods

Open symbols represent control dams and filled symbols represent dams exposed to hypoxia. * represents $p<0.01$ when compared to age-matched controls (Bonferroni post-hoc). n=16 per group.

3.4.2 Effect of maternal hypoxia on prenatal fetal oxygen availability

Histological sections of placentas and fetal segments of heart and liver prepared with Pimonidazole (Figure 3-2) demonstrated that maternal exposure to an oxygen concentration of 11.5% indeed caused a reduction in fetal oxygen availability as shown by increased levels of Pimonidazole staining.

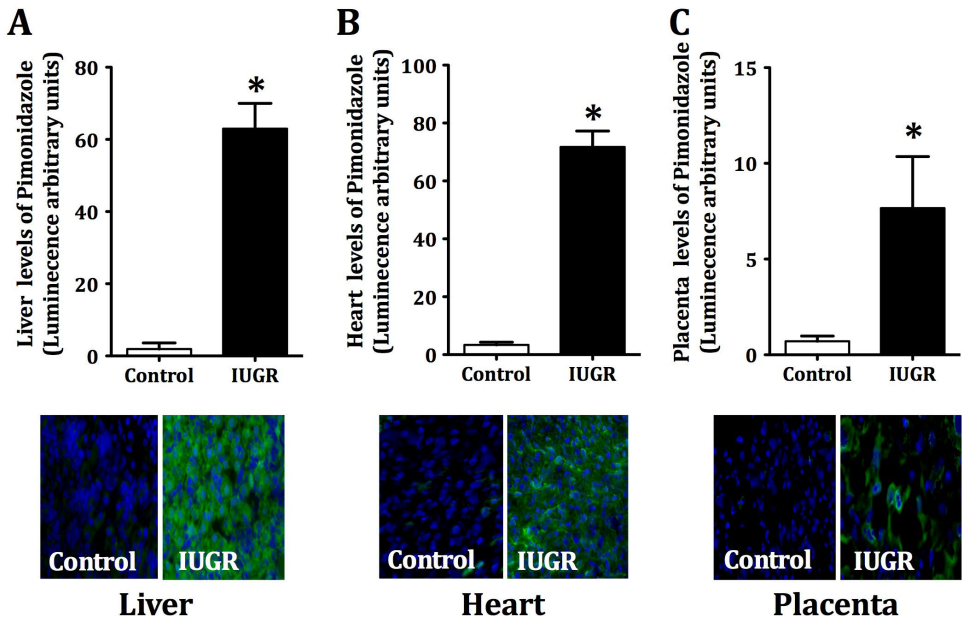


Figure 3-2 Levels of Pimonidazole in fetal tissues

Data obtained from 24 pups from eight litters (four intrauterine growth restricted; [IUGR] and four controls) euthanized at gestational day (G) 20.75. All dams were injected with hypoxia-reactive dye (Pimonidazole) at G20.5. Sections of (**A**) liver, (**B**) heart and (**C**) placenta were obtained from male pups and analyzed by immunohistochemistry. Nuclei are stained with DAPI (blue) and hypoxic tissue is labeled with Pimonidazole (green). Each panel presents summary figures and representative images of Control and IUGR offspring. * represents $p<0.001$ when compared to controls using an unpaired t-test.

Additional confirmation of the hypoxic status of the fetuses was obtained using a different hypoxia marker (Hypoxyprobe F6), which has a longer half-life (Figure 3-3). In addition, concentrations of HIF-1α, an intracellular hypoxia-mediated signaling molecule, were found to be increased in the livers of offspring exposed to hypoxia *in utero* relative to controls (Figure 3-3).

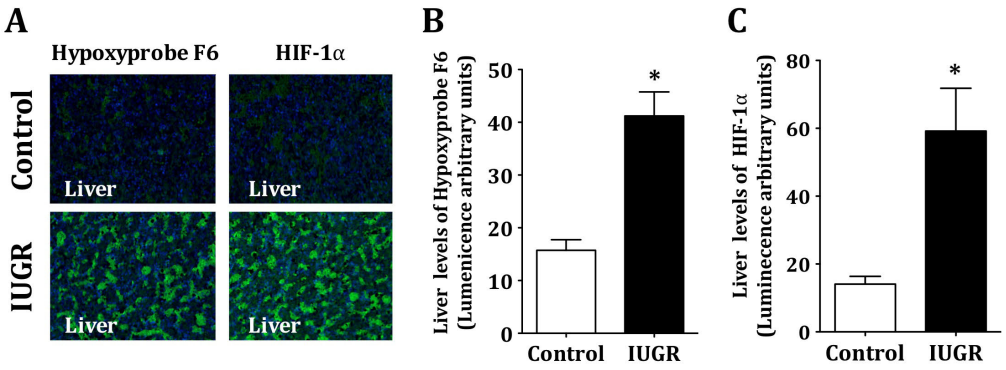


Figure 3-3 Effect of maternal hypoxia on fetal tissue levels of Hypoxyprobe F6 and hypoxia inducible factor-1α

Data obtained from 18 pups from six litters (three intrauterine growth restricted; [IUGR] and three controls) euthanized at G20.75. All dams were injected with a hypoxia-reactive dye (Hypoxyprobe F6) at G19.5. (**A**) Representative images of liver sections. Nuclei are stained with DAPI (blue) and hypoxic tissue is labeled with antibodies against either Hypoxyprobe F6 or HIF1-α (green). Summary figures of liver levels of (**B**) Hypoxyprobe F6 and (**C**) HIF-1α of both control and IUGR offspring are shown. * represents $p<0.001$ when compared to controls using an unpaired t-test.

3.4.3 Prenatal outcomes

Just prior to birth there were no differences in litter size (Controls 17±0.7 vs. IUGR 18±0.7 pups, p=0.7) or sex distribution (proportion of male offspring: Controls 51% vs. IUGR 47%, p=0.9) between experimental groups. Moreover, in this group of experiments, no stillborn fetuses were observed *in utero* in any of the dams used for the prenatal studies. Fetuses from dams exposed to hypoxia exhibited a reduction not only in body weight, but also in absolute placental size (Figure 3-4). Moreover, prenatal hypoxia caused an increase in relative cardiac weight both in male and female fetuses. Interestingly, no differences were observed in tibia length either in male or female IUGR offspring relative to sex-matched controls (Figure 3-4).

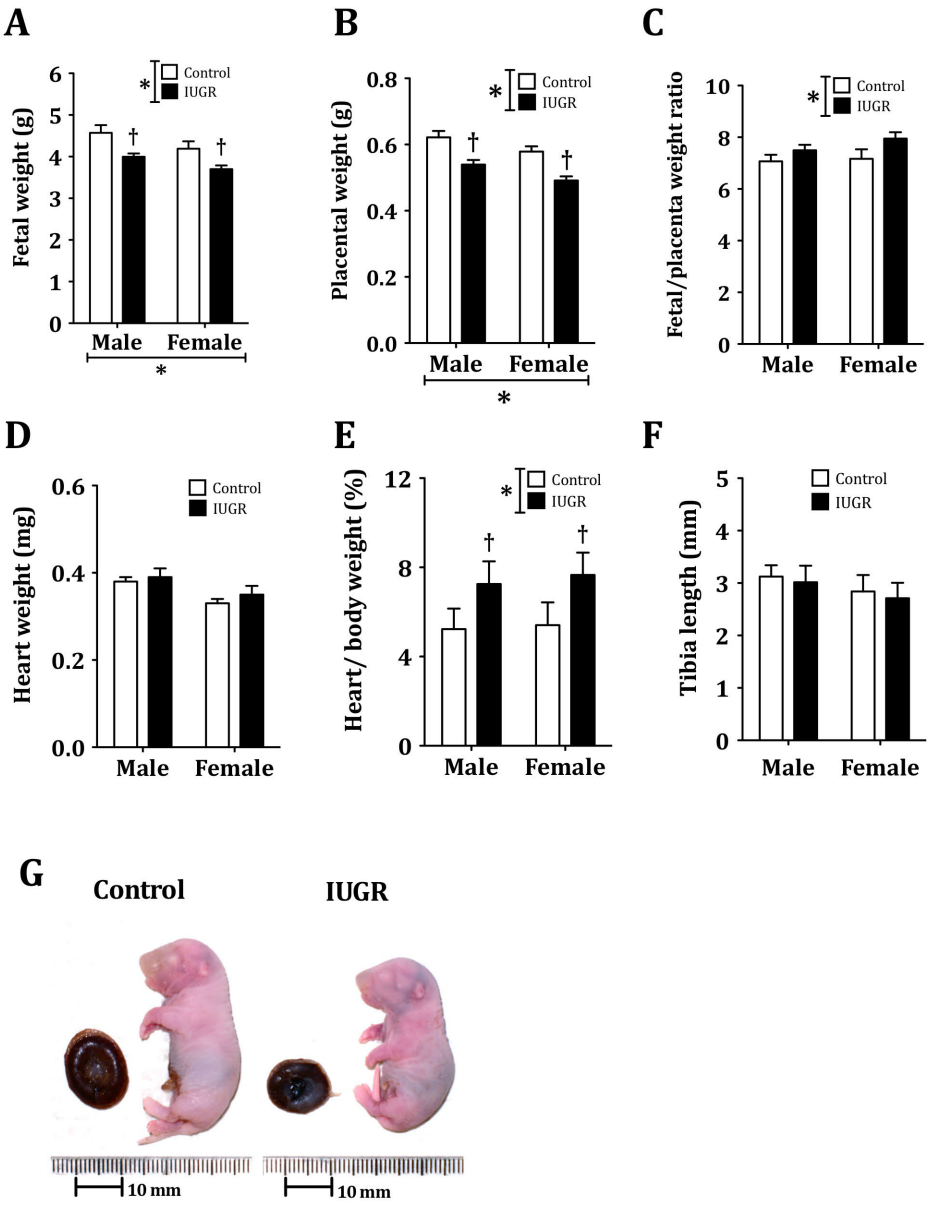


Figure 3-4 Effect of prenatal exposure to hypoxia from G15 to G20.75

Data obtained from 24 pups from eight litters (four intrauterine growth restricted; [IUGR] and four controls) euthanized at G20.75. Summary figures of (**A**) fetal body weight, (**B**) placental weight, (**C**) fetal/placenta weight ratio, (**D**) heart weight, (**E**) heart/body weight ratio and (**F**) tibia length. Panel **G** presents a representative image of both Control and IUGR male fetuses and placentas at G20.75d. * represents values of $p<0.05$ for the respective sources of variation (sex or prenatal intervention) using two-way ANOVA. † represents $p<0.05$ vs. controls of the same sex after a Bonferroni post-hoc test.

3.4.4 Postnatal outcomes

Following parturition, perinatal parameters such as litter size (Controls 16±2 vs. IUGR 15±2 pups, p=0.54), proportion of stillborn offspring (Controls 0.6% vs. IUGR 1.7%, p=0.3) and sex distribution (proportion of male offspring: Controls 53% vs. IUGR 45%, p=0.95) were comparable between groups. Consistent with data obtained from prenatal studies, results from pups culled during litter reductions showed that prenatal IUGR was associated with a reduced body weight and an increase in the relative cardiac weight (Figure 3-5).

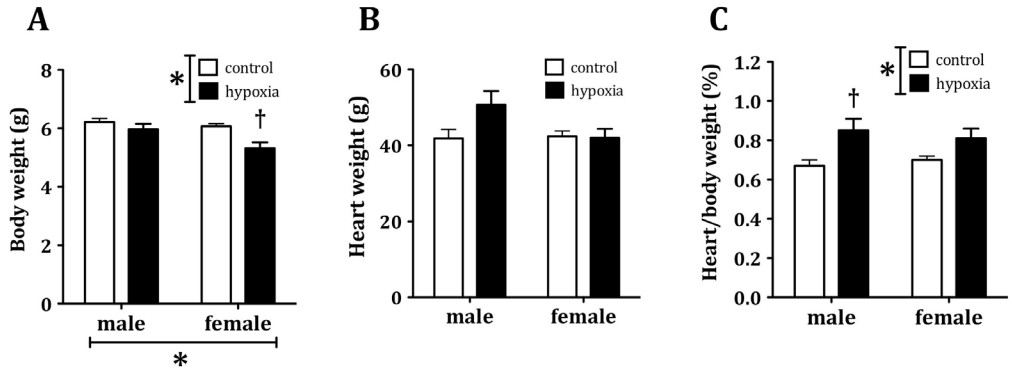


Figure 3-5 Body and heart weight from offspring culled after litter reductions.

Data obtained from 39 pups from at least eight litters from each group euthanized 6 to 12 hours after birth. Summary figures of (**A**) fetal body weight, (**B**) heart weight and (**C**) heart to body weight ratio. * represents values of p<0.05 for the respective sources of variation (sex or prenatal intervention) using two-way ANOVA. † represents p<0.05 vs. controls of the same sex after a Bonferroni post-hoc test.

3.4.5 Postnatal growth trajectory

Both male and female newborns exposed to hypoxia *in utero* rapidly caught up with controls in terms of body weight. Within four days after birth there were no differences in body weight between sex- and age-matched experimental groups (Figure 3-6).

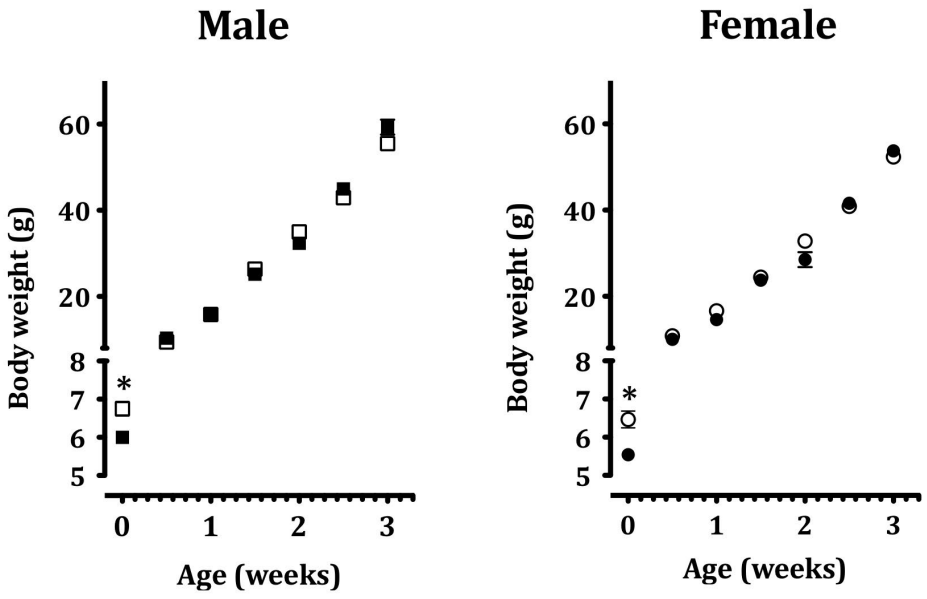


Figure 3-6 Effect of prenatal exposure to hypoxia on offspring body weight from birth to weaning

Open symbols represent controls and filled symbols represent animals exposed to hypoxia. * represents $p<0.01$ when compared to age-matched controls. Data obtained from at least nine different litters in each group.

3.5 Discussion

Relative to other common prenatal insults used to induce IUGR in animal models, maternal hypoxia constitutes the only non-invasive method to reduce fetal oxygen availability. Hypoxia constitutes an interesting tool that simulates many common clinical conditions that cause IUGR in developed countries like Canada (such as preeclampsia, pulmonary diseases and placental insufficiency).[9]

3.5.1 Hypoxia-induced IUGR and maternal weight gain

Using the same animal model, our group has previously described that despite having *ad libitum* access to food and water, dams housed under hypoxic conditions exhibited a significant reduction (~40%) in food intake.[10] Moreover, we have also observed that rats exposed to hypoxia also exhibit a decrease in physical activity. However this change in physical activity has not

been documented by standardized methods. Interestingly, at the end of parturition, rats exposed to hypoxia had an approximately 16% reduction in body weight relative to controls, which suggests that the decrease in energy requirements attributable to changes in physical activity may not account for the total change in food intake observed in these animals. Some potential anorexigenic factors that could be associated with maternal hypoxia and could cause a decrease in maternal weight gain include maternal stress and respiratory alkalosis (secondary to compensatory hyperventilation). These and other important considerations of this animal model are discussed in more detail in Section 9.6.2.

3.5.2 Fetal hypoxia

As mentioned, previous clinical studies suggest that a healthy placenta is capable of adapting when exposed to low oxygen concentrations.[8] The results obtained with hypoxia-sensitive dyes, however, demonstrated that exposing pregnant rats to moderately hypoxic conditions (11.5% oxygen) during the last week of pregnancy produced a sustained and significant decrease in fetal oxygen availability (below 10 mmHg) and led to the activation of hypoxia-mediated transcriptional signals such as HIF-1α. One of the challenges of evaluating the long-term effects of hypoxic prenatal insults is to determine the maximum hypoxic insult that can be tolerated by the litter without fatal perinatal outcomes. Although dams can survive hypoxic insults with oxygen concentrations as low as 6% oxygen, preliminary experiments demonstrated that maternal exposure to oxygen concentrations below 11% during the last week of pregnancy often resulted in perinatal mortality.

3.5.3 Maternal hypoxia, fetal birth weight, gestational length and offspring growth catch-up

Similar to other experimental models of IUGR,[11-19] maternal hypoxia caused a consistent reduction in prenatal fetal weight in both male (-12.5%) and female (-13.0%) offspring. Although this absolute reduction in birth

weight is small, it is clinically significant since it places IUGR newborns below the 15th percentile§ of normal body weight for sex-matched controls. Similar to observations made in clinical studies[11, 14, 20] and different animal models of IUGR,[13, 18, 21] offspring born IUGR as a result of a hypoxic insult rapidly "caught up" with controls and, a few days after birth, there were no differences in body weight relative to sex- and age-matched controls. This catch-up phenomenon is one of the hallmarks of IUGR and, for some authors, is thought to have more deleterious effects (on future cardiovascular and metabolic health) than IUGR by itself.[13, 19, 22-25]

Prenatal exposure to hypoxia did not affect absolute cardiac weight at birth; however, due to changes in body weight, it caused a significant increase in the relative cardiac weight. These results are consistent with previous studies showing that prolonged fetal hypoxic insults may cause asymmetric IUGR.[26] This type of IUGR is commonly seen in conditions causing placental insufficiency and is characterized by the induction of circulatory adaptations leading to redistribution of the cardiac output to maintain oxygen supply to the brain and other vital organs (such as heart and adrenal glands).[27-29] Interestingly, the prenatal hypoxic insult that we used did not affect tibia length in the offspring, which further supports the fact that not all organs are equally affected by this kind of prenatal insult.[30]

Some clinical conditions causing IUGR are also associated with preterm birth.[31] Pregnant rats exposed to hypoxia, however, had comparable or slightly longer (six to eight hours) gestational periods than controls, therefore, the differences in body weight observed in this model of IUGR cannot be attributed to differences in gestational length.

3.5.4 Maternal hypoxia and placental development

Placental ability to support fetal growth is determined by three main factors: total surface area (determined by placental size and the width of the

§ based on records of 171 offspring from 52 different litters

folded region of the feto/maternal interface), the distance across the epithelial/endothelial cell layer (because this influences the rate of diffusion from mother to fetus) and the presence of structures and transporters influencing nutrient exchange across the feto/maternal interface.[32] As expected, fetuses exposed to hypoxia exhibited a reduction in both body weight and placental weight. Interestingly, the relative placental weight (adjusted to the respective fetal body weight) was also reduced in IUGR offspring relative to controls. This result is contrary to previous clinical observations made in fetuses with severe IUGR, in which the relative placental weight was bigger than that in normal weight fetuses.[33-35] A possible interpretation of these results is that placenta from fetuses exposed to hypoxia undergo structural and functional adaptations that improve placental efficiency and render them capable of sustaining relatively larger fetuses. Another potential interpretation of these results is that under physiological conditions, the placental capacity to transport oxygen and nutrients to the fetus is higher than fetal demand (placental reserve) and in the presence of hypoxic insults, placental development is initially compromised without affecting fetal development, until placental capacity is overcome by fetal demands and the placental reserve is depleted.[36]

3.6 References

1. Morton JS, Rueda-Clausen CF, Davidge ST. Mechanisms of Endothelium-Dependent Vasodilation in Male and Female, Young and Aged Offspring Born Growth Restricted. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R930-938.

2. Williams SJ, Hemmings DG, Mitchell JM, McMillen IC, Davidge ST. Effects of maternal hypoxia or nutrient restriction during pregnancy on endothelial function in adult male rat offspring. *J Physiol*. 2005;565:125-135.

3. Hemmings DG, Williams SJ, Davidge ST. Increased myogenic tone in 7-month-old adult male but not female offspring from rat dams exposed to hypoxia during pregnancy. *Am J Physiol Heart Circ Physiol*. 2005;289:H674-682.

4. Williams SJ, Campbell ME, McMillen IC, Davidge ST. Differential effects of maternal hypoxia or nutrient restriction on carotid and femoral vascular function in neonatal rats. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R360-367.

5. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

6. Xue Q, Zhang L. Prenatal hypoxia causes a sex-dependent increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring: role of protein kinase C epsilon. *J Pharmacol Exp Ther*. 2009;330:624-632.

7. Zhang L. Prenatal hypoxia and cardiac programming. *J Soc Gynecol Investig*. 2005;12:2-13.

8. Zamudio S. High-altitude hypoxia and preeclampsia. *Front Biosci*. 2007;12:2967-2977.

9. Neerhof MG. Causes of intrauterine growth restriction. *Clin Perinatol*. 1995;22:375-385.

10. Williams SJ, Hemmings DG, Mitchell JM, McMillen IC, Davidge ST. Effects of maternal hypoxia or nutrient restriction during pregnancy on endothelial function in adult male rat offspring. *J Physiol*. 2005;565:125-135.

11. Ong KK, Ahmed ML, Emmett PM, Preece MA, Dunger DB. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. *BMJ*. 2000;320:967-971.

12. Hales CN, Ozanne SE. The dangerous road of catch-up growth. *J Physiol*. 2003;547:5-10.

13. Morrison JL, Duffield JA, Muhlhausler BS, Gentili S, McMillen IC. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol*. 2010;25:669-677.

14. Hales CN. Metabolic consequences of intrauterine growth retardation. *Acta Paediatr Suppl*. 1997;423:184-187; discussion 188.

15. Halliday HL. Neonatal management and long-term sequelae. *Best Pract Res Clin Obstet Gynaecol*. 2009;23:871-880.

16. Coupe B, Amarger V, Grit I, Benani A, Parnet P. Nutritional programming affects hypothalamic organization and early response to leptin. *Endocrinology*. 2010;151:702-713.

17. Desai M, Gayle D, Babu J, Ross MG. Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R91-96.

18. Coupe B, Grit I, Darmaun D, Parnet P. The timing of "catch-up growth" affects metabolism and appetite regulation in male rats born with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R813-824.

19. Jimenez-Chillaron JC, Patti ME. To catch up or not to catch up: is this the question? Lessons from animal models. *Curr Opin Endocrinol Diabetes Obes*. 2007;14:23-29.

20. Forsen T, Eriksson JG, Tuomilehto J, Osmond C, Barker DJ. Growth in utero and during childhood among women who develop coronary heart disease: longitudinal study. *Bmj*. 1999;319:1403-1407.

21. Bol VV, Delattre AI, Reusens B, Raes M, Remacle C. Forced catch-up growth after fetal protein restriction alters the adipose tissue gene expression program leading to obesity in adult mice. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R291-299.

22. Ong KK, Dunger DB. Birth weight, infant growth and insulin resistance. *Eur J Endocrinol*. 2004;151 Suppl 3:U131-139.

23. Reusens B, Ozanne SE, Remacle C. Fetal determinants of type 2 diabetes. *Curr Drug Targets*. 2007;8:935-941.

24. Ross MG, Desai M. Gestational programming: population survival effects of drought and famine during pregnancy. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R25-33.

25. Samaras TT, Elrick H, Storms LH. Birthweight, rapid growth, cancer, and longevity: a review. *J Natl Med Assoc*. 2003;95:1170-1183.

26. Lockwood CJ, Weiner S. Assessment of fetal growth. *Clinics in Perinatology*. 1986;13:3-35.

27. Yoshimura S, Masuzaki H, Miura K, Gotoh H, Ishimaru T. Fetal blood flow redistribution in term intrauterine growth retardation (IUGR) and post-natal growth. *Int J Gynaecol Obstet*. 1998;60:3-8.

28. Cohn HE, Sacks EJ, Heymann MA, Rudolph AM. Cardiovascular responses to hypoxemia and acidemia in fetal lambs. *Am J Obstet Gynecol*. 1974;120:817-824.

29. Rurak DW, Richardson BS, Patrick JE, Carmichael L, Homan J. Blood flow and oxygen delivery to fetal organs and tissues during sustained hypoxemia. *Am J Physiol*. 1990;258:R1116-1122.

30. Peleg D, Kennedy CM, Hunter SK. Intrauterine growth restriction: identification and management. *Am Fam Physician*. 1998;58:453-460, 466-457.

31. Fang S. Management of preterm infants with intrauterine growth restriction. *Early Human Development*. 2005;81:889-900.

32. Leiser R, Dantzer V. Structural and functional aspects of porcine placental microvasculature. *Anat Embryol (Berl)*. 1988;177:409-419.

33. Chang YL, Chang SD, Chao AS, Hsieh PC, Wang CN, Tseng LH. The individual fetal weight/estimated placental weight ratios in monochorionic twins with selective intrauterine growth restriction. *Prenat Diagn*. 2008;28:217-221.

34. Chang YL, Chang SD, Chao AS, Hsieh PC, Wang CN, Wang TH. Clinical outcome and placental territory ratio of monochorionic twin pregnancies and selective intrauterine growth restriction with different types of umbilical artery Doppler. *Prenat Diagn*. 2009;29:253-256.

35. Chauhan SP, Shields D, Parker D, Sanderson M, Scardo JA, Magann EF. Detecting fetal growth restriction or discordant growth in twin gestations stratified by placental chorionicity. *J Reprod Med*. 2004;49:279-284.

36. Heinonen S, Taipale P, Saarikoski S. Weights of placentae from small-for-gestational age infants revisited. *Placenta*. 2001;22:399-404.

CHAPTER 4 LONG-TERM EFFECTS OF HYPOXIA-INDUCED IUGR ON CARDIOPULMONARY STRUCTURE AND FUNCTION**

4.1 Introduction

Nowadays, CVDs are still the main cause of death and disability in developed countries.[1] In consideration of the high prevalence of prenatal conditions that could induce cardiac programming,[2] the study of the long-term effects of prevalent prenatal insults on cardiac structure and function constitutes a highly relevant research area that could favor the development of novel strategies to prevent and treat CVDs.[3] As mentioned in Section 1.3 of this thesis, our group has previously reported that offspring born IUGR (secondary to a prenatal hypoxic insult) exhibit a cardiac phenotype characterized by changes in patterns of collagen deposition, altered myosin composition, decreased activity of MMP-2 and increased susceptibility to I/R injury (*ex vivo).*[4] However, the *ex vivo* study of the cardiac phenotype developed by these animals has some limitations: firstly, the equipment used evaluates cardiac function under artificially controlled conditions lacking the physiological components that could modulate cardiac function (such as autonomic modulation and hormonal factors). Moreover, the study of cardiac phenotypical characteristics *ex vivo* has a very limited translation to clinical scenarios. Interestingly, no studies have been performed to determine whether the prenatal hypoxic insults that are emulated by this animal model have any long-term implications for cardiac function *in vivo*.

***Most components of this chapter have previously been published in:*

- *"**Rueda-Clausen CF**, Morton JS and Davidge ST. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. Cardiovascular Research 2009;81:713-722.*

***Contribution:** Rueda-Clausen CF. was the project coordinator, performed all the experiments and data analyses, wrote the first draft of the review article (including all the figures) and coordinated with the other authors in compiling the final version of the manuscript.*

As mentioned in Section 1.4.1, the normal aging process is characterized by an increased prevalence and severity of a number of cardiovascular pathologies including hypertension,[5, 6] coronary heart disease,[7] and heart failure.[2] Interestingly, most of these aging-associated conditions are also known to appear earlier and/or with more severity in subjects born from pregnancies complicated with IUGR.[7-10] Based on this evidence, it has been suggested that exposure to adverse environmental conditions during early stages of life may accelerate the development of chronic diseases during adulthood by affecting the response of different tissues to the normal aging process.[11] Actual data supporting this hypothesis, however, is still very limited.

Moreover, and given the paucity of data regarding sex differences in the early programming of CVDs, a more extensive description of the sex differences in the context of early cardiovascular programming needs to be made. This will allow us to firstly better characterize this particular animal model and secondly, reach a better understanding of the fundamental sex differences in the pathophysiology of CVDs from an early programming perspective.

4.2 Objectives

In order to answer these questions, the objectives of this chapter are: to study the long-term consequences of hypoxia-induced IUGR on *in vivo* cardiac function, to determine the interaction between hypoxia-induced IUGR and aging in the context of cardiac structure and function and to explore potential sex differences in the long-term effects of prenatal hypoxic insults.

Methods

4.2.1 Animal model of hypoxia induced IUGR

Female Sprague Dawley rats were mated within the animal facility and

treated as described in Section 2.1.1. At birth, litters were reduced to eight pups (four male and four female) and offspring were treated following the IUGR/aging interaction protocol described in Section 2.1.1.2.

4.2.2 In vivo *evaluation of cardiopulmonary function*

At 4 or 12 months of age, both male and female offspring were anesthetized and a complete cardiopulmonary function and structure evaluation was performed using a high-resolution UBM system as described in Section 2.2.1.

4.2.3 Isolated working heart preparation and evaluation of left ventricular function

At either 4 or 12 months of age, hearts were excised from anesthetized rats and perfused using a working heart setup as described in Section 2.2.3. *Ex vivo* LV function was evaluated using a Millar catheter as described in Section 2.2.3.2.

4.2.4 Pulmonary histology and morphometry

In a separate set of 12 month old rats (both control and IUGR), lungs were prepared as described in Section 2.2.2 and used for histological studies.

4.3 Results

4.3.1 Effects of hypoxia-induced IUGR on body weight during adulthood

At four months of age, offspring born IUGR (both male and female) had comparable body weight to age- and sex-matched controls. As they age, however, bodyweight of adult male offspring born IUGR exhibit a decrease of approximately 10% relative to their respective controls. Interestingly, these differences in body weight during adulthood were not observed in female offspring (Figure 4-1).

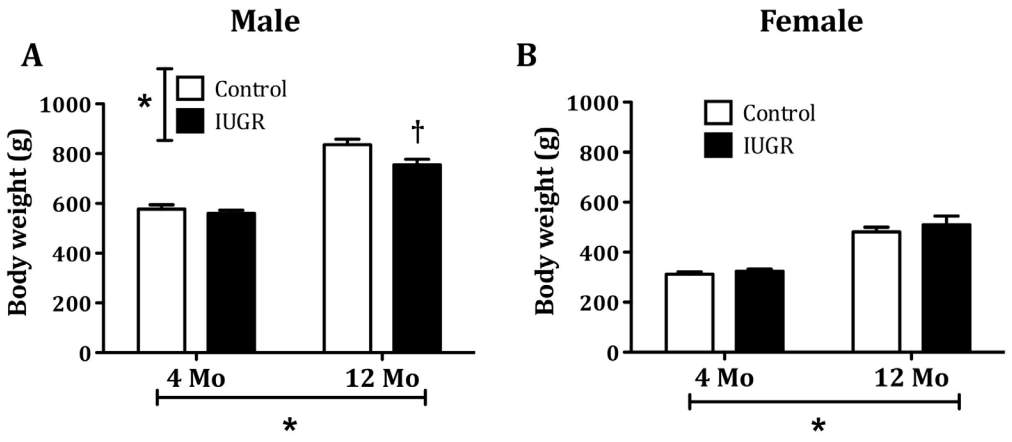


Figure 4-1 Body weight of young adult and aged offspring

Body weight of (**A**) male and (**B**) female offspring from different experimental groups either control (open bars) or IUGR (solid bars) at both 4 and 12 months (Mo) of age. Data obtained from at least 11 litters for each experimental group. * represents values of $p<0.05$ for the respective sources of variation (sex or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same sex following a Bonferroni post-hoc test.

4.3.2 Effects of hypoxia-induced IUGR on cardiac and left ventricle weights

As mentioned in Section 3.4.4, newborn male, but not female, offspring had an increased adjusted heart weight compared to controls. At four months of age, however, these differences in heart weight were not observed and the heart/body weight ratio was comparable between IUGR and sex- age-matched controls in both male and female offspring (Figure 4-2). At 12 months of age, male but not female offspring exposed to prenatal hypoxia had a greater heart/body weight ratio than their respective controls. Since there were differences in the body weight of male animals from the prenatal hypoxia group, changes in LV mass are depicted after adjusting for the absolute heart weight (Figure 4-2). Only aged (12 month old) male animals exposed to hypoxia before birth had a higher relative ventricular weight when compared to their respective controls.

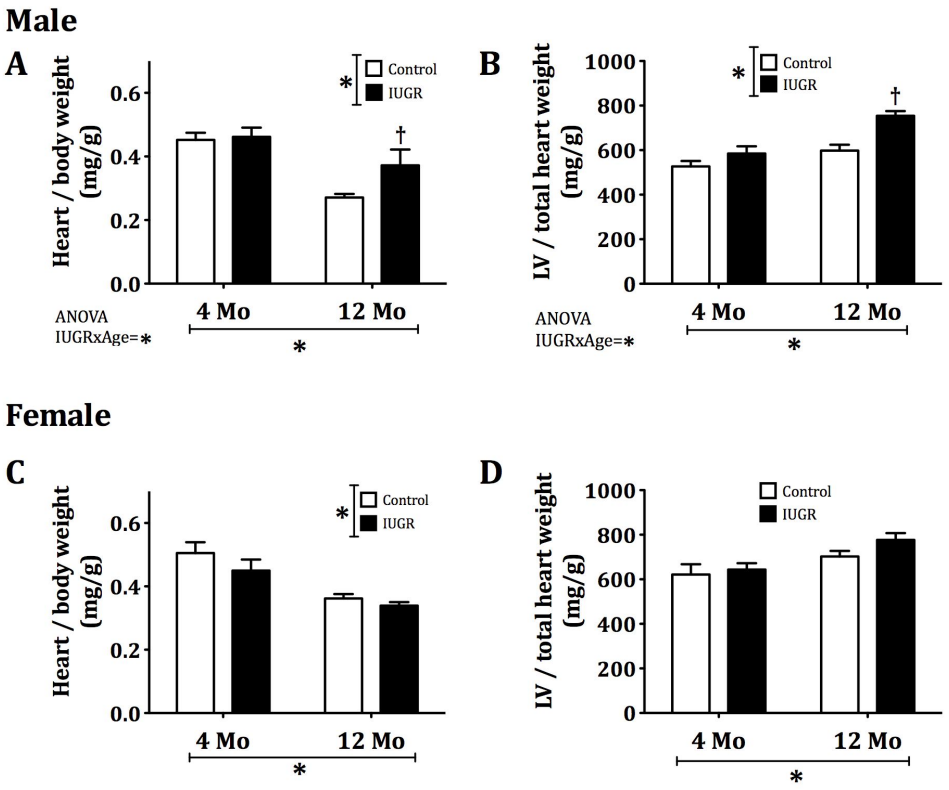


Figure 4-2 Effect of prenatal exposure to hypoxia on heart/body weight ratio and left ventricle/total heart weight in male and female adult offspring

(**A** and **C**) heart/body weight ratio (wet weight), (**B** and **D**) LV weight (as determined by UBM)/total heart wet weight, (solid bars) offspring from dams exposed to hypoxia during pregnancy and (open bars) controls. Data obtained from at least 11 litters in each experimental group at 4 and 12 months (Mo) of age. * represents values of $p<0.05$ for the respective sources of variation (sex or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same sex after a Bonferroni post-hoc test.

4.3.3 Effects of hypoxia-induced IUGR on cardiopulmonary structure and function.

Figure 4-3 shows representative images illustrating the most significant *in vivo* findings observed in adult offspring born IUGR. These findings included: increased LV wall thickness (Column A), LV diastolic dysfunction (Column B) and pulmonary hypertension (Column C). Detailed information regarding each of these findings and other cardiopulmonary parameters evaluated by UBM are presented in more detail in the following sections.

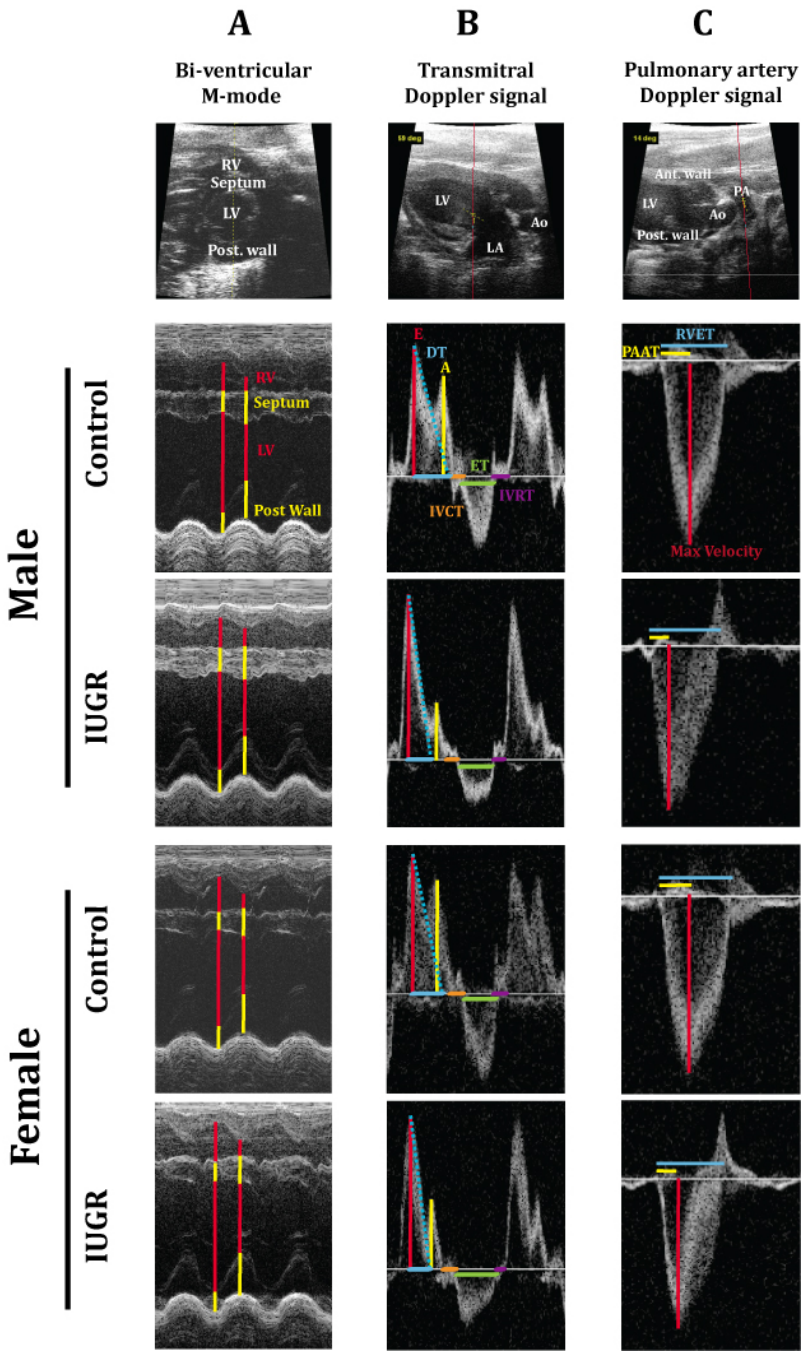


Figure 4-3 Representative images obtained by ultrasound biomicroscopy in aged offspring

Images obtained from 12 month old offspring using (**A**) Bi-ventricular M-mode, (**B**) trans-mitral Doppler signaling and (**C**) trans-pulmonary Doppler signaling in both offspring born IUGR or controls from both sexes. RV: right ventricle, LV: left ventricle, Post wall: posterior wall of the left ventricle, Ant Wall: anterior wall of the left ventricle PA: pulmonary artery, Ao: aorta, E: E mitral (passive filling) wave, A: A mitral (active filing) wave, DT: deceleration time, IVCT: isovolumetric contraction time, ET: ejection time, IVRT: isovolumetric relaxation time, PAAT: pulmonary artery acceleration time, RVET: right ventricle ejection time.

4.3.4 Hypoxia-induced IUGR and changes in left ventricular function

Consistent with the *ex-vivo* findings, only aged male rats exposed to hypoxia had an augmented septal and posterior wall thickness (evaluated *in vivo*) when compared to controls (Table 4-1 and column A, Figure 4-3). Interestingly, offspring prenatally exposed to hypoxia did not exhibit any changes in UBM parameters evaluating LV systolic function or aortic diameter at either 4 or 12 months of age (Table 4-2).

At 12 month of age, however, offspring from both sexes exposed to hypoxia *in utero* presented with echocardiographic signs of LV diastolic dysfunction; including decreased mitral A-wave maximum velocity, isovolumetric relaxation time and deceleration time of the mitral valve as well as increased E/A and Tei indexes (Table 4-3 and column B, Figure 4-3).

In order to complement these UBM findings, cardiac hemodynamic parameters were evaluated during *ex vivo* heart perfusion experiments (as described in Section 2.2.3.2). Consistent with the *in vivo* findings suggestive of LV diastolic dysfunction, the primary significant difference observed in the aerobic function of hearts obtained from IUGR animals was an increased LVEDP relative to sex- and age-matched controls (Table 4-4). This difference was significant in both male and female animals at 12 months of age. Interestingly, aging and prenatal exposure to hypoxia were also associated with a decrease in heart rate (measurements made *in vivo* and under general anesthesia, Table 4-2).

Table 4-1 Summary of the most relevant cardiac morphometric measurements obtained from biventricular M-mode imaging performed at 4 and 12 months of age using ultrasound biomicroscopy

	4 Months		12 Months		ANOVA		
Male offspring	**Control n=14**	**IUGR n=12**	**Control n=13**	**IUGR n=10**	**Age**	**IUGR**	**Int**
$RVID_{dias}$	2.3 (0.16)	2.6 (0.21)	2.2 (0.27)	3.2 (0.25) †		*	
ST_{dias}	1.8 (0.08)	1.7 (0.11)	2 (0.04)	2.2 (0.11)			
ST_{sys}	2.42 (0.10)	2.36 (0.12)	2.53 (0.14)	3.13 (0.15) †	*	*	
$LVID_{dias}$	7.7 (0.29)	7.8 (0.25)	7.9 (0.5)	8.1 (0.48)			
$LVID_{sys}$	4.11 (0.17)	3.85 (0.18)	4.27 (0.17)	3.99 (0.16)		*	
$LVPW_{dias}$	2.1 (0.07)	2.4 (0.18)	2.4 (0.08)	2.7 (0.05)	*		
$LVPW_{sys}$	3.1 (0.07)	3.5 (0.15)	3.7 (0.11)	4.5 (0.2) †	*		
LVV_{dias}	316 (16)	355 (30)	352 (19)	368 (23)	*		
LVV_{sys}	79 (8)	80 (12)	83 (7)	82 (8)			
Stroke vol	237 (9)	256 (14)	271 (15)	286 (16)	*		
LVmass/BW	1.81 (0.04)	2.07 (0.1)	1.59 (0.08)	2.04 (0.12) †			
LVmass/HW	527 (24)	585 (32)	597 (26)	754 (21) †	*	*	
Female offspring	**n=12**	**n=13**	**n=10**	**n=10**			
$RVID_{dias}$	1.9 (0.14)	2.0 (0.21)	2.2 (0.24)	2.5 (0.3)			
ST_{dias}	1.6 (0.12)	1.5 (0.12)	1.7 (0.08)	1.7 (0.15)			
ST_{sys}	2.12 (0.12)	2.31 (0.05)	2.45 (0.11)	2.49 (0.13)			
$LVID_{dias}$	6.8 (0.16)	6.9 (0.27)	7.4 (0.28)	7.3 (0.26)			
$LVID_{sys}$	3.25 (0.16)	3.03 (0.2)	3.61 (0.3)	3.32 (0.18)			
$LVPW_{dias}$	1.9 (0.21)	2.0 (0.07)	2.4 (0.09)	2.6 (0.08)	*		
$LVPW_{sys}$	2.8 (0.11)	2.7 (0.12)	3.5 (0.12)	3.9 (0.2)	*		
LVV_{dias}	224 (14)	233 (23)	246 (30)	282 (27)	*		
LVV_{sys}	49 (9)	45 (13)	49 (11)	55 (12)			
Stroke vol	175 (9)	187 (13)	196 (20)	226 (19)			
LVmass/BW	2.55 (0.2)	2.34 (0.16)	2.16 (0.09)	2.12 (0.08)			
LVmass/HW	621 (45)	643 (28)	702 (25)	776 (30)	*		

Data presented as mean (SE). $RVID_{dias}$: right ventricle internal diameter in diastole (mm), ST_{dias}: end-diastolic septal thickness (mm), ST_{sys}: end-systolic septal thickness (mm), $LVID_{dias}$: lef venticle end-diastolic internal diameter (mm), $LVID_{sys}$: left ventricle end-systolic internal diameter (mm), $LVPW_{dias}$: left ventricle end-diastolic posterior wall thickness (mm), $LVPW_{sys}$: left ventricle end-systolic posterior wall thickness (mm), LVV_{dias}: left ventricle end-diastolic volume determined by M-mode trace (μL), LVV_{sys}: left ventricle end-systolic volume determined by M-mode trace (μL), Stroke vol: stroke volume (μL), LVmass/BW: left ventricle estimated mass/body weight (mg/g), LVmass/HW: left ventricle estimated mass/heart weight (mg/g), * represents values of $p<0.01$ for the respective sources of variation such as prenatal hypoxia (IUGR), aging (Age) or their interaction (Int) using two-way ANOVA.
† represents a $p<0.01$ vs. controls of the same age after a Bonferroni post-hoc test.

Table 4-2 Summary of the most relevant measurements of left ventricular function obtained from the parasternal short axis and aortic Doppler signals at 4 and 12 months of age using utrasound biomicroscopy

	4 Months		12 Months		ANOVA		
Male Offspring	**Control n=14**	**IUGR n=12**	**Control n=13**	**IUGR n=10**	**Age**	**IUGR**	**Int**
HR	349 (10)	340 (11)	307 (7)	291 (9)	*	*	
Parasternal short-axis							
CO/BW	140 (7)	150 (10)	160 (22)	150 (26)			
LV shortening frac	43 (1)	45 (1.4)	46 (1.3)	48 (1.7)			
LV ejection frac	71 (1.2)	74 (1.5)	73 (1.4)	78 (1.7)			
Parasternal long-axis aortic view							
Ao $outf_{int}$	8.4 (0.9)	9.6 (1.1)	7.7 (0.7)	7.9 (1.6)			
Ao peak ejec vel	1.3 (0.1)	1.2 (0.2)	1.17 (0.1)	1.0 (0.2)			
Ao ejection time	71 (2.5)	74 (3.7)	79 (4)	74 (5)			
Ao $diam_{dias}$	3.2 (0.1)	3.1 (0.1)	3.5 (0.1)	3.6 (0.1)	*		
Ao $diam_{sys}$	3.5 (0.08)	3.4 (0.14)	3.8 (0.2)	4 (0.1)	*		
Female Offspring	**n=12**	**n=13**	**n=10**	**n=10**			
HR	338 (14)	325 (14)	290 (14)	280 (6)	*	*	
Parasternal short-axis							
CO/BW	200 (20)	211 (12)	172 (20)	186 (26)	*		
LV shortening frac	46 (1.6)	43 (2)	47 (1.6)	47 (2.2)			
LV ejection frac	79 (2.6)	73 (2.2)	78 (2.1)	77 (2.3)			
Parasternal long-axis aortic view							
Ao $outf_{int}$	8.3 (1)	9.1 (1.7)	7.5 (1.3)	8 (1.3)	*		
Ao peak ejec vel	1.5 (0.16)	1.5 (0.2)	1.1 (0.1)	1.2 (0.8)	*		
Ao ejection time	77 (5)	78 (4)	81 (3)	82 (2.4)			
Ao $diam_{dias}$	3.1 (0.3)	2.9 (0.2)	3.3 (0.1)	3.4 (0.1)			
Ao $diam_{sys}$	3.3 (0.33)	3 (0.08)	3.3 (0.1)	3.6 (0.2)			

Data presented as mean (SE). HR: heart rate (bpm), CO/BW: cardiac output adjusted by body weight ($ml \bullet min^{-1} \bullet Kg^{-1}$), LV shortening frac: left ventricle shortenong fraction (%), LV ejection frac: left ventricle ejection fraction (%), Ao $outf_{int}$: aortic outflow time/velocity first integral (m/s^2), Ao peak ejec vel: aortic outflow maximum ejection velocity (m/s), Ao ejection time: aortic ejection time (ms), Ao $diam_{dias}$: aortic root internal diameter at the end of diastole (mm), Ao $diam_{sys}$: aortic root diameter at the end of systole (mm), * represents values of $p<0.01$ for the respective sources of variation such as prenatal hypoxia (IUGR), aging (Age) or their interaction (Int) using two-way ANOVA.

Table 4-3 Summary of the most relevant measurements of the left ventricular function obtained from the mitral Doppler signal in a modified parasternal long-axis view obtained at 4 and 12 months of age using ultrasound biomicroscopy

	4 Months		12 Months		ANOVA		
Male offspring	**Control n=14**	**IUGR n=12**	**Control n=13**	**IUGR n=10**	**Age**	**IUGR**	**Int**
$MitE_{max}$	1160 (257)	1275 (206)	1327 (130)	1309 (116)			
$MitA_{max}$	688 (94)	697 (98)	911 (73)	573 (58) †			
E/A index	1.8 (0.1)	1.8 (0.2)	1.4 (0.1)	2.2 (0.18) †		*	*
Mit decel	49.3 (3.5)	45.6 (5.9)	41.2 (5.2)	32.1 (4.1) †	*	*	*
Tei index	0.37 (0.04)	0.37 (0.03)	0.33 (0.03)	0.48 (0.04) †			
Mit IVRT	17.5 (2.0)	14.3 (3.5)	16.3 (2.3)	9.5 (5.2) †	*	*	*
Mit IVCT	13.5 (3.5)	12.2 (2.1)	9.9 (2.4)	10.1 (4.4)	*		*
Female offspring	**n=12**	**n=13**	**n=10**	**n=10**			
$MitE_{max}$	1319 (244)	1307 (148)	1481 (250)	1252 (198)			
$MitA_{max}$	696 (95)	796 (76)	945 (102)	670 (75) †			
E/A index	1.9 (0.2)	1.7 (0.2)	1.6 (0.1)	2.1 (0.1) †			
Mit decel	49.2 (3.5)	45 (5.2)	41.7 (4.1)	32.6 (3.9) †	*		*
Tei index	0.33 (0.04)	0.37 (0.04)	0.31 (0.03)	0.43 (0.03) †		*	
Mit IVRT	13.8 (2.3)	14.6 (3.2)	11.2 (2.3)	7.3 (3.1) †		*	
Mit IVCT	11.2 (3.3)	13.2 (4.23)	10.0 (3.6)	12.4 (3.7)			

Data presented as mean (SE). $MitE_{max}$: Mitral vave E wave maximum velocity (mm/s), $MitA_{max}$: Mitral valve A wave maximum velocity (mm/s), E/A index: Mitral waves E/A index, Mit decel: Mitral flow deceleration time (ms), (Tei Index) Myocardial performance index also known as Tei index, (Mit IVRT) Mitral isovolumetric relaxation time (ms), (Mit IVCT) Mitral isovolumetric constriction time (ms). *: $p<0.01$ when effect of hypoxia (IUGR), aging (Age) or their interaction (Int) was evaluated by two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

Table 4-4 Parameters of left ventricular function determined *ex vivo* using a Millar catheter during aerobic perfusion in a working heart set up

	4 months		12 months		ANOVA		
Male Offspring	**Control n=4**	**IUGR n=4**	**Control n=5**	**IUGR n=4**	**Age**	**IUGR**	**Int**
Paced HR	304 (6)	301 (5)	275 (6)	279 (4)	*		
SBP	95.2 (1.7)	99.2 (3.3)	99.1 (2.2)	103.1 (3.1)			
DBP	77.8 (0.5)	79.2 (0.12)	71.8 (2.9)	72.9 (3.04)	*		
Cardiac work	1086 (157)	1193 (294)	950 (152)	1035 (77)			
LV dP/dt_{max}	4704 (676)	5240 (640)	4653 (707)	4283 (257)			
LVEDP	3.6 (0.9)	6.1 (1.2)	6.5 (1.7)	11.8 (1.1) †	*	*	
Female offspring	**n=4**	**n=4**	**n=4**	**n=6**			
Paced HR	303 (9)	300 (6)	271 (11)	276 (4)	*		
SBP	90.1 (0.5)	85.3 (2.4)	85.9 (6.5)	92.1 (4.7)			
DBP	80.3 (0.6)	75.3 (2.4)	68.5 (4.7)	71.6 (3.1)	*		
Cardiac work	672.9 (102)	523 (66)	576 (53)	690 (124)			
LV dP/dt_{max}	3692 (579)	3141 (451)	4466 (279)	4140 (574)	*	*	
LVEDP	4.3 (0.81)	5.6 (0.69)	7.6 (0.71)	13.2 (1.2) †	*	*	

Data presented as mean (SE). HR: heart rate (bpm), SBP: systolic blood pressure (mmHg), DBP: diastolic blood pressure (mmHg), LV dP/dt_{max}: maximum first derivative of the LV pressure over time (mmHg/s), Cardiac work is reported in ($mL \bullet min^{-1} \bullet mmHg^{-1} \bullet g\ dry\ wt^{-1}$). LVEDP: left ventricle end-diastolic pressure (mmHg), * represents values of $p<0.05$ for the respective sources of variation such as prenatal hypoxia (IUGR), aging (Age) or their interaction (Int) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

4.3.5 Hypoxia-induced IUGR and pulmonary hypertension

At 12 months of age, both male and female animals exposed to hypoxia exhibited echocardiographic signs of pulmonary hypertension; including decreased pulmonary artery acceleration time and increased right ventricle diameter in diastole when compared to age- and sex-matched controls (Table 4-5 and column C, Figure 4-3). In addition, histological studies of the lungs showed that the media thickness of small pulmonary arteries was increased in aged animals following prenatal exposure to hypoxia when compared to controls (Figure 4-4).

Table 4-5 Summary of the most relevant cardiopulmonary measurements obtained at 4 and 12 months of age using ultrasound biomicroscopy

	4 Months		12 Months		ANOVA		
Male offspring	**Control n=14**	**IUGR n=12**	**Control n=13**	**IUGR n=10**	**Age**	**IUGR**	**Int**
LAD_{dias}	3.3 (0.1)	3.1 (0.1)	3.5 (0.1)	3.9 (0.14)	*		
LAD_{sys}	3.7 (0.1)	3.9 (0.2)	3.8 (0.2)	4 (0.1)			
PA_{int}	4.54 (0.18)	4.95 (0.3)	4.42 (0.17)	4.88 (0.17)			
PA_{max}	848 (19)	853 (24)	771 (28)	820 (38)			
RVET	84 (3)	85 (2.1)	89 (3)	94 (3.3)	*		
PAAT	37 (2.3)	36 (2.1)	38 (2.3)	28 (2.6) †		*	
PAAT/HR	0.11 (0.07)	0.12 (0.01)	0.12 (0.09)	0.08 (0.01) †			
PAAT/RVET	0.43 (0.01)	0.42 (0.02)	0.43 (0.02)	0.35 (0.02) †		*	*
Female offspring	**n=14**	**n=12**	**n=13**	**n=10**			
LAD_{dias}	3.1 (0.2)	2.8 (0.2)	3 (0.1)	3.2 (0.16)			
LAD_{sys}	3.6 (0.3)	2.9 (0.08)	3.3 (0.1)	3.6 (0.2)			
PA_{int}	4.43 (0.24)	4.83 (0.26)	4.15 (0.2)	4.75 (0.2)		*	
PA_{max}	806 (29)	839 (40)	705 (43)	862 (37)			
RVET	78 (4)	86 (2.8)	85 (3.8)	88 (3)			
PAAT	31 (3.1)	31 (2.9)	38 (2.1)	30 (1.9)†		*	
PAAT/HR	0.10 (0.01)	0.11 (0.01)	0.13 (0.01)	0.11 (0.006)			*
PAAT/RVET	0.40 (0.03)	0.39 (0.02)	0.44 (0.02)	0.34 (0.02) †		*	

Data presented as mean (SE). LAD_{dias}: left atrium end-diastolic diameter (mm), LAD_{sys}: left atrium end-systolic diameter (mm), PA_{int}: pulmonary artery velocity/time integral (mmHg/s), PA_{max}: pulmonary artery maximum velocity (mm/s), RVET: right ventricle ejection time (ms), PAAT: pulmonary artery acceleration time (ms), HR: heart rate (bps), * represents values of $p<0.05$ for the respective sources of variation such as prenatal hypoxia (IUGR), aging (Age) or their interaction (Int) using two-way ANOVA.† represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

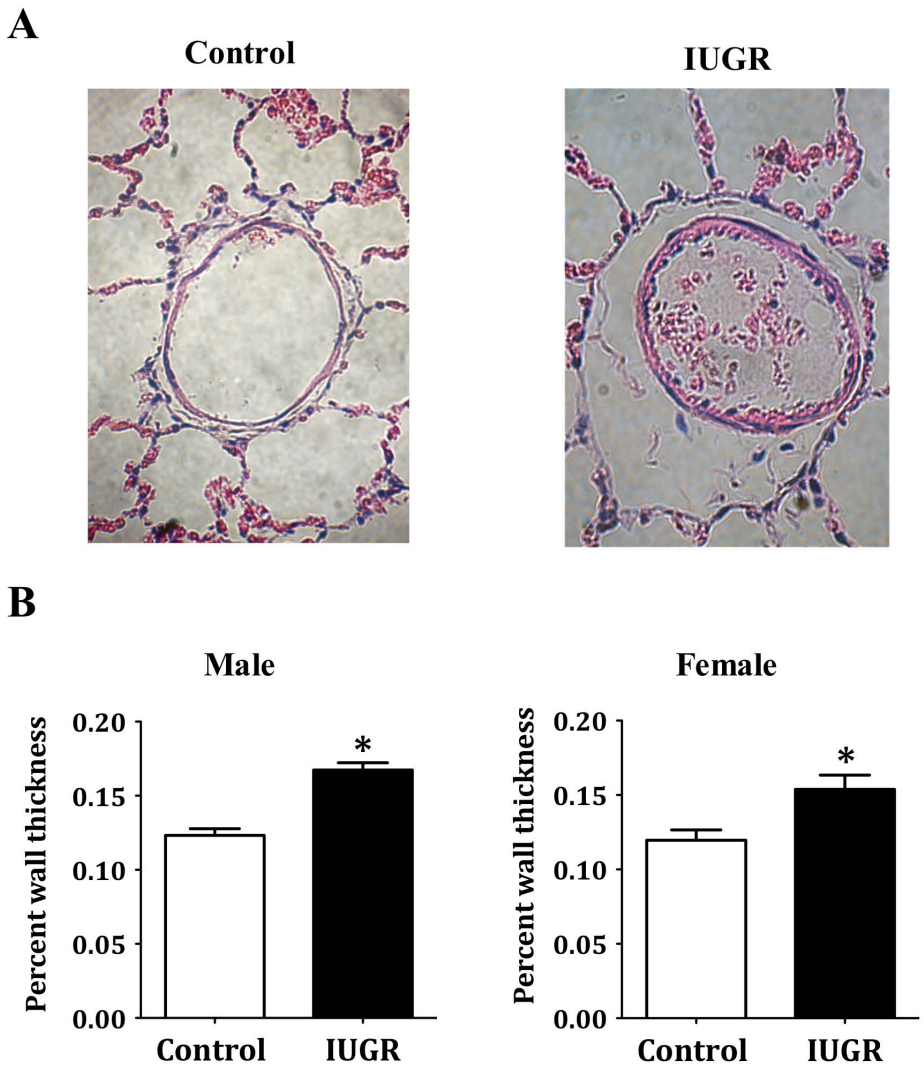


Figure 4-4 Effect of hypoxia induced IUGR on pulmonary arteriolar wall thickness at 12 months of age

Panel **A** shows representative images of histological preparations of lungs from aged (12 month old) male offspring exposed to hypoxia and normal conditions during fetal development. Panel **B** summarizes information of arterial media thickness in the lung of 12 months old animals (n≥3 for each group). *: $p<0.01$ when compared to controls.

4.4 Discussion

4.4.1 Effects of hypoxia-induced IUGR on postnatal body weight

Contrary to other models of IUGR, adult offspring prenatally exposed to hypoxia did not exhibit an exaggerated weight gain after birth. In fact,

female adult offspring prenatally exposed to hypoxia had the same body weight as their respective controls and male offspring exposed to hypoxia during fetal development had a statistically significant lower body weight than their respective controls at both 4 and 12 months of age. This finding is interesting because is demonstrates that changes in long-term cardiac function are not necessarily associated with changes in body weight or fat composition as described in other models of IUGR.[12, 13]

4.4.2 Long-term effects of hypoxia-induced IUGR on left ventricular structure and function

Our study provides the first *in vivo* description of the long-term effects of hypoxia-induced IUGR on cardiopulmonary function in young adult and aged offspring. These data further support the previously observed micro-structural and enzymatic changes in myocardial tissue evaluated *ex vivo.* Moreover, it is the first study in mammals to show that a prenatal hypoxic insult can induce the spontaneous development of LV diastolic dysfunction and pulmonary hypertension as the animal ages.

Despite the changes in LV diastolic function observed in hypoxic animals, LV systolic function (evaluated by ejection fraction, shortening fraction and cardiac output) remained comparable to that of controls at 4 and 12 months of age. The presence of abnormalities in mechanical function during diastole that do not compromise the ejection fraction is also known as isolated LV diastolic dysfunction[14] and, in this particular animal model, could be explained by the changes in collagen deposition, impaired ß/αMHC and decreased activity of MMP-2 previously described.[15]

4.4.3 Hypoxia-induced IUGR and development of pulmonary hypertension

Another adverse finding in aged offspring prenatally exposed to hypoxia was pulmonary hypertension. Low birth weight and conditions associated with low oxygen availability during fetal development have been

extensively linked with acute respiratory distress, bronchopulmonary dysplasia and persistent pulmonary hypertension of the newborn.[reviewed in 16] To the best of our knowledge, however, this is the first study showing an association between hypoxia-induced IUGR and the development of primary pulmonary hypertension in aged rats. Moreover, in our model, pregnant dams were always placed in normal oxygen conditions before birth. Therefore, the chronic effects of this hypoxic insult during fetal development cannot be attributed to newborn exposure to a hypoxic environment during the early stages of postnatal life.

Hypoxia-induced IUGR and aging interaction

In our study, signs of both LV diastolic dysfunction and pulmonary hypertension were evident at 12 but not at 4 months of age; suggesting that in this particular model, the aging process may act as a stressor that is less well tolerated by animals which were prenatally exposed to hypoxia. Additionally, none of the echocardiographic or direct *ex vivo* examinations of the hearts from adult animals prenatally exposed to hypoxia exhibited any kind of malformation. Therefore, the observed effects of prenatal hypoxia on cardiac function cannot be attributed to potential teratogenic effects of the hypoxic insult used.[17]

4.4.4 Long-term effects of hypoxia-induced IUGR on heart rate

We also found that aged animals had a slight but significant decrease in heart rate when compared to young adults, which is consistent with previous animal studies showing a reduction in heart rate associated with aging in both males and females.[18] Interestingly, we also observed that IUGR offspring of any age or sex had a statistically significant reduction in heart rate. These results suggest that dysregulation of the mechanisms that control heart rate (such as the autonomic nervous system), could be involved in the pathophysiology of the long-term cardiovascular consequences of early prenatal insults. Autonomic dysregulation has previously been described in

other models of early programming.[19, 20] The information available on hypoxia-mediated early programming of the autonomic function, however, is very limited.

4.4.5 Sex differences in long-term effects of hypoxia–induced IUGR

Findings in male and female animals were consistent in terms of the development of LV diastolic dysfunction and pulmonary hypertension. Interestingly, male but not female offspring exposed to hypoxia had greater ventricular mass at 12 months of age when compared to their sex-matched controls. This suggests that the female sex may confer some form of protection against this particular long-term effect of IUGR. Consistent with this finding, *ex vivo* vascular studies previously conducted by our group described that male, but not female, offspring exposed to hypoxia during fetal development expressed changes in vascular myogenic tone during adulthood.[21] Given that antecedent, one potential mechanism that could explain the development of increased thickness of the LV wall in male aged animals exposed to hypoxia is an increase in blood pressure. Interestingly, previous results obtained using the tail-cuff technique in the same animal model suggest that the hypoxic insult has no effect on the blood pressure of adult male offspring.[15]

4.4.6 Study limitations

The results described herein should be interpreted with caution; rats were anesthetized during the UBM studies and the potential acute effects of anesthetics on cardiovascular function must be considered. It has been described that thiopental has a direct negative inotropic effect on rats,[22] and that at high doses can cause a temporal drop in cardiac output.[23] These described acute effects of thiopental, however, would not likely be related to the changes in ventricular morphometry, ventricular diastolic function or pulmonary hypertension described in IUGR animals.

4.4.7 Conclusions

In conclusion, the results presented in this chapter demonstrate that prenatal exposure to hypoxia has important deleterious consequences on the cardiopulmonary function of the offspring later in life. This includes the development of LV diastolic dysfunction, pulmonary hypertension and an increased LV mass. Being female seems to confer some degree of protection against the development of increased LV wall thickness but not against any of the other cardiopulmonary findings associated with hypoxia-induced IUGR. The mechanisms involved in this long-term programming phenomenon still need to be determined. One of the relevant implications of these findings from a clinical perspective is the fact that perinatal diagnosis could be potentially useful as a predictor of cardiopulmonary outcomes (such as left ventricle diastolic dysfunction and pulmonary hypertension) during adulthood.

4.5 References

1. Dahlof B. Cardiovascular disease risk factors: epidemiology and risk assessment. *Am J Cardiol*. 2010;105:3A-9A.

2. Godfrey KM, Barker DJ. Fetal programming and adult health. *Public Health Nutr*. 2001;4:611-624.

3. Thornburg KL. Hypoxia and cardiac programming. *J Soc Gynecol Investig*. 2003;10:251.

4. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

5. Schwartz J, Thornburg KL. The influence of various physiological challenges on permanent changes to the cardiovascular system. *Arch Physiol Biochem*. 2003;111:3-7.

6. Ojeda NB, Grigore D, Alexander BT. Intrauterine growth restriction: fetal programming of hypertension and kidney disease. *Adv Chronic Kidney Dis*. 2008;15:101-106.

7. Louey S, Thornburg KL. The prenatal environment and later cardiovascular disease. *Early Hum Dev*. 2005;81:745-751.

8. Thomas R, Kaskel FJ. It's not over till the last glomerulus forms. *Kidney International*. 2009;76:361-363.

9. Dotsch J, Plank C, Amann K, Ingelfinger J. The implications of fetal programming of glomerular number and renal function. *J Mol Med*. 2009;87:841-848.

10. Morrison JL, Duffield JA, Muhlhausler BS, Gentili S, McMillen IC. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol*. 2010;25:669-677.

11. Nilsson PM, Lurbe E, Laurent S. The early life origins of vascular ageing and cardiovascular risk: the EVA syndrome. *J Hypertens*. 2008;26:1049-1057.

12. Coupe B, Amarger V, Grit I, Benani A, Parnet P. Nutritional programming affects hypothalamic organization and early response to leptin. *Endocrinology*. 2010;151:702-713.

13. De Blasio MJ, Blache D, Gatford KL, Robinson JS, Owens JA. Placental restriction increases adipose leptin gene expression and plasma leptin and alters their relationship to feeding activity in the young lamb. *Pediatr Res*. 2010;67:603-608.

14. Zile MR, Brutsaert DL. New concepts in diastolic dysfunction and diastolic heart failure: Part I: diagnosis, prognosis, and measurements of diastolic function. *Circulation*. 2002;105:1387-1393.

15. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

16. Tuder RM, Yun JH, Bhunia A, Fijalkowska I. Hypoxia and chronic lung disease. *J Mol Med*. 2007;85:1317-1324.

17. Webster WS, Abela D. The effect of hypoxia in development. *Birth Defects Res C Embryo Today*. 2007;81:215-228.

18. Roberts J, Goldberg PB. Changes in basic cardiovascular activities during the lifetime of the rat. *Exp Aging Res*. 1976;2:487-517.

19. Frasch MG, Muller T, Wicher C, Weiss C, Lohle M, Schwab K*, et al.* Fetal body weight and the development of the control of the cardiovascular system in fetal sheep. *J Physiol*. 2007;579:893-907.

20. Massin MM, Withofs N, Maeyns K, Ravet F. The influence of fetal and postnatal growth on heart rate variability in young infants. *Cardiology*. 2001;95:80-83.

21. Hemmings DG, Williams SJ, Davidge ST. Increased myogenic tone in 7-month-old adult male but not female offspring from rat dams exposed to hypoxia during pregnancy. *Am J Physiol Heart Circ Physiol*. 2005;289:H674-682.

22. Kanaya N, Zakhary DR, Murray PA, Damron DS. Thiopental alters contraction, intracellular Ca2+, and pH in rat ventricular myocytes. *Anesthesiology*. 1998;89:202-214.

23. Wada DR, Harashima H, Ebling W, Osaki EW, Stanski DR. Effects of thiopental on regional blood flows in the rat. *Anesthesiology*. 1996;84:596-604.

CHAPTER 5 LONG-TERM EFFECTS OF IUGR ON CARDIAC ENERGY METABOLISM AND SUSCEPTIBILITY TO ISCHEMIA/REPERFUSION INJURY ††

5.1 Introduction.

As previously mentioned (Section 1.3.1.4), several mechanisms have been proposed to explain the described increased myocardial susceptibility to ischemia observed in adult offspring born IUGR.[1-4] From this constellation of proposed pathways, the potential participation of mechanisms that regulate cardiac energy metabolism is particularly interesting (as discussed in Section 1.3.1.5).

Remarkably, preliminary results obtained by our group using the rodent model of hypoxia-induced IUGR, demonstrated that the myocardium from adult IUGR offspring exhibits an increase in the expression of mitochondrial enzymes such as the F1F0-proton ATPase and aconitase, which are both involved in mitochondrial energy production.[5] Moreover, conditions that have been previously described in our animal model of IUGR, such as increased LV wall thickness and diastolic dysfunction (see Section 4.3.2), are also known to be associated with changes in myocardial energy substrate selection.[6]

These results, together with previous studies demonstrating the importance of energetic substrate selection in determining cardiac susceptibility to I/R injury,[7-12] suggest that the hypoxic insult that we are

†† *Most components of this chapter have been submitted for publication as:*

- *"**Rueda-Clausen CF**, Morton JS, Lopaschuk GD and Davidge ST. Long-term effects of intrauterine growth restriction on cardiac metabolism and susceptibility to ischemia reperfusion. In press, Cardiovascular Research 2010."*

Contribution: *Rueda-Clausen CF. was the project coordinator, performed all the experiments and data analyses, wrote the first draft of the manuscript (including all the figures) and coordinated with the other authors in compiling the final version of the manuscript.*

using in our animal model could have fundamental effects on cardiac energy metabolism.

5.2 Objectives

Despite available evidence suggesting that changes in cardiac energy metabolism could be involved in the increased cardiac susceptibility to ischemia,[3] the presence of long-term changes in cardiac metabolism in this or any other animal model of IUGR is still unknown. Based on previous findings, the primary objective of this study was to determine whether hypoxia-induced IUGR leads to long-term changes in the selection of energetic substrates used by the heart to produce energy and whether these changes prevent the effective recovery of the heart from an I/R injury.

In addition, our previous results also suggest that being born IUGR may accelerate the normal aging process in terms of cardiovascular function (Chapter 4).[13] Consequently, one of the secondary objectives of this study was to evaluate the interaction of these two factors (hypoxia-induced IUGR and aging) in the development of an undesirable cardiac metabolic phenotype. Moreover, and despite well-described differences between males and females in the pathophysiology of chronic CVDs,[14, 15] most studies of cardiac function in this field have been conducted only in male animals. Therefore, potential sex differences in this condition remain to be explored and have been incorporated into our experimental design.

5.3 Methods

5.3.1 Animal model

Experimental animals were treated as described in detail in Section 2.1.1 following the IUGR/aging interaction protocol (Section 2.1.1.2)

5.3.2 Isolated working heart preparation, cardiac ischemia/reperfusion protocols and cardiac metabolism evaluation

At 4 or 12 months of age, hearts were excised from anesthetized rats and perfused using a working heart setup as described in Section 2.2.3. Protocols for I/R were performed as described in Section 2.2.3 and evaluation of cardiac metabolism was performed as described in Section 2.2.3.4.

5.4 Results

5.4.1 Long-term effects of hypoxia-induced IUGR on cardiac susceptibility to ischemia/reperfusion injury

During the initial pre-ischemic period of the I/R protocol, hearts from all offspring developed comparable levels of cardiac power, independent of their sex, age or *in utero* exposure to hypoxia. During reperfusion, however, both male and female offspring born IUGR exhibited a remarkable decrease in cardiac performance recovery when compared to age- and sex-matched controls (Figure 5-1). In male offspring (both IUGR and controls), aging had an additional deleterious effect on cardiac susceptibility to I/R injury. Interestingly, aging had no additional effect on the susceptibility to I/R injury in female offspring. Additional information regarding cardiac function experiments (including cardiac output, coronary flows and LV developed pressure) is presented in Table 5-1.

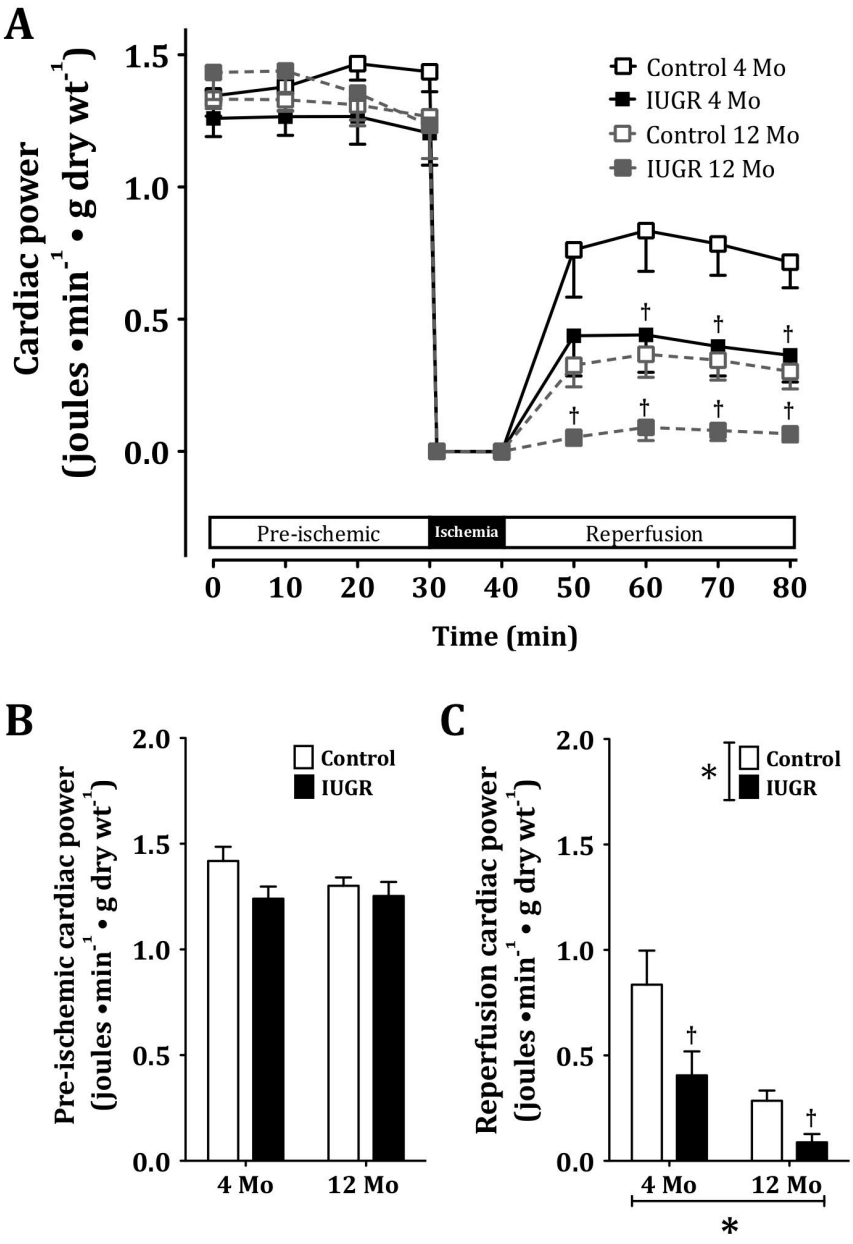


Figure 5-1 ***Ex vivo*** **cardiac power development during pre-ischemic and reperfusion periods in adult male offspring**

(**A**) Average cardiac power developed over time during *ex vivo* cardiac aerobic perfusion (pre-ischemia) and after 10 minutes of no-flow ischemia (reperfusion) from different experimental groups, (**B**) average maximal cardiac power developed during pre-ischemia period, (**C**) average maximal cardiac power developed during reperfusion. Values obtained from 9 to 11 animals from different litters per group at 4 and 12 months (Mo) of age. * represents a value of $p<0.05$ for the respective sources of variation (age or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

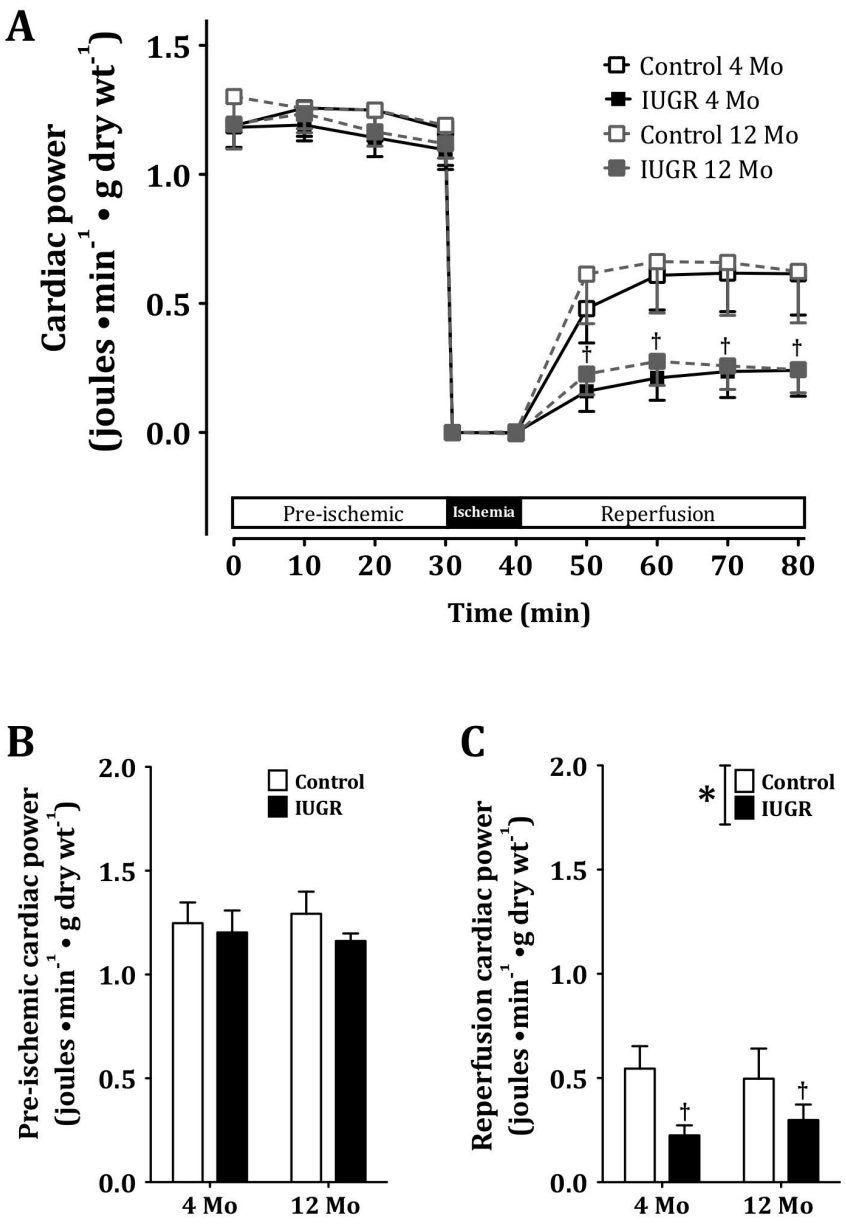


Figure 5-2 *Ex vivo* cardiac power development during pre-ischemic and reperfusion periods in adult female offspring

(**A**) Average cardiac power developed over time during *ex vivo* cardiac aerobic perfusion (pre-ischemia) and after 10 minutes of no-flow ischemia (reperfusion) from different experimental groups, (**B**) average maximal cardiac power developed during pre-ischemia period, (**C**) average maximal cardiac power developed during reperfusion. Values obtained from 9 to 11 animals from different litters per group at 4 and 12 months (Mo) of age. * represents a value of $p<0.05$ for the respective sources of variation (age or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

Table 5-1 ***Ex-vivo*** **cardiac function parameters**

	4 Months		12 Months		ANOVA		
Male offspring	**Control n=14**	**IUGR n=15**	**Control n=19**	**IUGR n=14**	**Age**	**IUGR**	**Int**
CO Pre-ischemia	129.6 (7.3)	126.4 (6.1)	115.0 (3.4)	119.5 (11.1)			
CO Reperfusion	71.9 (14.7)	48.5 (12.8)	36.8 (6.4)	12.6 (7.7)	*	*	
CF Pre-ischemia	58.4 (4.6)	78.6 (15.7)	64.2 (5.0)	65.2 (9.9)			
CF Reperfusion	50.5 (5.5)	53.5 (14.5)	42.7 (4.6)	20.7 (6.7)	*		
LVDP Pre-ischemia	28.7 (2.5)	27.6 (2.2)	34.7 (2.4)	26.9 (3.1)			
LVDP Reperfusion	29.1 (3.9)	23.0 (2.1)	29.1 (2.7)	18.1 (2.5)		*	
Female offspring							
	n=14	**n=14**	**n=16**	**n=16**			
CO Pre-ischemia	127.0 (2.5)	135.0 (6.2)	137.5 (11.8)	133.2 (7.1)			
CO Reperfusion	45.5 (4.2)	29.3 (8.6)	48.6 (13.9)	26.1 (8.0)		*	
CF Pre-ischemia	59.6 (6.2)	82.9 (6.4)	76.3 (7.1)	77.0 (10.2)			
CF Reperfusion	38.0 (10.2)	52.5 (10.7)	45.3 (8.9)	44.8 (10.0)			
LVDP Pre-ischemia	18.7 (2.3)	16.9 (2.0)	23.7 (1.9)	23.4 (1.6)	*		
LVDP Reperfusion	21.1 (4.5)	14.5 (2.4)	20.1 (1.6)	18.7 (2.5)			

Data presented as mean (SE). *Ex vivo* hemodynamic parameters measured during pre-ischemic and reperfusion periods including CO: cardiac output (reported as $mL \bullet min^{-1} \bullet g^{-1}$), CF: coronary flow (reported as $mL \bullet min^{-1} \bullet g^{-1}$) and LVDP: left ventricle developed pressure (reported as mmHg; LVDP) in both male and female offspring. * represents values of $p<0.05$ for the respective sources of variation (age or prenatal intervention) using two-way ANOVA.

5.4.2 Effects of hypoxia-induced IUGR on cardiac energy metabolism

During the pre-ischemic period, the relative contribution of each metabolic substrate to total myocardial ATP production was comparable among all groups regardless of their sex, age or prenatal intervention (Figure 5-3).

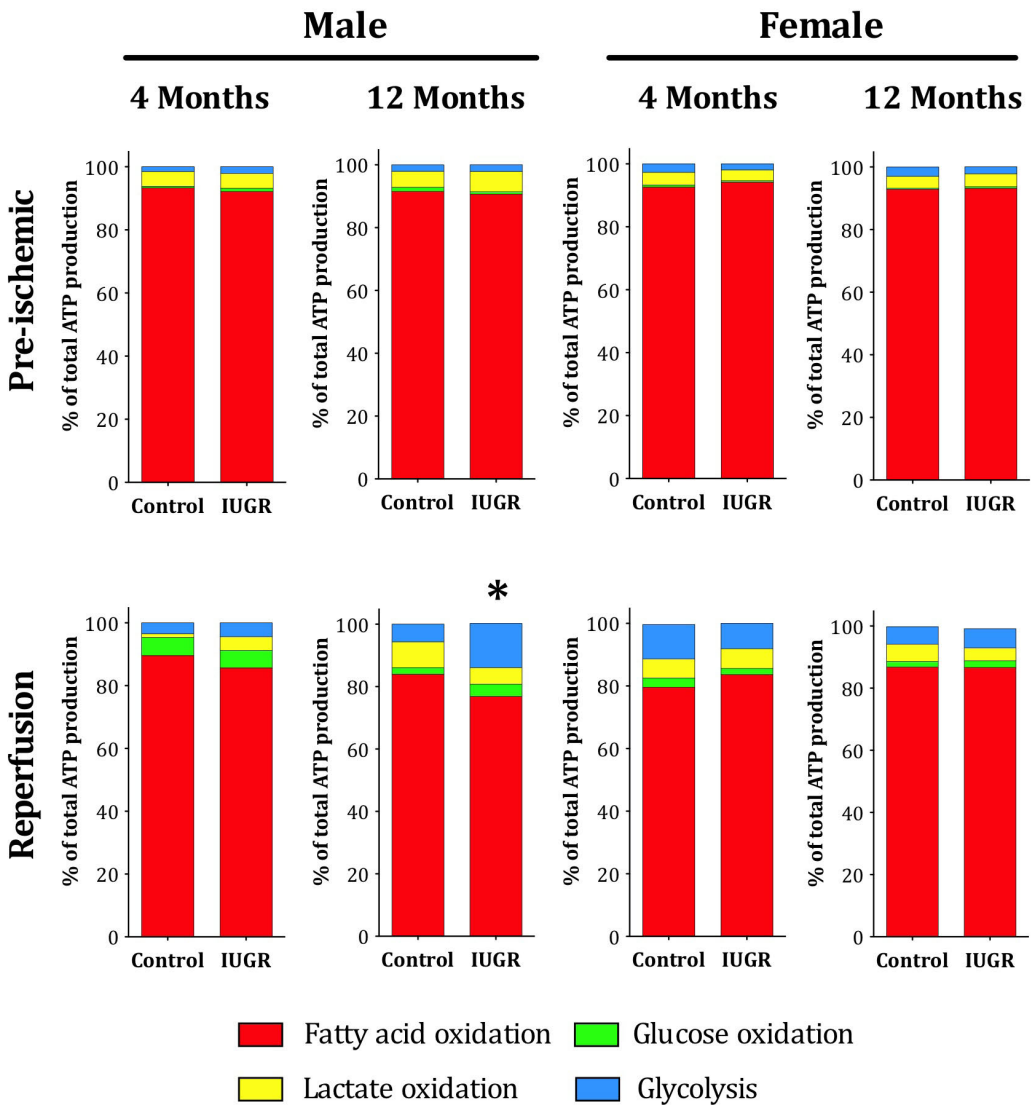


Figure 5-3 Relative contribution of each of the major myocardial energetic substrates in all experimental groups during pre-ischemic and reperfusion periods

* represents significant differences in the proportion of glycolysis relative to controls in the same experimental group during reperfusion. Values of n≥7 per group. Detailed information available in Appendix Table 10.3.

During reperfusion, however, all groups exhibited a decrease in the relative proportion of ATP derived from fatty acid oxidation and a relative increase in the proportion of ATP derived from both the catabolism of carbohydrates and glycolysis. Interestingly, the proportion of energy derived from lactate oxidation remained unchanged in all groups of animals following

I/R injury. Moreover, during this reperfusion period, the proportional change in glycolysis rates relative to glucose oxidation rates (glucose metabolism uncoupling) was consistently higher in 12 month old IUGR male offspring when compared to sex- and age-matched controls (Figure 5-3). Moreover, IUGR offspring from both ages and sexes exhibited an increased production of H^+ during reperfusion when compared to age- and sex-matched controls (Figure 5-4).

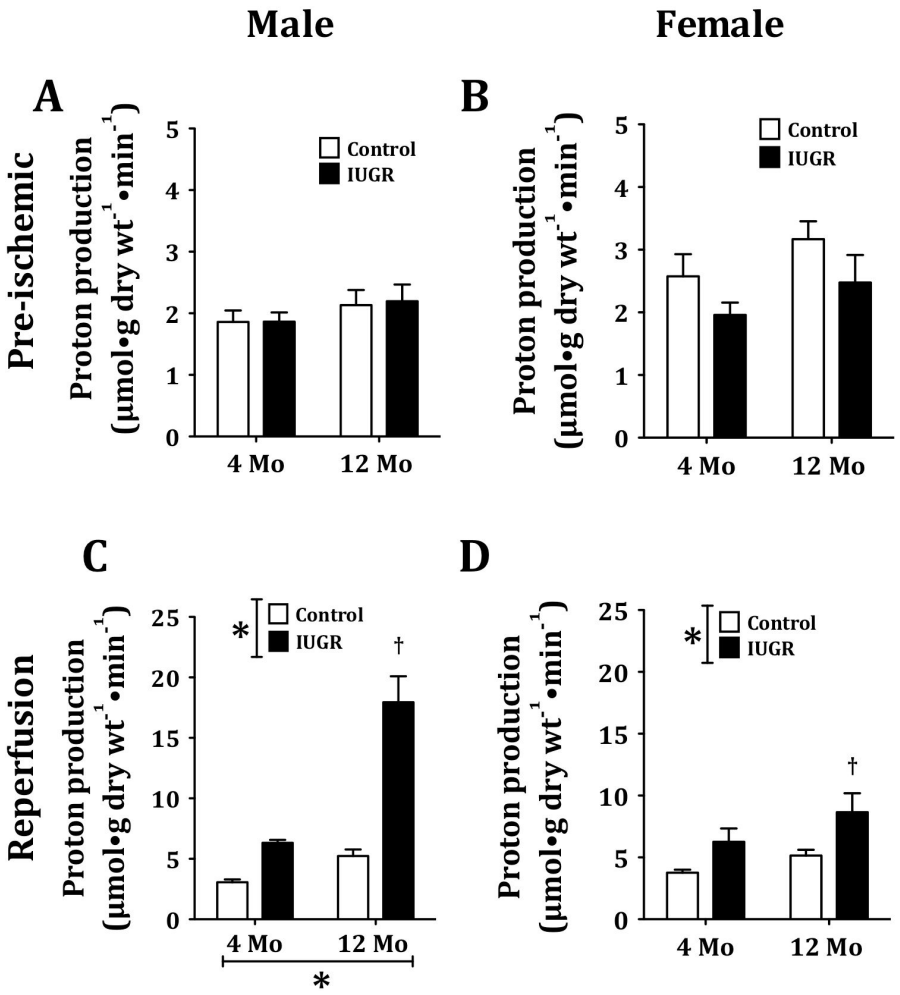


Figure 5-4 Myocardial proton production

Proton production derived from glucose metabolism uncoupling during both the pre-ischemic period (in male (**A**) and female (**B**) offspring) and during reperfusion after 10 minutes of no-flow ischemia (in male (**C**) and female (**D**) offspring). Values obtained from at least seven animals from different litters at both 4 and 12 months (Mo) of age. * represents values of $p<0.05$ for the respective sources of variation (age or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

Interestingly, in male but not female offspring, aging was also associated with a higher post-ischemic degree of glucose metabolism uncoupling and H^+ production independent of the prenatal history of IUGR.

Despite the observed changes in myocardial energy substrate selection, the overall energy production capacity of hearts from IUGR offspring was not compromised. During the aerobic period, the myocardial production of acetyl-CoA was comparable among all experimental groups independent of sex, age or prenatal exposure to hypoxia (Figure 5-5).

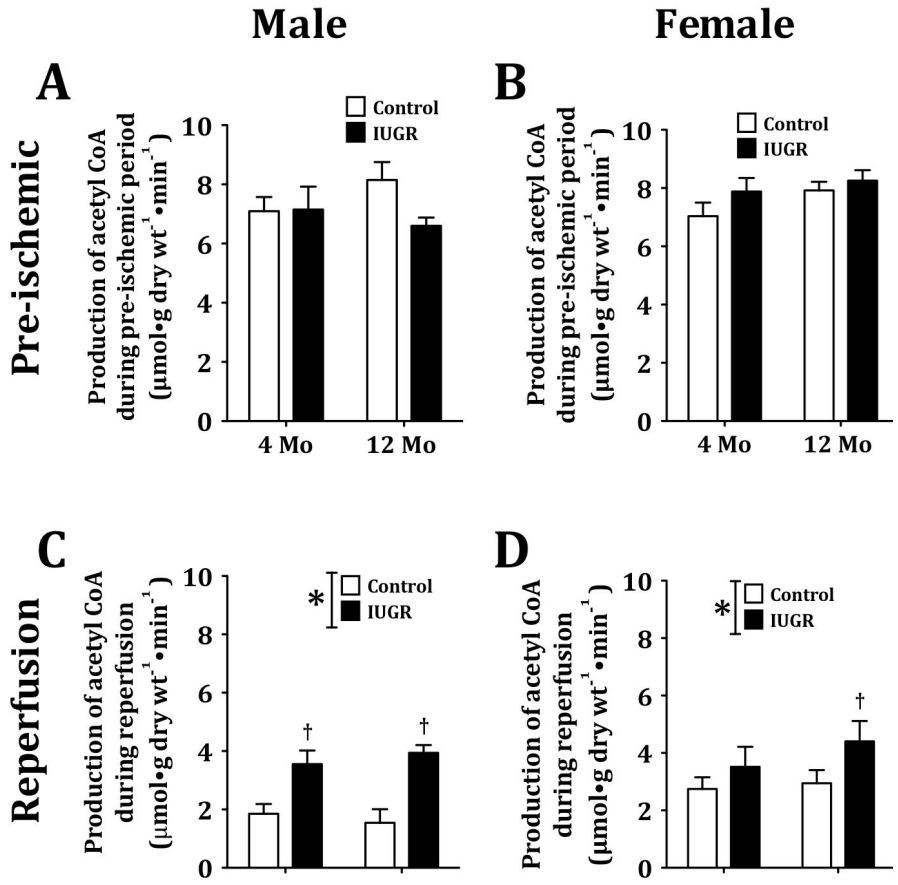


Figure 5-5 Myocardial acetyl-CoA production during both the pre-ischemic and reperfusion periods

Myocardial production of acetyl-CoA during the pre-ischemic period (in male (**A**) and female (**B**) offspring) and during reperfusion after 10 minutes of no-flow ischemia (in male (**C**) and female (**D**) offspring). Values obtained from at least seven animals from different litters at both 4 and 12 months (Mo) of age. * represents values of $p<0.05$ for the respective sources of variation (age or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

As expected, during reperfusion all groups of animals exhibited a decrease in myocardial energy production. However, during this period, offspring born IUGR exhibited an increased myocardial production of acetyl-CoA relative to sex- and age-matched controls (Figure 5-5). The mismatch between the post-ischemic energy production and the amount of cardiac work developed indicates that, in IUGR offspring, cardiac efficiency during reperfusion was notably decreased relative to their respective age-matched controls (Figure 5-6).

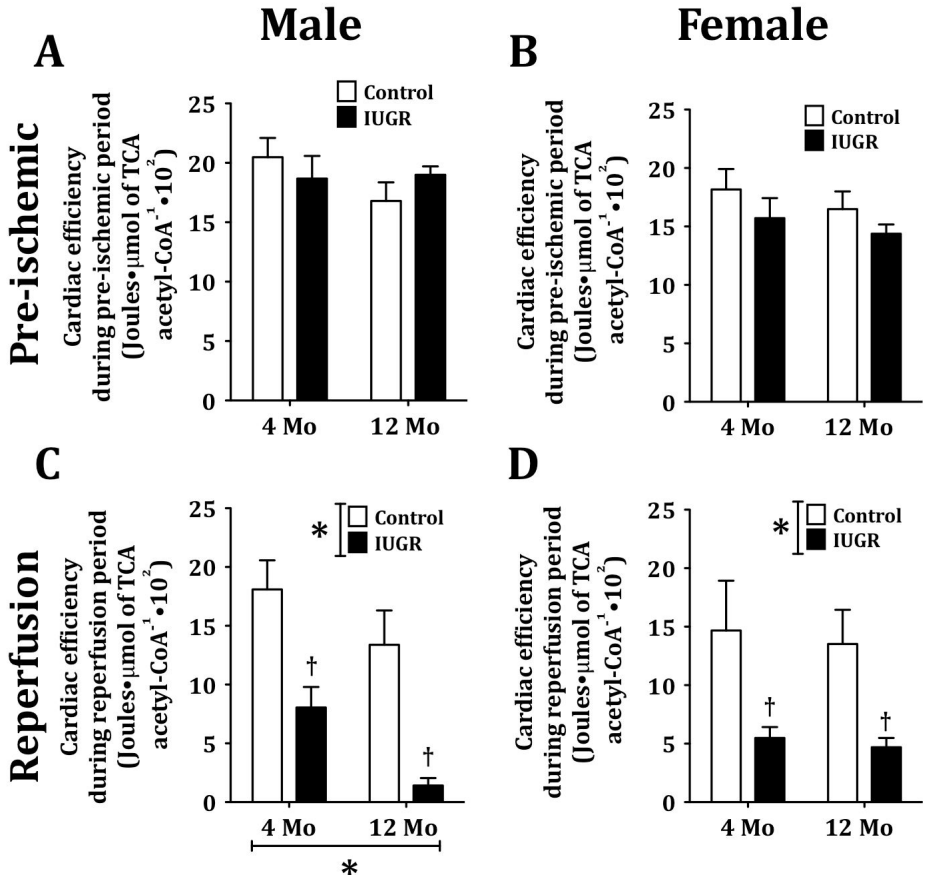


Figure 5-6 Myocardial energetic efficiency during both pre-ischemic and reperfusion periods

Myocardial energetic efficiency reported as amount of work developed per unit of acetyl-CoA during the pre-ischemic period (male (**A**) and female (**B**) offspring) and during reperfusion after 10 minutes of no-flow ischemia (in male (**C**) and female (**D**) offspring. Values obtained from at least seven animals from different litters at both 4 and 12 months (Mo) of age. * represents values of $p<0.05$ for the respective sources of variation (age or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

5.5 Discussion

Consistent with our previous results,[3] we confirmed that the myocardium of IUGR offspring was more susceptible to I/R injury. As a novel contribution, the results presented in this chapter constitute not only the first description of the cardiac metabolic profile in a fetal programming model, but also the first characterization of cardiac metabolism in both male and female offspring at different stages in life (early adulthood and aging). Therefore, it provides valuable information for the understanding of potential interactions between aging and sex in both the physiological changes in cardiac metabolism and the pathophysiology of increased susceptibility to I/R injury observed in adult offspring born IUGR.

5.5.1 Hypoxia-induced IUGR and increased susceptibility to myocardial ischemia/reperfusion injury

One interesting characteristic of this fetal programming model was that cardiac performance during the pre-ischemic period was comparable among groups; which means that the long-term consequences of the prenatal hypoxic insult do not affect baseline cardiac function. However, after exposure to an I/R challenge, adult offspring exposed to prenatal hypoxia demonstrated differences in the cardiac phenotype, which were evident in both male and female offspring as early as four months of age.

Experiments conducted to evaluate sex differences in the cardiac response to I/R injury are controversial. Several studies using the Langendorff perfusion technique have shown that due to the influence of hormones, hearts from female animals are more resistant to I/R injury than males.[16] Moreover, it has been shown that in the presence of pathophysiological conditions such as increased LV wall thickness and hypercontractile states, sex differences can exacerbate the susceptibility to I/R injuries.[17, 18] In contrast, others have described no sex differences in the myocardial susceptibility to ischemia.[16, 19] In our work, we described that in

male but not female offspring, aging was associated with a further increase in susceptibility to I/R injury. However, the deleterious effect of being born IUGR on the myocardial susceptibility to ischemia was very similar in all offspring regardless of their sex or age.

5.5.2 Long-term effects of hypoxia–induced IUGR on cardiac energy metabolism

In terms of cardiac energy metabolism, one of the major findings reported in this chapter was that, relative to controls, IUGR offspring had a significant increase in the amount of glucose that underwent glycolysis relative to the amount of glucose that was oxidized during the reperfusion period. As previously proposed by Neely and coworkers,[20] this uncoupling in myocardial glucose metabolism causes an increase in the amount of H^+ in the cytoplasm; triggering a cascade of compensatory mechanisms to re-establish cellular ionic and acid/base homeostasis, which unfortunately causes an increase in energy expenditure.[21] To prevent cellular damage resulting from a H^+ accumulation and the respective shift in pH, compensatory mechanisms such as Na^+/H^+ exchangers are rapidly activated.[22] These transporters reduce H^+ without using energy but cause an intracellular Na^+ overload. To deal with this overload of Na^+, the myocardium uses two major mechanisms; activation of ATP-dependent ion transporters such as Na/K ATPase, and activation of non ATP-dependent mechanisms such as the Na^+/Ca^{2+} exchanger [23] (which reduce intracellular Na^+ without depleting ATP but cause an increase in intracellular Ca^{2+}). Since an increase in Ca^{2+} levels constitutes a serious hazard for cardiac viability and is one of the major mediators of I/R injury,[24] increased intracellular levels of this ion require the intervention of additional transporters such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to maintain cellular homeostasis at the expense of ATP depletion.[25] Generally speaking, an increase in H^+ production constitutes a major ionic imbalance that requires large amounts of energy to resolve. Therefore, the potential benefits of using the glycolytic pathway to produce energy under anaerobic

conditions may be diminished by the increase in the energetic demands of ion homeostasis. One additional component that could be involved in the cardiac programming phenomenon that we have described, is the activity of membrane-transporters such as the Na/K ATPase pump, Na/H exchanger type 1 (NHE-1), Na/Ca exchangers (NCX) and type 2A SERCA channels. These transporters are responsible for maintaining homeostasis in the cardiomyocyte and could be involved in both the increased susceptibility to I/R injury as well as the increased post-ischemic H^+ production observed in offspring born IUGR. Future experiments are required to evaluate the potential involvement of these particular homeostatic mechanisms in the development of the cardiac phenotype that we have described.

We also observed that despite recovering less than 50% of their initial cardiac work during the reperfusion period, control animals from all groups recovered approximately 70% of their basal energy production capacity. These results are consistent with previous reports showing that during reperfusion, the cardiac activity of the TCA cycle recovers rapidly and to a greater extent than cardiac work.[26, 27] Interestingly, when compared to sex- and age-matched controls, IUGR offspring exhibited an increased capacity to produce energy during reperfusion. Therefore, this finding suggests that the post-ischemic reduction in developed cardiac work described in IUGR offspring cannot be attributed to a decreased ability of the myocardium to produce energy during the post-ischemic period but rather to a higher expenditure of energy in non-contractile processes such as those required to maintain ion balance and cellular homeostasis.

The fact that aged male offspring born IUGR exhibited an increased glucose metabolism uncoupling and increased H^+ production is not completely unexpected given that these animals are known to develop an increase in LV wall thickness (Chapter 4)[13] and similar metabolic alterations have previously been described in hypertrophic hearts.[28] However, that fact that hearts from female offspring born IUGR exhibited similar metabolic

changes in the absence of alterations in myocardial mass or function (Chapter 4)[13] suggests that the changes in myocardial metabolism associated with being born IUGR are not necessarily linked to the development of increased LV wall thickness. Therefore, these two conditions may have parallel and synergistic deleterious effects on cardiac metabolism leading to an increased susceptibility to I/R insult later in life.

5.5.3 Study limitations

One of the limitations of the technique used to evaluate cardiac metabolism in this study was that it only determines the amount of glucose, lactate and fatty acids converted to acetyl-CoA. However, not necessarily all of the acetyl-CoA generated in a cell is used to produce ATP. In fact, there are multiple mechanisms by which the H^+ gradient generated in the membrane of mitochondria can be consumed without producing ATP (a phenomenon that is known as proton leak)[29, 30] which could compromise cardiac efficiency in post-ischemic states.[30] Some authors have suggested that ischemic insults could compromise membrane permeability and increase proton leak in the mitochondria.[31, 32] We chose to use a 10 minute ischemic insult based on preliminary results showing that hearts from aged rats do not recover when exposed to longer ischemic periods. The ischemic insult used in our studies was significantly shorter than insults commonly used in other studies (up to 45 minutes) and may not be enough to cause a significant proton leak in the mitochondria. Moreover, there was a remarkable consistency between the changes in cardiac efficiency and the increase in H^+ production. Together, these results suggest that extra-mitochondrial H^+ production (and not mitochondrial proton leak) may play a major role in the pathophysiology of increased suceptibility to I/R injury observed in IUGR offspring.[33]

5.5.4 Conclusions

Our results suggest that a prenatal hypoxic insult causing IUGR has long-term effects on cardiac susceptibility to I/R injury by causing glucose

metabolism uncoupling. These changes in cardiac metabolism lead to a post-ischemic H^+ overproduction and accumulation in the myocardium that requires significant amounts of energy to compensate for. Our findings could have several important clinical implications. First, it suggests that identifying the population with a history of IUGR or other pregnancy complications leading to fetal hypoxia may be useful for the screening of subjects more susceptible to myocardial ischemic events. Moreover, our results suggest that therapeutic approaches aimed at improving glucose oxidation and decreasing intracellular H^+ accumulation during reperfusion could be particularly beneficial in the management of adults born IUGR who are undergoing a myocardial ischemic episode.

5.6 References

1. Bubb KJ, Cock ML, Black MJ, Dodic M, Boon WM, Parkington HC*, et al.* Intrauterine growth restriction delays cardiomyocyte maturation and alters coronary artery function in the fetal sheep. *J Physiol*. 2007;578:871-881.

2. Xu Y, Armstrong SJ, Arenas IA, Pehowich DJ, Davidge ST. Cardioprotection by chronic estrogen or superoxide dismutase mimetic treatment in the aged female rat. *Am J Physiol Heart Circ Physiol*. 2004;287:H165-171.

3. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

4. Li G, Xiao Y, Estrella JL, Ducsay CA, Gilbert RD, Zhang L. Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *J Soc Gynecol Investig*. 2003;10:265-274.

5. Xu Y, Williams SJ, Armstrong SJ, ST. D. Effects of maternal hypoxia on expression of cardiac mitochondrial F1F0-proton atpaseand postischemic recovery in adult offspring rats. . *Can J Cardiol*. 2005;21:81C (Abstract).

6. Semeniuk LM, Kryski AJ, Severson DL. Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hGLUT4 mice. *Am J Physiol Heart Circ Physiol*. 2002;283:H976-982.

7. Allard MF, Lopaschuk GD. Ischemia and reperfusion injury in the hypertrophied heart. *EXS*. 1996;76:423-441.

8. Broderick TL, Quinney HA, Barker CC, Lopaschuk GD. Beneficial effect of carnitine on mechanical recovery of rat hearts reperfused after a transient period of global ischemia is accompanied by a stimulation of glucose oxidation. *Circulation*. 1993;87:972-981.

9. Cheng JF, Huang Y, Penuliar R, Nishimoto M, Liu L, Arrhenius T*, et al.* Discovery of potent and orally available malonyl-CoA decarboxylase inhibitors as cardioprotective agents. *Journal of Medicinal Chemistry*. 2006;49:4055-4058.

10. Dyck JR, Barr AJ, Barr RL, Kolattukudy PE, Lopaschuk GD. Characterization of cardiac malonyl-CoA decarboxylase and its putative role in regulating fatty acid oxidation. *Am J Physiol*. 1998;275:H2122-2129.

11. Dyck JR, Cheng JF, Stanley WC, Barr R, Chandler MP, Brown S*, et al.* Malonyl coenzyme a decarboxylase inhibition protects the ischemic heart by inhibiting fatty acid oxidation and stimulating glucose oxidation. *Circ Res*. 2004;94:e78-84.

12. Dyck JR, Lopaschuk GD. Malonyl CoA control of fatty acid oxidation in the ischemic heart. *J Mol Cell Cardiol*. 2002;34:1099-1109.

13. Rueda-Clausen CF, Morton JS, Davidge ST. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. *Cardiovasc Res*. 2009;81:713-722.

14. Miller AA, De Silva TM, Jackman KA, Sobey CG. Effect of gender and sex hormones on vascular oxidative stress. *Clin Exp Pharmacol Physiol*. 2007;34:1037-1043.

15. Ordovas JM. Gender, a significant factor in the cross talk between genes, environment, and health. *Gend Med*. 2007;4 Suppl B:S111-122.

16. Gabel SA, Walker VR, London RE, Steenbergen C, Korach KS, Murphy E. Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury. *J Mol Cell Cardiol*. 2005;38:289-297.

17. Zhai P, Eurell TE, Cotthaus RP, Jeffery EH, Bahr JM, Gross DR. Effects of dietary phytoestrogen on global myocardial ischemia-reperfusion injury in isolated female rat hearts. *Am J Physiol Heart Circ Physiol*. 2001;281:H1223-1232.

18. McCully JD, Toyoda Y, Wakiyama H, Rousou AJ, Parker RA, Levitsky S. Age- and gender-related differences in ischemia/reperfusion injury and cardioprotection: effects of diazoxide. *Annals of Thoracic Surgery*. 2006;82:117-123.

19. Cross HR, Murphy E, Koch WJ, Steenbergen C. Male and female mice overexpressing the beta(2)-adrenergic receptor exhibit differences in ischemia/reperfusion injury: role of nitric oxide. *Cardiovasc Res*. 2002;53:662-671.

20. Tani M, Neely JR. Role of intracellular Na+ in Ca2+ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H+-Na+ and Na+-Ca2+ exchange. *Circ Res*. 1989;65:1045-1056.

21. Lopaschuk GD, Wambolt RB, Barr RL. An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts. *J Pharmacol Exp Ther*. 1993;264:135-144.

22. Hata K, Takasago T, Saeki A, Nishioka T, Goto Y. Stunned myocardium after rapid correction of acidosis. Increased oxygen cost of contractility and the role of the Na(+)-H+ exchange system. *Circ Res*. 1994;74:794-805.

23. Kihara Y, Sasayama S, Inoko M, Morgan JP. Sodium/calcium exchange modulates intracellular calcium overload during posthypoxic reoxygenation in mammalian working myocardium. Evidence from aequorin-loaded ferret ventricular muscles. *Journal of Clinical Investigation*. 1994;93:1275-1284.

24. Cross HR, Lu L, Steenbergen C, Philipson KD, Murphy E. Overexpression of the cardiac Na+/Ca2+ exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. *Circ Res*. 1998;83:1215-1223.

25. Talukder MA, Kalyanasundaram A, Zhao X, Zuo L, Bhupathy P, Babu GJ, *et al.* Expression of SERCA isoform with faster Ca2+ transport properties improves postischemic cardiac function and Ca2+ handling and decreases myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2007;293:H2418-2428.

26. Opie LH. Myocardial ischemia--metabolic pathways and implications of increased glycolysis. *Cardiovasc Drugs Ther*. 1990;4 Suppl 4:777-790.

27. Liu B, el Alaoui-Talibi Z, Clanachan AS, Schulz R, Lopaschuk GD. Uncoupling of contractile function from mitochondrial TCA cycle activity and MVO2 during reperfusion of ischemic hearts. *Am J Physiol*. 1996;270:H72-80.

28. Sambandam N, Lopaschuk GD, Brownsey RW, Allard MF. Energy metabolism in the hypertrophied heart. *Heart Fail Rev*. 2002;7:161-173.

29. Brand MD. The proton leak across the mitochondrial inner membrane. *Biochimica et Biophysica Acta*. 1990;1018:128-133.

30. Brand MD, Chien LF, Ainscow EK, Rolfe DF, Porter RK. The causes and functions of mitochondrial proton leak. *Biochimica et Biophysica Acta*. 1994;1187:132-139.

31. Muscari C, Bonafe F, Gamberini C, Giordano E, Lenaz G, Caldarera CM. Ischemic preconditioning preserves proton leakage from mitochondrial membranes but not oxidative phosphorylation during heart reperfusion. *Cell Biochemistry and Function*. 2006;24:511-518.

32. Nadtochiy SM, Tompkins AJ, Brookes PS. Different mechanisms of mitochondrial proton leak in ischaemia/reperfusion injury and preconditioning: implications for pathology and cardioprotection. *Biochemical Journal*. 2006;395:611-618.

33. Borutaite V, Mildaziene V, Brown GC, Brand MD. Control and kinetic analysis of ischemia-damaged heart mitochondria: which parts of the oxidative phosphorylation system are affected by ischemia? *Biochimica et Biophysica Acta*. 1995;1272:154-158.

CHAPTER 6 EFFECTS OF HYPOXIA-INDUCED IUGR ON CARDIAC SIDEROSIS AND OXIDATIVE STRESS ‡‡

6.1 Introduction

Oxidative stress occurs when there is an excess of free radicals or ROS relative to the amount of antioxidants, leading to damage of DNA, proteins, and lipid membranes.[1] ROS-induced cellular damage has been implicated in the development of many pathological conditions, such as aging, T2DM, cardiovascular disease and atherosclerosis among others.[2, 3]

Certain levels of ROS are fundamental for the normal function of the cell and have been implicated in several physiological processes including proliferation, immunity, intra-cellular signaling, transcriptional activation and apoptosis among others.[4] Under certain conditions (both physiological and pathological), ROS production can overcome the buffering capacity of the cell and this condition is known as oxidative stress. Increased levels of oxidative stress can lead to DNA damage and alterations in the structure of several lipids and proteins; which may lead to cellular dysfunction, apoptosis and necrosis.[5] In order to protect themselves from the deleterious effects of ROS, cells have developed several mechanisms to remove excess ROS such as thiol reducing elements (glutathione and thioredoxion) and enzymes such as superoxide dismutase, catalase and glutathione peroxidase.[6]

As previously mentioned, we have demonstrated that adult offspring born IUGR secondary to a prenatal hypoxic insult, exhibit an increase in left-ventricular mass, early *in vivo* signs of heart failure (Chapter 4)[7] and impaired tolerance to myocardial ischemia (Chapter 5). These phenotypical characteristics are compatible with cardiac remodeling[8, 9] and suggest that

‡‡ ***Contribution:*** *Rueda-Clausen C.F. was the project coordinator, performed all the experiments and data analyses.*

myocardial fibrosis could play a role in the development of cardiac pathology observed in these animals. Moreover, previous studies performed by our group using the same animal model,[10] showed that adult male offspring born IUGR exhibit myocardial structural changes characterized by increased deposition of type I and III collagen, an increase in the ß/αMHC ratio and decreased myocardial activity of MMP-2; all of which suggest the presence of an active cardiac fibrosis/remodeling process in offspring born IUGR.[10]

From the assortment of mechanisms that could induce oxidative stress and cardiac remodeling, we decided to study the potential role of iron metabolism and myocardial iron accumulation for a number of reasons: *i*) iron is an essential element with a high reduction-oxidation potential and when accumulated in excess, can cause oxidative stress,[11] *ii*) iron has potential toxic effects and there are no physiological mechanisms to eliminate excess iron accumulations, therefore, the uptake, transport and storage of this element are closely regulated by the organism through a number of mechanisms, some of which can be triggered by hypoxia,[12] *iii*) pathological conditions such as hemochromatosis, in which tissue and plasma iron levels are substantially increased, are characterized by the deposition of iron in multiple organs including the heart[13] and *iv*) experimental models of hemochromatosis in rodents[14] have shown that these animals exhibit a cardiac phenotype that is very similar to that which we have described in adult offspring born IUGR.[7] Moreover, these features are compatible with the most common manifestations of chronic iron toxicity in humans.[13, 15]

6.2 Hypotheses and objectives

Given that hypoxia is one of the major regulators of iron absorption and metabolism,[16] we hypothesize that hypoxia-induced IUGR offspring have long-term changes in their ability to regulate iron metabolism leading to

myocardial iron deposition, induction of myocardial oxidative stress, cardiac remodeling and cardiac dysfunction later in life.

In order to approach this hypothesis, this chapter presents a study designed to assess the presence of myocardial oxidative stress in the myocardium of adult offspring born IUGR, characterize the presence of collagen deposits in the myocardium and clarify the potential role of iron metabolism and myocardial siderosis in the development of the cardiac phenotype that we have previously described in adult offspring born IUGR.

6.3 Methods

6.3.1 Animal model of hypoxia induced IUGR

Female Sprague Dawley rats were mated within the animal facility and treated as described in Section 2.1.1. At birth, litters were reduced to eight pups (four male and four female) and offspring were treated following the IUGR/aging interaction protocol described in Section 2.1.1.2.

6.3.2 Experimental measurements

At 4 or 12 months of age, both male and female rats were anesthetized and blood samples were collected as described in Section 2.2.5. Simultaneously, hearts were excised and perfused using a working heart setup as described in Section 2.2.3. Following perfusion, hearts were either fixed in formalin as described in Section 0 or frozen at -80 °C. Samples fixed in formalin were used to perform histological preparations with either Masson's trichrome or Prussian blue staining as described in Sections 2.2.3.6 and 2.2.3.7, respectively. At the completion of experiments, plasma samples were used to determine circulating markers of systemic iron homeostasis as described in Section 2.2.6. Myocardial samples of frozen specimens were processed to determine myocardial levels of oxidized (GSSG) and reduced (GSH) glutathione (used as a marker of oxidative stress) as well as myocardial iron content as described in Sections 2.2.7 and 2.2.4, respectively.

6.4 Results

6.4.1 Effects of IUGR on myocardial oxidative stress

Overall, in both male and female offspring, IUGR and aging were associated with an increase in the myocardial levels of total glutathione (GSH+GSSG). (Figure 6-1 A and B). The myocardial concentration of GSH, however, presented a dimorphic behavior depending on the sex of the offspring. In male, but not in female offspring, aging was associated with a decrease in the reduced fraction of glutathione (Figure 6-1C and D). This reduction was particularly notable in male offspring born IUGR. However, this change did not reach statistical significance. By presenting these results as the ratio between the oxidized and reduced fractions of glutathione (GSSG/GSH), it is evident that in male, but not in female, offspring both aging and IUGR were associated with an increased presence of myocardial oxidative stress (Figure 6-1G and H).

6.4.2 Intra-myocardial collagen deposition

To better characterize the increase in collagen deposition that has been described in the myocardium from offspring born IUGR,[10] we performed qualitative histological studies with Masson's trichrome staining. Neither in young offspring nor aged female offspring was IUGR associated with changes in cardiac structure or collagen deposition that could be detected in the histological preparations. Aged male offspring born IUGR, on the other hand, exhibited a particular histological pattern characterized by star-shaped collagen depositions in random locations throughout the ventricular wall (Figure 6-2)

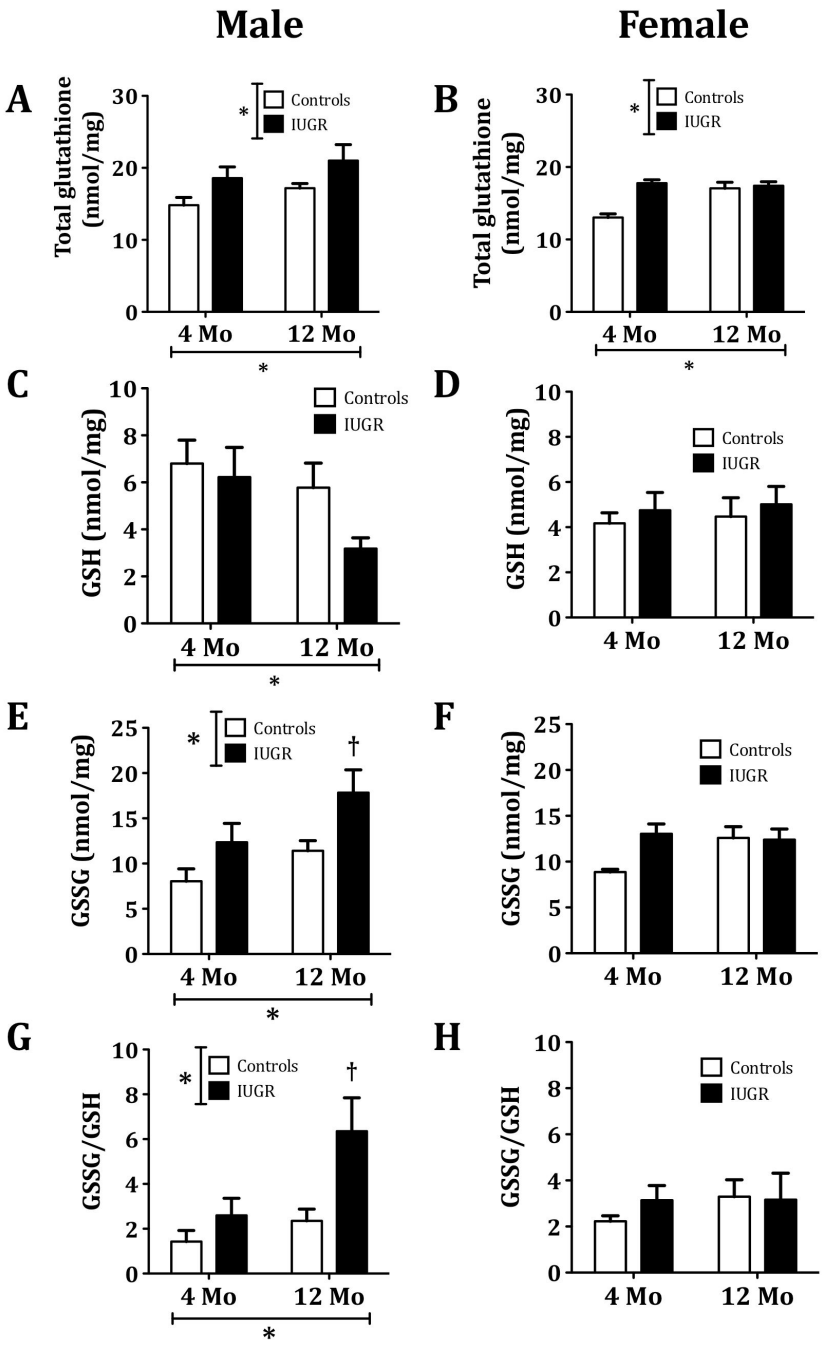


Figure 6-1 Long-term effects of IUGR on the myocardial levels of glutathione

Measurements performed in myocardial samples from Control and IUGR offspring of both sexes at 4 and 12 months (Mo) of age. (**A** and **B**) Total glutathione levels (GSH+GSSG), (**C** and **D**) levels of reduced glutathione (GSH), (**E** and **F**) oxidized glutathione (GSSG) and (**G** and **H**) ratio of reduced to oxidized glutathione (GSH/GSSG). * represents values of $p<0.05$ for the respective sources of variation (IUGR or Age) using two-way ANOVA. † $p<0.05$ vs. controls after a Bonferroni post-hoc test comparing IUGR and control offspring of the same age (n=6 per group).

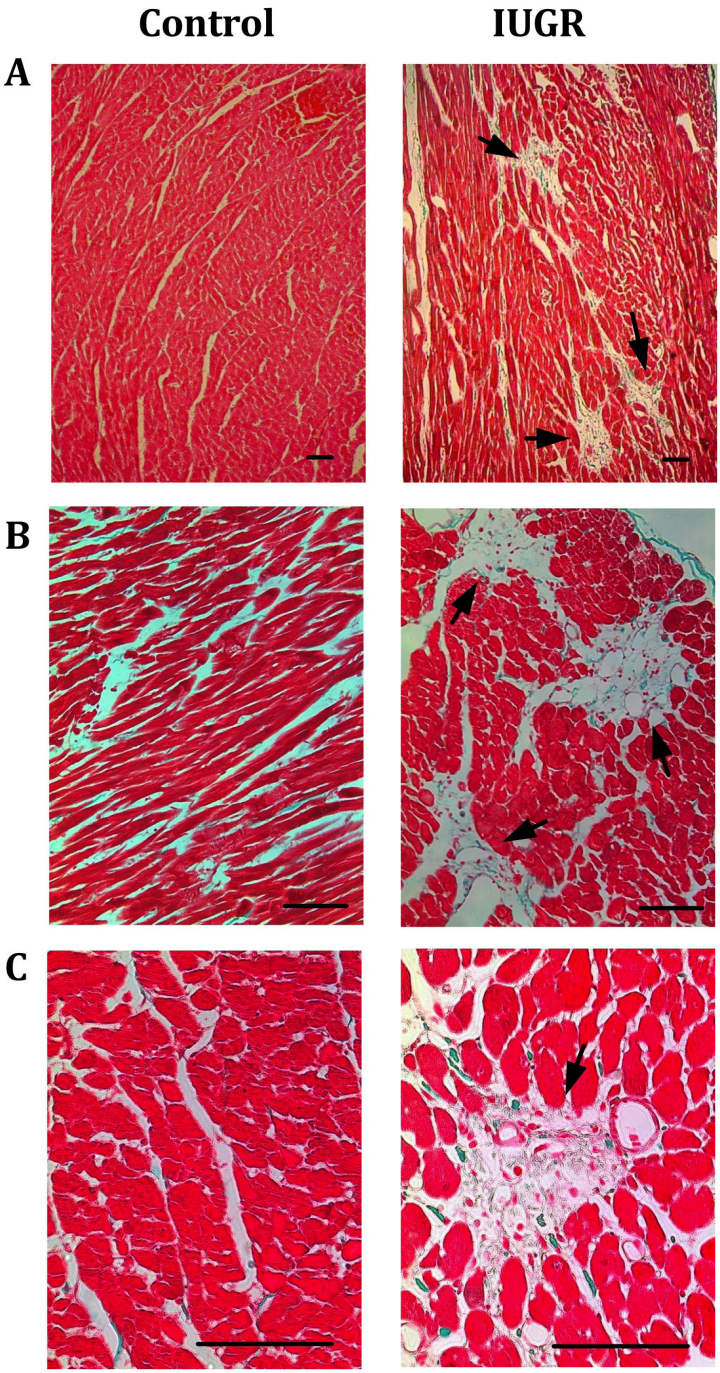


Figure 6-2 Myocardial deposition of collagen in offspring born IUGR at 12 months of age

Representative images of myocardial samples stained with Masson's trichrome in which muscle fibers are stained red, collagen is green, cytoplasm is pink and cell nuclei is dark brown. Images obtained from the inter-ventricular septum of male offspring at 12 months of age using (**A**) low (**B**) mid or (**C**) high magnification. Arrows indicate intra-myocardial areas of collagen deposition that were observed only in aged, male offspring born IUGR. Calibration bars represents 50 μm.

6.4.3 Effect of IUGR on plasma markers of iron homeostasis and myocardial iron accumulation

Deposition of iron in the myocardium was determined in histological preparations with Prussian blue in samples from 10 aged, male offspring (five IUGR and five controls). Interestingly, iron deposits were identified in four of the five IUGR offspring and only in minimal amounts in one of the controls. Moreover, the pattern of iron deposition was characterized by being restricted to regions comparable with fibrotic areas that were randomly distributed throughout the ventricular wall of these animals (Figure 6-3). We also evaluated areas of normal collagen deposition such as the papillary insertion of the tendinous cords from control animals (Figure 6-3D) and observed that iron staining was not present in those regions. This demonstrated that Prussian blue staining did not have a cross-reaction with collagen-rich areas within histological sections.

Neither IUGR nor aging were associated with changes in any of the plasma markers of iron metabolism evaluated in this project (Figure 6-4). Moreover, it was notable that in female offspring, both plasma concentrations of free iron and total iron binding capacity were substantially greater (~50%; $p<0.01$) than those in male rats regardless of which experimental group they belonged to (Figure 6-4A, B, E and F). Both ferritin levels and iron saturation indexes showed a trend towards an increase in male offspring born IUGR, however, these differences did not reach statistical significance.

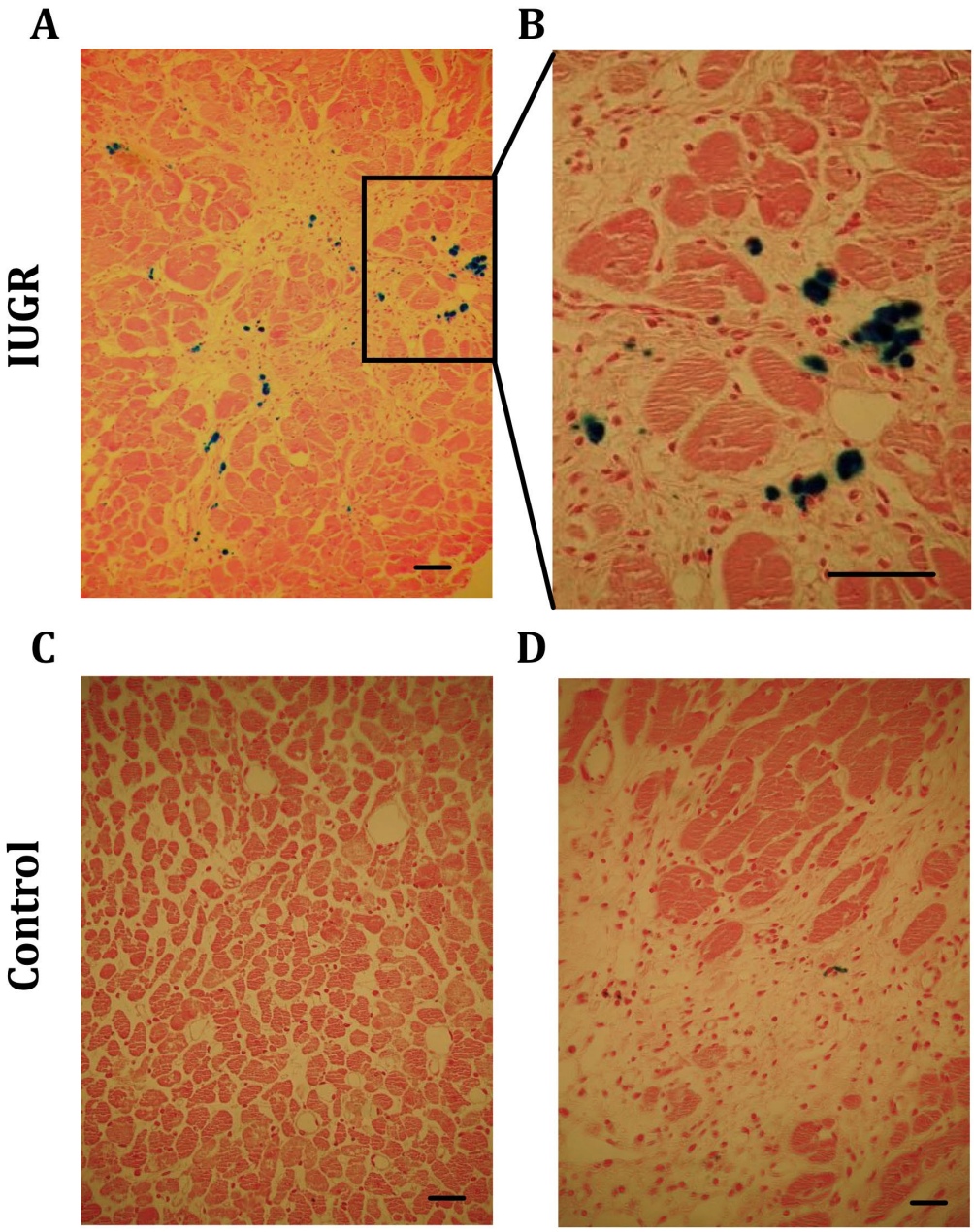


Figure 6-3 Myocardial accumulation of iron in offspring born IUGR at 12 months of age

Representative images of myocardial slides stained with Prussian blue to detect iron accumulation (blue). (**A** and **B**) Representative images showing isolated iron deposits in the intra-ventricular septum of IUGR offspring. **C** Representative image obtained in the intra-ventricular septum of a male control of the same age. **D** Representative image obtained in the papillary insertion of the tendinous cord in the left ventricle free wall of a male control. Calibration bars represents 30 μm.

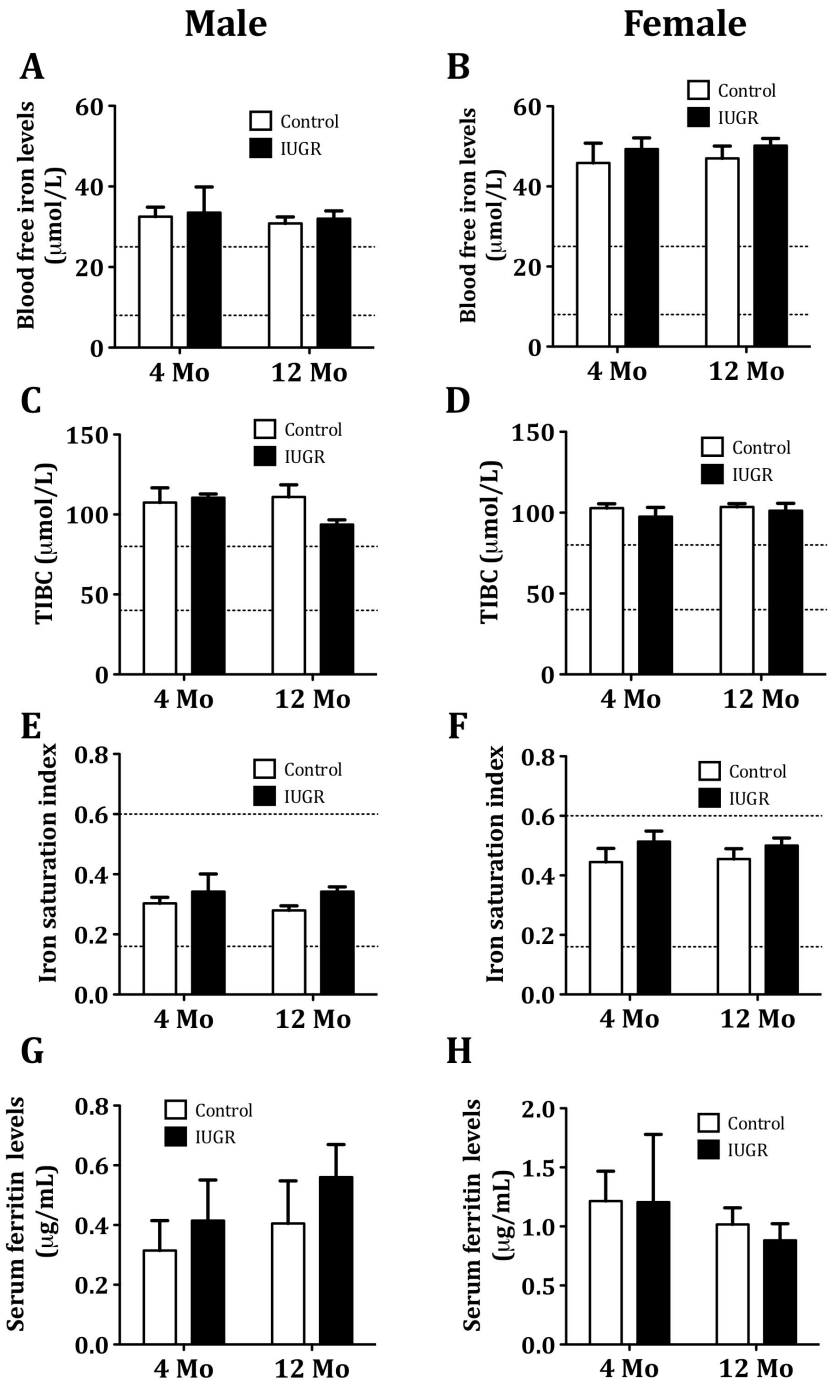


Figure 6-4 Long-term effect of IUGR on plasma levels of iron homeostasis markers

Measurements performed in serum samples from Control and IUGR offspring of both sexes at 4 and 12 months (Mo) of age (n=6 per group). (**A** and **B**) blood free iron levels, (**C** and **D**) total iron binding capacity (TIBC), (**E** and **F**) iron saturation index, (**G** and **H**) serum ferritin levels. **Dashed lines** represent maximum and minimum normal values reported for humans.

In order to confirm the increased tissue deposition of iron in the myocardium described by histological techniques, we performed measurements of iron in myocardial samples from male offspring in all experimental groups using the gold standard technique (mass spectrometry). As summarized in Figure 6-5, aging was associated with an increase in the total iron content; however, contrary to our hypothesis, IUGR did not affect total myocardial iron content.

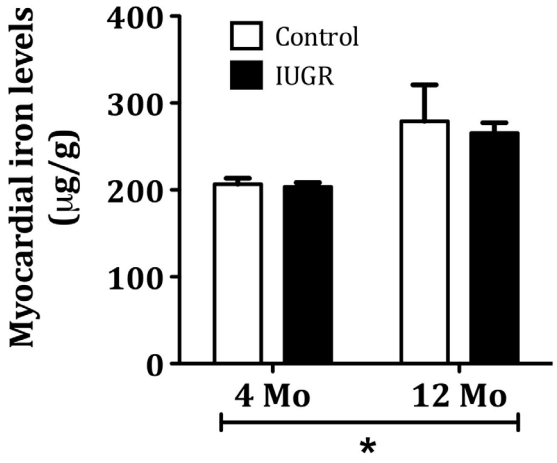


Figure 6-5 Long-term effect of IUGR on myocardial accumulation of iron in male offspring

Measurements performed in left ventricle tissue from Control and IUGR offspring at 4 or 12 months (Mo) of age. * represents values of $p<0.05$ for the respective sources of variation (IUGR or Age) using two-way ANOVA. (n=6 per group) Determinations performed by the Trace Elements Laboratory in London, ON using a High Resolution Sector Field Inductively Coupled Plasma Mass Spectrometer.

6.5 Discussion

6.5.1 IUGR and oxidative stress

To the best of our knowledge, our study presents the first experimental evidence showing that hypoxia-induced IUGR was associated with increased levels of oxidative stress in the myocardium. Previous clinical studies have reported that babies born IUGR, as a result of maternal undernourishment, exhibit increased levels of oxidative stress at birth[17]

Moreover, experimental evidence has demonstrated that being born IUGR as a result of a maternal nutritional restriction, was associated with increased susceptibility to cardiac I/R injury.[18] Interestingly, the same authors also showed that inducing antioxidant mechanisms in the offspring could reverse susceptibility to ischemia.[19] Together, these results suggest that programmed increases in myocardial oxidative stress (either as a result of excessive production of ROS or decreased activity of antioxidant mechanisms) could play a fundamental role in the phenotypical characteristics observed in offspring born IUGR.

Glutathione is a key intracellular tripeptide thiol composed of glutamic acid, cysteine, and glycine.[20] Glutathione helps protect cells from free radical damage by acting as an antioxidant. Within cells, glutathione exists in reduced (GSH) and oxidized (GSSG) states.[21] Reduced glutathione's thiol group provides reducing equivalents to other unstable ROS, which in turn then becomes unstable itself. This unstable GSH readily reacts with another unstable GSH to form a stable GSSG molecule.[21]

In our study we found that IUGR was associated with a consistent and significant increase in the total levels of glutathione, which suggests that these tissues exhibit an up-regulation of the expression of this tripeptide probably as a result of being exposed to higher levels of oxidative stress.[22, 23] In healthy myocardial cells, more than 90% of the total glutathione pool is normally found in the reduced form due to the activity of the enzyme glutathione reductase.[24] This enzyme is constitutively active and, in addition, can be induced by oxidative stress.[25] An increased GSSG/GSH ratio is considered a marker of oxidative stress.[25] Interestingly, our results showed that close to 50% of all the glutathione in the myocardium of all our experimental groups was in the oxidized form. This finding could be a consequence of the measurement technique; since the tissues used for these determinations were collected after reperfusion, a condition in which oxidative stress is known to be increased.[26, 27] Additionally, it is possible that

the increased susceptibility to ischemic injuries described in these animals may be due to increased levels of oxidative stress in the pre-ischemic heart, or that increased levels of oxidative stress in the myocardium are the result of the decreased recovery after ischemia. The results from female aged offspring provides an interesting finding given that, in this particular group of animals, IUGR was associated with a decreased recovery after a 10 minute ischemic challenge but was not associated with changes in intra-myocardial markers of oxidative stress.

6.5.2 IUGR cardiac fibrosis and iron deposition

The results obtained using Masson's trichrome histological preparations demonstrated that, in male offspring, IUGR and aging interact to produce a particular cardiac phenotype characterized by multiple isolated areas of intra-ventricular collagen deposition. These results are consistent with our previous observations demonstrating that IUGR produced a change in the collagen deposition pattern in adult offspring.[28] Moreover, histological preparations with Prussian blue offered a promising preliminary result showing that myocardial iron accumulation could be involved in the etiology of the cardiac phenotype observed in animals born IUGR.

In addition to these histological results, the strong and close association between hypoxia and iron metabolism supported the hypothesis that hypoxic prenatal insults could produce long-term effects on iron metabolism.[16, 29] Contrary to our hypothesis, and despite the histological findings suggesting that IUGR could be associated with an increased accumulation of iron in the myocardium, we could not identify differences in the levels of iron in the myocardium of IUGR offspring as measured using high resolution inductively coupled plasma mass spectrometry, which is the gold standard technique to perform this kind of measurement. It is plausible that the samples of myocardium used for these determinations (100 mg per sample) were not representative of the total myocardial tissue and failed to

include areas of iron deposition. However, all samples were collected from approximately the same anatomical location in the LV free wall where fibrotic clusters and iron deposits were observed.

6.5.3 Conclusions

The present study suggests that hypoxic insults leading to IUGR produce long-term effects on the levels of oxidative stress in the post-ischemic myocardium of male offspring. We also demonstrated that in male offspring, IUGR and aging interact and produce an interesting cardiac phenotype characterized by isolated intra-myocardial clusters of collagen and iron deposition. Contrary to these histological results, studies to evaluate cardiac siderosis showed no changes in total iron accumulation in the myocardium. However, the potential effects of IUGR on myocardial oxidative stress and siderosis warrant further investigation. Moreover, further studies are required to understand the specific mechanisms leading to the observed changes in the cardiac structure of offspring born IUGR.

6.6 References

1. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev*. 2002;82:47-95.

2. Montuschi P, Barnes P, Roberts LJ, 2nd. Insights into oxidative stress: the isoprostanes. *Curr Med Chem*. 2007;14:703-717.

3. Atabek ME, Vatansev H, Erkul I. Oxidative stress in childhood obesity. *J Pediatr Endocrinol Metab*. 2004;17:1063-1068.

4. Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med*. 2010;48:749-762.

5. Sedelnikova OA, Redon CE, Dickey JS, Nakamura AJ, Georgakilas AG, Bonner WM. Role of oxidatively induced DNA lesions in human pathogenesis. *Mutation Research*. 2010;704:152-159.

6. White JF, Jr., Torres MS. Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? *Physiol Plant*. 2010;138:440-446.

7. Rueda-Clausen CF, Morton JS, Davidge ST. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. *Cardiovasc Res*. 2009;81:713-722.

8. Dukanovic N, Jakovljevic V, Mujovic VM. Evaluation of myocardial relaxation in conditions of cardiac remodeling. *Med Pregl*. 2009;62:555-568.

9. Paulus WJ, van Ballegoij JJ. Treatment of heart failure with normal ejection fraction: an inconvenient truth! *J Am Coll Cardiol*. 2010;55:526-537.

10. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

11. Liu Q, Sun L, Tan Y, Wang G, Lin X, Cai L. Role of iron deficiency and overload in the pathogenesis of diabetes and diabetic complications. *Curr Med Chem*. 2009;16:113-129.

12. Gray SG, Crowe J, Lawless MW. Hemochromatosis: as a conformational disorder. *Int J Biochem Cell Biol*. 2009;41:2094-2097.

13. Shander A, Cappellini MD, Goodnough LT. Iron overload and toxicity: the hidden risk of multiple blood transfusions. *Vox Sang*. 2009;97:185-197.

14. Oudit GY, Sun H, Trivieri MG, Koch SE, Dawood F, Ackerley C*, et al.* L-type Ca2+ channels provide a major pathway for iron entry into cardiomyocytes in iron-overload cardiomyopathy. *Nat Med*. 2003;9:1187-1194.

15. Lee PL, Beutler E. Regulation of hepcidin and iron-overload disease. *Annu Rev Pathol*. 2009;4:489-515.

16. Anderson GJ, Frazer DM, McLaren GD. Iron absorption and metabolism. *Curr Opin Gastroenterol*. 2009;25:129-135.

17. Gupta P, Narang M, Banerjee BD, Basu S. Oxidative stress in term small for gestational age neonates born to undernourished mothers: a case control study. *BMC Pediatr*. 2004;4:14.

18. Elmes MJ, Gardner DS, Langley-Evans SC. Fetal exposure to a maternal low-protein diet is associated with altered left ventricular pressure response to ischaemia-reperfusion injury. *Br J Nutr*. 2007;98:93-100.

19. Elmes MJ, McMullen S, Gardner DS, Langley-Evans SC. Prenatal diet determines susceptibility to cardiac ischaemia-reperfusion injury following treatment with diethylmaleic acid and N-acetylcysteine. *Life Sci*. 2008;82:149-155.

20. Henderson BC, Tyagi SC. Oxidative mechanism and homeostasis of proteinase/antiproteinase in congestive heart failure. *J Mol Cell Cardiol*. 2006;41:959-962.

21. Parodi O, De Maria R, Roubina E. Redox state, oxidative stress and endothelial dysfunction in heart failure: the puzzle of nitrate-thiol interaction. *J Cardiovasc Med (Hagerstown)*. 2007;8:765-774.

22. Sharma S, Dewald O, Adrogue J, Salazar RL, Razeghi P, Crapo JD*, et al.* Induction of antioxidant gene expression in a mouse model of ischemic cardiomyopathy is dependent on reactive oxygen species. *Free Radic Biol Med*. 2006;40:2223-2231.

23. Yeh CT, Ching LC, Yen GC. Inducing gene expression of cardiac antioxidant enzymes by dietary phenolic acids in rats. *J Nutr Biochem*. 2009;20:163-171.

24. Fineschi V, Baroldi G, Centini F, Cerretani D, Fiaschi AI, Micheli L*, et al.* Markers of cardiac oxidative stress and altered morphology after intraperitoneal cocaine injection in a rat model. *Int J Legal Med*. 2001;114:323-330.

25. Tsutsui H, Kinugawa S, Matsushima S. Mitochondrial oxidative stress and dysfunction in myocardial remodelling. *Cardiovasc Res*. 2009;81:449-456.

26. Hariharan N, Zhai P, Sadoshima J. Oxidative Stress Stimulates Autophagic Flux during Ischemia/Reperfusion. *Antioxid Redox Signal*. 2010.

27. Zhao Y, Zhao B. Protective Effect of Natural Antioxidants on Heart Against Ischemia-Reperfusion Damage. *Curr Pharm Biotechnol*. 2010.

28. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

29. Ben-Yosef Y, Miller A, Shapiro S, Lahat N. Hypoxia of endothelial cells leads to MMP-2-dependent survival and death. *Am J Physiol Cell Physiol*. 2005;289:C1321-1331.

CHAPTER 7 EFFECT OF HYPOXIA-INDUCED IUGR ON LATER SUSCEPTIBILITY TO HIGH-FAT DIET-INDUCED METABOLIC SYNDROME [§§]

7.1 Introduction

As mentioned in the introduction (Section 0), the proportion of young adults with obesity has increased dramatically in recent decades.[1] Moreover, several obesity-related pathologies (most of which are components of the MetS) are reaching record prevalence among youth and are resulting in substantial costs for the health care system.[2] Although most elements of the MetS have a complex pathophysiology characterized by both a strong influence of inherited factors[3] and environmental variables,[4] it is clear that behaviors such as decreased physical activity and consuming high-fat (HF) and hypercaloric western diets also play a major role in their pathophysiology.[5]

Interestingly, there is a tremendous variability in the responses of different individuals to similar environmental, nutritional and behavioral conditions;[6] which have primarily been attributed to the effect of genetic differences.[7] A growing body of evidence, however, suggests that adverse environmental conditions during crucial periods of development may also predispose individuals to exhibit different components of the MetS in adulthood.[8] In addition, several animal models using a variety of prenatal nutritional insults (such as global food restriction, protein restriction,

---

[§§] *Most components of this chapter have previously been submitted for publication as:*

- *"**Rueda-Clausen CF***[*]*, Dolinsky VW*[*]*, Morton JS, Dyck JRB and Davidge ST. Intrauterine growth restriction increases the susceptibility of rats to high-fat diet-induced metabolic syndrome. in press, Diabetes 2011" (*: shared first authorship)*

Contribution: *Rueda-Clausen CF. was the project coordinator. Except for glucose metabolism studies, lipid determinations and all western blots, which were performed by Dolinsky VW, Rueda-Clausen CF performed all the experiments and data analyses, wrote the first draft of the manuscript and coordinated with the other authors in compiling the final version of the manuscript.*

micronutrient restriction and excess fat feeding) have been used to validate this observation.[9-12] Interestingly, less is known regarding the long-term effects of other prenatal insults such a fetal hypoxia.[13] In fact, there are no studies designed to identify the potential long-term effects of hypoxic insults on the susceptibility to develop obesity and components of the MetS later in life.

While performing the *ex vivo* experiments described in Chapters 4 to 6 of this thesis, we observed that, relative to sex- and age-matched controls, male IUGR offspring tended to accumulate more pericardial and intra-abdominal fat as they aged (Appendix Figure 10-1).

These preliminary results raised the question of whether prenatal hypoxic insults leading to IUGR could have long-term effects on the mechanisms that regulate fat accumulation and deposition as well as other metabolic responses to western lifestyles during adulthood.

7.2 Objectives

Based on our preliminary observations, the objectives of the study presented in this chapter were to investigate whether hypoxia-induced IUGR increased susceptibility to obesity, dyslipidemia and insulin resistance in young adult rats exposed to a HF diet and to examine signal transduction pathways that may be involved in the long-term metabolic effects of being born from pregnancies complicated by hypoxia-induced IUGR.

7.3 Methods

7.3.1 Animal model

Female Sprague Dawley rats were mated within the animal facility and treated as described in Section 2.1.1. At birth, offspring were randomized to receive either low-fat (LF; 10% fat) or high-fat (HF; 45% FAT) diets as indicated in the IUGR/diet interaction protocol described in Section 2.1.1.3. In total, 48 offspring were included in this study (12 animals from six

different litters in each of the four experimental groups).

7.3.2 Experimental measurements

After weaning, food intake and body weight were measured for all animals twice per week. Following nine weeks of nutritional intervention (at 12 weeks of age) *in vivo and ex-vivo* measurements were performed; including whole body composition analysis (Section 2.3.4), indirect calorimetry and physical activity (Sections 2.3.4 and 2.3.5), blood pressure measurements (Section 2.2.8), glucose and insulin tolerance tests (Section 2.3.7), lipid profiles (Section 2.3.12), tissue levels of lipids (Section 2.3.13), evaluation of intra-abdominal adiposity and adipocyte morphometry (Sections 2.3.6 and 2.3.14), evaluation of pancreatic weight and insulin content (Sections 2.3.6 and 2.3.9), determination of circulating factors such as insulin and adipokines (Sections 2.3.8 and 2.3.11) and insulin signaling studies (Section 2.3.10).

7.4 Results

7.4.1 Body weight gain, blood pressure, energy intake and physical activity

As expected, after nine weeks of feeding, offspring on a HF diet gained more weight than those on a LF diet (Figure 10-1A). Exposure to prenatal hypoxia had no effect on body weight gain (Figure 7-1A) or body weight/tibia length ratio of the offspring (Figure 7-1B). Neither IUGR nor HF diet were associated with changes in blood pressure before stress (Table 7-1). However, HF diet was associated with an increase in SBP after an air-puff stress regardless of whether they were IUGR or control offspring. Interestingly, there was a consistent decrease in heart rate in offspring born IUGR relative to controls, and these differences were more noticeable in the HF diet group, particularly following acute stress (Table 7-1).

Despite having similar body weights, food intake was decreased in IUGR offspring independently of the diet received (Figure 7-1C and D). At the same time, however, physical activity was also modestly but significantly reduced in IUGR offspring independent of diet (Figure 7-1E and F); a factor which could account for the lack of differences in weight gain compared to controls.

Table 7-1 Blood pressure and heart rate before and after an air-puff stress in control and IUGR rats fed a low- or high-fat diet for nine weeks

	LF diet		HF diet		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Diet	Int
Before stress							
SBP (mmHg)	143 (10)	132 (5)	148 (9)	140 (7)			
MBP (mmHg)	110 (9)	106 (5)	118 (7)	115 (7)			
Heart rate (bpm)	392 (10)	366 (9)	388 (19)	356 (7)	*		
After stress							
SBP (mmHg)	156 (7)	149 (7)	171 (12)	166 (7)		*	
MBP (mmHg)	122 (6)	120 (7)	132 (11)	133 (6)			
Heart rate (bpm)	396 (8)	377 (9)	412 (12)	364 (13)†	*		
MBP delta after stress (mmHg)	13.3 (5)	17.1 (5)	22.9 (9)	25.0 (7)			

Data presented as mean (SE). Measurements made after nine weeks of either LF: low-fat diet or HF: high-fat diet. SBP: systolic blood pressure, MBP: mean blood pressure. ***** represents values of $p<0.05$ for the respective sources of variation (IUGR and diet) using two-way ANOVA. **†** represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

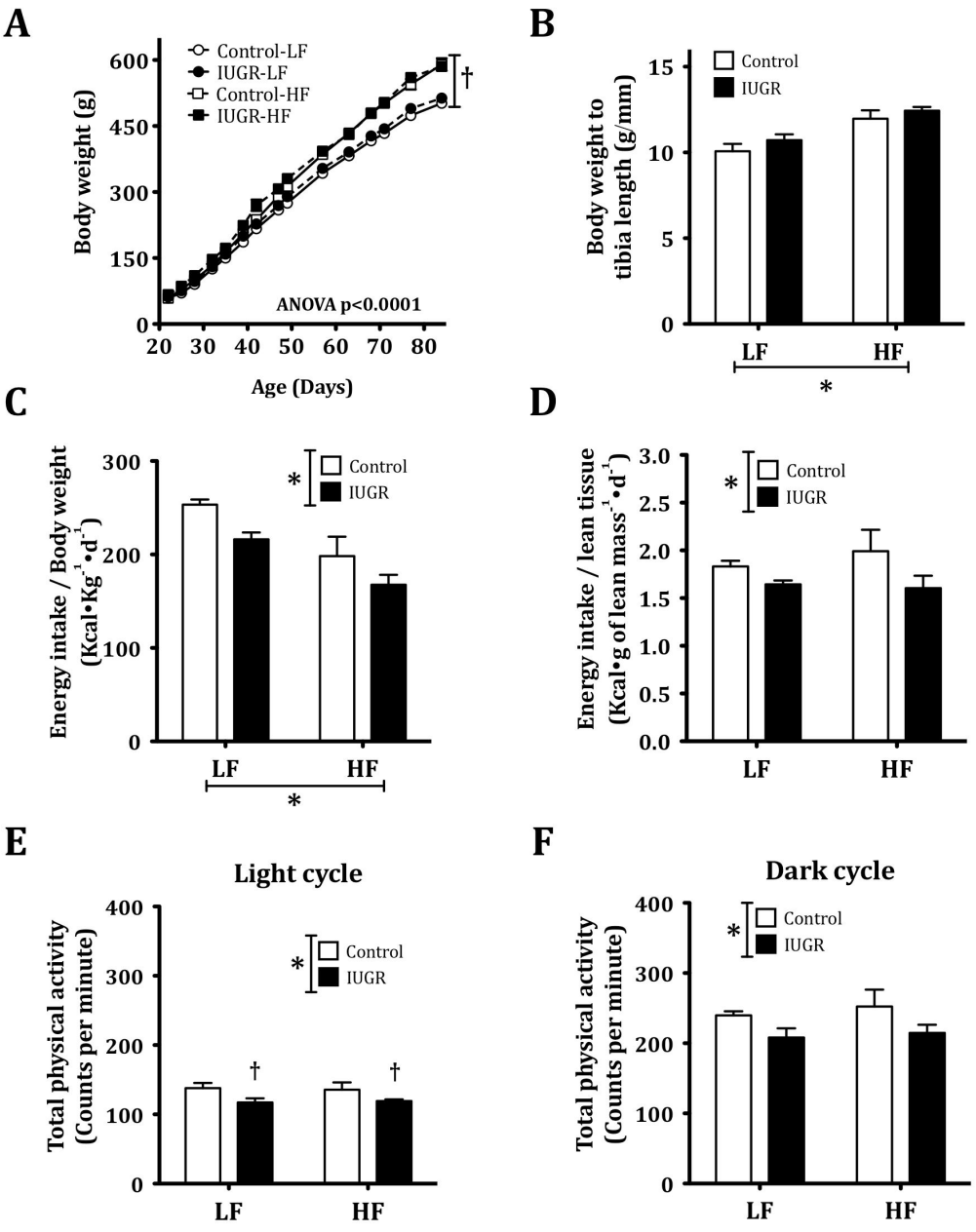


Figure 7-1 Body weight, energy intake and physical activity after nine weeks of nutritional intervention

Effect of intrauterine growth restriction (IUGR) and diet on (**A**) change in body weight over time, (**B**) body weight measured under anesthesia adjusted by tibia length, (**C**) absolute energy intake adjusted by body weight, (**D**) absolute energy intake adjusted by body lean tissue weight calculated by TD-SPEC, total physical activity during (**E**) light and (**F**) dark cycles. LF: low-fat diet, HF: high-fat diet. * represents values of $p<0.05$ for the respective sources of variation (diet or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

Additional assessments of physical activity and energy consumption were performed after four weeks of nutritional intervention (at seven weeks of age). At this point, however, statistically significant differences were not observed among the groups in either of these parameters (Table 7-2). This suggests that reduced physical activity and energy consumption observed after nine weeks of nutritional intervention in offspring born IUGR resulted from an interaction of prenatal conditions and the normal aging process.

Table 7-2 Physical activity and energy intake after four weeks of nutritional intervention

	LF diet		HF diet	
	Control	**IUGR**	**Control**	**IUGR**
Total physical activity light cycle (Counts per hour)	138 (18)	119 (11)	142 (21)	141 (10)
Total physical activity dark cycle (Counts per hour)	297 (26)	250 (28)	268 (31)	263 (15)
Energy intake ($Kcal \bullet Kg^{-1} \bullet day^{-1}$)	263 (33)	290 (14)	243 (25)	259 (19)

Data presented as mean (SE). LF: low-fat diet, HF: high-fat diet. No statistically significant differences were observed by two-way ANOVA (n=6 per group).

All rats receiving a HF diet had a significant decrease in the RER, indicative of greater fatty acid utilization for energy; however, differences were not associated with IUGR when compared to controls receiving the same diet (Table 7-3).

Table 7-3 Gas exchange ratio and heat production in control and IUGR rats fed a low- or high-fat diet

	LF diet		HF diet		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Diet	Int
Light cycle							
VO_2 ($ml \bullet Kg^{-1} \bullet h^{-1}$)	1154 (24)	1050 (23)	1071 (43)	975 (63)	*	*	
VCO_2 ($ml \bullet Kg^{-1} \bullet h^{-1}$)	1143 (37)	1030 (29)	855 (47)	774 (51)	*	*	
RER	0.98 (0.01)	0.97 (0.01)	0.78 (0.02)	0.79 (0.01)		*	
Heat (Kcal/h)	2.83 (0.06)	2.68 (0.06)	2.92 (0.06)	2.74 (0.17)	*	*	
Dark cycle							
VO_2 ($ml \bullet Kg^{-1} \bullet h^{-1}$)	1412 (28)	1322 (27)	1239 (73)	1140 (61)	*	*	
VCO_2 ($ml \bullet Kg^{-1} \bullet h^{-1}$)	1142 (26)	1317 (21)	1027 (79)	942 (55)	*	*	
RER	1.02 (0.01)	0.99 (0.07)	0.82 (0.02)	0.82 (0.01)			
Heat (Kcal/h)	3.5 (0.07)	3.4 (0.08)	3.4 (0.15)	3.2 (0.18)	*	*	

Data presented as mean (SE). LF: low-fat diet, HF: high-fat diet, VO_2: oxygen consumption, VCO_2: carbon dioxide production, RER: respiratory exchange ratio, * represents values of $p<0.01$ for the respective sources of variation such as prenatal hypoxia (IUGR), HF diet (Diet) or their interaction (Int) using two-way ANOVA (n=6 per group).

To further investigate the potential interaction between diet, IUGR, physical activity and energy metabolism we also measured the expression of peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC1-α) and 5' AMP-activated protein kinase (AMPK) in skeletal muscle (Figure 7-2). However, differences were not observed among the experimental groups. These results indicate that changes in the expression/activity of these particular proteins may not be involved in the phenotype observed in these animals.

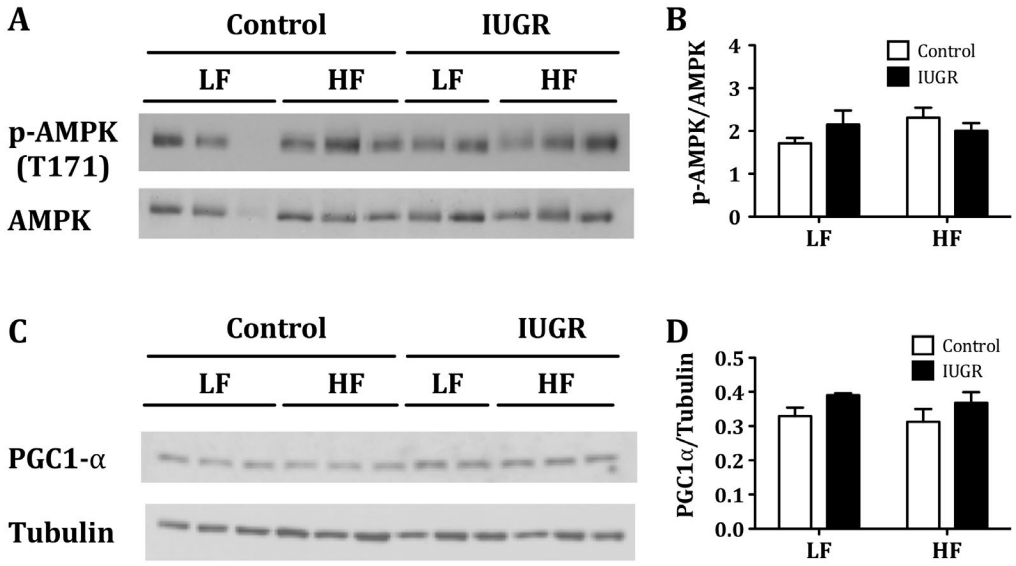


Figure 7-2 Markers of physical activity and energy metabolism in skeletal muscle

Effect of intrauterine growth restriction (IUGR) and diet on markers of physical activity and energy metabolism; including (**A** and **B**) phosphorylation of 5' AMP-activated protein kinase (AMPK) expressed as ratio of p-AMPK/AMPK and (**C** and **D**) expression of peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC1-α) in gastrocnemius muscle from offspring receiving either low-fat (LF) or high-fat (HF) diets for nine weeks (n=6 per group).

7.4.2 Body composition and fat distribution

As predicted, both control and IUGR offspring that received the HF diet had a higher proportion of total body fat mass relative to those receiving the LF diet (Figure 7-3A). In the LF diet group, we did not observe differences in body composition between IUGR and control offspring. Interestingly, the proportion of total body fat in the HF diet group was lower in IUGR offspring with a corresponding increase in the proportion of total lean body mass relative to controls (Figure 7-3A).

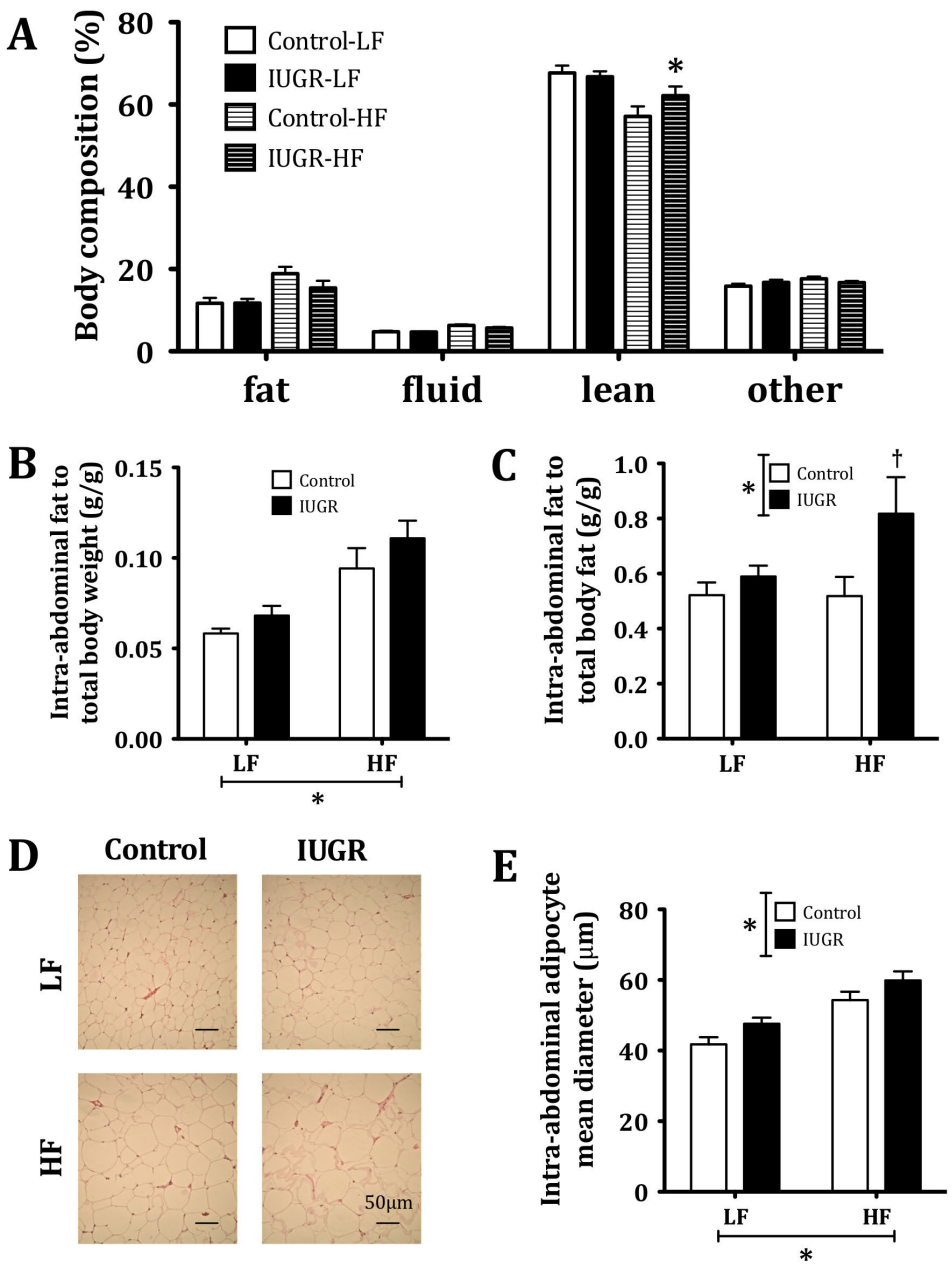


Figure 7-3 Body composition, intra-abdominal fat content and adipocyte size

Effect of intrauterine growth restriction (IUGR) and nine weeks of diet on (**A**) body composition at 12 weeks of age determined by TD-SPEC, (**B**) total intra-abdominal fat adjusted by body weight and (**C**) total body fat weight estimated by TD-SPEC. (**D**) Representative pictures of omental fat tissue (magnification 40X) and (**E**) average intra-abdominal adipocyte diameter. LF: low-fat diet, HF: high-fat diet. * represents values of $p<0.05$ for the respective sources of variation (diet or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

In addition to the reduction in the proportion of body fat, a redistribution of fat tissue was apparent in IUGR offspring; as indicated by the increased intra-abdominal body fat (Figure 7-3B and C). Consistent with this observation, HF diet and IUGR were also associated with a significant increase in the intra-abdominal adipocyte diameter (Figure 7-3D and E).

7.4.3 Circulating adipokines and inflammatory markers

Corresponding to the increased fat mass in the HF-fed groups, plasma concentrations of leptin were elevated in both control and IUGR offspring compared to their respective LF-fed controls (Table 7-4). Moreover, the increase in plasma leptin was more substantial in IUGR offspring fed a HF diet (1.42-fold) compared to HF-fed controls. This finding, together with the observed change in fat distribution, is comparable with previous studies showing that, relative to other fat deposits, intra-abdominal fat may be the most important source of adipokines and cytokines, and may contribute directly to obesity-related metabolic co-morbidities.[14] Interestingly, no differences in circulating concentrations of adiponectin, IL-1β or IL-6 were observed among the experimental groups (Table 7-4). Plasma concentrations of TNFα were below the detectable range in most animals.

Table 7-4 Circulating concentrations of glucose and adipokines in control and IUGR rats fed a low-fat or high-fat diet

	LF diet		HF diet		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Diet	Int
Glucose (mmol/L)	5.1 (0.24)	5.2 (0.18)	5.4 (0.17)	5.6 (0.24)			
Leptin (pg/ml)	4.03 (0.41)	4.36 (0.65)	9.10 (1.3)	12.9 (1.2)†	*	*	
Adiponectin (μg/ml)	2.74 (0.22)	2.90 (0.17)	3.06 (0.18)	3.10 (0.17)			
IL-1β (pg/ml)	145.0 (43.9)	112.7 (47.2)	230.8 (45.9)	181.6 (56.2)			
IL-6 (pg/ml)	46.2 (7.5)	47.2 (7.8)	47.7 (6.2)	35.2 (8.6)			

Data presented as mean (SE). IL: Interleukin, * represents values of $p<0.05$ for the respective sources of variation (diet or prenatal intervention) using two-way ANOVA. † represents $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

7.4.4 Circulating and tissue lipids

As expected, nine weeks of HF diet increased plasma and tissue lipid concentrations in both control and IUGR rats (Table 7-5). However, consistent with the elevated abdominal adiposity in growth-restricted animals, IUGR rats displayed more severe hyperlipidemia than control rats. Indeed, circulating concentrations of TG and FFA were significantly higher in HF-fed IUGR rats compared to HF-fed control rats. In addition, HF-fed IUGR rats also exhibited higher concentrations of TG and ceramides in their livers and gastrocnemius muscles than HF-fed control offspring (Table 7-5). As lipid accumulation is often associated with peripheral insulin resistance,[15] these data suggested that HF-fed IUGR rats could be more glucose intolerant and/or insulin resistant.

Table 7-5 Circulating and tissue lipid concentrations in control and IUGR rats fed a low- or high-fat diet

	LF diet		HF diet		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Diet	Int
Plasma							
Triglycerides (mg/dL)	86 (18)	86 (14)	196 (25)	298 (31)†	*	*	
Cholesterol esters (mg/dL)	48.4 (8.9)	55.7 (3.9)	71.6 (4.2)	72.9 (3.1)		*	
Cholesterol (mg/dL)	29.0 (2.6)	30.0 (1.2)	41.5 (4.2)	43.6 (5.6)		*	
FFA (mmol/L)	0.53 (0.01)	0.51 (0.01)	0.60 (0.01)	0.69 (0.02)†	*	*	*
Liver							
Triglycerides (μg/mg protein)	47 (14)	154 (26)†	158 (20)	249 (32)†	*	*	
Cholesterol esters (μg/mg protein)	80.8 (10.2)	86.7 (2.3)	86.2 (4.6)	84.1 (6.3)			
Cholesterol (μg/mg protein)	22.5 (3.3)	27.0 (2.4)	20.0 (2.0)	27.1 (2.1)	*		
Ceramides (μg/mg protein)	274 (14)	271 (16)	334 (21)	394 (16)†		*	
Gastrocnemius muscle							
Triglycerides (μg/mg protein)	15 (3)	29 (7)	97 (18)	132 (33)†	*	*	*
Cholesterol esters (μg/mg protein)	22.0 (3.3)	26.1 (6.7)	39.1 (7.6)	45.3 (10.4)		*	
Cholesterol (μg/mg protein)	4.4 (0.4)	6.98 (2.7)	8.9 (0.5)	8.1 (1.3)			
Ceramides (μg/mg protein)	60.5 (4.3)	62.2 (3.1)	68.1 (4.6)	86.5 (6.0)†	*	*	

Data presented as mean (SE). FFA: free fatty acids,* $p<0.05$ for the respective source of variation such as prenatal hypoxia (IUGR), nine weeks of diet (Diet) or their interaction (Int) using two-way ANOVA. † represents a $p<0.05$ vs. Control receiving the same diet after a Bonferroni post-hoc test (n=4 to 6 per group).

7.4.5 Glucose homeostasis

Although nine weeks of HF diet did not result in fasting hyperglycemia in any of the experimental groups (Table 7-4), offspring receiving a HF diet had higher fasting plasma insulin concentrations than rats fed a LF diet (Figure 7-4A). Among rats receiving a HF diet, those born IUGR exhibited a substantial increase in both circulating concentrations of insulin (Figure 7-4A) and HOMA index (Figure 7-4B), suggesting that IUGR may predispose

young HF-fed rats to develop whole body insulin resistance earlier than control rats. To investigate this, control and IUGR rats fed either a LF or HF diet were subjected to GTT and ITTs.

Consistent with the normal fasting plasma glucose and insulin concentrations observed, whole body glucose disposal and insulin tolerance were similar in both groups of rats receiving a LF diet (Figure 7-4C and E). In contrast, HF diet reduced glucose tolerance and impaired the response to insulin in both groups of rats relative to those fed a LF diet. However, it is interesting to note that impaired glucose disposal and insulin sensitivity were significantly more pronounced in rat offspring born IUGR and receiving HF diet than in controls under similar dietary conditions (Figure 7-4D and F); further supporting our initial observation that IUGR increases the susceptibility of rats to HF diet-induced insulin resistance.

Although β-cell insulin content can play a role in whole body glucose homeostasis, it is unlikely that this pathway contributed substantially in the IUGR model as differences in the pancreas size and insulin content were not observed among groups (Figure 7-4G and H).

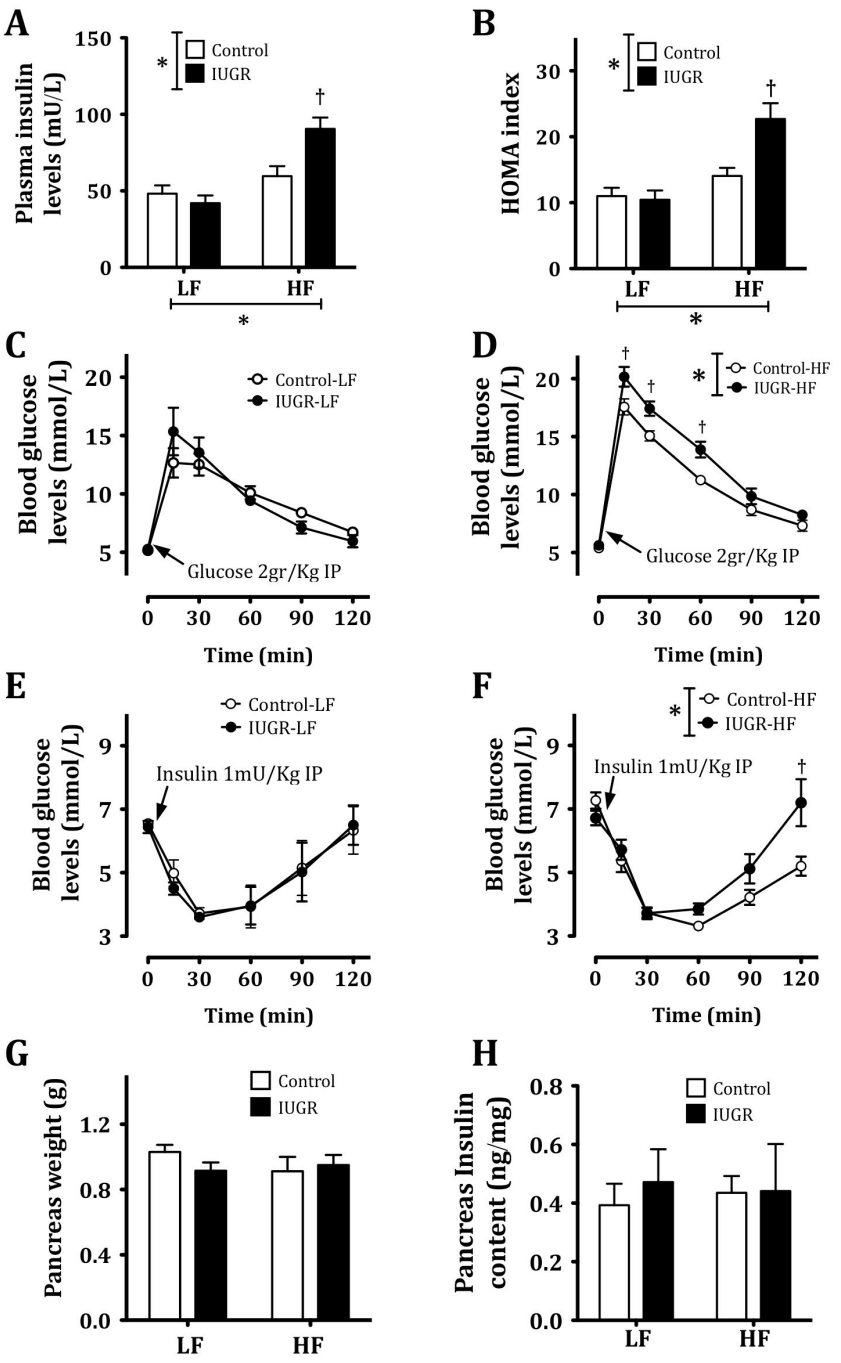


Figure 7-4 Glucose homeostasis and pancreas insulin content

Effect of intrauterine growth restriction (IUGR) and nine weeks of diet on (**A**) plasma insulin concentrations (**B**) HOMA index, (**C** and **D**) glucose tolerance test (GTT) and (**E** and **F**) insulin tolerance test (ITT) in offspring fed a low-fat (LF) or high-fat (HF) diet. (**G**) Pancreas weight and (**H**) pancreas insulin content. * $p<0.05$ for the respective source of variation such as prenatal hypoxia (IUGR) or HF diet (Diet) using two-way ANOVA. † represents a $p<0.05$ vs. Control receiving the same diet after a Bonferroni post-hoc test (n=6 per group).

7.4.6 Insulin signaling

While the precise signaling pathways involved in the induction of insulin resistance are incompletely understood, the major effects appear to be mediated through dysfunctional regulation of the insulin signaling cascade.[16] Therefore, we examined whether the insulin signaling pathway was impaired in HF-fed IUGR rats. First, we determined whether the activity of signaling molecules that regulate insulin-stimulated glucose uptake were altered in HF-fed control and IUGR rats following insulin administration. In agreement with the interpretation of data from GTT and ITT experiments, feeding a HF diet to IUGR rats impaired insulin-stimulated Akt phosphorylation at Ser473 (activation) in both liver and gastrocnemius muscle (Figure 7-5A and B).

To further characterize the source of the signaling defect, we measured phosphorylation of upstream regulators of Akt. Since phosphorylation of insulin receptor substrate 1 (IRS-1) at Ser1101 inhibits the ability of IRS-1 to activate PI-3 kinase and subsequently Akt, we measured phosphorylation at this site. Consistent with our hypothesis, phosphorylation of IRS-1 at Ser1101 in both liver and skeletal muscle was significantly higher in HF-fed IUGR rats compared to HF-fed control rats (Figure 7-5C and D). Furthermore, in the absence of tissue lipid accumulation and subsequent glucose intolerance and insulin resistance, these signaling pathways were not altered in LF-fed IUGR rats relative to controls on the same diet (Figure 7-5).

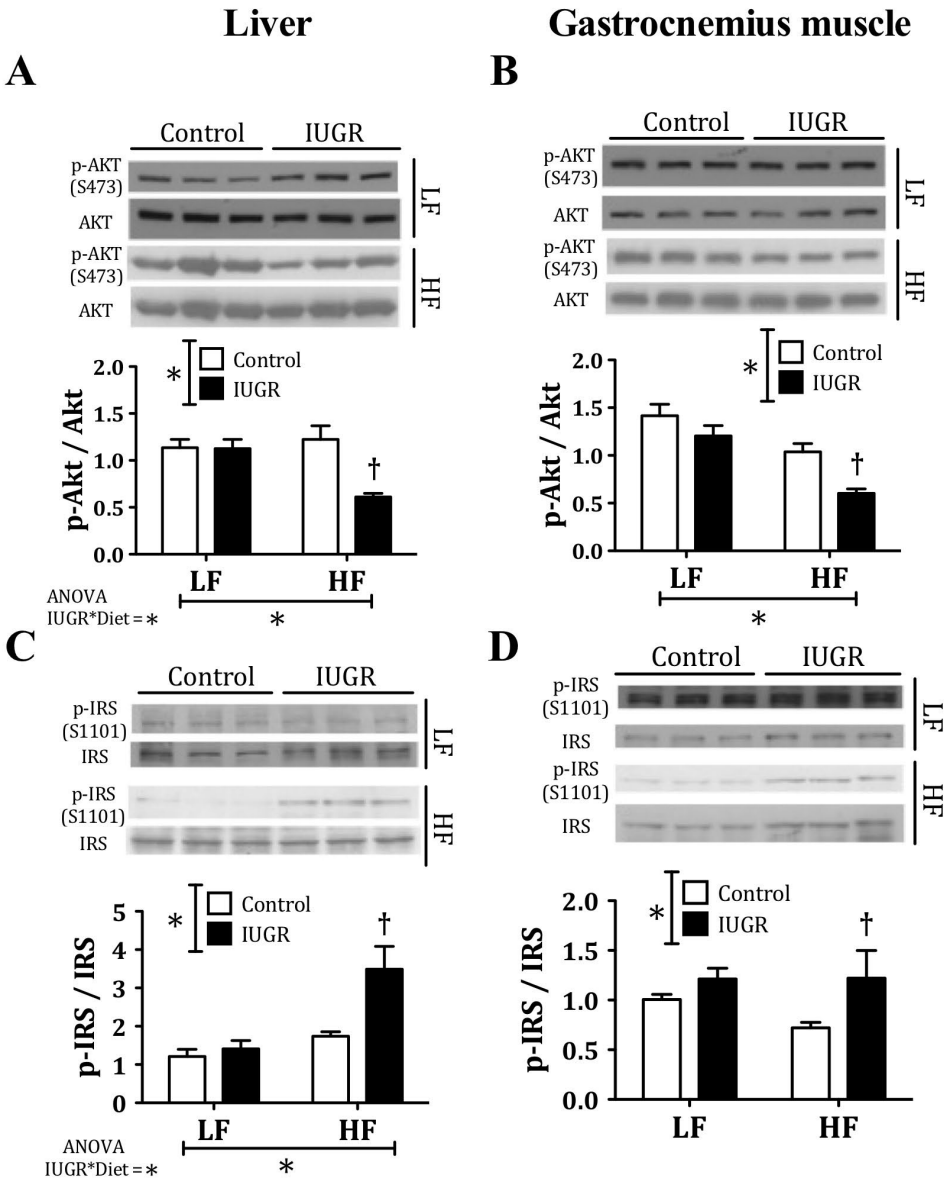


Figure 7-5 Effect of IUGR and diet on phosphorylation of Akt and phosphorylation of the insulin receptor substrate-1 of offspring receiving either a low-fat or high-fat diet

Effect of intrauterine growth restriction (IUGR) and nine weeks of diet on hepatic and skeletal muscle insulin signaling elements; including (**A** and **B**) phosphorylation of AKT (ratio p-Akt/Akt;), (**C** and **D**) phosphorylation of the insulin receptor substrate-1 (ratio p-IRS/IRS) in offspring fed a low-fat (LF) or high-fat (HF) diet. * represents a $p<0.05$ for the respective source of variation such as prenatal hypoxia (IUGR), diet (Diet) or their interaction (ANOVA IUGRxDiet) using two-way ANOVA. † represents a $p<0.05$ vs. Control receiving the same diet after a Bonferroni post-hoc test (n=6 per group).

As phosphorylation of IRS-1 at Ser1101 was known to be directly caused by protein kinase C theta (PKCθ),[17] we measured the phosphorylation of PKCθ at Thr538, which is a requirement for PKCθ activation.[18] Consistent with increased IRS-1 phosphorylation at Ser1101, we observed that liver and muscle P-PKCθ were increased in HF-fed IUGR rats compared to HF-fed control rats and there were no differences in offspring receiving a LF diet (Figure 7-6).

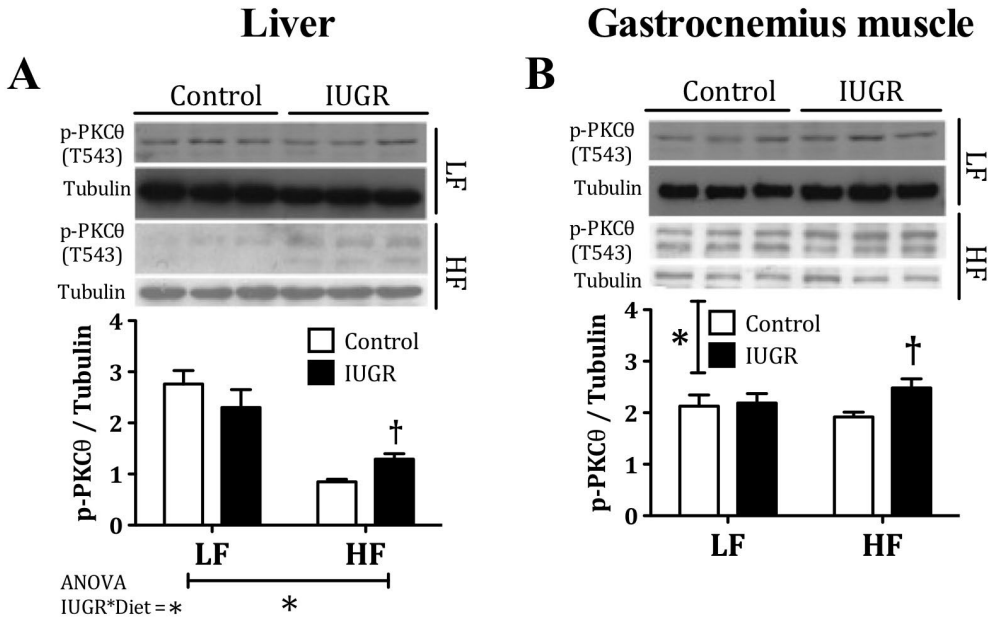


Figure 7-6 Effect of IUGR on phosphorylation of protein kinase C-θ of offspring receiving either a low- or high fat diet

Effect of intrauterine growth restriction (IUGR) and nine weeks of diet on phosphorylation of protein kinase C-θ of (**A**) liver and (**B**) muscle in offspring fed a low-fat (LF) or high-fat (HF) diet. * represents a $p<0.05$ for the respective source of variation such as prenatal hypoxia (IUGR), diet (Diet) using two-way ANOVA. † represents a $p<0.05$ vs. Control receiving the same diet after a Bonferroni post-hoc test (n=6 per group).

7.5 Discussion

This is the first study showing that a hypoxic fetal insult resulting in IUGR can impact the postnatal response to a HF diet. Specifically, we demonstrated that hypoxia-induced IUGR increases the susceptibility to HF diet induced alterations of fat distribution, lipid metabolism and insulin

signaling pathways. The data presented herein provide four important findings. These include: i) IUGR significantly influences fat deposition, with HF-fed IUGR rats having significantly elevated amounts of intra-abdominal fat and larger adipocyte diameters compared to control rats in the absence of changes in whole body adipose tissue mass; ii) IUGR results in a significant increase in plasma and tissue lipid concentrations in response to a HF diet compared to HF-fed controls; iii) IUGR contributes to an increased susceptibility to HF diet-induced insulin resistance by altering skeletal muscle and liver insulin signaling pathways and iv) IUGR results in decreased physical activity relative to control rats independent of diet.

Collectively, our findings highlight the importance of IUGR in the development of MetS and provide considerable insight into why subjects in the western world born IUGR may require closer clinical monitoring and could obtain greater benefit from nutritional interventions designed to reduce the prevalence of cardiovascular risk factors. The important clinical implications of these findings are discussed below.

7.5.1 IUGR, high-fat diet, fat distribution and lipid metabolism

Contrary to the results reported by other groups using nutritional restriction to induce IUGR,[9] offspring exposed to hypoxia did not exhibit an exacerbated weight gain after birth. Moreover, feeding a HF diet to offspring born IUGR did not cause rats to gain more weight or increase total body fat content during the early stages of their lifespan. However, relative to all other groups, rats born growth restricted exhibited a notable increase in the proportion of fat located in the intra-abdominal cavity and had a larger adipocyte diameter when fed a HF diet. Interestingly, these changes in fat distribution and morphology were associated with a significant impairment in lipid profile and glucose homeostasis, which suggests that special attention should be paid to the distribution of fat rather than body weight or total body fat content when evaluating cardiovascular and metabolic risks. Most efforts

toward understanding the regulation of adipogenesis and adipose differentiation have been made *ex vivo* using cultures of pre-adipocytes.[19] Based on previous studies, it has been suggested that adipocyte differentiation is a complex process that can be modulated by multiple stimuli including transcription factors and hormonal signals.[20] Although little is known about the mechanisms that determine adipose tissue distribution or the differential development of fat deposits,[21] our data highlight the potential involvement of prenatal growth as one of the regulators of this process.

Previous studies in rats also demonstrated that, at the time of weaning, the majority of body fat is located in subcutaneous deposits, but as rats age (2 months later) the majority of body fat is located in the abdominal cavity.[22] Although subcutaneous fat weight increases 6-fold between weaning and adulthood, intra-abdominal fat increases 116-fold during the same period of time.[22] Since intra-abdominal fat has such a dramatic rate of increase during postnatal stages, it is possible that the catch-up growth of IUGR pups following their birth may contribute to the greater accumulation of abdominal fat and contribute to later stage metabolic dysfunction in IUGR rats. In addition, IUGR rats exposed to a hypercaloric HF diet (but not a LF diet) developed high circulating concentrations of TG and FFAs, as well as an accumulation of TG and ceramides in the liver and skeletal muscles.[23] These observations demonstrate that the synergistic effects of both IUGR and diet are associated with an early onset of dyslipidemia in this model.

7.5.2 Effects of IUGR and high-fat diet on feeding behavior and physical activity

Previous studies that have evaluated the association between IUGR and the development of obesity concluded that exposure to prenatal insults that lead to IUGR and early postnatal catch-up growth produce long-term changes in neuroendocrine appetite regulation.[24] Interestingly, our study

also describes an increase in circulating concentrations of leptin and reduced calorie intake in offspring born IUGR. These results suggest that, in our IUGR model at 12 weeks of age, the appetite regulatory system and the central effects of leptin were at least partially preserved. Despite the reduced consumption of food, abdominal adiposity was still increased in HF-fed IUGR rats, together with elevated tissue and circulating levels of lipids.

Another interesting finding was that IUGR offspring had a modest decrease in physical activity relative to controls independent of the diet they were receiving. The reduced physical activity was associated with decreased oxygen consumption (VO_2). Since energy consumption was also diminished in IUGR rats, the development of intra-abdominal adiposity and other metabolic changes cannot be entirely attributed to reduced energy expenditure. However, it is well known that exercise can improve dyslipidemia and glucose tolerance[25] and it is plausible that decreased physical activity could be associated with an accumulation of lipids in the tissues and impaired glucose homeostasis in IUGR offspring fed a HF diet. Although increased adiposity and reduced physical activity in adult IUGR rats may be perceived as a pathological response to the hypoxic insult, it is also reasonable to consider that these adaptations could represent compensatory mechanisms programmed during fetal growth. These compensatory mechanisms may be an adaptation aimed to prepare the developing organism to survive in an oxygen depleted environment after birth by increasing the ability to accumulate energy and reduce energy demand through decreased physical activity. Consistent with this observation, it has been reported that teenagers born with extremely low birth weight participate much less in sports and are more sedentary than normal birth weight teenagers, despite reporting an equal enjoyment of physical activity.[26] Whether or not the compensatory adaptation to a growth-restricting environment could also determine the site of adipose tissue deposition is currently unknown.

7.5.3 IUGR, high-fat diet and impaired glucose metabolism

Insulin resistance has previously been associated with both the consumption of a HF diet[27] and being born IUGR.[28] In the present study, we clearly identified an interaction between these two factors by showing that IUGR increases the severity of the hyperinsulinemia and insulin resistance resulting from the challenge of a HF diet. We also show that changes in glucose homeostasis cannot be attributed to the long-term effects of a hypoxic insult on pancreatic development since pancreas size and pancreatic insulin content were comparable among all experimental groups. In the absence of changes in pancreatic insulin content, the increase in circulating levels of insulin could be attributed to an increase in the secretory activity of the pancreatic ß-cells. Moreover, the data presented herein identified defects in the insulin signaling cascade in relevant organs that play pivotal roles in the development of insulin resistance, such as liver and skeletal muscle. As such, these data suggest that nutritional interventions that decrease the consumption of foods high in fat and sucrose, combined with increased physical activity, could be even more beneficial in preventing or ameliorating the detrimental long-term effects of IUGR on lipid and glucose homeostasis. The fact that IUGR animals on a HF diet presented a pronounced hyperinsulinemia without exhibiting changes in pancreatic insulin content is quite an interesting result.

Considering that inflammation has been identified as a potential mediator of obesity induced insulin resistance,[29] it is interesting that no differences were observed in inflammatory markers among the groups. Since these parameters were measured early in life following a short nutritional intervention (nine weeks of diet), it is possible that a pro-inflammatory phenotype had not yet been established in these animals. Interestingly, changes in insulin homeostasis were notable at this point, which suggests that the major synergistic effects of IUGR and HF diet on glucose homeostasis may not be mediated via inflammatory pathways.

Although we have not investigated the temporal relationship between adipose tissue dysfunction, increased circulating TG and FFA concentrations and the development of insulin resistance, we propose a model where alterations in adipose function contribute to the accumulation of lipids in the circulation and eventually the peripheral tissues, which ultimately alters insulin signaling (Figure 7-7).

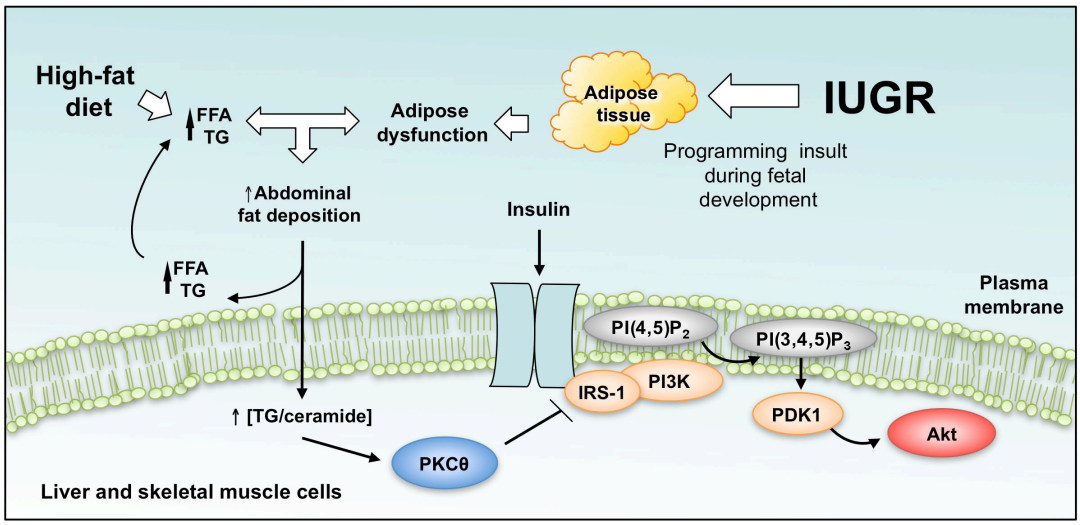


Figure 7-7 A proposed mechanism for the effect of IUGR and high-fat diet on peripheral insulin signaling

FFA: free fatty acids, TG: triacylglycerol, PKCθ: protein kinase C theta, IRS-1: insulin receptor substrate-1, PI3K: phosphoinositide 3-kinase. Prenatal events causing hypoxia-induced IUGR resulted in a long-term change in the adipose tissue function rendering it less capable to respond to increased dietary lipids. Consequently, circulating and tissue concentrations of FFA and other lipid sub-products like TG and ceramides are increased when exposed to a high-fat diet. Elevated tissue lipids activate PKCθ and inhibit IRS-1, which reduces insulin sensitivity.

One limitation of our hypoxia-induced IUGR rat model is that the hypoxic insult affects not only the fetuses but also the dam, therefore the potential interaction between fetal and maternal responses to hypoxia should also be considered. Despite these potential confounding variables, the hypoxia-induced IUGR rat model utilized in this study has been extensively characterized[30-33] and is the only non-invasive technique available to induce isobaric fetal hypoxia in rodents.

In conclusion, the results presented in this chapter provide strong evidence to support the programming effects of hypoxia-induced IUGR on metabolic function by causing a change in the adipose tissue response to a HF diet and increased the severity of diet-induced adipocyte dysfunction and insulin resistance. Our results suggest that prenatal insults causing IUGR could play a fundamental role in determining the long-term systemic response to HF diets. Indeed, our results suggest that early metabolic programming could contribute to the observed variability in the response to nutritional interventions and could further the development of cardiovascular and metabolic diseases later in life, particularly in environments where HF diets are prevalent.[34]

7.6 References

1. Andreassi MG. Metabolic syndrome, diabetes and atherosclerosis: influence of gene-environment interaction. *Mutation Research*. 2009;667:35-43.

2. Renna F, Thakur N. Direct and indirect effects of obesity on U.S. labor market outcomes of older working age adults. *Soc Sci Med*. 2010:[Epub ahead of print].

3. Wardle J, Carnell S, Haworth CM, Plomin R. Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment. *Am J Clin Nutr*. 2008;87:398-404.

4. Hawkins SS, Cole TJ, Law C, Millennium Cohort Study Child Health G. An ecological systems approach to examining risk factors for early childhood overweight: findings from the UK Millennium Cohort Study. *J Epidemiol Community Health*. 2009;63:147-155.

5. He Y, Ma G, Zhai F, Li Y, Hu Y, Feskens EJ*, et al.* Dietary patterns and glucose tolerance abnormalities in Chinese adults. *Diabetes Care*. 2009;32:1972-1976.

6. Boutcher SH, Dunn SL. Factors that may impede the weight loss response to exercise-based interventions. *Obes Rev*. 2009;10:671-680.

7. Vaag A, Jensen CB, Poulsen P, Brons C, Pilgaard K, Grunnet L*, et al.* Metabolic aspects of insulin resistance in individuals born small for gestational age. *Horm Res*. 2006;65 Suppl 3:137-143.

8. Shankar K, Kang P, Harrell A, Zhong Y, Marecki JC, Ronis MJ*, et al.* Maternal Overweight Programs Insulin and Adiponectin Signaling in the Offspring. *Endocrinology*. 2010;151:2577-2589.

9. Taylor PD, Poston L. Developmental programming of obesity in mammals. *Exp Physiol*. 2007;92:287-298.

10. Desai M, Gayle D, Babu J, Ross MG. Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R91-96.

11. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *Journal of Biological Chemistry*. 1995;270:17513-17520.

12. Vickers MH. Developmental programming and adult obesity: the role of leptin. *Curr Opin Endocrinol Diabetes Obes*. 2007;14:17-22.

13. Armitage JA, Taylor PD, Poston L. Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. *J Physiol*. 2005;565:3-8.

14. Esposito K, Giugliano G, Giugliano D. Metabolic effects of liposuction - yes or no? *N Engl J Med*. 2004;351:1354-1357.

15. Pickersgill L, Litherland GJ, Greenberg AS, Walker M, Yeaman SJ. Key role for ceramides in mediating insulin resistance in human muscle cells. *J Biol Chem*. 2007;282:12583-12589.

16. Petersen KF, Shulman GI. Pathogenesis of skeletal muscle insulin resistance in type 2 diabetes mellitus. *Am J Cardiol*. 2002;90:11G-18G.

17. Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, Lee D*, et al.* Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes*. 1999;48:1270-1274.

18. Sparatore B, Passalacqua M, Pedrazzi M, Ledda S, Patrone M, Gaggero D*, et al.* Role of the kinase activation loop on protein kinase C theta activity and intracellular localisation. *FEBS Lett*. 2003;554:35-40.

19. Mur C, Arribas M, Benito M, Valverde AM. Essential role of insulin-like growth factor I receptor in insulin-induced fetal brown adipocyte differentiation. *Endocrinology*. 2003;144:581-593.

20. Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. *Endocrinology*. 2005;146:1328-1337.

21. Giorgino F, Laviola L, Eriksson JW. Regional differences of insulin action in adipose tissue: insights from in vivo and in vitro studies. *Acta Physiol Scand*. 2005;183:13-30.

22. Caluwaerts S, Lambin S, van Bree R, Peeters H, Vergote I, Verhaeghe J. Diet-induced obesity in gravid rats engenders early hyperadiposity in the offspring. *Metabolism*. 2007;56:1431-1438.

23. Jacob S, Machann J, Rett K, Brechtel K, Volk A, Renn W*, et al.* Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. *Diabetes*. 1999;48:1113-1119.

24. Coupe B, Amarger V, Grit I, Benani A, Parnet P. Nutritional programming affects hypothalamic organization and early response to leptin. *Endocrinology*. 2010;151:702-713.

25. Tuomilehto J. Nonpharmacologic therapy and exercise in the prevention of type 2 diabetes. *Diabetes Care*. 2009;32 S189-193.

26. Whitfield MF, Grunau RE. Teenagers born at extremely low birth weight. *Paediatr Child Health*. 2006;11:275-277.

27. Boden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes*. 1997;46:3-10.

28. Simmons RA. Developmental origins of diabetes: the role of epigenetic mechanisms. *Curr Opin Endocrinol Diabetes Obes*. 2007;14:13-16.

29. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*. 1993;259:87-91.

30. Morton JS, Rueda-Clausen CF, Davidge ST. Mechanisms of Endothelium-Dependent Vasodilation in Male and Female, Young and Aged Offspring Born Growth Restricted. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R930-938.

31. Rueda-Clausen CF, Morton JS, Davidge ST. Effects of hypoxia-induced intrauterine growth restriction on cardiopulmonary structure and function during adulthood. *Cardiovasc Res*. 2009;81:713-722.

32. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

33. Hemmings DG, Williams SJ, Davidge ST. Increased myogenic tone in 7-month-old adult male but not female offspring from rat dams exposed to hypoxia during pregnancy. *Am J Physiol Heart Circ Physiol*. 2005;289:H674-682.

34. Benson L, Baer HJ, Kaelber DC. Trends in the diagnosis of overweight and obesity in children and adolescents: 1999-2007. *Pediatrics*. 2009;123:e153-158.

CHAPTER 8 POSTNATAL ADMINISTRATION OF RESVERATROL PREVENTS DIET-INDUCED METABOLIC SYNDROME IN YOUNG OFFSPRING BORN IUGR[***]

8.1 Introduction

In the previous chapter, we demonstrated that offspring born IUGR as a result of a prenatal hypoxic insult exhibit long-term metabolic changes that make them more susceptible to develop several components of the MetS (including a relative increase in intra-abdominal fat deposition, dyslipidemia and insulin resistance) when exposed to a HF diet. In a clinical scenario, there are not many therapeutic alternatives to treat subjects diagnosed with IUGR.[1] In fact, the main objective of most medical interventions, in these cases, is to prevent the development of acute perinatal complications in the mother and the baby. In that context, and given our previous results, we decided to study whether the programmed susceptibility to HF diet observed in our model could be treated using pharmacological interventions.

Resveratrol, which is a natural polyphenol produced by plants,[2] has been used in several studies both in animals,[3-5] and human volunteers,[6-9] to treat several chronic conditions. Several beneficial effects have been attributed to the oral administration of this molecule; including prolongation of lifespan and protection against the development of diet-induced obesity and insulin resistance.[10-12] Moreover, preliminary toxicological studies

[***] *Most components of this chapter have previously been submitted for publication as:*

- *"Dolinsky VW[*], **Rueda-Clausen CF[*],** Morton JS, Dyck JRB and Davidge ST. Postnatal Administration Of Resveratrol Prevents Diet Induced Metabolic Syndrome In Young Offspring Born IUGR. In preparation for Diabetes 2011"(*: shared first authorship)*

Contribution: *Rueda-Clausen CF. was the project coordinator. Except for glucose metabolism studies, lipid determinations and all western blots, which were performed by Dolinsky VW, Rueda-Clausen CF performed all the experiments and data analyses, wrote the first draft of the manuscript and coordinated with the other authors in compiling the final version.*

suggest that therapeutic doses of Resveratrol are non-toxic and easily absorbed by humans,[13-15] which makes Resveratrol an interesting candidate for the management of the long-term deleterious metabolic consequences of being born IUGR.

8.2 Objectives

Based on these antecedents, this chapter presents a study designed to evaluate the usefulness of nutritional supplementation with Resveratrol to prevent the early development of adverse metabolic effects associated with the interaction between prenatal hypoxic insults leading to IUGR and postnatal exposure to a HF diet.

8.3 Methods

8.3.1 Animal model

Female Sprague Dawley rats were mated within the animal facility and treated as described in Section 2.1.1. At birth, litters were treated as indicated in the IUGR plus HF diet/Resveratrol interaction protocol described in Section 2.1.1.4. In total, 48 male offspring were included in this study (12 animals from six different litters in each of the four experimental groups).

8.3.2 Experimental measurements

After weaning, food intake and body weight were measured in all animals twice per week. After nine weeks of nutritional intervention with either a HF diet or a HF diet plus Resveratrol 4g/Kg of food (at 12 weeks of age), several *in vivo* and *ex vivo* measurements were performed; including whole body composition analysis (Section 2.3.4), indirect calorimetry and physical activity (Sections 2.3.4 and 2.3.5), blood pressure measurements (Section 2.2.8), glucose and insulin tolerance tests (Section 2.3.7), lipid profile (Section 2.3.12), evaluation of intra-abdominal adiposity and

adipocyte morphometry (as described in sections 2.3.5, 2.3.6 and 2.3.14 respectively), and determination of circulating levels of insulin (Sections 2.3.8 and 2.3.11).

8.4 Results

8.4.1 Body weight gain, energy intake and physical activity

Consistent with our previous results, exposure to prenatal hypoxia had no effect on body weight gain (Figure 8-1A) or body weight/tibia length ratio (Figure 8-1B) of the offspring after nine weeks of HF diet. Despite similar body weights, food intake was decreased in IUGR offspring independent of the diet received (Figure 8-1C). At this time, however, physical activity was also significantly reduced in IUGR offspring, this difference being more notable in IUGR offspring receiving Resveratrol (Figure 8-1D). Together, these two findings are similar to our previous experience with this model and could explain the lack of differences in weight gain compared to controls despite a decreased energy intake. Interestingly, neither IUGR nor Resveratrol administration had any significant effect on the total body composition among groups (Figure 8-1E).

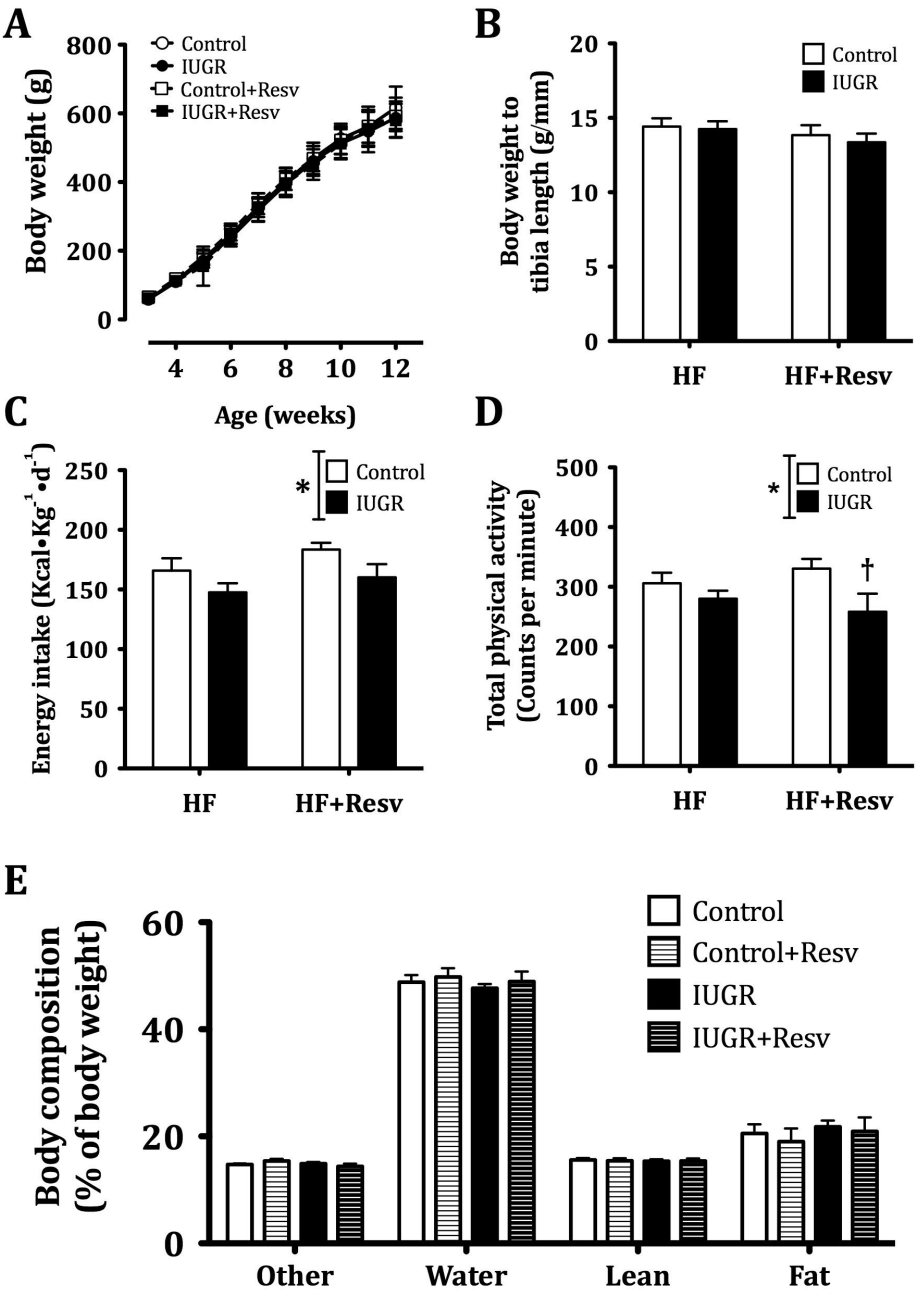


Figure 8-1 Effect of IUGR and administration of Resveratrol on body weight energy intake and physical activity after nine weeks of high-fat diet

Effect of intrauterine growth restriction (IUGR) and supplementation with Resveratrol (Resv; 4g/Kg of diet) on (**A**) change in body weight over time, (**B**) body weight measured under anesthesia adjusted by tibia length, (**C**) absolute energy intake adjusted by body weight, (**D**) total physical activity in 24 hours (**E**) total body composition estimated by TD-SPEC. HF: high-fat diet. * represents values of $p<0.05$ for the respective sources of variation (IUGR and Resveratrol administration) using two-way ANOVA. † represents a $p<0.05$ vs. controls after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

Consistent with our previous results, offspring born IUGR exhibited no statistically significant changes in the RER. However, they exhibited a consistent and significant decrease in VO_2, VCO_2 and heat production during both light and dark cycles relative to controls regardless of whether they were receiving Resveratrol in their diet (Table 8-1).

Table 8-1 Gas exchange ratio and heat production in control and IUGR rats fed a high-fat diet with or without Resveratrol

	HF diet		HF diet + Resv		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Resv	Int
Light cycle							
VO_2 ($mL \bullet Kg^{-1} \bullet h^{-1}$)	1124 (39)	1007 (34)	1098 (30)	1036 (27)	*		
VCO_2 ($mL \bullet Kg^{-1} \bullet h^{-1}$)	760 (23)	691 (23)	758 (28)	699 (26)	*		
RER	0.67 (0.01)	0.67 (0.01)	0.69 (0.01)	0.66 (0.02)			
Heat (Kcal/h)	3.2 (0.13)	2.81 (0.11)	3.04 (0.10)	2.63 (0.09)	*		
Dark cycle							
VO_2 ($mL \bullet Kg^{-1} \bullet h^{-1}$)	1233 (41)	1120 (35)	1221 (26)	1195 (34)	*		
VCO_2 ($mL \bullet Kg^{-1} \bullet h^{-1}$)	856 (27)	784 (24)	857 (28)	817 (33)	*		
RER	0.69 (0.01)	0.68 (0.02)	0.70 (0.01)	0.68 (0.02)			
Heat (Kcal/h)	3.54 (0.1)	3.18 (0.1)	3.4 (0.1)	3.0 (0.1)	*		

Experiments performed after nine weeks of nutritional intervention. VO_2: oxygen consumption, VCO_2: carbon dioxide production, RER: respiratory exchange ratio, * represents values of $p<0.05$ for the respective sources of variation (IUGR: Intrauterine growth restriction and Resv: Resveratrol administration) using two-way ANOVA. † $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

8.4.2 Intra-abdominal fat distribution and adipocyte morphometry

Despite having similar body weight and total body compositions, offspring born IUGR and exposed to a HF diet exhibited an increase in total and relative (adjusted by total body fat) intra-abdominal fat distribution as determined by both abdominal tomographic imaging and mechanical fat extraction (Figure 8-2A to D). Administration of Resveratrol caused a reduction in the absolute abdominal fat content regardless of whether the rats were IUGR or control (Figure 8-2C). Interestingly, in control offspring, the administration of Resveratrol did not affect the relative intra-abdominal fat distribution, whereas in IUGR offspring the administration of Resveratrol reduced the relative intra-abdominal fat distribution to levels comparable to those observed in controls (Figure 8-2D). Neither IUGR nor administration of Resveratrol had any statistically significant effect on fat distribution among the different intra-abdominal sites of fat deposition (Figure 8-2E). Consistent with our previous results, IUGR was also associated with an increase in omental adipocyte diameter (Figure 8-2F and G). Resveratrol administration reduced adipocyte diameter to a similar extent in both IUGR and control offspring (Figure 8-2F and G).

IUGR had no effect on liver, pancreas or spleen absolute or relative (adjusted by body weight) weights (Table 8-2). However, IUGR was associated with a decrease in absolute and relative weights of the heart and kidneys (Table 8-2). Resveratrol was associated with an overall decrease in the absolute, but not relative, kidney weight regardless of the diet the offspring were receiving (Table 8-2).

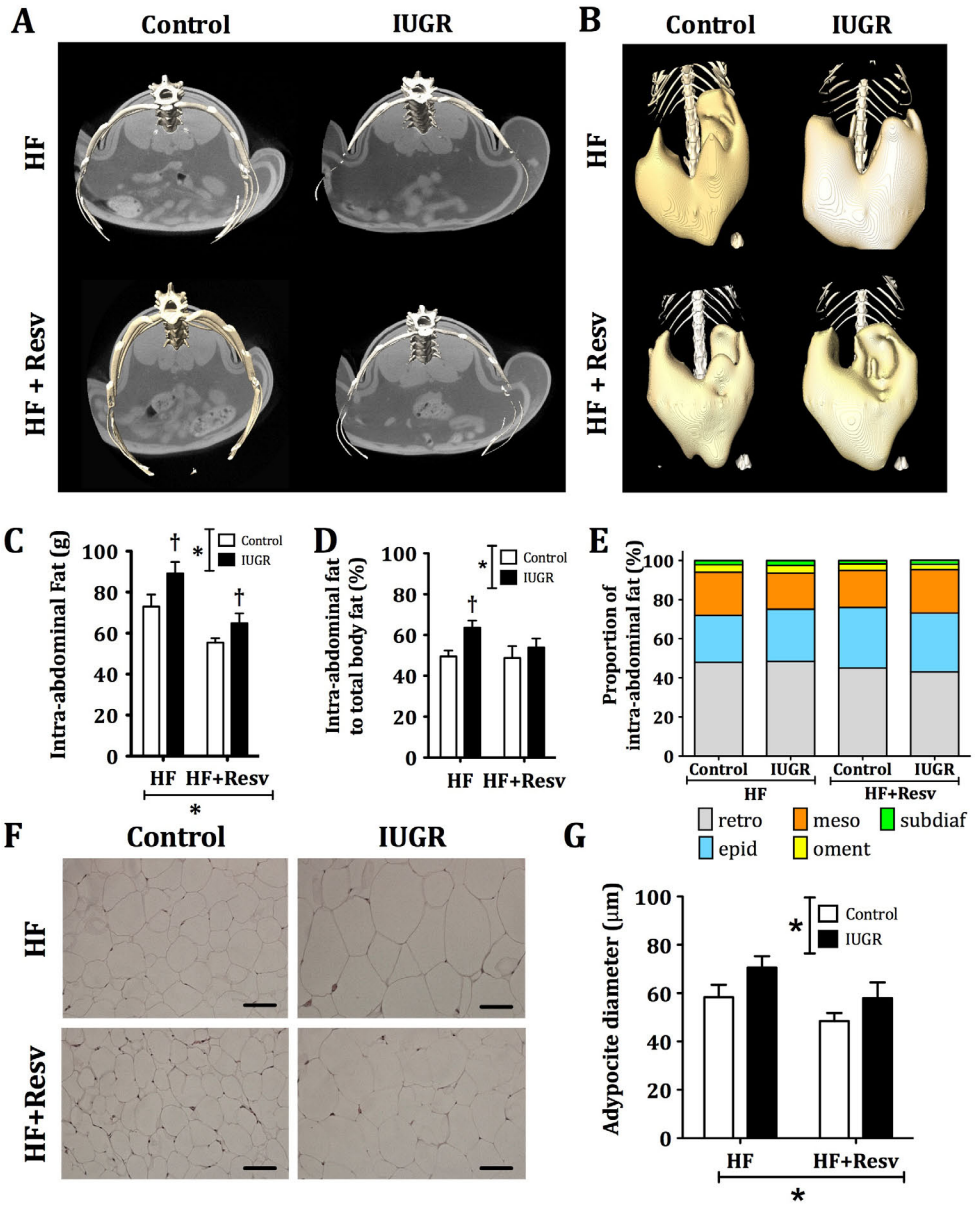


Figure 8-2 Effect of IUGRand administration of Resveratrol on body composition, intra-abdominal fat content and adipocyte size

Measurements made after nine weeks of HF: High-fat diet with or without (Resv) Resveratrol 4g/Kg of diet. (**A**) representative axial views and (**B**) 3D reconstructions of intra-abdominal fat deposits. (**C**) total and (**D**) relative intra-abdominal fat adjusted by total body fat determined by TD-SPEC (**E**) distribution of intra-abdominal fat among the different fat deposits: retro: retroperitoneal, epid: epididymal, meso: mesometrial, omen: omental and subdiaf: subdiafragmatic. (**F**) representative pictures of omental fat tissue histological preparations (hematoxylin-eosin, bar 50 μm), (**G**) average intra-abdominal adipocyte diameter. * represents values of $p<0.05$ for the respective sources of variation (IUGR or Resveratrol) using two-way ANOVA. † represents a $p<0.05$ vs. controls after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

Table 8-2 Organ weights in control and IUGR rats fed a high-fat diet with or without Resveratrol

	HF diet		HF diet + Resv		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Resv	Int
Light cycle							
Liver weight (g)	22.8 (1.6)	21.3 (1.1)	21.4 (1.5)	19.9 (1.3)			
Liver relative weight (mg/g body weight)	33.4 (1.1)	32.6 (1.5)	32.8 (0.7)	32.5 (1.0)			
Heart weight (g)	2.68 (0.11)	2.71 (0.10)	2.58 (0.08)	2.46 (0.09)			
Heart relative weight (mg/g body weight)	3.98 (0.22)	4.15 (0.26)	4.00 (0.13)	4.07 (0.25)			
Pancreas weight (g)	1.36 (0.03)	1.15 (0.08)	1.26 (0.07)	1.30 (0.16)			
Pancreas relative weight (mg/g body weight)	2.02 (0.1)	1.76 (0.13)	1.96 (0.14)	2.13 (0.27)			
Spleen weight (g)	0.95 (0.05)	1.01 (0.07)	0.94 (0.05)	0.92 (0.05)			
Spleen relative weight (mg/g body weight)	1.4 (0.08)	1.54 (0.09)	1.45 (0.03)	1.51 (0.07)			
Kidneys weight (g)	4.08 (0.14)	3.58 (0.08)†	3.84 (0.09)	3.30 (0.08)†	*	*	
Kidneys relative weight (mg/g body weight)	6.02 (0.15)	5.48 (0.16)	5.95 (0.22)	5.43 (0.18)	*		

Measurements made after nine weeks of HF: high-fat diet with or without (Resv) Resveratrol 4g/Kg of diet.,* $p<0.05$ for the respective source of variation such as intrauterine growth restriction (IUGR), Resveratrol or their interaction (Int) using two-way ANOVA. † represents a $p<0.05$ vs. Control receiving the same diet after a Bonferroni post-hoc test (n=6 per group).

8.4.3 Lipid profile, lipid accumulation and glucose homeostasis

Similar to previous results, administration of a HF diet caused a greater increase in circulating levels of TG and FFA in IUGR compared to controls (Table 8-3). Administration of Resveratrol had no effect on Control offspring. However, it completely reversed the impaired lipid profile observed in IUGR offspring; decreasing circulating levels of TG and FFA to levels comparable to those observed in control animals receiving a HF diet (Table 8-3).

Table 8-3 Circulating and tissue lipid concentrations of control and IUGR rats fed a high-fat diet with or without Resveratrol

	HF diet		HF diet +Resv		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Resv	Int
Triglycerides (mmol/L)	2.5 (0.5)	6.2 (0.8)†	2.4 (0.2)	3.0 (0.5)	*	*	*
Cholesterol esters (mmol/L)	3.6 (0.5)	4.1 (0.5)	3.0 (0.3)	3.6 (0.2)			
Cholesterol (mmol/L)	0.87 (0.07)	1.05 (0.07)	0.97 (0.17)	1.00 (0.007)			
FFA (mmol/L)	0.51 (0.07)	0.86 (0.12)†	0.4 (0.01)	0.42 (0.03)	*	*	*

Measurements made after nine weeks of HF: High-fat diet with or without (Resv) Resveratrol 4g/Kg of diet. FFA: free fatty acids, * $p<0.05$ for the respective source of variation such as prenatal hypoxia (IUGR), Resveratrol (Resv) or their interaction (Int) using two-way ANOVA. † $p<0.05$ vs. Control receiving the same diet after a Bonferroni post-hoc test (n=6 per group).

As previously described, IUGR had no effect on fasting blood glucose levels (Figure 8-3A) but caused an elevation in fasting insulin levels (Figure 8-3B) and consequently in HOMA index (Figure 8-3C). Moreover, IUGR offspring also exhibited impaired responses to glucose (Figure 8-3D and F) and insulin (Figure 8-3G and I) administration. Interestingly, and consistent with the effects on lipid profile, Resveratrol had no effect on glucose metabolism in control offspring but reversed the long-term deleterious effects of IUGR on glucose metabolism; decreasing all of these parameters to levels comparable to those observed in control offspring receiving a HF diet (Figure 1-3B and C, E and F, H and I).

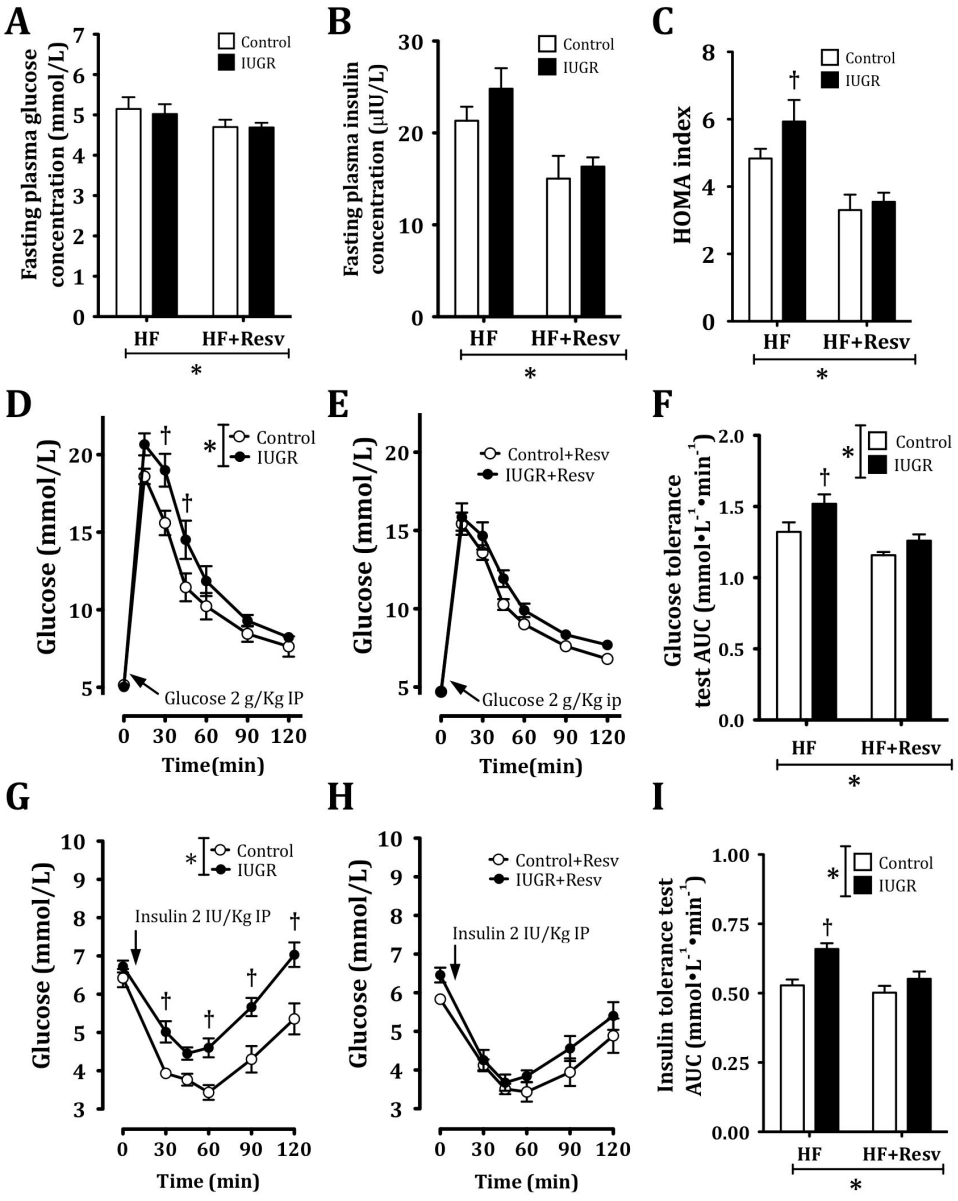


Figure 8-3 Effects of IUGR and administration of Resveratrol on glucose homeostasis parameters

Measurements made after nine weeks of HF: High-fat diet with or without (Resv) Resveratrol 4g/Kg of diet on: (**A**) fasting blood glucose levels (**B**) fasting plasma levels of insulin, (**C**) HOMA index. (**D** and **E**) glucose tolerance test (GTT) and (**F**) summary information presented as area under the curves (AUC). (**G** and **H**) insulin tolerance test (ITT) and (**I**) summary information presented as AUC. * represents $p<0.05$ for the respective source of variation (IUGR or Resv) using two-way ANOVA (bar graphs) or a repeated measurements ANOVA (GTT and ITT). † represents a $p<0.05$ vs. Control receiving the same diet after a Bonferroni post-hoc test (n=6 per group).

8.4.4 Blood pressure and heart rate

Offspring born IUGR exhibited an increase of ~10mmHg in SBP regardless of whether or not they were receiving Resveratrol. However, no significant changes in MBP were observed among the experimental groups (Table 8-4). Consistent with previous results, IUGR was also associated with a statistically significant decrease in heart rate. Interestingly, this difference seemed to be ameliorated by the administration of Resveratrol in the diet (Table 8-4).

Table 8-4 Blood pressure and heart rate in control and IUGR rats fed a high-fat diet with or without Resveratrol

	HF diet		HF diet + Resv		2-way ANOVA		
	Control	IUGR	Control	IUGR	IUGR	Diet	Int
Light cycle							
SBP (mmHg)	155 (2)	162 (5)	159 (3)	168 (5)	*		
MBP (mmHg)	112 (2)	116 (3)	116 (4)	113 (3)			
Heart rate (bpm)	411 (8)	377 (4)†	414 (9)	410 (9)	*	*	

Measurements made after nine weeks of HF: High-fat diet with or without (Resv) Resveratrol 4g/Kg of diet. SBP: systolic blood pressure, MBP: mean blood pressure. * represents values of $p<0.05$ for the respective sources of variation (IUGR and Resveratrol administration) using two-way ANOVA. † represents a $p<0.05$ vs. controls after a Bonferroni post-hoc test comparing IUGR and control offspring receiving the same diet (n=6 per group).

8.5 Discussion

This study demonstrates that administration of Resveratrol in the diet can reverse some of the deleterious long-term effects of being born IUGR. Specifically, we showed that Resveratrol can reduced the increased susceptibility to HF diet-induced metabolic alterations in fat distribution, adipocyte size, lipid metabolism and glucose homeostasis that we have previously described in young-adult offspring born IUGR as consequence of a prenatal hypoxic insult. Collectively, our findings suggest that Resveratrol

may have an application for preventing the development of undesirable metabolic outcomes in subjects born IUGR. The details of these findings and their important clinical implications are discussed below.

8.5.1 Physical activity and energy homeostasis of IUGR offspring exposed to a high-fat diet

Consistent with our previous findings, offspring exposed to a HF diet demonstrated no changes in body weight-gain relative to controls receiving the same diet but exhibited a decrease of ~10% in total energy intake relative to controls. Interestingly, supplementation with Resveratrol did not cause any statistically significant effect on either body weight or food intake. Previous studies performed in Sprague Dawley rats receiving similar doses of Resveratrol (60 mg•Kg BW^{-1}•d^{-1}, for six weeks), obtained similar results showing that Resveratrol did not affect total body weight or energy intake in controls.[16] Also consistent with our previous observations, offspring born IUGR were characterized by a modest decrease (~15%) in physical activity relative to controls. This decrease in physical activity associated with IUGR was more pronounced in animals receiving Resveratrol; which suggests that the effects of Resveratrol on other metabolic parameters cannot be attributed to an increase in energy requirements resulting from changes in physical activity.

As expected, rats fed a HF diet exhibited a low RER as a result of increased availability of fat as an energy source. Interestingly, and consistent with the observed changes in physical activity, offspring born IUGR also demonstrated a slight (~10%) but statistically significant decrease in VO_2, VCO_2 and heat production rates both during light and dark cycles. Laboratory rats are normally confined to cages that markedly restrict their physical activity, and therefore, the resting energy expenditure of these animals accounts for 90% of the total daily energy expenditure, and daily physical activities only account for the remaining 10%.[17, 18] Consequently, the

decrease in the overall metabolic rate (described as changes in VO_2 and heat production) are unlikely to be the result of changes in physical activity and suggest that offspring born IUGR had a long-term decrease in their baseline metabolic rates. Previous studies in rodents have shown that mice selectively bred for a high level of physical activity (after 30 generations), are less susceptible to develop diet-induced obesity due not only to changes in their spontaneous physical activity, but mainly due to an increase in their baseline metabolic rates.[19] However, to the best of our knowledge, our results are the first evidence showing that significant changes in baseline metabolic rates can be programmed depending on the fetal oxygen availability during pregnancy. This constitutes a highly relevant observation that may contribute to the understanding of mechanisms linking IUGR and the increased risk of developing diet-induced obesity and MetS later in life. Interestingly, addition of Resveratrol to the diet had no effect on any of these metabolic parameters.

8.5.2 Effect of Resveratrol on body composition, fat distribution and lipid profile of IUGR offspring exposed to high-fat diet

Consistent with our previous results, prenatal hypoxia had no effect on total body composition but resulted in increased absolute and relative intra-abdominal fat content as well as the intra-abdominal adipocyte diameter in young-adult offspring. Administration of Resveratrol did not alter body composition either in control or IUGR offspring. However, it had a beneficial effect on fat distribution by decreasing the amount of fat located in the abdominal cavity and reducing adipocyte diameter. Interestingly, this effect of Resveratrol affected all intra-abdominal fat deposits similarly. Together, these results are consistent with an extensive and convincing body of evidence showing that oral administration of Resveratrol (in doses similar to those used in this study) to rodents on a HF diet can ameliorate the deleterious effects of such nutritional interventions, not only by increasing their survival and motor function[20] but also diminishing the total body fat

content and decreasing the amount of epididymal, inguinal and retroperitoneal white adipose tissue.[20, 21]

Despite the lack of effect of Resveratrol on total body weight, the effect of this molecule on the body fat distribution of offspring born IUGR is remarkable. Specifically, Resveratrol's effect on the relative amount of fat located in the intra-abdominal cavity, which was decreased to levels comparable to control animals, was noteworthy. This observation is particularly interesting considering the relatively short length of the intervention (only nine weeks) compared to other studies showing the beneficial effects of Resveratrol on fat distribution when administered for up to 60 weeks.[22-24] Similar to our findings, previous studies in rodents have also shown that the effect of Resveratrol on total body weight or body weight gain is minimal.[4, 25] Despite being ineffective in reducing body weight of obese Zucker rats, administration of Resveratrol has several beneficial effects on the metabolism of these animals, including improvement of lipid profile and a slight decrease in body fat content.[26]

Another interesting result of our study was that the administration of Resveratrol completely reversed the increased susceptibility to diet induced dyslipidemia that we previously described in IUGR offspring. Similar to our results, studies in obese Zucker rats showed that Resveratrol resulted in a significant reduction in plasma TG, FFA, cholesterol and liver TG compared to controls.[26] Studies *in vitro* suggested that the anti-dyslipidemic effects of Resveratrol could be attributed to direct effects of this molecule on hepatic function. In isolated hepatocytes, for instance, Resveratrol was found to reduce activity of acetyl-CoA carboxylase, inhibit fatty acid synthesis and decrease the hepatic accumulation of TG.[21, 27] Direct effects of Resveratrol on adipocytes have also been demonstrated and could play a fundamental role in the mechanisms of action of this molecule. Incubation of isolated adipocytes with Resveratrol for instance has been shown to produce a reduction in lipogenic and an increase in lipolytic rates; probably due to an

increase in levels of cyclic adenosine monophosphate (cAMP) in the adipose tissue associated with the administration of Resveratrol.[28]

8.5.3 Effects of Resveratrol on glucose metabolism in IUGR offspring receiving a high-fat diet

The long-term effects of IUGR on glucose tolerance and insulin response in offspring exposed to HF is one of the most remarkable phenotypical characteristics observed in offspring born IUGR as a result of a maternal hypoxic insult. These results are consistent not only with our previous results using the same animal model (Chapter 7) but also with the results obtained by other authors using different prenatal insults to induce IUGR.[29]

The administration of Resveratrol demonstrated beneficial effects on glucose metabolism in both controls as well as IUGR offspring exposed to a HF diet. These beneficial effects, however, were more notable in IUGR offspring in whom the administration of this molecule reversed all parameters of glucose homeostasis to values comparable with those observed in control offspring receiving Resveratrol. The literature supporting the beneficial effects of Resveratrol on glucose homeostasis in rodents receiving a HF diet is quite extensive. Previous studies using rodents receiving a HF diet, demonstrated that addition of Resveratrol to the diet substantially diminished blood insulin compared to animals consuming a HF diet without Resveratrol.[20, 30] In addition, studies in rats receiving a high cholesterol–fructose diet[31], as well as obese Zucker rats with hyperinsulinemia[26] showed that administration of Resveratrol produced a significant reduction in plasma insulin levels relative to controls receiving the same diet without Resveratrol. However, to the best of our knowledge our study provides the first evidence supporting the beneficial metabolic effects of Resveratrol in animals born IUGR and exposed to a HF diet.

The mechanisms by which Resveratrol exerts its favorable metabolic effects are not completely understood. However, several candidates have been proposed including the direct activation of glycogen synthase and inhibition of glycogen phosphorylase,[32] activation of a NAD^+-dependent protein deacetylase (SIRT1) induction and the consequent activation of PGC-1α, [33, 34] activation of the estrogen receptor leading to increased Akt phosphorylation and consequent increase in glucose uptake.[31] Other proposed mechanisms include the induction of genes for oxidative phosphorylation and mitochondrial biogenesis,[20] increased expression of uncoupling protein-1 (UCP-1) in brown adipose tissue[35] and phosphorylation/activation AMPK leading to inhibition of acetyl-CoA carboxylase which stimulates the oxidation of fatty acids and decreases their synthesis.[36] Interestingly, the beneficial effects of Resveratrol have also been be attributed to its direct effect on the central nervous system since intracerebral injection of this molecule can normalize hyperglycemia and reduced hyperinsulinemia in mice receiving a high-energy diet.[37] It is, therefore, possible that the multiple beneficial effects of Resveratrol are due to a combination of both long-term (such as changes in gene expression) and short-term (such as changes in enzyme activities) effects produced by this molecule.

8.5.4 Conclusions

Our results confirmed our previous results showing that prenatal hypoxic insults causing IUGR could play a fundamental role in determining the long-term systemic response to HF diets, which are highly prevalent in western societies. Moreover, this is the first evidence showing that postnatal administration of Resveratrol could have relevant clinical applications since it can ameliorate the increased susceptibility to diet-induced MetS observed in IUGR offspring.

8.6 References

1. Rizzo G, Arduini D. Intrauterine growth restriction: diagnosis and management. A review. *Minerva Ginecol*. 2009;61:411-420.

2. Signorelli P, Ghidoni R. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *J Nutr Biochem*. 2005;16:449-466.

3. Akar F, Pektas MB, Tufan C, Soylemez S, Sepici A, Ulus AT*, et al.* Resveratrol Shows Vasoprotective Effect Reducing Oxidative Stress Without Affecting Metabolic Disturbances in Insulin-dependent Diabetes of Rabbits. *Cardiovasc Drugs Ther*. 2010.

4. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A*, et al.* Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*. 2006;444:337-342.

5. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG*, et al.* Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature*. 2003;425:191-196.

6. Ghanim H, Sia CL, Abuaysheh S, Korzeniewski K, Patnaik P, Marumganti A*, et al.* An antiinflammatory and reactive oxygen species suppressive effects of an extract of Polygonum cuspidatum containing resveratrol. *J Clin Endocrinol Metab*. 2010;95:E1-8.

7. Kennedy DO, Wightman EL, Reay JL, Lietz G, Okello EJ, Wilde A*, et al.* Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. *Am J Clin Nutr*. 2010;91:1590-1597.

8. la Porte C, Voduc N, Zhang G, Seguin I, Tardiff D, Singhal N*, et al.* Steady-State pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects. *Clin Pharmacokinet*. 2010;49:449-454.

9. Spaak J, Tomlinson G, McGowan CL, Soleas GJ, Morris BL, Picton P*, et al.* Dose-related effects of red wine and alcohol on heart rate variability. *Am J Physiol Heart Circ Physiol*. 2010;298:H2226-2231.

10. Hao HD, He LR. Mechanisms of cardiovascular protection by resveratrol. *J Med Food*. 2004;7:290-298.

11. Harikumar KB, Aggarwal BB. Resveratrol: a multitargeted agent for age-associated chronic diseases. *Cell Cycle*. 2008;7:1020-1035.

12. Knutson MD, Leeuwenburgh C. Resveratrol and novel potent activators of SIRT1: effects on aging and age-related diseases. *Nutr Rev*. 2008;66:591-596.

13. Biswas S, Rahman I. Modulation of steroid activity in chronic inflammation: a novel anti-inflammatory role for curcumin. *Mol Nutr Food Res*. 2008;52:987-994.

14. Brisdelli F, D'Andrea G, Bozzi A. Resveratrol: a natural polyphenol with multiple chemopreventive properties. *Curr Drug Metab*. 2009;10:530-546.

15. Das DK, Maulik N. Resveratrol in cardioprotection: a therapeutic promise of alternative medicine. *Mol Interv*. 2006;6:36-47.

16. Macarulla MT, Alberdi G, Gomez S, Tueros I, Bald C, Rodriguez VM*, et al.* Effects of different doses of resveratrol on body fat and serum parameters in rats fed a hypercaloric diet. *J Physiol Biochem*. 2009;65:369-376.

17. Miyasaka K, Ichikawa M, Kawanami T, Kanai S, Ohta M, Sato N*, et al.* Physical activity prevented age-related decline in energy metabolism in genetically obese and diabetic rats, but not in control rats. *Mech Ageing Dev*. 2003;124:183-190.

18. Ichikawa M, Kanai S, Ichimaru Y, Funakoshi A, Miyasaka K. The diurnal rhythm of energy expenditure differs between obese and glucose-intolerant rats and streptozotocin-induced diabetic rats. *J Nutr*. 2000;130:2562-2567.

19. Vaanholt LM, Jonas I, Doornbos M, Schubert KA, Nyakas C, Garland T, Jr.*, et al.* Metabolic and behavioral responses to high-fat feeding in mice selectively bred for high wheel-running activity. *Int J Obes (Lond)*. 2008;32:1566-1575.

20. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F*, et al.* Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell*. 2006;127:1109-1122.

21. Shang J, Chen LL, Xiao FX, Sun H, Ding HC, Xiao H. Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase. *Acta Pharmacol Sin*. 2008;29:698-706.

22. Frojdo S, Durand C, Pirola L. Metabolic effects of resveratrol in mammals--a link between improved insulin action and aging. *Curr Aging Sci*. 2008;1:145-151.

23. Rockenfeller P, Madeo F. Ageing and eating. *Biochim Biophys Acta*. 2010;1803:499-506.

24. Szkudelska K, Szkudelski T. Resveratrol, obesity and diabetes. *Eur J Pharmacol*. 2010;635:1-8.

25. Rocha KK, Souza GA, Ebaid GX, Seiva FR, Cataneo AC, Novelli EL. Resveratrol toxicity: effects on risk factors for atherosclerosis and hepatic oxidative stress in standard and high-fat diets. *Food Chem Toxicol*. 2009;47:1362-1367.

26. Rivera L, Moron R, Zarzuelo A, Galisteo M. Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats. *Biochem Pharmacol*. 2009;77:1053-1063.

27. Gnoni GV, Paglialonga G. Resveratrol inhibits fatty acid and triacylglycerol synthesis in rat hepatocytes. *Eur J Clin Invest*. 2009;39:211-218.

28. Szkudelska K, Nogowski L, Szkudelski T. Resveratrol, a naturally occurring diphenolic compound, affects lipogenesis, lipolysis and the antilipolytic action of insulin in isolated rat adipocytes. *J Steroid Biochem Mol Biol*. 2009;113:17-24.

29. Thompson NM, Norman AM, Donkin SS, Shankar RR, Vickers MH, Miles JL*, et al.* Prenatal and postnatal pathways to obesity: different underlying mechanisms, different metabolic outcomes. *Endocrinology*. 2007;148:2345-2354.

30. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov*. 2006;5:493-506.

31. Deng JY, Hsieh PS, Huang JP, Lu LS, Hung LM. Activation of estrogen receptor is crucial for resveratrol-stimulating muscular glucose uptake via both

insulin-dependent and -independent pathways. *Diabetes*. 2008;57:1814-1823.

32. Palsamy P, Subramanian S. Modulatory effects of resveratrol on attenuating the key enzymes activities of carbohydrate metabolism in streptozotocin-nicotinamide-induced diabetic rats. *Chem Biol Interact*. 2009;179:356-362.

33. Bai L, Pang WJ, Yang YJ, Yang GS. Modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes. *Mol Cell Biochem*. 2008;307:129-140.

34. Baur JA. Biochemical effects of SIRT1 activators. *Biochim Biophys Acta*. 2010;1804:1626-1634.

35. Mayers JR, Iliff BW, Swoap SJ. Resveratrol treatment in mice does not elicit the bradycardia and hypothermia associated with calorie restriction. *Faseb J*. 2009;23:1032-1040.

36. Hardie DG, Pan DA. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem Soc Trans*. 2002;30:1064-1070.

37. Ramadori G, Gautron L, Fujikawa T, Vianna CR, Elmquist JK, Coppari R. Central administration of resveratrol improves diet-induced diabetes. *Endocrinology*. 2009;150:5326-5333.

CHAPTER 9 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The volume of basic, clinical and epidemiological evidence demonstrating the long-term effects of certain prenatal conditions is extensive.[1, 2] For example, many initiatives in the past have focused on the study of prenatal insults such as maternal obesity,[3, 4] stress,[5] restriction of macro and micro nutrients[4, 6] or the administration of corticosteroids.[7] However, models of IUGR created by decreasing fetal oxygen availability (hypoxia) constitute an interesting, underused tool for the investigation of a prenatal insult that requires better understanding.

In the face of a hypoxic environment, the fetus has no alternative source to compensate for a reduced oxygen supply. Therefore, hypoxia is one of the most deleterious and common prenatal insults leading to IUGR. In fact, hypoxia models emulate the most common conditions causing IUGR in industrialized countries (such as placental insufficiency, pre-eclampsia, placenta previa, placenta acreta, anemia and maternal smoking among others).[8] Despite the clinical relevance, and the fact that hypoxia could have fundamentally different long-term consequences and mechanisms of action, little work has been done with models of hypoxia-induced IUGR. Consequently, this thesis provides an important contribution to the understanding of this fascinating phenomenon.

9.1 Summary of the most significant findings

Following a characterization of the rodent model of hypoxia-induced IUGR used throughout this thesis (Chapter 3), we provided a detailed description of the long-term effects of prenatal hypoxia on both cardiovascular function and structure *in vivo* (Chapter 4). We demonstrated that IUGR has multiple long-term effects on cardiac structure and function leading to the later development of multiple distinctive manifestations of early heart failure such as increased LV wall thickness (in male offspring

only), diastolic dysfunction and pulmonary hypertension (both in male and female offspring).

In a subsequent set of experiments, we described the *ex vivo* cardiac susceptibility to I/R injury as well as cardiac energy metabolism during both pre-ischemic and reperfusion conditions (Chapter 5). In these experiments we observed that, the myocardium of adult offspring born IUGR was more susceptible to I/R injury and exhibited an increase in proton production during reperfusion due to an uncoupling in glucose metabolism. We also identified that this cardiac phenotype is present regardless of sex differences; however, it is more pronounced in aged male offspring.

Continuing with the exploration of potential pathways that could explain the cardiac phenotype described in adult offspring born IUGR, Chapter 6 presents a study designed to evaluate the potential involvement of myocardial iron accumulation and oxidative stress. In this chapter, we demonstrated that offspring born IUGR exhibit an increase in myocardial oxidative stress independent of their sex or age. We also described a very characteristic histological pattern of collagen and iron deposition in the myocardium of adult offspring born IUGR. However, we did not detect differences in total myocardial iron accumulation between control and IUGR offspring.

While performing the *ex vivo* cardiac function experiments presented in Chapters 4 and 5, we observed that offspring born IUGR had an increased intra-abdominal fat deposition. This observation gave the foundation for the project presented in Chapter 7, in which we demonstrated that offspring born IUGR are more susceptible to develop components of MetS when exposed to a HF diet after weaning. Finally, the project presented in Chapter 8 showed that these deleterious effects of IUGR on the response to HF diets can be reversed by administering Resveratrol in the diet. These final chapters present an interesting new direction for future research that may have important clinical implications considering the current obesity pandemic and

the high cost of obesity related pathologies. Although this research is still in its early stages, our results provide a promising perspective for the early intervention of subjects born IUGR to prevent the later development of metabolic complications.

The integration of all these projects raise some interesting questions and arguments regarding the models used, as well as the clinical implications and relevance of the results, some of which are discussed below.

9.2 Sex differences and hypoxia-induced IUGR

One of the strengths of this thesis was the ability to make comparisons between male and female offspring exposed to similar prenatal and postnatal conditions. In this regard, there are some results that deserve mention.

In some cases, sexual dimorphisms in the long-term effects of hypoxia-induced IUGR were quite evident. These cases included; the effects of IUGR on body weight gain over time, the development of increased LV wall thickness in aged animals, the synergistic effect of aging and IUGR on post-ischemic cardiac recovery, and the presence of clusters of collagen deposits in the myocardium of aged offspring born IUGR. Given that estrogens are known to have beneficial effects on cardiac function,[9, 10] it is not surprising that female offspring exhibited some degree of protection against certain deleterious long-term effects of IUGR. Interestingly, there were other phenotypical manifestations associated with IUGR that were consistent both in male and female offspring; such as the development of diastolic dysfunction, increased pulmonary artery pressure gradients, increased susceptibility to I/R and increased production of protons during cardiac reperfusion.

Together, these results illustrate the complexity of the mechanisms behind the early programming phenomenon. Moreover, they suggest that the association between IUGR and undesirable outcomes may be mediated by a variety of mechanisms and that only some of those mechanisms could be

modulated by sex differences. These results have important implications, particularly in the understanding of cardiovascular pathophysiolgy of female subjects, a population in which the cardiovascular risk tends to be underestimated. In fact, this thesis demonstrated that non-menopausal female offspring could have the same susceptibility to some of the early cardiovascular programming phenomenon as their male counterparts.[11-13]

In this context, one aspect of the model used in this study that needs to be considered is that female rats do not undergo menopause. As they age, female rats become infertile by undergoing ovarian senescence[14] and, at this stage, their estrogen levels remain constant compared to the normal decrease seen with human menopause. Therefore, in the particular case of females, the model used in our study emulates an aging-premenopausal stage. To have a better understanding of the cardiac programming phenomenon and its consequences for future cardiovascular health in women, further studies in aged, ovariectomized rats may be required. This may be particularly important when considering that females demonstrate an increased incidence of cardiovascular diseases following menopause.

Finally, studies designed to evaluate the long-term effects of IUGR on the increased susceptibility to a HF diet did not include female animals due to the complexity of the design; which already included two sources of variation (IUGR and diet). The preliminary results used to support these studies suggested that increased abdominal fat deposition associated with IUGR was more evident in male than in female offspring. Based on these results, we decided to use only male offspring in this set of studies. However, the strong phenotypical characteristics of male offspring born IUGR and fed a HF diet suggest that the characterization of this phenomenon in female offspring may provide valuable additional information.

9.3 Programming and susceptibility to a second insult

One interesting finding that was consistent throughout this thesis was that in the early stages of life, IUGR offspring exhibited very little or no differences in their phenotypical characteristics when compared to sex- and age-matched controls. However, when exposed to additional stressors, either physiological (such as age) or pathological (such as I/R injury or HF diet), differences among groups became evident. The *in vivo* cardiac function studies, for instance, showed that functional and structural changes associated with IUGR were only evident in aged offspring (at 12 months of age); which illustrates the synergistic effect of aging and hypoxia-induced IUGR on the development of cardiac pathologies. The *ex vivo* cardiac function evaluation also showed similar results. During aerobic cardiac function assessment, no differences were observed between experimental groups, however, after a period of no-flow ischemia, an increased susceptibility to I/R injury in IUGR offspring became evident. In this set of experiments, in which the hearts were challenged with ischemia, differences between control and IUGR offspring were evident at both 4 and 12 months of age. Interestingly, and consistent with the UBM results, in male but not in female offspring IUGR and aging had a synergistic deleterious effect on cardiac metabolism and susceptibility to I/R. Finally, and consistent with both *in vivo* and *ex vivo* cardiac studies, the response of offspring to postnatal administration of a HF diet showed similar results. In fact, an increased susceptibility to develop metabolic abnormalities in offspring born IUGR was evident in those offspring receiving a HF diet but not in the ones receiving a LF diet.

All together, these results are similar to other reports made in the literature using different prenatal insults,[15-18] and suggest that postnatal exposure to physiological or pathological "stressors" favor the development of phenotypical differences between experimental groups. Our results also suggest that IUGR may influence the future development of cardiac and

metabolic pathologies by reducing physiological reserves and, therefore, the ability of the offspring to properly respond when exposed to different stressors later in life.

These observations have very important clinical implications: firstly they suggest that the long-term consequences of being exposed to prenatal hypoxia could be actively affecting the offspring's health since early stages in life despite a lack of phenotypical manifestations of diseases. Moreover, these results suggest that postnatal interventions directed at reducing the exposure to some postnatal stressors may be effective in reducing the later development of undesirable cardiovascular and metabolic outcomes; despite having no effect on the early-programmed condition itself. Therefore, the identification of, and the potential for early intervention in subjects with some degree of "early programming" could have a tremendous potential for the prevention of chronic diseases from the perspective of health care providers and policy makers.

9.4 Pharmacological intervention to reduce cardiac susceptibility to ischemia/reperfusion injury

The results presented in Chapter 5, in which we assessed cardiac function *ex vivo*, suggest that pharmacological interventions designed to modulate cardiac energy metabolism and improve glucose metabolism coupling during reperfusion could offer an additional benefit to adult offspring born IUGR. However, further experiments with specific pharmacological agents are needed to test this hypothesis.

In addition to its antioxidant, anti-oncogenic, anti-aging and metabolic effects, previous studies have described that administration of Resveratrol can protect the heart from I/R injury by inducing preconditioning of the heart.[19-21] The specific mechanisms by which this molecule can cause cardiac conditioning are still unknown, however, a number of mechanisms have been proposed including induction of nitric oxide dependent pathways,[21]

activation of adenosine receptors, PI3 kinase and MAPK.[20, 22, 23] However, the extrapolation of these previous studies to our model is limited for a number of reasons. Firstly, most of these previous studies have been performed by acutely administering Resveratrol in the perfusion fluid instead of evaluating the effects of the long-term administration of this compound in the diet. Additionally, the doses of Resveratrol added to the perfusate in these studies were considerably higher (10 mmol/L) relative to the plasma concentrations reached by supplementing the diet as we did (in the μmol/L range). Moreover, none of the above-mentioned studies have included either animals born IUGR or fed with a HF diet.

Although it was not one of the main objectives of this thesis, we took the opportunity to evaluate the effects of Resveratrol administration on myocardial susceptibility to I/R injury in both control and IUGR offspring receiving a HF diet (Appendix Figure 10-2). Interestingly, we observed that at 12 weeks of age, offspring born IUGR and fed a HF diet exhibited a ~30% decrease in the cardiac work during the pre-ischemic period. This finding is particularly relevant when considering that offspring born IUGR and fed standard rat chow (4% fat) have no changes in pre-ischemic cardiac performance relative to controls receiving the same diet (either at 4 or 12 months of age). During reperfusion, after a 10-minute no-flow ischemic injury, control offspring receiving a HF diet recovered approximately 40% of their initial cardiac function. Moreover, in this group of animals, the administration of Resveratrol had no effect on post-ischemic cardiac recovery. On the other hand, and consistent with the results from offspring receiving standard rat chow, IUGR offspring on a HF diet showed a decrease in their cardiac function recovery during reperfusion relative to controls under the same nutritional intervention. Interestingly, in the IUGR group, the administration of Resveratrol caused a modest but significant improvement in the post-ischemic cardiac performance (Appendix Figure 10-2). These results from I/R experiments mirror those from other metabolic

determinations such as lipid profile and glucose tolerance, in which IUGR but not control offspring exhibited a benefit of receiving Resveratrol. Together with the fact that Resveratrol had no effect on the post-ischemic cardiac performance of control offspring, these results suggest that the beneficial effects of this molecule on the cardiac susceptibility to I/R in offspring born IUGR may be attributable to its beneficial effects on lipids and glucose metabolism, rather than to its direct effects on cardiac function.

9.5 Early cardiovascular programming and changes in autonomic regulation

One fascinating observation reported in this thesis was that offspring born IUGR exhibited a decrease in heart rate relative to sex- and age-matched controls. This finding was consistent throughout different *in vivo* experiments regardless of whether it was measured under anesthesia (during *in vivo* UBM cardiac studies) or in conscious animals (during blood pressure evaluation both before and after an air puff stress). Moreover, similar results were obtained when cardiac function was evaluated *ex vivo.* Preliminary data presented in Appendix Figure 10-3 shows the spontaneous heart rates of isolated working hearts from offspring receiving HF diet with or without Resveratrol and demonstrates that being born IUGR was associated with a decrease in the *ex vivo* spontaneous heart rate regardless of which nutritional intervention they were receiving.

Among the different mechanisms that regulate the heart rate, the autonomic nervous system (ANS) is among the most important. The ANS is a component of the central nervous system functioning below the level of consciousness and controlling several components of cardiovascular function such as heart rate and vascular tone.[24] Many of the phenotypical characteristics that have been associated with early programming have also been linked to some degree of autonomic dysfunction.[25, 26] Therefore, changes in factors that determine autonomic tone constitute interesting

pathophysiological mechanisms that could be involved in early cardiovascular programming. Clinical studies have previously shown a significant correlation between fetal growth and the presence of an increased sympathetic tone that was present beyond the neonatal period.[27] Although these results are in contradiction to our results (since a decreased heart rate would suggest a decrease in sympathetic tone) they suggest that the regulation of autonomic parameters can be influenced by prenatal conditions. Moreover, and consistent with our findings, other authors have previously shown that IUGR induced by maternal protein restriction also has several effects on the autonomic regulation of the offspring's cardiovascular system. These effects include a decreased density of β_1-adrenergic receptors leading to an impaired response to β agonists[28] and increased levels of circulating epinephrine.[29] However, evidence linking early programming and long-term changes in autonomic regulation is very limited and requires further research.

9.6 Consideration of the experimental models

While interpreting and extrapolating our results, we made some considerations regarding the limitations and characteristics of the experimental models used in this thesis that should be discussed.

9.6.1 Interspecies differences

In addition to the rather evident and well-described interspecies differences between humans and rats (such as metabolic rates, gestational length, litter size, type of placentation, etc) there are other substantial differences that are particularly relevant to the experiments presented in this thesis and deserve a special mention. The fetal development and maturity of rat offspring at birth, has important differences relative to humans and other species (Section 1.2.3.1). The pulmonary development of the rat, for instance, notably differs from humans. In fact, the degree of pulmonary development in a term rat is comparable to that observed in a 32 week old human fetus.

Similarly, the degree of cardiac and adipose tissue maturity and development at birth differs between species. Therefore, it is plausible that fetal exposure to hypoxia during certain stages of fetal development would have a different effect in rats and humans.

In addition to the differences in developmental stages at birth, laboratory rats have some peculiarities in terms of lifespan and normal growth trajectories that need to be considered when interpreting the results presented in this thesis. The mean lifespan of Sprague Dawley rats is ~720 days (24 months) for males and ~840 days (28 months) for females. Under normal conditions, newborn rats double their body weight in the first five days after birth. Pups grow rapidly and at weaning their average body weight is about 10 times their birth weight (~45 g).[30] Growth rate deceleration continues throughout life reaching a plateau by 250-300 days (9 to 10 months) of life. Although these rats will continue to grow throughout life, most of the growth after 10 months of age is due to fat deposition.

For the purposes of metabolic studies, there are other characteristics of laboratory rats that need to be considered. Rats have a well-defined nocturnal rhythm, being active during the dark and sleeping and resting during light hours. Feeding occurs at night and digestion during early daylight hours. Moreover, laboratory rats are normally confined to cages that markedly restrict their physical activity, and therefore resting energy expenditure accounts for 90% of the total daily energy expenditure while daily physical activities only account for the remaining 10%.[31, 32]

Additionally, it has to be mentioned that there are other anatomical and functional considerations regarding species differences in the cardiac structure and function that have to be made when interpreting our results. In healthy young rats and mice, for instance, the E/A index of the mitral valve is <1, while in healthy humans this parameter is expected to be between 1.2 and 1.5 and values <1 are considered to be a diagnostic criteria for severe LV diastolic dysfunction. Despite these differences, the *ex vivo* confirmation of

the results obtained by UBM supports the validity of the echocardiographic findings and their interpretation in the context of this animal model.

9.6.2 Maternal hypoxia as a prenatal insult to induce IUGR

One limitation of this rodent model of IUGR was that hypoxic insults during pregnancy affected not only the fetuses but also the dam. Therefore, this model mimics conditions such as high-altitude pregnancy or smoking during pregnancy but has some limitations emulating conditions like placental insufficiency or preeclampsia in which the fetus, but not the mother, has restricted oxygen availability. Exposure of adult rats to hypoxic environments activates a cascade of physiological responses[33] that could impact fetal development independent of fetal growth or oxygen availability. Acute physiological responses to hypoxia include blood flow redistribution[34] (with vasoconstriction of pulmonary beds and vasodilatation of coronary and cerebral blood vessels) and hyperventilation leading to respiratory alkalosis and compensatory metabolic acidosis. Within minutes, of being placed under hypoxic conditions, maternal protein and RNA production in organs with high metabolic demands are substantially decreased[35] and several transcription factors (such as HIF-1α) are activated; leading to multiple cellular and systemic effects such as increased glycolytic activity, erythropoietin secretion and hematopoiesis.[36, 37]

In addition to the previously mentioned effects of hypoxia on maternal physiology, it is also plausible that this kind of intervention may cause some degree of maternal stress, leading to increased secretion of catecholamines in the maternal circulation, which could also have a direct effect on fetal development and could have long-term deleterious effects on the offspring.[38, 39] Despite all these considerations, the rodent model of hypoxia induced-IUGR used in this thesis offers a reliable, viable and consistent model of IUGR that requires no surgical interventions or administration of medications.

One characteristic of this animal model of IUGR that our group has described previously and requires further discussion relies is the fact that pregnant rats exposed to hypoxia exhibited a significant decrease (~40%) in food intake relative to pregnant rats under normal oxygen conditions. In order to control the potential confounding effect caused by differences in maternal food intake, our group have performed studies in the past using the same fundamental hypoxic intervention but including a second control group of pregnant animals housed in normoxic conditions but exposed to food restriction (-40% food intake in controls) during the last week of pregnancy.[40-43]

Interestingly, these previous results suggest that this particular group of offspring born from dams exposed to nutritional restriction may not be an appropriate control group. Rats under hypoxic conditions, for instance, are less active than pregnant rats under normoxic conditions. Therefore, it is plausible that the observed decrease in food intake in these animals is at least partially attributable to a decrease in the basal energy requirements of the dam. Supporting this theory, our group has previously shown that offspring born from dams exposed to nutritional restriction (but housed in normoxic conditions) have a smaller birth weights that those born from dams consuming comparable amounts of food but exposed to hypoxia during pregnancy.[41] Moreover, our previous studies measuring the offspring organ weights at birth, demonstrated that hypoxia and nutritional restriction have different effects on the relative weight of certain organs at birth (including brain, kidney and heart) suggesting that the blood flow redistribution phenomenon may be different in each of these experimental groups.[41]

Finally, it is plausible that the long-term effects of prenatal hypoxic insults on cardiac and metabolic function resulted from a combined effect of different factors (reduced oxygen availability, decreased nutritional intake, maternal stress, etc).

9.6.3 Choosing the right diet

In chapters 7 and 8 of this thesis we evaluated the effect of a HF diet on the development of phenotypical differences between experimental groups. The selection of this particular intervention was not arbitrary and deserves further discussion. Nutritional habits characterized by HF, hypercaloric nutritional intake were initially associated with wealthy western societies.[44, 45] However, the rapid transition and massive industrialization of the food industry has expanded widely regardless of the classic socioeconomic limiting conditions. This has led to global changes in nutritional habits and a pandemic of diet-induced obesity and other metabolic complications affecting populations all around the world; regardless of their socioeconomical status.[46-48] Therefore, understanding the potential mechanisms leading to programmed increases in the susceptibility to develop diet-induced obesity are highly relevant in today's society.

Since 1940 when the first animal models of diet-induced obesity were described,[49] a vast range of diets have been used to induce obesity.[50] Today, it is well accepted that a HF diet is an insult that is capable of inducing a phenotype in rodents that emulates many aspects of the MetS. However, the correct definition for a HF diet is rather controversial. Different authors have used a variety of nutritional interventions with different fat fractions (between 20 and 60%), fat sources (lard, fish oil and vegetable oil) and lengths of intervention (two weeks to several months).[50] No consensus exists regarding the best intervention to emulate the conditions observed in clinical scenarios. Regardless of this controversy, it is generally accepted that the models which better emulate the clinical characteristics of MetS are obtained with nutritional interventions lasting more than six weeks and using diets in which 40 to 50% of the caloric content is derived from lard.[50] Our results, using a diet with 45% energy derived from lard, are quite interesting because after nine weeks of nutritional intervention we did not observe differences in body weight or total body composition between control and IUGR offspring.

However, after this short period of time, several components of the MetS were clearly present in IUGR but not in control offspring. The HF diet used in our studies was shown to be effective in accelerating the apparition of metabolic outcomes in IUGR offspring. However, future studies aimed to identify the postnatal response to other common nutritional patterns (such as high-fructose diets) may provide further useful information to understand the interaction between hypoxia-induced IUGR and the postnatal environment in the development of metabolic diseases.

9.7 Future directions from a basic-research perspective

9.7.1 Characterization of the hypoxia-induced IUGR model

Although the model of hypoxia-induced IUGR that we used for this study has been extensively used by both our own and other groups, there are still some additional elements of this model that would benefit from further characterization. Some of these elements include:

- Evaluation of maternal stress by measuring plasma glucocorticoid levels before and after the hypoxic insult.
- Evaluation of maternal physical activity, oxygen consumption and heat production during hypoxic insults.
- A more complete evaluation of the fetal morphometry including abdominal circumference, crown-rump length, internal organ weights adjusted by tibia length, dry placenta weight and umbilical cord length.
- *In vivo* evaluation of uterine arteries and umbilical vasculature flow patterns during hypoxia as well as evaluation of placental structure (complete histological evaluation of placental structures and morphometry) and function (changes in placental active transporters efficiency).

- Assessment of additional fetal parameters such as blood hematocrit, hemoglobin and erythropoietin levels and other eritrocitary parameters (e.g. mean corpuscular volume, and mean corpuscular hemoglobin content).

Future investigation of these parameters would contribute to the understanding of the fetal/neonatal phenotype described in this particular model of IUGR and may suggest new avenues for the understanding of the early programming phenomenon.

9.7.2 Further characterization of the cardiovascular phenotype in adult offspring exposed to hypoxia in utero*.*

The work presented in this thesis highlights multiple phenotypical changes observed in adult offspring exposed to hypoxia *in utero*. However, it also describes other interesting findings that may require further investigation:

9.7.2.1 Spontaneous bradycardia

One interesting finding in adult offspring exposed to hypoxia, was that they exhibit a significant decrease in heart rate, both *in vivo* (during echocardiographic studies and blood pressure measurements) and *ex vivo* (during non-paced heart perfusion). This interesting finding was present in all offspring exposed to hypoxia regardless of their age, sex or level of activity (under anesthesia, awake or after air-puff stress). The potential effect of prenatal insults on the later regulation of sympathetic tone has been widely described in different clinical and animal models.[51-54] However, the specific mechanisms and the potential implications of this condition on the future development of cardiovascular pathology still require to be clarified. Different components of autonomic regulation, such as the peripheral density of adrenergic receptors,[55-58] long-term changes in sympathetic tone[59, 60] and neuro-endocrine factors,[61-63] have previously been proposed as potential elements susceptible to early programming. Future studies should focus on

the determination of adrenergic receptor density in the heart, hypothalamus and other organs as well as the *in vivo* evaluation of sympathetic tone (either through spectral analysis of electrocardiographic traces or spontaneous baroreflex and power).

9.7.2.2 Blood pressure and renal function

One of the cardiovascular parameters that should be studied in more detail in adult offspring prenatally exposed to hypoxia is blood pressure. In all of the studies reported in this thesis, blood pressure measurements were performed by the tail-cuff technique in previously trained animals. Our results were variable depending on the model used. However, this non-invasive technique for the evaluation of blood pressure has several known limitations such as having important inter- and intra-assay variability, being influenced by animal restriction during measurements (despite being trained) and the lack of accuracy in determining diastolic blood pressure.

Having a more accurate evaluation of the blood pressure in our animal model is fundamental for the interpretation and understanding of the observed phenotype. As motioned in Chapter 4 of this thesis, aged male but not female offspring born IUGR exhibited an increase in the left ventricular wall thickness. This particular finding could be the result of a number of possible factors including alterations in the mechanisms that regulate the extracellular matrix turn over[43, 64] or idiopathic immunological factors.[65, 66] However, the most common condition leading to left ventricular hypertrophy is cardiac overload.[67] Interestingly, observations made by other groups have shown that prenatal insults (such as maternal stress and nutritional restriction) can lead to a long-term increase in offspring's blood pressure.[38, 68-70] The results presented in this thesis suggest that adult offspring born IUGR do not exhibit changes in blood pressure. It is plausible that the mechanisms causing left ventricle remodeling in these animals are not mediated by sustained hemodynamic changes. However, more accurate measurement of blood pressure need to be made before changes in

hemodynamic parameters can be ruled out as the ethological factor for the left ventricular remodeling. Therefore, future studies should focus on the evaluation of blood pressure in these animals using alternative techniques, such as telemetry or invasive blood pressure evaluation.

Renal structure and function constitutes an additional component involved in blood pressure regulation that may deserve further study. The effect of certain prenatal insults on renal development and function has been extensively published.[71-75] Interestingly, little is known regarding the effects of prenatal hypoxic insults on renal development and function later in life. Our previous results suggest that newborn offspring exposed to hypoxia exhibit a decrease in the relative kidney weight and that those differences remain during adulthood (Table 8-2). Considering the important role of the kidneys in vascular tone regulation and the previously described effects of other prenatal insults on renal structure and function, future studies should focus on obtaining a better description of the renal phenotype of these offspring including nephron density and renal function (creatinin excretion and microproteinuria).

9.7.2.3 Pulmonary hypertension

One interesting result obtained by UBM and confirmed by histology was that adult offspring born IUGR, as a result of a hypoxic insult, exhibit signs of pulmonary hypertension; including alterations in the pulmonary artery flow pattern, increased right ventricle internal diameters and increased media thickness in pulmonary small vessels. The long-term effects of prenatal hypoxia on the pulmonary circulation have been previously reported in many animal models.[76-78] However, the mechanisms underlying this particular finding still need to be clarified. A recent study made in sheep provides further information that could be relevant to our model. Using a hypobaric hypoxic insult, placing ewes at high altitude during pregnancy and returning them to low altitude before birth, the authors demonstrated that offspring exposed to hypoxia during pregnancy exhibited a persistent

increase in pulmonary artery pressure and changes in pulmonary arterial function characterized by increased vascular responses to endothelin-1, increased vascular expression of eNOS and increased levels of the nitric oxide second messenger cGMP.[79] Future studies to explore this particular finding, including an *ex vivo* evaluation of right ventricle morphology, estimation of pulmonary water content (pulmonary edema) and a more complete evaluation of pulmonary vascular structure and function may provide a better understanding of this phenotypical characteristic observed in adult offspring born IUGR.

9.7.2.4 Effect of sex hormones

As previously mentioned, one of the limitations of this animal model was that female rats do not undergo menopause as they age and, therefore, may not reflect the physiological condition of the aging human female characterized by decreased hormonal levels. The observed differences in the phenotypes developed by male and female offspring prenatally exposed to hypoxia, and the influence of sex hormones on the development of these phenotypes, constitutes an interesting question that should be investigated in more detail. To do so, further experiments could be performed in which aged female offspring from different experimental groups are ovariectomized and randomized to receive either placebo or hormonal supplementation.

9.7.2.5 Thyroid function

One additional component that could potentially be involved in some of the phenotypical characteristics observed in adult offspring exposed to hypoxia *in utero*, is thyroid function. Tyrosine-based hormones produced by the thyroid gland, such as thyroxine (T4) and triiodothyronine (T3), are essential for proper development and differentiation and have direct effects on multiple organs and systems: including basal metabolic rate,[80] cardiac function,[81, 82] iron[83] and carbohydrate metabolism,[80, 84, 85] body fat distribution[86] neuronal maturation and sensitivity to catecholamines. The

effect of prenatal insults, such as exposure to alcohol[87] or corticoids,[88, 89] has on the hypothalamic-pituitary-thyroid function been previously reported. However, little is known about the potential long-term effects of a hypoxic insult on thyroid function later in life.

9.7.3 Understanding the mechanisms impairing cardiac metabolism and increasing proton production

Data presented in Chapter 5 of this thesis demonstrated that the myocardium from offspring born IUGR produced more protons during periods of reperfusion. As mentioned in the discussion of that chapter, an increase in proton production could have severe implications on the cardiac response to I/R injury by affecting the ion homeostasis of the myocardium. In order to have a better understanding of the cardiac phenotype observed in these animals, it would be important to characterize the activity of the ion mobilization mechanisms that respond to an increased proton production. Therefore, a better description of ion homeostasis in the myocardium of these animals could provide a better understanding of this phenomenon. Among the ion homeostasis mechanisms that could be involved, intracellular pH and levels of calcium during I/R injury and the activity of membrane-transporters (such as the Na/K ATPase pump, Na/H exchanger type 1 (NHE-1), Na/Ca exchangers (NCX) and type 2A SERCA channels) are particularly relevant. A more detailed description of these components could improve our understanding of the mechanisms leading to decreased cardiac energy efficiency during reperfusion and may be fundamental for choosing an adequate pharmacological intervention with the potential to prevent the increased susceptibility to I/R observed in the myocardium of offspring born IUGR.

Another interesting metabolic component that should be explored in the myocardium from offspring born IUGR is the characteristics of the mitochondria (number, density and function); particularly when considering

preliminary results suggesting that the hearts from offspring born IUGR may have an increased expression of mitochondrial proteins such as the F_1F_0 APTase.[90] Finally, and given that the excessive proton production observed in these animals resulted from an uncoupling of glucose metabolism during reperfusion, it may worthwhile to evaluate the beneficial effect of interventions designed to increase glucose oxidation during reperfusion. Pharmacological interventions that may be interesting for this particular purpose include inhibitors of malonyl coenzyme A decarboxylase (MCD) or inhibitors of pyruvate dehydrogenase kinase (PDHK). MCD inhibitors, such as CBM-301940 and CBM-300864, increase glucose oxidation by increasing myocardial levels of malonyl CoA. Malonyl CoA is an endogenous inhibitor of carnitine palmitoyltransferase-1 (CPT-1) that is one of the key regulators of mitochondrial fatty acid uptake. By inhibiting fatty acid uptake, this approach increases pyruvate oxidation through the Randle cycle. [91-95] An alternative intervention that could be use to modulate the substrate selection in the hearts of these animals is the inhibition of PDHK with agents such as dichloroacetate (DCA), which promote glucose oxidation by increasing the activity of the pyruvate dehydrogenase complex (PDC), which increases the rate of mitochondrial pyruvate oxidation and decreases the production of protons from glycolysis.

In addition to the effects of hypoxia-induced IUGR on cardiac metabolism there are other potential mechanisms that could be involved in the cardiac phenotype observed in our model. Fetal exposure to alkaloids like cocaine has proven to impair the myocardial sensitivity to I/R injury during adulthood by decreasing expression of cardio protective factors such as protein kinase C epsilon (PKC-ε).[96] [97] Future studies should focus in describing the contribution of this particular pathway in the development of the cardiac phenotype described in adult offspring exposed to hypoxia *in utero*.

Finally, there is evidence that several genes regulating cellular energy metabolism can be affected by epigenetic mechanisms.[98-100] The mitochondrial DNA encodes over 30 different genes, most of which are related to energy metabolism regulation which have a very low mutation rate but are highly regulated by histone phosphorylation and acetylation, which modulates not only mitochondrial function, biogenesis and growth.[98] Therefore, the study of potential epigenetic mechanisms, both in the cellular and mitochondrial DNA, should be aimed in the future.

9.7.4 Mechanisms of increased susceptibility to diet-induced MetS

Although preliminary data from our 4 and 12 month old animals receiving a regular diet suggests that male but not female offspring prenatally exposed to hypoxia develop an increased intra-abdominal fat deposition later in life (Figure 10-1), investigating whether this particular susceptibility to diet-induced obesity can be modulated by sex differences could be an interesting direction for future experiments.

Future directions for the study of the obesogenic phenotype developed by offspring born IUGR, may include the use of diets rich not only in fat but also in carbohydrates (more similar to the modern western diet pattern[101-103]), performing nutritional interventions for longer periods of time, or determining whether obese offspring prenatally exposed to hypoxia lose weight at the same rate as obese offspring born from normal pregnancies when exposed to a restricted nutritional intake. Results obtained from metabolic cages and presented in chapters 7 and 8 suggest that offspring born IUGR are less physically active than those born from normal pregnancies. Future studies to evaluate tolerance to exercise in adult offspring born from either control dams or dams exposed to hypoxia during pregnancy could provide further information regarding the long-term effects of prenatal hypoxia on physical activity and exercise tolerance and may contribute to the understanding of this interesting phenomenon.

The use of resveratrol to prevent the susceptibility to diet-induced MetS observed in offspring born IUGR showed very promising results. From a translational perspective, Resveratrol is a very attractive molecule, not only because it is a natural compound, but also because it has been used at high doses in adult populations and has been shown to be safe and well-tolerated.[104, 105] From the mechanistic perspective, however, Resveratrol constitute an intervention difficult to study because of the number of effects that it has on different metabolic pathways including AMPK, Sirt1, CPG-1 and Akt among many others.[106] Therefore, future directions pointed toward the understanding of the mechanisms behind this finding should consider the use of more selective pharmacological interventions.[106, 107] One additional characteristic of Resveratrol that could be explored in more details, is that it can facilitate the availability of nitric oxide through an Akt-dependent pathway.[108] This linking between Resveratrol and nitric oxide production could be related to the mechanisms by with this natural compound exert its beneficial effects and should be considered as a future research direction.

As previously mentioned, we proposed that prenatal hypoxia could induce a long-term increased susceptibility to MetS by impairing the function of mature adipocytes in the intra-abdominal cavity leading to increased circulating fatty-acids and peripheral lipid accumulation (Figure 7-7). However, this theory was not supported by our results. Further data to support or refute this hypothesis could be obtained by performing cross-transplantation of intra-abdominal fat between adult offspring from different experimental groups.[109, 110]

9.8 Future directions from a clinical-research perspective

Due in part to its biomedical and clinical relevance, the early programming concept remains a source of scientific interest and discussion; particularly so because it opens new doors not only for the understanding of

species adaptability and evolution[111] but also to novel pathophysiological and therapeutic approaches for highly prevalent medical conditions.[112]

One common criticism of the early programming theory results from the known association of being born with a low birth weight and long-term exposure to stress, poverty and infections;[113] which raised the question of whether the association between low birth weight and chronic diseases was simply an artifact of being born and raised under low socioeconomic conditions rather than a true programming phenomenon. A limited number of clinical studies, such as those describing the long-term effects of the twin-to-twin transfusion syndrome,[29] and many studies in animal models[114-117] have provided valuable information to support the early programming theory by separating the long-term effects of being born small from those linked to prematurity or associated with postnatal life. However, despite the advances made so far in the description and understanding of the early programming phenomenon, more efforts need to be made to understand the pathophysiological mechanisms before screening techniques and therapeutic alternatives can be implemented.[118] Moreover, the clinical evidence available to support the principles of early programming is very limited and needs to be expanded.

9.8.1 The challenge of identifying those who are IUGR during pregnancy

One of the reasons why the early programming theory is controversial relies on certain characteristics of the evidence that Barker and his predecessors used to support it. Most of the epidemiological data behind the initial proposal of this theory included several cohorts of subjects in whom birth anthropometric data was collected (for non-research purposes) and who could be followed up several decades later when they started developing cardiovascular outcomes.[17, 119-121] The biggest pitfall in the interpretation of this evidence is the impossibility of identifying which

subjects were born with low birth weight because some prenatal condition restricted their growth potential, which of them were born premature (and therefore smaller) and which were constitutively small. Some recent epidemiological studies, including more detailed anthropometrical and obstetric information, support the initial observation made by Barker and showed that being born growth restricted was associated with an increased susceptibility to develop chronic diseases later in life independent of the gestational age.[122] However, these studies need to be expanded.

Even though being born small for gestational age (SGA) has a very simplistic clinical definition (babies born below the 10th percentile of the expected weight for any given gestational age[123]), there are many other considerations that need to be made when evaluating gestational outcomes.[124, 125] In the absence of chromosomal abnormalities, two major groups of conditions that may cause fetus growth to be less than average and should be considered are: i) the fetus has a constitutional and physiological small size and ii) the presence of some pathological condition has restricted the ability of the developing fetus to reach its growth potential. However, only the latter of these can be considered IUGR.[126]

The birth-weight cut-off points used to define IUGR and SGA are based on epidemiological studies and do not consider the individual and physiological differences in some other important determinants of intrauterine growth such as genetic background and maternal height.[127] Therefore, these definitions fail to correctly identify those fetuses or newborns who are constituently small (lack of specificity) or those who are IUGR but still above the body weight 10th percentile limit (lack of sensitivity).[128] Despite these limitations, and due to pragmatic considerations, fetal and newborn weight and length are still the most commonly used parameters to screen fetuses and newborns for IUGR; and the lower 10th percentile is still the most commonly used cut-off point to identify those fetuses and babies who are small.

Fetal growth occurs on a continuum that starts very early in pregnancy and has variable rates throughout pregnancy. Generally speaking, fetal growth can be divided into two major overlapping stages, an initial organogenesis stage where differentiation of the tissues is more significant than the actual increase in embryonic mass, and a latter stage where physical structure abounds and better differentiated tissues exhibit a rapid growth trajectory.[129] Following a normal fetal growth chart, it is evident that there is less variability in fetal growth during the first third of pregnancy when outer percentiles of fetal weight (5th and 95th percentiles) are very close to each other (Supplemental Figure 10-4).[130] Interestingly, as the pregnancy progresses, the growth trajectory exhibits a more pronounced slope and the percentiles start to diverge. Based on this observation, most clinicians and epidemiologist agree that variations in birth weight that are susceptible to being prevented are a consequence of epiphenomenon occurring mainly during the last stages of pregnancy, especially during the last trimester.[131] Therefore, most attention in the study of the associations between birth weight and later development of health outcomes has focused on pathologies that affect fetal growth during this period of pregnancy.[132] However, it is plausible that certain prenatal insults affecting early stages of fetal development, or even those affecting maternal physiology before pregnancy, may have important long-term consequences on the future function of organs or systems regardless of whether or not they have a direct impact on fetal growth.[5, 133, 134] Given these considerations, one potential future direction for the understanding of the fetal programming phenomenon, and more importantly, the translation of basic fundamental knowledge that we have into a clinical scenario, would involve the challenge of correctly identifying babies who are IUGR and the severity of their condition.

A number of previous studies have proposed that it is the intrauterine growth trajectory rather than the birth weight that determines the level of IUGR in any given pregnancy.[135-138] In fact, it is well accepted that follow-up of growth trajectories *in utero* using echographic tools provides the most

accurate technique to determine gestational age and changes in fetal growth trajectories associated with IUGR. Recent publications have reported an association between changes in growth trajectories *in utero* and the presence of cardiac function abnormalities at birth.[139-142] However, little has been done to identify the association between these fetal growth patterns and the future development of cardiac and metabolic diseases. Moreover, the appropriate criteria to define changes in the growth trajectories are very controversial and require further investigation. Some authors have postulated that the evaluation of growth trajectories during the first years of life can provide a useful tool to identify those subjects in whom intrauterine conditions were suboptimal.[143-145] However, the usefulness of this information in therapeutic decision-making is very limited.

9.8.2 The challenge of identifying adults who were born IUGR

Following the same line of thinking, another interesting future perspective that could improve the clinical application of fetal programming is the definition of clinical or biochemical parameters that could identify adult subjects who were exposed, early in life, to conditions leading to programming. One characteristic shared by both animal and clinical models of early programming is that, after weaning, offspring who underwent early programming-inducing events exhibit no clear phenotypical characteristics that differentiate them from controls before the exposure to second insults and the consequent apparition of pathological outcomes. Therefore, in the absence of complete perinatal information, the early identification of these subjects at risk of developing chronic diseases is not possible. The identification and validation of early programming markers could have tremendous implications not only by improving techniques for clinical research in programming related areas, but also as a diagnostic tool that may have an important application in the evaluation of cardiovascular and metabolic risk in clinical scenarios.

9.8.3 Implications of early programming in children with obesity

As previously mentioned, childhood overweight and obesity constitutes a current health problem affecting 20% of children between 2 and 18 years old in North America.[146] Relative to adults, the association between obesity and the presence of other components of the MetS in children is less strong. Whereas in adults close to 95% of obese subjects have at least one other component of the MetS, only 50% of children with obesity have some other metabolic condition.[147-149] The fact that obese children are less likely to have other metabolic alterations than adults could be attributed to a larger metabolic reserves in children. However, since there is no consensus regarding the definition of the presence of most components of the MetS in children,[150] it is possible that the criteria used may underestimate the prevalence of these risk factors in a pediatric population. Regardless of whether the cut-off point used to define metabolic alterations is the most appropriate, the fact that 50% of obese children fulfilled those criteria suggests a heterogeneity in the metabolic changes associated with pediatric obesity; an observation that is greatly interesting in the perspective of our results.

In our studies, we discovered that offspring born IUGR as a result of a prenatal hypoxic insult were more likely to develop several components of the MetS when exposed to a HF diet (Chapter 7). Although all animals receiving a HF diet had increased body weights and total body fat, we did not observe any differences in these parameters when comparing control and IUGR offspring receiving the same diet. By extrapolating these results, we hypothesize that within a population of obese children, those born from pregnancies complicated by conditions leading to IUGR are likely to develop more severe obesity-related metabolic alterations when compared to children with the same degree of obesity but born from non-complicated pregnancies. To test this hypothesis we contacted members of the Pediatric Centre for Weight and Health at the University of Alberta with whom we

started a collaborative project to study the association between perinatal clinical parameters and the presence of metabolic alterations in a group of children with severe obesity. As a product of this collaboration, we designed a format to record all clinically relevant information from these children (Appendix 10.4) that may help us test this hypothesis in the future.

9.9 Conclusions

To conclude, the studies presented in this thesis provide valuable information for understanding the long-term consequences of hypoxia-induced IUGR from the perspective of cardiac and metabolic function. These results suggest that hypoxic prenatal insults leading to IUGR may affect the response to "second insults" such as aging, myocardial ischemia and HF diet.

In addition, we also described important interactions between hypoxia-induced early programming and sex differences, which suggest that some, but not all, mechanisms linking hypoxia-induced IUGR, and the later development of pathological conditions may be modulated by sex differences. Finally, our studies suggest that postnatal modulation of this prenatal preconditioning is possible through pharmacological interventions and offered a foundation for the development of ongoing collaborations toward the translation of this knowledge to clinical scenarios.

9.10 References

1. Kanaka-Gantenbein C. Fetal origins of adult diabetes. *Ann N Y Acad Sci*. 2010;1205:99-105.

2. Xita N, Tsatsoulis A. Fetal origins of the metabolic syndrome. *Ann N Y Acad Sci*. 2010;1205:148-155.

3. Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J Physiol*. 2004;561:355-377.

4. Armitage JA, Taylor PD, Poston L. Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. *J Physiol*. 2005;565:3-8.

5. Beydoun H, Saftlas AF. Physical and mental health outcomes of prenatal maternal stress in human and animal studies: a review of recent evidence. *Paediatr Perinat Epidemiol*. 2008;22:438-466.

6. Coupe B, Grit I, Darmaun D, Parnet P. The timing of "catch-up growth" affects metabolism and appetite regulation in male rats born with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol*. 2009;297:R813-824.

7. Bertram CE, Hanson MA. Prenatal programming of postnatal endocrine responses by glucocorticoids. *Reproduction*. 2002;124:459-467.

8. Chakraborty S, Joseph DV, Bankart MJ, Petersen SA, Wailoo MP. Fetal growth restriction: relation to growth and obesity at the age of 9 years. *Arch Dis Child Fetal Neonatal Ed*. 2007;92:F479-483.

9. Murphy E, Steenbergen C. Cardioprotection in females: a role for nitric oxide and altered gene expression. *Heart Fail Rev*. 2007;12:293-300.

10. Teede HJ. Sex hormones and the cardiovascular system: effects on arterial function in women. *Clin Exp Pharmacol Physiol*. 2007;34:672-676.

11. Arnal JF, Laurell H, Fontaine C, Billon A, Calippe B, Lenfant F, *et al.* Estrogen receptor actions on vascular biology and inflammation: implications in vascular pathophysiology. *Climacteric*. 2009;12 Suppl 1:12-17.

12. Ostadal B, Netuka I, Maly J, Besik J, Ostadalova I. Gender differences in cardiac ischemic injury and protection--experimental aspects. *Exp Biol Med (Maywood)*. 2009;234:1011-1019.

13. Xing D, Nozell S, Chen YF, Hage F, Oparil S. Estrogen and mechanisms of vascular protection. *Arterioscler Thromb Vasc Biol*. 2009;29:289-295.

14. Hunter JC, Kostyak JC, Novotny JL, Simpson AM, Korzick DH. Estrogen deficiency decreases ischemic tolerance in the aged rat heart: Roles of PKCdelta, PKCepsilon, Akt, and GSK3beta. *Am J Physiol Regul Integr Comp Physiol*. 2007;292:R800-809.

15. Stocker CJ, Arch JR, Cawthorne MA. Fetal origins of insulin resistance and obesity. *Proc Nutr Soc*. 2005;64:143-151.

16. Thornburg KL. Hypoxia and cardiac programming. *J Soc Gynecol Investig*. 2003;10:251.

17. Barker DJ. In utero programming of cardiovascular disease. *Theriogenology*. 2000;53:555-574.

18. Schwartz J, Thornburg KL. The influence of various physiological challenges on permanent changes to the cardiovascular system. *Arch Physiol Biochem*. 2003;111:3-7.

19. Bradamante S, Barenghi L, Piccinini F, Bertelli AA, De Jonge R, Beemster P, *et al.* Resveratrol provides late-phase cardioprotection by means of a nitric oxide- and adenosine-mediated mechanism. *Eur J Pharmacol*. 2003;465:115-123.

20. Das S, Cordis GA, Maulik N, Das DK. Pharmacological preconditioning with resveratrol: role of CREB-dependent Bcl-2 signaling via adenosine A3 receptor activation. *Am J Physiol Heart Circ Physiol*. 2005;288:H328-335.

21. Hattori R, Otani H, Maulik N, Das DK. Pharmacological preconditioning with resveratrol: role of nitric oxide. *Am J Physiol Heart Circ Physiol*. 2002;282:H1988-1995.

22. Das DK, Maulik N. Resveratrol in cardioprotection: a therapeutic promise of alternative medicine. *Mol Interv*. 2006;6:36-47.

23. Das DK, Sato M, Ray PS, Maulik G, Engelman RM, Bertelli AA*, et al.* Cardioprotection of red wine: role of polyphenolic antioxidants. *Drugs Exp Clin Res*. 1999;25:115-120.

24. Zipes DP, Barber MJ, Takahashi N, Gilmour RF, Jr. Influence of the autonomic nervous system on the genesis of cardiac arrhythmias. *Pacing Clin Electrophysiol*. 1983;6:1210-1220.

25. Ceconi C, Curello S, Ferrari R. Catecholamines: the cardiovascular and neuroendocrine system. *Eur Heart J*. 1998;19 Suppl F:F2-6.

26. Finsterer J, Stollberger C. The heart in human dystrophinopathies. *Cardiology*. 2003;99:1-19.

27. Massin MM, Withofs N, Maeyns K, Ravet F. The influence of fetal and postnatal growth on heart rate variability in young infants. *Cardiology*. 2001;95:80-83.

28. Fernandez-Twinn DS, Ekizoglou S, Wayman A, Petry CJ, Ozanne SE. Maternal low-protein diet programs cardiac beta-adrenergic response and signaling in 3-mo-old male offspring. *Am J Physiol Regul Integr Comp Physiol*. 2006;291:R429-436.

29. Petry CJ, Dorling MW, Wang CL, Pawlak DB, Ozanne SE. Catecholamine levels and receptor expression in low protein rat offspring. *Diabet Med*. 2000;17:848-853.

30. Furnes MW, Zhao CM, Stenstrom B, Arum CJ, Tommeras K, Kulseng B*, et al.* Feeding behavior and body weight development: lessons from rats subjected to gastric bypass surgery or high-fat diet. *J Physiol Pharmacol*. 2009;60 Suppl 7:25-31.

31. Miyasaka K, Ichikawa M, Kawanami T, Kanai S, Ohta M, Sato N*, et al.* Physical activity prevented age-related decline in energy metabolism in genetically obese and diabetic rats, but not in control rats. *Mech Ageing Dev*. 2003;124:183-190.

32. Ichikawa M, Kanai S, Ichimaru Y, Funakoshi A, Miyasaka K. The diurnal rhythm of energy expenditure differs between obese and glucose-intolerant rats and streptozotocin-induced diabetic rats. *J Nutr*. 2000;130:2562-2567.

33. Michiels C. Physiological and pathological responses to hypoxia. *Am J Pathol*. 2004;164:1875-1882.

34. Singer D. Metabolic adaptation to hypoxia: cost and benefit of being small. *Respir Physiol Neurobiol*. 2004;141:215-228.

35. Lynn EG, Lu Z, Minerbi D, Sack MN. The regulation, control, and consequences of mitochondrial oxygen utilization and disposition in the heart and skeletal muscle during hypoxia. *Antioxid Redox Signal*. 2007;9:1353-1361.

36. Gunaratnam L, Bonventre JV. HIF in kidney disease and development. *J Am Soc Nephrol*. 2009;20:1877-1887.

37. Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp Physiol*. 2006;91:807-819.

38. Cottrell EC, Seckl JR. Prenatal stress, glucocorticoids and the programming of adult disease. *Front Behav Neurosci*. 2009;3:19.

39. de Bruijn AT, van Bakel HJ, Wijnen H, Pop VJ, van Baar AL. Prenatal maternal emotional complaints are associated with cortisol responses in toddler and preschool aged girls. *Dev Psychobiol*. 2009;51:553-563.

40. Hemmings DG, Williams SJ, Davidge ST. Increased myogenic tone in 7-month-old adult male but not female offspring from rat dams exposed to hypoxia during pregnancy. *Am J Physiol Heart Circ Physiol*. 2005;289:H674-682.

41. Williams SJ, Campbell ME, McMillen IC, Davidge ST. Differential effects of maternal hypoxia or nutrient restriction on carotid and femoral vascular function in neonatal rats. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R360-367.

42. Williams SJ, Hemmings DG, Mitchell JM, McMillen IC, Davidge ST. Effects of maternal hypoxia or nutrient restriction during pregnancy on endothelial function in adult male rat offspring. *J Physiol*. 2005;565:125-135.

43. Xu Y, Williams SJ, O'Brien D, Davidge ST. Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *Faseb J*. 2006;20:1251-1253.

44. Zaccaria M, De Palo E, Zago E, Sicolo N, Erle G, Federspil G. Metabolic and endocrine responses to a standard mixed meal. A physiologic study. *Acta Diabetol Lat*. 1979;16:45-53.

45. Zimmet P. Epidemiology of diabetes and its macrovascular manifestations in Pacific populations: the medical effects of social progress. *Diabetes Care*. 1979;2:144-153.

46. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA*, et al.* Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr*. 2005;81:341-354.

47. Robson AA. Preventing diet induced disease: bioavailable nutrient-rich, low-energy-dense diets. *Nutr Health*. 2009;20:135-166.

48. Simopoulos AP. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomed Pharmacother*. 2006;60:502-507.

49. Banerji GG. Effect of a high-fat diet on the excretion of bisulphite-binding substances in the urine of rats deficient in vitamin B(1). *Biochem J*. 1940;34:1329-1333.

50. Buettner R, Scholmerich J, Bollheimer LC. High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity (Silver Spring)*. 2007;15:798-808.

51. Boychuk CR, Fuller DD, Hayward LF. Sex differences in heart rate variability during sleep following prenatal nicotine exposure in rat pups. *Behav Brain Res*. 2010.

52. Graf AV, Maslova MV, Trofimova LK, Dunaeva TY, Sokolova NA, Kudryashova NY*, et al.* Effect of antenatal hypoxia on age-specific dynamics of ECG parameters and content of biogenic amines in the central nervous system. *Bull Exp Biol Med*. 2007;144:188-191.

53. Schneider U, Fiedler A, Schroder B, Jaekel S, Stacke A, Hoyer D*, et al.* The effect of antenatal steroid treatment on fetal autonomic heart rate regulation revealed by fetal magnetocardiography (fMCG). *Early Hum Dev*. 2010;86:319-325.

54. Schuetze P, Eiden RD, Danielewicz S. The association between prenatal cocaine exposure and physiological regulation at 13 months of age. *J Child Psychol Psychiatry*. 2009;50:1401-1409.

55. Bunag RD, Tomita T, Krizsan D. Renovascular beta adrenergic hypersensitivity and hyperinsulinemia in rats with dietary-induced obesity. *J Pharmacol Exp Ther*. 1990;255:325-332.

56. Chen W, Srinivasan SR, Hallman DM, Berenson GS. The relationship between birthweight and longitudinal changes of blood pressure is modulated by beta-adrenergic receptor genes: the Bogalusa Heart Study. *J Biomed Biotechnol*. 2010;2010:543514.

57. Dygalo NN, Kalinina TS, Shishkina GT. Neonatal programming of rat behavior by downregulation of alpha2A-adrenoreceptor gene expression in the brain. *Ann N Y Acad Sci*. 2008;1148:409-414.

58. Kudlacz EM, Navarro HA, Eylers JP, Slotkin TA. Adrenergic modulation of cardiac development in the rat: effects of prenatal exposure to propranolol via continuous maternal infusion. *J Dev Physiol*. 1990;13:243-249.

59. Longo LD, Pearce WJ. Fetal cerebrovascular acclimatization responses to high-altitude, long-term hypoxia: a model for prenatal programming of adult disease? *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R16-24.

60. Schaffer L, Burkhardt T, Muller-Vizentini D, Rauh M, Tomaske M, Mieth RA*, et al.* Cardiac autonomic balance in small-for-gestational-age neonates. *Am J Physiol Heart Circ Physiol*. 2008;294:H884-890.

61. Brummelte S, Grunau RE, Zaidman-Zait A, Weinberg J, Nordstokke D, Cepeda IL. Cortisol levels in relation to maternal interaction and child internalizing

behavior in preterm and full-term children at 18 months corrected age. *Dev Psychobiol*. 2010.

62. Brunton PJ, Russell JA. Neuroendocrine control of maternal stress responses and fetal programming by stress in pregnancy. *Prog Neuropsychopharmacol Biol Psychiatry*. 2011.

63. Harris A, Seckl J. Glucocorticoids, prenatal stress and the programming of disease. *Horm Behav*. 2010.

64. Janicki JS, Brower GL, Gardner JD, Chancey AL, Stewart JA, Jr. The dynamic interaction between matrix metalloproteinase activity and adverse myocardial remodeling. *Heart Fail Rev*. 2004;9:33-42.

65. Satpathy HK, Frey D, Satpathy R, Satpathy C, Fleming A, Mohiuddin SM*, et al.* Peripartum cardiomyopathy. *Postgrad Med*. 2008;120:28-32.

66. Liu HR, Zhao RR, Jiao XY, Wang YY, Fu M. Relationship of myocardial remodeling to the genesis of serum autoantibodies to cardiac beta(1)-adrenoceptors and muscarinic type 2 acetylcholine receptors in rats. *J Am Coll Cardiol*. 2002;39:1866-1873.

67. Gaddam KK, Verma A, Thompson M, Amin R, Ventura H. Hypertension and cardiac failure in its various forms. *Med Clin North Am*. 2009;93:665-680.

68. Bogdarina I, Haase A, Langley-Evans S, Clark AJ. Glucocorticoid effects on the programming of AT1b angiotensin receptor gene methylation and expression in the rat. *PLoS One*. 2010;5:e9237.

69. Ligi I, Grandvuillemin I, Andres V, Dignat-George F, Simeoni U. Low birth weight infants and the developmental programming of hypertension: a focus on vascular factors. *Semin Perinatol*. 2010;34:188-192.

70. Vohr BR, Allan W, Katz KH, Schneider KC, Ment LR. Early predictors of hypertension in prematurely born adolescents. *Acta Paediatr*. 2010;99:1812-1818.

71. Bagby SP. Maternal nutrition, low nephron number, and hypertension in later life: pathways of nutritional programming. *J Nutr*. 2007;137:1066-1072.

72. Barker DJ, Bagby SP, Hanson MA. Mechanisms of disease: in utero programming in the pathogenesis of hypertension. *Nat Clin Pract Nephrol*. 2006;2:700-707.

73. Baum M. Role of the kidney in the prenatal and early postnatal programming of hypertension. *Am J Physiol Renal Physiol*. 2010;298:F235-247.

74. McMullen S, Langley-Evans SC. Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms. *Am J Physiol Regul Integr Comp Physiol*. 2005;288:R85-90.

75. Nuyt AM, Alexander BT. Developmental programming and hypertension. *Curr Opin Nephrol Hypertens*. 2009;18:144-152.

76. Villamor E, Kessels CG, Ruijtenbeek K, van Suylen RJ, Belik J, de Mey JG*, et al.* Chronic in ovo hypoxia decreases pulmonary arterial contractile reactivity and induces biventricular cardiac enlargement in the chicken embryo. *Am J Physiol Regul Integr Comp Physiol*. 2004;287:R642-651.

77. Zoer B, Kessels L, Vereijken A, De Mey JG, Bruggeman V, Decuypere E*, et al.* Effects of prenatal hypoxia on pulmonary vascular reactivity in chickens prone to pulmonary hypertension. *J Physiol Pharmacol*. 2009;60:119-130.

78. Jones RD, Morice AH, Emery CJ. Effects of perinatal exposure to hypoxia upon the pulmonary circulation of the adult rat. *Physiol Res*. 2004;53:11-17.

79. Herrera EA, Riquelme RA, Ebensperger G, Reyes RV, Ulloa CE, Cabello G*, et al.* Long-term exposure to high-altitude chronic hypoxia during gestation induces neonatal pulmonary hypertension at sea level. *Am J Physiol Regul Integr Comp Physiol*. 2010;299:R1676-1684.

80. Kaltsas G, Vgontzas A, Chrousos G. Fatigue, endocrinopathies, and metabolic disorders. *PM R*. 2010;2:393-398.

81. Gerdes AM, Iervasi G. Thyroid replacement therapy and heart failure. *Circulation*. 2010;122:385-393.

82. Nabbout LA, Robbins RJ. The cardiovascular effects of hyperthyroidism. *Methodist Debakey Cardiovasc J*. 2010;6:3-8.

83. Zimmermann MB. The influence of iron status on iodine utilization and thyroid function. *Annu Rev Nutr*. 2006;26:367-389.

84. Cioffi F, Lanni A, Goglia F. Thyroid hormones, mitochondrial bioenergetics and lipid handling. *Curr Opin Endocrinol Diabetes Obes*. 2010;17:402-407.

85. Pisarev MA. Interrelationships between the pancreas and the thyroid. *Curr Opin Endocrinol Diabetes Obes*. 2010;17:437-439.

86. Symonds ME, Mostyn A, Pearce S, Budge H, Stephenson T. Endocrine and nutritional regulation of fetal adipose tissue development. *J Endocrinol*. 2003;179:293-299.

87. Wilcoxon JS, Redei EE. Prenatal programming of adult thyroid function by alcohol and thyroid hormones. *Am J Physiol Endocrinol Metab*. 2004;287:E318-326.

88. Slone-Wilcoxon J, Redei EE. Maternal-fetal glucocorticoid milieu programs hypothalamic-pituitary-thyroid function of adult offspring. *Endocrinology*. 2004;145:4068-4072.

89. Weinstock M. The potential influence of maternal stress hormones on development and mental health of the offspring. *Brain Behav Immun*. 2005;19:296-308.

90. Xu Y, Williams SJ, Armstrong SJ. Effects of maternal hypoxia on expression of cardiac mitochondrial F1F0- proton ATPase and post-ischemic recovery in adult offspring. *The Canadian Journal of Cardiology*. 2005;21:81C-82C.

91. Cheng JF, Huang Y, Penuliar R, Nishimoto M, Liu L, Arrhenius T*, et al.* Discovery of potent and orally available malonyl-CoA decarboxylase inhibitors as cardioprotective agents. *J Med Chem*. 2006;49:4055-4058.

92. Cuthbert KD, Dyck JR. Malonyl-CoA decarboxylase is a major regulator of myocardial fatty acid oxidation. *Current hypertension reports*. 2005;7:407-411.

93. Lopaschuk GD, Stanley WC. Malonyl-CoA decarboxylase inhibition as a novel approach to treat ischemic heart disease. *Cardiovasc Drugs Ther*. 2006;20:433-439.

94. Ussher JR, Lopaschuk GD. The malonyl CoA axis as a potential target for treating ischaemic heart disease. *Cardiovasc Res*. 2008;79:259-268.

95. Ussher JR, Lopaschuk GD. Targeting malonyl CoA inhibition of mitochondrial fatty acid uptake as an approach to treat cardiac ischemia/reperfusion. *Basic Res Cardiol*. 2009;104:203-210.

96. Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D*, et al.* Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc Natl Acad Sci U S A*. 2001;98:11114-11119.

97. Bae S, Zhang L. Prenatal cocaine exposure increases apoptosis of neonatal rat heart and heart susceptibility to ischemia-reperfusion injury in 1-month-old rat. *Br J Pharmacol*. 2005;144:900-907.

98. Wallace DC. Mitochondria, bioenergetics, and the epigenome in eukaryotic and human evolution. *Cold Spring Harb Symp Quant Biol*. 2009;74:383-393.

99. Wallace DC. Bioenergetics and the epigenome: interface between the environment and genes in common diseases. *Dev Disabil Res Rev*. 2010;16:114-119.

100. Wallace DC, Fan W. Energetics, epigenetics, mitochondrial genetics. *Mitochondrion*. 2010;10:12-31.

101. Delisle HF, Vioque J, Gil A. Dietary patterns and quality in West-African immigrants in Madrid. *Nutr J*. 2009;8:3.

102. Denova-Gutierrez E, Castanon S, Talavera JO, Gallegos-Carrillo K, Flores M, Dosamantes-Carrasco D*, et al.* Dietary patterns are associated with metabolic syndrome in an urban Mexican population. *J Nutr*. 2010;140:1855-1863.

103. Paradis AM, Godin G, Perusse L, Vohl MC. Associations between dietary patterns and obesity phenotypes. *Int J Obes (Lond)*. 2009;33:1419-1426.

104. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov*. 2006;5:493-506.

105. Szkudelska K, Szkudelski T. Resveratrol, obesity and diabetes. *European Journal of Pharmacology*. 2010;635:1-8.

106. Resveratrol. Monograph. *Altern Med Rev*. 2010;15:152-158.

107. Kelly G. A review of the sirtuin system, its clinical implications, and the potential role of dietary activators like resveratrol: part 1. *Altern Med Rev*. 2010;15:245-263.

108. Gresele P, Cerletti C, Guglielmini G, Pignatelli P, de Gaetano G, Violi F. Effects of resveratrol and other wine polyphenols on vascular function: an update. *J Nutr Biochem*. 2010.

109. Park BH, Wang MY, Lee Y, Yu X, Ravazzola M, Orci L*, et al.* Combined leptin actions on adipose tissue and hypothalamus are required to deplete adipocyte fat in lean rats: implications for obesity treatment. *J Biol Chem*. 2006;281:40283-40291.

110. Wang MY, Orci L, Ravazzola M, Unger RH. Fat storage in adipocytes requires inactivation of leptin's paracrine activity: implications for treatment of human obesity. *Proc Natl Acad Sci U S A*. 2005;102:18011-18016.

111. Soto AM, Rubin BS, Sonnenschein C. Interpreting endocrine disruption from an integrative biology perspective. *Mol Cell Endocrinol*. 2009;304:3-7.

112. Joss-Moore LA, Lane RH. The developmental origins of adult disease. *Curr Opin Pediatr*. 2009;21:230-234.

113. Holland ML, Kitzman H, Veazie P. The effects of stress on birth weight in low-income, unmarried black women. *Womens Health Issues*. 2009;19:390-397.

114. Li G, Bae S, Zhang L. Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. *Am J Physiol Heart Circ Physiol*. 2004;286:H1712-1719.

115. Han HC, Austin KJ, Nathanielsz PW, Ford SP, Nijland MJ, Hansen TR. Maternal nutrient restriction alters gene expression in the ovine fetal heart. *J Physiol*. 2004;558:111-121.

116. Bogdarina I, Murphy HC, Burns SP, Clark AJ. Investigation of the role of epigenetic modification of the rat glucokinase gene in fetal programming. *Life Sci*. 2004;74:1407-1415.

117. Bertram CE, Hanson MA. Animal models and programming of the metabolic syndrome. *Br Med Bull*. 2001;60:103-121.

118. Barker DJ. In utero programming of chronic disease. *Clin Sci (Lond)*. 1998;95:115-128.

119. Fall CH, Barker DJ. The fetal origins of coronary heart disease and non-insulin dependent diabetes in India. *Indian Pediatr*. 1997;34:5-8.

120. Barker DJ. Fetal origins of coronary heart disease. *Br Heart J*. 1993;69:195-196.

121. Barker DJ. The origins of the developmental origins theory. *J Intern Med*. 2007;261:412-417.

122. Barker DJ, Osmond C, Simmonds SJ, Wield GA. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. *Bmj*. 1993;306:422-426.

123. Rizzo G, Arduini D. Intrauterine growth restriction: diagnosis and management. A review. *Minerva Ginecol*. 2009;61:411-420.

124. Falo AP. Intrauterine growth retardation (IUGR): prenatal diagnosis by imaging. *Pediatr Endocrinol Rev*. 2009;6 Suppl 3:326-331.

125. Manning FA. Antepartum fetal testing: a critical appraisal. *Curr Opin Obstet Gynecol*. 2009;21:348-352.

126. Marsal K. Intrauterine growth restriction. *Curr Opin Obstet Gynecol*. 2002;14:127-135.

127. Sacks DA. Determinants of fetal growth. *Curr Diab Rep*. 2004;4:281-287.

128. Ananth CV, Vintzileos AM. Distinguishing pathological from constitutional small for gestational age births in population-based studies. *Early Hum Dev*. 2009;85:653-658.

129. Lockwood CJ, Weiner S. Assessment of fetal growth. *Clinics in Perinatology*. 1986;13:3-35.

130. Shinozuka N, Nakamura T, Hirayama M. Standard Growth Curve of Japanese using Non-Linear Growth Model *Acta Neanatoligica Japonica*. 1994;30:433-441.

131. Deorari AK, Agarwal R, Paul VK. Management of infants with intra-uterine growth restriction. *Indian J Pediatr*. 2008;75:171-174.

132. Gardosi J. New definition of small for gestational age based on fetal growth potential. *Horm Res*. 2006;65 Suppl 3:15-18.

133. Chmurzynska A. Fetal programming: link between early nutrition, DNA methylation, and complex diseases. *Nutrition Reviews*. 2010;68:87-98.

134. MacLaughlin SM, Walker SK, Roberts CT, Kleemann DO, McMillen IC. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *J Physiol*. 2005;565:111-124.

135. Deter RL. Individualized growth assessment: evaluation of growth using each fetus as its own control. *Semin Perinatol*. 2004;28:23-32.

136. Lee W, Deter RL, McNie B, Goncalves LF, Espinoza J, Chaiworapongsa T*, et al.* The fetal arm: individualized growth assessment in normal pregnancies. *J Ultrasound Med*. 2005;24:817-828.

137. Lee W, Deter RL, McNie B, Goncalves LF, Espinoza J, Chaiworapongsa T*, et al.* Individualized growth assessment of fetal soft tissue using fractional thigh volume. *Ultrasound Obstet Gynecol*. 2004;24:766-774.

138. Marlow N. Teasing out the effects of different fetal growth trajectories: commentary on the article by van Batenburg-Eddes et al. on page 132. *Pediatr Res*. 2010;67:128-129; discussion 132.

139. Crispi F, Bijnens B, Figueras F, Bartrons J, Eixarch E, Le Noble F*, et al.* Fetal growth restriction results in remodeled and less efficient hearts in children. *Circulation*. 2010;121:2427-2436.

140. Tsyvian PB, Markova TV, Mikhailova SV, Hop WC, Wladimiroff JW. Left ventricular isovolumic relaxation and renin-angiotensin system in the growth restricted fetus. *Eur J Obstet Gynecol Reprod Biol*. 2008;140:33-37.

141. Verburg BO, Jaddoe VW, Wladimiroff JW, Hofman A, Witteman JC, Steegers EA. Fetal hemodynamic adaptive changes related to intrauterine growth: the Generation R Study. *Circulation*. 2008;117:649-659.

142. Verkauskiene R, Beltrand J, Claris O, Chevenne D, Deghmoun S, Dorgeret S*, et al.* Impact of fetal growth restriction on body composition and hormonal status at birth in infants of small and appropriate weight for gestational age. *Eur J Endocrinol*. 2007;157:605-612.

143. de Beer M, van Eijsden M, Vrijkotte TG, Gemke RJ. Early growth patterns and cardiometabolic function at the age of 5 in a multiethnic birth cohort: the ABCD study. *BMC Pediatr*. 2009;9:23.

144. Dulloo AG. Thrifty energy metabolism in catch-up growth trajectories to insulin and leptin resistance. *Best Pract Res Clin Endocrinol Metab*. 2008;22:155-171.

145. Dulloo AG, Jacquet J, Seydoux J, Montani JP. The thrifty 'catch-up fat' phenotype: its impact on insulin sensitivity during growth trajectories to obesity and metabolic syndrome. *Int J Obes (Lond)*. 2006;30 Suppl 4:S23-35.

146. Han JC, Lawlor DA, Kimm SY. Childhood obesity. *Lancet*. 2010;375:1737-1748.

147. Benson L, Baer HJ, Kaelber DC. Trends in the diagnosis of overweight and obesity in children and adolescents: 1999-2007. *Pediatrics*. 2009;123:e153-158.

148. Flodmark CE, Ohlsson T. Childhood obesity: from nutrition to behaviour. *Proc Nutr Soc*. 2008;67:356-362.

149. Hudson CE. Being overweight and obese: Black children ages 2-5 years. *ABNF J*. 2008;19:89-91.

150. Mancini MC. Metabolic syndrome in children and adolescents - criteria for diagnosis. *Diabetol Metab Syndr*. 2009;1:20.

CHAPTER 10 APPENDICES

10.1 Standard rodent echocardiography form

Technician name:	Echo date:
Study name:	Echo start time:
Species/strain:	Echo end time:
Group/ID: Date of birth/age:	Anesthetic induction:
Scan head:	Anesthetic maintenance
Rectal temperature at beginning: at the end:	Anesthetic gas (oxygen) or (air)

Biventricular (short-axis)	1st Reading	2nd Reading	3rd Reading
rvidd - right ventricle internal diam in diastole (mm)			
ivsd - interventricular septum in diastole (mm)			
lvidd -LV internal diameter in diastole (mm)			
lvpwd -LV posterior wall diastole (mm)			
ivss - interventricular septum in systole (mm)			
lvids - LV internal diameter in systole (mm)			
lvpws - LV posterior wall in systole (mm)			
Left vent (long-axis)	**1st Reading**	**2nd Reading**	**3rd Reading**
lvidtraces – LV internal diam by trace systole (mm)			
lvidtraced -LV internal diam by trace in diastole (mm)			
lvvols - LV vol in systole (µl)			
lvvold - LV vol in diastole (µl)			
stroke - stroke volume (µl)			
eftrace - ejection fraction by trace (%)			
fstrace - fractional shortening by trace (%)			
cotrace –cardiac output by trace (ml/min)			
Ao	**1st Reading**	**2nd Reading**	**3rd Reading**
aovti - aortic valve vel time integral (cm)			
aovtimeanvel - aortic valve mean vel (cm/s)			
aomenagrad - aortic valve mean gradient (mmHg)			
aopeakvel - aortic valve peak velocity (mm/s)			
aopeaKgrad – Ao valve peak gradient (mmHg)			
aoejectime - aortic valve ejection time (ms)			
Aoexitdiamd – aortic outflow diam in diastole (mm)			
aoexitdiams – aortic outflow diameter in systole (mm)			
latriums – Left atrium diameter in systole (mm)			
latriumd – Left atrium diameter in diastole (mm)			
aorootsys - Aortic root internal diam in systole (mm)			
aorootdias - Aortic root internal diam in diastole (mm)			
hr – heart rate			

Mitral	1st Reading	2nd Reading	3rd Reading
mved – mitral E vel by regular Doppler (mm/s)			
mvad – mitral A velocity by regular Doppler (mm/s)			
mvetd – mitral E vel by tissue Doppler (mm/s)			
mveetd – mitral E' vel by tissue Doppler (mm/s)			
mvdecelt – mitral acceleration (mm/s^2)			
mvdec –mitral decel time (ms)			
ivrt – isovolumetric relaxation time (ms)			
ivct – isovolumetric contraction time (ms)			
meaind – mitral e/a index			
Tricuspid	**1st Reading**	**2nd Reading**	**3rd Reading**
tve – tricuspid valve E vel (mm/s)			
tva – tricuspid valve A vel (mm/s)			
regtime – regurgitation time (ms)			
tved – tricuspid valve E vel (tissue Doppler) (mm/s)			
Tve'd – tricuspid valve E' vel (tissue Doppler) (mm/s)			
Pulmonary	**1st Reading**	**2nd Reading**	**3rd Reading**
pvvti – pulmonary valve volume time integral (cm)			
pvmeanvel - pulmonary valve mean vel (mm/s)			
pvmeangrad – pulmonary valve mean grad (mmHg)			
pvpeakvel – pulmonary peak velocity (mm/s)			
pvpeaKgrad – pulmonary peak gradient (mmHg)			
Pdopacel – pulmonary valve acceleration time (ms)			
Rvejecttime – right ventricle acceleration time (ms)			
Function on biventricular evaluation	**1st Reading**	**2nd Reading**	**3rd Reading**
Fs - fractional shortening (%)			
ef - ejection fraction (%)			
lvmass - left vent mass (mg)			
lvmascor - LV mass corrected (mg)			
lvvold - LV vol in diastole (µL)			
lvvols - LV vol in systole (µL)			

Notes:

10.2 Perfusion report form

Animal Code[1]: **DAM** [ ] [ ] [ ] - [ ]
Consecutive | Sex (M/F) | Age (Months) | Number

D.O.B.[2] [d][d] / [m][m] / [y][y][y][y]

Treatment[3] Hypoxia [1] Control [0] **Total Body Weight[4]** [ ][ ][ ] . [ ] grs

Echo[5] Yes [1] No [0] **Date / time of echo[6]** [ ]

Heparin[7] Yes [1] No [0] **Anesthesia[9]** Ketamin/Xilacin [1] Isofluorane [2] Pentotal [3]

Dosage[8] [1000 U/Kg IP] **Dosage[10]** [75 mg/Kg / 10 mg/Kg I.P] [5%] [50-60mg/Kg IP]

Langendorf Solution

NaCl	120	mmol/L	$NaHCO_3$	25	mmol/L	KCl	1.2	mmol/L	KH_2PO_4	1.2	mmol/L
$MgSO_4$	1.2	mmol/L	CaCl	2.5	mmol/L	Glucose	5.5	mmol/L	Insulin	0	mU/L
Lactate	0.5	mmol/L	**Volume prepared[12]**					mL	**Ph[13]**		

Working Heart Solution

NaCl	120	mmol/L	$NaHCO_3$	25	mmol/L	KCl	1.2	mmol/L	KH_2PO_4	1.2	mmol/L
$MgSO_4$	1.2	mmol/L	CaCl	1.2 / 2.5	mmol/L	Glucose	5 / 5.5 / 10	mmol/L	Insulin	100	mU/L
Lactate	0.5	mmol/L	BSA	3	%	**Palmitate[14]**		mg	**HCO3[15]**		mg
						Palmitate[16]	0.6 / 1.2	mmol/L			

Volume prepared[17] [200] mL **Volume against dialized[18]** [1800] mL **Dialysis Time[19]** [ ] Hrs

Ph[20] [ ] **Radioisotopes[21]** Glucose ^{14}C [mC] Glucose ^{3}H [mC] Lactate ^{14}C [mC] Palmitate ^{3}H [mC]

Perfusion

Perfusion Date[22] [d][d] / [m][m] / [y][y][y][y] **Perfused by:[23]** [ ]

Time to Langerdorf[24] [ ] s **Time to Working[25]** [ ] m **Pacing[26]** [1] Yes rate: [0] No

F.A[27] [1] Yes [0] No **Technique roblem[28]** [1] Yes [0] No

IV Millar catheter[29] [1] Mitral [2] Aortic [0] None

Atrium cannula Leak[30] [0] None [1] Minor [2] Major **Time of mechanical support after ischemia[31]** [ ] m

Working initial Vol[32] [ ] mL **Working Final Vol[33]** [ ] mL

Number of solution samples[34] [ ] **Vol of each solution samples[35]** [ ] mL

Total heart frozen weight 36 [Al] [Al+♥] g **Heart fragment frozen weight** [Al] [Al+♥] g **Heart fragment dry weight38** [Al+♥] g

Notes:[39]

Suitable for analyses[40] [0] No [1] Yes [2] Partially

10.3 Cardiac metabolism details

Male offspring

4 Months

	Pre-ischemia		Reperfusion	
	Control (n=7)	**IUGR (n=9)**	**Control (n=7)**	**IUGR (n=9)**
FOx	91.0 (1.00)	90.6 (0.40)	84.8 (5.47)*	80.6 (2.38)*
GOx	1.31 (0.64)	0.77 (0.09)	5.15 (2.11)*	4.63 (1.69)*
LOx	5.08 (0.96)	6.47 (0.28)	3.73 (0.45)	5.02 (1.06)
Glyco	2.61 (0.73)	2.10 (0.22)	6.25 (1.04)*	9.71(1.42)*

12 Months

	Pre-ischemia		Reperfusion	
	Control (n=9)	**IUGR (n=7)**	**Control (n=9)**	**IUGR (n=7)**
FOx	92.2 (0.76)	90.4 (1.35)	81.9 (2.56)*	72.1 (4.24)*
GOx	0.49 (0.06)	0.99 (0.15)	3.17 (0.79)*	4.03 (1.65)*
LOx	4.61 (0.73)	4.62 (1.74)	5.21 (1.61)	5.17 (1.3)
Glyco	2.66 (0.44)	3.98 (0.56)	9.65 (1.35)*	18.6 (1.84)*†

Female offspring

4 Months

	Pre-ischemia		Reperfusion	
	Control (n=8)	**IUGR (n=8)**	**Control (n=8)**	**IUGR (n=8)**
FOx	92.6 (0.34)	94.1 (0.78)	85.8 (2.79)*	83.8 (1.46)*
GOx	0.66 (0.13)	0.43 (0.04)	2.52 (0.69)*	1.86 (0.29)
LOx	4.02 (0.50)	3.45 (0.620	5.04 (2.01)	6.00 (1.15)*
Glyco	2.71 (0.30)	1.95 (0.30)	6.55 (0.68)*	8.33 (1.29)*

12 Months

	Pre-ischemia		Reperfusion	
	Control (n=9)	**IUGR (n=12)**	**Control (n=9)**	**IUGR (n=12)**
FOx	92.8 (0.31)	93.1 (0.31)	86.3 (1.32)*	87.5 (1.74)*
GOx	0.38 (0.03)	0.57 (0.07)	0.61 (0.12)	2.34 (0.63)*
LOx	3.78 (0.35)	4.10 (0.38)	5.04 (1.35)	1.93 (0.45)
Glyco	2.98 (0.27)	2.27 (0.36)	8.08 (1.22)*	8.18 (1.07)*

Contribution of the four main energy substrates used by the myocardium to produce ATP under pre-ischemic conditions and during reperfusion after a 10-minute no-flow ischemia. Values presented as mean (SE) % relative to the total ATP produced under ideal Acetyl-CoA / ATP coupling. FOx: palmitate oxidation, GOx: glucose oxidation, LOx: lactate oxidation, Glyco: glycolysis. * $p<0.05$ when compared to basal values in the same group of animals. † represents a $p<0.05$ using a χ^2 test, when compared to controls of the same age and sex.

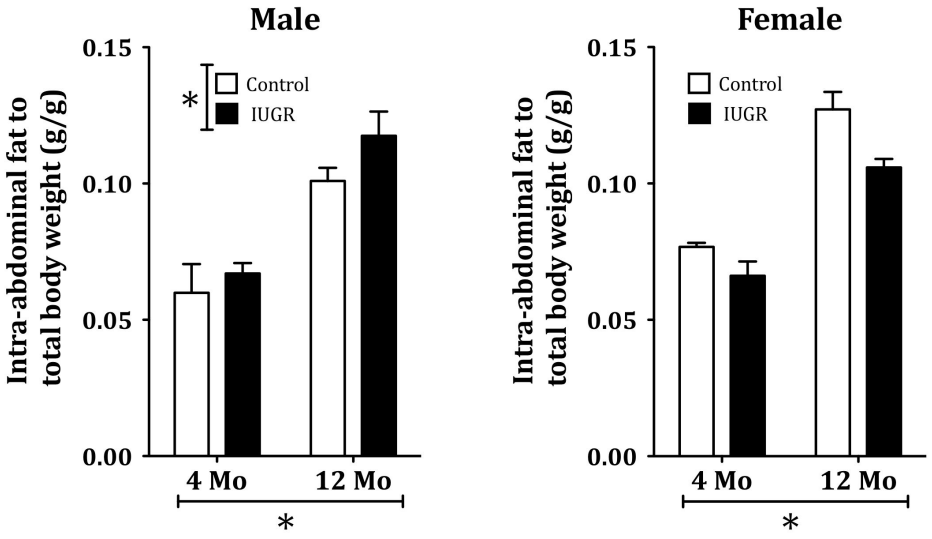


Figure 10-1 Effect of IUGR on relative intra-abdominal fat content of male and female offspring at 4 and 12 months of age (Pilot study)

Data obtained from experimental animals used for studies presented in chapters 4 to 6 of this thesis. Following cardiac extraction and perfusion, a complete laparotomy was performed, pancreas was extracted and intra-abdominal fat pads (mesenteric-epiploic, epididymal, retroperitoneal and sub-diaphragmatic) were extracted and weighed. Data is presented as total intra-abdominal fat weight corrected by total body weight (n=2 to 5). Pilot study.

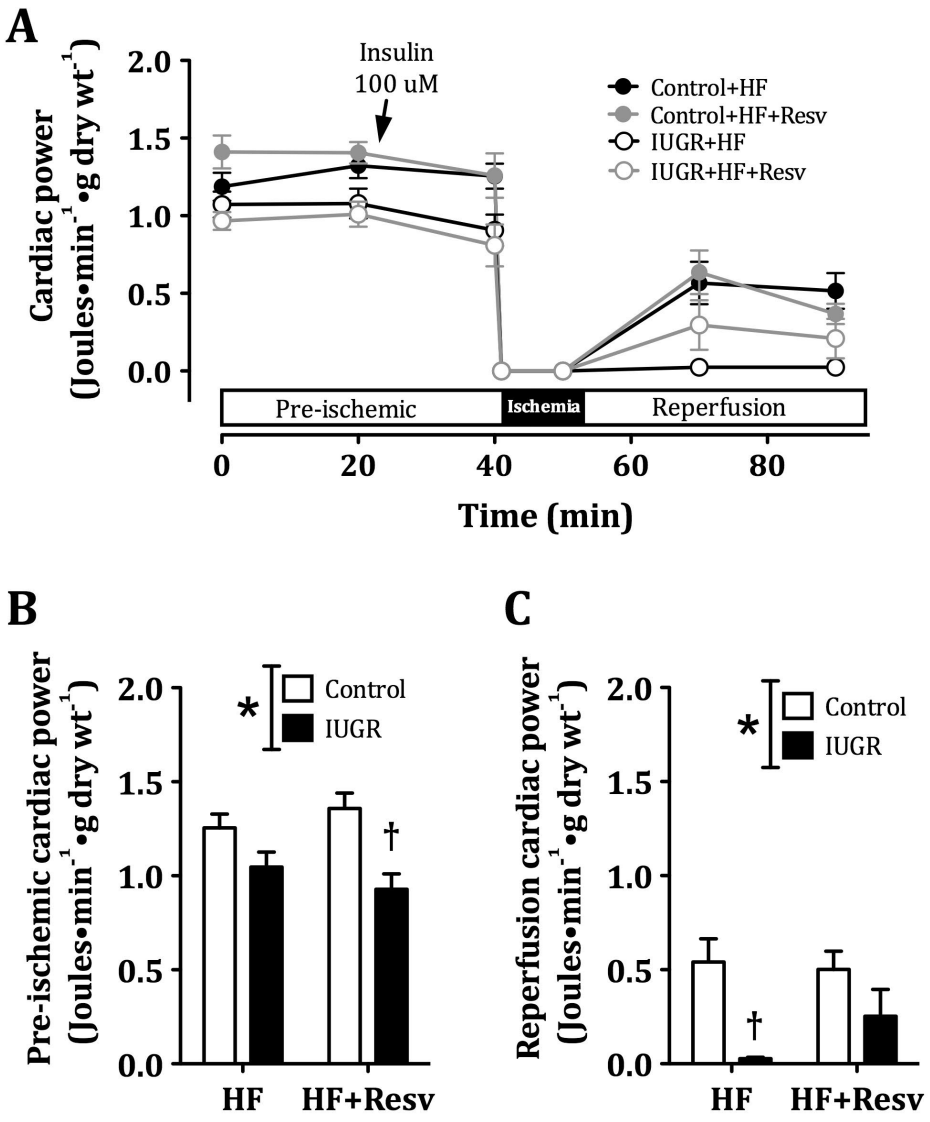


Figure 10-2 Effect of IUGR and administration of Resveratrol on myocardial susceptibility to ischemia/reperfusion injury in offspring receiving a high-fat diet

(**A**) Average cardiac power developed over time during *ex vivo* cardiac aerobic perfusion (pre-ischemic) and after 10 minutes of no-flow ischemia (reperfusion) from different experimental groups, (**B**) average maximal cardiac power developed during pre-ischemic period, (**C**) average maximal cardiac power developed during reperfusion. Values obtained from five to six animals from different litters per group after nine weeks receiving high-fat (HF) diet with or without Resveratrol (Resv; 4 g/Kg of diet). * represents a value of $p<0.05$ for the respective sources of variation (age or prenatal intervention) using two-way ANOVA. † represents a $p<0.05$ vs. controls of the same age after a Bonferroni post-hoc test.

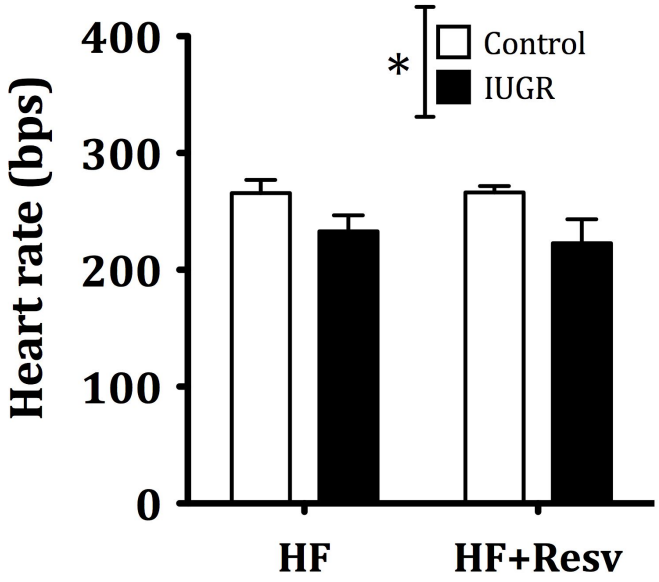


Figure 10-3 Effect of IUGR and administration of Resveratrol on heart rate evaluated *ex vivo* in young adult offspring receiving a high-fat diet (pilot study)

Data obtained from experimental animals used for the studies presented in Chapter 8 of this thesis. Once the hearts were placed in working mode and had a stable rhythm and cardiac output, surface electrodes were placed in the DI Einthoven's axis and an electrocardiographic trace was recorded for one minute. Heart rate during that period of time was average for each animal. * represents $p<0.05$ for the respective source of variation (IUGR or Resveratrol) using two-way ANOVA (n=5 to 7) Pilot study.

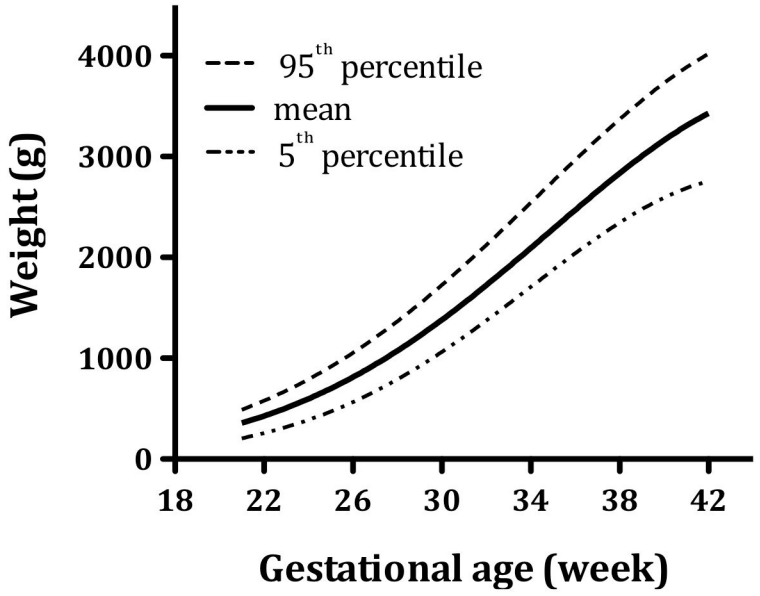


Figure 10-4 Normal fetal growth chart

SD: Standard deviation. Modified from Shinozuka N, Nakamura T, Hirayama M. Standard Growth Curve of Japanese using Non-Linear Growth Model *Acta Neanatoligica Japonica*. 1994;30:433-441.

10.4 Medical history and physical exam initial assessment form

Perinatal History:

1. Age of the mother when she gave birth to the children (years): ____
2. Marital status during pregnancy:
   Single ❑1 Married ❑2 Widowed ❑3 Common law ❑4 Other ❑5
3. How many prenatal controls did the mother go to during pregnancy: ____

During Pregnancy, did the mother: If YES, please explain:

4. Smoke	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
4.1. If yes please explain:_____					
5. Drink alcohol	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
5.1. If yes please explain:_____					
6. Use medications	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
6.1. If yes please explain:_____					
7. Use drugs	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
7.1. If yes please explain:_____					
8. Use hormones	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
8.1. If yes please explain:_____					
9. Have X-rays	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
9.1. If yes please explain:_____					
10. Have vaginal bleeding	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
10.1. If yes please explain:_____					
11. Vomit excessively (More than twice a day most days during 3 months)	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
11.1. If yes please explain:_____					
12. Have infections (systemic infections that require antibiotic treatment)	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
12.1. If yes please explain:_____					
13. Develop toxemia (high blood pressure or pre-eclampsia)	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
13.1. If yes please explain:_____					
14. Have seizures	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
14.1. If yes please explain:_____					
15. Have thyroid problems	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
15.1. If yes please explain:_____					
16. Develop diabetes	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)
16.1 If yes did she required insulin?	No ❑(0)	Yes ❑(1)	1st Trim ❑(2)	2nd Trim ❑(3)	3rd Trim ❑(4)

17. Did the mother undergo any stressful situation during pregnancy (such as: divorce, death of a relative or a close friend, illness of a relative, financial problems, unemployment, domestic violence, victim of vandalism, assault or natural catastrophes, or any other stressful situation): No ❑(0) Yes ❑(1)
17.1. If yes, please explain _____
18. If yes, during which period of pregnancy was the mother exposed to stress: (select all that apply)
1st trimester ❑(18.1) 2nd trimester ❑(18.2) 3rd trimester ❑(18.3)
19. What was the mother's weight before getting pregnant: ____ Kg (or) ____Pounds(convert to Kg)
20. What was the mother's weight after delivery: _____ Kg (or) ____Pounds(convert to Kg)
21. How long was the pregnancy: _____weeks (or) ____months (convert to weeks) Don't know❑(.)
22. Was the child born premature (before time): No ❑(0) Yes ❑(1) Don't know❑(.)

23. Did the mother receive any medication to induce contractions (pitosin)

No ❑(0) Yes ❑(1) Don't know❑(.)

24. Number of babies: Single ❑(1) Twins❑(2) Triplets❑(3) More than 3❑(4)
25. What was the birth weight of the patient?
_____g (or)____ pounds(convert to g) Don't know❑(.)
26. What was the birth length? _____cm (or) _____inches(convert to cm) Don't know❑(.)
27. What was the head perimeter? _____cm (or) _____inches(convert to days) Don't know❑(.)
28. Was the birth: Normal / vaginal ❑(1) C-section or surgery ❑(2)
29. Any complications during the labor or delivery? No ❑(0) Yes ❑(1) Don't know❑(.)
29.1. If yes, please explain _____
30. Placental weight _____ (g)
31. Were there any problems during the first month, such as jaundice or feeding problems?
No ❑(0) Yes ❑(1) Don't know❑(.)
31.1. If yes, please explain _____
_____
32. Did the baby required to be hospitalized on an Intensive Care Unit (ICU) after birth?
No ❑(0) Yes ❑(1) Don't know❑(.)
32.1. If yes, please explain _____
_____
33. Did the baby receive any breast feeding: No ❑(0) Yes ❑(1) Don't know❑(.)
34. Age when complimentary feeding was started: ____days (or) ____weeks (or) ____months (convert to days)
35. Age of weaning: ____days (or) ____weeks (or) ____months (convert to days)

Developmental milestones:

At what age did these happen?
36. Sat up_____ Months
37. Said Mama/Dada_____ Months
38. Walked alone_____ Months
39. Talked in sentences_____ Months
40. Toilet trained_____ Months
41. Did you have any concerns about your child's development?
_____
_____
_____

42. What grade is your child in?

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Not in school	Nursery school	Elementary					Junior-high				High school			University		

43. How are your child's grades?

Extremely below average (1)	Below average (2)	Average (3)	Above average (4)	Extremely above average (5)

44. Are there concerns about school performance? No ❑(0) Yes ❑(1)
44.1 if yes, please explain _____

Passive smoking

45. How many of the caregivers of the child smoke? _____ #.#. Which _____
46. How many of the caregivers of the child smoke inside the house or the car? _____

Physical activity recall

47. Does the child have any phisical condition that reduces his/her mobility?

No ❑(0) Yes ❑(1)

47.1 If yes, please explain _____

	Weekdays (Monday to Friday)	Weekends (Saturday Sunday)
Sleeping (including naps)	48	49
School activities (classes, reading, homework, bus to school)	50	51
Moderate physical activity (see examples) Walking, Riding a bike or skating slowly, Playing in a swimming pool Gymnastics, ballet, dancing, Playing inside the house (dolls, cars) , Playing in the park (with a ball or a pet), Playing an instrument	52	53
Intense physical activity (see examples) Running fast, Carrying heavy objects, Riding a bike or skating fast, Competition sports Hockey, football	54	55
TV, PC, videogames, internet.	56	57
TOTAL	24	24

Please write down the number of hours per day that the child spends doing each one of the activities listed below in an average week (they must total 24)

Fiscal exam and biochemistry

58. Date of birth: dd / mmm / yyyy 59. Sex: Male ❑(1) Female ❑(2)

60. Date of examination: dd / mmm / yyyy

General Exam:

61. Racial background (chose only one, the most predominant)

Caucasian❑(1) African-American ❑(2) Asiatic❑(3) Can. Aboriginal ❑(4) Hispanic ❑(5)
Indian❑(6) Other❑(7)

62. Predominant phenotype: Normal ❑(0) , Bardet-Biedl ❑(1), Prader-Willi ❑(2) , Alstrom ❑(3) , Cohen ❑(4) , Albright's hereditary osteodystrophy ❑(5), Carpenter ❑(6) , MOMO ❑(7) , Rubinstein-Taybi ❑(8) Börjeson-Forssman-Lehma❑(9) , Other❑(10)

63. Weight :_____(Kg) Height: _____(cm)

64. Height to age and sex percentile	1	2	3	4	5	6	7	8
	p ≤ 5	p5-9	p10-24	p25-49	p50-74	p75-89	p90-95	p≥ 95

65. Body mass index _____(Kg/m²)

66. BMI to age and sex percentile	1	2	3	4	5	6	7	8
	p ≤ 5	p5-9	p10-24	p25-49	p50-74	p75-89	p90-95	p≥ 95

67. Temperature: _____(°C) 68. Heart rate: ____(bps) 69. Respiratory rate ____(bps)

Blood pressure Sit 1 70.1_____/ 70.2_____ (mmHg) Sit 2 70.3_____/ 70.4_____ (mmHg)

Stand 1 70.5_____/ 70.6_____ (mmHg) Stand 2 70.7_____/ 70.8_____ (mmHg)

71. Blood presure percentile by height and age (if more than 1 category select highest)	1	2	3	4	5
	P <50	P 50-89	P 90-94	P 95-99	P >99

72. Waist circumference 1 _____cm
73. Waist circumference 2 _____cm
74. Hip circumference 1 _____cm
75. Hip circumference 2 _____cm
76. Brachial circumference 1 _____cm
77. Brachial circumference 2 _____cm

78. Waist circumference to sex and age percentile

1	3	4	5	6	8
p ≤ 10	p10-24	p25-49	p50-74	p75-89	p≥ 90

79 Bisipital skinfold 1 _____mm
80 Bicipital skinfold 2 _____mm
81 Tricipital skinfold 1 _____mm
82 Tricipital skinfold 2_____mm
83 Abdominal skinfold 1 _____mm
84 Abdominal skinfold 2 _____mm

Blood work

85. Blood withdrawn at ___:___(time) of ____(dd/mmm/yyyy) after ___ hours of fasting
86. Hemoglobin _____ (mg/dL or gr/L)
87. Hematocrit _____(%)
88. Mean Corpuscular Volume (MCV) _____ (fL)
89. Leucocytes _____(cells 10^3per uL)
90. Plasma fasting glucose _____(mg/dL) or _____(mmol/L)
91. Plasma glucose levels 2 hours after 75g glucose load _____(mg/dL) or _____(mmol/L)
92. Fasting insulin plasma levels _____(mU/L)
93. Total Cholesterol _____(mg/dL) or _____ (mmol/L)
94. LDL Cholesterol _____(mg/dL) or _____ (mmol/L)
95. VLDL Cholesterol _____(mg/dL) or _____ (mmol/L)
96. HDL Cholesterol _____(mg/dL) or _____ (mmol/L)
97. Tryglycerides _____(mg/dL) or _____ (mmol/L)
98. Creatinin _____(mg/dL) or _____ (mmol/L)
99. Uric Acid _____(mg/dL) or _____ (mmol/L)
100. TSH _____(μIU/mL or mIU/L)
101. Free T3 _____(μIU/mL) or _____ (pmol/L)
102. Free T4 _____(μIU/mL) or _____ (pmol/L)
103. Cortisol _____(nmol/L) or _____ (ug/dL)
104. Leptin _____(mg/dL) or _____ (mmol/L)