A MURINE MODEL OF DEVELOPMENTAL PROGRAMMING OF ATHEROSCLEROSIS

by

Nima Goharkhay, MD

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

May, 2009

To my family for their enduring support.

To all those who mentored and taught me throughout the years.

Acknowledgements

I want to express my gratitude for my mentor Dr. George Saade for nurturing and guiding my research with patience and with care. He gave me the freedom to think and work independently, and was always there to help and support when I needed him. I am indebted to Drs. Garland Anderson, Gary Hankins and George Saade for their encouragement to join UTMB and for their continuous support throughout my time at this great institution. I am thankful to Dr. Monica Longo, Dr. Lu Fangxian, Dr. Egle Bytautiene, Dr. Elena Sbrana, Esther Tamayo, Phyllis Orise, Ancizar Betancourt, M.Sc., and Huaizhi Yin, M.Sc., for their selfless backing and sharing of their expertise with me both in addressing problems as well as hands-on support over the last several years. I am very grateful to my committee members Dr. Irina Buhimschi, Dr. Concepcion Diaz-Arrastia, Dr. Gary Hankins, Dr. Monica Longo, Dr. Wayne Snodgrass and Dr. George Saade. A very special thanks belongs to my program director, Dr. Golda Leonard, head of the Cell Biology Program. She has been extremely accommodating and supportive throughout my studies to allow the best possible accommodation of my dual needs and responsibilities as a student and practicing clinician. I thank Dr. Dorian Coppenhaver for his guidance. I am indebted to my family for their unconditional love and support that has guided me through my efforts to achieve my educational goals.

A MURINE MODEL OF DEVELOPMENTAL PROGRAMMING OF ATHEROSCLEROSIS

Publication No._____

Nima Goharkhay, MD

The University of Texas Medical Branch, 2009

Supervisor: Dr. George R. Saade

Early life is increasingly being recognized as an important period of development during which environmental changes can lead to long term effects on an individual's health. The association between poor nutrition prior to birth and an increased risk to develop coronary heart disease, hypertension and the metabolic syndrome is well established. Animal models are a central tool to investigate the details and mechanistic basis of the effects of the early life milieu.

Coronary artery disease secondary to atherosclerosis remains a major cause of death in most societies. Limited human studies indicate a strong association between maternal hypercholesterolemia and increased rates of formation of atherosclerotic lesions in children. It is conceivable that exposure to a high lipid environment during intrauterine development and early postnatal life may emerge as one of the principal risk factor for premature atherosclerosis.

These studies were performed to determine the effect of maternal hypercholesterolemia on the risk of atherosclerotic lesion formation in the offspring in a

homogenous small animal model. The apoprotein E (apoE)-deficient mouse strain was chosen because of its well described propensity to spontaneously manifest hypercholesterolemia and atherosclerosis. A strong correlation between maternal hypercholesterolemia and an increase in serum cholesterol levels was revealed in chow fed heterozygous litters born to hyperlipidemic dams at both 4 and 8 months of age. In addition, 8-month old heterozygote animals born to apoE-deficient mothers ($apoE^{+/-mat}$) showed higher rates of atherosclerosis and evidence of liver and kidney damage as compared with their $apoE^{+/-pat}$ counterparts. In contrast, at day 21 of life $apoE^{-/-KO}$ and $apoE^{+/-mat}$ pups showed lower total cholesterol and triglyceride levels than $apoE^{+/+WT}$ or $apoE^{+/-pat}$ litters.

Studies in liver tissue from offspring at 8 months of age suggest activation of the endogenous cholesterol synthetic pathway in $apoE^{+/-mat}$ offspring. This may be one of the mechanisms responsible for the observed programming effects. In-vivo activity and blood pressure measurements and vascular reactivity experiments in 4-month old animals did not demonstrate significant differences among study groups. No marked variation in serum cholesterol levels among genetically similar dams was detected.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACh	acetylcholine
apoE	apoprotein E
ATP	adenosine triphosphate
BP	blood pressure
cAMP	cyclic adenosine monophosphate
CO	cardiac output
COX	cyclooxygenase
CpG	cytodine-guanosine site
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
EDRF	endothelium dependent relaxing factor
eNOS	endothelial nitric oxide synthase
FELIC	Fate of Early Lesions in Children
FRET	fluorescence resonance energy transfer
GLUT4	glucose transporter-4
GR	glucocorticoid receptor
HDL	high density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HR	heart rate
IACUC	Institutional Animal Care and Use Committee
iNOS	inducible nitric oxide synthase
IUGR	intrauterine growth restriction
kDa	kilo Dalton
LDL	low density lipoprotein
LDLR	low-density lipoprotein receptor
L-NAME	L-N^G-nitroarginine methyl ester hydrochloride
LXR-α	liver-X receptor-α
MAP	mean arterial blood pressure
mRNA	messenger ribonucleic acid
NO	nitric oxide
NOS	nitric oxide synthase
NZW	New Zealand White
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PE	phenylephrine
PKA	protein kinase A
PLSD	posthoc least significant difference
PP	pulse pressure
PPAR-α	peroxisome proliferator-activated receptor-α
PPAR-γ	peroxisome proliferator-activated receptor-γ

PPAR-δ	peroxisome proliferator-activated receptor-δ
PSS	physiologic salt solution
PVR	peripheral vascular resistance
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SBP	systolic blood pressure
SCAP	SREBP-cleavage activator protein
SEM	standard error of the mean
SNP	sodium nitroprusside
SREBP-1a	sterol response element binding protein-1a
SREBP-2	sterol response element binding protein-2
SV	stroke volume
VIP	vasoactive inhibitory peptide

CHAPTER 1. INTRODUCTION

Mortality from cardiovascular disease remains the most important cause of death in all industrialized nations (Lloyd-Jones et al., 2009; National Center for Health Statistics, 2008; World Health Organization, 2008). Over the last several decades improvements in health care, as well as adverse changes in diet and lifestyle, have caused a significant change in disease patterns in developing countries in such a way that presently heart disease has become the number one cause of death in almost all societies around the globe (World Health Organization, 2008). Atherosclerosis, or the formation of lipid plaques within the vascular wall that can lead to vessel narrowing and thrombus formation, is the main culprit leading to diseases of the cardiovascular system (Kadar and Glasz, 2001).

Developmental programming is an area of science that deals with the effects of the environment during early life on long-term health outcomes in individuals (Barker, 1998; Barker, 2002; Barker and Osmond, 1986; Gluckman et al., 2008; Nijland et al., 2008). Given the importance of atherosclerosis and the significance of environmental factors in its etiology, the role of developmental programming in the development of this disease is one that deserves close scrutiny.

Atherosclerosis

Atherosclerotic disease has plagued humanity since at least the time of the Egyptian pharaohs, as is evident from advanced lesions described in preserved mummies from that era (Shattock, 1909). It is an insidious disease that can start as early as before one is born, and one that can take the entire lifespan to fully develop (Insull, 2009; Napoli et al., 1999b; Napoli et al., 1997).

The development of atherosclerosis is characterized by progressive formation of atherotic lesions, or plaques. Although the speed at which such lesions develop is subject to great individual variability, the course of the formation of such plaques generally follows a sequence of events that is characteristic and has been well described (Hansson, 2005; Insull, 2009; Stary, 1990; Stary, 2003; Sternby et al., 1999).

CLASSIFICATION OF ATHEROSCLEROSIS

The continuous progression of atherosclerotic lesions occurs over as a sequence that begins with early lesions that are found in most individuals after puberty and develops into more advanced lesions that can become clinically significant (Insull, 2009; Stary, 2003). The first lesions are mainly found at sites with a high predilection, such as the main bifurcation of the left coronary artery, the bifurcation of the carotid arteries, and the distal portion of the descending aorta leading to the iliac arteries (Stary, 2003). Although all the specific factors that lead to a higher vulnerability for plaque formation in each vascular bed are not understood, it is evident that areas exposed to higher mechanical shear forces (e.g., more turbulent blood flow) are more susceptible (Caro et al., 1969; Stary, 2003; VanderLaan et al., 2004; Zarins et al., 1983). Other regions, such as the ventral portion of the thoracic aorta, show a marked resistance to formation of atherosclerotic lesions under normal conditions.

Different classification systems can be used to categorize atherosclerotic and pre-atherosclerotic lesions (Insull, 2009; Stary, 2003). From a clinical standpoint, atheromas can be divided into preclinical and clinical lesions, with the latter ones exhibiting a significant risk for clinical complications. Histologically, preclinical lesions are also referred to as early or minimal lesions versus the clinically relevant advanced or raised lesions. Another important feature of early lesions is the fact that they can be reversible and reabsorbed, e.g., in cases in which circulating lipid levels are lowered through drug therapy or changes in diet and exercise, or when

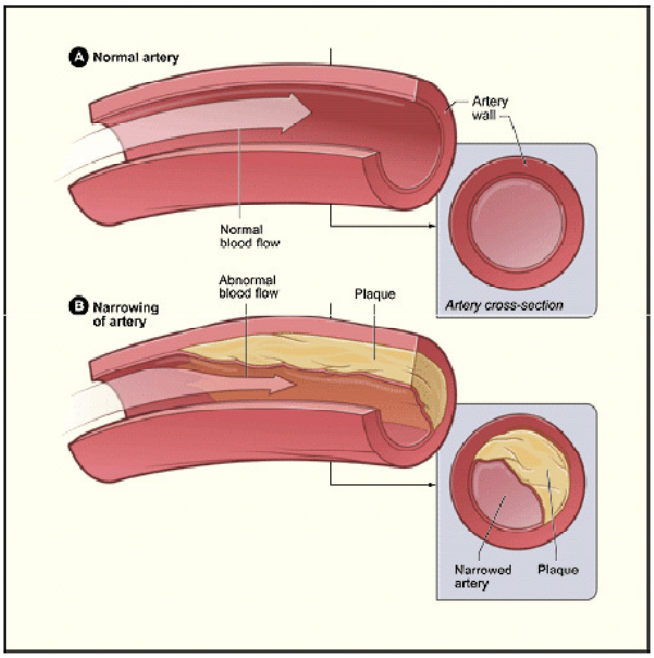
a pathologic pattern of blood flow is reversed to the normal state. A more detailed histologic classification of pre- and atherosclerotic lesions based on morphological characteristics, along with the descriptive terminology used for different lesion types is shown in table 1 (Insull, 2009; Stary, 1992; Stary, 2003). It is further to be noted that Types VI-VIII are different variants of advanced plaques that evolve from the same common precursor lesions. These represent different possible variants of advanced plaques and, although they may interconvert to each other under certain conditions, their number designation does not necessarily signify an increasing degree of severity.

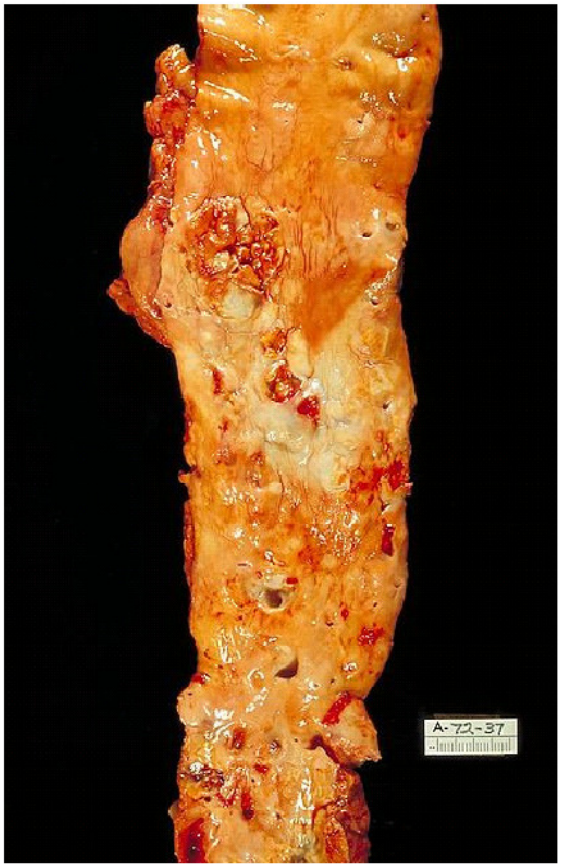
Table 1. Histological and clinical classification of the types and stages of atherosclerosis (modified after Stary, 2003).

Histological Classification		Other terms used	
Type I	Isolated macrophage foam cells	Fatty dot, fatty streak	***Early lesions***
Type II	Multiple layers of foam cells		
Type III	Preatheroma		
Type IV	Atheroma	Fibrolipid plaque, fibrous plaque, plaque	***Advanced lesions***
Type V	Fibroatheroma		
Type VI	Fissured, ulcerated or hemorrhagic lesion	Complicated lesion	
Type VII	Calcified lesion	Calcific lesion	
Type VIII	Fibrotic lesion		

Figure 1 depicts a comparison between a healthy vessel versus one that is affected by atherosclerosis. It further shows an ex-vivo specimen from a human aorta that is severely affected by atherosclerosis.

Figure 1. Effects of atherosclerosis on blood vessels. ***Left:*** comparison between normal blood vessel (A) and an artery showing significant plaque formation and some narrowing of the lumen (B)(source: National Heart Lung and Blood Institute Diseases and Conditions Index, http://www.nhlbi.nih.gov/health/dci/Diseases/Hbc/HBC_WhatIs.html, accessed 02/20/2009). ***Right:*** Example of an aorta at autopsy showing severe atherosclerosis with ulcerated lesions (source: Wikimedia Commons/Dr. Edwin P. Ewing, Jr., http://commons.wikimedia.org/wiki/File:Atherosclerosis,_aorta,_gross_pathology_PHIL_846_lores.jpg, accessed 02/19/2009).





ETIOLOGY OF COMPLICATIONS OF ATHEROSCLEROSIS

The progression from early to advanced lesions corresponds to the different steps in the development of atherosclerosis (Insull, 2009; Stary, 1990; Stary, 2003). The earliest steps include the extravasation of lipids into the intimal layer of the vessel. This primarily occurs at high predilection sites, where the integrity of the vessel wall may be diminished due to excessive shearing forces and continuous remodeling. The entering lipids undergo modification and oxidation in the intima, which in turn initiates an inflammatory cascade. The local immune response recruits inflammatory cells to the area (Hansson, 2005; Libby et al., 2002; Weber et al., 2008). Invading monocytes transform into macrophages that engulf lipid particles and convert into so-called foam cells containing lipid vesicles, which are the hallmarks of early lesions. Subsequently, areas of extracellular lipid accumulation start to appear in the intimal layer of affected vessels.

Increasing lipid accumulation promotes further inflammation which in turn stimulates the growth of smooth muscle cells in the intima. These cells secrete increasing amounts of proteoglycans and lead to the formation of fibrous areas or “fibrous caps”. Extracellular lipids continue to accumulate together with debris from cell necrosis below the fibrous cap. This constellation constitutes a fibroatheroma.

Further deposition of lipids disrupts the smooth muscle layer of the vascular media and causes a weakening in the structural strength of the vascular wall. Accumulation of cell debris and lipids can result in precipitation of lipid and calcium crystals. Fibrous degeneration on the luminal surface predisposes it to increased susceptibility to mechanical damage in response to mechanical shear.

Disruption of the compromised vessel wall ordinarily occurs along the periphery of the atherosclerotic plaque where the fibrous cap is usually the thinnest (Insull, 2009; Kadar and Glasz, 2001; Stary, 2003). Fissure or ulceration of the surface of a plaque can lead to local thrombosis. The blood clot may then be absorbed and integrated into the atheroma. This leads to

further growth of the plaque and can cause gradual narrowing of the vessel lumen, as may be the case in coronary arteries. Such narrowing can correspond to clinical symptoms of angina in affected individuals.

Alternatively, the thrombus may expand and lead to complete occlusion of the vessel. If such an occlusion occurs suddenly in an area with no established collateral circulation, it may cause complete cessation of blood flow. This is the mechanism by which atherosclerosis leads to myocardial infarcts or strokes in many cases.

The aforementioned weakening and stiffening of the vessel wall predisposes to other types of complications of atherosclerosis. The thinned out muscle layer may yield to the pressure of the circulation over time which will cause the formation of potentially life-threatening aneurysms (Guo et al., 2006). Such aneurysms may eventually burst and cause serious hemorrhage, e.g., in the form of a stroke.

RISK FACTORS

Certain risk factors predispose to the development of atherosclerotic lesions in individuals (Bostom et al., 1999; Craig et al., 1998; Fruchart et al., 2004; Hokanson and Austin, 1996; Kannel and McGee, 1979; The Expert Panel, 2002; van der Meer et al., 2002). These classic risk factors are listed in table 2. Some of these exert their adverse influence through a direct effect on the vascular endothelium, facilitating vessel wall injury and the subsequent entry of lipids and inflammatory cells into the vascular wall. Examples include hypertension, diabetes and elevated LDL cholesterol levels. Female gender, in general, has a protective effect on the predisposition to develop atherosclerosis. This observation had classically been attributed to a protective effect of estrogens against cardiovascular disease, although this notion appears to have been refuted in the face of finding from recent clinical studies (American College of Obstetricans and Gynecologists Committee on Gynecologic Practice, 2008; Rossouw et al., 2002).

In recent years advances in the understanding of lipid physiology and the inflammatory mechanisms involved in the pathogenesis of atherotic lesions have led to the characterization of new measures that correlate with morbidity risk from atherosclerosis. Table 2 also contains a list of some of the more recently recognized risk factors for plaque formation currently under investigation.

Table 2. Classic and newly recognized risk factors of atherosclerosis (modified after: Fruchart et al, 2004). LDL: low density lipoprotein, HDL: high density lipoprotein, CRP: C-reactive protein.

Classic	New
Age	High-sensitivity CRP
Male gender	Hyperhomocysteinemia
Family history of premature cardiovascular disease	High oxidized LDL level
High LDL cholesterol	Elevated lipoprotein(a)
Low HDL cholesterol	Antibodies against oxidized LDL
Hypertriglyceridemia	Hyperfibrinogenemia
Hypertension	
Diabetes	
Smoking	
Obesity	

ApoE Deficient Mouse Model of Atherosclerosis

As in most areas of biology, the use of animal models has been of uttermost value to the study of the pathogenesis and treatment of atherosclerosis and its complications. Apoprotein E (apoE) is a 34 kDa protein comprised of 299 amino acids (Mahley et al., 2008). It is primarily synthesized in the liver, with the brain being the second most common site of apoE synthesis, and is present in other tissues (Elshourbagy et al., 1985). ApoE has a central role in the transport

of lipids between tissues, and is a component of most lipoproteins. It binds strongly to the LDL receptor, as well as to heparin sulfate proteoglycans (HSGP). These interactions form the basis of two important mechanisms by which lipoprotein remnants are removed from the circulation in the liver (Fazio et al., 2000; Mahley and Rall, 2000; Mahley and Huang, 2007; Mahley et al., 2008; Meir and Leitersdorf, 2004). The apoE deficient mouse strain is the most widely used species utilized in atherosclerosis research today (Jawien et al., 2004; Meir and Leitersdorf, 2004; Reardon and Getz, 2001; Whitman, 2004; Wouters et al., 2005; Zadelaar et al., 2007).

DEVELOPMENT OF ANIMAL MODEL

Mice lacking a functional gene for apoprotein E were produced almost instantaneously by two different groups and first described in 1992 (Piedrahita et al., 1992; Plump et al., 1992). The commercially available apoE deficient animals (strain name: B6.129P2-Apoe^{tm1Unc}/J, The Jackson Laboratory, Bal Harbor, ME (The Jackson Laboratory, 2009b)) represent the progeny of the mice originally developed by Piedrahita and coworkers.

Piedrahita and associates achieved inactivation of the gene for apoE through insertion of plasmid constructs containing a neomycin resistance gene into mouse embryonic stem cells by homologous recombination after electroporation (Piedrahita et al., 1992). The insert disrupts the structure of the apoE gene and causes functional deactivation of apoE. The cell lines expressing the recombinant gene were then introduced into C57BL/6J blastocysts in order to produce germ line chimera. The chimera expressing the disrupted gene in the germline were then crossbred with wild-type mice and heterozygous offspring lacking a functional apoE gene were obtained. Mice homozygous for the deficient apoE gene were then produced through breeding of heterozygous mice with each other. It was an important achievement to document that both heterozygous, as well homozygous, mice carrying the disrupted apoE variant are able to thrive and capable of reproduction (Jawien et al., 2004; Piedrahita et al., 1992).

RELEVANT CHARACTERISTICS OF THE APOE STRAIN

The hallmark of the phenotype of apoE deficient mice is the occurrence of spontaneous hypercholesterolemia and the development of atherosclerotic lesions (Meir and Leitersdorf, 2004; Plump et al., 1992; Reardon and Getz, 2001; Wouters et al., 2005; Zadelaar et al., 2007; Zhang et al., 1992). Total cholesterol levels in homozygous apoE deficient mice on regular mouse chow are about fivefold as compared to wild-type C57/BL6 controls (Piedrahita et al., 1992; Plump et al., 1992). Heterozygous mice fed regular chow do not show an increase in cholesterol levels, suggesting that under normal conditions one functional copy of the apoE gene is sufficient to maintain normal cholesterol homeostasis. Serum triglyceride levels, on the other hand, have been shown to be elevated by 68% in homozygous animals, and to a lesser degrees in heterozygous mice (Zhang et al., 1992).

The degree of hypercholesterolemia is exponentially higher in mice lacking the functional apoE gene when fed a high-fat diet or atherogenic diets containing cholate. Using a Western-type diet, total mean cholesterol levels are four-fold higher in homozygous mice as compared to the same animals on regular chow (1821 mg/dl vs. 494 mg/dl) (Plump et al., 1992). When an atherogenic diet containing saturated fats and cholate is utilized, the difference becomes even more marked, with levels reaching 2712±768 mg/dl after 12 weeks of feeding (Zhang et al., 1994). Of interest, a high-fat atherogenic diet also causes a slight but significant increase in total cholesterol levels in heterozygous animals.

The earliest evidence of preatherosclerotic lesions in apoE deficient mice can be found after a few weeks of life (Nakashima et al., 1994; Reddick et al., 1994). Adhesion of monocytes to the endothelial lining is apparent at 5-6 weeks of age. Early precursors of atheromas, fatty streak lesions, can be demonstrated by 6-10 weeks of age. Intermediate lesions and fibrous plaques are present in apoE deficient mice fed regular chow by about 15 and 20 weeks,

respectively. These lesions occur earlier in mice being fed a Western-type diet, at around 9 and 15 weeks of age, respectively. Other associated findings characteristic of advanced atherosclerotic plaques, such as calcification and thinning of the vessel wall, are encountered in a portion of the lesions in older animals. These findings are especially significant considering the fact that the mouse as a species is highly resistant to the development of atherosclerosis under normal circumstances.

Although the formation and progression of atherosclerotic plaques in apoE deficient mice closely resembles those seen in human and other species, spontaneous rupture of atherotic lesions is rarely observed in these animals (Calara et al., 2001; Jackson, 2006; Schwartz et al., 2007). Since clinical syndromes in humans are frequently associated with plaque rupture and thrombosis, understanding the mechanisms of plaque rupture and the availability of an experimental model to study the effect of medications is of uttermost importance. Several approaches to induce plaque instability and rupture have been developed by different groups of investigators in recent years (Bea et al., 2002; Johnson et al., 2005; Sasaki et al., 2006; von der Thusen et al., 2002).

Developmental Programming

The classic concept of the etiology of disease involves the interaction between external environmental factors with inherent genetic predisposition (Kumar et al., 2005; McCance and Huether, 2002). In this model, the propensity to develop certain disorders is dependent upon the interaction between environmental stimuli, such as infectious agents, toxins or nutritional deficiencies, with the individual susceptibility of each person, which depends on the genetic background. Since presently our ability to modify our genetic makeup is limited, knowledge of the specific environmental factors that lead to disease in humans is extremely important in preventing and treating conditions that affect our health. Until not long ago the environment

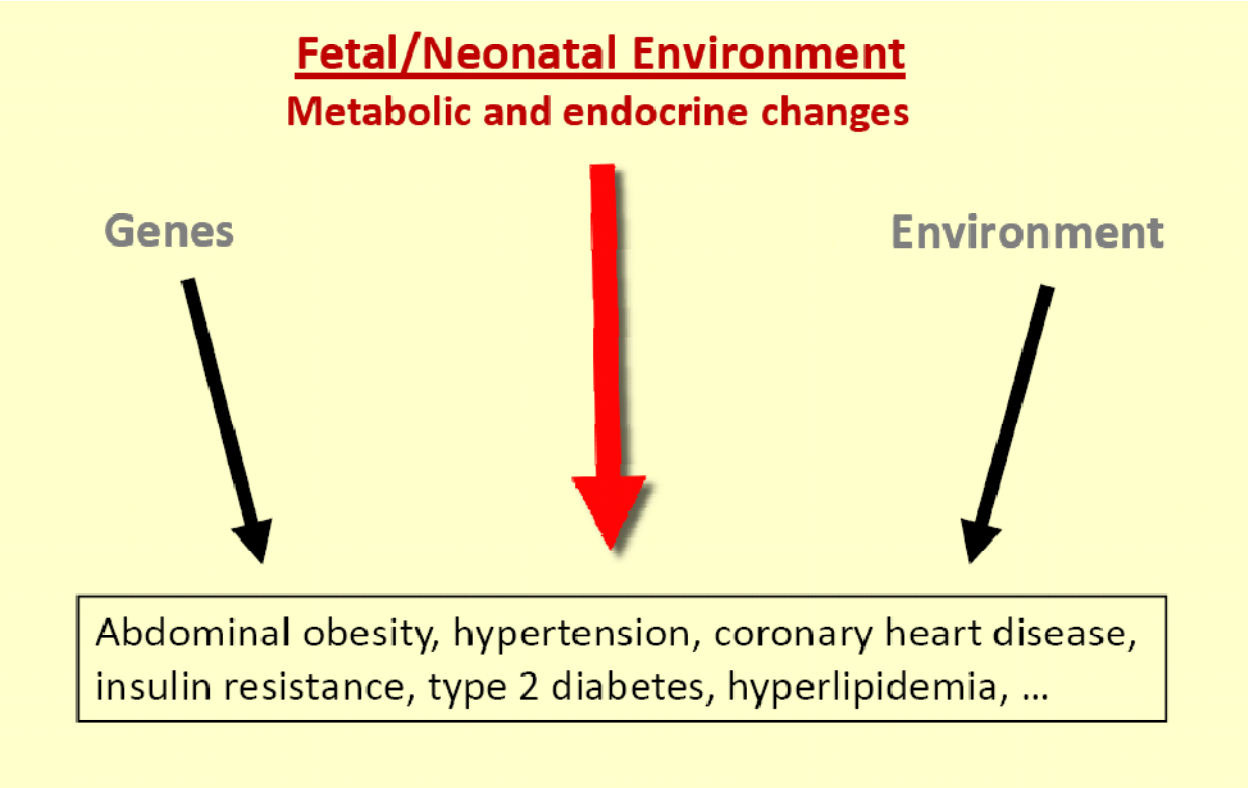
during adulthood has been the major area of interest with respect to external causes of morbidity (Barker, 1995; Byrne and Phillips, 2000; Gluckman and Hanson, 2005; Gluckman and Hanson, 2006; Gluckman et al., 2008).

More recently, it has become evident that the environment during early life, including the period of in-utero development as well as early postnatal life, may have a very significant contribution to the etiology of major human chronic disorders (Alexander, 2006; Barker, 1995; Barker, 1998; Barker, 2002; Barker and Osmond, 1986; Byrne and Phillips, 2000; Eriksson et al., 1999; Fowden and Forhead, 2004; Gluckman and Hanson, 2005; Gluckman and Hanson, 2006; Gluckman et al., 2008; Holmang, 2001; Ingelfinger, 2004; Ismail-Beigi et al., 2006; Kapoor et al., 2006; Lau and Rogers, 2004; Leon et al., 1998; McMillen and Robinson, 2005; Morris, 1998; Myatt, 2006; Nijland et al., 2008; Novak et al., 2006; Schwartz and Morrison, 2005; Stein et al., 1996; Zandi-Nejad et al., 2006). The diagram in figure 2 illustrates the basic concept underlying developmental programming, or as it also referred to, "Developmental Programming of Health and Disease (DOHaD)".

OVERVIEW OF DEVELOPMENTAL PROGRAMMING

The observation that environmental influences during in-utero life can have s significant health impact of the offspring is not new. The association between teratogenic effects occurring prior to birth and congenital structural malformations has been known in humans since the 1940s (Lau and Rogers, 2004; Warkany and Nelson, 1940). In contrast to this "classic teratology", groups of teratologists have focused on the functional rather than structural effects of teratogens demonstrable after birth (Slotkin et al., 1985a; Slotkin et al., 1985b).

Figure 2. Concept of developmental programming. Classically, the interaction between an individual genotype and the specific adult environmental has been thought to be the basis of disease etiology in humans. Developmental programming emphasizes the importance of environmental factors during early life as determinants of an individual's health in adulthood (modified after: Holmang, 2001)



In 1986 Barker and Osmond published their observations on an increased rate of mortality from ischemic heart disease during adulthood in individuals who were born small (Barker and Osmond, 1986). They concluded that undernutrition during in-utero life, as indicated by a low birthweight, may predispose individuals subsequently exposed to an affluent diet to develop coronary artery disease several decades later. Although this association has been disputed by some, several large scale studies in different geographic regions and ethnic populations have subsequently confirmed the relationship between low birthweight and

morbidity from coronary artery disease (Eriksson et al., 1999; Frankel et al., 1996; Leon et al., 1998; Osmond et al., 1993; Stein et al., 1996; Valdez et al., 1994).

In addition to coronary artery disease, it has become apparent that the early life environment may have important contributions to the development of a variety of chronic diseases in adulthood. Evidence is accumulating for an association between fetal undernutrition with adult hypertension (Curhan et al., 1996; Gennser et al., 1988; Law et al., 1993; Leon et al., 1996), type II diabetes mellitus and glucose intolerance (Barker et al., 1993; Hales et al., 1991; Lithell et al., 1996; McKeigue et al., 1998; Rich-Edwards et al., 1999), obesity (Kensara et al., 2005; Law et al., 1992; Phillips et al., 2000; Sayer et al., 2004), and a variety of other disorders (Cannon et al., 2002; Lau and Rogers, 2004; Phillips et al., 2000; Stein et al., 1997; Wahlbeck et al., 2001). In recognition of the pivotal contribution of Dr. David Barker to the discovery of fetal undernutrition as a risk factor for chronic disease this association has also been termed the Barker hypothesis.

A phenomenon that is common to many of the disease associations with low birthweight is an exponentially higher susceptibility if small birthweight is combined with accelerated weight gain during early childhood and adolescence, also referred to as “catch-up growth” (Fagerberg et al., 2004; Huxley et al., 2000; Lau and Rogers, 2004; Ozanne and Hales, 2004). It is hypothesized that the undernourished fetus develops a “thrifty phenotype” in response to the poor nutritional environment before birth. Once exposed to an affluent environment, the “thrifty” individual is programmed to attempt to maximize nutritional intake which may lead to the phenomenon of catch-up growth and cause the resultant adverse health effects. This maladaptive programming effect has been suggested to exemplify the basic concept common to all developmental programming phenomena. This basic principle is a “predictive adaptive response (PAR)”, according to which the fetus or neonate undergoes changes in response to the adverse environment it encounters during early life. These responses protect the individual as long as the environment remains constant. Once the subject is removed from the adverse milieu it is

presumed that some of the adaptive modifications have become permanent and "engrained" in such a way that they become harmful since they are no longer commensurate with the new environment (Gluckman and Hanson, 2004; Gluckman and Hanson, 2006).

Although the initial focus of research in the area of developmental programming mainly focused on the effects of generalized undernutrition during early life on long-term health consequences, the spectrum of environmental stimuli that may affect the offspring has been widely expanded. Some examples of alternate injury models that have been studied are as follows: Fetal hypoxia in a rat model causes increased susceptibility to ischemia-reperfusion injury and remodeling of the cardiac muscle cells (Li et al., 2003). Maternal restriction of iron intake leads to decreased body weight and elevated blood pressure in adult offspring (Lewis et al., 2002). In a mouse model, Longo and coworkers have shown abnormal vascular reactivity in offspring bred in hypertensive dams (Longo et al., 2005). Experimentally induced fetal anemia in sheep has been associated with changes in cardiac function in adulthood (Broberg et al., 2003). In humans, maternal hyperglycemia has been correlated with increased incidence of obesity and the metabolic syndrome in the offspring (Boney et al., 2005). Along the same line of observation, it has been shown that formula feeding in infants increases the risk of obesity later in life (Harder et al., 2005). This latter report, along with the phenomenon of "catch-up growth", underlines the fact that not only the intrauterine environment, but the early postnatal period as well may be important critical periods of insult. Clark and associates have demonstrated this in a functional assessment of vascular response patterns: crossfostering offspring between high-risk and low-risk mothers resulted in a significant change of vascular phenotype in the offspring (Clark et al., 2007). Undoubtedly, the use of animal models has been extremely valuable in designing and testing experimental designs and hypotheses related to developmental programming.

One of the characteristics of developmental programming effects is that, in many cases,

the outcome will depend on the gender of the offspring. While this is not uniformly the case, several examples of such a phenomenon have been reported in the literature (Hemmings et al., 2005; Khan et al., 2004; Lu et al., 2007; Zambrano et al., 2005). Interestingly, in various settings the programming effect has been found to be inheritable across future generations (transgenerational effect) (Costantine et al., 2008; Oh et al., 1991; Zambrano et al., 2005). Nathanielsz has compiled a list of the "ten principles of developmental programming" which is shown in table 3 (Nathanielsz, 1999).

Table 3. The ten principles of developmental programming (Nathanielsz, 1999)

Principle 1	During development, there are critical periods of vulnerability to suboptimal conditions. Vulnerable periods occur at different times for different tissues. Cells dividing rapidly at the time of exposure are at greatest risk. Risk factors include too much of a normal chemical such as a hormone, critical nutrient or vitamin; deficiency of a normal chemical such as a hormone, critical nutrient or vitamin; abnormal chemicals such as alcohol or nicotine; abnormal physical forces, such as high blood pressure.
Principle 2	Programming has permanent effects that alter responses in later life and can modify susceptibility to disease.
Principle 3	Fetal development is dependent on fetal physical activity. Normal development is dependent on continuing normal activity. Each phase of development provides the required conditions for subsequent development.
Principle 4	Programming may involve structural changes in important organs. The absolute numbers of cells in the organ may increase or decrease; the relative proportions and distribution of different types of cell within the organ may be unbalanced; the normal blood supply to the organ may be compromised; too many or too few hormone receptors may form with a resultant resetting of feedback and other control mechanisms.
Principle 5	The placenta plays a key role in some forms of programming.
Principle 6	Compensation carries a price. In an unfavorable environment, the developing baby makes attempts to compensate for deficiencies. Following compensation, birth weight may be normal or only slightly decreased. However, the compensatory effort carries a price.
Principle 7	Attempts made after birth to reverse the consequences of programming may have their own unwanted consequences. When postnatal conditions prove to be other than those for which the fetus prepared, problems may arise.
Principle 8	Fetal cellular mechanisms often differ from adult processes. Fetuses react differently to suboptimal conditions than do newborn babies or adults.
Principle 9	The effects of programming may pass across generations by mechanisms that do not necessarily involve changes in the genes.
Principle 10	Programming often has different effects in males and females.

Some of the animal models mentioned above and their findings give clues to tissue-specific and cellular mechanisms that may underlie the process of developmental programming under various circumstances. Although considerable effort has been expanded on delineating these pathways, our understanding on this subject is still very rudimentary. It is generally assumed that developmental programming is a consequence of perturbance of developing organ systems during a "critical", or vulnerable period of development that leads to a permanent change in the affected individual (Gluckman and Hanson, 2006; Gluckman et al., 2008). As such, it is presumed that the involvement of epigenetic mechanisms is pivotal to such processes. This may also explain the transgenerational passage of some of the phenotypes alluded to earlier.

Some of the most compelling proof of permanent organ involvement in response to an adverse early life environment comes from histological studies on kidneys in nutrition restricted models across several species, as well as in human autopsies from formerly low birthweight individuals (Bassan et al., 2000; Bauer et al., 2002; Langley-Evans et al., 1999; Manalich et al., 2000; Pham et al., 2003). What emanates from these reports is a decrease in the number of nephrons in undernourished individuals. At the same time, the average size of the glomeruli increases in what is believed to be a compensatory response. These alterations lead to a decrease in the rate of glomerular filtration. Similar changes have been commonly described among individuals from certain populations that are at a high risk to develop hypertension around the globe, and it is believed that a causal relationship exists between these findings and the increased incidence of elevated blood pressures in nutrition restricted individuals (Abdi et al., 2003; Zandi-Nejad et al., 2006).

It is further believed that developmental programming effects may alter organ development by restricting the size of organ systems that are essential to glucose and lipid metabolism, namely the liver and skeletal muscle (Cleasby et al., 2003; Gluckman et al., 2008). These changes may in part explain the increased incidence of metabolic disorders and diabetes seen in affected populations.

At a more fundamental level, evidence is accumulating for differential activation of gene responses subsequent to various environmental challenges in early life. Ozanne and coworkers found changes in protein expression patterns of mediators of insulin signaling, including the GLUT4 glucose transporter, in skeletal muscle tissue from nondiabetic adults who had been small for gestational age at birth (Ozanne et al., 2005). Similar changes were further noticed in the adipose tissue within the same study population (Ozanne et al., 2006).

The transcriptional activity of genes is controlled by epigenetic processes. Two mechanisms are among the best described epigenetic pathways to control gene activity (Allis et al., 2007; Gluckman et al., 2008): Methylation of DNA through the action of the enzyme DNA methyltransferase occurs at cytodine-guanosine (CpG) sites. Generally, increased levels of DNA methylation at promoter sites are associated with decreased transcriptional activity. A second regulatory mechanism involves acetylation of core histone proteins that form nucleosome units with DNA. Higher level of histone acetylation is commonly associated with increased expressional activity of the adjacent gene. In a protein restricted rat model, Lillycrop and coworkers found a decrease in methylation levels at the promoter sites of the glucocorticoid receptor (GR) and the peroxisomal proliferator-activated receptor-alpha (PPAR-α) (Lillycrop et al., 2005). This change in methylation pattern was associated with a marked elevation in mRNA expression of these genes. Supplementation of the protein restricted diet with folic acid was sufficient to prevent the observed differences in methylation and protein transcription levels.

Pham and associates analyzed epigenetic effects in the kidney of rats in a model using bilateral ligation of uterine arteries to produce intrauterine growth restriction (IUGR) (Pham et al., 2003). They encountered decreased methylation at the gene and promoter sites of p53 associated with an increase in apoptotic activity. They suggest this pathway as one possible mechanism for decreased glomerular numbers observed in this and other studies. In a recent report utilizing Japanese macaque monkeys Aagaard-Tillery et al showed evidence for changes in the acetylation pattern of hepatic histone proteins and associated alterations in protein

expression levels in the offspring in response to a high-fat diet administered to pregnant dams (Aagaard-Tillery et al., 2008). Altogether, it is becoming apparent that unraveling the epigenetic processes involved will be the key to obtaining a better understanding of the responses to the environment during early life in the various models of developmental programming of disease.

A further level of epigenetic control is provided by so-called microRNAs, small strands of RNA that are complementary to mRNA sequences and interfere with protein translation. The role of microRNA regulation of protein synthesis in processes involving developmental processing has so far not been well studied.

DEVELOPMENTAL PROGRAMMING OF ATHEROSCLEROSIS

Although cardiovascular disease from atherosclerosis remains the main cause of morbidity and mortality, and despite the fact that ischemic heart disease was the first condition in which an association between early life conditions and later adverse outcome was established, the question of whether offspring of hypercholesterolemic mothers are at an increased risk to develop high cholesterol levels and premature atherosclerosis as a consequence of exposure to the maternal environment has not been studied well. This question is of special significance, not only since hypercholesterolemia is generally a common condition that affects a large number of women of reproductive age, but also because pregnancy itself is associated with a temporary but significant increase in circulating cholesterol levels in most women (Chiang et al., 1995; Loke et al., 1991; Martin et al., 1999). Cholesterol levels in pregnant women are significantly increased as compared with their nonpregnant counterparts beginning in the first trimester of gestation. The average total cholesterol level during the second trimester is elevated by about 30% versus the baseline. This elevation reaches the level of an almost twofold increase by the third trimester of pregnancy. Although there is a sharp decline in circulating serum cholesterol levels postpartum, a decrease to baseline levels may not occur until several weeks later (Loke et al., 1991; Martin et

al., 1999). The elevation in circulating triglyceride levels in response to a normal pregnancy is even more marked than is the case for cholesterol.

Although it is postulated that the placenta is impermeable to the flux of cholesterol from the mother to the fetus (and vice versa) this concept is not proven and has been questioned in view of recent findings (McConihay et al., 2001; Napoli et al., 1999b; Palinski and Napoli, 2002; Yoshida and Wada, 2005). Furthermore, the placenta has been shown to have the capacity to form and secrete lipid containing lipoprotein particles (Madsen et al., 2004). This may constitute a highly efficient route for the transfer of lipid-containing particles between mother and fetus.

A major effort to delineate the possible effect of a hyperlipidemic maternal environment on the risk of the offspring to develop atherosclerosis was undertaken through the collaboration between Dr. Wulf Palinski from the University of California at San Diego and Dr. Claudio Napoli from the University of Naples in Italy, leading to the publication of the "Fate of Early Lesions in Children (FELIC)" study in 1999 and its predecessor (Napoli et al., 1999b; Napoli et al., 1997). These studies were an important undertaking, and one that only was made possible through the particular and unique contributions from each of the participating institutions: In Italy, routine autopsy examinations were feasible in most cases of death due to any cause in children, which allowed the collection of a large number of autoptic specimens. Also of importance was the accessibility of comprehensive, longitudinal health data from a centralized source in Italy on the mothers. On the other hand, a well established basic cardiovascular research unit with expertise in atherogenesis and an interest in the effects of the maternal environment was established and participated in San Diego. Tissue and serum samples collected during the FELIC study continue to provide important material for current studies on marker identification and the underlying mechanisms (Liguori et al., 2008). This group to date remains one of the few and leading teams involved in the study of developmental contributions to the etiology of atherosclerosis. Their interest also includes the development and assessment of animal models for this purpose.

The first autoptic study to analyze the effect of maternal hypercholesterolemia was published by Napoli and associates in 1997 (Napoli et al., 1997). In this analysis a total of 82 stillborn fetuses and cases of neonatal death after preterm labor were reviewed. The specimens were categorized based on the cholesterol levels in the mothers during and before pregnancy as follows: normocholesterolemia (n=22), temporary hypercholesterolemia (hypercholesterolemia only during pregnancy; n=27) and hypercholesterolemia (present both before and during gestation; n=33). Histologic analysis of aortic specimens revealed that fetuses born to mothers with permanent hypercholesterolemia had significantly larger fatty streak lesions in the walls of the aortic arch and the abdominal aorta as compared with offspring of normocholesterolemic mothers. Remarkably, they also found an increase in fatty streak lesions in offspring born to mothers showing increased levels of cholesterol only during pregnancy, albeit this was to a slightly lesser degree as compared with those born to mothers with permanent hypercholesterolemia. Another important result of this investigation was a progressive decrease in fetal serum cholesterol levels with advancing gestation. Furthermore, these authors encountered a strong correlation of serum total cholesterol levels in the mother with those in the fetus. This correlation was only valid in fetuses up to 6 months of age and was not present thereafter.

In the subsequent FELIC study a larger number of children between the ages of 1 and 13 years (n=156) were included (Napoli et al., 1999b). The purpose of this effort was to assess the longer term effect of maternal hypercholesterolemia on the progression of atherotic lesions in the offspring, given that the fatty streak lesions described in the prior study are theoretically reversible, and since some still doubt their validity as precursor lesions for atherosclerotic plaques. In order to have a clear distinction between study groups, in the FELIC study only mothers showing evidence of permanent hypercholesterolemia were included. This diagnosis was based on repeat lipid measurements that were documented throughout pregnancy, as well as a serum measurement at the time of enrollment (around the time of death of the child). Although

there was some indication of a decrease in lesions size in young children as compared with fetuses in the aortic arch, this change was very slight and was more than offset by increases in lesion size in other areas of the aorta. The extent of pre- and atherosclerotic areas increased in all children with advancing age, and in some children older than 10 years frank atheromas were found. The rate of lesion progression was significantly higher in children born to hypercholesterolemic mothers in both the aortic arch as well as in the abdominal aorta. Of note, this difference was not accompanied by elevations in cholesterol levels among children born to mothers with abnormally high cholesterol levels. In a multivariate analysis age, group and birthweight were identified as independent risk factors for atherosclerotic lesions formation. Although low birthweight correlated with increased lesion area, the effect of birthweight did not differ between children born to hypercholesterolemic mothers versus those born to normocholesterolemic women. No marked difference in lesion size or progression was noted between female or male gender offspring in this or the prior study.

More recently, this group has shown evidence for an oxidative profile in the cord blood and placenta in a group of hypercholesterolemic mothers as compared to their normal counterparts (Liguori et al., 2008).

In view of the difficulty to obtain any prospective data in humans these are the only data available so far on the effect of hypercholesterolemia in the mother on the development of atherosclerosis in humans. This may change in future when results from the National Children's Study in the United States become available (Landrigan et al., 2006; Washam, 2009). The other source of information on the characteristics and mechanisms of developmental programming of atherosclerosis has been the use of various animal models.

Animal Models

The group around Drs. Palinski and Napoli has also pioneered the use of animal

correlates in order to determine the magnitude and mechanistic basis of developmental programming of atherosclerosis in response to a hypercholesterolemic maternal environment (Napoli et al., 2000; Napoli et al., 2002; Palinski and Napoli, 2002; Yamashita et al., 2006). They utilized New Zealand White (NZW) rabbits in which the dams were fed a normal diet, an atherogenic diet, or an atherogenic diet containing the cholesterol lowering agent cholestyramine and/or the antioxidant vitamin E (Napoli et al., 2000). The dams were started on the respective diets two weeks before mating and were continued on the same until two weeks postpartum. Litters were given regular chow and studied at birth or at 4 months of age. Lesion sizes in the offspring were significantly larger in pups born to hypercholesterolemic mothers. Cholestyramine was found to decrease lesion formation approximately to the degree that it lowered maternal cholesterol levels during pregnancy. On the other hand, vitamin E was found to lower lesion production, although it did not have a cholesterol-lowering effect in the mother. The authors concluded that oxidative damage from transfer of oxidized lipids from the mother to the fetus may be the basis of a reprogramming of the vascular endothelium in the offspring which explains the higher susceptibility to develop atherosclerosis during adulthood. The protective effect of vitamin E would thus be the result of diminished oxidative damage even in the presence of higher circulating lipid levels in the mother.

In a follow-up study this group of researchers followed pregnant dams that were given regular or an atherogenic chow, or an atherogenic chow containing vitamin E or cholestyramine, respectively (Palinski et al., 2001). Subsequently, the litters were given a mildly atherogenic diet containing cholesterol and followed for 12 months. Again, an important proatherogenic influence of maternal hypercholesterolemia on subsequent predisposition for lesion formation in the offspring was observed. Maternal therapy with either cholestyramine or vitamin E during gestation alone, with no further intervention in the offspring after birth, though, was sufficient to nullify the adverse effect seen in litters born to hypercholesterolemic mothers.

A further approach to this problem was undertaken by these researchers when they

switched to a model utilizing mice deficient for the low-density lipoprotein receptor (LDLR) (Napoli et al., 2002). After exposure to maternal hypercholesterolemia there was an increase in aortic root lesions in the male offspring fed regular chow at 3 months of age. RNA micro-array studies from unaffected areas of the aorta showed a differential gene activation pattern in mice born to hypercholesterolemic mothers versus controls.

A recent report from this group indicates that maternal immunization against oxidized LDL particles confers protection against atherosclerosis formation in offspring regardless of exposure to maternal hypercholesterolemia (Yamashita et al., 2006).

Two groups of investigators have studied the effect of the maternal environment on the subsequent risk of offspring to suffer from atherosclerosis using a model of apoE deficient mice crossbred with C57BL/6J wild-type counterparts (Alkemade et al., 2007; Madsen et al., 2003). In the report by Madsen and coworkers heterozygous mice were obtained through crossbreeding experiments and the pups were fed an atherogenic diet containing cholate and evaluated at 6 months of age. Using this experimental protocol this group did not find a significant difference in atherosclerosis formation when comparing heterozygous offspring born to apoE deficient mothers versus those born to wild-type C57BL/6J mothers.

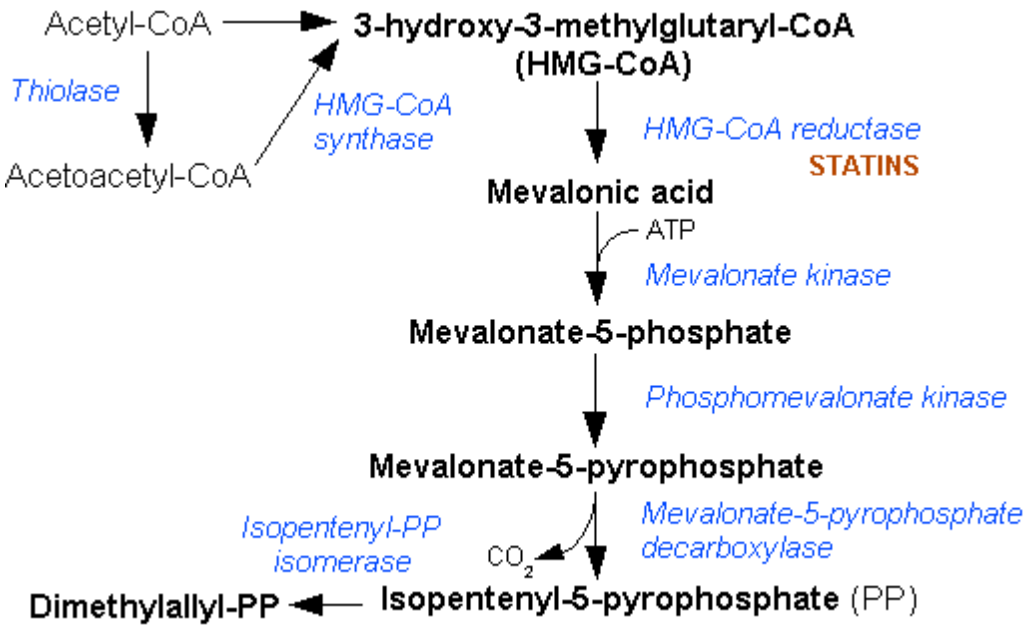
In the study by Alkemade et al, on the other hand, the investigators found a subtle but significant increase in carotid artery intimal thickness in heterozygous pups born to hypercholesterolemic apoE$^{-/-}$ mothers after a 16-week period on a Western style diet with a 1% cholesterol content (Alkemade et al., 2007). When vascular injury was then simulated by placing a constrictive collar on the carotid artery, the difference between the study groups of mice became much more prominent. Severe neointima formation along with a slight increase in medial thickness was observed only in the group of offspring born to apoE-deficient hyperlipidemic mothers. The authors concluded that this increased susceptibility was the result of maternal “imprinting” during the prenatal period.

HEPATIC CHOLESTEROL SYNTHESIS

The liver is one of the main sites for the metabolic regulation of lipids and cholesterol and a major site of de-novo cholesterol synthesis in the human body. Endogenous cholesterol synthesis occurs in the liver through the mevalonate pathway of cholesterol synthesis (see figure 3) and is primarily under the control of the SCAP-SREBP-HMGCR pathway (Bengoechea-Alonso and Ericsson, 2007; Horton et al., 2002). SCAP (SREBP-cleavage activator protein) and SREBP (sterol response element binding protein) are part of a cascade of proteins that are activated in response to decreased intracellular cholesterol levels. SREBP in turn stimulates the expression of lipogenic proteins, including 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase or HMGCR), the enzyme that catalyzes the committed step in the endogenous cholesterol synthetic pathway. SREBP exists as three distinct isoforms in humans, SREBP-1a, SREBP-1c and SREBP-2. It is believed that activation of isoforms SREBP-1a and SREBP-2 preferentially lead to increased endogenous cholesterol synthesis. In contrast, SREBP-1c overactivation does not increase cholesterol production in transgenic animals, and is believed to be mainly involved in the activation of the synthesis of fatty acids. The LDL receptor (LDLR) is a main mediator for cholesterol uptake into the liver. LDLR expression is also stimulated through SCAP and SREBPs when intracellular cholesterol levels are decreased.

Besides the influence of the SCAP-SREBP-HMGCR pathway and LDLR on cholesterol metabolism, cellular lipid homeostasis in the liver and elsewhere in the body is under the control of a variety of other factors. Among these mediators is a class of compounds referred to as the nuclear receptor family (Li and Glass, 2004). Members of this family present in liver tissue include the liver-X receptor-α (LXR-α), the peroxisome proliferator-activated receptors (PPARs) PPAR-α, PPAR-γ and PPAR-δ, among others. These substances are believed to play important roles in the process of the development of atherosclerotic lesions, and they are the target of intense study and evaluation for drug development (Finck, 2007; Li and Glass, 2004).

Figure 3. The mevalonate pathway of endogenous cholesterol synthesis. The various intermediates are indicated in black and corresponding enzymes are shown in blue letters. Note that 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase or HMGCR) is the enzyme catalyzing the committed, or rate-limiting, step in this pathway. HMG-CoA reductase is also the target of the most commonly prescribed class of agents to lower serum cholesterol in clinical use, the statins, as indicated in the figure. (source: Wikipedia Commons, http://commons.wikimedia.org/wiki/File:Mevalonate_pathway.png, accessed 02/27/2009).



Vascular Physiology

Blood pressure is a reflection of the pumping action of the heart to maintain the perfusion of the vascular bed throughout the organism. The flow of blood from the heart into the lungs through the pulmonary circulation serves the purpose of gas exchange, while the systemic circulation, originating in the outflow from the left ventricle, provides most of the body with oxygen-rich blood and a route for transport of nutrients, immune cells and endocrine mediators.

Blood pressure (BP) is positively proportional to cardiac output (CO) and peripheral vascular resistance (PVR) (Guyton, 1991).

Because of the inverse exponential relationship between vessel diameter and resistance to flow (Poiseuille's Law) peripheral vascular resistance is primarily dependent upon the wall tension within small arteries and arterioles at the distal end of the systemic circulation, the so-called resistance arteries. Cardiac output, on the other hand, is a function of the heart rate (HR) multiplied by the volume of blood propelled with each contraction, or the stroke volume (SV). The regulation of blood pressure, peripheral vascular resistance and each of their components is subject to a variety of complex and interacting control loops within the human body.

REGULATION OF VASCULAR RESPONSE

The contractile status of vessels is determined by a variety of factors that may act on the vascular smooth muscle directly, or indirectly through an effect on the vascular endothelium, the thin monolayer of cells lining the inside lumen of arteries that fulfills a multitude of physiologic functions (Rubanyi, 1993).

Among one of the most important endothelium-dependent mediators of vasorelaxation is the small molecule nitric oxide (NO), formerly referred to as endothelium dependent relaxing factor (EDRF). It is produced within endothelial cells from L-arginine through the action of the enzyme nitric oxide synthase (NOS). Several isoforms of NOS are known, with the constitutively expressed endothelial NOS (eNOS or NOS3), a membrane associated form that requires Ca^{2+} and calmodulin for its function, and the inducible Ca^{2+}-independent inducible NOS (iNOS or NOS2) believed to play major roles in the regulation of vascular function (Forstermann et al., 1994; Longo, 2005). The endothelium-dependent vasorelaxation in response to acetylcholine is mainly a function of NO production through NOS3 (Arnal et al., 1999; Furchgott and Zawadzki, 1980). Longo and coworkers have extensively studied developmental

programming effects of lack of NOS3 in dams on the offspring, as well as transgenerational effects, in a mouse model (Clark et al., 2007; Costantine et al., 2008; Ghulmiyyah et al., 2007; Longo, 2005; Longo et al., 2004).

A separate class of compounds affecting vascular contractile state are the derivatives of arachidonic acid that are produced through the initial action of the enzyme cyclooxygenase (COX) followed by further processing. Among these, Prostacyclin leads to vascular relaxation via stimulation of cyclic adenosine monophosphate (cAMP) and stimulation of protein kinase A (PKA), as well as membrane hyperpolarization by activation of ATP-sensitive K^+-channels (Jackson et al., 1993; Vegesna and Diamond, 1986). Another unstable metabolite of arachidonic acid, thromboxane A_2, is found in high concentrations in platelets. It has a proinflammatory effect in addition to its marked influence on vascular smooth muscle leading to a vasoconstrictive response. The reactions to thromboxane A_2 are mediated through its action on specific G-protein coupled membrane receptors (Nakahata, 2008).

A variety of circulating factors are known to influence vascular reactivity (Longo, 2005). Vasoactive inhibitory peptide (VIP), atrial natriuretic peptide and bradykinin are examples of circulating peptides with a vasorelaxatory effect. Vasopressin, epinephrine and angiotensin II are among important substances that lead to vasoconstriction. The specific hormone receptors for each of these mediators and the subsequent second messenger pathways leading to their vascular effects are in most cases well established. In the case of epinephrine the primary effect consists in smooth muscle contraction through activation of α-adrenergic receptors, with a smaller vasodilatory response via activation of β–receptors.

Many of the discussed vasoactive substances also function as neurotransmitters. Among these, epinephrine and acetylcholine are also the main transmitters in the autonomous nervous system. It is apparent that neuronal control of blood pressure and vascular regulation is essential in many circumstances, such as in a “fight or flight” situation. It is well established that most

portions of the vascular system receive an extensive supply of innervation, and that denervation of arteries has untoward consequences for the regulation of vascular tone (Burnstock, 2008).

Research Project Objectives

The data presented in the previous sections demonstrate wide-ranging evidence from experimental and observational data across different species (humans, rabbits, mice) supporting the notion that a hypercholesterolemic maternal environment during early life promotes the creation and progression of premature atherosclerotic lesions in the offspring. The proof is least consistent among the few studies reported so far in mice. This is partly due to specific factors peculiar to the individual protocols, and may be partially due to other circumstances, such as the inherent variability of biological systems. Nonetheless, the mouse serves as one of the most important model species. Providing stronger proof that hypercholesterolemia in dams can lead to premature atherosclerosis in the litters will be a significant contribution to this area of research, and it will open up vast possibilities to study the mechanisms and design targeted interventions in order to ameliorate the negative effect of an adverse early life environment. The following section describes the general and specific hypotheses and aims set out to study in this project:

HYPOTHESES AND AIMS

Our **general hypothesis** for the proposed studies was that the maternal environment plays a key role in determining the susceptibility of the offspring to develop atherosclerotic lesions over the long term. This general hypothesis was tested in a transgenic animal model by examining the following specific hypotheses:

Specific Hypothesis 1: Heterozygous offspring born to apoE deficient mothers develop premature atherosclerosis as compared to genetically similar offspring born to wild-type mothers.

Specific Hypothesis 2: The hypercholesterolemia and early atherosclerosis seen in heterozygous offspring born to hypercholesterolemic mothers is associated with abnormal cardiovascular function.

Specific Hypothesis 3: The cellular basis of fetal programming of atherosclerosis in the apoE heterozygous mouse model lies in developmental reprogramming of hepatic cholesterol metabolism.

Specific Hypothesis 4: Differences in serum lipid concentrations in the offspring are not related to variations in serum cholesterol levels between genetically similar dams.

According to this, the following specific aims were proposed to test the corresponding hypotheses:

Specific Aim 1: Levels of total blood cholesterol and triglyceride levels, the presence and extent of atherosclerotic plaques formation in the aorta, and possible histopathological end organ damage (affecting the kidney or liver) will be determined to evaluate the effect of early life exposure to high cholesterol levels.

Specific Aim 2: Blood pressure and vascular reactivity will be evaluated to determine the long term effect of exposure to high cholesterol levels during early life on cardiovascular function.

Specific Aim 3: The expression levels of genes involved in hepatic cholesterol homeostasis (SCAP, SREBP-1a, SREBP-2, HMG-CoA reductase, LDLR, PPAR-α, PPAR-γ, PPAR-δ and LXR-α) will be determined to evaluate the specific changes in the hepatic cholesterol metabolism underlying developmental programming of atherosclerosis.

Specific Aim 4: To determine serum cholesterol and triglyceride levels in dams mothering the different groups of offspring.

The proposed animal model represents a novel tool to study the importance of the maternal milieu and to distinguish the relative contributions of the intrauterine versus the early postnatal environment. The suitability of the proposed animal model is based on the following:

1) The genetically altered mice lack expression of a functional apoprotein E (apoE). The absence of apoE leads to a spontaneous predilection to develop hypercholesterolemia and premature atherosclerosis even on regular chow.

2) Atherosclerotic lesions in these animals initiate and progress closely paralleling those observed in humans.

3) A short gestation (19-20 days), ease of breeding, small size, availability of well described methods for embryo transfer experiments, crossfostering studies, nutritional and medical interventions and standardized and widely available histopathological and laboratory evaluation techniques.

4) The homogenous genetic background of apoE mice, along with availability of a suitable genetically identical control strain, allow a unique model to dissect different contributions introduced from genetic versus environmental factors.

SIGNIFICANCE

It is obvious that the burden from atherosclerotic disease and its complications represents one of the major causes of morbidity and mortality affecting humans worldwide. Advances in our understanding of the etiology and treatment of these conditions over the last few decades have led to a dramatic improvement in outcomes, and by one account, have prevented 815,000 deaths in the year 2000 alone (Landrigan et al., 2006). The scientific evidence strongly indicates that exposure to a high-cholesterol maternal environment may be an important early risk factor for the subsequent development of atherosclerotic lesions later in life. Treatment options are growing, and therapy of the hypercholesterolemic mother during pregnancy, or of children

during early childhood, is increasingly becoming feasible. Despite some differences in physiology, a genetically homogenous, well-described and easily breedable and reproducible small animal species offers the best tool to study the mechanistic basis and potential therapeutic approaches to this risk factor model.

CHAPTER 2. MATERIALS AND METHODS

Animal Care

Mature cycling female and male mice (between 4-6 weeks old) homozygous for disruption of the apoE gene (B6.129P2-Apoe^{tm1Unc}/J, stock number 002052 (The Jackson Laboratory, 2009b)) and their age-matched wild-type controls (strain C57BL/6J, stock number 000664 (The Jackson Laboratory, 2009a)) were purchased from The Jackson Laboratory (Bar Harbour, ME). The breeding colonies were maintained at a constant temperature of 25-26°C. The apoE deficient strain originated from the laboratories of Dr. Nobuyo Maeda in Chapel Hill, NC, as previously mentioned (Piedrahita et al., 1992). The C57BL/6J genetic background has been established by backcrossing the original mouse strain with C57BL/6J ten times (The Jackson Laboratory, 2009b). Mice homozygous for the apoE targeted mutation are viable and fertile. Further characteristics of this mouse strain were discussed in the previous chapter, and include a propensity to develop moderate hypercholesterolemia on regular diet and severe hypercholesterolemia when fed Western-type or atherogenic diets. The mice were maintained and bred in the animal care facility at the University of Texas Medical Branch in Galveston, Texas. All procedures were carried out after approval by and under the supervision of the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch. The mice were housed separately in temperature and humidity controlled quarters with constant light: dark cycles of 12h: 12h and provided with food and water ad libitum.

For the purposes of our experiments all mice were fed regular chow (7012 Teklad LM-485 mouse/rat sterilizable diet, Harlan Laboratories, Indianapolis, IN) (Harlan Laboratories, 2009). This diet consists of 19% crude protein, 5% crude oil, 5% crude fiber and does not contain any protein from animal sources. It has a crude energy rate of 4.05kcal/g, a digestible energy rate of 3.75kcal/g and a metabolizable energy rate of 3.41kcal/g, with 25% of the calories

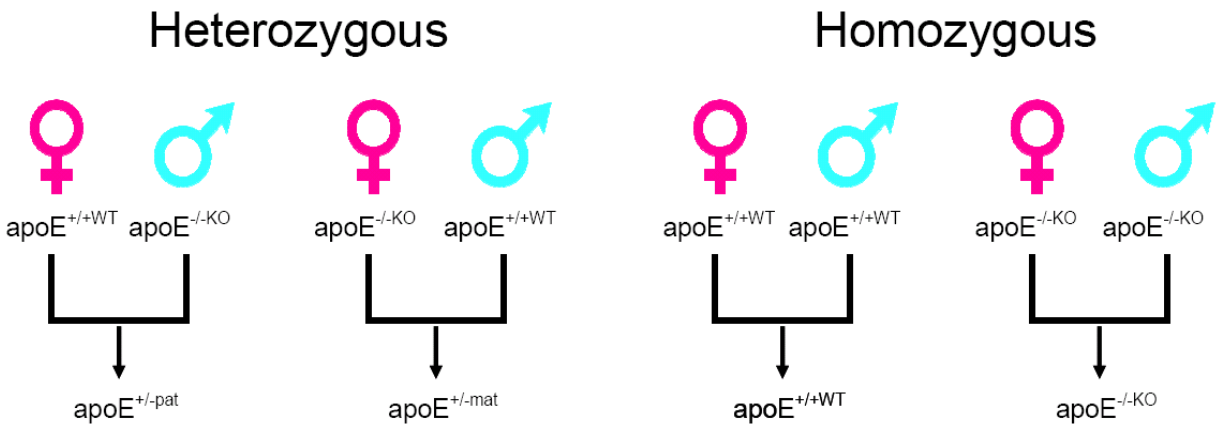
originating from protein, 17% from fat and 58% from carbohydrate origins. This diet is supplemented with vitamin A, vitamin K, thiamine and pantothenic acid and other vitamins and is autoclaveable. The same diet was used for all animals, including all dams before and during pregnancy as well as all offspring. Regular maintenance and care were provided by certified personnel and veterinary staff according to the guidelines of the IACUC. All surgical procedures were carried out by trained personnel according to the IACUC guidelines under anesthesia with ketamine (Ketalar®, Parke-Davis, Morris Plains, NJ) and xylazine hydrochloride (Gemini®, Rugby Laboratories, Rockville Center, NY). All animals were sacrificed by the CO_2 inhalation method per IACUC guidelines, which are in accordance with the guidelines set forward by the American Veterinary Medical Association guidelines (American Veterinary Medical Association, 2007).

Cross-Breeding

Female $apoE^{-/-}$ mice and their $apoE^{+/+}$ C57BL/6J controls at 7-8 weeks of age were cross-bred with $apoE^{-/-}$ and $apoE^{+/+WT}$ males to obtain maternally-derived ($apoE^{+/-mat}$) and paternally-derived ($apoE^{+/-pat}$) heterozygous, as well as $apoE^{+/+WT}$ and $apoE^{-/-KO}$ homozygous male and female offspring. The breeding scheme utilized to obtain all four groups of litters is outlined in figure 4.

Except for the parental origin of the single defective allele, both groups of heterozygous pups were genomically identical and had one functional apoE allele. The maternal environment, however, was different between the two heterozygous litters. In the $apoE^{+/-mat}$ litters, the female parent was homozygously deficient for apoE, and therefore expressed the corresponding phenotype. Therefore, the pups matured in a maternal environment lacking apoE and characterized by a significant level of hypercholesterolemia. Since all pups remained with their natural mothers during the period of breastfeeding, it is also possible (and likely) that the

Figure 4. Breeding scheme utilized to obtain the four different groups (two homozygous, two heterozygous) of offspring (modified after Goharkhay et al (2007) with permission (Goharkhay et al., 2007)).



maternal environment in apoE$^{+/-mat}$ litters also varied during this 21-day timeframe after birth, which may have adverse programming consequences by itself independently of the prenatal intrauterine environment.

In apoE$^{+/-pat}$ litters, on the other hand, the male parent was apoE$^{-/-KO}$ and the pups developed in a maternal environment expressing apoE, and lacking the high cholesterol environment present in the apoE$^{-/-}$ female mice in utero. Since these offspring breastfed from their apoE$^{+/+}$ mothers, they were further not subject to an apoE deficient hyperlipidemic maternal environment after birth. After day 21 of life, all litters were weaned from their mothers and placed in separate cages. Only mice of the same offspring group were housed together. These litters were then kept under the same standardized conditions and on regular chow for different durations (8 months, 4 months and 21 days, respectively) and subsequently utilized for the in-vitro vascular, histological and gene expression studies. The dams used for studies towards specific aim 4 were continued on regular chow after delivery.

Serological Studies

In the offspring the blood collection consisted of direct cardiac puncture after euthanasia with CO_2 suffocation and opening of the thoracic and abdominal cavities. To obtain samples from dams postpartum, a small section of the tail was removed using a surgical blade (Hoff, 2000).

After collection of whole blood, the sample was placed on ice for about 30 minutes and the spun for about 20 minutes and the serum was collected. Measurements of total cholesterol and triglyceride levels were done using a colorimetric enzymatic method. For the measurements of blood samples in 8-month old animals an automated machine was utilized (Fusion 5.0, Ortho Clinical Diagnostics, Rochester, NY). This method necessitated a larger sample volume (about 500µl per reaction), causing the loss of some data as some of the specimens required a secondary dilution but were exhausted after the initial measurement, as well as the inability to measure triglyceride levels in several animals because the entire sample had been utilized for the measurement of total cholesterol. In subsequent studies (analyses in 4-month and 21-day old animals) a modified approach was taken and an in-house assays were developed. The in-house total cholesterol assay was based on a colorimetric reaction that associates the chemical conversion of cholesterol into a color reaction, with a maximum wavelength of 600nm. The reagents were obtained from the Cholesterol E kit (Wako Chemicals U.S.A., Richmond, VA). The in-house assay to determine levels of triglycerides in serum samples was based on reagents from the LabAssay® triglyceride kit (Wako Chemicals U.S.A., Richmond, VA). This test was also based on a colorimetric enzymatic reaction, whereby triglycerides were hydrolyzed to glycerol and free fatty acids by the action of the enzyme lipoprotein lipase. Hydrogen peroxide (H_2O_2) is then produced from glycerol through the subsequent actions of the enzymes glycerolkinase and glycerol-3-phosphate oxidase. Finally, H_2O_2 mediates a color reaction that produces a blue pigment with a maximum absorption wavelength of 600nm. The in-house assay

method allowed the use of very small quantities of serum specimens (as low as 2-10µl). All samples were analyzed as duplicates. The inter- and intraassay coefficients of variation of the in-house assays for total cholesterol and triglycerides were both determined and were found to be less than 12%. Data are presented as means ± standard error of the mean (SEM).

Examination of the Aortic Arch

At the time of sacrifice the aortic arch in each animal was identified and dissected off. It was stored in an ethanol solution until further processing. Thereafter, staining of the specimens for fat deposits and atherosclerotic lesions was performed using oil red O stain and the *en face* method using a modification of the technique described by Nunnari and coworkers (Nunnari et al., 1989; Xu, 2006). A freshly prepared saturated solution of oil red O was made and filtered in order to remove any particulate debris. Prior to staining the aortic arch specimens were reexamined carefully and any attached adventitial tissue was removed under the microscope. The aortic arches were then stained in the oil red O solution for 30 minutes, rinsed off and placed in sterile water. Then each sample was bisected longitudinally, opened up, and laid flat on a glass slide. The specimen was then mounted on the glass side, coverslipped and stored for further analysis.

The samples were examined under the microscope after staining. Areas of aortic lesions that stained orange and red were classified as advanced plaques. Smaller areas of fat deposition that stained yellowish were classified as early lesions. Specimens that contained areas of advanced plaques were categorized as positive for the presence of atherosclerosis. Further image analysis was performed using the Image J image analysis software package (version 1.37h, National Institutes of Health, available at http://rsb.info.nih.gov/ij/, accessed 02/01/2009). The surface area of advanced lesions alone, followed by the surface area of advanced and early lesions combined, were measured in each specimen. The relative surface area of advanced and

advanced + early lesions was then calculated separately based on the total surface area of each aortic arch specimen and reported.

Histological Examination of Liver and Kidney

Livers and kidneys were removed at the time of sacrifice and fixed in 10% buffered formalin solution. These specimens were subsequently prepared as paraffin blocks, and sectioned into 5μm slices. These slices were then placed on slides and stained with hematoxylin and eosin. The slides were subsequently reviewed by a trained pathologist, who was blinded to the sample origin. Any pathologic changes noted were recorded in descriptive terms as well as graded according to the observed severity. For quantitative assessment of severity degrees a grading system was utilized with the following characteristics: grade 0 = no pathologic change (normal), grade 1 = negligible or nonspecific change, grade 2 = mild change, grade 3 = moderate change, grade 4 = severe change.

Gene Expression Studies

Sample Preparation and RNA Isolation

Gene expression assays were performed from fresh frozen liver samples. After CO_2 suffocation, the mice were dissected. Fresh blood was collected immediately through direct cardiac puncture. Organs were collected expeditiously, washed in phosphate buffered solution (PBS) until no visible trace of blood remained, placed into pre-labeled individually marked tubes and immediately flash frozen in liquid nitrogen. They were subsequently placed into freezers at –80°C and stored for mRNA processing. RNA extraction was then performed using the phenol-free RNAqueous® kit (Applied Biosystems/Ambion, Austin, TX). This kit is based on the use of a guanidium thiocyanate containing chaotropic agent that lyses the tissue and leads to the

inactivation of endogenous and exogenous ribonucleases. Subsequently, the lysate is suspended in an ethanol containing solution and passed through an RNA-binding filter composed of glass fibers. After multiple washing steps, the bound RNA is eluted from the glass fiber filter and can be used in applications requiring RNA.

Prior to the application of the RNA extraction kit a small fragment of tissue, weighing about 50-75mg, was broken off the frozen sample and placed into a pre-chilled mortar and pestle containing a small amount of liquid nitrogen. Special attention was paid to prevent any thawing of the specimen prior to its complete homogenization and stabilization in the lysis/binding solution. Approximately 300µl of the lysis/binding solution were then added to the sample fragment and the tissue was completely pulverized/homogenized using the pestle. An equal volume of a 64% ethanol solution was then added and the homogenate was passed through the filter. The filter was then washed using the supplied wash solution to remove debris and DNA. Finally, 40-60µl of the elution solution was added and the RNA was collected and saved at –80°C for further processing. The integrity of the extracted RNA was assured through two different methods: Firstly, an aliquot of the specimen was removed and its absorbance in the ultraviolet range measured using a spectrophotometer (Beckman Instruments, Palo Alto, CA). The samples were considered non-degraded when the ratio of their absorbance measured at 260nm divided by the absorbance at 280nm fell in the range of 1.8-2.0. Secondly, a denaturing agarose gel was run using each sample and the presence of 28S and 18S ribosomal RNA as specific bands was demonstrated after staining with ethidium bromide. If a specimen failed to satisfy these criteria then the extraction was repeated for that sample. After each 4-6 specimens the entire procedure was performed on a blank sample containing RNA-free water as a negative control. These negative controls for RNA extraction were then subjected to RT-PCR reactions with various probes and the absence of any amplified sequences assured that no cross-contamination between samples was present.

REVERSE TRANSCRIPTION (RT) REACTION

The reverse transcription reaction to produce complementary DNA (cDNA) from the RNA extracted during the previous step utilized the High-Capacity cDNA Reverse Transcription Kit® (Applied Biosystems, Foster City, CA). This reaction involved the use of an RT mix containing MultiScribe® reverse transcriptase enzyme, RNAse inhibitor and a blend of random primers. Approximately 1µg of total RNA were then added to the mixture. The RT reaction was performed by exposing the mixture containing the RNA to a temperature of 25ºC for 10 minutes, followed by 37ºC for 120 minutes, and then finally 85ºC for 5 seconds. The mRNA hereby obtained was either directly used for real-time polymerase chain reaction (PCR) or kept at -20ºC to -80ºC until further use. Each set of RT samples included at least two wells with no mRNA as negative controls.

SEMIQUANTITATIVE REAL-TIME PCR

Real-time PCR reactions were performed with the TaqMan Fast Universal PCR Master Mix® (Applied Biosystems, Foster City, California) using an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems, Foster City, California). This technique involves the use of Taq DNA polymerase, a DNA-dependent DNA polymerase with additional 5' exonuclease activity, and of specifically designed oligonucleotides (primers) that are complementary to the cDNA sequence of interest. It further applies an oligonucleotide probe that attaches to the cDNA of the gene of interest between the binding sites of the two specific primers and carries a reporter molecule. The probe carries a reporter fluorescent dye attached to its 5' end (the fluorescent dye FAM® for most genes of interest, and the dye VIC®, in the case of the 18S internal reference probe). Furthermore, the probe contains a quencher dye at the 3' end, which prevents the fluorescent dye from emanating light. This quenching activity is mediated through the

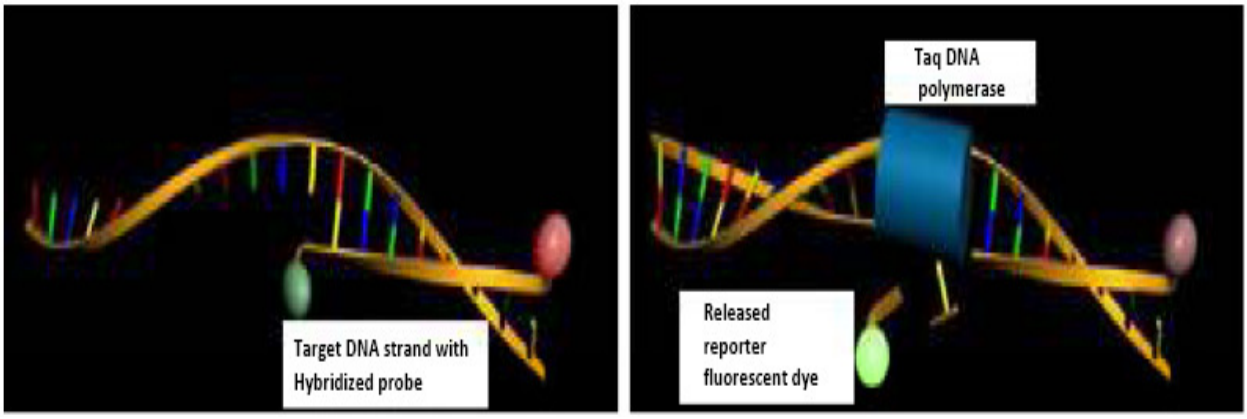
mechanism of fluorescence resonance energy transfer (FRET), and is dependent on a close spatial proximity of the fluorescent reporter dye with the quencher dye. During the annealing phase of the PCR cycle, the oligonucleotide primers as well as the probe attach to their complementary sites along the cDNA molecule. With primer extension, the Taq DNA polymerase, which its fork-like structure-dependent 5' nuclease activity, splits the probe from its attachment site on the cDNA molecule and cuts it into smaller pieces, thereby removing the close spatial relationship between the fluorescent and the quencher dyes. This allows the fluorescent dye to respond to light stimulation with emittance of a fluorescent signal (figure 5). As the process of the PCR goes on, the increased availability of free fluorescent dye molecules signals increasing amounts of replicated cDNA copies. The reaction wells also contain an additional dye, ROX®, which serves a passive reference dye. ROX® itself does not participate in the PCR reaction. The function of ROX® is to supply an internal reference to which the reporter dye signal can be normalized, or in other words as a "background" dye.

The original amount of cDNA in each sample determines after how many amplification cycles the total quantity of replicated cDNA copies exceeds a set threshold level (C_t). This level is then used to quantitate the original mRNA content between the various samples.

Probes and Primers

Specific TaqMan® Gene Expression Assays for the genes of interest (SCAP, SREBP-1a, SREBP-2, HMG-CoA reductase, LDLR, PPAR-α, PPAR-γ, PPAR-δ and LXR-α) were purchased from Applied Biosystems (Foster City, CA). The expression level of 18S ribosomal RNA (18S) in each sample was used as an internal reference ("housekeeping gene"), and the primers and probes for 18S were also obtained commercially (Applied Biosystems,

Figure 5. Principles of the probe containing the fluorescent reporter dye (green) and the quencher dye (red) used during real-time PCR with the Taq DNA polymerase ***Left:*** the probe is intact containing both dyes. Under these circumstances the fluorescent activity of the reported dye is quenched by the quencher dye through fluorescence resonance energy transfer (FRET). ***Right:*** The 5' exonuclease activity of Taq DNA polymerase removes the fluorescent dye attached to the 5' end of the probe, disrupting the close spatial relationship between the reporter and the quencher dyes, thereby allowing the reporter dye to exert its fluorescent effect (source: Wikimedia Commons/S. Jähnichen, http://commons.wikimedia.org/wiki/File:TaqMan_Probes.jpg, accessed 02/26/2009).



Foster City, CA). The TaqMan® Gene Expression Assays contain the Taq DNA polymerase, reaction buffer, specific primers and the dye-labeled oligonucleotide primers. The primers are designed to span exon-exon junctions, so that amplification of complementary sequences of genomic DNA is avoided. These assays have been optimized and verified for each specific gene of interest. Table 4 lists the assays used for each of the genes of interest and the 18S internal reference.

Table 4. Gene names, National Center for Biotechnology Information Reference Sequence (RefSeq) ID, and Taqman® Gene Expression Assay probe ID for the genes of interest during RT-PCR procedures

Gene Name	RefSeq ID	Assay ID
18S ribosomal RNA	n/a	**Hs99999901_s1**
SCAP	NM_001001144, NM_001103162	**Mm01250183_G1**
SREBP-1a	NM_011480.3	**Mm01138344_m1**
SREBP-2	NM_033218.1	**Mm01306294_m1**
HMGCR	NM_008255.2	**Mm01282497_g1**
LDLR	NM_010700.2	**Mm00440169_m1**
PPAR-α	NP_001106889.1, NP_035274.2	**Mm00440939_m1**
PPAR-γ	NP_001120802.1, NP_035276.2	**Mm00440945_m1**
PPAR-δ	NM_011145.3	**Mm00803186_g1**
LXR-α	NM_013839.3	**Mm00443454_m1**

One microliter of cDNA obtained from reverse transcription as described previously was utilized for each real-time PCR reaction. The total volume of each well was 10µl, and each sample was run in duplicates (8-month specimens) or triplicates (4-month and 21-day specimens). Several wells containing no cDNA template were included in each set of PCR reaction and served as negative controls to exclude the possibility of cross-contamination of samples or reagents. All negative controls for RNA extraction, reverse transcription and real-time PCR were negative for amplification of specific targets (data not shown). The cycling settings for real-time PCR using the 7500 Fast Real Time PCR equipment were as follows: Stage

1 included one initial denaturation step at 95 ^{o}C for 20 seconds; stage 2 included a repetition cycle of two steps, with step one consisting of denaturation at 95 ^{o}C for 3 seconds, and step two of an annealing/extension step at 60 ^{o}C over 30 seconds. The stage 2 cycle was repeated over for a total of 40 times. Relative expression levels (RQ) for each of gene of interest, as compared to the expression of the 18S housekeeping RNA, were calculated based on the C_t method using the factory provided 7500 Software® versions 1.5 and 2.0.1 (Applied Biosystems, Foster City, CA).

Telemetric In-Vivo Blood Pressure Measurements

We utilized a telemetric method for an in-vivo blood pressure and activity recording that allows the measurement of these parameters in conscious, unrestrained animals on a continuous basis for prolonged periods using a Data Sciences International Telemetry System® (Data Sciences International, St. Paul, MN) (Lu et al., 2007). The protocol utilizes an implantable catheter with a radiotransmitter of 0.4 mm diameter (PA-C20®, Data Sciences International, St. Paul, MN) that is inserted into the left the carotid artery during a surgical procedure. The transmitter forwards real-time recorded data to a radio receiver that is placed underneath each animal cage. The information is then processed and stored in a data acquisition and analysis system and can be analyzed using the appropriate software (A.R.T.3.1®, Data Sciences International, St. Paul, MN).

The surgical placement of the implantable catheter into the carotid artery was performed as follows: Mice were anesthetized with a mixture of ketamine (Ketalar®, Parke-Davis, Morris Plains, NJ) and xylazine hydrochloride (Gemini®, Rugby Laboratories, Rockville Center, NY). After sterilization of the operative site a vertical midline skin incision was made along the neck using a sterile surgical scalpel and the left common carotid artery was carefully dissected and isolated. The vessel was then ligated both proximally and distally to the catheter insertion site. A small incision was then placed into the wall of the carotid artery and the catheter tail including

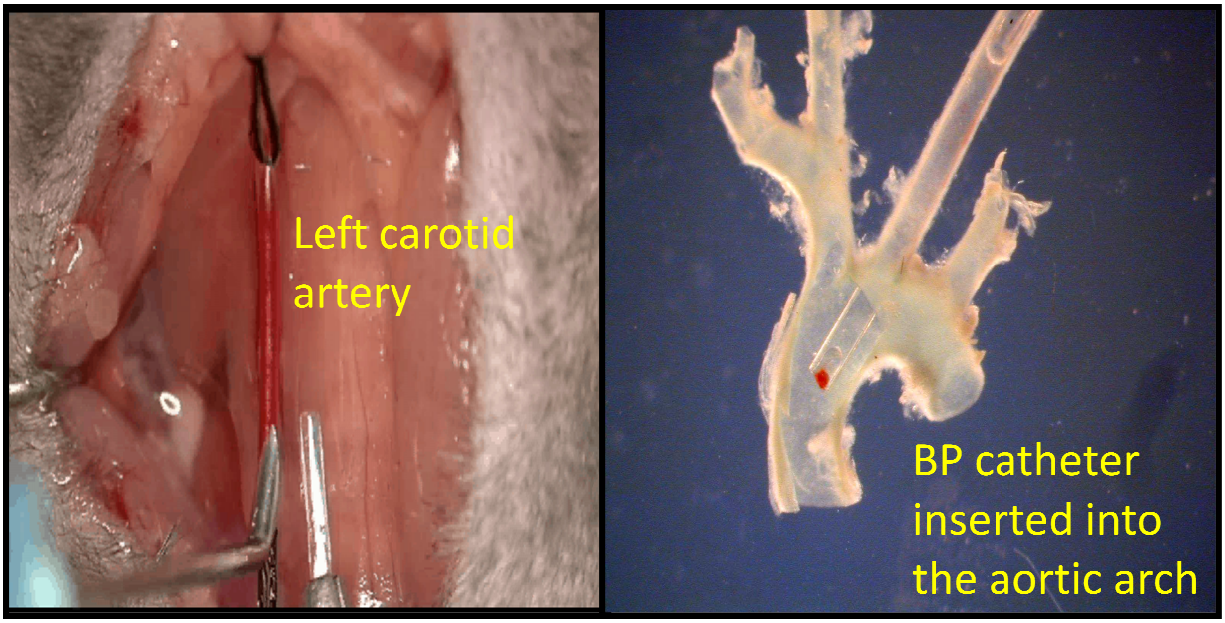
the sensing components was threaded into the vascular lumen. The tip of the sensor was advanced into the aortic arch in order to directly record central systemic blood pressures. The proximal and distal ligatures were then removed from the carotid vessel and good bleeding control was observed. The body of the transducer was then secured in a subcutaneous pouch along the animal's right flank through the same ventral neck incision in a position such that it would not interfere with the animal's movements or regular activities. The skin incision was subsequently closed using a 6–0 silk suture. Mice were kept under a heating lamp in a warm environment, closely monitored, and allowed to recover fully from anesthesia. All animals survived the procedure without significant morbidity. Figure 6 demonstrates exposure of the left carotid artery during the procedure and the final position of the catheter tip at the end of surgery.

After recovery, the animals were placed into a temperature, light and noise controlled dedicated room containing the telemetric system and placed in individual cages positioned on top of the radio receiving units. Food and water were provided ad libitum. No activity or blood pressure measurements were recorded during the initial 24-hour period in this setting, in order to allow them to accommodate to the new environment. Thereafter, activity and the following blood pressure parameters were measured and stored in a continuous fashion over a 5-9 day period: Activity Index, Mean arterial blood pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP). The recorded data were synchronized according to the time of the day, so that all readings were compared at the same circadian time points, and statistical analyses were performed.

Vascular Reactivity Studies

Vascular reactivity studies at isometric tension were performed using the technique described by Longo and associates (Longo, 2005; Longo et al., 2005). Mature cycling female and male offspring obtained according to the breeding protocol laid out earlier were utilized for these

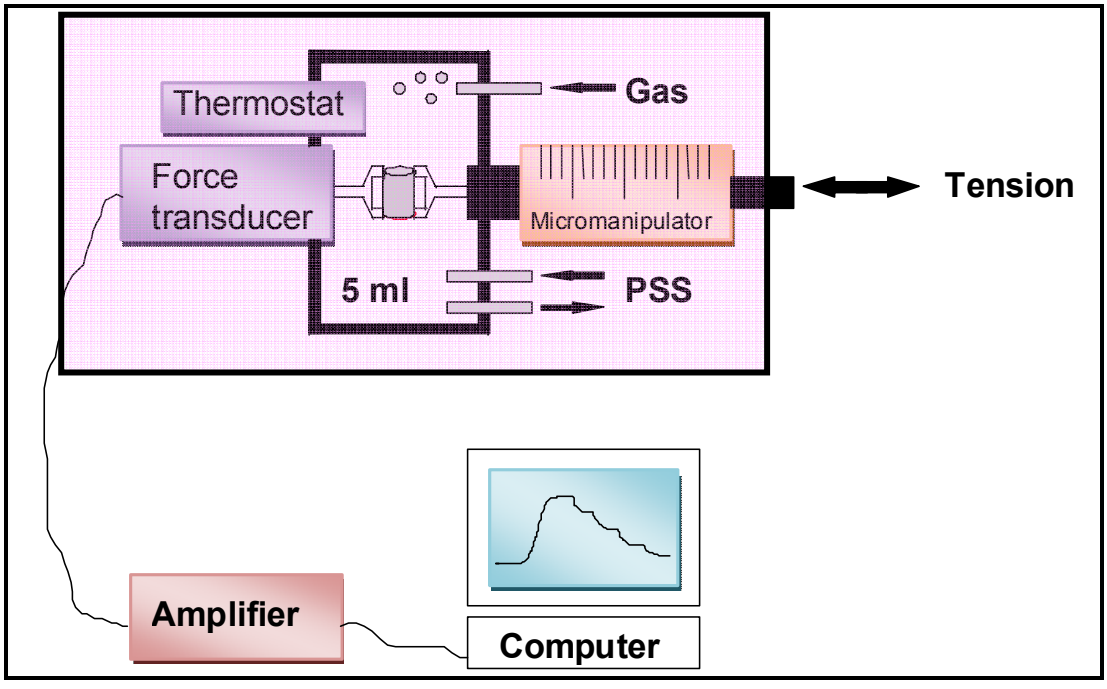
Figure 6. Catheter placement for activity and blood pressure recordings. ***Left:*** in-vivo photograph demonstrating the exposure of the left common carotid artery which has been ligated proximally and distally to the incision site. ***Right:*** ex-vivo aortic arch and main arteries showing the catheter insertion through the left carotid artery and placement of the catheter tip within the lumen of the aortic arch. (photographs courtesy of Esther H. Tamayo, University of Texas Medical Branch, Galveston, Texas)



studies. The carotid arteries were separated from the surrounding connective tissue, avoiding direct contact with the inside lumen of the vessel or undue pressure, thereby taking care not to damage the endothelium or the intima. The carotid arteries were separated from the segment just proximal to their bifurcation into internal and external carotid arteries. The internal diameters of the carotid vessels used were in the magnitude of about 100µm. After trimming both ends of the dissected vessels to remove nonviable portions, two-millimeter segments of the vessels were mounted between the two jaws of a wire myograph (Model 410A, J.P. Trading I/S, Aarhus, Denmark) on tungsten wires of a diameter of 25µm. One of the jaws in this system is connected

to a micromanipulator for adjustment of tension in the vessel while the other jaw is attached to an isometric force transducer. The preparations were bathed in physiological salt solution that was maintained at 37°C, with a pH of 7.4. A mixture of 95% O_2 and 5% CO_2 was infused continuously through the solution. The force was continuously recorded through the isometric force transducer and subsequently analyzed using the PowerLab® system and Chart 5® data acquisition and playback software (AD Instruments, Castle Hill, Australia). The setup of the isometric vessel myograph system is illustrated in figure 7.

Figure 7. In vitro contractility measurement in the carotid artery. Vascular segments of the arteries were mounted in a small vessel myograph chambers containing 5ml physiologic salt solution (PSS) and infused with a gas mixture (95% O_2 and 5% CO_2). The temperature was maintained constant at 37ºC. The vessels were suspended between the jaws of the myograph with 25µm tungsten wires. One jaw was attached to a micromanipulator allowing changes in the distance between the wires and hence adjusting the tension in the vessel while the other was connected to the force transducer for measurement. The signal was amplified and recorded with a data acquisition system (modified after Longo, 2005, with permission).



DRUGS AND SOLUTIONS

The drugs used in the in-vitro experiments were acetylcholine hydrochloride (ACh), phenylephrine hydrochloride (PE), sodium nitroprusside (SNP) and L-N^G-nitroarginine methyl ester hydrochloride (L-NAME) (Sigma-Aldrich, St. Louis, MO). We prepared stock solutions of each of the drugs (10^{-2} mol/L) in deionized water which were then stored at -20°C. The composition of physiological salt solution utilized in the isometric vessel myograph chamber was as follows: NaCl (115 mmol/l), KCl (5 mmol/l), NaH_2PO_4 (1.2 mmol/l), $NaHCO_3$ (25 mmol/l), $MgCl_2$ (1.2 mmol/l), $CaCl_2$ 2.5 (mmol/l), ethylenediaminetetraacetic acid (EDTA) 0.026 (mmol/l), glucose (11 mmol/l).

VASCULAR REACTIVITY PROTOCOL

After suspending the vessels in the isometric myograph chamber and increasing the tension slowly over about 15 minutes to a predefined level (3.5nN), the vessels were allowed to equilibrate slowly for approximately one hour. Subsequently the vessels were stimulated with a 60mM concentration of KCl for 10 minutes. The arteries were then washed several times, rested for 30 minutes and the KCl stimulation was repeated a second time. The purpose of this procedure was to "reset" all vessels to a common contractile state. This maneuver also assured that all suspended arteries were viable and were capable of mounting an appropriate contractile response. If this was not the case, then the affected artery was discarded and no data were used for further analysis. The second contraction in response to the KCl challenge further served as the reference contraction in the final calculations. The response to KCl confirmed the viability and functional capability of the vascular smooth muscle. Since the contribution of the endothelium, and possible changes in endothelial function, are an important outcome in our studies, we also assured the viability of the endothelial lining through the following procedure:

The vessels were contracted with phenylephrine (10^{-6} M) and then relaxation was tested through exposure to the endothelium-dependent vasodilator acetylcholine (10^{-6} M). Again, if the vessel failed to respond adequately it was discarded from further studies.

After a rest period in physiological salt solution the response to each of the vascular mediators was examined using a dose response protocol, starting with the lowest concentration and proceeding with increasingly higher concentrations in a half-logarithmic fashion. This was the case for all of the analyzed mediators with the exception of L-NAME. L-NAME was only administered in a single concentration (10^{-4} M) during a dose response determination using phenylephrine. Responses to the vasorelaxant agents acetylcholine and sodium nitroprusside were obtained after precontraction with phenylephrine at a dose of 10^{-6} M. The various compounds utilized for the determination of vascular responses in our studies, along with their respective concentration(s), and their primary modes of action on the vascular wall, are listed in Table 5.

Vascular response data were expressed as means ± SEM. The response to the second KCl stimulus after initial vessel equilibration was used as a reference to calculate the percent of contraction achieved by phenylephrine. Responses to acetylcholine and SNP were expressed as percentage relaxation of the phenylephrine induced precontraction. Statistical analyses were performed as described below.

Statistical Analysis

Continuous data were tested for normality with normal probability plots using the SigmaStat statistical software package (version 3.0, Systat Software, San Jose, California). Differences between groups for normally distributed data were analyzed with Student's *t* test,

Table 5. Compounds used to investigate selected pathways involved in the regulation of vascular function, major modes of action, and concentration ranges used in the study of vascular response patterns

Compound	*Type*	*Action*	*Dose*
Phenylephrine (PE)	α_1-adrenergic agonist	smooth muscle contraction	10^{-10}-10^{-5} M
Acetylcholine (Ach)	muscarinic-receptor agonist	endothelium-dependent-vasorelaxation	10^{-10}-10^{-5} M
Sodium nitroprusside (SNP)	NO donor	endothelium-independent-vasorelaxation	10^{-10}-10^{-5} M
L-NAME	inhibition of NOS function	prevents endothelium-dependent-vasorelaxation	10^{-4} M

one-way ANOVA, two-way ANOVA and repeat-measures ANOVA as appropriate. Post hoc comparisons were done using the Holm-Sidak method, Dunn's method or the Fisher's posthoc least significant difference (Fisher's PLSD) test. Nonparametric means were compared using the chi square test. Linear regression analysis was utilized for analysis of correlation data. A p-value < 0.05 was considered statistically significant.

CHAPTER 3. RESULTS

Specific Hypothesis 1

Heterozygous offspring born to apoE deficient mothers develop premature atherosclerosis as compared to genetically similar offspring born to wild-type mothers.

SPECIFIC AIM 1

Levels of total blood cholesterol and triglyceride levels, the presence and extent of atherosclerotic plaques formation in the aorta, and possible histopathological end organ damage (affecting the kidney or liver) will be determined to evaluate the effect of early life exposure to high cholesterol levels.

INTRODUCTION

It is evident from the evidence presented in the introduction that there is a paucity of data on the possible correlation of maternal hypercholesterolemia with an increased risk in the offspring to develop premature and/or more advanced atherosclerosis as adults. The enigmatic relationship between maternal and fetal/neonatal serum cholesterol levels, incomplete understanding of the mechanisms of cholesterol metabolism in fetus, and the difficulty to establish long-term follow-up and to obtain clinical outcome data in mother-child pairs in humans are some of the obstacles that lay in the way of unraveling developmental contributions towards the etiology of ischemic heart disease. Furthermore, clearly recognized adult risk factors magnetize the attention of researchers in the area and detract from possible influences earlier in life that cannot be that easily identified.

In the human data obtained during the FELIC study and its predecessor, and since then,

through the work of Drs. Palinski, Napoli, and their coworkers are consistent with the hypothesis that maternal hypercholesterolemia is associated with increased lesion formation in the offspring (Liguori et al., 2008; Napoli et al., 1999b; Napoli et al., 1997; Yamashita et al., 2006). These findings have not been unequivocally replicated by other investigators. Additionally, the increased lesion formation in these studies was not found to be associated with an elevation of cholesterol levels during childhood or adolescence. These investigators offer as one possible explanation for the increased propensity for lesion formation the transfer of oxidized lipids and/or other humoral factors from the mother that lead to passive damage in the offspring and result in permanent reprogramming effects.

Data from animal studies collectively agree with the evidence in humans that maternal hypercholesterolemic environment may raise the susceptibility in the offspring to develop lesions prematurely (Alkemade et al., 2007; Madsen et al., 2003; Napoli et al., 2000; Napoli et al., 2002; Palinski and Napoli, 2002). On the other hand, besides rabbit data from the Palinski and Napoli group, the support from experiments in animals is more ambiguous. Results in the apoE deficient mouse model, the most commonly used and arguably the most adequate model for atherosclerosis research, have been scarce and limited by study design limitations and inconclusive outcomes (Alkemade et al., 2007; Madsen et al., 2003).

EIGHT MONTH-OLD OFFSPRING

Study Design

Female apoE$^{-/-}$ mice and their apoE$^{+/+}$ C57BL/6J control counterparts of approximately 7-8 weeks of age were cross-bred with apoE$^{-/-KO}$ and apoE$^{+/+WT}$ males according to the breeding scheme outlined in figure 4 (page 35). The resultant offspring included maternally-derived (apoE$^{+/-mat}$) and paternally-derived (apoE$^{+/-pat}$) heterozygous litters, as well as apoE$^{+/+WT}$ and

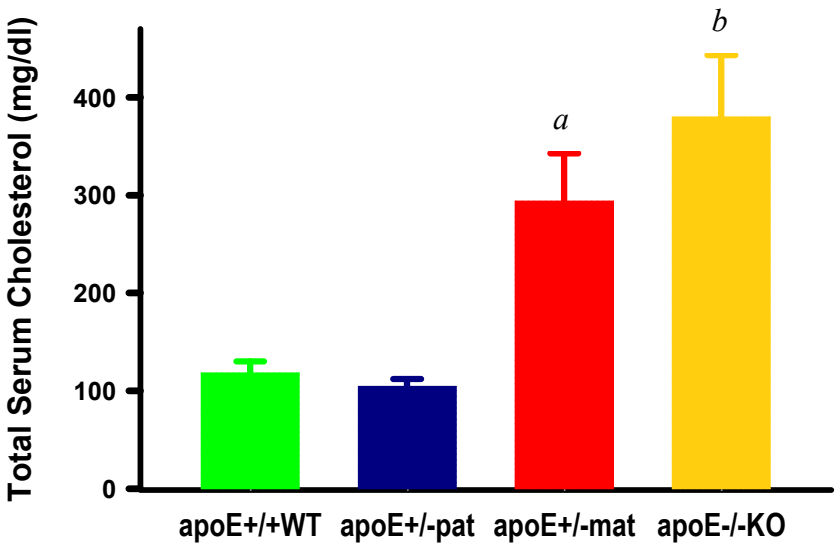
apoE$^{-/-KO}$ homozygous male and female offspring. The numbers of offspring obtained and followed in each group for this portion of the studies were as follows: apoE$^{+/+WT}$: n=12; apoE$^{+/-pat}$: n=7; apoE$^{+/-mat}$: n=18; apoE$^{-/-KO}$: n=14, for a total of 51 offspring (Goharkhay et al., 2007).

These animals remained with their natural dams for the duration of breastfeeding, and were then maintained on regular chow until they reached the age of 8 months. After killing the animals, serum was obtained and total cholesterol and triglyceride levels were determined as described earlier. The aortic arch was stained with oil red O and analyzed for the presence of pre- and atherosclerotic lesions. For the evaluation of liver and kidney histopathology, sections of hepatic and kidney tissue were stained with hematoxylin and eosin and assessed for the presence and severity of abnormal lesions.

Serum Cholesterol Levels

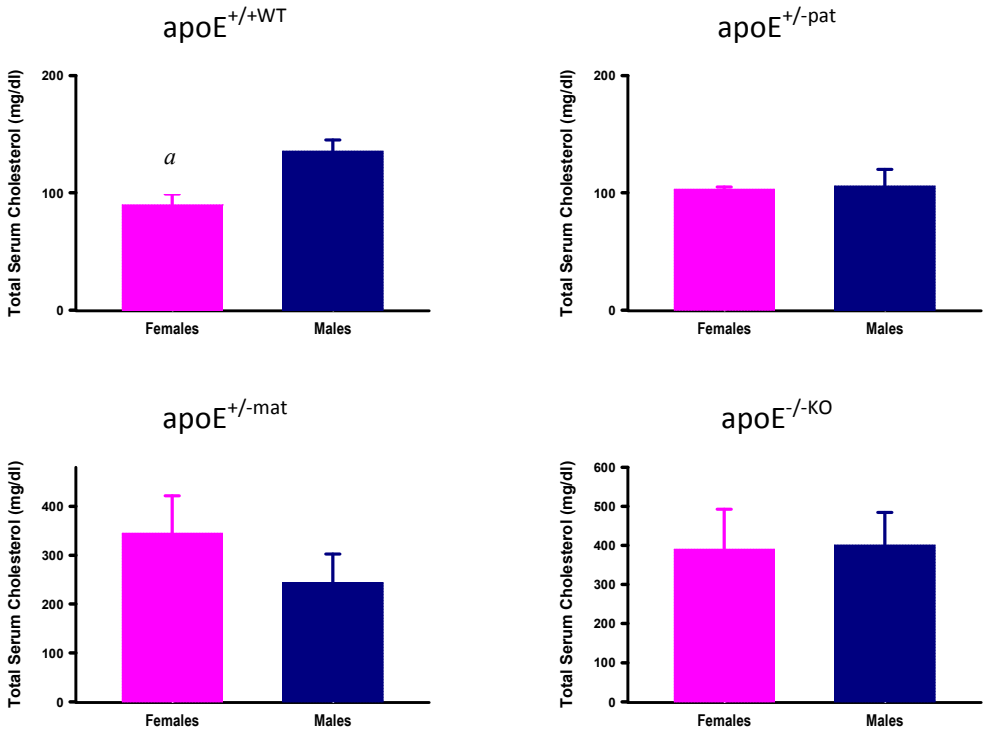
Serum cholesterol levels were higher in both the maternally heterozygote apoE$^{+/-mat}$ (289±47 mg/dl), as well as in the homozygously transgenic apoE$^{-/-KO}$ (396±62 mg/dl) groups as compared with apoE$^{+/-pat}$ (105±8 mg/dl) and apoE$^{+/+WT}$ (105±11 mg/dl) offspring (p=0.0021; figure 7). Circulating cholesterol levels in the apoE$^{+/-mat}$ group of offspring were significantly higher than in the apoE$^{+/+WT}$ (p=0.037) and apoE$^{+/-pat}$ offspring mice (p=0.027), respectively. Cholesterol levels were also found to be significantly elevated in apoE$^{-/-KO}$ animals as compared with both apoE$^{+/+WT}$ and the apoE$^{+/-pat}$ groups (p=0.002 and p=0.001). Although apoprotein E-deficient apoE$^{-/-KO}$ litters displayed somewhat higher levels of circulating cholesterol concentrations versus the maternally deficient heterozygous apoE$^{+/-mat}$ animals, this difference did not reach the level of statistical significance (p=0.110).

Figure 8. Serum total cholesterol levels in 8-month old offspring according to study group. *a* p=0.037 versus apoE$^{+/+WT}$ and p=0.027 versus apoE$^{+/-pat}$; *b* p=0.002 versus apoE$^{+/+WT}$ and p=0.001 versus apoE$^{+/-pat}$, respectively (modified from Goharkhay et al., 2007, with permission).



No significant variation between genders was noted, except in the apoE$^{+/+WT}$ group in which the male offspring had higher total cholesterol levels than females (136 ±10 mg/dl versus 89±9 mg/dl, p=0.038; figure 8).

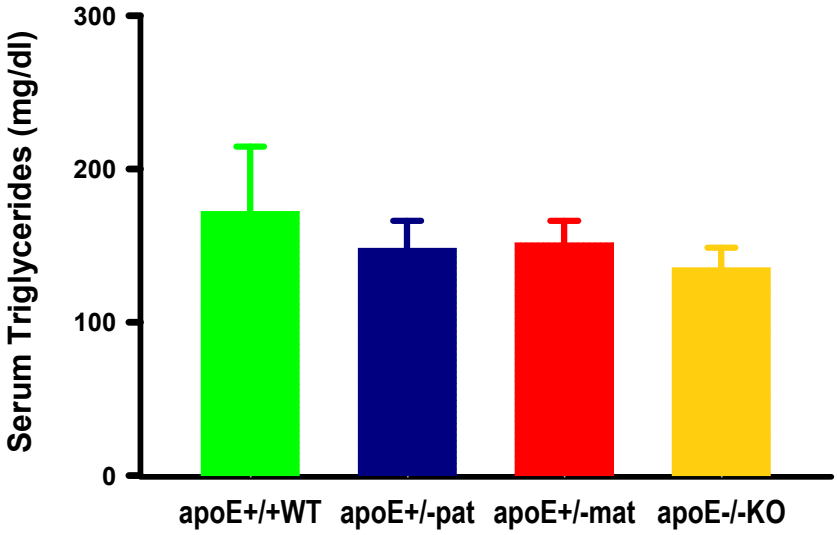
Figure 9. Serum total cholesterol levels in 8-month old offspring according to study group and gender. *a* p=0.038 versus males (Goharkhay et al., 2007).



Serum Triglyceride Levels

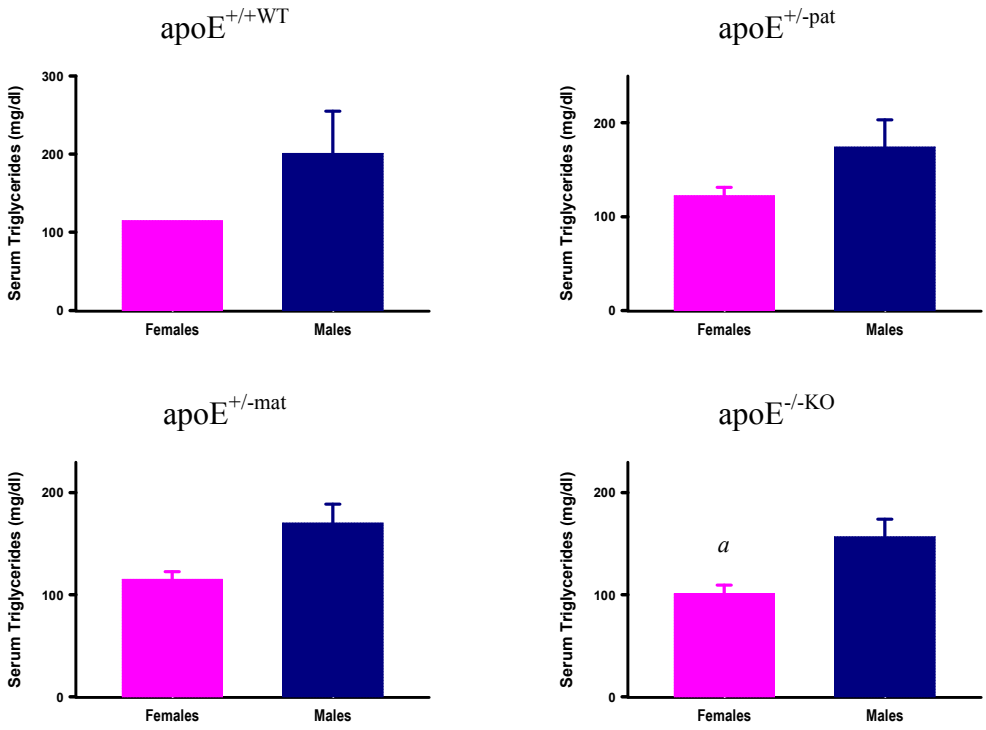
No significant difference in the triglyceride levels were found between the offspring in the four different study groups at 8 months of age (172±42 mg/dl in the apoE$^{+/+WT}$, 148±14 mg/dl in the apoE$^{+/-pat}$, 152±14 mg/dl in the apoE$^{+/-mat}$ and 135±13 mg/dl in the apoE$^{-/-KO}$ offspring; figure 9).

Figure 10. Serum triglyceride levels in 8-month old offspring according to study group (Goharkhay et al., 2007).



Male offspring had higher levels of triglycerides than females among the apoE$^{-/-KO}$ group of offspring (167±16 versus 99±7 mg/dl, p=0037), but not in any of the other remaining three groups of litters at 8 months, although an overall tendency towards higher levels in males was noted (figure 11).

Figure 11. Serum triglyceride levels in 8-month old offspring according to study group and gender. *a* p=0.037 versus males (Goharkhay et al., 2007).

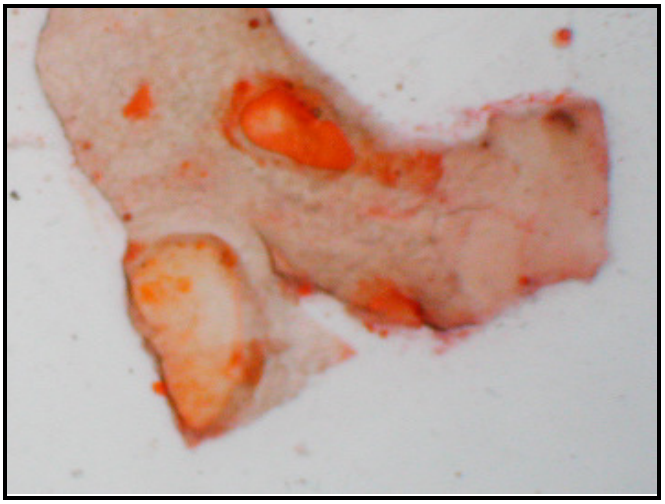


Atherosclerotic Plaque Formation

As noted above, the incidence, severity and relative surface areas of pre- and atherosclerotic lesions were recorded in the aortic arch of all offspring animals at 8 months of age utilizing the en face method of analysis after oil red O staining.

Figure 12 demonstrates an example of an aortic arch specimen containing more advanced lesions.

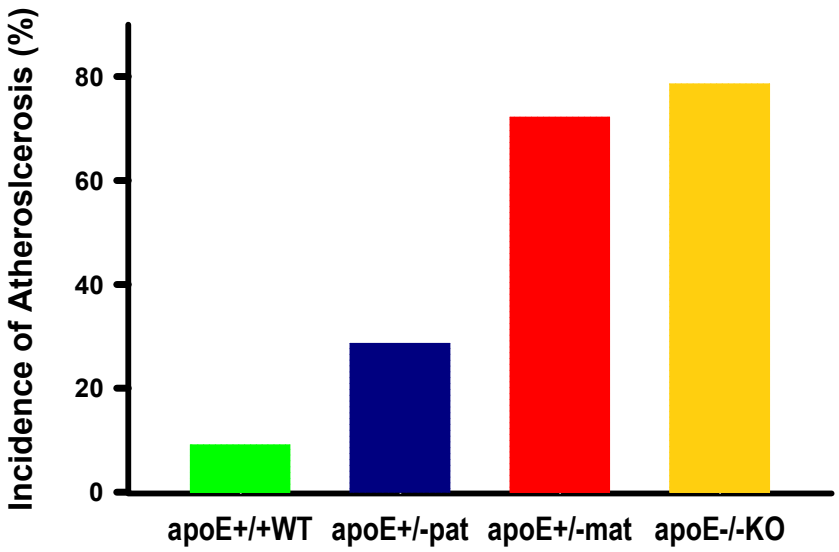
Figure 12. Example of an aortic arch after staining with oil red O demonstrating the presence of atherosclerotic plaques (orange and red lesions) (from Goharkhay et al., 2007, with permission).



Incidence of Atherosclerosis

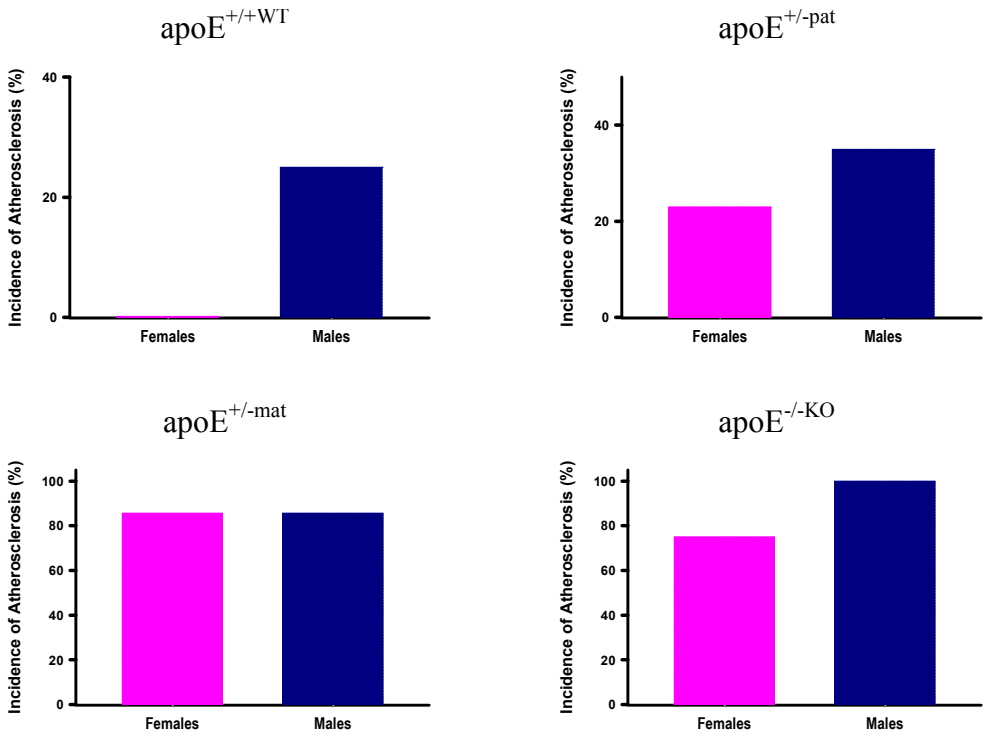
There was a significant difference in the incidence of atherosclerosis (advanced lesions) between the groups: 78.6% in $apoE^{-/-KO}$, 72.2% in $apoE^{+/-mat}$, 28.6% in $apoE^{+/-pat}$ and 9.1% in $apoE^{+/+WT}$ ($p<0.0001$; figure 13). Only in one $apoE^{+/+WT}$ animal an advanced lesions was observed, whereas the aortic arches in most $apoE^{+/-mat}$ and $apoE^{+/-pat}$ offspring demonstrate evidence of atherosclerotic plaques of varying sizes.

Figure 13. Incidence of advanced atherosclerotic lesions in the aortic arch by offspring group (p<0.0001) (Goharkhay et al., 2007).



Although the numbers in some subgroups were not sufficient to perform a meaningful statistical analysis, no significant effect of gender on the propensity to develop plaque formation was demonstrable in any of the study groups (figure 14).

Figure 14. Incidence of advanced atherosclerotic lesions in the aortic arch in each study group by gender of offspring.



Correlation of Cholesterol Levels with Plaque Formation

We found an association between serum cholesterol levels and the presence of atherosclerotic lesions. Overall in this study, animals showing atherosclerotic plaques had mean cholesterol levels of 385±41 mg/dl as compared to 104±4.9 mg/dl in those offspring which did not show any evidence of such lesions ($p<0.0001$). This relationship remained true when we analyzed each study group of animal offspring separately, albeit the differences within individual groups of litters was not statistically significant except in the case of the apoE$^{-/-KO}$ offspring (table 5).

Table 6. Total serum cholesterol levels by presence or absence of advanced atherosclerotic plaques within each study group (Goharkhay et al., 2007).

Group	Plaque	No Plaque	*p*
$apoE^{+/+WT}$	145	97±10	n/a
$apoE^{+/-pat}$	117±15	99±9	0.35
$apoE^{+/-mat}$	307±65	117±10	0.065
$apoE^{-/-KO}$	484±54	101±10	0.003

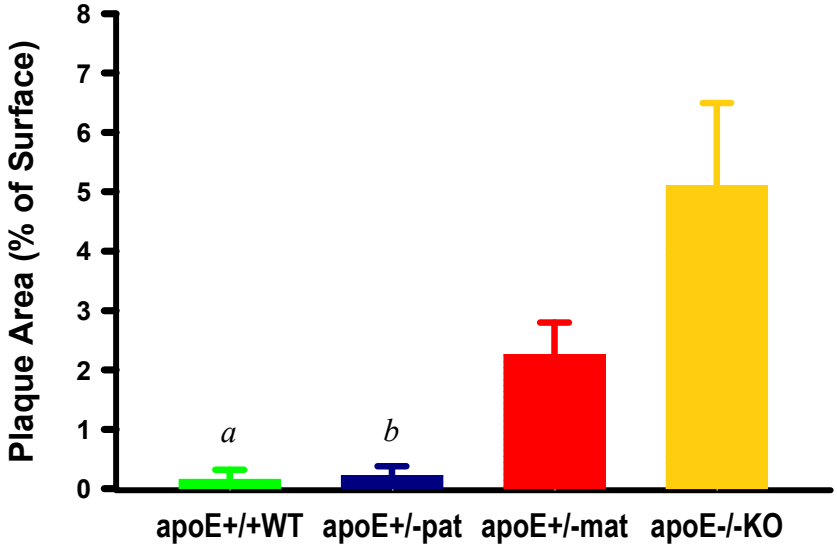
Analysis of Lesion Surface Areas

As alluded to earlier, the size of atherosclerotic lesions relative to the total surface area of the aortic arch was assessed for each sample. This analysis was done in two steps: In a first step solely advanced plaque lesions were quantitated. Subsequently, the combination of early and advanced lesion areas was determined and compared to the total surface area of the specimen and analyzed.

Advanced Plaque Lesion Areas

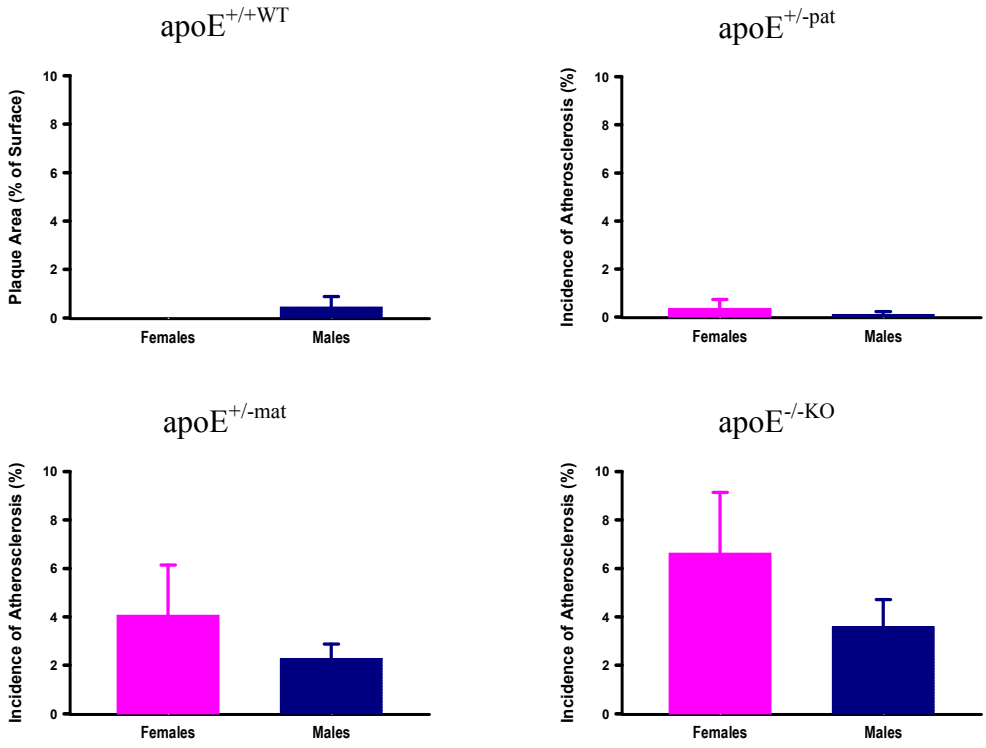
The ratio of the surface area of advanced atherosclerotic plaques relative to the total surface of the aortic arch specimens varied significantly when comparing the offspring groups. The percentage area covered with advanced lesions for each subgroup was as follows: 0.16±0.16% in $apoE^{+/+WT}$, 0.22±0.16% in $apoE^{+/-pat}$, 2.99±0.89% in $apoE^{+/-mat}$ and 5.10±1.39% in $apoE^{-/-KO}$ (p=0.0031; figure 15). In the posthoc analysis, differences in plaque areas were statistically significantly different between $apoE^{+/+WT}$ and $apoE^{+/-mat}$ (p=0.047), $apoE^{+/+WT}$ and $apoE^{-/-KO}$ (p=0.001), and between $apoE^{+/-pat}$ and $apoE^{-/-KO}$ offspring (p=0.004), respectively.

Figure 15. Relative surface area of advanced plaque lesions as compared to total surface area of aortic arch specimen by study group of offspring (p=0.0031) (Goharkhay et al., 2007). *a* p=0.047 versus apoE$^{+/-mat}$ and p=0.001 versus apoE$^{-/-KO}$, respectively; *b* p=0.004 versus apoE$^{-/-KO}$.



No significant effect of gender on the surface area covered by more advanced lesions in the aortic arch was observed in 8 month-old offspring (figure 16).

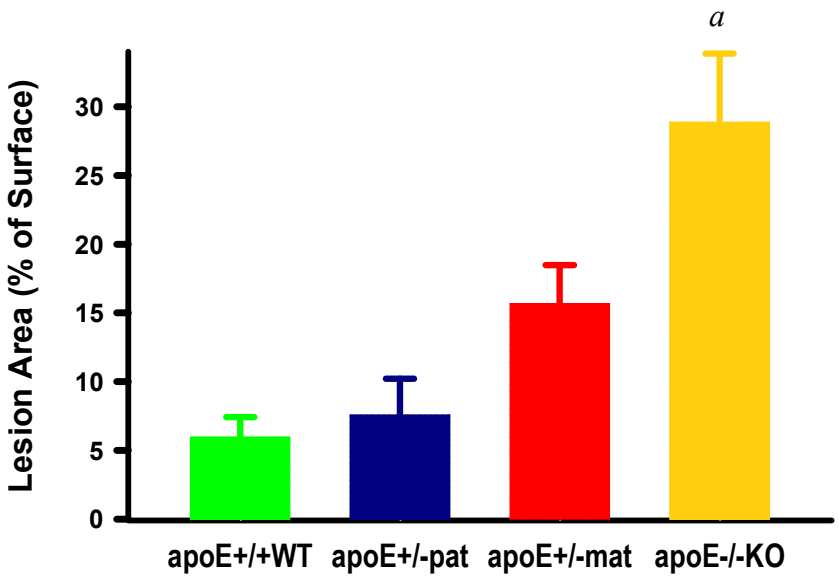
Figure 16. Relative surface are of advanced atherosclerotic lesions in the aortic arch in each study group by gender of offspring.



Early + Advanced Plaque Lesion Areas

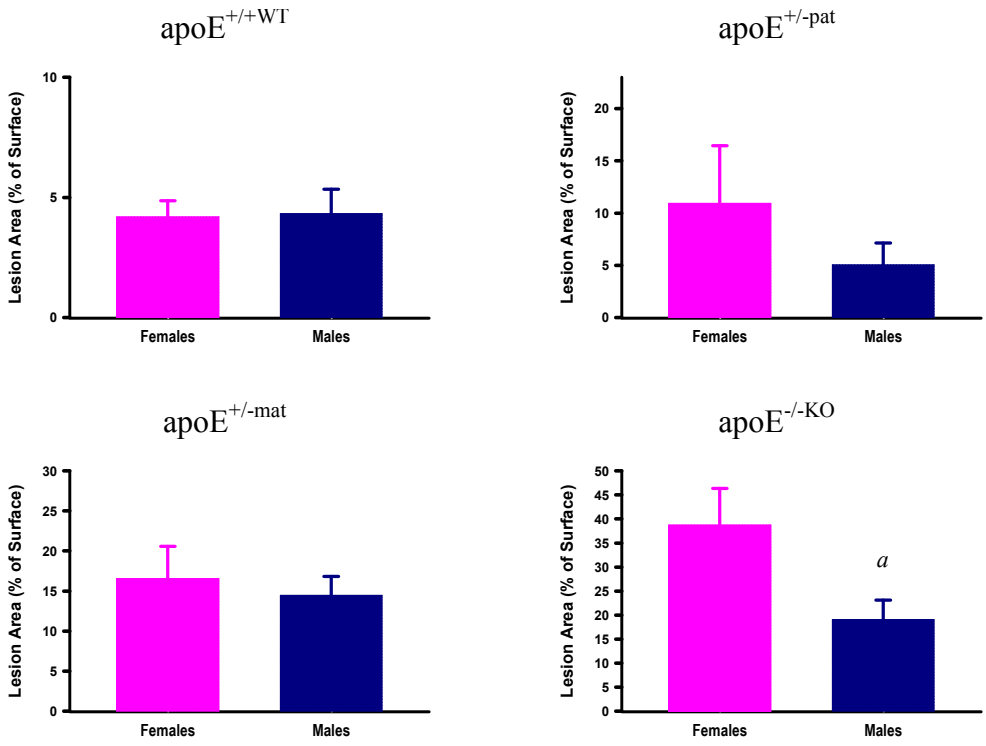
We further analyzed the total surface area covered by the plaque combining the early and advanced lesions within each group. The mean total area of atherosclerotic changes in the apoE$^{+/+WT}$ offspring was 5.96 ±1.45% versus 7.57±2.65% for the apoE$^{+/-pat}$, 15.3±2.07% for the apoE$^{+/-mat}$ and 28.9±4.98% for apoE$^{-/-KO}$ groups (p<0.0001, figure 17). When looking at the variation in surface area of aortic arch specimens affected between the different study groups the following discrepancies were noted: Differences in lesion areas were statistically significantly different between apoE$^{+/+WT}$ and apoE$^{-/-KO}$ (p<0.0001), between apoE$^{+/-pat}$ and apoE$^{-/-KO}$ (p=0.001), and between apoE$^{+/-pat}$ and apoE$^{+/-mat}$ groups (p=0.033), respectively.

Figure 17. Relative surface area of the combination of early and advanced lesion areas as compared to total surface area of aortic arch specimen by study group of offspring (p<0.0001) (Goharkhay et al., 2007). *a* p<0.0001 versus apoE$^{+/+WT}$, and p=0.0003 versus apoE$^{+/-pat}$, and p=0.0033 versus apoE$^{+/-mat}$, respectively.



Gender did not account for any significant difference in the relative surface areas covered by lesions in the various study groups except for within the apoE$^{-/-KO}$ group. In this group the total lesion area was somewhat larger in female offspring as compared with their male counterparts (38.7% in females versus 19.0% in males, p=0.042). The gender distribution of these lesions for each offspring type is demonstrated in figure 18.

Figure 18. Relative surface area of the combination of early and advanced lesion areas as compared to total surface area of aortic arch specimen in each study group of offspring by gender. *a* p=0.042 versus females.



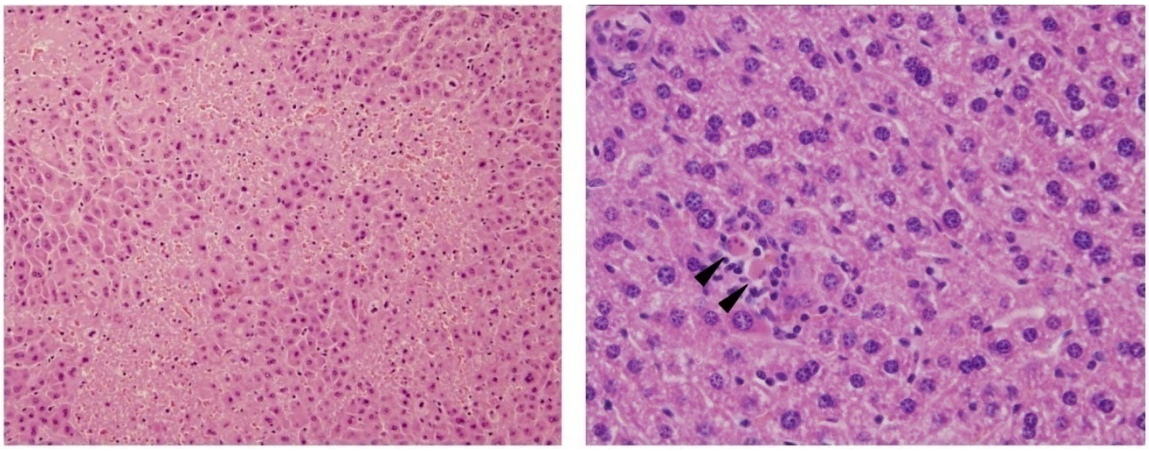
Evidence of End Organ Damage

Prolonged hyperlipidemia may lead, besides its effect on the vasculature, to widespread organ damage throughout other parts of the body. Vascular damage itself can indirectly lead to organ damage through its adverse effect on organ perfusion. In order to determine whether such effects were demonstrable in the 8 month old offspring in the various groups in this study liver and kidney sections were studied for the presence and severity of pathologic lesions.

Liver Lesions

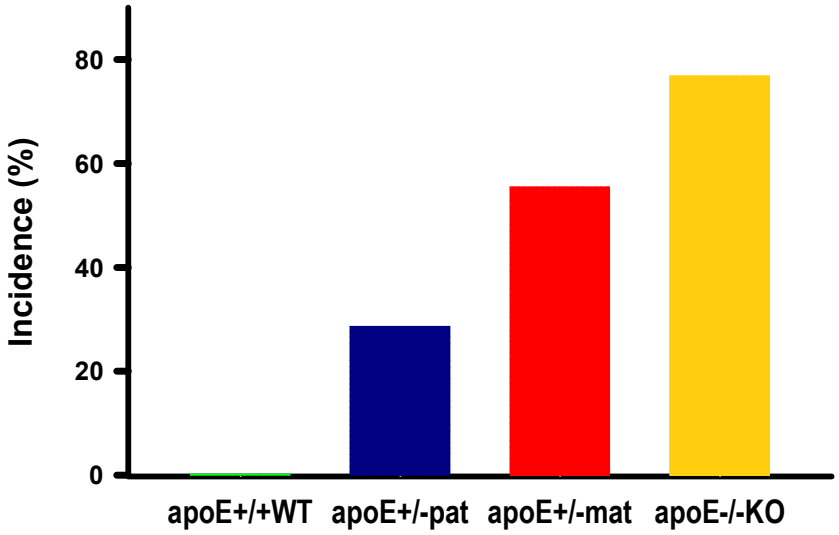
Two predominant changes were observed in animals that showed evidence of abnormalities in the liver: Regions showing evidence of hepatocellular necrosis, as well as areas containing apoptotic hepatocytes (Councilman bodies) (figure 19).

Figure 19. Examples of liver lesions observed in the animals examined. ***Left:*** hepatocellular necrosis. ***Right:*** acidophilic necrotic bodies (arrows; from Goharkhay et al., 2007, with permission).



Such lesions were predominantly found in the liver of $apoE^{+/-mat}$ and $apoE^{-/-KO}$ animals. The combined incidence of such anomalies in each study group was the following: 0% in $apoE^{+/+WT}$, 28.6% in $apoE^{+/-pat}$, 55.5% in $apoE^{+/-mat}$ and 76.9% in $apoE^{-/-KO}$ offspring mice (p=0.009, figure 20).

Figure 20. Incidence of histopathological lesions in the liver by study group (p=0.009) (Goharkhay et al., 2007).



The severity of the lesions noted in the various groups of offspring was predominantly low-grade (table 7).

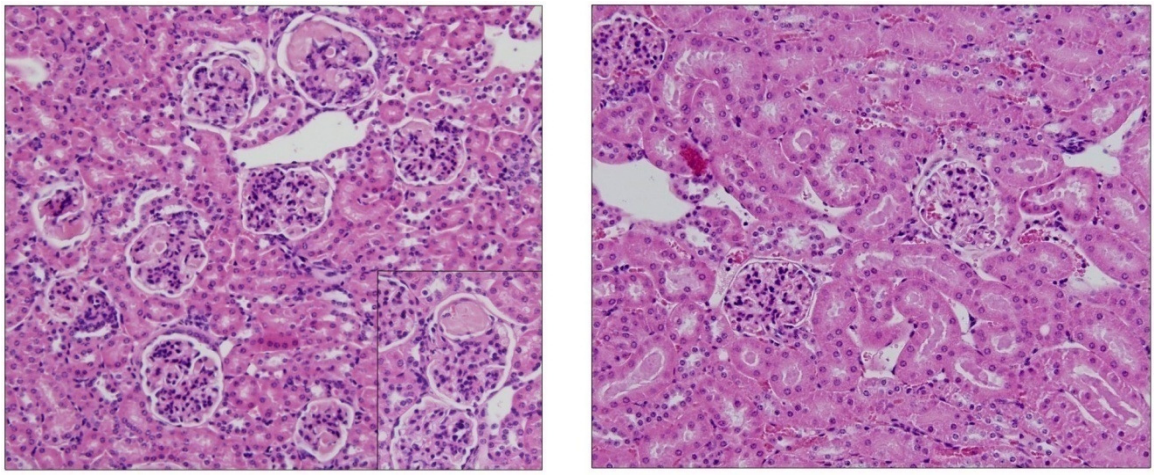
Table 7. Severity distribution of histopathologic liver changes in each litter group percent)(modified from Goharkhay et al., 2007).

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
$apoE^{+/+WT}$	100	-	-	-	-
$apoE^{+/-pat}$	71.4	28.6	-	-	-
$apoE^{+/-mat}$	44.4	44.4	11.1	-	-
$apoE^{-/-KO}$	23.1	61.5	15.4	-	-

Kidney Lesions

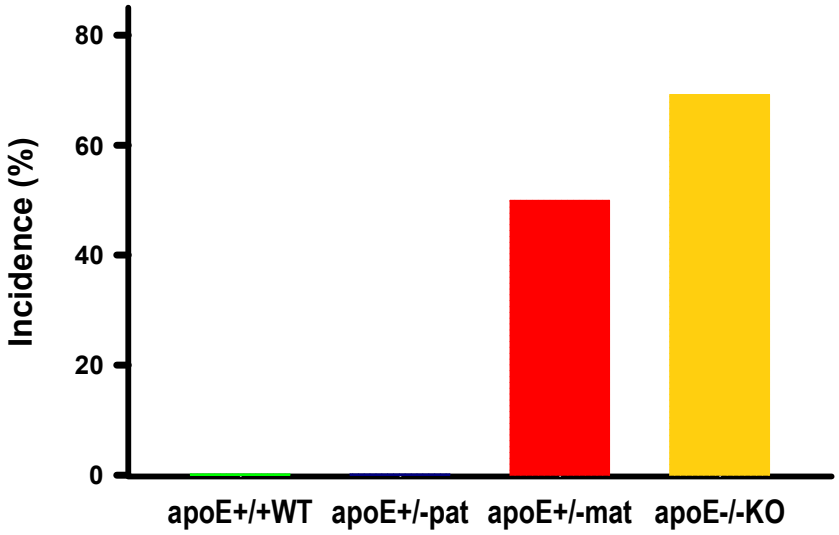
The histological examination of the kidneys did not show any pathologic changes in either apoE$^{+/+WT}$ or apoE$^{+/-pat}$ groups of offspring. The predominant lesions found in the kidneys of apoE$^{+/-mat}$ and apoE$^{-/KO-}$ animals were of two types: Glomerulosclerosis with Kimmelstiel-Wilson type lesions, as well as proteinaceous tubular casts (figure 21).

Figure 21. Examples of kidney lesions observed in 8-month old offspring. ***Left (and insert):*** glomerulosclerosis with Kimmelstiel-Wilson type lesion. ***Right:*** proteinaceous tubular casts (from Goharkhay et al., 2007, with permission).



Such changes were found in 50% of apoE$^{+/-mat}$ and in 69.2% of apoE$^{-/-KO}$ offspring mice. (figure 22).

Figure 22. Incidence of histopathological lesions in the kidney by study group (p=0.028) (Goharkhay et al., 2007).



The distribution of the severity of the anomalies found in kidney specimens from 8-month offspring in the various groups is depicted in table 8.

Table 8. Severity distribution of histopathologic kidney changes in each litter group (percent)(modified from Goharkhay et al., 2007).

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
$apoE^{+/+WT}$	100	-	-	-	-
$apoE^{+/-pat}$	100	-	-	-	-
$apoE^{+/-mat}$	50	22.2	16.7	-	22.2
$apoE^{-/-KO}$	30.8	61.5	15.4	-	-

FOUR MONTH-OLD OFFSPRING

Study Design

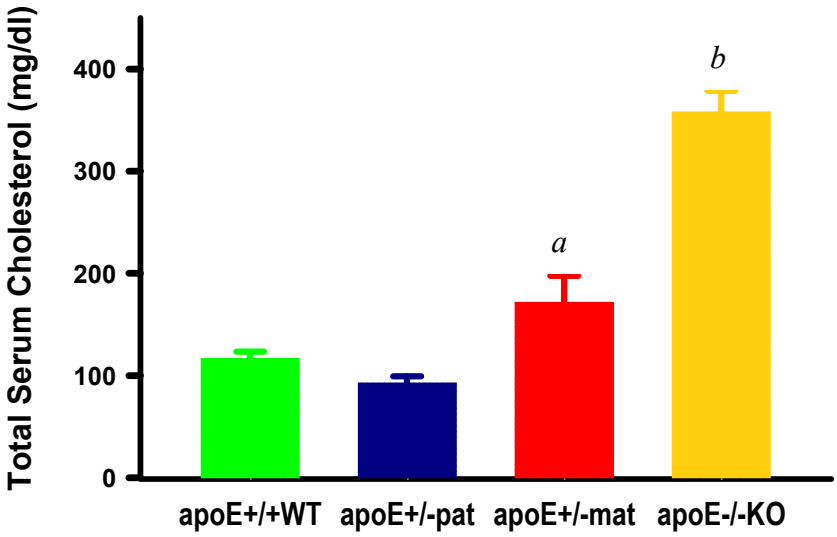
The study design in the case of the 4-month old offspring was similar to that utilized for 8-month old animals described above. Female $apoE^{-/-}$ mice and their $apoE^{+/+}$ C57BL/6J control counterparts of approximately 7-8 weeks of age were cross-bred with $apoE^{-/-KO}$ and $apoE^{+/+WT}$ males according to the breeding scheme outlined in figure 4. The resultant offspring included maternally-derived ($apoE^{+/-mat}$) and paternally-derived ($apoE^{+/-pat}$) heterozygous litters, as well as $apoE^{+/+WT}$ and $apoE^{-/-KO}$ homozygous male and female offspring. The numbers of offspring obtained and followed in each group for this portion of the studies were as follows: $apoE^{+/+WT}$: n=17; $apoE^{+/-pat}$: n=17; $apoE^{+/-mat}$: n=17; $apoE^{-/-KO}$: n=18, for a total of 69 offspring.

These animals remained with their natural dams for the duration of breastfeeding (21-22 days), and were then maintained on regular chow over a duration of 4 months. After the sacrifice of the animals, serum was obtained and total cholesterol and triglyceride levels were determined as described earlier. In the case of the 4-month old offspring the serological test used was the in-house assay as outlined. Liver and kidney sections from these animals were obtained and evaluated in the same manner as in the 8-month old offspring. The presence and severity of pathological lesions were recorded.

Serum Cholesterol Levels

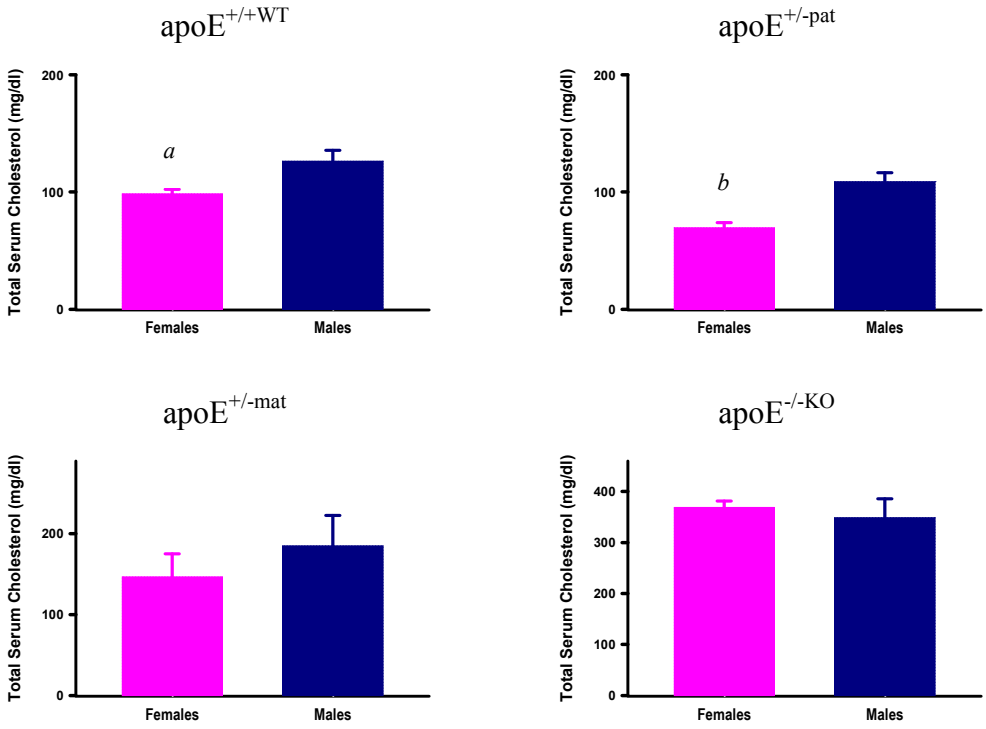
Serum cholesterol levels were higher in the maternally heterozygote $apoE^{+/-mat}$ (166±18 mg/dl), as well as in the homozygously transgenic $apoE^{-/-KO}$ (359±17 mg/dl) offspring as compared with $apoE^{+/-pat}$ (112±19 mg/dl) and $apoE^{+/+WT}$ (89±18 mg/dl) litters ($p<0.001$; figure 23). Circulating cholesterol levels in the $apoE^{+/-mat}$ group of offspring were significantly higher than in the $apoE^{+/+WT}$ ($p=0.047$) and $apoE^{+/-pat}$ offspring mice ($p=0.004$). Serum cholesterol levels in $apoE^{-/-KO}$ litters were significantly elevated versus $apoE^{+/+WT}$ ($p<0.001$), $apoE^{+/-pat}$ ($p<0.001$), as well as $apoE^{+/-mat}$ litters ($p<0.001$).

Figure 23. Serum total cholesterol levels in 4-month old offspring according to study group. *a* p=0.047 versus $apoE^{+/+WT}$, p=0.004 versus $apoE^{+/-pat}$ and p<0.001 versus $apoE^{-/-KO}$; *b* p<0.001 versus $apoE^{+/+WT}$, p<0.001 versus $apoE^{+/-pat}$, respectively.



Although in all animals combined serum cholesterol levels did not vary significantly by gender, this was not true for each study group analyzed separately. In wild-type apoE+/+WT and paternally deficient heterozygous apoE+/-pat litters male gender correlated with elevated serum cholesterol measurements (p=0.024 and p=0.001, respectively; figure 24).

Figure 24. Serum total cholesterol levels in 4-month old offspring according to study group and gender. *a* p=0.024 versus males; *b* p=0.001 versus males.

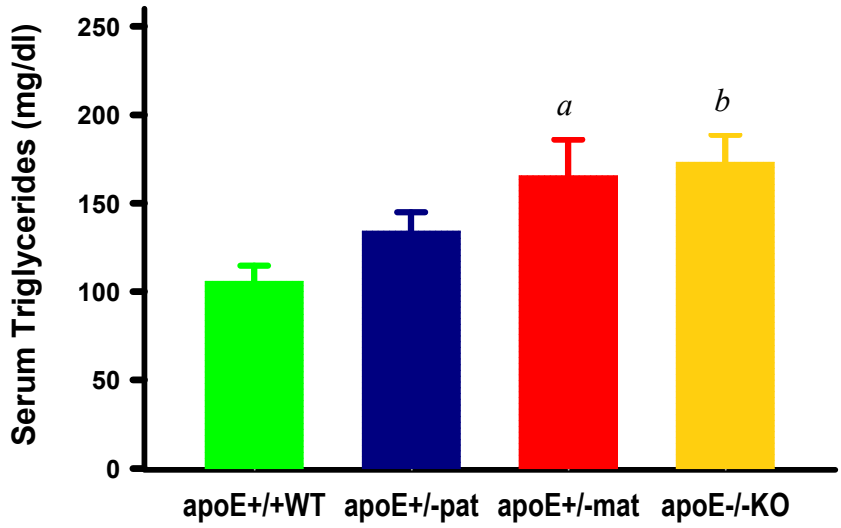


There was no significant interaction between gender and type of offspring (p=0.24).

Serum Triglyceride Levels

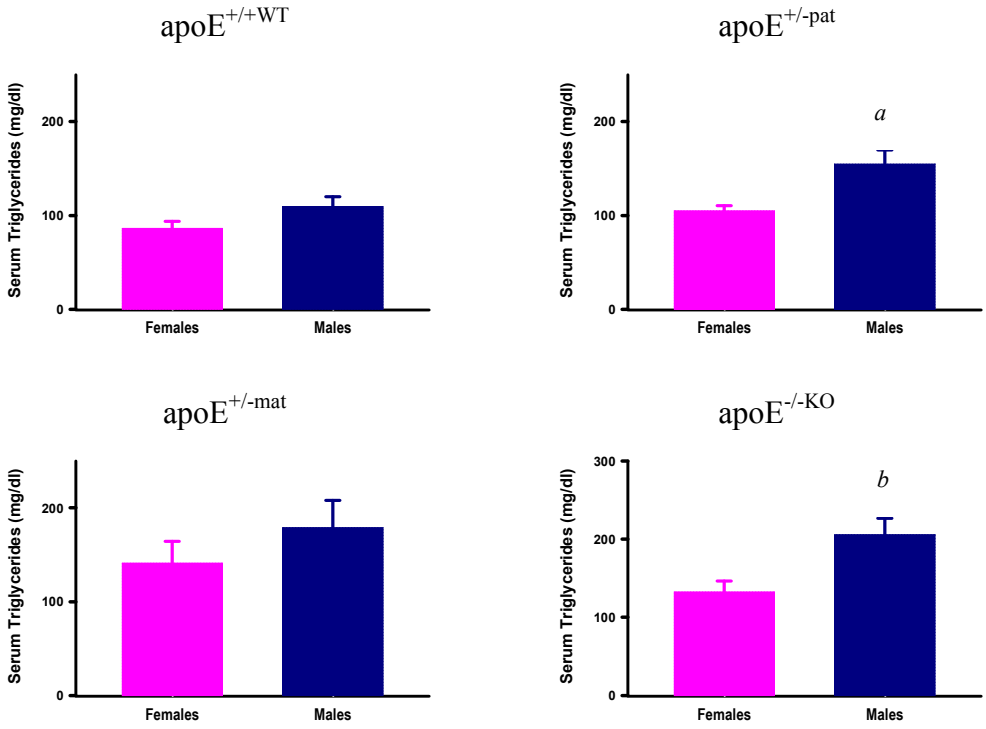
In 4-month old offspring it was found that serum triglyceride levels were significantly differentially distributed across the study groups (p=0.005). The highest triglyceride levels were encountered in apoE$^{-/-KO}$ group (169±13 mg/dl), followed by apoE$^{+/-mat}$ (160±14 mg/dl), apoE$^{+/-pat}$ (130±14 mg/dl) and finally apoE$^{+/+WT}$ (101±14 mg/dl) litters (figure 25). Serum triglyceride levels in the apoE$^{+/-mat}$ group of offspring were significantly higher than in the apoE$^{+/+WT}$ offspring (p=0.005). apoE$^{-/-KO}$ litters displayed a significant elevation of triglyceride levels as compared with the apoE$^{+/+WT}$ (p=0.001) and the apoE$^{+/-pat}$ (p=0.047) groups.

Figure 25. Serum triglyceride levels in 4-month old offspring according to study group. *a* p=0.005 versus apoE$^{+/+WT}$; *b* p=0.001 versus apoE$^{+/+WT}$, and p=0.047 versus apoE$^{+/-pat}$.



In two-way ANOVA analysis, male gender was generally associated with elevated levels of serum triglyceride levels in all animals combined (p=0.001). This overall difference was mainly due to the fact that, among individual litter types of 4-month old offspring, male animals showed significantly higher triglyceride levels as compared with females in the apoE$^{+/-pat}$ (155±18 mg/dl versus 105±21 mg/dl, p=0.016) and apoE$^{-/-KO}$ groups (205±18 mg/dl versus 132±20 mg/dl, p=0.013; figure 26).

Figure 26. Serum triglyceride levels in 4-month old offspring according to study group and gender. *a* p=0.016 versus females; *b* p=0.013 versus females.



There was no significant interaction between gender and type of offspring (p=0.71).

Evidence of End Organ Damage

In contrast to the histopathological examination of liver and kidney samples in the 8-month old offspring groups, the analysis of these tissues in 4-month old litters revealed a small proportion of animals with any abnormal findings.

Liver Lesions

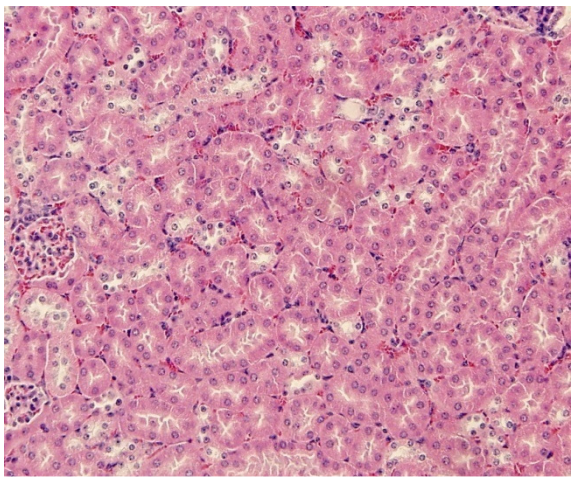
Among all hepatic samples studied from 4-month old litters only three cases (4.3% of all animals) were noted to contain any pathologic abnormalities. The lesions discovered consisted of

one case of grade one hepatocellular necrosis in an apoE$^{+/+WT}$ male offspring, as well as two total cases of grade 2 hepatocellular necrosis noted in one apoE$^{+/+WT}$ female as well as another apoE$^{+/-pat}$ animal.

Kidney Lesions

Abnormal kidney lesions were absent from all 4-month old animals analyzed in our studies. Figure 27 depicts an example of tubular areas in an apoE$^{+/-mat}$ animal showing normal architecture.

Figure 27. Absence of histopathologic changes in a kidney specimen from a 4-month old apoE$^{+/-mat}$ animal.



21-DAY OLD OFFSPRING

Study Design

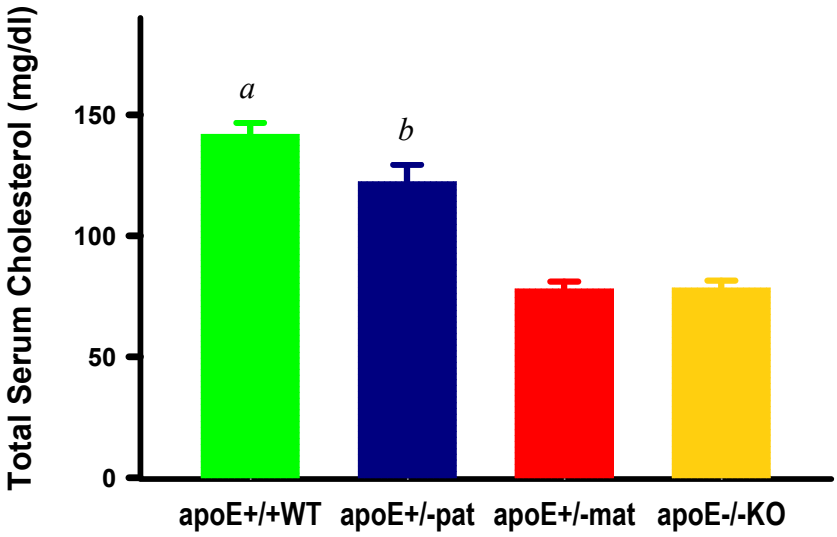
Female apoE$^{-/-}$ mice and their apoE$^{+/+}$ C57BL/6J control counterparts of approximately 7-8 weeks of age were cross-bred with apoE$^{-/-KO}$ and apoE$^{+/+WT}$ males according to the breeding scheme outlined in figure 4. The resultant offspring included maternally-derived (apoE$^{+/-mat}$) and paternally-derived (apoE$^{+/-pat}$) heterozygous litters, as well as apoE$^{+/+WT}$ and apoE$^{-/-KO}$ homozygous male and female offspring. The numbers of offspring obtained and followed in each group for this portion of the studies were as follows: apoE$^{+/+WT}$: n=11; apoE$^{+/-pat}$: n=12; apoE$^{+/-mat}$: n=10; apoE$^{-/-KO}$: n=8, for a total of 41 offspring.

These animals remained with their natural dams for the duration of breastfeeding (21-22 days). They were subsequently removed and sacrificed. Serum was obtained through cardiac puncture and total cholesterol and triglyceride levels were determined using the in-house assay as described.

Serum Cholesterol Levels

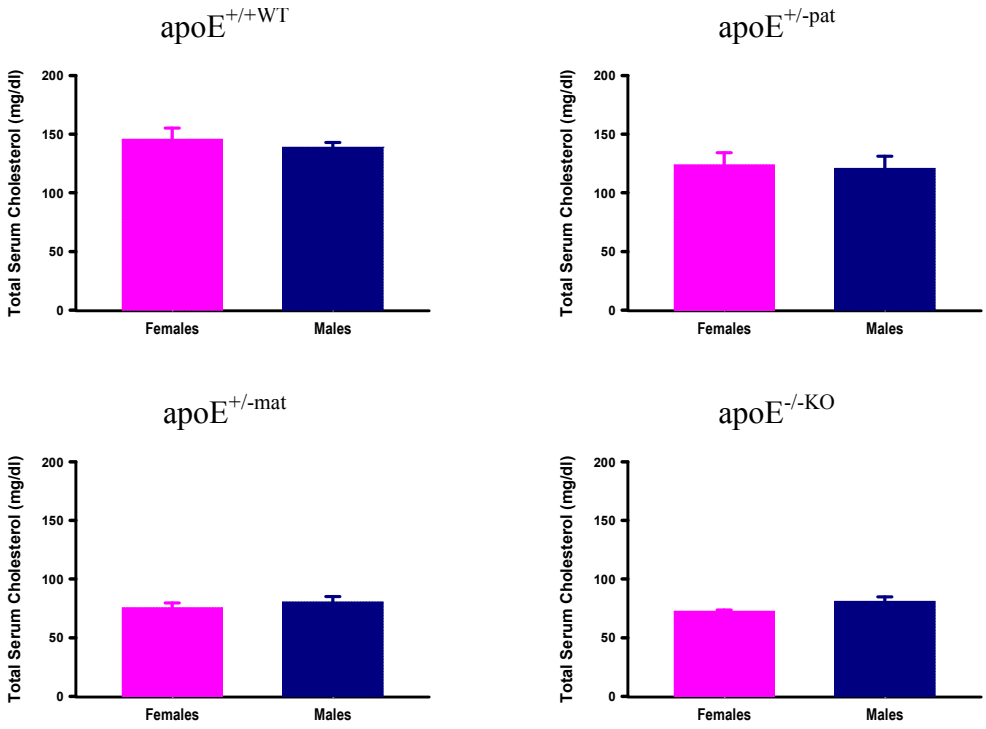
In 21-day old breastfeeding pups there was an overall significant variation in serum cholesterol levels across the study groups ($p<0.001$). The highest total serum cholesterol concentrations were obtained in apoE$^{+/+WT}$ litters (142±5 mg/dl), followed by apoE$^{+/-pat}$ (122±5 mg/dl), apoE$^{+/-mat}$ (78±5 mg/dl) and apoE$^{-/-KO}$ (77±7 mg/dl) groups (figure 28). In individual comparisons between study groups the differences were significant between apoE$^{+/+WT}$ and apoE$^{+/-pat}$ ($p=0.008$), apoE$^{+/+WT}$ and apoE$^{+/-mat}$ ($p<0.001$), apoE$^{+/+WT}$ and apoE$^{-/-KO}$ ($p<0.001$) groups, as well as between apoE$^{+/-pat}$ and apoE$^{+/-mat}$ ($p<0.001$), and apoE$^{+/-pat}$ and apoE$^{-/-KO}$ ($p<0.001$) litters, respectively.

Figure 28. Serum total cholesterol levels in 21-day old offspring according to study group. *a* p=0.008 versus $apoE^{+/-pat}$, p<0.001 versus $apoE^{+/-mat}$ and p<0.001 versus $apoE^{-/-KO}$; *b* p<0.001 versus $apoE^{+/-mat}$ and p<0.001 versus $apoE^{-/-KO}$, respectively.



Overall there was no significant difference in total cholesterol levels based on gender (p=0.894), and there was no statistically significant interaction between gender and offspring type (p=0.746). Within individual study groups no significant difference in cholesterol levels existed between female and male offspring (figure 29).

Figure 29. Serum total cholesterol levels in 21-day old offspring according to study group and gender.

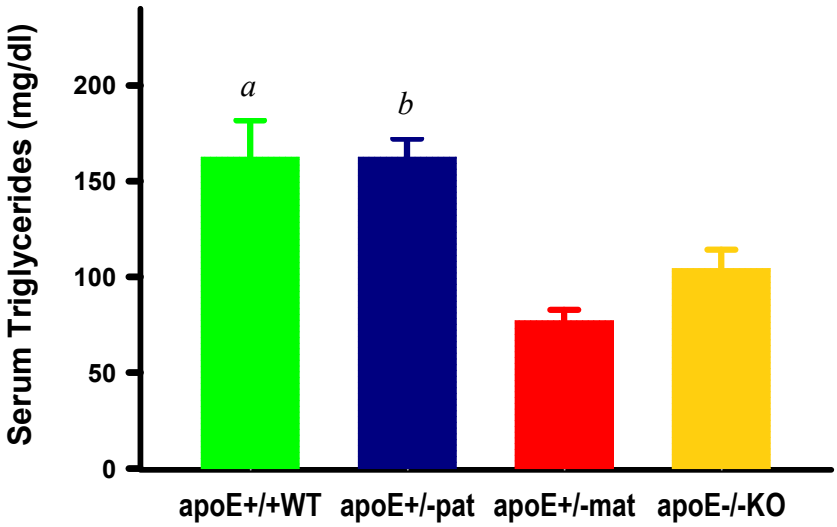


Serum Triglyceride Levels

Alike total serum cholesterol levels, there was a statistically significant difference across the 4 study groups with regards to serum triglyceride levels in 21-day old offspring ($p<0.001$). $apoE^{+/-pat}$ and $apoE^{+/+WT}$ animals displayed the highest serum triglyceride levels (163±12 mg/dl and 161±12 mg/dl, respectively), while $apoE^{-/-KO}$ and $apoE^{+/-mat}$ pups had relatively lower amounts of circulating triglycerides (99±14 mg/dl and 75±14 mg/dl, respectively; figure 30). $apoE^{+/+WT}$ litters displayed a significant elevation of triglyceride levels as compared with the $apoE^{+/-mat}$ ($p<0.001$) and the $apoE^{-/-KO}$ ($p=0.002$) offspring. Similarly, $apoE^{+/-pat}$ animals showed

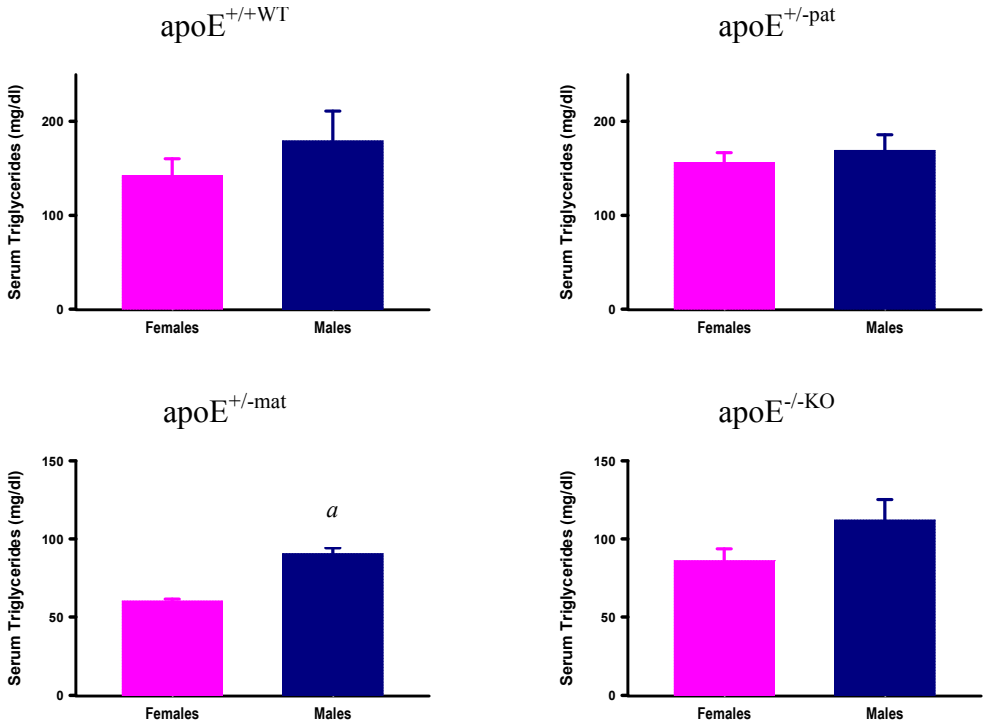
higher levels of triglycerides as compared with apoE$^{+/-mat}$ (p<0.001) and the apoE$^{-/-KO}$ (p=0.001) litters.

Figure 30. Serum triglyceride levels in 21-month old offspring according to study group. *a* p<0.001 versus apoE$^{+/-mat}$ and p=0.002 versus apoE$^{-/-KO}$; *b* p<0.001 versus apoE$^{+/-mat}$ and p=0.001 versus apoE$^{-/-KO}$, respectively.



At day 21 of life male offspring were found to have significantly higher levels of serum triglyceride levels than their female counterparts overall (138±8 mg/dl versus 111±10 mg/dl, p=0.048). When analyzing individual groups separately, only apoE$^{+/-mat}$ animals were found to show a statistically significant variation of triglyceride levels in male versus female animals (91±4 mg/dl versus 60±1 mg/dl, p<0.001, figure 31). There was no significant interaction between gender and type of offspring.

Figure 31. Serum triglyceride levels in 21-day old offspring according to study group and gender. *a* $p<0.001$ versus females.



Specific Hypothesis 2

The hypercholesterolemia and early atherosclerosis seen in heterozygous offspring born to hypercholesterolemic mothers is associated with abnormal cardiovascular function.

SPECIFIC AIM 2

Blood pressure and vascular reactivity will be evaluated to determine the long term effect of exposure to high cholesterol levels during early life on cardiovascular function.

INTRODUCTION

The findings obtained through experiments performed towards specific aim 1, as outlined in the previous section, demonstrate clearly that a high-cholesterol maternal environment during in-utero development and the early postnatal life leads to an increase in serum cholesterol levels in offspring 4 months or older of age. Atherosclerosis and high circulating cholesterol levels are associated with alterations in vascular response patterns, e.g., a decrease in available nitric oxide levels within the endothelium (Davignon and Ganz, 2004; John et al., 2005; John et al., 1998; Mason, 2008). The impairment of the vascular endothelium is an effect that can be apparent prior to the onset of overt lesions or clinical symptoms, and is associated with the presence of risk factors for cardiovascular disease in humans (Celermajer et al., 1994; Davignon and Ganz, 2004). The presence of a significant incidence of secondary organ damage in the liver and kidney in 8-month old animals suggests prior longstanding exposure to chronic hypercholesterolemia. The lower severity of the hypercholesterolemia found in $apoE^{+/-mat}$ offspring at four months of age, together with the observation that end organ damage in this group of offspring was virtually absent, suggested that earlier effects on the cardiovascular system may be demonstrable with the study of blood pressure parameters and the vascular response patterns in these animals.

MEASUREMENT OF ACTIVITY AND BLOOD PRESSURE PARAMETERS

Study Design

For the purpose of the measurement of activity and blood pressure parameters, 4-month old offspring from crossbreeds between $apoE^{-/-KO}$ and $apoE^{+/+WT}$ animals were obtained as laid out earlier. The numbers of offspring animals analyzed in each group for this portion of the studies were as follows: $apoE^{+/+WT}$: n=9; $apoE^{+/-pat}$: n=8; $apoE^{+/-mat}$: n=10; $apoE^{-/-KO}$: n=9, for a total of 36 offspring.

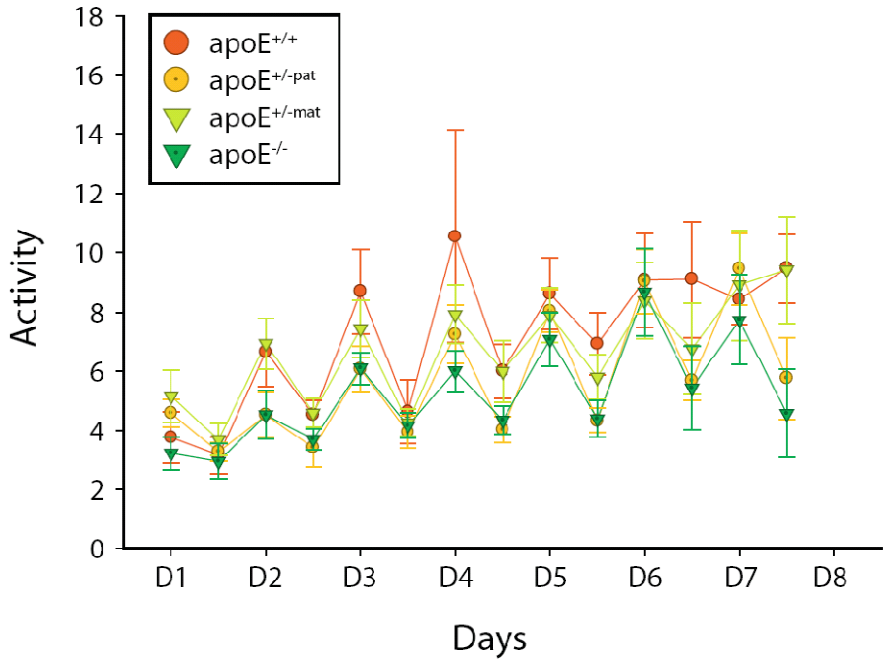
These animals remained with their natural dams for the duration of breastfeeding (21-22 days), and were then maintained on regular chow until they reached 4 months of age. At this

time point life, animals underwent a surgical procedure for the placement of the transmitting catheter into the aortic arch via the left carotid artery, as outlined previously. After complete recovery from the surgery, the offspring were placed in the telemetric blood pressure measurement system in individual cages and observed for another 24 hours. Thereafter, recording of activity of blood pressure measurements were started, which were performed continuously over a 5-9 day period. For the analysis of results the readings from different animals were synchronized to the same time points within the 24-hour cycle.

Activity Indices

Activity indices were recorded in all four groups of offspring and analyzed. As expected, a diurnal variation in activity levels was apparent in all groups of litters. When comparing the measurement of activity among the study groups no significant differences were noted (figure 32).

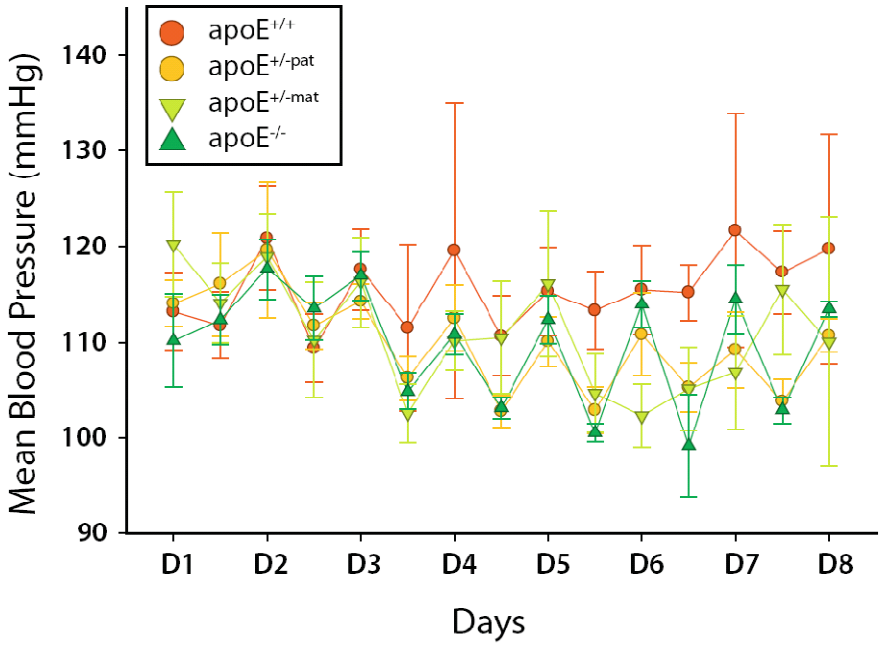
Figure 32. Comparison of activity indices in 4-month old offspring according to study group.



Mean Blood Pressure

No significant variation in mean blood pressure readings was found between the four different study groups. As with activity, there was diurnal variation of mean blood pressure values, with higher levels observed during the awake phase (figure 33).

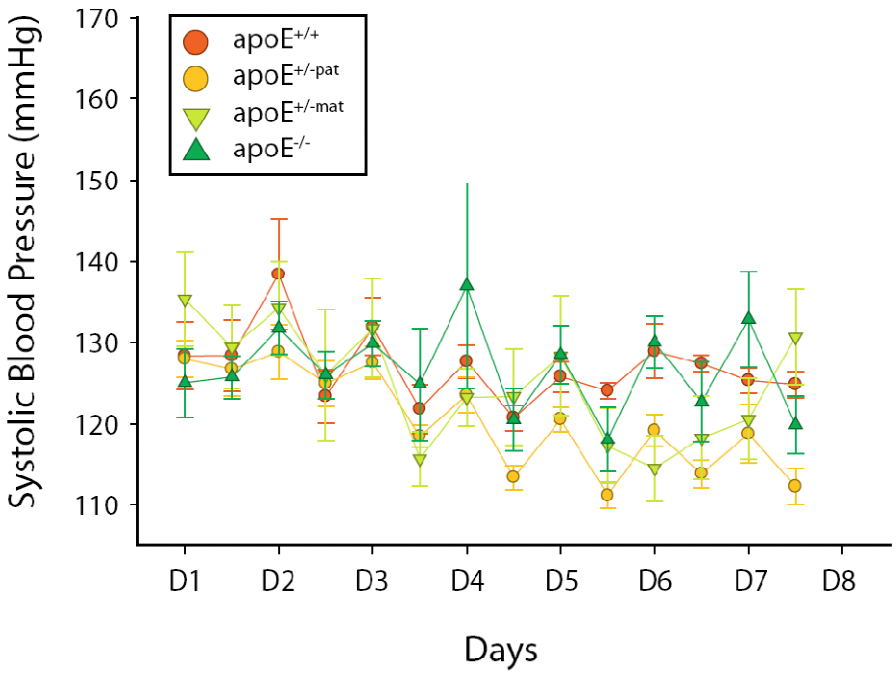
Figure 33. Comparison of mean blood pressure in 4-month old offspring according to study group.



Systolic Blood Pressure

There was no significant difference in reading of the systolic blood pressure over the course of the experiments in the four groups of offspring (figure 34).

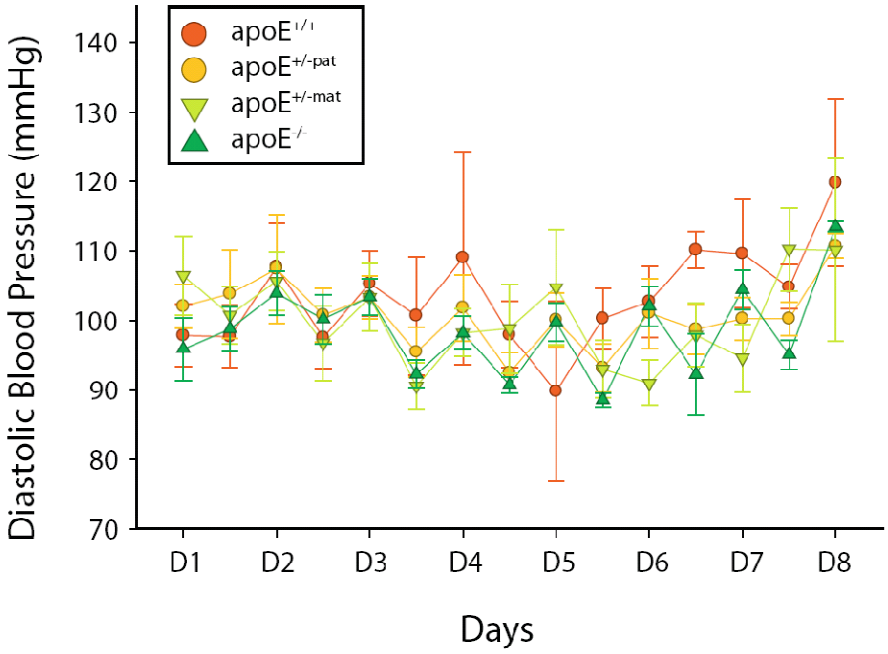
Figure 34. Comparison of systolic blood pressure in 4-month old offspring according to study group.



Diastolic Blood Pressure

No significant variation in diastolic blood pressure readings was observed among the four different groups in this study, although a trend was noted in the apoE$^{+/-pat}$ litters towards lower diastolic blood pressure levels between days 4 to 7 (figure 35).

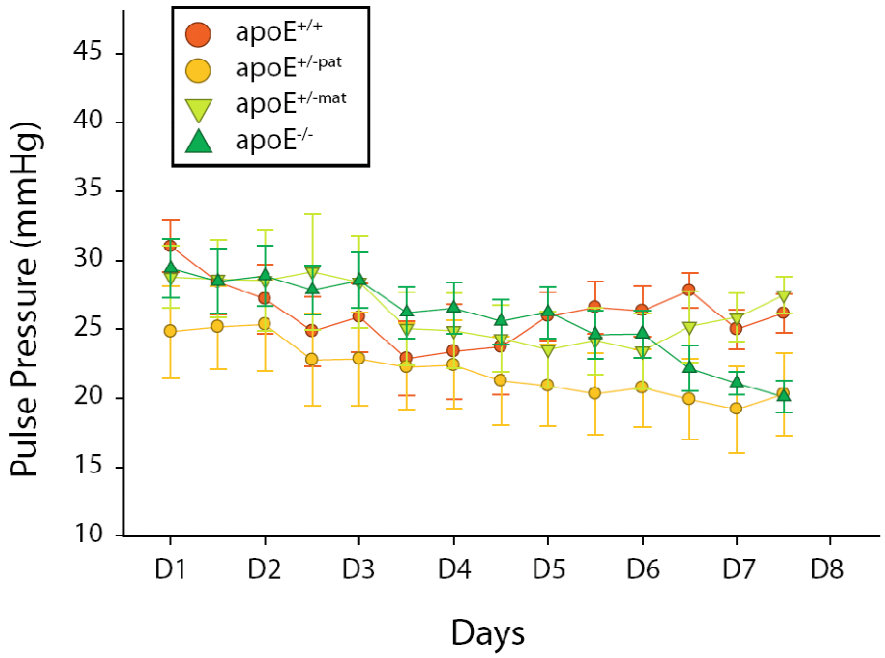
Figure 35. Comparison of diastolic blood pressure in 4-month old offspring according to study group.



Pulse Pressure

Four-month old offspring groups did not demonstrate different levels of pulse pressure (the difference between the systolic and diastolic blood pressure readings) during this study (figure 36). As with activity levels and mean, systolic and diastolic blood pressure, gender did not have a significant effect on the values of the pulse pressure reading during these experiments (data not shown).

Figure 36. Comparison of pulse pressure readings in 4-month old offspring according to study group.



VASCULAR REACTIVITY STUDIES

Study Design

Vascular reactivity studies were performed on carotid arteries from all 4 groups of offspring at 4 months of age. The number of animals in these experiments available for analysis in each group was as follows: apoE$^{+/+WT}$: n=5; apoE$^{+/-pat}$: n=6; apoE$^{+/-mat}$: n=9; apoE$^{-/-KO}$: n=4, a total of 24 animals.

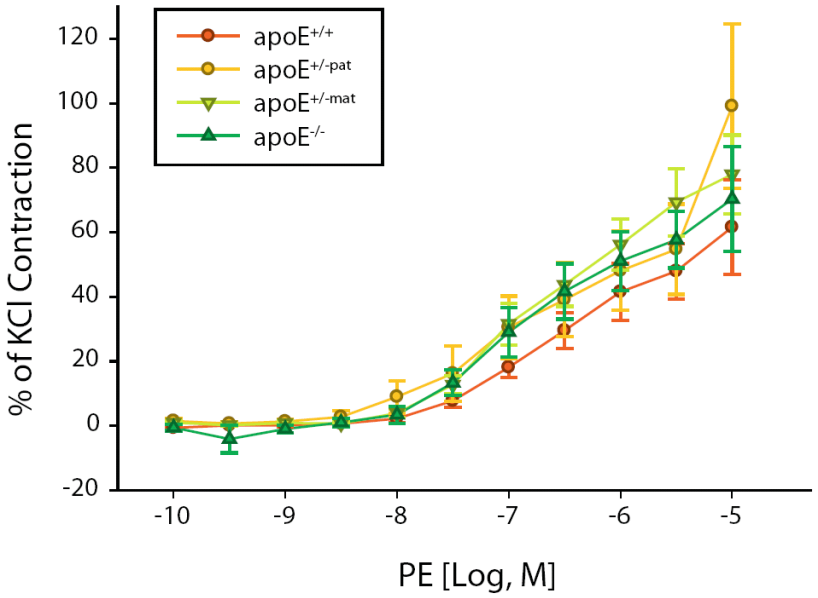
The mice utilized for carotid vascular studies were the same animals that underwent telemetric activity and blood pressure measurements as described in the previous section. After birth, these animals remained with their natural dams during the breastfeeding period (21-22 days), and were then maintained on regular chow for duration of 4 months. At 16 weeks,

catheters were surgically placed trough the left carotid artery as described. After a duration of 5-8 days of in-vivo blood pressure recordings these animals were sacrificed and the right carotid artery was utilized for vascular reactivity studies. In preliminary studies we established that placement of the catheter via the left carotid artery did not affect vascular reactivity patterns in the contralateral carotid vessel.

Phenylephrine Contractile Response

The concentration response curve to the effect of the α_1-adrenergic agonist phenylephrine (PE) was determined in the carotid artery of 4-month old offspring over a dose range of 10^{-10} to 10^{-5}M (figure 37). The contractile reply of the smooth muscle in the carotid artery was similar in the different types of offspring at all concentrations utilized.

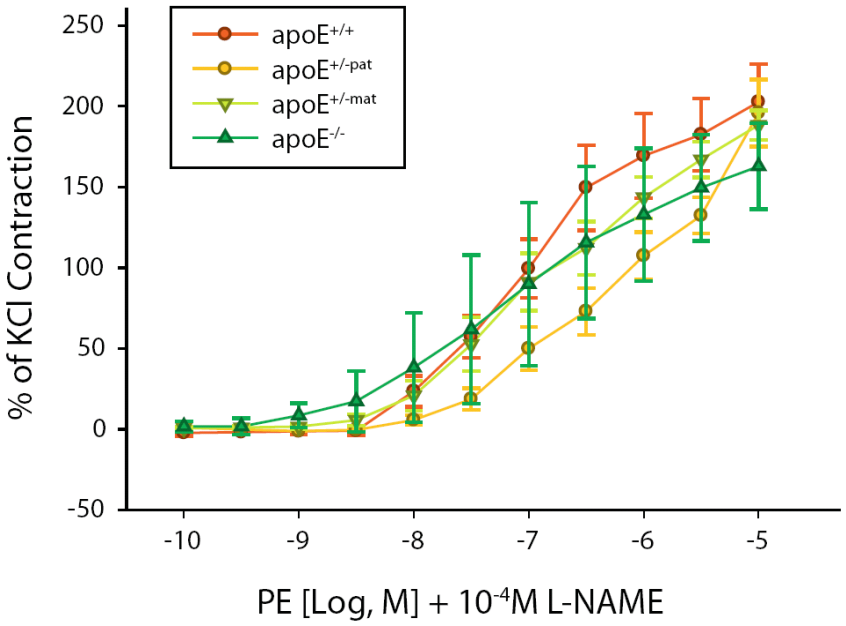
Figure 37. Contractile response to phenylephrine (PE) in the carotid artery by study group.



Contractile Response to Phenylephrine in the Presence of L-NAME

When a 10^{-4} M concentration of L-NAME was added to the buffer solution before applying the increasing doses of phenylephrine, the overall contractile response relative to the KCl contraction was increased as compared to when no L-NAME was present. As with the response curve to phenylephrine alone, the reaction to phenylephrine exposure in the presence of L-NAME did not vary considerably in the different groups of animals at 4-months (figure 38).

Figure 38. Contractile response to phenylephrine (PE) in the carotid artery in the presence of 10^{-4} M L-NAME by study group.

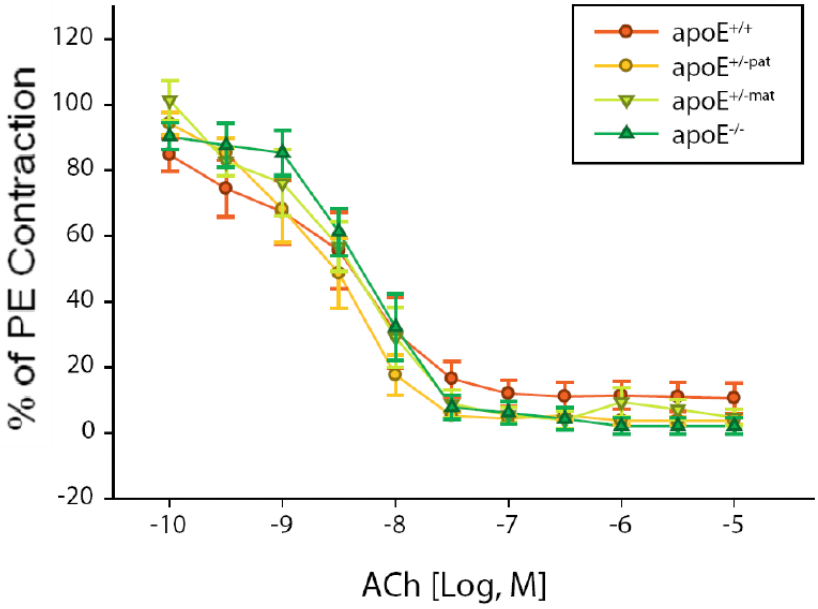


Relaxatory Response to Acetylcholine

As described in the methods, the relaxatory response was also determined in carotid vessels after precontraction with a 10^{-6} M solution of phenylephrine (PE). This was done in the form a dose response curve to the endothelium-dependent agent acetylcholine, as shown below

in figure 40. There was no significant discrepancy in the dilational reaction of the carotid artery to acetylcholine (ACh) among the study groups.

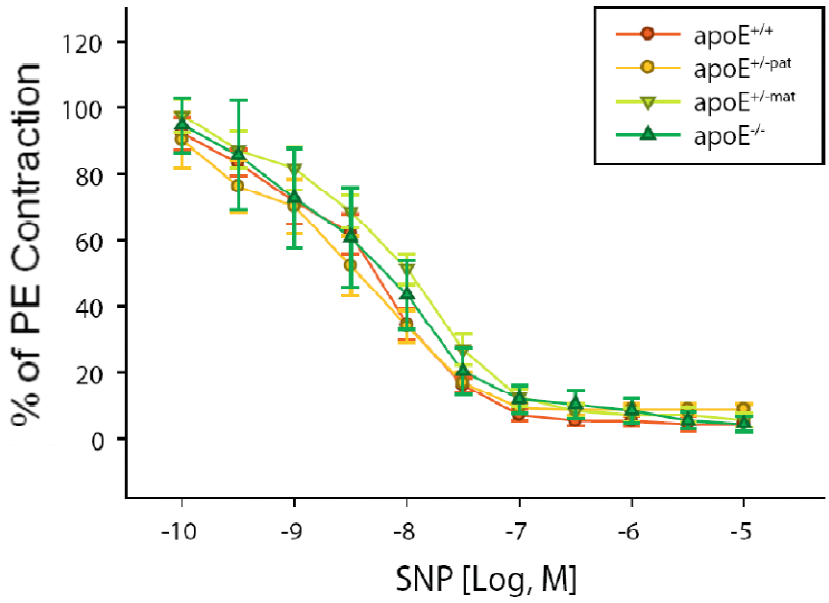
Figure 39. Relaxatory response to acetylcholine (ACh) in the carotid artery by study group.



Relaxatory Response to Sodium Nitroprusside (SNP)

A concentration response curve was obtained in the 4-month old offspring to a dose range of 10^{-10} to 10^{-5} M of sodium nitroprusside (SNP), an endothelium-independent mediator of vasorelaxation (figure 40). No significant difference in the response pattern was noted among the study groups at any of the utilized concentrations.

Figure 40. Relaxatory response to sodium nitroprusside (SNP) in the carotid artery by study group.



Specific Hypothesis 3

The cellular basis of fetal programming of atherosclerosis in the apoE heterozygous mouse model lies in developmental reprogramming of hepatic cholesterol metabolism.

SPECIFIC AIM 3

The expression levels of genes involved in hepatic cholesterol homeostasis (SCAP, SREBP-1a, SREBP-2, HMG-CoA reductase, LDLR, PPAR-α, PPAR-γ, PPAR-δ and LXR-α) will be determined to evaluate the specific changes in the hepatic cholesterol metabolism underlying developmental programming of atherosclerosis.

INTRODUCTION

The findings from specific aim 1 indicate that at eight months of age heterozygous offspring born to apoE deficient mothers had a near threefold increase in total cholesterol levels as compared to heterozygous mice born to wild-type dams (Goharkhay et al., 2007). This elevation of cholesterol levels was accompanied by a significantly increased rate of atherosclerotic plaque formation in heterozygous animals born to hypercholesterolemic mothers at this age. A similar elevation of circulating lipid levels was also noted at an earlier age in mouse offspring, as shown from the results on 4-month old animals detailed above. Since increased cholesterol levels are clearly associated with the development of atherosclerosis in mice, it was hypothesized that the premature occurrence of atherotic lesions in this model was secondary to the developmental programming effect leading to elevated lipid levels.

As mentioned, the liver is a main site for the regulation of cholesterol homeostasis, including endogenous synthesis of cholesterol as well as its uptake. In the 8-month old offspring, we aimed to determine the effect of maternal hypercholesterolemia on the transcriptional activation of SCAP, SREBP-1a, SREBP-2, HMG-CoA reductase (HMGCR) and the LDL receptor (LDLR), and to study the correlations between the expression of these mediators.

EIGHT MONTH-OLD OFFSPRING

Study Design

The liver tissue used for the experiments conducted under specific aim 3 originated from the 8-month old offspring utilized under specific aim 1. The number of animals included in each study group was as follows: $apoE^{+/+WT}$: n=15; $apoE^{+/-pat}$: n=7; $apoE^{+/-mat}$: n=16; $apoE^{-/-KO}$: n=10, for a total of 48 offspring (Goharkhay et al., 2008). The animals remained with their natural dams for the duration of breastfeeding, and were fed regular chow for a duration of 8 months. At

euthanasia, liver samples were collected immediately and flash frozen as described earlier. Real-time RT-PCR was performed for SCAP, SREBP-1a, SREBP-2, HMGCR and LDLR using the Taqman® Gene Expression Assays listed in table 4, and the equipment and reaction conditions laid out in the methods section.

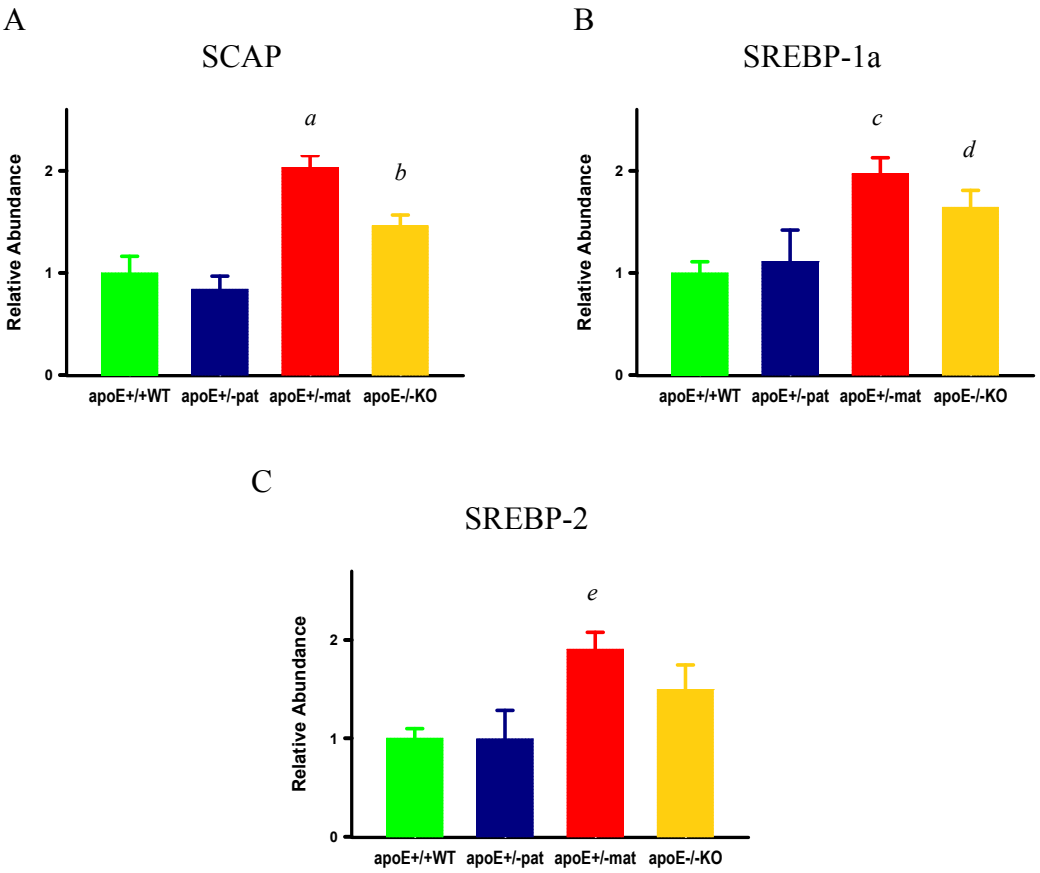
Comparison of Gene Expression Levels

There was a significant variation in SCAP message mRNA levels across the study groups at 8 months of age ($p<0.0001$)(Goharkhay et al., 2008). The gene expression was highest in the $apoE^{+/-mat}$ offspring, followed by the $apoE^{-/-KO}$, the $apoE^{+/+WT}$, and $apoE^{+/-pat}$ groups (figure 41A). In the posthoc analysis, the individual differences between groups were significant when comparing $apoE^{+/+WT}$ with $apoE^{+/-mat}$ offspring ($p<0.0001$), $apoE^{+/+WT}$ with $apoE^{-/-KO}$ ($p=0.352$), $apoE^{+/-pat}$ with $apoE^{+/-mat}$ ($p<0.0001$), as well as $apoE^{-/-KO}$ and $apoE^{+/-mat}$ animals ($p<0.001$).

The pattern of SREBP-1a expression resembled that observed for SCAP: The variation in mRNA levels was overall statistically significant ($p=0.0001$, figure 41B). The highest levels were again noted in $apoE^{+/-mat}$ offspring. The difference in SRBEP-1a-specific message expression was significant between $apoE^{+/-mat}$ as compared with $apoE^{+/+WT}$ and $apoE^{+/-pat}$ groups ($p<0.0001$ and $p=0.0027$, respectively), and between $apoE^{+/-pat}$ and $apoE^{+/-mat}$ animals ($p=0.0027$). $apoE^{-/-KO}$ animals showed intermediate levels of SREBP-1a mRNA, albeit the levels found were not statistically different from the $apoE^{+/+WT}$ and $apoE^{+/-pat}$ groups ($p=0.6912$ and $p=0.0742$, respectively).

The expression of SREBP-2 mRNA was significantly different across the four study groups at eight months of age ($p=0.0016$). $apoE^{+/-mat}$ animals showed the highest SREBP-2 expression, followed by $apoE^{-/-KO}$ offspring (figure 41C). The differences in SREBP-2 message levels were statistically significantly different between $apoE^{+/-mat}$ and $apoE^{+/+WT}$ groups ($p=0.0004$), as well between $apoE^{+/-mat}$ and $apoE^{+/-pat}$ animals ($p=0.0046$).

Figure 41. Relative mRNA expression levels in liver tissue of mediators of cholesterol metabolism in 8-month old offspring: **A** SCAP **B** SREBP-1a and **C** SREBP-2. *a* $p<0.0001$ versus $apoE^{+/+WT}$, $p<0.0001$ versus $apoE^{+/-pat}$ and $p<0.0001$ versus $apoE^{-/-KO}$; *b* $p=0.0352$ versus $apoE^{+/+WT}$ and $p<0.0001$ versus $apoE^{+/-mat}$; *c* $p<0.0001$ versus $apoE^{+/-pat}$ and $p<0.0027$ versus $apoE^{+/-pat}$; *d* $p=0.0086$ versus $apoE^{+/+WT}$, *e* $p=0.0004$ versus $apoE^{+/+WT}$ and $p=0.0046$ versus $apoE^{+/-pat}$ (Goharkhay et al., 2008).



When comparing the gene expression levels for SCAP, SREBP-1a and SREBP-2 in the liver of 8-month old offspring by gender no significant difference was noted except for SREBP-2 in the $apoE^{+/+WT}$ group (table 9). In these mice the level of SREBP-2 transcriptional activation was slightly higher in females as compared with males.

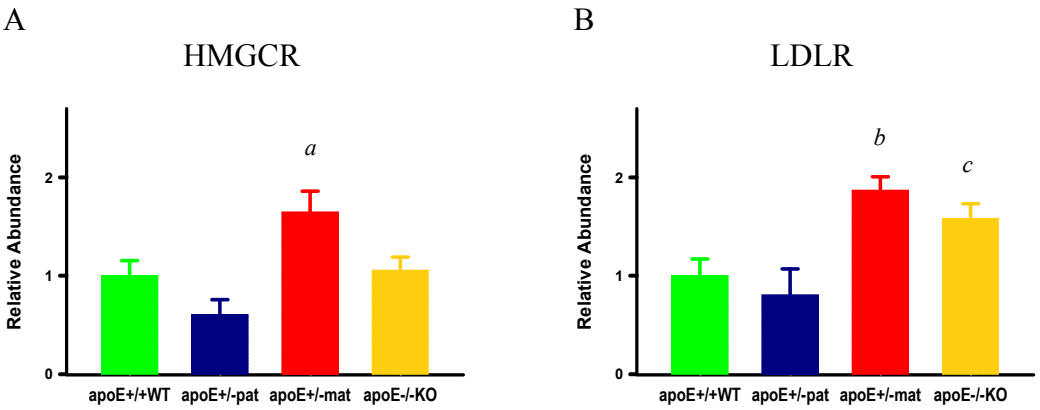
Table 9. Comparison of mRNA expression levels for SCAP, SREBP-1a and SREBP-2 in 8-month old offspring by gender. * designates a *p*-value less than 0.05.

SCAP			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.815±0.201	1.208±0.250	0.2381
$apoE^{+/-pat}$	1.275±0.495	0.829±0.098	0.3467
$apoE^{+/-mat}$	2.115±0.110	1.940±0.211	0.4742
$apoE^{-/-KO}$	1.554±0.108	1.311±0.215	0.3467
SREBP-1a			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.187±0.194	0.859±0.082	0.1444
$apoE^{+/-pat}$	1.819±0.835	0.794±0.134	0.1357
$apoE^{+/-mat}$	2.131±0.301	1.903±0.126	0.4971
$apoE^{-/-KO}$	1.795±0.254	1.510±0.182	0.4373
SREBP-2			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.209±0.149	0.791±0.083	0.0305*
$apoE^{+/-pat}$	0.930±0.689	0.794±0.208	0.8378
$apoE^{+/-mat}$	2.051±0.278	1.765±0.209	0.4242
$apoE^{-/-KO}$	1.636±0.394	1.290±0.237	0.5299

HMGCR mRNA expression levels varied significantly by study group at eight months of age (p=0.0109) (figure 42A). The quantity of HMGCR message was the highest in $apoE^{+/-mat}$ litters and this difference was statistically significant as compared with $apoE^{+/+WT}$ (p=0.0082), $apoE^{+/-pat}$ (p=0.0047) and $apoE^{-/-KO}$ offspring (p=0.0295). The expression of the mRNA message of HMGCR in $apoE^{-/-KO}$ animals was at comparable levels as in $apoE^{+/+WT}$ or $apoE^{+/-pat}$ litters.

There was differential expression of LDLR among the four study groups (p=0.002). LDLR mRNA levels were significantly elevated in apoE$^{+/-mat}$ versus apoE$^{+/+WT}$ (p=0.0004) and apoE$^{+/-pat}$ (p=0.0085) offspring (figure 42B). apoE$^{-/-KO}$ animals displayed higher LDLR expression levels than either apoE$^{+/+WT}$ or apoE$^{+/-pat}$ offspring, but the difference only reached statistical significance in comparison to wild-type (apoE$^{+/+WT}$) offspring of mice (p=0.03).

Figure 42. Relative mRNA expression levels in liver tissue of mediators of cholesterol metabolism in 8-month old offspring. **A** HMG-CoA reductase (HMGCR) **B** LDLR. *a* p=0.0082 versus apoE$^{+/+WT}$, p=0.0047 versus apoE $^{+/-pat}$ and p=0.0295 apoE$^{-/-KO}$; *b* p=0.0004 versus apoE$^{+/+WT}$, p=0.0085 versus apoE $^{+/-pat}$; *c* p=0.0300 versus apoE$^{+/+WT}$ (Goharkhay et al., 2008).



The message expression for HMGCR and LDLR was further assessed by gender in the 8-month old litters and no significant discrepancy was observed, as shown in table 10.

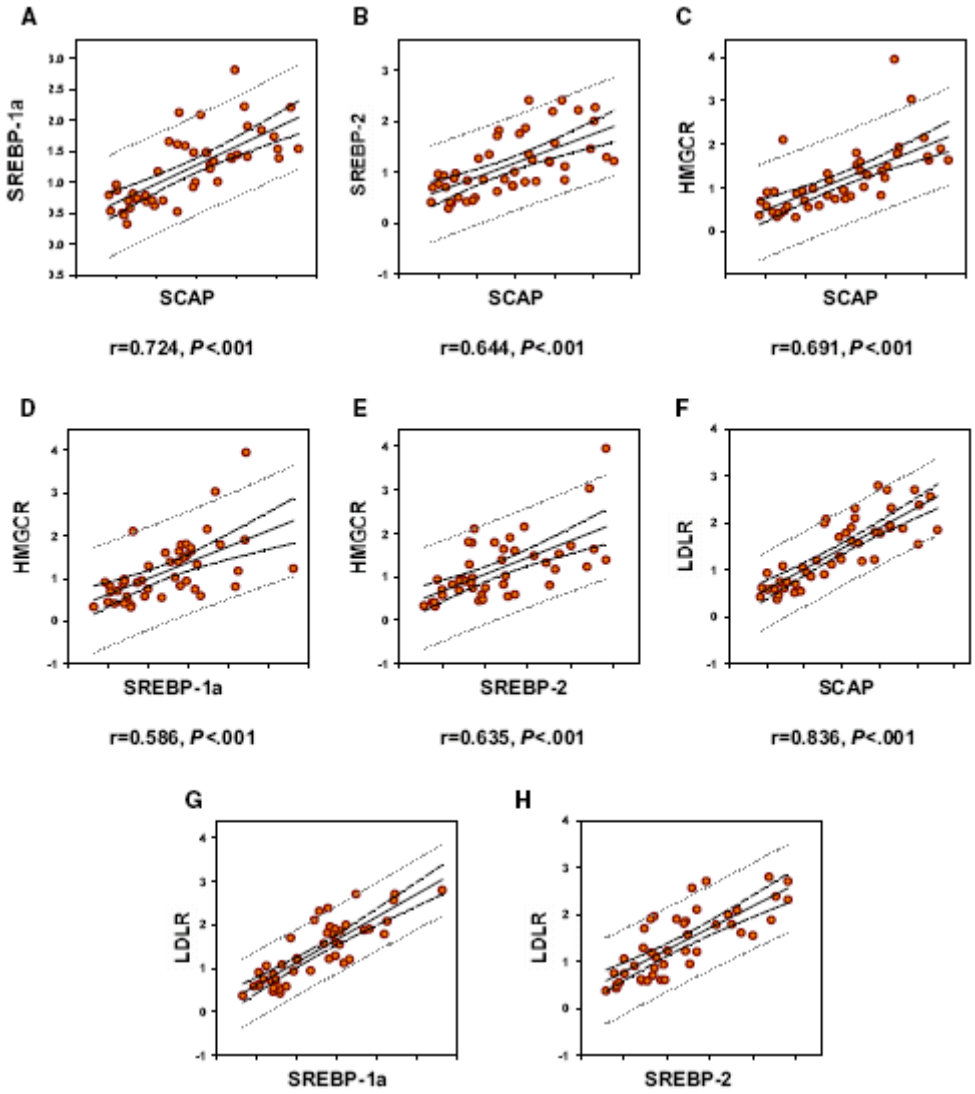
Table 10. Comparison of mRNA expression levels for HMGCR and LDLR in 8-month old offspring by gender.

HMGCR			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.168±0.266	0.808±0.108	0.2550
$apoE^{+/-pat}$	0766±0.403	0.523±0.154	0.5132
$apoE^{+/-mat}$	1.816±0.401	1.486±0.135	0.4482
$apoE^{-/-KO}$	0.932±0.151	1.245±0.242	0.2789
LDLR			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.275±0.280	0.688±0.089	0.0826
$apoE^{+/-pat}$	1.250±0.830	0.583±0.103	0.2776
$apoE^{+/-mat}$	1.885±0.234	1.859±0.157	0.9266
$apoE^{-/-KO}$	1.538±0.241	1.648±0.149	0.7432

Correlations between Gene Expression Levels

For further analysis the correlation of the level of message expression was analyzed between the mediators studied using linear regression analysis. The expression level of all the genes analyzed correlated significantly in this study. The corresponding correlation data are summarized in figure 43.

Figure 43. Correlations of relative levels of hepatic mRNA expression levels: between SCAP and SREBP-1a (**A**), SCAP and SREBP-2 (**B**), SCAP and HMGCR (**C**), SREBP-1a and HMGCR (**D**),SREBP-2 and HMGCR (**E**), SCAP and LDLR (**F**), SREBP-1a and LDLR (**G**), SREBP-2 and LDLR (**H**) in 8-month old offspring (from Goharkhay et al., 2008, with permission).



FOUR MONTH-OLD OFFSPRING

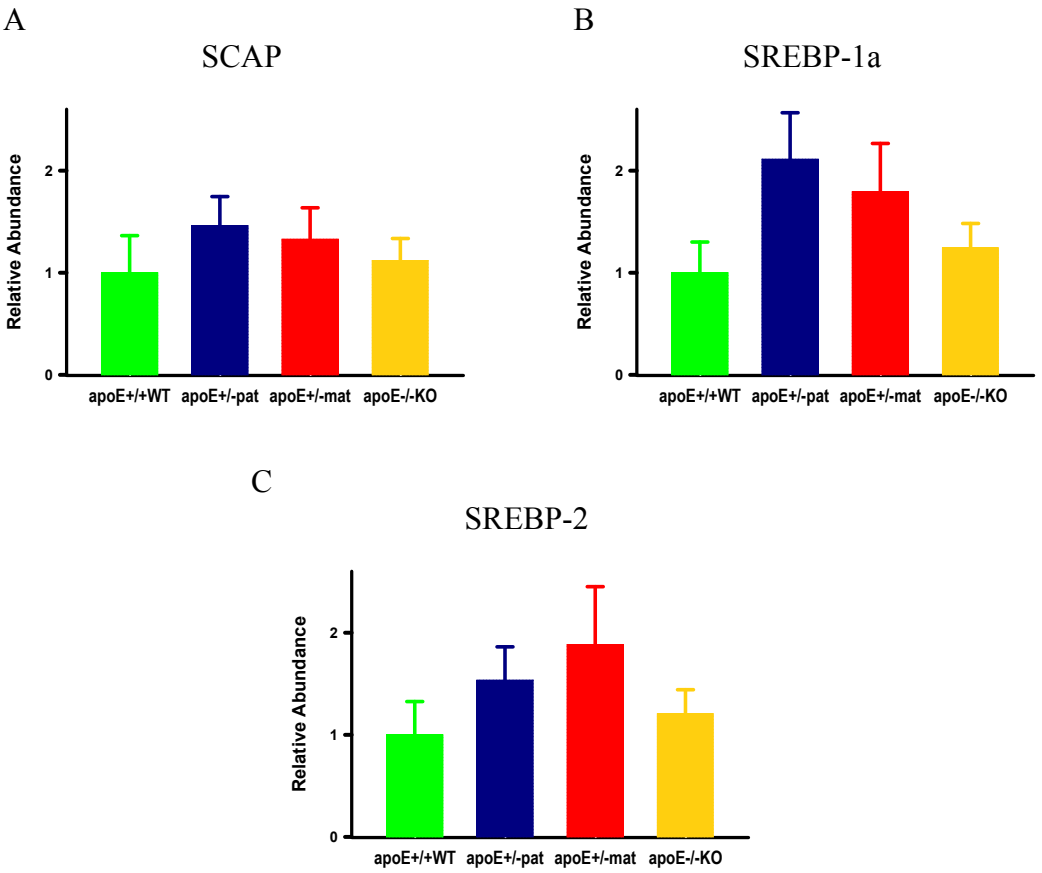
Study Design

The liver tissue used for the experiments conducted under specific aim 3 originated from the 4-month old offspring obtained as part of the serological experiments described under specific aim 1. The number of animals available for analysis of gene expression data in each study group was as follows: $apoE^{+/+WT}$: n=16; $apoE^{+/-pat}$: n=15; $apoE^{+/-mat}$: n=14; $apoE^{-/-KO}$: n=17, for a total of 62 offspring. As with the 8-month old offspring, pups remained with their natural dams for the duration of breastfeeding, and were fed regular chow until 4 months of age. At this time they were sacrificed, liver samples were collected, flash frozen and real-time RT-PCR was performed for SCAP, SREBP-1a, SREBP-2, HMGCR, LDLR, PPAR-α, PPAR-γ, PPAR-δ and LXR-α as described earlier.

Comparison of Gene Expression Levels

The analysis of relative mRNA expression levels for SCAP, SREBP-1a and SREBP-2 in offspring at 4 months of age did not reveal any significant discrepancy among the study groups, although a slight trend for higher levels of SREBP-2 expression was noted in the $apoE^{+/-mat}$ group (figure 44).

Figure 44. Relative mRNA expression levels in liver tissue of mediators of cholesterol metabolism at 4 months of age: **A** SCAP **B** SREBP-1a and **C** SREBP-2.



Gender did not significantly affect the expression levels of SCAP, SREBP-1a and SREBP-2 in the liver in 4-month old animals (table 11).

Table 11. Comparison of mRNA expression levels for SCAP, SREBP-1a and SREBP-2 in 4-month old offspring by gender.

SCAP			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.779±0.284	1.133±0.563	0.6516
$apoE^{+/-pat}$	1.470±0.226	1.449±0.519	0.9734
$apoE^{+/-mat}$	1.053±0.703	1.437±0.349	0.5940
$apoE^{-/-KO}$	0.859±0.242	1.261±0.306	0.3909
SREBP-1a			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.947±0.497	1.032±0.396	0.8962
$apoE^{+/-pat}$	2.919±0.724	1.405±0.476	0.0966
$apoE^{+/-mat}$	1.683±1.108	1.836±0.534	0.8897
$apoE^{-/-KO}$	1.221±0.405	1.254±0.313	0.9507
SREBP-2			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.056±0.548	0.966±0.428	0.8992
$apoE^{+/-pat}$	1.840±0.346	1.268±0.534	0.4004
$apoE^{+/-mat}$	2.005±1.486	1.834±0.594	0.8977
$apoE^{-/-KO}$	1.175±0.335	1.224±0.324	0.9234

Overall, the analysis of gene expression patterns for HMGCR and LDLR did not reveal a significant variation among the study groups, despite the fact that $apoE^{+/-mat}$ offspring displayed a trend towards elevated levels (figure 45).Gender differences were generally not of great magnitude for message levels of HMGCR and LDLR, although in $apoE^{+/-pat}$ mice female animals

had higher levels of HMGCR mRNA than males (table 12).

Figure 45. Relative hepatic mRNA expression levels in 4-month old litters for **A** HMGCR and **B** LDLR.

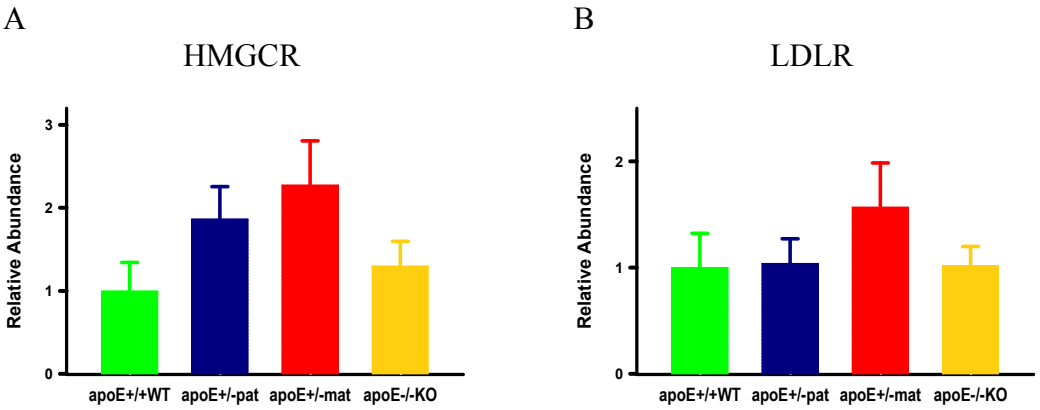


Table 12. Comparison of mRNA expression levels for HMGCR and LDLR in 4-month old offspring by gender. * designates a *p*-value less than 0.05.

HMGCR			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.035±0.567	0.977±0.455	0.9379
$apoE^{+/-pat}$	2.703±0.491	1.131±0.480	0.0400*
$apoE^{+/-mat}$	2.596±1.192	2.167±0.459	0.7453
$apoE^{-/-KO}$	1.291±0.490	1.302±0.391	0.9863
LDLR			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.770±0.368	1.153±0.489	0.5792
$apoE^{+/-pat}$	1.500±0.365	0.694±0.246	0.0814
$apoE^{+/-mat}$	1.496±1.123	1.601±0.404	0.9132
$apoE^{-/-KO}$	0.931±0.245	1.063±0.253	0.7390

mRNA levels of PPAR-α by real-time PCR were slightly elevated in apoE$^{+/-mat}$ mice, albeit not to a statistically significant degree (figure 46). There was no marked variation of PPAR-γ, PPAR-δ or LXR-α message expression across all four study groups. Gender of the offspring did not significantly affect the detected levels of message for PPAR-α, PPAR-γ, PPAR-δ or LXR-α (table 13).

Figure 46. Relative hepatic mRNA expression of nuclear receptor family members in 4-month old litters: **A** PPAR-α **B** PPAR-γ, **C** PPAR-δ and **D** LXR-α.

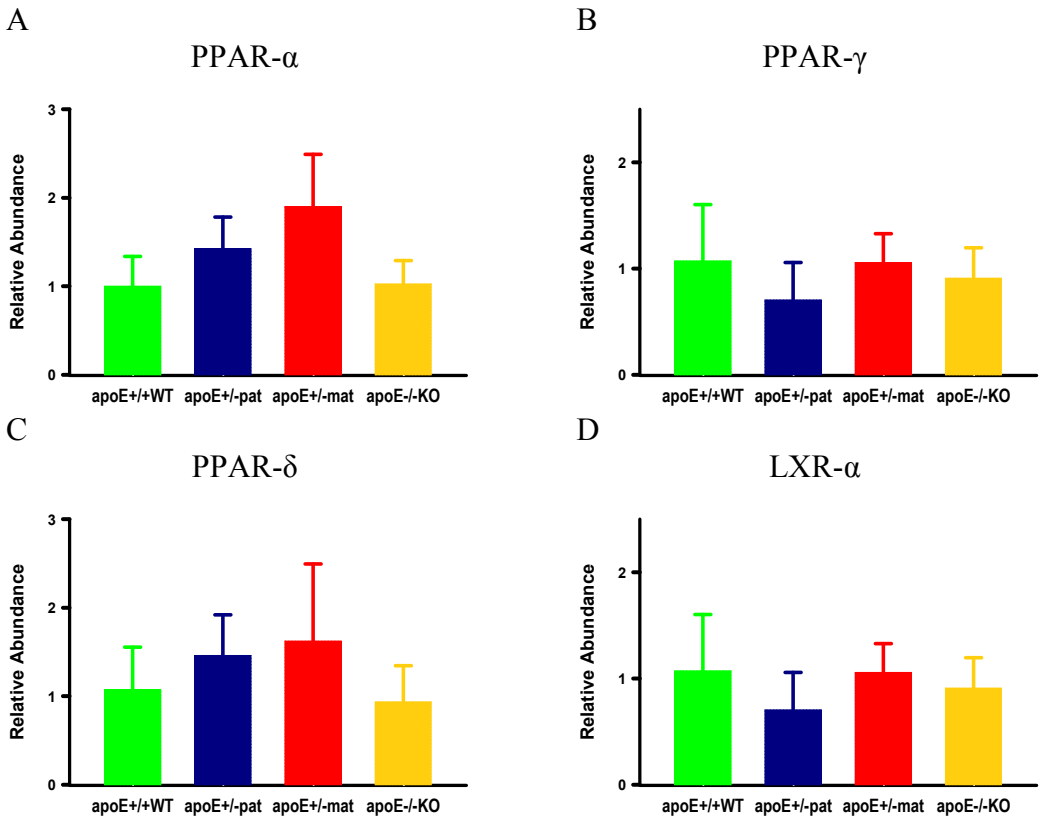


Table 13. Comparison of mRNA expression levels for PPAR-α, PPAR-γ, PPAR-δ and LXR-α in 4-month old offspring by gender.

PPAR-α			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.055±0.585	0.963±0.434	0.8994
$apoE^{+/-pat}$	1.440±0.317	1.407±0.643	0.9654
$apoE^{+/-mat}$	1.619±1.295	2.026±0.683	0.7650
$apoE^{-/-KO}$	0.768±0.234	1.166±0.389	0.4892
PPAR-γ			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.672±0.454	1.371±0.883	0.5374
$apoE^{+/-pat}$	0.374±0.090	0.894±0.553	0.5069
$apoE^{+/-mat}$	0.963±0.710	1.091±0.300	0.8459
$apoE^{-/-KO}$	0.739±0.314	0.996±0.405	0.6861
PPAR-δ			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.340±0.996	0.870±0.455	0.6472
$apoE^{+/-pat}$	1.498±0.651	1.426±0.692	0.9416
$apoE^{+/-mat}$	3.206±2.842	0.990±0.549	0.2668
$apoE^{-/-KO}$	0.861±0.338	0.979±0.642	0.8957
LXR-α			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.092±0.682	0.939±1.371	0.8481
$apoE^{+/-pat}$	1.294±0.360	1.233±0.570	0.9353
$apoE^{+/-mat}$	1.181±0.905	1.728±0.699	0.6617
$apoE^{-/-KO}$	0.917±0.329	0.992±0.270	0.8681

Correlations between Gene Expression Levels

As done previously for the 8-month old offspring, linear regression analysis was performed to assess the correlation of expression levels of the various mediators in this population of animals. There was a strong linear relationship for all studied mediators as indicated in the data presented in table 14. The correlation between the mediators PPAR-γ and PPAR-δ showed a somewhat lower level of statistical significance as compared with most other comparisons in this group.

Table 14. Linear regression data for the correlation between the various mediators in 4-month old offspring.

Mediators	Slope	R^2	*p*
SCAP vs. SREBP-1a	0.969	0.569	<0.0001
SCAP vs. SREBP-2	1.063	0.712	<0.0001
SCAP vs. HMGCR	1.051	0.658	<0.0001
SCAP vs. LDLR	0.790	0.703	<0.0001
SCAP vs. PPAR-α	1.108	0.742	<0.0001
SCAP vs. PPAR-γ	0.994	0.730	<0.0001
SCAP vs. PPAR-δ	1.085	0.357	<0.0001
SCAP vs. LXR-α	1.012	0.728	<0.0001
SREBP-1a vs. SREBP-2	0.855	0.760	<0.0001
SREBP-1a vs. HMGCR	0.884	0.770	<0.0001
SREBP-1a vs. LDLR	0.628	0.639	<0.0001
SREBP-1a vs. PPAR-α	0.774	0.599	<0.0001
SREBP-1a vs. PPAR-γ	0.553	0.339	<0.0001
SREBP-1a vs. PPAR-δ	0.963	0.464	<0.0001
SREBP-1a vs. LXR-α	0.831	0.705	<0.0001
SREBP-2 vs. HMGCR	0.941	0.835	<0.0001
SREBP-2 vs. LDLR	0.668	0.786	<0.0001
SREBP-2 vs. PPAR-α	0.966	0.890	<0.0001
SREBP-2 vs. PPAR-γ	0.730	0.535	<0.0001
SREBP-2 vs. PPAR-δ	1.121	0.600	<0.0001
SREBP-2 vs. LXR-α	0.905	0.910	<0.0001
HMGCR vs. LDLR	0.654	0.780	<0.0001
HMGCR vs. PPAR-α	0.854	0.738	<0.0001
HMGCR vs. PPAR-γ	0.604	0.450	<0.0001
HMGCR vs. PPAR-δ	1.057	0.556	<0.0001
HMGCR vs. LXR-α	0.806	0.744	<0.0001
LDLR vs. PPAR-α	1.174	0.738	<0.0001
LDLR vs. PPAR-γ	0.997	0.607	<0.0001
LDLR vs. PPAR-δ	1.285	0.449	<0.0001
LDLR vs. LXR-α	1.065	0.716	<0.0001
PPAR-α vs. PPAR-γ	0.790	0.638	<0.0001
PPAR-α vs. PPAR-δ	0.978	0.483	<0.0001
PPAR-α vs. LXR-α	0.876	0.904	<0.0001
PPAR-γ vs. PPAR-δ	0.715	0.198	0.0014
PPAR-γ vs. LXR-α	0.711	0.632	<0.0001
PPAR-δ vs. LXR-α	0.474	0.521	<0.0001

21-DAY OLD OFFSPRING

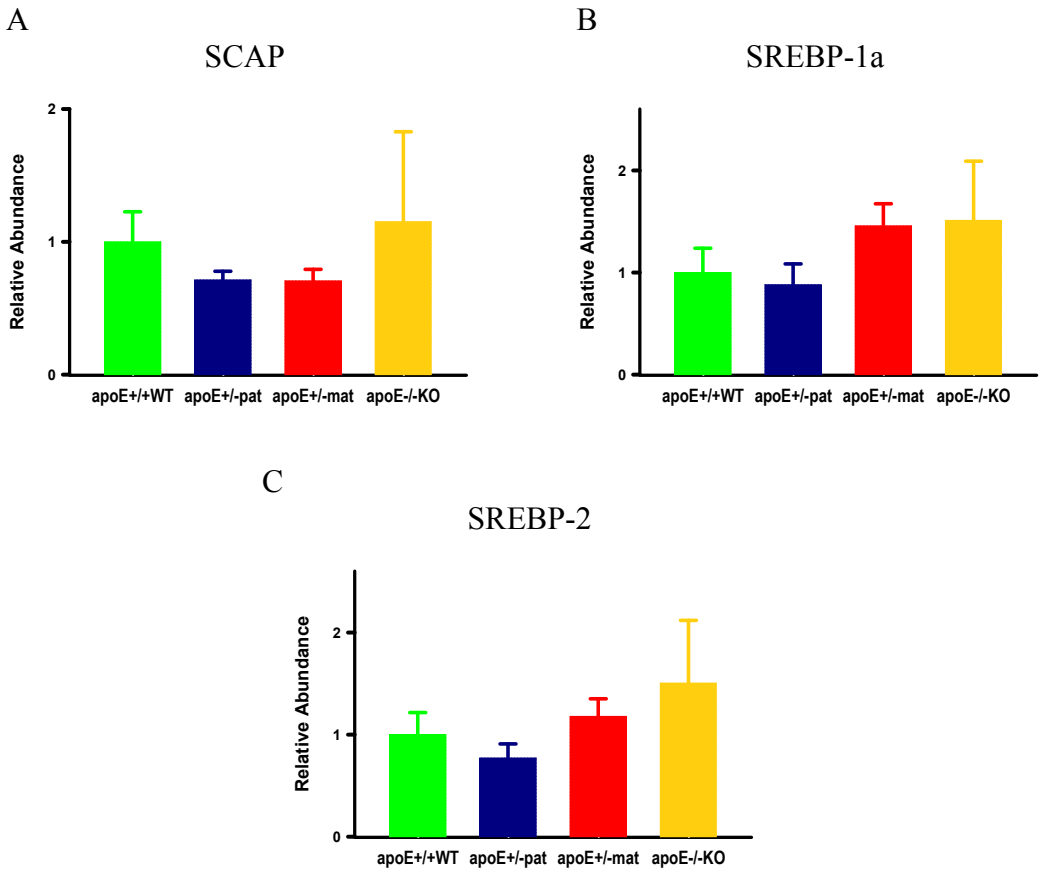
Study Design

The mice used for these experiments were from the same breed of animals utilized for serological studies at 21 days of age detailed earlier. The following numbers of animals were available in each of offspring: $apoE^{+/+WT}$: n=14; $apoE^{+/-pat}$: n=11; $apoE^{+/-mat}$: n=16; $apoE^{-/-KO}$: n=6, for a total of 42 offspring. The pups remained with their natural dams for the duration of breastfeeding. On day 21 (or 22) of life they were removed and euthanized, and liver samples were collected. Real-time RT-PCR was performed for SCAP, SREBP-1a, SREBP-2, HMGCR, LDLR, PPAR-α, PPAR-γ, PPAR-δ and LXR-α as described earlier.

Comparison of Gene Expression Levels

The analysis of relative mRNA expression levels for SCAP, SREBP-1a and SREBP-2 in 21-day old pups showed that there was no significant variations for these mediators across the different groups in the study (figure 47). A high variability was noted in the message level of SCAP, SREBP-1a and SREBP-2 in the $apoE^{-/-KO}$ group.

Figure 47. Relative mRNA expression levels in liver tissue of mediators of cholesterol metabolism in 21-day old offspring. **A** SCAP **B** SREBP-1a and **C** SREBP-2.



Analysis of the gender-specific levels of mRNA expression for SCAP, SREBP-1a and SREBP-2 did not reveal any marked dissimilarities in $apoE^{+/+WT}$, $apoE^{+/-pat}$ and $apoE^{+/-mat}$ animals (table 15). This comparison could not be made for the $apoE^{-/-KO}$ offspring, since only one female offspring was available for analysis in this group.

Table 15. Comparison of mRNA expression levels for SCAP, SREBP-1a and SREBP-2 in 21-day old offspring by gender.

SCAP			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.115±0.405	0.885±0.229	0.6290
$apoE^{+/-pat}$	0.705±0.072	0.717±0.0.114	0.9339
$apoE^{+/-mat}$	0.699±0.166	0.708±0.103	0.9591
$apoE^{-/-KO}$	0.214	1.386±0.823	n/a
SREBP-1a			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.111±0.324	0.889±0.364	0.6552
$apoE^{+/-pat}$	0.548±0.131	1.157±0.323	0.1397
$apoE^{+/-mat}$	1.655±0.400	1.337±0.257	0.4936
$apoE^{-/-KO}$	0.319	1.809±0.638	n/a
SREBP-2			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.189±0.328	0.811±0.281	0.3984
$apoE^{+/-pat}$	0.584±0.162	0.923±0.209	0.2462
$apoE^{+/-mat}$	1.380±0.363	1.055±0.175	0.3781
$apoE^{-/-KO}$	0.223	1.824±0.677	n/a

There was no significant variability in the gene expression patterns for HMGCR and LDLR among the study groups (figure 48). Gender differences were generally not found to be significant in neither of the $apoE^{+/+WT}$, $apoE^{+/-pat}$ and $apoE^{+/-mat}$ groups of animals (table 16).

Figure 48. Relative mRNA expression levels in liver tissue of mediators of cholesterol metabolism in 21-day old offspring. **A** HMGCR and **B** LDLR.

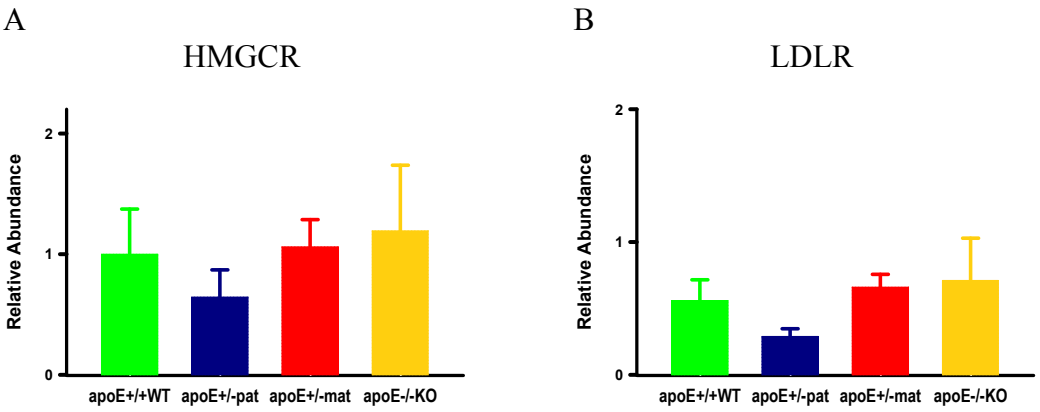


Table 16. Comparison of mRNA expression levels for HMGCR and LDLR in 21-day old offspring by gender.

HMGCR			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	1.465±0.684	0.535±0.245	0.2248
$apoE^{+/-pat}$	0.518±0.365	0.744±0.314	0.6511
$apoE^{+/-mat}$	1.091±0.365	1.042±0.303	0.9203
$apoE^{-/-KO}$	0.311	1.370±0.628	n/a
LDLR			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.565±0.174	0.553±0.278	0.9716
$apoE^{+/-pat}$	0.220±0.065	0.343±0.094	0.3285
$apoE^{+/-mat}$	0.803±0.208	0.575±0.087	0.2593
$apoE^{-/-KO}$	0.171	0.819±0.367	n/a

mRNA levels of PPAR-α by real-time PCR were the only ones found to differ significantly among the 21-day old animals in this study (p=0.0320, figure 49). In the post-hoc evaluation significant differences were noted between $apoE^{+/+WT}$ and $apoE^{+/-mat}$ as well between $apoE^{+/+WT}$ and $apoE^{-/-KO}$ animals (p=0.0075 and p=0.0275, respectively). There was no marked variation of PPAR-γ, PPAR-δ or LXR-α message expression across the four study groups. In the case of PPAR-γ and LXR-α a trend was noticeable towards higher levels of message expression in $apoE^{+/+WT}$ and $apoE^{-/-KO}$ animals. This difference did not reach the level of statistical significance, mainly due to the high variation noted in the $apoE^{-/-KO}$ group. Gender did not significantly affect the detected levels of message for PPAR-α, PPAR-γ, PPAR-δ or LXR-α (table 17).

Figure 49. Relative hepatic mRNA expression of nuclear receptor family members in 21-day old litters. **A** PPAR-α **B** PPAR-γ, **C** PPAR-δ and **D** LXR-α. *a* p=0.0075 versus apoE$^{+/-mat}$ and p=0.0275 versus apoE$^{-/-KO}$, respectively.

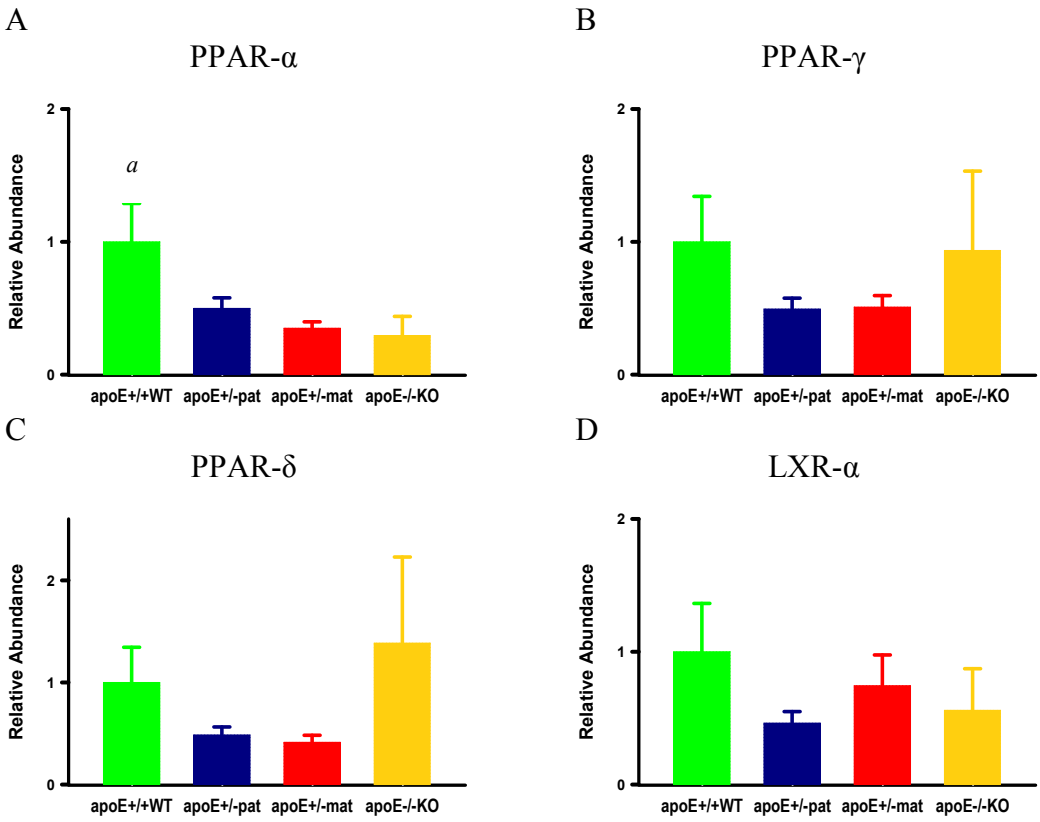


Table 17. Comparison of mRNA expression levels for PPAR-α, PPAR-γ, PPAR-δ and LXR-α in 21-day old offspring by gender.

PPAR-α			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.918±0.245	1.082±0.546	0.7879
$apoE^{+/-pat}$	0.495±0.149	0.502±0.100	0.9678
$apoE^{+/-mat}$	0.407±0.097	0.313±0.050	0.3532
$apoE^{-/-KO}$	0.032	0.346±0.164	n/a
PPAR-γ			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.805±0.227	1.195±0.665	0.5898
$apoE^{+/-pat}$	0.431±0.075	0.544±0.141	0.5240
$apoE^{+/-mat}$	0.568±0.149	0.471±0.109	0.6000
$apoE^{-/-KO}$	0.104	1.144±0.665	n/a
PPAR-δ			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.529±0.168	1.471±0.689	0.2089
$apoE^{+/-pat}$	0.433±0.102	0.486±0.141	0.7735
$apoE^{+/-mat}$	1.017±0.522	0.608±0.240	0.4240
$apoE^{-/-KO}$	0.089	0.676±0.375	n/a
LXR-α			
Group	**Female**	**Male**	***p***
$apoE^{+/+WT}$	0.729±0.144	1.316±740	0.4175
$apoE^{+/-pat}$	0.449±0.095	0.514±0.127	0.7013
$apoE^{+/-mat}$	0.413±0.108	0.412±0.091	0.9951
$apoE^{-/-KO}$	0.071	1.648±0.980	n/a

Correlations between Gene Expression Levels

Linear regression analysis was undertaken to assess the correlation of expression levels of the various mediators of 21-day old animals in this study (table 18). As seen previously in animals 8-months and 4-months of age, a strong linear correlation was noted across all the mediators studies in these analyses. A notable exception was the correlation between HMGCR and LXR-α which was the only one in the group of 21-day old litters that did not reach the level of statistical significance. A further observation was that in these young pups some of the other correlations had a smaller degree of statistical significance as compared to those found in young adult and middle-aged groups. Examples of these include the correlations between SREBP-1a and PPAR-α, HMGCR and PPAR-δ as well as PPAR-γ and PPAR-δ.

Table 18. Linear regression data for the correlation between the various mediators in 21-day old offspring.

Mediators	Slope	R^2	*p*
SCAP vs. SREBP-1a	0.868	0.467	<0.0001
SCAP vs. SREBP-2	0.991	0.669	<0.0001
SCAP vs. HMGCR	0.963	0.397	<0.0001
SCAP vs. LDLR	0.529	0.551	<0.0001
SCAP vs. PPAR-α	0.592	0.377	<0.0001
SCAP vs. PPAR-γ	0.920	0.561	<0.0001
SCAP vs. PPAR-δ	0.652	0.231	0.0006
SCAP vs. LXR-α	0.625	0.348	<0.0001
SREBP-1a vs. SREBP-2	0.816	0.866	<0.0001
SREBP-1a vs. HMGCR	0.983	0.645	<0.0001
SREBP-1a vs. LDLR	0.446	0.631	<0.0001
SREBP-1a vs. PPAR-α	0.246	0.103	0.0278
SREBP-1a vs. PPAR-γ	0.495	0.262	0.0002
SREBP-1a vs. PPAR-δ	0.416	0.512	0.0068
SREBP-1a vs. LXR-α	0.387	0.213	0.0011
SREBP-2 vs. HMGCR	1.158	0.686	<0.0001
SREBP-2 vs. LDLR	0.558	0.763	<0.0001
SREBP-2 vs. PPAR-α	0.432	0.244	0.0004
SREBP-2 vs. PPAR-γ	0.743	0.453	<0.0001
SREBP-2 vs. PPAR-δ	0.612	0.251	0.0003
SREBP-2 vs. LXR-α	0.522	0.300	<0.0001
HMGCR vs. LDLR	0.324	0.506	<0.0001
HMGCR vs. PPAR-α	0.215	0.116	0.0189
HMGCR vs. PPAR-γ	0.384	0.231	0.0007
HMGCR vs. PPAR-δ	0.306	0.119	0.0201
HMGCR vs. LXR-α	0.215	0.051	0.1313
LDLR vs. PPAR-α	0.874	0.403	<0.0001
LDLR vs. PPAR-γ	1.372	0.633	<0.0001
LDLR vs. PPAR-δ	1.089	0.328	<0.0001
LDLR vs. LXR-α	1.116	0.295	<0.0001
PPAR-α vs. PPAR-γ	1.112	0.776	<0.0001
PPAR-α vs. PPAR-δ	1.070	0.581	<0.0001
PPAR-α vs. LXR-α	0.872	0.335	<0.0001
PPAR-γ vs. PPAR-δ	0.906	0.674	0.0014
PPAR-γ vs. LXR-α	0.797	0.852	<0.0001
PPAR-δ vs. LXR-α	0630	0.644	<0.0001

Specific Hypothesis 4

Differences in serum lipid concentrations in the offspring are not related to variations in serum cholesterol levels between genetically similar dams.

SPECIFIC AIM 4

To determine serum cholesterol and triglyceride levels in dams mothering the different groups of offspring.

INTRODUCTION

It is presumed that maternal cholesterol levels are primarily a function of the genotype of the dam, regardless of the type of offspring. This assumption may be questioned based on two main circumstances: Firstly, there may be variation in circulating serum cholesterol levels among genetically similar dams giving rise to different types because of mere accidental variations inherent to biological systems, which are not related to the study design. Secondly, the contribution from the paternal genotype from the offspring may theoretically affect lipid metabolism and concentrations in the dam. For example, it is possible that wild-type mothers carrying $apoE^{+/+WT}$ litters have a different metabolic pattern than similar dams carrying $apoE^{+/-pat}$ pups. Should such differences indeed exist, it is conceivable that some of the variation in lipid levels and atherosclerosis rates observed in the offspring may be due to these variations.

STUDY DESIGN

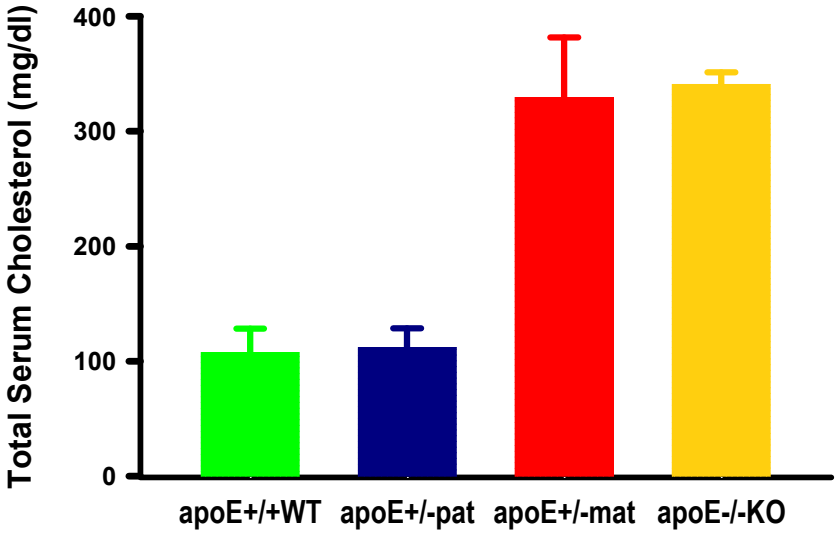
Vascular apoE knockout ($apoE^{-/-}$) and wild-type C57BL/6J ($apoE^{+/+}$) dams were used for the breeding of the following four types of offspring, as described earlier: heterozygous born to hypercholesterolemic $apoE^{-/-KO}$ mothers ($apoE^{+/-mat}$), heterozygous born to wild-type mothers

(apoE$^{+/-pat}$), homozygous knockout (apoE$^{-/-KO}$), and homozygous wild-type (apoE$^{+/+WT}$) offspring. Within 72 hours of delivery of the litters, the dams underwent blood collection from the tail vein. All dams received regular chow as their diet before, during, and after the pregnancy. This study included a total of 10 apoE-deficient dams (7 carrying apoE$^{-/-KO}$ pups, 3 carrying apoE$^{+/-mat}$ pups) and 7 wild-type dams (4 carrying apoE$^{+/+WT}$ pups, 3 carrying apoE$^{+/-pat}$ pups).

SERUM CHOLESTEROL LEVELS

Total cholesterol levels were significantly elevated in apoE$^{-/-}$ dams as compared with their apoE$^{+/+}$ counterparts (334 ±32mg/dl vs. 109±13mg/dl, $p<0.001$). Genetically identical apoE-deficient dams had similar serum cholesterol levels, regardless of the type of offspring they carried (340±10mg/dl in dams carrying apoE$^{+/-mat}$ pups versus 329±52mg/dl in dams carrying apoE$^{-/-KO}$ pups, $p=0.571$, figure 50). In a similar fashion, wild-type dams mothering apoE$^{+/+WT}$ and apoE$^{+/-pat}$ offspring had comparable mean total serum cholesterol concentrations (108±21mg/dl versus 112±17mg/dl, $p=0.888$, respectively).

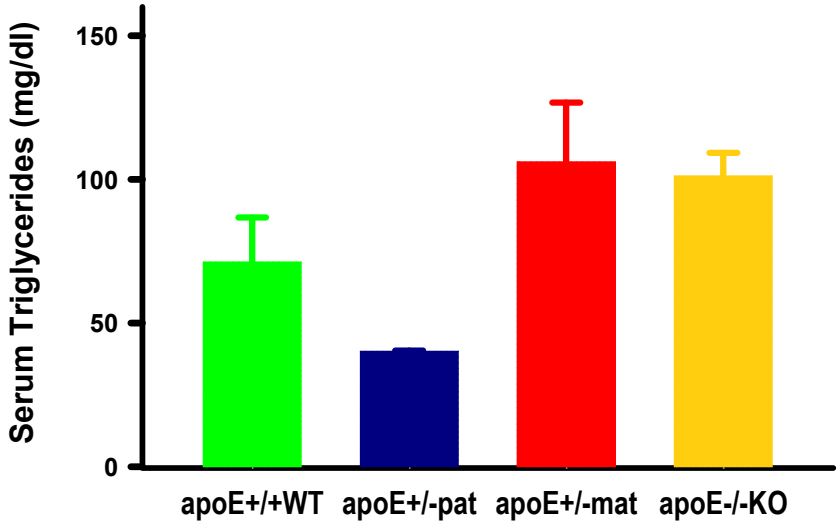
Figure 50. Serum total cholesterol levels in dams according to type of offspring.



SERUM TRIGLYCERIDE LEVELS

Overall, serum levels of triglycerides varied significantly in apoE$^{-/-}$ and apoE$^{+/+}$ dams (104 ±13mg/dl versus 58±10mg/dl, p=0.006). Genetically identical apoE-deficient dams had similar serum triglyceride levels, regardless of the type of offspring they carried (102±8mg/dl in dams carrying apoE$^{+/-mat}$ pups versus 106±17mg/21 in dams carrying apoE$^{-/-KO}$ pups, p=0.551, figure 51). In the same manner, wild-type dams mothering apoE$^{+/+WT}$ and apoE$^{+/-pat}$ displayed similar mean serum triglyceride concentrations (71±15mg/dl versus 40±0.3mg/dl, p=0.400, respectively).

Figure 51. Serum triglyceride levels in dams according to type of offspring.



CHAPTER 4. DISCUSSION

Maternal Hypercholesterolemia as a Risk Factor for the Offspring

The results from the maternally deficient heterozygote animals at 8 months of age indicate a strong correlation between a hyperlipidemic milieu in the mother and the risk of the offspring to develop hypercholesterolemia and premature atherosclerosis in adulthood. The near threefold increase in circulating cholesterol levels in $apoE^{+/-mat}$ mice is an effect of remarkable magnitude. The absence of any significant elevation in cholesterol levels in genetically identical $apoE^{+/-pat}$ animals is in strong support of the notion that the changes in lipid profile of the 8-month old $apoE^{+/-mat}$ mice are not due to genetic predisposition, but rather a response to the exposure to an adverse maternal environment. The observation that -while significantly elevated as compared with wild-type animals- the levels in $apoE^{+/-mat}$ offspring are below those found in apoE-deficient $apoE^{-/-KO}$ animals can be considered as evidence for biological plausibility in favor of a developmental programming effect.

The analysis of the blood specimens in the four groups of offspring at 4 months of age consolidate the validity of the finding of increased blood cholesterol levels in litters born to hypercholesterolemic mothers. Although the magnitude of the effect is smaller at this age, the level of significance remains exceedingly robust. The soundness of the results obtained at 4 months of age is further strengthened by the larger number of animals included at this age point. The use of the in-house assay to obtain serum total cholesterol and triglyceride readings that allowed the use of smaller sample volumes and additional dilutions was an improvement in methodology additionally supporting the strength of the results and the observed variations among study groups. The fact that the 4-month data replicate the relationship between maternal hypercholesterolemia on offspring litter cholesterol levels in a separate population of animals is

an important observation. The same considerations with regards to the biological plausibility and validity of the cholesterol concentration differences among the groups at 8 months also apply to the results in 4-month old offspring: As in the 8-month old groups, the levels in apoE$^{+/-pat}$ mice were not different from those in wild-type animals. The lack of an elevation in circulating cholesterol concentrations in apoE$^{+/-pat}$ mice in view of a significant increase in apoE$^{+/-mat}$ litters underscores the fact that absence of one functional copy of the apoE gene is likely not the reason for the discrepancy in lipid levels.

Although cholesterol concentrations in apoE$^{+/-mat}$ litters are lower at 4-months of age as compared to 8-months, the levels found in apoE$^{-/-KO}$ offspring do not vary considerably between these two time points. This is an expected finding, since the basis for the hypercholesterolemia in apoE$^{-/-KO}$ animals is an inherent genetic disorder which manifests itself within a short time after birth and is not dependent upon a slowly developing process (Zhang et al., 1992; Zhang et al., 1994). On the other hand, it is notable that the degree of increase in cholesterol levels in apoE$^{+/-mat}$ litters is lower at 4 months of age as compared with their older counterparts. In contrast to apoE-deficient mice, it can thus be postulated that the hypercholesterolemia in apoE$^{+/-mat}$ litters develops over a longer period of time and is a consequence of a process with an insidious progression as a consequence of a resetting of cholesterol homeostasis in response to an altered environment during prenatal and postnatal development. Such a gradual onset is a characteristic of other chronic disease models of developmental programming and is consistent with the theory of predictive adaptation (Barker, 1995; Gluckman and Hanson, 2004; Gluckman and Hanson, 2006; Novak et al., 2006). If this assumption is correct in the case of developmental programming of atherosclerosis, then the finding of an attenuated increase of cholesterol levels at 4 months of age as compared with older offspring lends further support to the validity of this disease model in mice.

The incidence and severity of atherosclerotic lesions in the root of the aorta in this model is consistent with the levels and the variations in cholesterol levels across the study groups. The

highest rates of plaque lesions were found in mice lacking any apoE-activity ($apoE^{-/-KO}$), followed by the $apoE^{+/-mat}$ offspring. This relationship held true when comparing the overall incidence rate of atherosclerosis, the relative surface area of advanced lesions only, as well as the relative sizes of all lesions combined (advanced plus early lesions), and affords an additional level of internal consistency within this study. Another measure that corroborates the findings is the piece of evidence that high cholesterol concentrations correlated with the presence of atherosclerotic lesions consistently in each group of animals studied.

Another line of evidence supporting the effect of maternal hypercholesterolemia on the development of hypercholesterolemia and atherosclerosis in the offspring comes from the findings from the analysis of liver and kidney histological sections. A large proportion of $apoE^{-/-KO}$ animals at 8 months of age showed lesions in these organs that are likely the result of damages from chronic exposure to high cholesterol levels and/or vascular damage from atherosclerosis. As expected, very few animals in the normocholesterolemic wild-type $apoE^{+/+WT}$ and paternally deficient heterozygous $apoE^{+/-pat}$ groups of litters demonstrated any evidence of histopathologic abnormalities. The few exceptions that showed such changes in these groups are likely related to age-dependent processes. In contrast, a large number of $apoE^{+/-mat}$ offspring at eight months had experienced significant injury to the liver and kidney. There was no marked discrepancy in the rates at which the liver versus the kidney were involved among the different study groups. This information, along with the evidence of elevated serum cholesterol levels in 4-month old $apoE^{+/-mat}$ offspring, is likely the result of longstanding exposure to high circulating lipid levels throughout adult life in these animals. The relatively lower proportion of individual animals in the $apoE^{+/-mat}$ group affected by such lesions -both in the liver and the kidney- is consistent with the differential degree of hypercholesterolemia between $apoE^{+/-mat}$ and $apoE^{-/-KO}$ offspring.

Albeit in few cases some disparity between female and male genders was evident, overall the effects observed in the present studies were not remarkably different between the two sexes.

This in important determination, since in a variety of models of developmental programming gender-specific differences have been described, as outlined earlier. A similar sensitivity to maternal hypercholesterolemia implies that preventive strategies or treatments may be effective for both genders to prevent the formation or progression of atherosclerosis. It is possible that future studies may reveal a more pronounced distinction in programming responses or in outcomes to specific interventions between genders, as has been suggested by some of the findings from the analyses reported here.

Examination of the experiments relating to activity and blood pressure parameters, as well as those performed to determine vascular reactivity patterns in the carotid vessels in 4-month old animals did not reveal significant differences between the four study groups. Several explanations may account for the lack of distinction among the litters. It is plausible that more subtle variations are present at 4 months of age as compared with in older animals, which were not of large enough magnitude to be detectable in the current analyses. Addition of larger numbers of mice, as well as analysis of 8-month old offspring will address these issues. A significant observation is the finding that activity levels do not appear to be influenced by the genotype or the maternal level of cholesterolemia. Since apoE has a variety of different functions within the body, including the nervous system, behavioral changes might have been assumed to be one of the possible explanations for phenotypic differences observed between the study groups.

Based on the maternal cholesterol and triglyceride levels obtained, variations in circulating lipid levels in dams mothering $apoE^{+/-mat}$ and $apoE^{-/-KO}$, or between dams carrying $apoE^{+/-pat}$ and $apoE^{+/+WT}$ litters, respectively, do not account for the differences subsequently noted in the offspring.

Comparison with Previous Studies

How do the present findings relate to those from the few other reports that have been conducted in mice in this area of research? Alkemade and coworkers bred mice in the same manner as was done in the present studies and obtained heterozygous offspring. They then allowed the offspring to breastfeed for a total duration of four weeks, followed by a Western-type diet containing 1% cholesterol over 16 weeks (Alkemade et al., 2007). Although the carotid vessels in animals so treated did not show any significant lesion formation, a thickening of the intimal layer was noted, which is consistent with very early stages of vessel injury. Placement of a compression cuff around the carotid vessels was associated with severe intimal thickening and moderate increase in medial size in apoE$^{+/-}$ offspring born to apoE-deficient mothers. These observations are in line with the increased incidence of lesion formation in apoE$^{+/-mat}$ litters observed in the current studies. The lack of more advanced lesion formation may be due to the choice of the carotid vessel, as well as the fact that the mice were relatively young at the time of the examination.

The results from the studies presented here contrast with the observations reported by Madsen and coworkers (Madsen et al., 2003). In their investigation there was no difference noted in atherosclerosis formation between heterozygous animals born to wild-type dams versus those born to apoE-deficient mothers. In contrast to the experiments presented currently, Madsen et al. utilized an atherogenic diet containing cholate. Consequently, a severe form of hypercholesterolemia ensued in all offspring. This circumstance likely accounts for the lack of difference observed in the offspring groups at 6 months of age. In addition, the proinflammatory effect of a cholate containing diet may have contributed to the formation of atherosclerosis in all animals, and thereby potentially blunted the differences that might otherwise have arisen between offspring groups exposed to different maternal environments.

The report by Napoli et al. in 2002 supports a role for maternal hyperlipidemia as a risk factor for premature atherosclerosis in the offspring in the LDLR-deficient mouse strain (Napoli

et al., 2002). At the same time, these authors report no discrepancy in circulating cholesterol levels between offspring of mice born to hypercholesterolemic versus normocholesterolemic mothers. This may be related to differences between the mouse strains and feeding protocols between the studies. On the other hand, the animals in the investigation by Napoli and associates were analyzed at 3 months of age, which may be too early for the variability in serum lipid levels to become manifest.

Another relevant question is the comparison of the findings from the current studies with the data obtained from human sources. Both the FELIC study and the earlier study performed by the group led by Drs. Palinski and Napoli have persistently demonstrated a significant influence of maternal lipid levels on the extent of atherosclerotic lesions in human offspring (Napoli et al., 1999a; Napoli et al., 1997). Cholesterol levels were not found to be increased in those children that were born to hypercholesterolemic mothers. This group has suggested and provided some evidence for increased levels of mediators of oxidative damage and inflammation in the circulation of offspring born to hypercholesterolemic mothers (Liguori et al., 2008; Napoli et al., 1999b; Napoli et al., 1997; Palinski and Napoli, 2002). The lack of more long term longitudinal data in humans is an important impediment to our ability to assess the consequences of chronic or pregnancy-induced hyperlipidemia on the development of atherosclerosis. Since atherosclerosis in humans is most commonly associated with elevated cholesterol levels, the question remains whether children of hypercholesterolemic mothers are more prone to manifest hyperlipidemia in adulthood. Other concerns are whether such offspring will show abnormalities in cholesterol and lipid homeostasis and/or in activation of inflammatory pathways that may be responsible for the increased propensity to develop atherotic lesions. The National Children's Study currently in its initial phase carries great expectations to produce some of the answers to these important questions (Landrigan et al., 2006; Washam, 2009).

Hypercholesterolemia in the Offspring – a Predictive Adaptive Response

A careful look at the temporal course of serum total cholesterol and triglyceride levels at the three time points examined reveals an important finding (figures 51 and 52).

Figure 51. Time course of total serum cholesterol levels by study group.

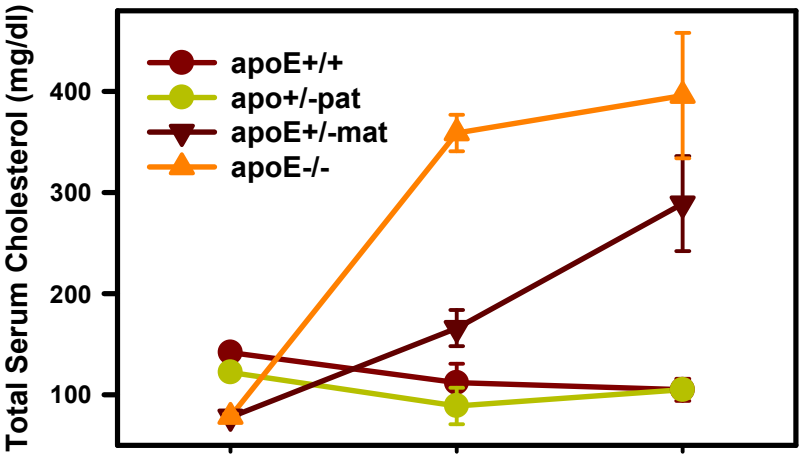
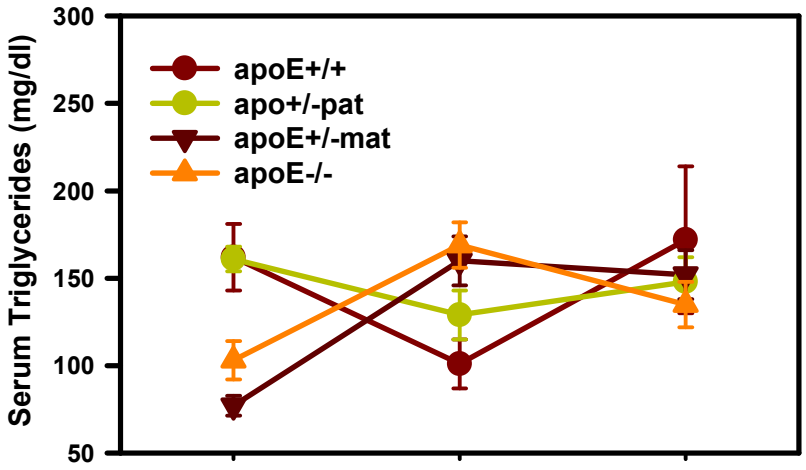


Figure 52. Time course of serum triglyceride levels by study group.



It is evident that the two groups of animals at highest risk to develop hypercholesterolemia and early atherosclerosis, apoE$^{+/-mat}$ and apoE$^{-/-KO}$, have significantly lower levels of serum cholesterol and triglyceride concentrations at day 21 of age. While this may be an unexpected finding, it is consistent with the suggested model of a predictive adaptive response assumed to be the evolutionary principle behind the majority of phenomena related to developmental programming effects (Gluckman and Hanson, 2004; Gluckman and Hanson, 2006). It appears that the continuation of the maternal environment through breastfeeding offers a protective effect in the high-risk groups of animals. This may be interpreted, according to the concept of predictive adaptive responses, in the following manner: The fetus develops an adaptive mechanism to cope with the adverse environment it encounters in utero within the hypercholesterolemic dam. This response mechanism continues to provide an adequate protective effect as long as the external environment remains unchanged – i.e., during the period of lactation, which occurs with the natural dams in these studies. When the external conditions change, the metabolic programming in the offspring has become irreversible and apoE$^{+/-mat}$

animals are unable to respond with a physiologically adequate reaction. The $apoE^{+/-mat}$ litters thus fail to readapt to a low-cholesterol environment after being removed from the hypercholesterolemic milieu, and begin to slowly develop chronic hypercholesterolemia.

It is unclear how the exposure to the mother during the early postpartum period confers a protection against hypercholesterolemia to the offspring. Secretion of apoE into breast milk is a possibility in the $apoE^{+/-pat}$ offspring, but not in $apoE^{-/-KO}$ or $apoE^{+/-mat}$ litters. This question, and whether apoE-deficient mothers produce breast milk with higher contents of lipids or oxidative and inflammatory mediators require further investigation.

Role of Triglycerides

In the 4-month old offspring a significant elevation in triglyceride levels was noted in both $apoE^{+/-mat}$ and $apoE^{-/-KO}$ litters. Along with the putative protective effect on offspring cholesterol levels noted in 21-day old pups, triglyceride levels were also noted to be significantly decreased in both of these groups in a similar fashion. The lack of a statistically significant difference in serum triglyceride concentrations in offspring groups at 8 months may be secondary to the limitations of the technique used for that group of animals. Hypertriglyceridemia is not generally recognized as one of the main characteristics of the apoE mouse model of atherosclerosis, although Zhang et al., in their original description of the model, reported a 68% increase in triglyceride levels in homozygously apoE-deficient animals (Zhang et al., 1992). This is in line with the observation from the present studies that $apoE^{-/-KO}$ dams possessed elevated levels of triglycerides. High triglyceride levels are ever more being recognized as a risk factor for the development of atherosclerotic disease (Fruchart et al., 2004; Hokanson and Austin, 1996). It is possible that the developmental programming effect noted in our studies is partially mediated through a maladaptive response to hypertriglyceridemia.

Possible Mechanisms of Developmental Programming of Atherosclerosis

One of the most important questions to be answered is the exact nature of the cellular and molecular events that underlie the evolution of the hyperlipidemic phenotype generated in $apoE^{+/-mat}$ offspring exposed to a hypercholesterolemic maternal environment. Studies performed under specific aim 3 are an attempt to address this issue. In 8-month old offspring there was consistent evidence for an activation of the endogenous SCAP-SREBP-HMGCR pathway of cholesterol production in the liver, suggesting this synthetic chain as a potential target for the programming effects occurring in response to the altered milieu during early life. This pattern of activation is not present in young pups on day 21 of life, consistent with the potential protective role of the continued maternal environment during the period of breastfeeding. In 4-month old $apoE^{+/-mat}$ animals the evidence for increased gene activation of these mediators is less convincing. Although there is a trend towards higher levels of mRNA expression for SREBP-2, HMGCR, LDLR and PPAR-α in $apoE^{+/-mat}$ litters, these associations do not reach the level of statistical significance. Further research is needed to reject or verify a role for abnormal stimulation of hepatic de-novo cholesterol synthesis in 4-month old offspring.

Besides the determination of the specific targets of epigenetic reprogramming in the pups, the specific factors in the maternal environment that lead to such effects need to be determined. As mentioned earlier, there is accumulating data indicating that transfer of lipids, including active secretion, between the maternal and fetal sides of the placenta exist (Madsen et al., 2004; Napoli et al., 1999b; Palinski and Napoli, 2002; Yoshida and Wada, 2005). Since in humans fetal and maternal blood cholesterol levels correlate up to the sixth month of gestation, a period during which most organogenesis in humans is completed, exposure to hypercholesterolemia during that period may be essential to the development of atherosclerosis risk in the offspring. Alternatively, or in combination with the above, passage of mediators of oxidative stress, such as oxidized LDL, oxidized fatty acids and inflammatory mediators from

the hypercholesterolemic mother to the fetus may play important roles (Liguori et al., 2008; Napoli et al., 2002; Palinski and Napoli, 2002; Palinski et al., 2001).

The protective function of the continued exposure to the maternal environment demonstrates another important point: The early postpartum period may be an essential component of the mechanisms leading to the occurrence of programming effects in the offspring. The importance of the early postpartum period has been established in other models of developmental programming (Clark et al., 2007).

Implications for Prevention and Therapy

The ultimate goal of experimental modeling is to produce results that are applicable to clinical practice and public health policies. Lessons learned from developmental programming studies are beginning to produce such information, such as the example of recommendations against bottle feeding in early childhood.

From the point of view of this mouse model of developmental programming of atherosclerosis further experimentation is necessary in order to define specific interventions that reduce risk for disease in the offspring. Such strategies can include the use of antioxidants or lipid lowering medications in dams during gestation, administration of special diets or pharmaceutical agents to pups, modification of the postnatal environment and other measures. These findings will guide the further development of procedures that can be appropriate for human use and hopefully lead to an overall reduction of the burden from atherosclerotic disease and its many complications.

Conclusions

A strong influence of a hypercholesterolemic maternal environment is evident on the predisposition of offspring to develop elevated cholesterol levels and premature atherosclerosis in this apoE model of developmental programming of atherosclerosis. This phenomenon is evident in early adulthood and becomes more pronounced later in life, and is not primarily related to differences in genetic predisposition of the offspring. Exposure to a hyperlipidemic maternal milieu during the breastfeeding period confers a protective effect on the litters during that time period. The differential effects observed in the offspring are not related to fluctuations in lipid levels among genetically identical dams carrying varying types of litters.

A better understanding of the basic mechanisms underlying the developmental programming effects in this mouse model will help to establish specific preventive and therapeutic interventions to ameliorate the impact of atherosclerosis as a global health problem.

REFERENCES

Aagaard-Tillery, K.M., K. Grove, J. Bishop, X. Ke, Q. Fu, R. McKnight, R.H. Lane, 2008. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J Mol Endocrinol 41, 91-102.

Abdi, R., V.M. Dong, J.R. Rubel, D. Kittur, F. Marshall, L.C. Racusen, 2003. Correlation between glomerular size and long-term renal function in patients with substantial loss of renal mass. J Urol 170, 42-44.

Alexander, B.T., 2006. Fetal programming of hypertension. Am J Physiol Regul Integr Comp Physiol 290, R1-R10.

Alkemade, F.E., A.C. Gittenberger-de Groot, A.E. Schiel, J.C. VanMunsteren, B. Hogers, L.S. van Vliet, R.E. Poelmann, L.M. Havekes, K. Willems van Dijk, M.C. DeRuiter, 2007. Intrauterine exposure to maternal atherosclerotic risk factors increases the susceptibility to atherosclerosis in adult life. Arterioscler Thromb Vasc Biol 27, 2228-2235.

Allis, C.D., T. Jenuwein, D. Reinberg, 2007. Epigenetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

American College of Obstetricans and Gynecologists Committee on Gynecologic Practice, 2008. ACOG Committee Opinion No. 420, November 2008: hormone therapy and heart disease. Obstet Gynecol 112, 1189-1192.

American Veterinary Medical Association, 2007. American Veterinary Medical Association Guidelines for Euthanasia, American Veterinary Medical Association, Schaumburg, IL.

Arnal, J.F., A.T. Dinh-Xuan, M. Pueyo, B. Darblade, J. Rami, 1999. Endothelium-derived nitric oxide and vascular physiology and pathology. Cell Mol Life Sci 55, 1078-1087.

Barker, D.J., 1995. Intrauterine programming of adult disease. Mol Med Today 1, 418-423.

Barker, D.J., 1998. In utero programming of chronic disease. Clin Sci (Lond) 95, 115-128.

Barker, D.J., 2002. Fetal programming of coronary heart disease. Trends Endocrinol Metab 13, 364-368.

Barker, D.J., C. Osmond, 1986. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1, 1077-1081.

Barker, D.J., C.N. Hales, C.H. Fall, C. Osmond, K. Phipps, P.M. Clark, 1993. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia 36, 62-67.

Bassan, H., L.L. Trejo, N. Kariv, M. Bassan, E. Berger, A. Fattal, I. Gozes, S. Harel, 2000. Experimental intrauterine growth retardation alters renal development. Pediatr Nephrol 15, 192-195.

Bauer, R., B. Walter, K. Bauer, R. Klupsch, S. Patt, U. Zwiener, 2002. Intrauterine growth restriction reduces nephron number and renal excretory function in newborn piglets. Acta Physiol Scand 176, 83-90.

Bea, F., E. Blessing, B. Bennett, M. Levitz, E.P. Wallace, M.E. Rosenfeld, 2002. Simvastatin promotes atherosclerotic plaque stability in apoE-deficient mice independently of lipid lowering. Arterioscler Thromb Vasc Biol 22, 1832-1837.

Bengoechea-Alonso, M.T., J. Ericsson, 2007. SREBP in signal transduction: cholesterol metabolism and beyond. Curr Opin Cell Biol 19, 215-222.

Boney, C.M., A. Verma, R. Tucker, B.R. Vohr, 2005. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 115, e290-296.

Bostom, A.G., H. Silbershatz, I.H. Rosenberg, J. Selhub, R.B. D'Agostino, P.A. Wolf, P.F. Jacques, P.W. Wilson, 1999. Nonfasting plasma total homocysteine levels and all-cause and cardiovascular disease mortality in elderly Framingham men and women. Arch Intern Med 159, 1077-1080.

Broberg, C.S., G.D. Giraud, J.M. Schultz, K.L. Thornburg, A.R. Hohimer, L.E. Davis, 2003. Fetal anemia leads to augmented contractile response to hypoxic stress in adulthood. Am J Physiol Regul Integr Comp Physiol 285, R649-655.

Burnstock, G., 2008. Autonomic Neurotransmission: 60 Years Since Sir Henry Dale. Annu Rev Pharmacol Toxicol 49, 1-30.

Byrne, C.D., D.I. Phillips, 2000. Fetal origins of adult disease: epidemiology and mechanisms. J Clin Pathol 53, 822-828.

Calara, F., M. Silvestre, F. Casanada, N. Yuan, C. Napoli, W. Palinski, 2001. Spontaneous plaque rupture and secondary thrombosis in apolipoprotein E-deficient and LDL receptor-deficient mice. J Pathol 195, 257-263.

Cannon, M., P.B. Jones, R.M. Murray, 2002. Obstetric complications and schizophrenia: historical and meta-analytic review. Am J Psychiatry 159, 1080-1092.

Caro, C.G., J.M. Fitz-Gerald, R.C. Schroter, 1969. Arterial wall shear and distribution of early atheroma in man. Nature 223, 1159-1160.

Celermajer, D.S., K.E. Sorensen, C. Bull, J. Robinson, J.E. Deanfield, 1994. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. J Am Coll Cardiol 24, 1468-1474.

Chiang, A.N., M.L. Yang, J.H. Hung, P. Chou, S.K. Shyn, H.T. Ng, 1995. Alterations of serum lipid levels and their biological relevances during and after pregnancy. Life Sci 56, 2367-2375.

Clark, S.M., M. Makhlouf, G.D. Hankins, G.D. Anderson, G.R. Saade, M. Longo, 2007. The effect of the postnatal environment on altered fetal programming of adult vascular function in mice that lack endothelial nitric oxide synthase. Am J Obstet Gynecol 196, 354 e1-7.

Cleasby, M.E., P.A. Kelly, B.R. Walker, J.R. Seckl, 2003. Programming of rat muscle and fat metabolism by in utero overexposure to glucocorticoids. Endocrinology 144, 999-1007.

Costantine, M.M., L.M. Ghulmiyyah, E. Tamayo, G.D. Hankins, G.R. Saade, M. Longo, 2008. Transgenerational effect of fetal programming on vascular phenotype and reactivity in endothelial nitric oxide synthase knockout mouse model. Am J Obstet Gynecol 199, 250 e1-7.

Craig, W.Y., L.M. Neveux, G.E. Palomaki, M.M. Cleveland, J.E. Haddow, 1998. Lipoprotein(a) as a risk factor for ischemic heart disease: metaanalysis of prospective studies. Clin Chem 44, 2301-2306.

Curhan, G.C., G.M. Chertow, W.C. Willett, D. Spiegelman, G.A. Colditz, J.E. Manson, F.E. Speizer, M.J. Stampfer, 1996. Birth weight and adult hypertension and obesity in women. Circulation 94, 1310-1315.

Davignon, J., P. Ganz, 2004. Role of endothelial dysfunction in atherosclerosis. Circulation 109, III27-32.

Elshourbagy, N.A., W.S. Liao, R.W. Mahley, J.M. Taylor, 1985. Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. Proc Natl Acad Sci U S A 82, 203-207.

Eriksson, J.G., T. Forsen, J. Tuomilehto, P.D. Winter, C. Osmond, D.J. Barker, 1999. Catch-up growth in childhood and death from coronary heart disease: longitudinal study. BMJ 318, 427-431.

Fagerberg, B., L. Bondjers, P. Nilsson, 2004. Low birth weight in combination with catch-up growth predicts the occurrence of the metabolic syndrome in men at late middle age: the Atherosclerosis and Insulin Resistance study. J Intern Med 256, 254-259.

Fazio, S., M.F. Linton, L.L. Swift, 2000. The cell biology and physiologic relevance of ApoE recycling. Trends Cardiovasc Med 10, 23-30.

Finck, B.N., 2007. The PPAR regulatory system in cardiac physiology and disease. Cardiovasc Res 73, 269-277.

Forstermann, U., E.I. Closs, J.S. Pollock, M. Nakane, P. Schwarz, I. Gath, H. Kleinert, 1994. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. Hypertension 23, 1121-1131.

Fowden, A.L., A.J. Forhead, 2004. Endocrine mechanisms of intrauterine programming. Reproduction 127, 515-526.

Frankel, S., P. Elwood, P. Sweetnam, J. Yarnell, G.D. Smith, 1996. Birthweight, adult risk factors and incident coronary heart disease: the Caerphilly Study. Public Health 110, 139-143.

Fruchart, J.C., M.C. Nierman, E.S. Stroes, J.J. Kastelein, P. Duriez, 2004. New risk factors for atherosclerosis and patient risk assessment. Circulation 109, III15-19.

Furchgott, R.F., J.V. Zawadzki, 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373-376.

Gennser, G., P. Rymark, P.E. Isberg, 1988. Low birth weight and risk of high blood pressure in adulthood. BMJ (Clin Res Ed) 296, 1498-1500.

Ghulmiyyah, L.M., E. Tamayo, S.M. Clark, G.D. Hankins, G.D. Anderson, G.R. Saade, M. Longo, 2007. Effect of a previous pregnancy on vascular function in endothelial nitric oxide synthase 3 knockout mice. Am J Obstet Gynecol 197, 279 e1-5.

Gluckman, P.D., M.A. Hanson, 2004. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr Res 56, 311-317.

Gluckman, P.D., M. Hanson, 2005. The fetal matrix : evolution, development, and disease / Peter Gluckman, Mark Hanson. Cambridge University Press, NY.

Gluckman, P.D., M.A. Hanson, 2006. Developmental origins of health and disease. Cambridge University Press, Cambridge ; NY.

Gluckman, P.D., M.A. Hanson, C. Cooper, K.L. Thornburg, 2008. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 359, 61-73.

Goharkhay, N., E.H. Tamayo, H. Yin, G.D. Hankins, G.R. Saade, M. Longo, 2008. Maternal hypercholesterolemia leads to activation of endogenous cholesterol synthesis in the offspring. Am J Obstet Gynecol 199, 273 e1-6.

Goharkhay, N., E. Sbrana, P.K. Gamble, E.H. Tamayo, A. Betancourt, K. Villarreal, G.D. Hankins, G.R. Saade, M. Longo, 2007. Characterization of a murine model of fetal programming of atherosclerosis. Am J Obstet Gynecol 197, 416 e1-5.

Guo, D.C., C.L. Papke, R. He, D.M. Milewicz, 2006. Pathogenesis of thoracic and abdominal aortic aneurysms. Ann N Y Acad Sci 1085, 339-352.

Guyton, A.C., 1991. Textbook of medical physiology, 8th ed. Saunders, Philadelphia, PA.

Hales, C.N., D.J. Barker, P.M. Clark, L.J. Cox, C. Fall, C. Osmond, P.D. Winter, 1991. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 303, 1019-1022.

Hansson, G.K., 2005. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352, 1685-1695.

Harder, T., R. Bergmann, G. Kallischnigg, A. Plagemann, 2005. Duration of breastfeeding and risk of overweight: a meta-analysis. Am J Epidemiol 162, 397-403.

Harlan Laboratories, 2009. 7012 Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet, source: http://www.harlan.com/download.axd/6fa19ca5c460411f97a58899866fd543.pdf?d=7012%20Tech%20Sheet, accessed 02/28/2009.

Hemmings, D.G., S.J. Williams, S.T. Davidge, 2005. 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 289, H674-682.

Hoff, J., 2000. Methods of blood collection in the mouse. Lab Animal 29, 47-53.

Hokanson, J.E., M.A. Austin, 1996. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. J Cardiovasc Risk 3, 213-219.

Holmang, A., 2001. Perinatal origin of adult disease. Scand Cardiovasc J 35, 178-185.

Horton, J.D., J.L. Goldstein, M.S. Brown, 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109, 1125-1131.

Huxley, R.R., A.W. Shiell, C.M. Law, 2000. The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: a systematic review of the literature. J Hypertens 18, 815-831.

Ingelfinger, J.R., 2004. Pathogenesis of perinatal programming. Curr Opin Nephrol Hypertens 13, 459-464.

Insull, W., Jr., 2009. The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. Am J Med 122, S3-S14.

Ismail-Beigi, F., P.M. Catalano, R.W. Hanson, 2006. Metabolic programming: fetal origins of obesity and metabolic syndrome in the adult. Am J Physiol Endocrinol Metab 291, E439-440.

Jackson, C.L., 2006. Ruptures of delight? A new mouse model of plaque rupture. Arterioscler Thromb Vasc Biol 26, 1191-1192.

Jackson, W.F., A. Konig, T. Dambacher, R. Busse, 1993. Prostacyclin-induced vasodilation in rabbit heart is mediated by ATP-sensitive potassium channels. Am J Physiol 264, H238-243.

Jawien, J., P. Nastalek, R. Korbut, 2004. Mouse models of experimental atherosclerosis. J Physiol Pharmacol 55, 503-517.

John, S., M.P. Schneider, C. Delles, J. Jacobi, R.E. Schmieder, 2005. Lipid-independent effects of statins on endothelial function and bioavailability of nitric oxide in hypercholesterolemic patients. Am Heart J 149, 473.

John, S., M. Schlaich, M. Langenfeld, H. Weihprecht, G. Schmitz, G. Weidinger, R.E. Schmieder, 1998. Increased bioavailability of nitric oxide after lipid-lowering therapy in hypercholesterolemic patients: a randomized, placebo-controlled, double-blind study. Circulation 98, 211-216.

Johnson, J., K. Carson, H. Williams, S. Karanam, A. Newby, G. Angelini, S. George, C. Jackson, 2005. Plaque rupture after short periods of fat feeding in the apolipoprotein E-knockout mouse: model characterization and effects of pravastatin treatment. Circulation 111, 1422-1430.

Kadar, A., T. Glasz, 2001. Development of atherosclerosis and plaque biology. Cardiovasc Surg 9, 109-121.

Kannel, W.B., D.L. McGee, 1979. Diabetes and cardiovascular disease. The Framingham study. JAMA 241, 2035-2038.

Kapoor, A., E. Dunn, A. Kostaki, M.H. Andrews, S.G. Matthews, 2006. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. J Physiol 572, 31-44.

Kensara, O.A., S.A. Wootton, D.I. Phillips, M. Patel, A.A. Jackson, M. Elia, 2005. Fetal programming of body composition: relation between birth weight and body composition measured with dual-energy X-ray absorptiometry and anthropometric methods in older Englishmen. Am J Clin Nutr 82, 980-987.

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

Kumar, V., A.K. Abbas, N. Fausto, S.L. Robbins, R.S. Cotran, 2005. Robbins and Cotran pathologic basis of disease, 7th ed. Elsevier Saunders, Philadelphia, PA.

Landrigan, P.J., L. Trasande, L.E. Thorpe, C. Gwynn, P.J. Lioy, M.E. D'Alton, H.S. Lipkind, J. Swanson, P.D. Wadhwa, E.B. Clark, V.A. Rauh, F.P. Perera, E. Susser, 2006. The National Children's Study: a 21-year prospective study of 100,000 American children. Pediatrics 118, 2173-2186.

Langley-Evans, S.C., S.J. Welham, A.A. Jackson, 1999. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. Life Sci 64, 965-974.

Lau, C., J.M. Rogers, 2004. Embryonic and fetal programming of physiological disorders in adulthood. Birth Defects Res C Embryo Today 72, 300-312.

Law, C.M., D.J. Barker, C. Osmond, C.H. Fall, S.J. Simmonds, 1992. Early growth and abdominal fatness in adult life. J Epidemiol Community Health 46, 184-186.

Law, C.M., M. de Swiet, C. Osmond, P.M. Fayers, D.J. Barker, A.M. Cruddas, C.H. Fall, 1993. Initiation of hypertension in utero and its amplification throughout life. BMJ 306, 24-27.

Leon, D.A., I. Koupilova, H.O. Lithell, L. Berglund, R. Mohsen, D. Vagero, U.B. Lithell, P.M. McKeigue, 1996. Failure to realise growth potential in utero and adult obesity in relation to blood pressure in 50 year old Swedish men. BMJ 312, 401-406.

Leon, D.A., H.O. Lithell, D. Vagero, I. Koupilova, R. Mohsen, L. Berglund, U.B. Lithell, P.M. McKeigue, 1998. Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29. BMJ 317, 241-245.

Lewis, R.M., A.J. Forhead, C.J. Petry, S.E. Ozanne, C.N. Hales, 2002. Long-term programming of blood pressure by maternal dietary iron restriction in the rat. Br J Nutr 88, 283-290.

Li, A.C., C.K. Glass, 2004. PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. J Lipid Res 45, 2161-2173.

Li, G., Y. Xiao, J.L. Estrella, C.A. Ducsay, R.D. Gilbert, L. Zhang, 2003. Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. J Soc Gynecol Investig 10, 265-274.

Libby, P., P.M. Ridker, A. Maseri, 2002. Inflammation and atherosclerosis. Circulation 105, 1135-1143.

Liguori, A., F.P. D'Armiento, A. Palagiano, W. Palinski, C. Napoli, 2008. Maternal C-reactive protein and developmental programming of atherosclerosis. Am J Obstet Gynecol 198, 281 e1-5.

Lillycrop, K.A., E.S. Phillips, A.A. Jackson, M.A. Hanson, G.C. Burdge, 2005. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr 135, 1382-1386.

Lithell, H.O., P.M. McKeigue, L. Berglund, R. Mohsen, U.B. Lithell, D.A. Leon, 1996. Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50-60 years. BMJ 312, 406-410.

Lloyd-Jones, D., R. Adams, M. Carnethon, G. De Simone, T.B. Ferguson, K. Flegal, E. Ford, K. Furie, A. Go, K. Greenlund, N. Haase, S. Hailpern, M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lackland, L. Lisabeth, A. Marelli, M. McDermott, J. Meigs, D. Mozaffarian, G. Nichol, C. O'Donnell, V. Roger, W. Rosamond, R. Sacco, P. Sorlie, R. Stafford, J. Steinberger, T. Thom, S. Wasserthiel-Smoller, N. Wong, J. Wylie-Rosett, Y. Hong, 2009.

Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 119, 480-486.

Loke, D.F., O.A. Viegas, L.P. Kek, M. Rauff, A.C. Thai, S.S. Ratnam, 1991. Lipid profiles during and after normal pregnancy. Gynecol Obstet Invest 32, 144-147.

Longo, M., 2005. In utero programming of vascular function in transgenic mice lacking endothelila nitric oxide synthase (dissertation), University of Texas Medical Branch, Galveston, TX.

Longo, M., V. Jain, J. Langenveld, Y.P. Vedernikov, R.E. Garfield, G.D. Hankins, G.D. Anderson, G.R. Saade, 2004. Enhanced growth and improved vascular function in offspring from successive pregnancies in endothelial nitric oxide synthase knockout mice. Am J Obstet Gynecol 191, 1470-1476.

Longo, M., V. Jain, Y.P. Vedernikov, R. Bukowski, R.E. Garfield, G.D. Hankins, G.D. Anderson, G.R. Saade, 2005. Fetal origins of adult vascular dysfunction in mice lacking endothelial nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 288, R1114-1121.

Lu, F., E. Bytautiene, E. Tamayo, P. Gamble, G.D. Anderson, G.D. Hankins, M. Longo, G.R. Saade, 2007. Gender-specific effect of overexpression of sFlt-1 in pregnant mice on fetal programming of blood pressure in the offspring later in life. Am J Obstet Gynecol 197, 418 e1-5.

Madsen, C., F. Dagnaes-Hansen, J. Moller, E. Falk, 2003. Hypercholesterolemia in pregnant mice does not affect atherosclerosis in adult offspring. Atherosclerosis 168, 221-228.

Madsen, E.M., M.L. Lindegaard, C.B. Andersen, P. Damm, L.B. Nielsen, 2004. Human placenta secretes apolipoprotein B-100-containing lipoproteins. J Biol Chem 279, 55271-55276.

Mahley, R.W., S.C. Rall, Jr., 2000. Apolipoprotein E: far more than a lipid transport protein. Annu Rev Genomics Hum Genet 1, 507-537.

Mahley, R.W., Y. Huang, 2007. Atherogenic remnant lipoproteins: role for proteoglycans in trapping, transferring, and internalizing. J Clin Invest 117, 94-98.

Mahley, R.W., K.H. Weisgraber, Y. Huang, 2008. Apolipoprotein E: structure determines function-from atherosclerosis to Alzheimer's disease to AIDS. J Lipid Res (epub ahead of print).

Manalich, R., L. Reyes, M. Herrera, C. Melendi, I. Fundora, 2000. Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. Kidney Int 58, 770-773.

Martin, U., C. Davies, S. Hayavi, A. Hartland, F. Dunne, 1999. Is normal pregnancy atherogenic? Clin Sci (Lond) 96, 421-425.

Mason, R.P., 2008. Scientific rationale for combination of a calcium channel antagonist and an HMG-CoA reductase inhibitor: a new approach to risk factor management. Drugs 68, 885-900.

McCance, K.L., S.E. Huether, 2002. Pathophysiology : the biologic basis for disease in adults & children, 4th ed. Mosby, St. Louis, MO.

McConihay, J.A., P.S. Horn, L.A. Woollett, 2001. Effect of maternal hypercholesterolemia on fetal sterol metabolism in the Golden Syrian hamster. J Lipid Res 42, 1111-1119.

McKeigue, P.M., H.O. Lithell, D.A. Leon, 1998. Glucose tolerance and resistance to insulin-stimulated glucose uptake in men aged 70 years in relation to size at birth. Diabetologia 41, 1133-1138.

McMillen, I.C., J.S. Robinson, 2005. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 85, 571-633.

Meir, K.S., E. Leitersdorf, 2004. Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 24, 1006-1014.

Morris, J.A., 1998. Fetal origin of maturity-onset diabetes mellitus: genetic or environmental cause? Med Hypotheses 51, 285-288.

Myatt, L., 2006. Placental adaptive responses and fetal programming. J Physiol 572, 25-30.

Nakahata, N., 2008. Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. Pharmacol Ther 118, 18-35.

Nakashima, Y., A.S. Plump, E.W. Raines, J.L. Breslow, R. Ross, 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb 14, 133-140.

Napoli, C., J.L. Witztum, F. Calara, F. de Nigris, W. Palinski, 2000. Maternal hypercholesterolemia enhances atherogenesis in normocholesterolemic rabbits, which is

inhibited by antioxidant or lipid-lowering intervention during pregnancy: an experimental model of atherogenic mechanisms in human fetuses. Circ Res 87, 946-952.

Napoli, C., J.L. Witztum, F. de Nigris, G. Palumbo, F.P. D'Armiento, W. Palinski, 1999a. Intracranial arteries of human fetuses are more resistant to hypercholesterolemia-induced fatty streak formation than extracranial arteries. Circulation 99, 2003-2010.

Napoli, C., C.K. Glass, J.L. Witztum, R. Deutsch, F.P. D'Armiento, W. Palinski, 1999b. Influence of maternal hypercholesterolaemia during pregnancy on progression of early atherosclerotic lesions in childhood: Fate of Early Lesions in Children (FELIC) study. Lancet 354, 1234-1241.

Napoli, C., F.P. D'Armiento, F.P. Mancini, A. Postiglione, J.L. Witztum, G. Palumbo, W. Palinski, 1997. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. J Clin Invest 100, 2680-2690.

Napoli, C., F. de Nigris, J.S. Welch, F.B. Calara, R.O. Stuart, C.K. Glass, W. Palinski, 2002. Maternal hypercholesterolemia during pregnancy promotes early atherogenesis in LDL receptor-deficient mice and alters aortic gene expression determined by microarray. Circulation 105, 1360-1367.

Nathanielsz, P.W., 1999. Life in the womb : the origin of health and disease. Promethean Press, Ithaca, NY.

National Center for Health Statistics, 2008. Health, United States, Dept. of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics, Hyattsville, MD.

Nijland, M.J., S.P. Ford, P.W. Nathanielsz, 2008. Prenatal origins of adult disease. Curr Opin Obstet Gynecol 20, 132-138.

Novak, D.A., M. Desai, M.G. Ross, 2006. Gestational programming of offspring obesity/hypertension. J Matern Fetal Neonatal Med 19, 591-599.

Nunnari, J.J., T. Zand, I. Joris, G. Majno, 1989. Quantitation of oil red O staining of the aorta in hypercholesterolemic rats. Exp Mol Pathol 51, 1-8.

Oh, W., N.L. Gelardi, C.J. Cha, 1991. The cross-generation effect of neonatal macrosomia in rat pups of streptozotocin-induced diabetes. Pediatr Res 29, 606-610.

Osmond, C., D.J. Barker, P.D. Winter, C.H. Fall, S.J. Simmonds, 1993. Early growth and death from cardiovascular disease in women. BMJ 307, 1519-1524.

Ozanne, S.E., C.N. Hales, 2004. Lifespan: catch-up growth and obesity in male mice. Nature 427, 411-412.

Ozanne, S.E., C.B. Jensen, K.J. Tingey, H. Storgaard, S. Madsbad, A.A. Vaag, 2005. Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. Diabetologia 48, 547-552.

Ozanne, S.E., C.B. Jensen, K.J. Tingey, M.S. Martin-Gronert, L. Grunnet, C. Brons, H. Storgaard, A.A. Vaag, 2006. Decreased protein levels of key insulin signalling molecules in adipose tissue from young men with a low birthweight: potential link to increased risk of diabetes? Diabetologia 49, 2993-2999.

Palinski, W., C. Napoli, 2002. The fetal origins of atherosclerosis: maternal hypercholesterolemia, and cholesterol-lowering or antioxidant treatment during pregnancy influence in utero programming and postnatal susceptibility to atherogenesis. FASEB J 16, 1348-1360.

Palinski, W., F.P. D'Armiento, J.L. Witztum, F. de Nigris, F. Casanada, M. Condorelli, M. Silvestre, C. Napoli, 2001. Maternal hypercholesterolemia and treatment during pregnancy influence the long-term progression of atherosclerosis in offspring of rabbits. Circ Res 89, 991-996.

Pham, T.D., N.K. MacLennan, C.T. Chiu, G.S. Laksana, J.L. Hsu, R.H. Lane, 2003. Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. Am J Physiol Regul Integr Comp Physiol 285, R962-970.

Phillips, D.I., B.R. Walker, R.M. Reynolds, D.E. Flanagan, P.J. Wood, C. Osmond, D.J. Barker, C.B. Whorwood, 2000. Low birth weight predicts elevated plasma cortisol concentrations in adults from 3 populations. Hypertension 35, 1301-1306.

Piedrahita, J.A., S.H. Zhang, J.R. Hagaman, P.M. Oliver, N. Maeda, 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc Natl Acad Sci U S A 89, 4471-4475.

Plump, A.S., J.D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J.G. Verstuyft, E.M. Rubin, J.L. Breslow, 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 71, 343-353.

Reardon, C.A., G.S. Getz, 2001. Mouse models of atherosclerosis. Curr Opin Lipidol 12, 167-173.

Reddick, R.L., S.H. Zhang, N. Maeda, 1994. Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. Arterioscler Thromb 14, 141-147.

Rich-Edwards, J.W., G.A. Colditz, M.J. Stampfer, W.C. Willett, M.W. Gillman, C.H. Hennekens, F.E. Speizer, J.E. Manson, 1999. Birthweight and the risk for type 2 diabetes mellitus in adult women. Ann Intern Med 130, 278-284.

Rossouw, J.E., G.L. Anderson, R.L. Prentice, A.Z. LaCroix, C. Kooperberg, M.L. Stefanick, R.D. Jackson, S.A. Beresford, B.V. Howard, K.C. Johnson, J.M. Kotchen, J. Ockene, 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 288, 321-333.

Rubanyi, G.M., 1993. The role of endothelium in cardiovascular homeostasis and diseases. J Cardiovasc Pharmacol 22 Suppl 4, S1-14.

Sasaki, T., M. Kuzuya, K. Nakamura, X.W. Cheng, T. Shibata, K. Sato, A. Iguchi, 2006. A simple method of plaque rupture induction in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 26, 1304-1309.

Sayer, A.A., H.E. Syddall, E.M. Dennison, H.J. Gilbody, S.L. Duggleby, C. Cooper, D.J. Barker, D.I. Phillips, 2004. Birth weight, weight at 1 y of age, and body composition in older men: findings from the Hertfordshire Cohort Study. Am J Clin Nutr 80, 199-203.

Schwartz, J., J.L. Morrison, 2005. Impact and mechanisms of fetal physiological programming. Am J Physiol Regul Integr Comp Physiol 288, R11-15.

Schwartz, S.M., Z.S. Galis, M.E. Rosenfeld, E. Falk, 2007. Plaque rupture in humans and mice. Arterioscler Thromb Vasc Biol 27, 705-713.

Shattock, S.G., 1909. A report upon the pathological condition of the aorta of King Menephtah, traditionally regarded as the Pharaoh of the Exodus. Proceedings of the Royal Society of Medicine 2 (Pathol Sect), 122-127.

Slotkin, T.A., S. Pachman, R.J. Kavlock, J. Bartolome, 1985a. Early biochemical detection of adverse effects of a neurobehavioral teratogen: influence of prenatal methylmercury exposure on ornithine decarboxylase in brain and other tissues of fetal and neonatal rat. Teratology 32, 195-202.

Slotkin, T.A., S. Pachman, J. Bartolome, R.J. Kavlock, 1985b. Biochemical and functional alterations in renal and cardiac development resulting from neonatal methylmercury treatment. Toxicology 36, 231-241.

Stary, H.C., 1990. The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. Eur Heart J 11 Suppl E, 3-19.

Stary, H.C., 1992. Composition and classification of human atherosclerotic lesions. Virchows Arch A Pathol Anat Histopathol 421, 277-290.

Stary, H.C., 2003. Atlas of atherosclerosis : progression and regression, 2nd ed. Parthenon Pub. Group, Boca Raton, FL.

Stein, C.E., C.H. Fall, K. Kumaran, C. Osmond, V. Cox, D.J. Barker, 1996. Fetal growth and coronary heart disease in south India. Lancet 348, 1269-1273.

Stein, C.E., K. Kumaran, C.H. Fall, S.O. Shaheen, C. Osmond, D.J. Barker, 1997. Relation of fetal growth to adult lung function in south India. Thorax 52, 895-899.

Sternby, N.H., J.E. Fernandez-Britto, P. Nordet, 1999. Pathobiological determinants of atherosclerosis in youth (PBDAY Study), 1986-96. Bull World Health Organ 77, 250-257.

The Expert Panel, 2002. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation 106, 3143-3421.

The Jackson Laboratory, 2009a. C57BL/6J, source: http://jaxmice.jax.org/strain/000664.html, accessed 02/26/2009.

The Jackson Laboratory, 2009b. B6.129P2-*Apoe*^{tm1Unc}/J, source: http://jaxmice.jax.org/strain/002052.html, accessed 02/26/2009.

Valdez, R., M.A. Athens, G.H. Thompson, B.S. Bradshaw, M.P. Stern, 1994. Birthweight and adult health outcomes in a biethnic population in the USA. Diabetologia 37, 624-631.

van der Meer, I.M., M.P. de Maat, M.L. Bots, M.M. Breteler, J. Meijer, A.J. Kiliaan, A. Hofman, J.C. Witteman, 2002. Inflammatory mediators and cell adhesion molecules as indicators of severity of atherosclerosis: the Rotterdam Study. Arterioscler Thromb Vasc Biol 22, 838-842.

VanderLaan, P.A., C.A. Reardon, G.S. Getz, 2004. Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. Arterioscler Thromb Vasc Biol 24, 12-22.

Vegesna, R.V., J. Diamond, 1986. Elevation of cyclic AMP by prostacyclin is accompanied by relaxation of bovine coronary arteries and contraction of rabbit aortic rings. Eur J Pharmacol 128, 25-31.

von der Thusen, J.H., B.J. van Vlijmen, R.C. Hoeben, M.M. Kockx, L.M. Havekes, T.J. van Berkel, E.A. Biessen, 2002. Induction of atherosclerotic plaque rupture in apolipoprotein E-/- mice after adenovirus-mediated transfer of p53. Circulation 105, 2064-2070.

Wahlbeck, K., T. Forsen, C. Osmond, D.J. Barker, J.G. Eriksson, 2001. Association of schizophrenia with low maternal body mass index, small size at birth, and thinness during childhood. Arch Gen Psychiatry 58, 48-52.

Warkany, J., R.C. Nelson, 1940. Appearance of Skeletal Abnormalities in the Offspring of Rats Reared on a Deficient Diet. Science 92, 383-384.

Washam, C., 2009. Children's health: National Children's Study begins recruitment. Environ Health Perspect 117, A19.

Weber, C., A. Zernecke, P. Libby, 2008. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol 8, 802-815.

Whitman, S.C., 2004. A practical approach to using mice in atherosclerosis research. Clin Biochem Rev 25, 81-93.

World Health Organization, 2008. World health statistics, World Health Organization, Geneva, Switzerland.

Wouters, K., R. Shiri-Sverdlov, P.J. van Gorp, M. van Bilsen, M.H. Hofker, 2005. Understanding hyperlipidemia and atherosclerosis: lessons from genetically modified apoe and ldlr mice. Clin Chem Lab Med 43, 470-479.

Xu, Q., 2006. A handbook of mouse models of cardiovascular disease. John Wiley & Sons, Chichester, England ; Hoboken, NJ.

Yamashita, T., S. Freigang, C. Eberle, J. Pattison, S. Gupta, C. Napoli, W. Palinski, 2006. Maternal immunization programs postnatal immune responses and reduces atherosclerosis in offspring. Circ Res 99, e51-64.

Yoshida, S., Y. Wada, 2005. Transfer of maternal cholesterol to embryo and fetus in pregnant mice. J Lipid Res 46, 2168-2174.

Zadelaar, S., R. Kleemann, L. Verschuren, J. de Vries-Van der Weij, J. van der Hoorn, H.M. Princen, T. Kooistra, 2007. Mouse models for atherosclerosis and pharmaceutical modifiers. Arterioscler Thromb Vasc Biol 27, 1706-1721.

Zambrano, E., P.M. Martinez-Samayoa, C.J. Bautista, M. Deas, L. Guillen, G.L. Rodriguez-Gonzalez, C. Guzman, F. Larrea, P.W. Nathanielsz, 2005. Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. J Physiol 566, 225-236.

Zandi-Nejad, K., V.A. Luyckx, B.M. Brenner, 2006. Adult hypertension and kidney disease: the role of fetal programming. Hypertension 47, 502-508.

Zarins, C.K., D.P. Giddens, B.K. Bharadvaj, V.S. Sottiurai, R.F. Mabon, S. Glagov, 1983. Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. Circ Res 53, 502-514.

Zhang, S.H., R.L. Reddick, J.A. Piedrahita, N. Maeda, 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 258, 468-471.

Zhang, S.H., R.L. Reddick, B. Burkey, N. Maeda, 1994. Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. J Clin Invest 94, 937-945.

VITA

Nima Goharkhay was born in Tehran, Iran, on September 21, 1973, to parents Mehri Samiee and Abdolali Goharkhay. Nima attended primary school in Tehran and lived in Vienna, Austria, thereafter. He attended the University of Vienna School of Medicine and from which he graduated in 1998. During his medical education he received the Dean's Scholar Award on several occasions, and he was given an ERASMUS scholarship of the European Union to attend the School of Medicine at the University of Alicante in Spain for 11 months in 1997. After graduation from medical school Nima worked as a postdoctoral fellow at the University of Southern California. He subsequently entered the residency program in Obstetrics and Gynecology at the University of Miami, Florida. In 2001 Nima became a clinical fellow in Maternal-Fetal Medicine and a entered the Cell Biology Program at the University of Texas Medical Branch as a graduate student. He finished his fellowship in 2008. Nima has received several awards and distinctions during his medical and graduate studies and has given important presentations and invited lectures at national and international meetings.

Education

M.D., May 1998, University of Vienna School of Medicine, Vienna, Austria

Publications

Original Articles:

1. Lehner R, **Goharkhay N**, Jirecek S. Gynecologic malignancies as a cause of acute abdomen. International Journal of Gynecology & Obstetrics 1997;59:263-64.
2. **Goharkhay N**, Stanczyk FZ, Gentzschein E, Wing DA. Plasma prostaglandin E-2 metabolite levels during labor induction with a sustained-release prostaglandin E-2 vaginal insert. Journal of the Society for Gynecologic Investigation 2000;7:338-42
3. Lehner R, Strohmer H, Jirecek S, **Goharkhay N**, Tringler B, Barrada M. Placental insufficiency and maternal death caused by advanced stage of breast cancer in third

trimester. European Journal of Obstetrics Gynecology and Reproductive Biology 2001;99:272-73.

4. **Goharkhay N**, Stanczyk FZ, Zhang L, Wing DA. Plasma progesterone, dehydroepiandrosterone sulfate and estriol levels during labor induction with a sustained-release prostaglandin E2 vaginal insert. J Matern.Fetal Med 2001;10:197-202.

5. Lehner R, Szabo S, **Goharkhay N**, Jirecek S, Tringler B, Sevelda P. Prognostic influence of delays between exploratory and definitive laparotomy in the treatment of malignant ovarian tumors. Arch.Gynecol Obstet 2001;265:36-39.

6. **Goharkhay N**, Wing DA, Pan V, McCausland V, Hanna M, Naidu YM et al. The expression of EP3-6 and inducible nitric oxide synthase messenger RNA are correlated in pregnant and misoprostol-treated but not in nongravid or menopausal myometrium. American Journal of Obstetrics and Gynecology 2002;186:1202-06.

7. Lehner R, Tringler B, Stengg K, **Goharkhay N**. Premature labor in a women with perforating appendicitis at 36 weeks of gestation - A case report. Journal of Reproductive Medicine 2002;47:327-+.

8. Pan VL, **Goharkhay N**, Felix JC, Wing DA. FGL2 prothrombinase messenger RNA expression in gravid and nongravid human myometrium. American Journal of Obstetrics and Gynecology 2003;188:1057-62.

9. Wing DA, **Goharkhay N**, Naidu YM, Kovacs BW, Felix JC. EP3-2 receptor mRNA expression is reduced and EP3-6 receptor mRNA expression is increased in gravid human myometrium. Journal of the Society for Gynecologic Investigation 2003;10:124-29.

10. Lehner R, **Goharkhay N**, Tringler B, Fasching C, Hengstschlager M. Pedigree analysis and descriptive investigation of three classic phenotypes associated with Holt-Oram syndrome. Journal of Reproductive Medicine 2003;48:153-59.

11. **Goharkhay N**, Verma U. Conservative management of cervical ectopic pregnancy. Journal of the Society for Gynecologic Investigation 2004;11:160A.

12. Wing DA, **Goharkhay N**, Felix JC, Rostamkhani M, Naidu YM, Kovacs BW. Expression of the oxytocin and V1a vasopressin receptors in human myometrium in differing physiologic states and following misoprostol administration. Gynecologic and Obstetric Investigation 2006;62:181-85.

13. Verma U, **Goharkhay N**, Beydoun S. Conservative management of preterm premature rupture of membranes between 18 and 23 weeks of gestation - Maternal and neonatal outcome. European Journal of Obstetrics Gynecology and Reproductive Biology 2006;128:119-24.

14. **Goharkhay N**, Sbrana E, Gamble PK, Tamayo EH, Betancourt A, Villarreal K et al. Characterization of a murine model of fetal programming of atherosclerosis. American Journal of Obstetrics and Gynecology 2007;197.

15. **Goharkhay N**, Lu J, Felix JC, Wing DA. Expression of calcitonin gene-related peptide-receptor component protein (CGRP-RCP) in human myometrium in differing physiological states and following misoprostol administration. American Journal of Perinatology 2007;24:497-500.

16. **Goharkhay N**, Verma U, Maggiorotto F. Comparison of CT- or ultrasound-guided drainage with concomitant intravenous antibiotics vs. intravenous antibiotics alone in the management of tubo-ovarian abscesses. Ultrasound in Obstetrics & Gynecology 2007;29:65-69.

17. Verma U, **Goharkhay N**. Conservative management of cervical ectopic pregnancy. Fertility and Sterility, 2008 (Epub ahead of print, March 2008)

18. **Goharkhay N**, Tamayo ET, Yin H, Hankins GDV, Saade GR, Longo M. Maternal Hypercholesterolemia Leads to Activation of Endogenous Cholesterol Synthesis in the Offspring. American Journal of Obstetrics and Gynecology 2008, 199(3):273.e1-6.

19. Byers BD, **Goharkhay N**, Mateus J, Ward KK, Munn MB, Wen TS. Pregnancy outcome after the ultrasound diagnosis of fetal intra-abdominal umbilical vein varix. Ultrasound in Obstetrics and Gynecology Ultrasound Obstet Gynecol. 2008 Dec 29. [Epub ahead of print].

Review Article:

1. Jain S, **Goharkhay N**, Saade G, Hankins GD, Anderson GD. Hepatitis C in pregnancy. American Journal of Perinatology 2007;24:251-56.

Abstracts

Oral Presentations:

1. McCausland V, **Goharkhay N**, Hanna M, Pan V, Felix JC, Wing DA. The expression of EP3-6 and iNOS mRNA are correlated in pregnant and misoprostol-treated, but not in nongravid or menopausal myometrium. Pacific Coast Obstetrical and Gynecological Society, 68th annual meeting, Ashland, Oregon, September 2001. Frank Lynch Memorial Prize for best oral presentation.

2. **Goharkhay N**, Felix JC, Pan V, Lu J, Hanna M, Naidu YM, Stanczyk FZ, Wing DA. Expression patterns for EP3 prostaglandin receptor splice variants in human myometrium. Society for Gynecologic Investigation, 49th annual meeting, Los Angeles, California, March 2002. Journal of the Society for Gynecologic Investigation 2002, 9(1[Suppl]):271A.

3. Lee H, **Goharkhay N**, Wing DA, Felix JC, Kovacs B. Vasopressin receptor in the gravid and nongravid myometrium. Twelfth Annual USC Residents Scientific Forum, Glendale, California, May 1999.

4. Saadat P, Boostanfar R, Poysky J, Buckwalter G, Zhang C, Saadat P, **Goharkhay N**, Guan C, Stanczyk FZ, Roy S. A randomized pilot study comparing the effects of hormone replacement therapy containing medroxyprogesterone acetate to micronized progesterone on mood, libido, and serum endocrine markers in postmenopausal women. Thirteenth Annual USC Residents Scientific Forum, Glendale, California, May 2000.

5. Melnik M, Boostanfar R, Amezcua CA, **Goharkhay N**, Roy S, Stanczyk FZ, Felix JC. The growth effects of Raloxifene, medroxyprogesterone acetate, and progesterone on human endometrial adenocarcinoma Ishikawa cells. Thirteenth Annual USC Residents Scientific Forum, Glendale, California, May 2000.

6. Parks K, Stanczyk FZ, Slater C, Zhang C, Guan C, **Goharkhay N**, Mishell DR. Androgen levels before and after prophylactic oopherectomy. Thirteenth Annual USC Residents Scientific Forum, Glendale, California, May 2000.

7. McCausland V, **Goharkhay N**, Pan VL, Felix JC, Wing DA. Inducible nitric oxide synthase in human myometrium. Fourteenth Annual USC Residents Scientific Forum, Glendale, California, May 2001.

8. **Goharkhay N**, Verma U, Gilles J, Beydoun S. Conservative management of preterm premature rupture of membranes (PPROM) between 18 and 23 weeks of gestation-neonatal outcome. The William A. Little Society – University of Miami Ob/Gyn Alumni, Annual Scientific Meeting, Key Biscayne, Florida, May 2002.

9. **Goharkhay N**. Prostaglandins and other mediators of labor. The William A. Little Society – University of Miami Ob/Gyn Alumni, Annual Scientific Meeting, Key Biscayne, Florida, May 2003.

10. **Goharkhay N**, Verma U. Conservative management of cervical ectopic pregnancy. Society for Gynecologic Investigation, 51st annual meeting, Houston, Texas, March 2004. Journal of the Society for Gynecologic Investigation 2004, 11(2[Suppl]):160A.

11. **Goharkhay N**. Treatment options for tubo-ovarian abscesses. The William A. Little Society – University of Miami Ob/Gyn Alumni, Annual Scientific Meeting, Key Biscayne, Florida, May 2005.

12. **Goharkhay N**, Longo M, Betancourt A, Lu F, Tamayo E, Gamble P, Bytautiene E, Vedernikov YP, Saade GR. Fetal programming of atherosclerosis – effects of the intrauterine milieu. Texas Association of Obstetricians and Gynecologists, 77th Annual Meeting, San Antonio, Texas, March 2006. Winner, Fellows' Presentation Award.

13. **Goharkhay N**, Saade GR, Longo M, Lu F, Bytautiene E, Vedernikov YP, Hankins GV, Anderson GD. Fetal programming of atherosclerosis in ApoE knockout mice. Women's Reproductive Health Research Symposium, Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, Texas, April 2006.

14. **Goharkhay N**, Villarreal K, Rodriguez S, Mareth A, Stisser K, Tamayo E, Gamble P, Makhlouf M, Anderson G, Longo M, Saade G. Changes in cellular cholesterol metabolism are associated with fetal programming of atherosclerosis. Fellows' Plenary

Session, Society for Maternal-Fetal Medicine, 27th Annual Meeting, San Francisco, California, February 2007. American Journal of Obstetrics and Gynecology 2006, 195(6[Suppl 1]):S18.

15. **Goharkhay N**, Lu F, Sbrana E, Gamble P, Betancourt A, Tamayo E, Villarreal K, Hankins G, Longo M, Saade G. A murine model of fetal programming of atherosclerosis: Morphologic and histologic characterization. Society for Maternal-Fetal Medicine, 27th Annual Meeting, San Francisco, California, February 2007. Fellows' Plenary Session. American Journal of Obstetrics and Gynecology 2006, 195(6[Suppl 1]):S23. Winner, Award of Research Excellence.

16. **Goharkhay N**. A murine model of fetal programming of atherosclerosis. Fourteenth Annual South Central Conference on Perinatal Research, Austin, Texas, October 2007.

17. Lu F, Mateus J, **Goharkhay N**, Yin H, Tamayo E, Anderson GD, Longo M, Saade GR. Placental and renal expression of hypoxic genes in a mouse model of preeclampsia induced by over-expression of SFLT-1. Plenary Session, Society for Maternal-Fetal Medicine, 28th Annual Meeting, Dallas, Texas, January 2008. American Journal of Obstetrics and Gynecology 2007, 197(6[Suppl 1]):S3.

18. **Goharkhay N**, Tamayo E, Lu F, Yin H, Hankins G, Saade G, Longo M. Adverse uterine environment leads to failure to downregulate endogenous cholesterol synthesis in a mouse model. Society for Maternal-Fetal Medicine, 28th Annual Meeting, Dallas, Texas, January 2008. American Journal of Obstetrics and Gynecology 2007, 197(6[Suppl 1]):S33.

19. **Goharkhay N**, Prasad M, Rouse D. Perinatal Transmission of Hepatitis C – Disease Prevalence, Risk Factors and Clearance Rate (concept proposal). Meeting of the National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network, Bethesda, Maryland, April 2008.

20. **Goharkhay N**. Developmental programming of atherosclerosis. Women's Reproductive Health Research (WRHR) Symposium, University of Texas Medical Branch, Galveston, Texas, April 2008.

21. **Goharkhay N.** Perinatal transmission of hepatitis C. Center for Hepatitis Research scientific meeting, University of Texas Medical Branch, Galveston, Texas, January 2009.

22. **Goharkhay N**, Prasad M, Rouse D. VERTICaL: Viral Hepatitis C - Examining Risk Factors for Transmission to Infants via Clinical and Laboratory Parameters (protocol proposal). Meeting of the National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network, Bethesda, Maryland, October 2008. Protocol approved (vote: 14:2).

23. Lu F, **Goharkhay N**, Bytautiene E, Tamayo E, Hankins GVD, Longo M, Saade GR. In-vitro vascular reactivity in a mouse model of preeclampsia induced by overexpression of sFlt-1. The Central Association of Obstetricians and Gynecologists, 75th Annual

Meeting, New Orleans, Louisiana, October 2008. Winner, President's Certificate of Merit Award.

24. **Goharkhay N**, Tamayo E, Yin H, Lu F, Hankins GDV, Longo M, Saade GR. Fetal programming of hypercholesterolemia and hypertriglyceridemia is evident in young adulthood. Fellows' Plenary Session, Society for Maternal-Fetal Medicine, 29th Annual Meeting, San Diego, California, January 2009. American Journal of Obstetrics and Gynecology 2008, 199(6[Suppl 1]):S19.

Poster Presentations:

1. **Goharkhay N**, Lehner R, Jirecek K, Loranth K, Egarter C. [Acute abdomen caused by gynecologic malignancies]. Common educational and annual meeting of the Austrian Society of Gynecology and Obstetrics and the Bavarian Society of Obstetrics and Gynecology, Graz, Austria, May 1997.
2. Stengg K, Jaindl M, Fasching C, Jirecek S, **Goharkhay N**, Lehner R. [Premature labor and acute abdomen in a case of acute perforated appendicitis in the third trimester]. Common educational and annual meeting of the Austrian Society of Gynecology and Obstetrics and the Bavarian Society of Obstetrics and Gynecology, Graz, Austria, May 1997.
3. Rabl M, Lehner R, **Goharkhay N**, Loranth K, Stengg K, Jaindl M. Unique case of maternal death from advanced breast cancer. Fifteenth FIGO World Congress of Gynecology and Obstetrics. Copenhagen, Denmark, August 1997. Acta Obstetricis et Gynecologica Scandinavica 1997, 76(Suppl):68.
4. Rabl K, Stengg K, Jirecek S, Lehner R, Jaindl M, **Goharkhay N**, Loranth K. Carcinoma of cervix uteri metastasis to the breast. Fifteenth FIGO World Congress of Gynecology and Obstetrics. Copenhagen, Denmark, August 1997. Acta Obstetricis et Gynecologica Scandinavica 1997, 76(Suppl):103.
5. **Goharkhay N**, Lehner R, Fasching C. [Meta analysis and genealogical analysis of the Holt-Oram syndrome]. Annual meeting of the Austrian Society of Gynecology and Obstetrics, Bad Gastein, Austria, June 1998. Speculum 1998, 16(Suppl 1):31.
6. **Goharkhay N**, Lehner R, Loranth K, Levin D, Jirecek S, Husslein P. [The decision-delivery time of acute cesarean sections]. Annual meeting of the Austrian Society of Gynecology and Obstetrics, Bad Gastein, Austria, June 1998. Speculum 1998, 16(Suppl 1):32.
7. Loranth K, **Goharkhay N**, Levin D, Jirecek S, Rabl M, Lehner R, Husslein P. [Complications after operative vaginal deliveries]. Annual meeting of the Austrian Society of Gynecology and Obstetrics, Bad Gastein, Austria, June 1998. Speculum 1998,16(Suppl 1):33.
8. **Goharkhay N**, Felix JC, Naidu Y, Wing DA. Differential expression of prostaglandin receptor EP3 isoforms in human myometrium. Society for Gynecologic Investigation,

47th annual meeting, Chicago, Illinois, March 2000. Journal of the Society for Gynecologic Investigation 2000, 7(Suppl):231A.

9. **Goharkhay N**, Stanczyk FZ, Gentzschein E, Wing DA. Plasma prostaglandin E2 (PGE2) metabolite levels during labor induction with a sustained release PGE2 vaginal hydrogel Insert. Society for Gynecologic Investigation, 47th annual meeting, Chicago, Illinois, March 2000. Journal of the Society for Gynecologic Investigation 2000, 7(Suppl):273A.

10. **Goharkhay N**, Stanczyk FZ, Zhang L, Wing DA. Plasma progesterone, DHEAS and unconjugated estriol (E3) levels during labor induction with a prostaglandin E2 vaginal insert Society for Gynecologic Investigation, 47th annual meeting, Chicago, Illinois, March 2000. Journal of the Society for Gynecologic Investigation 2000, 7(Suppl):274A.

11. Saadat P, Boostanfar R, Poysky J, Buckwalter G, Zhang C, Saadat P, **Goharkhay N**, Guan C, Stanczyk FZ, Roy S. A randomized pilot study comparing the effects of hormone replacement therapy containing medroxyprogesterone acetate to micronized progesterone on mood, libido, and serum endocrine markers in postmenopausal women. Obstetrical and Gynecological Assembly of Southern California, 56th annual meeting, Los Angeles, California, February 2001.

12. Boostanfar R, Tourgeman D, Amezcua C, **Goharkhay N**, Roy S, Stanczyk FZ, Felix J.The comparative effects of raloxifene and progestins on endometrial adenocarcinoma cells in vitro. Obstetrical and Gynecological Assembly of Southern California, 56th annual meeting, Los Angeles, California, February 2001.

13. **Goharkhay N**, Felix JC, Naidu Y, Hanna M, Wing DA. The Expression of the EP3-2 prostaglandin receptor is selectively reduced in gravid human myometrium. Society for Gynecologic Investigation, 48th annual meeting, Toronto, Canada, March 2001. Journal of the Society for Gynecologic Investigation 2001, 8(Suppl):126A.

14. **Goharkhay N**, Wing DA, Naidu Y, Hanna M, Felix JC. The human erythroleukemia (HEL) cell line as a model to study EP3 receptor isoforms Society for Gynecologic Investigation, 48th annual meeting, Toronto, Canada, March 2001 Journal of the Society for Gynecologic Investigation 2001, 8(Suppl):126A.

15. **Goharkhay N**, Felix JC, Naidu Y, Hanna M, Wing DA. Misoprostol inhibits the expression of the oxytocin receptor (OTR) and the V1aR vasopressin receptor in human erythroleukemia (HEL) cells. Society for Gynecologic Investigation, 48th annual meeting, Toronto, Canada, March 2001 Journal of the Society for Gynecologic Investigation 2001, 8(Suppl):126A.

16. **Goharkhay N**, Felix JC, Naidu Y, Hanna M, Wing DA. Expression of the oxytocin receptor (OTR) and the vasopressin V1aR receptor in human myometrium. Society for Gynecologic Investigation, 48th annual meeting, Toronto, Canada, March 2001. Journal of the Society for Gynecologic Investigation 2001, 8(Suppl):126-127A.

17. Pan V, **Goharkhay N**, Felix JC, Wing DA. Fgl2 prothrombinase expression in gravid and nongravid human myometrium. Society for Maternal-Fetal Medicine, 22nd annual

meeting, New Orleans, Louisiana, January 2002. American Journal of Obstetrics and Gynecology 2001, 185(Suppl):S230.

18. Lu J, **Goharkhay N**, Dahl G, Pan V, Mirhashemi R, Stanczyk FZ, Wing DA, Felix JC. Expression of calcitonin gene related protein-receptor component protein (CGRP-RCP) mRNA in human myometrium. Society for Gynecologic Investigation, 49th annual meeting, Los Angeles, California, March 2002. Journal of the Society for Gynecologic Investigation 2002, 9(1[Suppl]):305A.

19. Verma U, **Goharkhay N**, Gilles J, Beydoun S. Conservative Management of preterm premature rupture of membranes (PPROM) between 18 and 23 Weeks of Gestation-Neonatal Outcome. Society for Gynecologic Investigation, 49th annual meeting, Los Angeles, California, March 2002. Journal of the Society for Gynecologic Investigation 2002, 9(1[Suppl]):98A.

20. Pan V, **Goharkhay N**, Felix JC, Wing DA. The expression of the fgl2 prothrombinase compared to prostaglandin EP3 receptors and inducible nitric oxide synthase (iNOS) in human myometrium. Society for Gynecologic Investigation, 49th annual meeting, Los Angeles, California, March 2002. Journal of the Society for Gynecologic Investigation 2002, 9(1[Suppl]):306A.

21. Amezcua C, Lu J, **Goharkhay N**, Stanczyk FZ, Felix JC. Pituitary tumor transforming gene (PTTG1) expression in endometrial adenocarcinoma. Society for Gynecologic Investigation, 49th annual meeting, Los Angeles, California, March 2002. Journal of the Society for Gynecologic Investigation 2002, 9(1[Suppl]):345A.

22. **Goharkhay N**, Amezcua C, Lu J, Stanczyk FZ, Felix JC. Pituitary tumor transforming gene-1 (PTTG1) expression in endometrial adenocarcinoma. University of Miami – Sylvester Comprehensive Cancer Center, 2002 Cancer Research Poster Session, Miami, Florida, June 2002.

23. **Goharkhay N**, Costa C, Matos J, Salom E, Verma U. Comparison of CT or ultrasound guided drainage with concomitant intravenous antibiotics versus intravenous antibiotics alone in the management of tuboovarian abscesses. Society for Gynecologic Investigation, 50th annual meeting, Washington, DC, March 2003. Journal of the Society for Gynecologic Investigation 2003, 10(2[Suppl]):92A.

24. **Goharkhay N**. Conservative management of cervical ectopic pregnancy The William A. Little Society – University of Miami Ob/Gyn Alumni, Annual Scientific Meeting, Key Biscayne, Florida, May 2004.

25. **Goharkhay N**, Bytautiene E, Tamayo EH, Vedernikov YP, Longo M, Anderson GD, Saade GR. A murine model of fetal programming of atherosclerosis. Women's Health Week Scientific Poster Session, May 2006, Galveston, Texas. Winner, Second Prize (Basic Sciences).

26. **Goharkhay N** , Lu F, Tamayo EH, Gamble P, Betancourt A, Mareth A, Bytautiene E, Vedernikov YP, Anderson GD, Longo M, Saade GR. Fetal programming of

atherosclerosis in a mouse model. Cell Biology and Neuroscience Program Introduction, September 2006, Galveston, Texas.

27. Lu F, **Goharkhay N**, Bytautiene E, Tamayo E, Orise P, Anderson GD, Longo M, Saade GR. Fetal programming of adult vascular function in a preeclampsia-like animal model is gender-specific. American Journal of Obstetrics and Gynecology 2007, 197(6[Suppl 1]):S141.

28. Longo M, Lu F, Tamayo E, Gamble P, Sbrana E, **Goharkhay N**, Anderson GD, Hankins GDV, Saade GR. The effect of postnatal age on fetal vascular programming of blood pressure in a nitric oxide synthase knockout mouse model. American Journal of Obstetrics and Gynecology 2007, 197(6[Suppl 1]):S121.

29. **Goharkhay N**, Mateus J, Egbert K, Smith O, Gamble P, Yin H, Anderson G, Saade G, Longo M. Lack of hypercholesterolemia and of activation of cholesterol synthesis during infancy in a mouse model of developmental programming of atherosclerosis. Society for Maternal-Fetal Medicine, 28th annual meeting, Dallas, Texas, January 2008. American Journal of Obstetrics and Gynecology 2007, 197(6[Suppl 1]):S119.

30. **Goharkhay N**, Yin H, Tamayo E, Gamble P, Lu F, Betancourt A, Villarreal K, Hankins GDV, Saade GR, Longo M. A novel KISS-1 related peptide as indicator of murine pregnancy and potential prenatal diagnostic marker. Society for Maternal-Fetal Medicine, 28th annual meeting, Dallas, Texas, January 2008. American Journal of Obstetrics and Gynecology 2007, 197(6[Suppl 1]):S120.

31. **Goharkhay N**, Sbrana E, Moss JE, Tamayo E, Gamble P, Saade GR, Longo M. Attenuation of hepatic histopathologic damage in NOS3 deficient dams from the initial to the subsequent pregnancy. Society for Gynecologic Investigation, 55th annual meeting, San Diego, California, March 2008. Journal of the Society for Gynecologic Investigation 2008 15(2[Suppl]):197A.

32. Moss JE, Mateo RI, Hawkins HK, **Goharkhay N**, Longo M, Saade GR, Aronson JF, Sbrana E. Etiologic Agents Isolated in Cases of Fatal Neonatal Sepsis. Society for Gynecologic Investigation, 55th annual meeting, San Diego, California, March 2008. Journal of the Society for Gynecologic Investigation 2008 15(2[Suppl]):256A.

33. **Goharkhay N**, Mateus J, Egbert K, Smith O, Gamble P, Yin H, Anderson G, Saade G, Longo M. Lack of hypercholesterolemia and of activation of cholesterol synthesis during infancy in a mouse model of developmental programming of atherosclerosis. Center for Interdisciplinary Research in Women's Health (CIRWH) Poster Session, Galveston, Texas, April 2008. Winner, Third Prize, Translational Science.

34. **Goharkhay N**, Yin H, Tamayo E, Gamble P, Lu F, Betancourt A, Villarreal K, Hankins GDV, Saade GR, Longo M. A novel KISS-1 related peptide as indicator of murine pregnancy and potential prenatal diagnostic marker. Center for Interdisciplinary Research in Women's Health (CIRWH) Poster Session, Galveston, Texas, April 2008.

35. **Goharkhay N**, Sbrana E, Moss JE, Tamayo E, Gamble P, Saade GR, Longo M. Attenuation of hepatic histopathologic damage in NOS3 deficient dams from the initial to

the subsequent pregnancy. Center for Interdisciplinary Research in Women's Health (CIRWH) Poster Session, Galveston, Texas, April 2008.

36. **Goharkhay N**, Lu F, Bytautiene E, Tamayo E, Betancourt A, Hankins GDV, Longo M, Saade GR. Fetal programming of atherosclerosis: Effect on activity and blood pressure in young adult mice. Society for Maternal-Fetal Medicine, 29th Annual Meeting, San Diego, California, January 2009. American Journal of Obstetrics and Gynecology 2008, 199(6[Suppl 1]):S83.

37. Lu F, **Goharkhay N**, Bytautiene E, Tamayo E, Hankins GDV, Longo M, Saade GR. In vitro uterine artery reactivity in a pregnant mouse model of preeclampsia induced by over-expression of sFlt-1.Society for Maternal-Fetal Medicine, 29th Annual Meeting, San Diego, California, January 2009. American Journal of Obstetrics and Gynecology 2008, 199(6[Suppl 1]):S85.

38. **Goharkhay N**, Lu F, Bytautiene E, Gamble P, Tamayo E, Yin H, Hankins GDV, Longo M, Saade GR. Vascular reactivity patterns in the carotid artery of offspring in the apoE mouse model of developmental programming of atherosclerosis at 4 months of age. Society for Maternal-Fetal Medicine, 29th Annual Meeting, San Diego, California, January 2009. American Journal of Obstetrics and Gynecology 2008, 199(6[Suppl 1]):S169.

39. **Goharkhay N**, Yin H, Gamble P, Tamayo E, Lu F, Bytautiene E, Longo M, Saade GR. Developmental programming of atherosclerosis is unrelated to variations in serum cholesterol levels among genetically similar mothers. Society for Maternal-Fetal Medicine, 29th Annual Meeting, San Diego, California, January 2009. American Journal of Obstetrics and Gynecology 2008, 199(6[Suppl 1]):S173.

40. Mateus J, **Goharkhay N**, Longo M, Saade GR, Moss J, Sbrana E. Placental histopathology in mild versus severe hypertensive disorders in pregnancy: Implications for feto-placental perfusion. Society for Maternal-Fetal Medicine, 29th Annual Meeting, San Diego, California, January 2009. American Journal of Obstetrics and Gynecology 2008, 199(6[Suppl 1]):S215.

This dissertation was typed by Nima Goharkhay