OULU 2009
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ACTA UNIVERSITATIS OULUENSIS


*Mirella Hietaniemi*

# STUDIES ON NOVEL AND TRADITIONAL RISK FACTORS OF ATHEROSCLEROSIS

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# STUDIES ON NOVEL AND TRADITIONAL RISK FACTORS OF ATHEROSCLEROSIS

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium 8 of Oulu University Hospital, on 16 June 2009, at 12 noon

OULUN YLIOPISTO, OULU 2009

Acta Univ. Oul. D 1016, 2009

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ISBN 978-951-42-9128-9 (Paperback)
ISBN 978-951-42-9129-6 (PDF)
http://herkules.oulu.fi/isbn9789514291296/
ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)
http://herkules.oulu.fi/issn03553221/

Cover design
Raimo Ahonen

OULU UNIVERSITY PRESS
OULU 2009

**Hietaniemi, Mirella, Studies on novel and traditional risk factors of atherosclerosis**
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*Acta Univ. Oul. D 1016, 2009*
Oulu, Finland

## *Abstract*

The atherosclerotic plaques develop with the adhesion of inflammatory cells and lipids onto the innermost layer of the vessel. They may eventually occlude the vessel impairing blood flow. A severe complication is the rupture of a plaque resulting in the formation of a thrombus that can cause myocardial infarction or stroke. Though a large number of risk factors for atherosclerosis have been identified, the pathogenesis of atherosclerosis is far from unravelled. The aim of the present work was to study both traditional as well as potential novel risk factors of atherosclerosis.

The first study examined the relationship between IGF-I concentrations and carotid artery atherosclerosis and its metabolic risk factors. Low IGF-I concentrations were associated with several cardiovascular risk factors. A positive association was observed between IGF-I concentrations and carotid artery intima-media thickness in women. The results suggest that IGF-I may be involved in the pathogenesis of atherosclerosis. Interestingly, the effect may manifest differentially in men and women.

The second study focused upon the effects of obesity and weight loss on liver gene expression. A global decrease in gene expression was observed. The down-regulated genes included genes involved in the ubiquitin cycle, which may point to a reduction in oxidative stress due to the hypocaloric diet. The down-regulation of *peroxisome proliferator-activated receptor gamma cofactor 1 alpha (PGC-1α)* may be related to improved insulin sensitivity. Several novel genes not previously linked to obesity and weight loss were also discovered.

In the third and fourth studies, the developmental origins of atherosclerosis hypothesis was studied in a rat model of fetal undernutrition. Unfavourable changes in the obesity-related peptide hormones adiponectin and resistin were observed which could predispose to insulin resistance in later life. In addition, total cholesterol levels were elevated in the undernourished offspring. The gene expression changes in the rat pups suggest that the development of pancreas was affected, which might further contribute to disturbances in insulin and glucose metabolism.

*Keywords:* atherosclerosis, fetal development, gene expression, insulin-like growth factor I, obesity, peptide hormones, weight loss

**Hietaniemi, Mirella, Tutkimuksia ateroskleroosin uusista ja perinteisistä riskitekijöistä**
Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Sisätaudit, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto; Biocenter Oulu, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto; Kliinisen tutkimuksen keskus, Oulun yliopistollinen sairaala, PL 5000, 90014 Oulun yliopisto
*Acta Univ. Oul. D 1016, 2009*
Oulu

## *Tiivistelmä*

Ateroskleroosi eli valtimonkovettumatauti on sairaus, joka saa alkunsa verisuonen sisäseinämään kiinnittyvistä tulehdussoluista ja veren rasvapartikkeleista, joista muodostuu pitkän ajan kuluessa ateroskleroottisia plakkeja. Plakit voivat kasvaessaan heikentää veren virtausta valtimoissa ja pahimmillaan jopa tukkia suonen kokonaan. Mikäli plakki repeää, voi muodostua verihyytymä joka sydämessä aiheuttaa sydäninfarktin ja aivoissa aivoinfarktin. Vaikka useita ateroskleroosille altistavia tekijöitä tunnetaan, taudin syntymekanismit ovat vielä suurelta osin selvittämättä. Tämän väitöskirjatyön tarkoituksena oli tutkia sekä ateroskleroosin perinteisiä että mahdollisia uusia riskitekijöitä.

Ensimmäisessä osatyössä tutkittiin insuliininkaltaisen kasvutekijä I:n (IGF-I) yhteyttä kaulavaltimon ateroskleroosiin sekä perinteisiin ateroskleroosin riskitekijöihin. Matalat IGF-I pitoisuudet liittyivät moniin ateroskleroosin riskitekijöihin. Naisissa korkeammat IGF-I pitoisuudet kuitenkin yhdistyivät paksumpaan kaulavaltimoon, mikä viittaa ateroskleroosiin. Tulosten perusteella IGF-I saattaa liittyä ateroskleroosin kehitykseen ja mahdollisesti sen vaikutukset ilmenevät naisissa ja miehissä eri tavoin.

Toisessa osatyössä tutkittiin maksan geenien ilmentymistä lihavuudessa ja laihdutusjakson jälkeen. Laihduttaneessa ryhmässä 142:n geenin ilmentyminen oli vähentynyt ja vain yhden lisääntynyt suhteessa kontrolliryhmään. Ubikitiini-syklin geenien ilmentymisen väheneminen voi viitata vähentyneeseen oksidatiiviseen stressiin elimistössä dieetin seurauksena. Muun muassa diabetekseen liittyvän geenin, *peroxisome proliferator-activated receptor gamma cofactor 1 alpha*, väheneminen puolestaan voi liittyä parantuneeseen insuliiniherkkyyteen laihduttaneissa. Lisäksi tässä työssä tuli esiin monia uusia, mielenkiintoisia geenejä, joita ei aiemmin ole yhdistetty lihavuuteen tai ateroskleroosiin.

Kolmannessa ja neljännessä osatyössä selvitettiin ns. Barkerin hypoteesia, eli sitä, voisiko sairastumisalttius määräytyä jo sikiökauden ja varhaiskehityksen aikana. Rottakokeemme osoittivat, että sikiöaikaisen aliravitsemuksen seurauksena kolesteroliarvot olivat korkeammat ja että lihavuuteen liittyvien peptidihormonien, adiponektiinin ja resistiinin, pitoisuuksissa oli tapahtunut epäsuotuisia muutoksia, jotka voivat altistaa insuliiniresistenssille Tulokset viittasivat myös siihen, että aliravitsemus oli mahdollisesti vaikuttanut haiman kehitykseen, mikä voi myös osaltaan vaikuttaa mm. insuliini- ja sokeriaineenvaihduntaan. Tämänkaltaiset muutokset saattavat altistaa ateroskleroosille myöhemmällä iällä.

*Asiasanat:* ateroskleroosi, geenieksрessio, insuliininkaltainen kasvutekijä I, lihavuus, painonpudotus, peptidihormonit, sikiönkehitys

# Acknowledgements

This work was carried out at the Department of Internal Medicine and Biocenter Oulu, University of Oulu and Clinical Research Center, Oulu University Hospital.

I wish to express my sincere gratitude to Professor Antero Kesäniemi for the opportunity to become acquainted with scientific research under his expert guidance. He has provided excellent resources to conduct this work. My other supervisors, Maarit Jokela and Olavi Ukkola deserve my warmest thanks for their invaluable expertise and kind support through these years.

The official referees of this thesis, Professor Johan Eriksson and Professor Olli Raitakari are greatly acknowledged for their careful revision and constructive comments. Ewen MacDonald is thanked the language revision.

I am very grateful to everyone in the Department of Internal Medicine, Clinical Research Center and Biocenter Oulu who has, in one way or another, taken part or supported this work. My warmest thanks go to our skilful and hard-working ladies, Heidi Häikiö and Saija Kortetjärvi for their expert laboratory assistance and a positive attitude. Our secretaries Marita Koistinen and Anne Salovaara are greatly appreciated for their help; so far I have not managed to pose any question that these multiply skilled professionals could not help me with! The PhD students in our group, Anne, Merja, Johanna, Elina, Eija and Maritta as well as in the "neighbouring" group of Markku Savolainen, Tiia, Sanna, Antti, Tuija and post-docs Marja and Tuire are warmly acknowledged for their unselfish help in numerous matters as well as enjoyable conversations during lunch and coffee breaks. My special thanks go to Anne for being my friend and supporter already from the first days of my university studies. My close collaborators in the animal experiments, Elina and Merja are also warmly thanked for unforgettable and momentous conversations in our office.

I am deeply indebted to all my co-authors and collaborators. In particular, the help from Seppo Pöykkö in statistical problems in the early days of this thesis project has been essential and I highly value his patience in answering my never-ending questions. In addition, I am truly thankful to the warm-hearted and helpful personnel at the Oulu University Experimental Animal Center.

I want to express my heartfelt thanks to all my friends outside of the world of science. The cheerful and relaxing moments with you bring energy and sunshine into my life!

My deepest and warmest gratitude go to my family; my mother Marja, my father Markku and brother Manu, and last but not least my dear husband Raimo.

Thank you all for your love, encouragement and patience over the years and for standing by my side through the best and worst times.

This work was financially supported by the Research Council for Health of the Academy of Finland, the Finnish Foundation for Cardiovascular Research, Paavo Nurmi Foundation and Finnish Cardiac Society.

# Abbreviations

| | |
|---|---|
| A | adenine |
| ALAT | alanine aminotransferase |
| ANCOVA | analysis of covariance |
| ANOVA | analysis of variance |
| AP | alkaline phosphatase |
| ApoE | apolipoprotein E |
| ASAT | aspartate aminotransferase |
| BIF | bifurcation enlargement |
| BMI | body mass index |
| CCA | common carotid artery |
| CRP | C-reactive protein |
| CVD | cardiovascular disease |
| C | cytosine |
| DBP | diastolic blood pressure |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immuno sorbent assay |
| FDR | false discovery rate |
| G | guanine |
| GH | growth hormone |
| GHRH | growth hormone releasing hormone |
| GWA | genome-wide association |
| HDL | high-density lipoprotein |
| ICA | internal carotid artery |
| IGF-I | insulin-like growth factor-I |
| IGFBP | insulin-like growth factor binding protein |
| IL | interleukin |
| IMT | intima-media thickness |
| kcal | kilocalorie |
| kDa | kilodalton |
| LD | linkage disequilibrium |
| LDL | low-density lipoprotein |
| MJ | megajoule |
| NAFLD | non-alcoholic fatty liver disease |
| NEFA | non-esterified fatty acid |
| NO | nitric oxide |

| | |
|---|---|
| OPERA | Oulu Project Elucidating Risk of Atherosclerosis |
| PCR | polymerase chain reaction |
| PGC-1α | peroxisome proliferator-activated receptor gamma cofactor 1 alpha |
| qRT-PCR | relative quantitative real-time PCR |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| SBP | systolic blood pressure |
| SNP | single nucleotide polymorphism |
| SLCO1A2 | solute carrier organic anion transporter family member 1A2 |
| T | thymine |
| T2D | type 2 diabetes mellitus |
| TG | triglyceride |
| TNFα | tumour necrosis factor-α |
| VLDL | very-low-density lipoprotein |
| VSMC | vascular smooth muscle cell |

# List of original articles

The thesis is based on the following original articles, which are referred to in the text by their Roman numerals:

I Hietaniemi M, Pöykkö SM, Ukkola O, Päivänsalo M & Kesäniemi YA (2005) IGF-I concentrations are positively associated with carotid artery atherosclerosis in women. Ann Med 37(5): 373–82.

II Hietaniemi M, Jokela M, Rantala M, Ukkola O, Vuoristo JT, Ilves M, Rysä J & Kesäniemi YA (2009) The effect of a short-term hypocaloric diet on liver gene expression and metabolic risk factors in obese women. Nutr Metab Cardiovasc Dis. 19(3):177–83.

III Hietaniemi M*, Malo E*, Jokela M, Santaniemi M, Ukkola O & Kesäniemi YA (2009) The effect of energy restriction during pregnancy on obesity-related peptide hormones in rat offspring. Peptides 30: 705–709.

IV Hietaniemi M, Santaniemi M, Malo E, Ukkola O, Kesäniemi YA & Jokela M. Gene expression profiles in fetal and neonatal rat offspring of energy-restricted rat dams. Manuscript.

*Equal contribution

# Contents

# 1 Introduction

Atherosclerotic cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in Western countries and their prevalence is constantly increasing also in the developing countries. Extensive research has revealed numerous possible risk factors and candidate genes for atherosclerosis, but the molecular and genetic background of this disease in many respects is still undefined.

Atherosclerosis is an insidious, complex disease that may start already in childhood and often progresses over decades before manifesting as a clinical disease. It is a multifactorial disorder involving many cell types and circulating mediators. Therefore, in most cases it is usually impossible to determine only a limited number of causal factors for the development of this disease. In addition, it is possible that some particular factors may be operating in certain forms of atherosclerotic CVD but not in others (Arnett *et al.* 2007).

Conventional, independent risk factors for CVD include cigarette smoking, high total and low-density lipoprotein (LDL) cholesterol, low high-density lipoprotein (HDL) cholesterol, hypertension, type 2 diabetes mellitus (T2D) and advancing age. In addition, several predisposing risk factors, such as (abdominal) obesity, physical inactivity and elevated serum triglyceride (TG) concentrations are recognized. Their effects on disease risk are partly mediated through the conventional risk factors but they may have independent effects as well. So-called conditional risk factors such as homocysteine and lipoprotein(a) may also contribute to disease development in the presence of causative risk factors (Grundy *et al.* 1999). Currently, atherosclerosis is also considered having a major inflammatory component (reviewed by Ross 1999).

It is obvious that several predisposing genes are involved in the development of CVD. For example, a genetic predisposition to coronary artery disease and myocardial infarction has been demonstrated in several extensive studies (reviewed by Mayer *et al.* 2007). It is generally believed that complex diseases, like CVDs arise from the interactions between environmental factors and several predisposing gene variants. The effect of each of these risk factors alone is small, but an unfavourable combination of the risk factors results in disease. Recent studies have suggested that the individual risk of developing atherosclerosis may partly be determined already during the fetal and early postnatal development (Barker 2000).

The present study was carried out to explore the involvement of some recently identified, novel risk factors in the development of atherosclerosis. The

growth hormone (GH)/insulin-like growth factor I (IGF-I) axis has an effect on several biological processes that probably play an important role in the pathogenesis of atherosclerosis, but the results from previous studies have so far been inconclusive. Another controversial topic in CVD development is whether an unfavourable environment during the fetal period could lead to such permanent changes in physiology that a higher risk for CVD could be explained by these early life circumstances. We also studied the role of obesity and weight loss, since even though obesity is a fairly well established risk factor of CVD, still numerous unresolved questions related to the molecular mechanisms remain unanswered.

# 2 Review of the literature

## 2.1 The definition and pathogenesis of atherosclerosis

Atherosclerosis is a disease of the innermost layer of the vessel wall, the intima. It develops over decades and may eventually manifest as myocardial infarction (heart attack) or stroke. The atherosclerotic lesions are classified from type I to VI based on their morphological characteristics (Stary 2000). During the period when an initial lesion progressively turns into an advanced lesion, it accumulates novel features but generally also the characteristics of the previous stage are present. The characteristics that distinguish the next lesion type from the previous stage are shown in Figure 1 and described in more detail below.

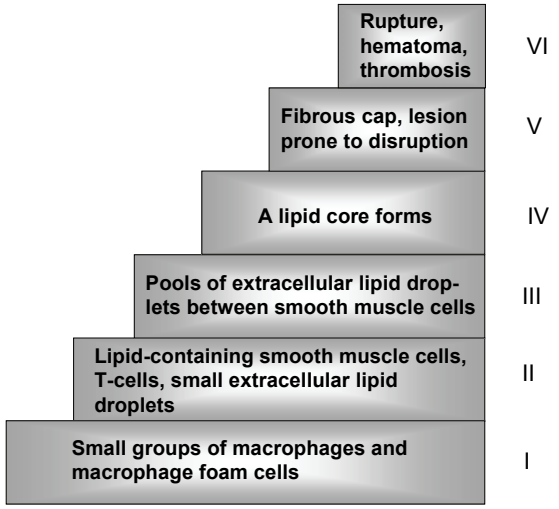


**Fig. 1. The progression of an atherosclerotic lesion from type I to VI (Stary 2000) with the essential characteristics that distinguish each type from the previous stage depicted.**

Type I lesions, also known as initial lesions, are observed already in infants and children. They consist of small isolated groups of macrophages, some of which have ingested lipids, turning them into so-called foam cells. When also lipid-containing vascular smooth muscle cells (VSMC) are found, the lesion is classified as a type II lesion. Even though most of the lipid in type II lesions is located inside the cells, also some extracellular lipid droplets are visible at this stage. T lymphocytes can be found in type II lesions though they are not very abundant.

Type III lesions may develop soon after puberty. In type III lesions, the extracellular lipid builds up, forming pools that can disrupt the connections between intimal smooth muscle cells.

A type IV lesion is known as an atheroma, since it is potentially symptom producing. These are found frequently from the third decade onwards. At this stage, the extracellular lipid has formed a large lipid core that causes substantial intimal disorganisation but not necessarily any significant narrowing of the lumen.

In type V lesions, the intima covering the lipid core is thickened by formation of fibrous connective tissue resulting in a fibroatheroma. Calcification of the lipid core and other parts of the lesion can occur at this stage. Both type IV and V lesions may develop fissures, hematomas and/or thrombi. If they contain one or more of these features they are classified as type VI lesions. Proteolytic enzymes released by the macrophages in the lesion or structural weakness related to lesion composition can promote plaque disruption. This may result in the formation of a thrombus which can lead to impairment of the blood supply or total occlusion of a vein, causing tissue death. (Stary *et al.* 1994, Stary *et al.* 1995)

Endothelial dysfunction is considered the earliest, fundamental step in atherosclerosis progression. The endothelium is a monolayer of cells covering the innermost, luminal side of the vessel wall. It has a key role in maintaining the normal function of the vessel wall. The properties of a normal, healthy endothelium are listed in Table 1.

**Table 1. Properties of the healthy endothelium (Stary *et al.* 1992).**

| The endothelium is: |
|---|
| a permeability barrier, regulating the transport of macromolecules |
| antithrombotic, inhibiting platelet adhesion |
| involved in inflammatory and immune response |
| a key regulator of vascular tone and contraction |

The increased production of adhesion factors by the endothelium can set into motion a series of events that lead to the formation of an atherosclerotic plaque. Several factors, such as hypercholesterolemia, oxidative stress, elevated blood glucose, cigarette smoking and infection, can trigger the increased expression of adhesion molecules and recruitment of macrophages, monocytes and T lymphocytes and in addition, endothelium adhesiveness towards platelets increases. Monocytes may migrate to the subendothelial space where they become macrophages that can ingest lipids, turning them into foam cells. The subendothelial macrophages are able to secrete chemoattractants and mitogens that attract additional smooth muscle cells and inflammatory cells to the intima. (reviewed by Ross 1999)

## 2.2 Methods for identifying genetic susceptibility and molecular markers for atherosclerosis

In the majority of cases, atherosclerosis is attributable to the synergism of several predisposing genetic and environmental factors as well as interactions between these factors. The individual effect of each of these factors is usually small as such, and thus far, the interactions between genes and environment are poorly understood.

### *2.2.1 Genetic studies*

The two most common approaches to study the genetic susceptibility to atherosclerosis have been linkage studies and association analyses. In linkage studies, known polymorphic markers are genotyped across the genome using family data. Those markers that appear to be identical by descent and show correlation to phenotypic data among family members can identify loci that may be important in the pathogenesis of atherosclerosis. However, these regions tend to be wide; encompassing numerous genes and thus fine mapping with additional markers is required. Even so, it may be very challenging to identify specific causal genes by linkage analysis, especially those having only a moderate individual effect. (Arnett *et al.* 2007)

Association studies have traditionally been done by genotyping common deoxyribonucleic acid (DNA) sequence variations, known as single nucleotide polymorphisms (SNP), from candidate genes using large groups of unrelated patients and controls. Differences in the frequencies of the genetic variants

between the affected and controls may point to the possible involvement of the SNP itself in disease development. Alternatively, it may be in linkage disequilibrium (LD) with the true functional variant, meaning that there is no meiotic recombination between these two loci and thus, they are inherited as an intact chromosomal region. A common drawback in association studies is the lack of replication. This can be explained at least partly by inappropriate choice and matching of patients and controls or by differences in the distribution of LD between populations. (Brookes 1999)

A novel genetic approach to identify causal genes in the development of complex diseases is genome-wide association (GWA) studies. These are based on high-throughput microarrays representing up to 1 million SNPs on one chip. This method has produced impressive amounts of novel data on genetic variants that may be related to disease development. GWA studies have begun to replace the traditional approaches based on genetic linkage and candidate genes, even though, for the time being, the financial costs are still fairly high. (Grant & Hakonarson 2008)

### *2.2.2 Expression studies*

Gene expression profiling is used for studying gene expression at a specific time point in a given tissue. By comparing the gene expression profiles of patients and controls, one can find genes that may be involved in disease pathogenesis. Alternatively, the differing gene expression patterns may be a consequence of pathogenic processes.

The expression of tens of thousands of genes can be assessed simultaneously by using microarrays. Relative quantitative real-time polymerase chain reaction (qRT-PCR) is used for determining the expression of individual genes at a specific time point in a given tissue. This method is commonly used also for verifying microarray results. (Arnett *et al.* 2007)

### *2.2.3 Animal studies*

Animal models, either conventional, outbred laboratory animals or genetically modified animals, are commonly used in studies attempting to elucidate the molecular background of atherosclerosis. These models are important when investigating the effect of a specific gene or environmental exposures *in vivo*. Even though these studies can offer invaluable knowledge about the causes,

consequences and interactions between molecular markers in whole living organisms, there are some limitations in extrapolating and applying the results obtained from animal experiments to humans. Rodents are the most widely used experimental animals in CVD studies, but it is important to remember that they differ in many key physiological processes when compared to humans. For example, the lipid metabolism in rodents differs from humans *i.e.* in rodents, it is based principally on HDL and not on LDL, as in humans (Russell & Proctor 2006).

## 2.3 The GH/IGF-I axis and atherosclerotic CVD

Numerous studies have attempted to clarify the role of the GH/IGF-I axis in the development of atherosclerotic CVD (Bayes-Genis *et al.* 2000, Frystyk *et al.* 2002, Klibanski 2003, Colao *et al.* 2006). Both GH deficiency and GH excess (acromegaly) are associated with increased risk for CVD (reviewed by Lombardi *et al.* 1997). Consequently, as GH is the main regulator of IGF-I synthesis (Schwander *et al.* 1983), studies on IGF-I have reported that both low and high IGF-I concentrations can be associated with increased risk for CVD (Kawachi *et al.* 2005, Colao *et al.* 2005). Hence, the relationship of the GH/IGF-I axis and CVD appears to be complex, which may reflect the complexity of the development of atherosclerosis itself, *i.e.* many different factors operating during different phases of disease progression could potentially be influenced by the GH/IGF-I axis.

### *2.3.1 GH action and regulation*

Human GH is expressed by the *hGH-N* gene which is a member of the GH gene family, a five-gene cluster of which the four other genes are expressed only in the placenta (Jones *et al.* 1995). GH is an anabolic hormone synthesized by the somatotroph cells of the anterior pituitary gland (reviewed by Ayuk & Sheppard 2006). The hypothalamic hormones, GH-releasing hormone (GHRH) and the inhibitory hormone somatostatin (SS), as well as the GH releasing peptide, also known as ghrelin, and IGF-I are the major regulators of GH secretion. However, the regulation of GH secretion is very complex, involving physiological and metabolic signals, including sleep, stress, exercise, gonadal, thyroid and glucocorticoid hormones as well as the concentrations of glucose, amino acids and non-esterified (free) fatty acids (NEFAs) (reviewed by Casanueva 1992).

In addition to promoting somatic growth, GH has several other stimulatory actions (reviewed by Angelin & Rudling 1994, Butler & Le Roith 2001 and Hattori 2009). These are shown in Figure 2.

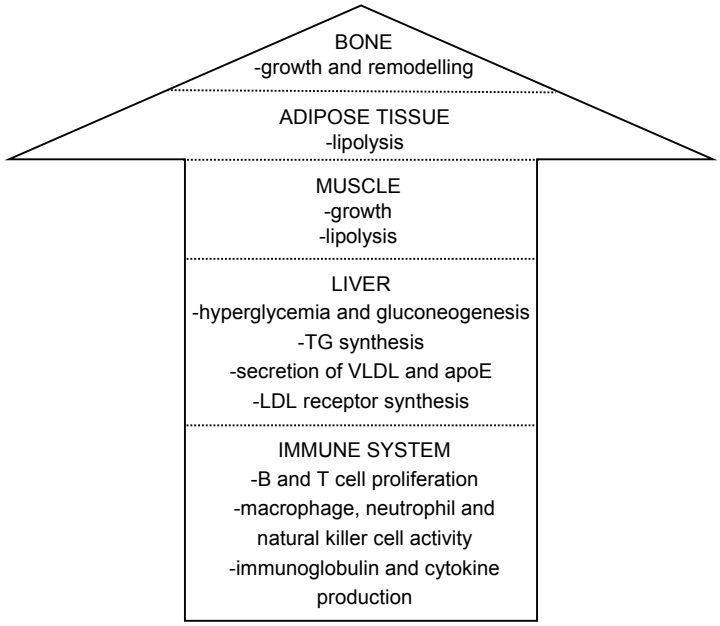


**Fig. 2. The major stimulatory effects of GH on different tissues and organs.**

### *2.3.2 The somatomedin hypothesis*

Already 50 years ago, experiments suggested that the effects of GH on growth were mediated through an intermediary substance which was termed "sulfation factor" and later "somatomedin" (Salmon & Daughaday 1957). In 1978, it was shown that the somatomedin substance regulated by GH was IGF-I, also known as somatomedin-C (Rinderknecht & Humbel 1978). This led to the original somatomedin hypothesis. It was postulated that GH could control growth by stimulating the liver production of IGF-I which, in turn, circulated to the target organs. In addition, it was observed that there was a negative feedback system between GH and IGF-I whereby increased IGF-I concentrations suppressed GH synthesis in the pituitary gland (Berelowitz *et al.* 1981).

The original somatomedin hypothesis was updated when it was discovered that IGF-I was produced, not only by the liver, but also locally by diverse tissues (D'Ercole *et al.* 1980). Subsequently it was observed that since GH was able to stimulate growth directly, it was possible that this growth-promoting effect could be mediated through locally produced IGF-I instead of circulating IGF-I (Isaksson *et al.* 1982). The autocrine and paracrine effects of locally produced IGF-I are now well recognised and it has been shown that GH has also direct effects not mediated by IGF-I. Furthermore, it has even been suggested that the GH stimulated, local IGF-I production would principally determine somatic growth, whereas the circulating IGF-I produced by the liver would function mainly to control GH secretion via a negative feedback loop (Le Roith *et al.* 2001).

### *2.3.3 The IGF-I system*

IGF-I is a ubiquitously expressed polypeptide that acts both as an endocrine hormone and a tissue growth factor (reviewed by Jones & Clemmons 1995). The best known functions of IGF-I are stimulation of growth (reviewed by Laron 2001) and its insulin-like effects, like lowering of blood glucose and amino acid uptake (reviewed by Clemmons 2006). IGF-I is synthesised by various tissues, but the liver accounts for the majority of the total serum IGF-I (Sjogren *et al.* 1999).

IGF-I mediates many of the metabolic actions of GH and GH is the primary determinant of circulating IGF-I. However, also (poor) nutritional status, thyroid hormones and insulin directly regulate IGF-I concentrations, and there are also major indirect determinants operating through the GH-IGF-I axis include aging, body composition, estrogens, androgens and exercise (reviewed by Rosen & Pollak 1999).

Genetic factors may also play an important role in determining IGF-I levels. These could include, in addition to polymorphic variation in the *IGF-I* gene itself, also other candidate genes, such as the genes encoding GH, GHRH and the receptors for these hormones (Rosen & Pollak 1999).

The activity and availability of IGF-I is modulated by the IGF binding proteins (IGFBPs) (reviewed by Hwa *et al.* 1999). Six mammalian IGFBPs have been well characterised (reviewed by Kelley *et al.* 1996). Most of the circulating IGF-I is bound to these proteins which prolong the half-life of IGF-I, thus constituting a stable circulating reservoir of IGF-I. However, it has been suggested that free IGF-I, which represents only about 1% of total IGF-I, may be the functionally active form (Janssen *et al.* 2003).

Most of the circulating IGF-I is carried in a ternary complex consisting of IGF-I, IGFBP-3 and a liver-derived glycoprotein called the acid-labile subunit (ALS). This large complex is apparently not able to cross the capillary wall, but the ability of the other smaller IGFBPs to do so probably is an essential aspect of IGF-I function (reviewed by Kelley *et al.* 1996). In particular, IGFBP-1 may be important in modulating the free IGF-I fractions, notably since it is the only IGFBP that is acutely regulated by metabolic status and its expression is inhibited by insulin (reviewed by Lee *et al.* 1993).

In addition to the IGFs and IGFBPs, the IGF system includes also the IGF receptors. Most of the actions of IGF-I are mediated by the type I IGF receptor which is similar to the insulin receptor. Due to the structural similarity between insulin and IGF-I as well as their receptors, IGF-I can bind to the insulin receptor, although with a 100-fold lower affinity than insulin. The close homology between these receptors enables the formation of hybrid IGF-I/insulin receptors on cells that express both receptor genes. These hybrid receptors have 15–50 times higher affinity for IGF-I than insulin. (reviewed by Jones & Clemmons 1995)

### *2.3.4 IGF-I and insulin: similarities and differences*

IGF-I and proinsulin share a 48% sequence homology and a significant structural homology, indicating that the genes for these peptides diverged from a common ancestor by duplication (Rinderknecht & Humbel 1978). In addition, the receptors show a high degree of sequence homology, but important structural differences do exist that result in quite specific binding of IGF-I and insulin to their respective receptors (reviewed by Siddle *et al.* 2001). Under normal physiological conditions, IGF-I is not able to stimulate insulin receptor activation and vice versa (reviewed by Clemmons 2006).

It has been shown *in vitro* and *in vivo* that both insulin and IGF-I can stimulate glucose uptake, promote glycogen synthesis and inhibit protein catabolism (Dimitriadis *et al.* 1992, Di Cola *et al.* 1997). However, the hypoglycaemic potency of IGF-I is only about 10% of that of insulin (reviewed by Hussain *et al.* 1995).

One important difference between insulin and IGF-I is the tissue distribution of their receptors. Skeletal muscle carries both receptor types but whereas hepatocytes and adipocytes are important targets for insulin action, they lack IGF-I receptors (reviewed by Navarro *et al.* 1999). This divergence leads to differences at the tissue level in the actions of insulin and IGF-I. In adipose tissue

and the liver, insulin regulates fatty acid metabolism and TG synthesis, which is not observed under physiologic concentrations of IGF. In skeletal muscle, both insulin and IGF-I stimulate protein synthesis and cellular hypertrophy, but IGF-I has a more potent role in this, while insulin stimulates more effectively glucose disposal in muscle. (reviewed by Clemmons 2006)

### *2.3.5 Effects of GH and IGF-I on cardiovascular risk factors*

GH and IGF-I interact with the cardiovascular system both directly by influencing the structure and function of the heart and vasculature, as well as indirectly via their metabolic actions. The GH/IGF-I axis appears to affect most of the well established risk factors of atherosclerosis: dyslipidemia, elevated blood pressure, insulin resistance, inflammation and obesity (Figure 3). These will be discussed in more detail below.

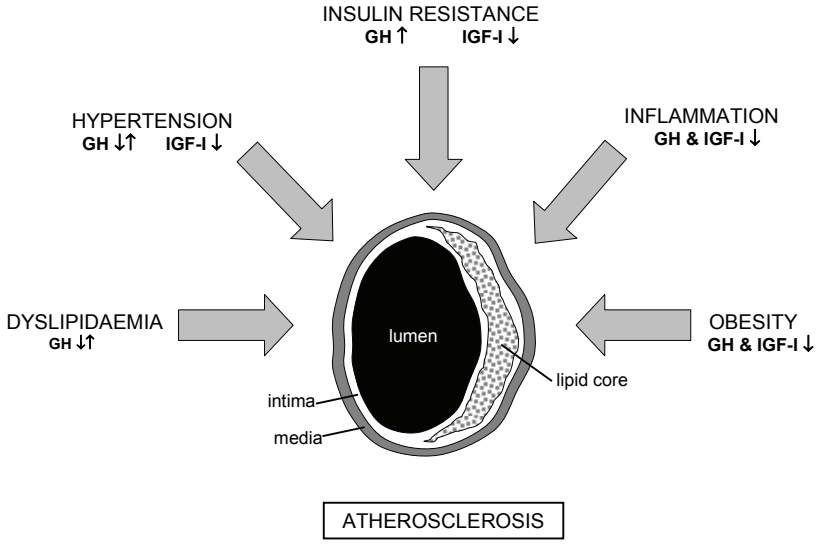


**Fig. 3. The association of the GH/IGF-I axis with risk factors of cardiovascular disease.**

GH has important effects on hepatic lipoprotein metabolism. GH reduces LDL cholesterol levels by increasing LDL clearance though this effect is probably not mediated by IGF-I (Lind *et al.* 2004). In addition, GH can stimulate TG biosynthesis and secretion of very-low-density lipoprotein (VLDL) by the liver (reviewed by Angelin & Rudling 1994). Interestingly, it has been shown that

acromegalic patients who have elevated GH levels, display higher total and LDL cholesterol and TG levels when compared to controls (Colao *et al.* 2002). Therefore, it seems that both GH deficiency and excess may lead to dyslipidemia.

Endothelial dysfunction is an early sign of atherosclerosis. An essential feature of normal endothelial function is the release of nitric oxide (NO) (reviewed by Giannotti & Landmesser 2007). IGF-I stimulates NO production by increasing NO synthase activity and NO, in turn, displays several anti-atherogenic effects including increased vasodilation, enhanced glucose uptake, anti-platelet function, scavenging of free oxygen radicals and anti-inflammatory properties (reviewed by Conti *et al.* 2004). Decreased NO synthesis is associated with GH deficiency (Boger *et al.* 1996). On the other hand, endothelial dysfunction has been reported also in acromegaly. This may be related to the clustering of several risk factors for endothelial dysfunction, such as insulin resistance and dyslipidemia, which are associated with acromegaly (reviewed by Clayton 2003).

VSMCs are also involved in the development of atherosclerosis. The proliferation and migration of VSMCs into the vessel wall contribute to the development of an atherosclerotic plaque and IGF-I appears to stimulate this process. On the other hand, diminished levels of IGF-I in an advanced plaque may lead to plaque instability and its rupture through apoptosis of VSMCs. Hence, it is possible that IGF-I has a dual role in the development of atherosclerotic plaques: it can promote the development of a plaque but it may also inhibit the destabilisation of an advanced plaque (Delafontaine *et al.* 2004).

The effects of GH and IGF-I on carbohydrate metabolism are differing. The insulin-like, glucose-lowering effect of IGF-I is well known (reviewed by Clemmons 2006). GH, in turn, has been associated with insulin resistance since it counteracts the effects of insulin (Dominici *et al.* 1999). On the other hand, in GH deficiency, GH therapy improves insulin sensitivity by promoting a reduction in the amount of visceral fat (Cenci *et al.* 2008).

GH deficiency is associated with a proinflammatory state and GH administration has been shown to reduce inflammatory markers in GH deficiency (reviewed by Klibanski 2003). GH exerts many actions on the immune system and immune cells, in turn, express GH and IGF-I receptors (Buul-Offers & Kooijman 1998). Therefore, it is plausible that both GH and IGF-I have direct effects on proatherogenic inflammatory processes. It has also been reported that in GH deficiency, macrophages secrete increased amounts of proinflammatory cytokines and are more susceptible to becoming foam cells (Serri *et al.* 2004). In addition, the lipolytic function of GH may in part explain the reduction of

inflammatory markers: it may be related to a decrease in the amount of abdominal fat which is the source of these proinflammatory markers (Franco *et al.* 2005).

Obesity, especially abdominal obesity, is associated with low levels of GH and IGF-I (Rudman *et al.* 1981, Marin *et al.* 1993). GH therapy increases lipolysis and causes changes in body composition by increasing protein synthesis and fat mobilisation and oxidation. These effects are presumably not mediated by IGF-I and the effects of IGF-I, in turn, depend on the duration of IGF-I administration: short-term treatment decreases oxidation of fat but long-lasting treatment with IGF-I enhances lipid oxidation in GH deficient adults (Mauras & Haymond 2005).

Clearly, there is an association between GH deficiency and obesity, but it is not always straightforward. In other words, it is not known which is the cause and which the consequence. Obesity itself may lead to GH deficiency through mechanisms that impair GH secretion through either metabolic factors like NEFAs and insulin or through central nervous system-related inhibitory factors. IGF-I secretion is not as greatly affected by obesity as GH secretion is, although both increased and decreased total IGF-I levels have been reported in obese subjects. (reviewed by Maccario *et al.* 2000)

Taken together, it appears that the relationship between the GH/IGF-I axis and metabolic risk factors as well as the development of CVD is U-shaped. Both GH deficiency and excess seem to associate with risk factors for atherosclerosis. The same applies for IGF-I. In addition, variations in the concentration of these hormones may demonstrate different effects at different stages of CVD development. The effects of locally produced IGF-I and IGF-I in the circulation add another layer to the complexity of this network and complicates interpreting the results from previous studies.

## 2.4 Obesity as a risk factor for atherosclerosis

It is well known that obesity is a major health problem that is reaching epidemic proportions, especially in the Western countries, though the prevalence is increasing rapidly also in the developing countries. The results from the latest national FINRISK Study 2007 survey, carried out every five years by the National Institute for Health and Welfare using independent, random and representative population samples from different parts of Finland, have demonstrated that Finns continue to gain weight. Only 33% of Finnish men and 48% of Finnish women

can be considered as normal weight and 20% of both genders are obese (http://www.thl.fi). Similar trends are observed all over the world.

Overweight and obesity can be defined on the basis of body mass index (BMI) which is determined by weight (kg) divided by height squared ($m^2$). An individual with a BMI between 25 and 29.9 is considered as overweight and a person with a BMI ≥30 as obese. However, this might be an oversimplified definition, since it fails to consider fat distribution (Sowers 2003). The location of adipose tissue may be essential in defining the associated risk, since an excess of abdominal fat is most clearly associated with metabolic risk factors. Hence, the best way to estimate obesity might be the measurement of waist circumference. Measuring percent body fat would provide a more accurate estimate of the proportion of fat mass in relation to body weight, but, for the time being, it is rarely used since there is no practical and rapid means to measure it reliably in clinical practice (Grundy 2004).

It is well established that obesity is associated with an increased incidence of atherosclerotic CVD. The reason for this association is that obesity is usually accompanied by risk factors for atherosclerotic CVD, like atherogenic dyslipidemia, insulin resistance, a proinflammatory state, a prothrombotic state and hypertension. A significant part of increased prevalence of CVD in obesity is mediated by T2D (Grundy 2004).

### *2.4.1 The link between obesity and type 2 diabetes*

Insulin resistance is associated with both obesity and T2D. In this state, the pancreatic β-cells are not able to compensate for the decreased insulin action by increasing their secretion of insulin. The abnormally high plasma NEFA concentrations are probably the single most critical factor modulating insulin sensitivity (Kahn *et al.* 2006). The excess NEFAs lead to insulin resistance in muscle and liver which further promotes the release of NEFA, as the insulin levels are high but not sufficient to suppress adipose tissue lipolysis (Grundy 2004). In this way, a vicious cycle is created.

There are various reasons why the hyperglycemia associated with T2D may promote atherosclerosis. These include harmful effects on the vascular wall that are caused by the activation and adhesion of monocytes, inhibition of NO production by endothelial cells, and the stimulation of VSMC proliferation. Diabetes is usually also associated with diabetic dyslipidemia that is clearly related to the development of atherosclerosis. It is characterised by reduced HDL

cholesterol concentrations, increased triglyceride-rich lipoprotein concentrations and abnormalities in the composition of HDL, LDL and triglyceride-rich lipoprotein particles. There is also evidence for a chronic subclinical inflammation in the vessel wall, although it is difficult to determine which is the cause and which the consequence, since both atherosclerosis and T2D are insidious diseases that progress gradually over decades. However, it seems that the endothelium directly responds to hyperglycemia and dyslipidemia with an inflammatory reaction. (reviewed by Mazzone *et al.* 2008)

### *2.4.2 Adipose tissue: an active endocrine organ*

Past and gone are the times when the adipose tissue was seen only as storage tissue for TG. At present, it is known that, in addition to storing and releasing energy, the adipocytes actively communicate with at least the central nervous system, the immune system and the cardiovascular system through the secretion of bioactive peptides, known as adipokines. The adipose tissue is also endowed with receptors that enable it to respond to external signals emitted from other hormonal systems and the central nervous system. The various factors secreted from the adipose tissue are involved in many crucial biological processes (reviewed by Klein *et al.* 2006) (Figure 4).

The endocrine function of the adipose tissue appears to depend on the anatomic location of the tissue. This may be related to the fact that the factors secreted by the visceral depots enter the portal vein, and hence, access directly the liver while the factors from the subcutaneous adipose tissue are secreted into the systemic circulation (Kershaw & Flier 2004).

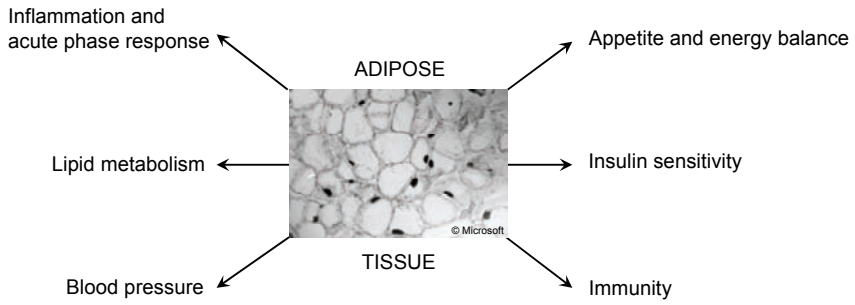


**Fig. 4. Examples of biological processes in which factors secreted by the adipose tissue are involved. Modified from Trayhurn & Wood (2004).**

### *2.4.3 Obesity and inflammation*

Some of the factors secreted by the adipose tissue are directly involved in inflammatory processes and it is well known that obesity is associated with a chronic, low-grade inflammation (reviewed by Fantuzzi 2005). A state of systemic inflammation is believed to mediate also the development of atherosclerosis (reviewed by Mullenix *et al.* 2005). No unequivocal view has yet been established on why obesity is associated with inflammation, but it has been argued that the increased levels of inflammatory markers reflect a spill-over from the inflamed adipose tissue rather than a systemic inflammation as such (Trayhurn & Wood 2004).

The inflammation can at least partly be explained by the inflammatory cytokines released by adipose tissue since their synthesis and secretion are increased in obesity. These cytokines include tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6), IL-1β, IL-10, IL-17D and transforming growth factor-β (TGF-β) as well as many acute-phase proteins, such as plasminogen activator inhibitor-1 (PAI-1), serum amyloid A (SAA) and C-reactive protein (CRP). (reviewed by Trayhurn & Wood 2005)

In addition, it has been demonstrated that in obesity, the adipose tissue is infiltrated by macrophages such that the macrophage content correlates with BMI and adipocyte size. Hence, it was suggested that the macrophages within the adipose tissue are the primary source of TNFα and other proinflammatory molecules in the tissue (Weisberg *et al.* 2003). In addition, several other

adipokines that are neither cytokines nor acute-phase proteins are believed to be involved in the inflammatory response (Trayhurn & Wood 2004). So far, the best characterised of these is adiponectin, which will be discussed in more detail below.

### *2.4.4 Obesity-related peptides*

The discovery of leptin in 1994 (Zhang *et al.* 1994) laid the groundwork for the view that adipocytes have an important role in the secretion of bioactive molecules. Since then, several other adipokines have been discovered and their function in many biological processes is now well characterised. In addition to the adipokines, also the levels of other peptides not secreted from the adipocytes, for example PYY 3–36 (reviewed by Karra *et al*. 2009), have been shown to correlate with obesity.

Leptin is a classical adipokine. It is the product of the *ob* gene and secreted predominantly from adipocytes, in proportion to adipose tissue mass. Leptin plays an important role in the regulation of energy homeostasis by decreasing energy intake and permitting energy expenditure. Paradoxally, obese subjects usually have high leptin levels and it has therefore been suggested that a leptin resistance develops with obesity. Leptin receptors are abundant in the hypothalamus and brainstem, which indicates that leptin functions mainly through the central nervous system to regulate satiety and energy expenditure. (reviewed by Myers *et al.* 2008)

Another well-known and widely studied adipokine is adiponectin, also known as apM1 (adipose tissue most abundant gene transcript), Acrp30 (adipocyte complement-related protein of 30 kDa) adipoQ and GBP28 (gelatine binding protein of 28 kDa). The inconsistency in terminology is due to the discovery of this peptide by four different groups at approximately the same time (Scherer *et al.* 1995, Maeda *et al.* 1996, Nakano *et al.* 1996, Hu *et al.* 1996).

In contrast to leptin and most other adipokines, the expression of adiponectin is decreased in obesity (Hu *et al.* 1996). Adiponectin seems to have beneficial effects on cardiovascular and metabolic functions, since it increases insulin sensitivity, improves endothelial function and has anti-inflammatory properties. (reviewed by Giannessi *et al.* 2007)

Resistin was initially thought to be expressed in adipose tissue based on the original findings in mice (Steppan *et al.* 2001). This view still holds true when one considers rodents, but the results in humans have been controversial and the

more recent studies have suggested that in humans, resistin may be produced mainly by leukocytes and thus, be an inflammatory marker (Reilly *et al.* 2005).

In rodents, resistin seems to be involved in the development of insulin resistance and T2D *i.e.* higher levels of resistin are associated with impaired glucose tolerance and reduced effects of insulin (Steppan *et al.* 2001). Even though it is expressed from different cell types in rodents and humans, resistin may well be associated with the development of insulin resistance and T2D also in humans, through the complex interplay between inflammation, obesity and T2D (Lehrke *et al.* 2004).

Interestingly, recently a transgenic mouse model with macrophage-specific expression of human resistin but lacking adipocyte-derived mouse resistin was generated. When fed a high-fat diet, these mice rapidly developed accelerated white adipose tissue inflammation, increased lipolysis and elevated serum NEFAs. An accumulation of lipids was observed in muscle, which presumably was the primary cause of insulin resistance in these animals. This study demonstrated that macrophage-derived human resistin can contribute to insulin resistance and it also identified the proinflammatory property of human resistin as a major contributor to its role in insulin resistance (Qatanani *et al.* 2009).

Ghrelin is a peptide hormone that was discovered in 1999 as an endogenous ligand for the growth hormone secretagogue receptor (Kojima *et al.* 1999). It is not an adipokine, since it is principally released from the stomach mucosa, but it plays an important role in regulating appetite and weight, and the circulating ghrelin levels are negatively correlated with body fat and BMI (Tschop *et al.* 2001).

Ghrelin is considered as a gut-brain hormone, since it is mainly released from the stomach and it is thought to exert its effects through accessing the brain, either directly or indirectly (reviewed by Higgins *et al.* 2007). Ghrelin increases appetite, but in obese subjects, ghrelin levels are lower when compared to normal weight subjects and also the normally occurring postprandial reductions in plasma ghrelin levels are less marked in obese subjects (le Roux *et al.* 2005). Therefore, it has been suggested that the lesser suppression of postprandial ghrelin levels in obesity may prolong the duration of feeling hungry. Moreover, since another known function of ghrelin is to reduce the speed of gastric emptying, this effect may be faint in obese persons, contributing additionally to a reduced feeling of satiety and thus, increased food intake (Higgins *et al.* 2007). In addition to a reported disturbed ghrelin function in obesity, lower fasting ghrelin concentrations have been observed in T2D even after adjustments for BMI

(Poykko *et al.* 2003) and low plasma ghrelin concentrations may be an indicator of the metabolic syndrome (Ukkola *et al.* 2006).

### *2.4.5 Obesity and the liver*

A very common condition associated with obesity is the development of non-alcoholic fatty liver disease (NAFLD). The term NAFLD encompasses conditions ranging from simple steatosis (fat accumulation in the liver) to steatohepatitis (steatosis accompanied by inflammation and necrosis with or without fibrosis) up to the final stage, liver cirrhosis (reviewed by Schreuder *et al.* 2008). The pathogenesis of NAFLD is at present most commonly explained by the "two-hit" hypothesis. According to this theory, the initial "first hit" is the accumulation of TG within hepatocytes which predisposes the hepatocytes to the "second hits" that cause cellular injury and/or inflammation (Day & James 1998).

It has been proposed that insulin resistance may be a central factor contributing to the accumulation of TG into hepatocytes but, on the other hand, steatosis itself may cause insulin resistance. However, a disturbance between the supply, formation and consumption (through mitochondrial β-oxidation, ketone body production and secretion in VLDL particles) of TG leads to lipotoxicity (Schreuder *et al.* 2008). Lipotoxicity can result in cell death either directly or via the liberation of oxidised lipids. This can trigger the inflammatory response characteristic of steatohepatitis (Farrell & Larter 2006).

Even though the mechanisms leading to NAFLD are only partly understood, the importance of prevention, slowing down the progression or even reversing the state of NAFLD should not be overlooked. Not only is NAFLD the third most important indication for liver transplantation, but also a correlation between hepatic steatosis and CVD has been demonstrated (Schreuder *et al.* 2008).

There is yet no direct drug treatment for hepatic steatosis as such, but some very promising results have been obtained using antidiabetic agents, such as rosiglitazone. It has been reported that these agents decrease liver fat at least in patients with T2D (Tiikkainen *et al.* 2004, Juurinen *et al.* 2008). However, at present, it seems that a gradual weight loss either through lifestyle modifications or anti-obesity surgery may represent the best option for the management of this disease (Farrell & Larter 2006). It was shown for example that a mean of 8% weight loss resulted in an average of 49% decrease in liver fat in obese women (Tiikkainen *et al.* 2003).

### 2.4.6 Management of obesity and the effects of weight loss

The association between obesity and increased cardiovascular risk is well established and it has been shown that there is a dose-dependent effect of BMI on the clustering of cardiovascular risk factors, with atherogenic risk factor clustering worsening in parallel with weight gain (Wilson *et al.* 1999). In addition, obesity debilitates the prognosis of patients with CVD (Dagenais *et al.* 2005). Therefore, it is encouraging to know that even a modest weight reduction of 5–10% has been associated with improvements in glycemic control, systolic and diastolic blood pressure and plasma lipid profile (reviewed by Vidal 2002).

Unfortunately, the regain of the lost weight is a common feature in obese subjects. Follow-up studies on subjects who have lost weight, through behavioural interventions, medications and even surgery, highlight that the typical pattern is a gradual weight regain and stabilisation of a body weight either slightly under pre-weight-loss levels or even exceeding the baseline weight (reviewed by Levy *et al.* 2007 and Mauro *et al.* 2008). In addition there are also risks associated with weight loss, especially with pharmacotherapy (reviewed by Vincent & le Roux 2007) and surgical interventions (reviewed by Bult *et al.* 2008). However, when all the conceivable risk factors are considered, on the whole, the benefits of weight loss are unquestionably greater than its possible negative consequences.

A model of energy homeostasis has been proposed to explain the unsuccessful treatment of obesity. According to this theory, a neurohumoral regulatory system involving catabolic and anabolic pathways is programmed to maintain rather stable adipose stores over long time periods. Catabolic pathways reduce food intake and increase energy expenditure while anabolic pathways promote food intake and weight gain. (Schwartz *et al.* 2003)

Leptin and insulin are probably important signals that stimulate the catabolic pathways during the basal state and as caloric restriction lowers the concentration of these signals, the anabolic pathways become activated and catabolic pathways inhibited. Furthermore, this hypothesis suggests that the anabolic pathways are more sensitive than the catabolic pathways. This means that the system would be more tolerant of weight gain than any corresponding weight loss. (Schwartz *et al.* 2003)

The development of this homeostatic regulatory mechanism can be explained from an evolutionary perspective. When food availability was irregular it was important to develop the ability to store fat to utilize during periods of energy deprivation (Bjorntorp 2001). Since the body resists weight loss, it is possible that

obesity is a chronic disease and hence, the treatment of obese patients should be recognized as involving lifelong intervention (Mauro *et al.* 2008).

## 2.5 Developmental origins of atherosclerosis

For several decades it has been suspected that environmental factors, such as intrauterine undernutrition, can influence early development and that the resultant changes may be permanent and predispose to disease in later life. Animal experiments directly demonstrated that nutrient restriction during gestation led to morphological and/or enzymatic abnormalities in several organs (Shrader & Zeman 1969). The first indirect evidence of this phenomenon in humans were from studies showing that poverty in childhood followed by prosperity was a risk factor for coronary heart disease (Forsdahl 1977). It was also reported that men exposed to the Dutch famine (1944–45) *in utero* during the first half of pregnancy were more obese at the age of 19 years (Ravelli *et al.* 1976).

Subsequent epidemiological studies have shown that undernutrition during fetal life is associated with a higher risk of developing several non-communicable diseases and metabolic disorders. One important landmark was the studies by Barker and co-workers in Southampton, United Kingdom, and hence, the "developmental origins of disease" hypothesis is broadly also known as the Barker hypothesis.

Barker and co-workers observed that certain areas which in the early 20$^{th}$ century suffered high newborn mortality, a phenomenon in those days commonly caused by low birth weight, had the highest numbers in death from coronary heart disease 5 to 6 decades later. This geographical association suggested that intrauterine undernutrition, manifested as low birth weight, increased the risk of having coronary heart disease later in life. (Barker & Osmond 1986)

Further studies have shown an association between low birth weight and CVD (Martyn *et al.* 1998, Barker *et al.* 2002), T2D (Forsen *et al.* 2000, Jones & Ozanne 2007), the metabolic syndrome (Cottrell & Ozanne 2007) as well as several risk factors predisposing to these diseases, such as obesity (McMillen *et al.* 2005, Taylor & Poston 2007), dyslipidemia (Barker *et al.* 1993, Tenhola *et al.* 2000), insulin resistance (Geremia & Cianfarani 2004) and elevated blood pressure (Mogren *et al.* 2001).

These finding have led to the concept that the susceptibility to certain diseases can be "programmed" during development by an insult or a stimulus that has a permanent effect on physiology (Lucas 1991). It is generally accepted that

undernutrition is a factor that can contribute to fetal programming. It is thought that when the fetus is undernourished, it initiates adaptations to ensure its survival and the development of essential organs, like the brain. These adaptations include endocrine modifications and the redistribution of blood flow (Barker 2000). A flowchart of the hypothesis of the programming of atherosclerotic disease is shown in Figure 5.

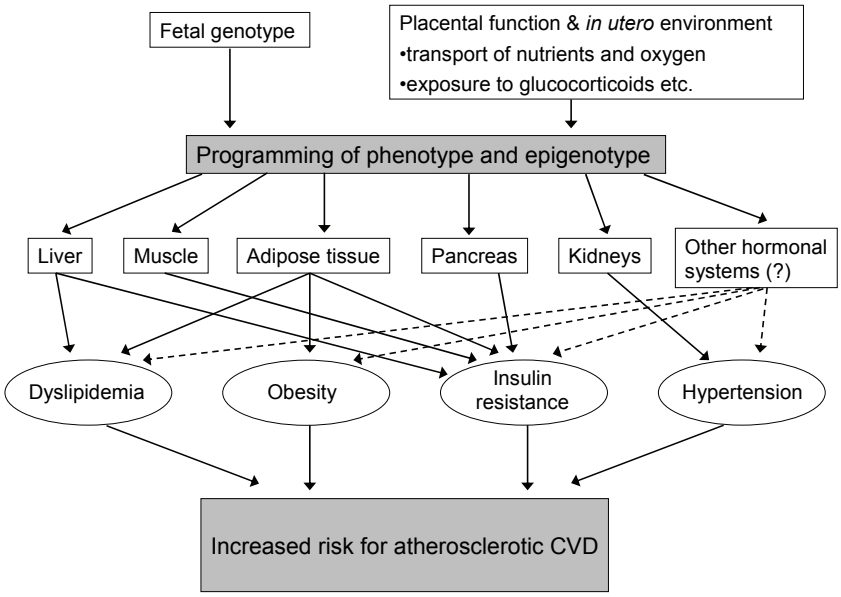


**Fig. 5. A hypothetical scenario about the developmental origins of atherosclerotic disease. Modified from Kajantie *et al.* (2003).**

The "thrifty phenotype" hypothesis introduced by Hales & Barker (Hales & Barker 1992) suggests that inadequate fetal nutrition leads to adaptations aimed at ensuring survival under poor nutritional conditions but problems arise when postnatal nutrition is adequate or even in surplus, leading to the development of insulin resistance and eventually to T2D.

### *2.5.1 Factors influencing fetal growth*

Mechanisms that influence fetal growth include maternal, placental and fetal factors with both environmentally and genetically acting elements (Table 2).

**Table 2. Factors influencing fetal growth. Adapted from Lee *et al.* (2003).**

| Fetal | Maternal | Uterine/placental | Demographic/other |
|---|---|---|---|
| Karyotypic abnormalities | Medical conditions | Placental structural abnormality | Maternal age |
| Other chromosomal abnormalities | Infections | Suboptimal implantation site | Maternal height |
| Congenital anomalies | Nutritional status | Placental abruption | Maternal weight |
| | Substance (ab)use | | Ethnicity |
| | | | Parity |
| | | | Multiple gestation |

Genomic regulation is estimated to determine 40–80% of fetal growth (reviewed by Maulik *et al.* 2006). It is believed that the maternal genetic influence dominates over paternal genetic factors. Environmentally induced epigenetic mechanisms (discussed later in more detail) are also included in the genomic regulation processes. However, it has been shown that in pregnancies after ovum donation, small women have small babies even if the egg donor is a large woman, which suggests that environmental factors would, after all, be more important than genetic factors in determining birth size (Brooks *et al.* 1995).

### *2.5.2 The fetal origins of disease hypothesis updated*

With the accumulating evidence from human and animal studies, the original Barker hypothesis has gradually been modified into a general "developmental plasticity" hypothesis. This means that the same genotype can give rise to varied phenotypes in response to different environmental factors (West-Eberhard 2005) and this period of plasticity probably extends beyond intrauterine life.

Programming may extend through infancy, since many organs continue to develop after birth (Barker 2001). In addition, it is not only nutritional factors that are able to induce programming, but also excess cortisol exposure (Edwards *et al.* 1993) and oxidative stress (Luo *et al.* 2006) have been postulated as being important.

The "fetal insulin hypothesis" has suggested that the association between impaired fetal growth and insulin resistance, along with hypertension and vascular disease, could be caused by an insulin-resistant genotype. That is to say,

impaired insulin-mediated fetal growth and insulin resistance would be manifestations of the same genotype (Hattersley & Tooke 1999).

### *2.5.3 The implication of catch-up growth*

The majority of babies born small for gestational age show catch-up growth that is usually completed by 2 years of age (Albertsson-Wikland & Karlberg 1997). It has been shown that low birth weight accompanied by rapid weight gain in childhood has adverse outcomes, *i.e.* not only low birth weight alone necessarily leads to disease, but the effect is modified by post-natal (nutritional) influences. These findings have led to the "catch-up growth" (Cianfarani *et al.* 1999) or the "growth acceleration" (Singhal & Lucas 2004) hypothesis.

The concept of catch-up growth and its consequences on later health is somewhat controversial. It may be essential to distinguish between early and late catch-up growth in order to evaluate whether it is detrimental or beneficial (Eriksson 2001). While in general it is thought that catch-up growth may increase the risk of developing obesity in later life (reviewed by Ong 2007), it has also been shown that *early* catch-up growth can be advantageous. Both short-term benefits (Victora *et al.* 2001) as well as long-term positive consequences in relation to coronary heart disease (Eriksson *et al.* 2001) have been reported.

During childhood, BMI normally decreases from about the age of 2 years until about 6 years of age. The increase in BMI after this period is known as the adiposity rebound. It has been reported that an early adiposity rebound is associated with high BMI in later childhood and T2D in adult life (Eriksson *et al.* 2003). Furthermore, children that had a low weight gain between birth and 1 year of age and who were thin at the age of 1 year rebounded at an earlier age. This supports the view that promoting early growth is beneficial.

A high-nutrient diet in infancy can program metabolism in a direction that promotes the development of the metabolic syndrome, obesity, dyslipidemia and insulin resistance (Singhal & Lucas 2004). The rapid catch-up growth and possible obesity in later life may be related to more effective physiological mechanisms in storing energy and programming of hyperphagia by intrauterine undernutrition as well as perinatal nutrition (Vickers *et al.* 2000).

It is not known why rapid catch-up growth is detrimental to health, but several explanations have been proposed. One reason might be that it could lead to an unfavourable balance in the lean vs. adipose tissue ratio, since thin babies lack muscle and there is little muscle cell replication after birth, Therefore, a rapid

weight gain would be attributable to the accumulation of excess fat mass. Furthermore, it is possible that intrauterine growth restriction has led to a reduced cell number in several relevant organs and this limited number of cells is then exhausted by the metabolic demands of the growing body. This would lead to disturbances in cell function (Eriksson *et al.* 1999). The latter explanation is related to the "stem cell" hypothesis, stating that intrauterine malnutrition reduces the number of stem cells and the rapid catch-up growth would lead to an early overconsumption of the stem cell reservoir (Cianfarani 2003).

### *2.5.4 The role of epigenetics*

It is likely that persistent phenotypic adaptations programmed during development are the result of permanent changes in gene expression. Epigenetic modifications are defined as heritable changes in gene expression that are not mediated by alterations in the DNA sequence (reviewed by Jaenisch & Bird 2003). At present, DNA methylation is the most extensively studied covalent modification of chromatin that is associated with gene silencing.

DNA methylation denotes the addition of a methyl group at the carbon 5' position of a cytosine (C) base within a CpG dinucleotide. About 70% of all CpGs in the mammalian genome are methylated, but there are small genomic CpG regions called CpG islands where 50% of CpG dinucleotides are unmethylated. Most of the CpG islands are found in the promoter regions for genes, where transcription factors bind in order to initiate transcription of a gene. Methylation can prevent transcription by either hindering the binding of transcription factors or by promoting the binding of a transcriptional repressor, MECP2, that recognises methylated DNA making the promoter stably silent. (reviewed by Robertson & Wolffe 2000)

During early development, the DNA methylation pattern is reprogrammed, starting with a demethylation that erases the methylation patterns inherited from both gametes (with the exception of imprinted genes) by the blastocyst stage, followed by a cell lineage-specific *de novo* remethylation after implantation. The maintenance of methylation patterns is essential for embryonic development. (reviewed by Li 2002)

The nutritional status can directly affect DNA methylation, since it is dependent on dietary methyl group donors and cofactors carrying 1-carbon units (reviewed by Ulrey *et al.* 2005). The DNA methyltransferases that bring about this process are dependent on folate, choline and methionine as methyl donors

and at least vitamins B-6 and B-12, riboflavin and zinc are essential co-enzymes (reviewed by Oommen *et al.* 2005). The molecular effects of nutrients on methylation and gene expression are shown in Figure 6.

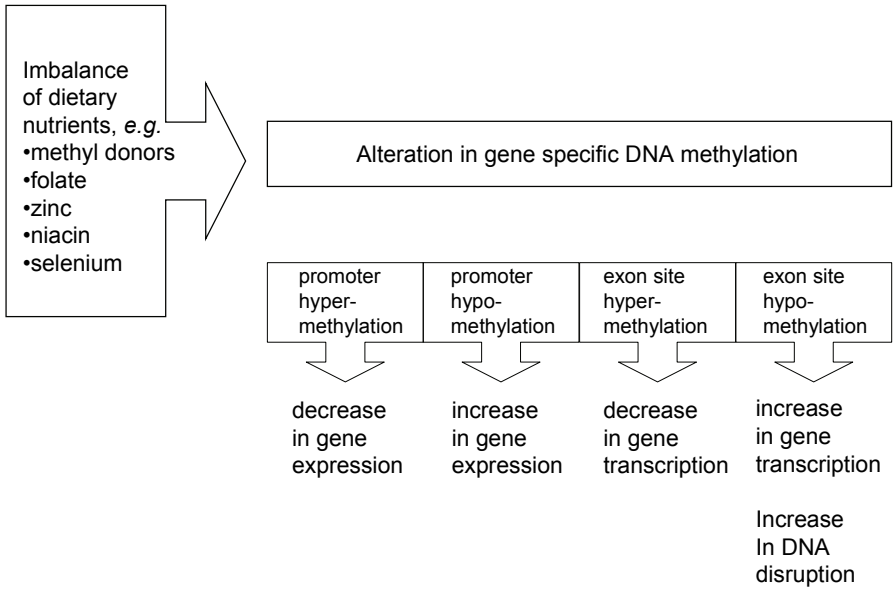


**Fig. 6. The molecular effects of nutrients on gene expression regulation via methylation. Modified from Friso & Choi (2002).**

The Agouti mouse is one of the best studied and characterised rodent models of nutritional influence on DNA methylation in early development (Wolff *et al.* 1998, Waterland & Jirtle 2003). The *agouti* gene regulates the production of mouse hair colour pigment and is usually transcribed only in the skin during a short period. Mutations in different regions of the *agouti* gene produce several alleles of the *agouti* gene with different phenotypes displaying varying coat colours. It has been shown that maternal dietary supplementation affects the methylation regulated transcription status of the *agouti* gene. Hypomethylation is associated with a ubiquitously expressed transcript causing the yellow, obese phenotype (Wolff *et al.* 1998). These results show how diet can have a stable effect on gene expression.

### 2.5.5 Can an unfavourable forecast be reversed?

Even though a large body of evidence seems to confirm the hypothesis that the susceptibility to a certain disease may have been programmed already in early life, several studies suggest that this unfavourable outcome is not inevitable, but can be influenced by therapeutic and lifestyle interventions.

It is likely that promoting women's health during pregnancy and even pre-pregnancy might have long term positive effects. Hence, this stage could be considered as the first target for intervention.

The next target for intervention could be the placenta, since placental function is an essential regulator of fetal growth. Potential intervention strategies to promote fetal growth *e.g.* by maternal IGF-I administration have been discussed but at present, these are only speculative options in humans and require substantially more studies to be done (reviewed by Jansson & Powell 2007).

If an intrauterine growth-restricted baby is born, it should be considered whether promotion of early growth in small babies is beneficial or harmful. Currently, it is a common public health policy to promote the growth of small babies though some studies have indicated that a rapid post-natal growth spurt may, on the contrary, be deleterious (Singhal *et al.* 2004). However, it should be borne in mind that the timing of the catch-up growth may be a crucial factor and hence, promoting *early* growth may be beneficial.

Experiments in rats have shown that the adverse outcome programmed by fetal undernutrition can be prevented or reversed by postnatal IGF-I, GH or leptin administration, even at an adult age (Vickers *et al.* 2001, Vickers *et al.* 2002, Vickers *et al.* 2005). However, these kinds of treatments should be considered carefully with all possible side-effects borne in mind, since GH treatment has evoked a further increase in hyperinsulinaemia in a rat model of fetal undernutrition (Vickers *et al.* 2002). Importantly, for the time being it is unclear when would be the optimal time to undertake these kinds of hypothetical interventions in humans.

Lifestyle modifications may also improve the outcome of individuals with a small body size at birth, which predisposes them to T2D, among others, because of their relatively higher fat to lean mass ratio. In humans, regular exercise has been shown to be related to lower rates of glucose intolerance in subjects with small birth size (Eriksson *et al.* 2004).

Although in the report by Eriksson *et al*, men born small exercised more in adulthood, an animal experiment by Vickers *et al* suggested that an inactive

lifestyle may be programmed by an adverse prenatal environment (Vickers *et al.* 2003). However, a follow-up study demonstrated that this could be reversed by neonatal leptin treatment from day 3 to day 13 which increased locomotor activity in the programmed animals (Vickers *et al.* 2005).

# 3 Aims of the present study

The pathogenesis of atherosclerosis is complex, involving both genetic and environmental factors as well as interactions between these factors. Our aim was to study different molecular mechanisms that could be underlying causes in the development of atherosclerotic CVD.

The specific aims of the present work were:

1. to clarify the role of the GH/IGF-I axis in the development of atherosclerotic CVD by exploring the relation of IGF-I concentrations to carotid artery atherosclerosis and traditional risk factors of atherosclerotic CVD in a large, population based cohort.
2. to study the well-known but inadequately understood association between obesity and atherosclerosis through hepatic gene expression changes induced by a hypocaloric diet in a group of obese women.
3. to explore the effect of fetal undernutrition on obesity-related peptide hormones and plasma lipids in rat offspring
4. to investigate which genes could be involved in the developmental origins of atherosclerotic CVD by examining the consequences of fetal undernutrition on overall gene expression in a rat model.

# 4 Subjects, animals and methods

The study designs and methods used are briefly described below and have been depicted in more detail in the original papers (I-IV).

## 4.1 Subjects (I, II)

### *4.1.1 The GH/IGF-I study*

This study was carried out in a population-based study group (n=1045) consisting of middle-aged (40–59 years) subjects. This was originally collected as a population-based, epidemiological case-control study to address the risk factors and disease end-points of atherosclerotic CVD. Approximately half of the subjects (n=519) were entitled to a special reimbursement for antihypertensive medication. For each hypertensive subject, an age and sex-matched control subject was randomly selected from the Social Insurance Institute register excluding subjects with the right to reimbursement for antihypertensive medication. This OPERA (Oulu Project Elucidating Risk of Atherosclerosis) study group has previously been described in more detail (Rantala *et al.* 1999). However, in the present study, this was treated as one study population whenever possible and the statistical analyses were controlled for study group.

### *4.1.2 The diet intervention study*

The subjects were middle-aged, overweight (BMI≥25 kg/m$^2$) women with gallstone disease scheduled for an elective gallbladder operation within 8 weeks. The weight reduction program was based on dietary counselling. The intervention subjects (n=19) consumed a hypocaloric AHA (American Heart Association) step I diet (Nicklas *et al.* 1997) with a recommended daily energy intake of 5.0 MJ (1200 kcal). The objective was to achieve a reduction of 0.5 kg of body weight per week. The control subjects (n=12) were instructed to continue their habitual diet and not to try to lose weight. During the study period, the subjects visited the laboratory at two week intervals for a clinical follow-up, fasting blood sample collection and body weight recording on all occasions. At the end of the study, surgical liver biopsies were obtained during the gallbladder operation.

## 4.2 Experimental animals (III, IV)

The pilot study was initiated by determination of normal daily food intake during gestation. Individually caged Sprague-Dawley rats (n=4) were fed standard laboratory chow *ad libitum*. The weight of the food consumed was recorded daily throughout gestation. Next, 9 weeks old. first-time-pregnant Sprague-Dawley rats were randomly assigned to three dietary treatment groups on day 4 of gestation. A group of control dams (n=6) was fed *ad libitum* throughout the pregnancy. The calorie-restricted groups received either 75% (n=9) or 50% (n=9) of the previously determined *ad libitum* intake.

Fetuses were collected on gestational days 13 and 17. After parturition, all dams and pups had free access to food. Litter sizes were equalized to 6 pups per litter on post-natal day 1. The excess pups were collected for later analyses. Rat pups were weighed 3 times per week from postnatal day 1 until 1 month of age. The lengths from snout to anus were measured in 1 and 15 days old pups.

At days 1 and 15 post-partum, the dams were sedated and hand-milked. At 1 month of age, blood was obtained from the pups by cardiac puncture after which the pups were killed by decapitation. The experimental design was approved by the Animal Care and Use Committee of the University of Oulu, Finland.

## 4.3 Methods

### *4.3.1 Clinical methods (I-II)*

Body height, weight (I, II) and waist and hip circumference (I) were measured by trained physicians and nurses. In the diet intervention trial (II), the weights were recorded twice during the baseline period and every second week during the diet intervention period. Systolic and diastolic blood pressures were measured with an automatic, oscillometric device (I, II). In the diet intervention study, blood pressure measurements were performed on all appointments.

### *4.3.2 Blood sample assays (I-IV)*

Fasting plasma glucose (I, II), insulin (I-III), cholesterol and TG (I-III) concentrations were measured by routinely used laboratory methods or commercially available kits. Plasma total IGF-I, IGFBP-1 and CRP were measured by commercially available kits according to the manufacturer's

instructions (I). Commercial ELISA (enzyme-linked immune sorbent assay) kits were used to measure rat serum adiponectin, leptin, total ghrelin and resistin concentrations (III).

### 4.3.3 Genotyping (I)

One SNP in the *GH1* gene, T1169A, was genotyped (n=1003) by the restriction fragment length polymorphism (RFPL) method. The genotyping protocol was based on a previously published article (Le Marchand *et al.* 2002). However, the results were not unambiguous since it was sometimes hard to determine the genotype only by looking at the image from the electrophoresis gel. Thus, sequencing was used to confirm the results. Sequencing was performed on an ABI Prism 377 sequencer. The obtained sequences were analysed using the Chromas 2.23 program.

### 4.3.4 Carotid ultrasonography (I)

The intima-media thickness (IMT) of the carotid arteries and the number of plaques were measured by a single trained radiologist using a duplex ultrasound system with 7.5 MHz scanning frequency in B-mode, pulsed Doppler mode and colour mode. IMT was defined as the distance between the media-adventitia interface and the lumen-intima interface. The IMT was measured at five locations; the internal carotid artery (ICA), the bifurcation enlargement (BIF) and three locations on the common carotid artery (CCA) on both sides of the throat, the near and the far wall of the vessel. The examiner searched for the thickest point of IMT at each site avoiding plaques. Only the 10 far wall measurements were used in our study because the near wall dimensions are known to be difficult to measure accurately (Wendelhag *et al.* 1991). The mean IMT was defined as the mean of ICA, BIF and the highest three of CCA measurements.

### 4.3.5 RNA extraction, microarray analysis and qRT-PCR (II, IV)

Total RNA was extracted from liver samples (II) and whole rat fetuses and 1 day old pups (IV). The microarray experiments were performed at the Biocenter Oulu Microarray Core Facility using Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays (II) or GeneChip Rat Expression Array 230 2.0 Arrays (IV). The microarray data was analyzed by GeneSpring 7.2 (II) or DNA-Chip Analyser

(dChip) (IV) software packages. Genes were defined as being differentially expressed if the fold change was at least 2.0-fold.

The microarray data was validated by performing qRT-PCR using TaqMan chemistry on an ABI 7700 Sequence Detection System (II) or by SYBR Green chemistry on an iQ5 real-time PCR detection system (IV).

### *4.3.6 Statistical methods (I-IV)*

The statistical analyses were performed using the statistical analysis software SPSS (versions 11.0, 14.0 and 15.0) except for the analysis of the microarray data in article IV, where the dChip software was used. ANOVA and ANCOVA were used when appropriate, *i.e.* when the data was normally distributed, the variances within each group were approximately equal and the observations were independent. When the data was not normally distributed even after a logarithmic transformation, the non-parametric Mann-Whitney test was used (II, III). In study I, the hypertensive and normotensive groups were treated as one study population and only if any interaction was observed between study group and IGF-I tertile, the groups were analysed separately. The correlation analyses were explored by partial correlation analysis controlled for study group. Linear and logistic regression analyses where performed when appropriate (I). Student's t-test was used to compare gene expression data between groups (II, IV). A *P* value smaller than 0.05 was considered statistically significant. In study I, the false discovery rate (FDR) method was used to control for the problem of multiple comparisons, since the possibility of finding a false positive associating increases along with the number of tests performed. The FDR method takes into account the possibility of erroneous rejections in addition to the possibility of accepting a false positive result (Benjamini & Hochberg 1995).

# 5 Results

The main results of each study are stated below. Additional findings of each study are described in more detail in the original papers (I-IV).

## 5.1 The influence of IGF-I on atherosclerotic CVD (I)

The aim was to study the relationship between IGF-I concentrations and carotid artery atherosclerosis (determined as carotid IMT) as well as the association between traditional cardiovascular risk factors and IGF-I concentrations. In addition, a SNP in the *GH* gene was genotyped.

IGF-I concentrations were positively associated with IMT in women (Figure 7). In men, on the contrary, the association between IGF-I and IMT was negative, although weaker than in women. In addition, IGF-I concentrations were negatively associated with many traditional CVD risk factors in both genders, such as BMI, TG concentrations and CRP.

Genotyping of the *GH* SNP *T1169A* showed that the *A* allele was associated with a favourable metabolic profile, with lower systolic blood pressure in women and lower LDL concentration in the whole study group. The SNP was not related to IGF-I concentrations in our study.

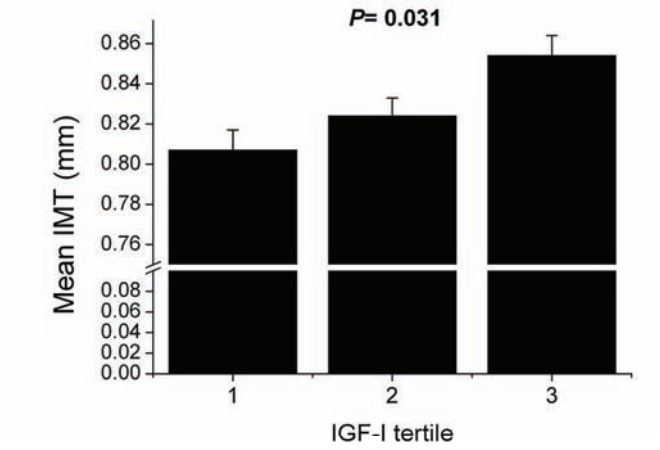


**Fig. 7. The relationship between IGF-I concentrations and IMT in women. The IMT values were adjusted for age, BMI, systolic blood pressure, LDL cholesterol and pack-years of cigarette smoking.**

## 5.2 The effect of weight loss on liver gene expression (II)

We studied hepatic gene expression after a weight reduction trial in overweight and obese women by comparing the microarray results to those obtained in a group of overweight and obese women who maintained their weight.

The most notable effect of the diet intervention was a global down-regulation of gene expression. A total of 142 genes were down-regulated with only one up-regulated in the diet intervention group. The only up-regulated gene was *solute carrier organic anion transporter family member 1A2 (SLCO1A2)*. The distribution of the down-regulated genes in different categories is shown in Figure 8. Interesting examples of the down-regulated genes included *peroxisome proliferator-activated receptor gamma cofactor 1 alpha PGC-1α* and several genes of the ubiquitin cycle.

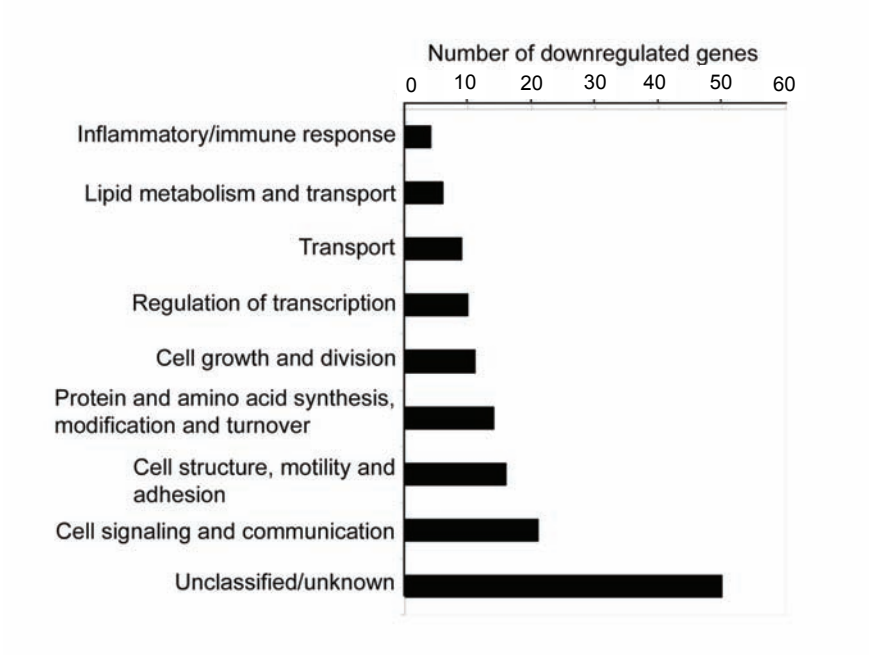


**Fig. 8. The functional classification of the down-regulated genes in the diet intervention group.**

In addition, the diet intervention resulted in decreased fasting plasma TG and insulin concentrations which is in accordance with many previous weight-loss studies.

## 5.3 The consequences of undernutrition during fetal and early postnatal development (III, IV)

The gene expression profiles of rat fetuses on gestational days 13 and 17 as well as 1 day old pups of 25% or 50% energy-restricted and control dams were studied. Overall, the number of genes that were differentially expressed between the feeding groups was small. Interestingly, the amount of genes increased in conjunction with the development of the offspring (Table 3). A change in the expression of many pancreatic digestion enzymes was observed in the 1 day old pups. The dams in the different feeding groups were milked, but no differences in milk protein or fat content were observed between the groups.

**Table 3. Functional classification of the genes differentially expressed between the feeding groups at each time point. The number of genes represents a minimum of 2-fold change in gene expression in the energy-restricted group compared with the control (ad libitum) group.**

| Gene function/process | 13 day old fetuses | | 17 day old fetuses | | 1 day old pups | |
|---|---|---|---|---|---|---|
| | 75% | 50% | 75% | 50% | 75% | 50% |
| Cell growth, communication and adhesion | 1 | 1 | 1 | 2 | 4 | 12 |
| Protein metabolism | 3 | 1 | 1 | 2 | 12 | 8 |
| Carbohydrate metabolism | - | - | - | - | 1 | - |
| Lipid metabolism | - | 1 | - | - | 4 | 2 |
| Cartilage and skeletal development | - | - | - | 3 | - | 2 |
| Unclassified/unknown function | 6 | 5 | 15 | 8 | 8 | 25 |
| Total | 10 | 8 | 17 | 15 | 30 | 49 |

Plasma concentrations of lipids, insulin and obesity-related peptides were measured from 1 month old offspring. Serum cholesterol levels were higher in both food-restricted groups when compared to the control pups. Serum adiponectin concentrations were lower in the 50% food-restricted group and serum resistin concentrations were higher in both food-restricted groups (Figure 9 A & B).

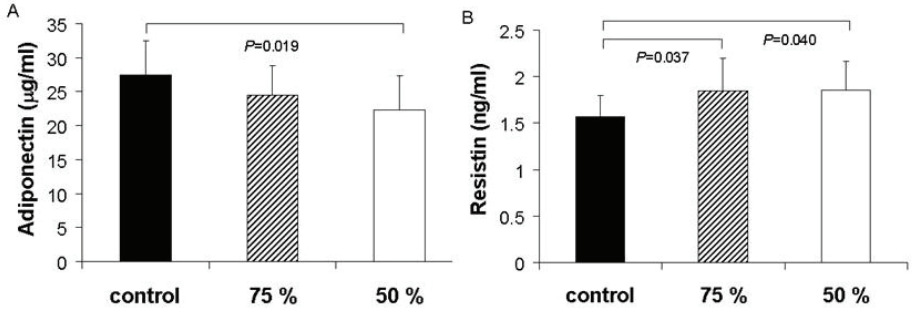


**Fig. 9. Serum adiponectin (A) and resistin (B) concentrations in 1 month old rat pups.**

# 6 Discussion

This work was conducted in order to explore both traditional risk factors of CVD as well as potential novel factors that may be involved in the pathogenesis and progression of atherosclerosis.

## 6.1 The effects of the GH/IGF-I axis on atherosclerosis and its risk factors

The involvement of the GH/IGF-I axis in the pathogenesis of atherosclerotic CVD has been studied widely without any consensus being reached at present. We wanted to investigate the relationship between IGF-I concentrations and carotid artery atherosclerosis and its metabolic risk factors using a population-based, middle-aged Finnish study group.

### *6.1.1 Features of the study population*

This study was carried out with the OPERA study group. It consists of a so-called hypertensive group and age- and gender-matched control subjects. The classification was based on the reimbursement criteria for antihypertensive medication in 1990. Therefore, the control group cannot be considered as normotensive, but nonetheless, the main characteristics of the control group are well comparable to the Finnish population in general. Even though this study was originally collected as a case-control study with a hypertensive and a normotensive group, only if an interaction between the study group and IGF-I tertile was observed, then the analyses were to be performed separately for both groups in the present study. When no interaction was observed, the study groups were treated as one study population and not as two separate groups. In addition, the correlation analyses were controlled for the study group. It was also ensured that the allele frequencies for the *GH* SNP *T1169A* did not differ between the study groups.

### *6.1.2 The measurement of IGF-I*

The total IGF-I concentration was measured from plasma samples by an ELISA kit that recognizes the IGF-I molecule independent of the IGF-binding proteins. The advantages of measuring total or free IGF-I have been discussed widely; for

example it has been argued that free IGF-I may reflect IGF-I bioactivity better than circulating total IGF-I levels (Janssen *et al.* 2003). However, the measurements in our study were performed at a time when no kits for the measurement of free IGF-I were available. It has been shown by correlation studies that under normal conditions total IGF-I is the strongest predictor of free IGF-I (Frystyk 2004). Still, it cannot be ruled out that our results could have been different if free IGF-I had been measured in addition to total IGF-I.

It can also be speculated that the peripheral IGF-I concentrations would be more informative than circulating IGF-I concentrations under certain conditions since locally produced IGF-I acts also through paracrine and autocrine mechanisms (Frystyk 2007). In addition, at least one study has shown a significant individual variability between two IGF-I measurements done within a two week interval (Milani *et al.* 2004). These aspects should be taken into account when designing future studies and they may have influenced the results of our study.

### *6.1.3 IMT as a measure of atherosclerosis*

Several studies have shown that carotid IMT is a strong predictor of future cardiovascular events, such as myocardial infarction and stroke (reviewed by Lorenz *et al.* 2007). In addition, a strong correlation has been demonstrated between IMT values obtained by ultrasound measurements and those determined by histology (Pignoli *et al.* 1986, Persson *et al.* 1994). Therefore, IMT is considered as a valid surrogate marker of atherosclerosis (de Groot *et al.* 2004).

IMT is a non-invasive imaging method and hence, relatively convenient to perform However, there is controversy regarding the implementation of IMT measurements as a screening tool in routine clinical practice (del Sol *et al.* 2001). It is possible that at the individual level, it is not useful in a clinical setting but when many study subjects are examined and the performance is well controlled, it represents a valuable research technique (Ibanez *et al.* 2009).

The lack of any standardized imaging and image analysis protocol is a drawback that can make the comparison between studies difficult. In clinical trials, the measurement most commonly used is from the common carotid artery only, since this is the most reproducible approach. The combination of information gathered from several different segments, as in the present study, probably improves the accuracy of this measure (Ibanez *et al.* 2009).

Abnormal thickening of carotid IMT is considered a marker of atherosclerotic disease. However, more advanced techniques are required if the intention is to analyse vessel wall structure and composition in more detail. Sophisticated non-invasive imaging techniques include multidetector computed tomography which provides excellent visualisation of the coronary arteries (Achenbach *et al.* 2004). A limitation of this approach is the relatively high dose of ionic radiation (Ibanez *et al.* 2009). Magnetic resonance imaging is another example of an advanced non-invasive method. It can be used for the evaluation of the structure and composition of plaques. Intraplaque hemorrhage and the integrity of the fibrous cap can be visualised by this technique (Yuan *et al.* 2001).

### *6.1.4 The GH polymorphism*

The SNP that was genotyped from the *GH* gene had previously been reported to associate with peak GH and IGF-I concentrations (Hasegawa *et al.* 2000, Le Marchand *et al.* 2002). We did not observe any association between this SNP and IGF-I concentrations but GH concentrations were not measured in our study.

IGF-I concentrations are generally believed to reflect GH concentrations, but since IGF-I is bound to IGFBPs that prolong its half-life, the pulsatile release of GH may not be reflected in circulating total IGF-I concentrations. Hence, it would not have been inconsistent that while the SNP has been previously reported to associate with peak GH concentrations, in our study, no association to total IGF-I concentrations was observed. However, the fact that this SNP has been reported to also associate with IGF-I concentrations and this was not seen in our study suggests that this SNP is not a true functional variant. Instead, it is more likely that it was in LD with some other SNP that has affected the IGF-I concentrations in the previous studies.

In our study, the *A* (adenine) allele was associated with a more favourable metabolic profile compared to the *T* (thymine) allele *i.e.* with lower systolic blood pressure in women and lower LDL concentration in the whole study group. The associations to lower LDL cholesterol in the whole study group and lower systolic blood pressure in women were statistically significant. It remains to be investigated whether the observed associations are directly related to this SNP or whether it was in LD with another variant that influenced these factors.

*Limitations of association studies*

Our results may reflect a phenomenon that is commonly seen in SNP studies; the results from one study cannot be replicated in another study group. In fact, one could state that it is not even very likely that the same study results could be expected to be replicated in two study groups from different continents. In the previous studies where the association to IGF-I levels was found, the subjects were Japanese (Hasegawa *et al.* 2000) or Japanese, Caucasian and Native Hawaiian (Le Marchand *et al.* 2002). The pattern of LD in these populations is likely to be very different when compared to our study group because of the population specific demographic history, like bottlenecks, admixture, inbreeding and migration (Terwilliger *et al.* 1998). This could at least partly explain the discrepancy between our results and those reported previously.

It should also be borne in mind that a statistically significant association may be a false positive result, a type I error in statistical terms. The problem of multiple comparisons is well known and relevant. In each test, there is a possibility of 5% of finding a false positive association if the significance level is set at $P<0.05$. As the number of tests increases, so does the probability of making type I errors. Therefore, it is widely argued that statistical adjustments for multiple tests should be made. However, some of these, like the Bonferroni adjustment, have been considered to be too strict and may even lead to a type II errors; rejecting a true association (Perneger 1998). Therefore, the FDR adjustment that was developed to avoid a "too high cost paid for the control of multiplicity" (Benjamini & Hochberg 1995) was used in the present study.

### 6.1.5 IGF-I may have gender specific effects

A positive association between IGF-I concentrations and carotid IMT was observed in women in the present study. It has been proposed that IGF-I might induce IMT thickening at least partly through direct effects of IGF-I on VSMCs (Brevetti *et al.* 2002). This is a possible explanation, since IMT is an imaging method that is unable to distinguish between the intima and media layers of the vessel wall.

The gender difference in the relation of IGF-I concentrations to IMT observed in the present study is intriguing. All statistical analyses showed a positive association between IGF-I concentrations and IMT in women. In men, on the contrary, the association was negative although weaker than the positive

association in women. Some traditional risk factors for atherosclerosis, like age, high BMI, elevated fasting insulin, TG and CRP concentrations were, however, negatively associated with IGF-I concentrations in both genders. These results point to a gender-specific effect of IGF-I on IMT, but no involvement of hormonal factors could be demonstrated in our study. The menopausal status or the use of hormone replacement therapy in either pre- or postmenopausal women did not have any statistical effect on the association between IGF-I concentrations and carotid IMT.

It has been shown that estrogen administration can diminish the concentration of free IGF-I (Veldhuis *et al.* 2005), but since free IGF-I was not measured in our study, it is impossible to know whether there was some discrepancy in the ratio of free/total IGF-I between the genders and if the results could be explained through this effect. It is possible that there exists a complex interplay between sex steroid hormones and IGF-I that would need further examination.

Another possible explanation for the positive association between IGF-I concentrations and IMT in women is that IGF-I by itself is a rather weak risk factor for atherosclerosis and hence, its effect appears more clearly in a study group where the overall disease risk is small. It is generally known that women have a lower risk for CVD, at least until postmenopause (reviewed by Collins 2006). Likewise, the Japanese have lower risk for CVD when compared to the Western countries (Ueshima 2007). Therefore, it is interesting that a positive association between IGF-I concentrations and IMT has also been demonstrated in Japanese men without any overt carotid artery atherosclerosis (Kawachi *et al.* 2005).

## 6.2 The effect of obesity and dieting on liver gene expression

The effects of obesity and weight loss on liver gene expression were studied to understand better at the molecular level why obesity increases the risk of CVD and what happens during weight loss *i.e.* why weight loss improves the metabolic risk profile.

### *6.2.1 Concerns related to the study design*

In this study, a group of overweight (BMI≥25) and obese (BMI≥30) women were on a hypocaloric diet while the controls were asked to maintain their current diet and weight. They all were scheduled for a gall bladder operation which at that

time was not always performed by laparoscopic surgery. Therefore, a liver sample could be obtained during the surgery.

One major limitation of this study design is that no liver samples could be obtained before the diet intervention was initiated. However, it was thought that a comparison of gene expression profiles could be made by using samples from the control subjects, who were equally overweight and obese, since their gene expression profiles would represent the baseline situation.

Liver samples that yielded RNA of good quality were available from 7 diet intervention subjects and 8 control subjects. Of these, 4 samples from each group were selected for the microarray analysis. In the selection process, we tried to select "pairs" between the two groups. The major criteria were overweight or obesity of similar degrees and approximately same age of the subjects.

In a small study group like this it is to be expected that relatively large heterogeneity will be observed among the subjects. Therefore, one should be careful in drawing conclusions from the results, since they may simply reflect random variation (between-individual variation) and not be related to the treatment.

This heterogeneity can make it difficult to detect any statistically significant results since common trends in small groups may not be as easy to observe as in larger study groups. In other words, too small samples lack statistical power to detect biologically important results. On the other hand, the results may be biased only by change in a small group by only one or two exceptional samples.

One more consideration in this study design is how the liver condition is reflected in the gene expression profile. It is well known that NAFLD is common in obesity and the pathologist's comments on liver histology, when available, indicated that different stages of NAFLD were indeed present in the study subjects. Unfortunately, this data was not available from all the subjects in our study and hence, this aspect can only be speculated without any definite knowledge about its relevance.

### *6.2.2 The novel results warrant further studies*

We expected to see expression changes in genes that have been associated with lipid and/or glucose metabolism in previous studies, and perhaps an indication of reduced expression of inflammatory genes, since obesity has been associated not only with various metabolic disturbances but also with low grade inflammation.

Instead, a global decrease in gene expression as a consequence of the hypocaloric diet was observed in the present study.

It is possible that the reduction in energy intake resulted in an overall diminished metabolic rate. This would represent a compensatory mechanism of the body striving to resist the weight loss. Over the course of evolution, this strategy has been essential in order to ensure survival of humans during periods of energy deprivation (Björntorp 2001).

One interesting example of the down-regulated genes was a group of genes involved in the ubiquitin cycle. The ubiquitin-proteasome system participates in the degradation of misfolded or otherwise damaged, *e.g.* oxidised proteins (Nandi *et al.* 2006). Hence, our results may be related to a reduced amount of damaged proteins in response to the hypocaloric diet. Consistent with this, it has been shown that dietary restriction and weight loss resulted in a decrease in reactive oxygen species generation by leucocytes and oxidative damage to lipids, proteins, and amino acids in obese subjects (Dandona *et al.* 2001). In addition, a similar result has been reported in animal studies where caloric restriction was associated with 55% lower levels of endogenous ubiquitin-protein conjugates in soluble liver supernatants (Scrofano *et al.* 1998). Our results could thus be considered as a marker of reduced oxidative stress which has been proposed to have a causal role in the pathogenesis of atherosclerotic CVD (Singh & Jialal 2006, Esposito *et al.* 2006).

Another noteworthy gene was *peroxisome proliferator-activated receptor gamma cofactor 1 alpha (PGC-1α)*. This is a transcriptional coactivator that plays a role in many biological responses related to energy homeostasis and its expression is increased in insulin resistance and diabetes (Finck & Kelly 2006). In our study, the level of fasting insulin was significantly reduced after the diet intervention, indicating that insulin sensitivity had improved and this reduction in the expression of *PGC-1α* is in accordance with this hypothesis.

The only up-regulated gene in our study was *solute carrier organic anion transporter family member 1A2 (SLCO1A2)*. The gene product is a multispecific transporter that mediates hepatic uptake of organic anions from sinusoidal blood and its substrates include bile acids (Kullak-Ublick *et al.* 1995). Therefore, the upregulation of this gene may be related to unfavourable changes in the gallbladder bile composition that have previously been demonstrated during dieting (Shiffman *et al.* 1992). Such changes are associated with the formation of gall stones and the up-regulation of *SLCO1A2* could reflect an effort to protect the hepatocytes against the altered bile composition evoked by weight loss.

Taken together, our results suggest that the regulation of energy metabolism in the liver is very complex, affecting several genes that are not so self-evidently involved in the processes that are known to be modified in obesity and/or weight loss. It remains to be investigated how these gene expression changes could affect biological pathways and whether they can be demonstrated also at the protein level.

## 6.3 Energy restriction-related molecular changes in rat fetuses and pups

A rat model of gestational undernutrition was used to explore the molecular changes in gene expression profiles and plasma peptide concentrations of the offspring. This study was based on the previously reported associations between low birth weight (assumed to be a manifestation of inadequate nutrition supply during fetal development) and future predisposition to metabolic disorders that may be involved in the development of CVD.

### *6.3.1 The importance of a pilot study*

Several different animal models of fetal undernutrition have been studied previously. In these studies, the undernutrition may have lasted for a limited time span during gestation (early or late gestation), throughout the entire gestation or may even have been prolonged into postnatal life. In some cases, the diet restriction has been specific, that is, a particular macro- or micronutrient like protein or folate may have been reduced while the energy content has been similar to control chow. Limiting the food supply is only one way of restricting the nutrient supply of the fetuses. Another relatively common method is ligation of the uterine artery. (reviewed by Vuguin 2007)

Due to the variability in practices of inducing fetal energy restriction it was necessary to conduct a pilot study. The aim was to obtain preliminary data to assess whether a larger-scale project would be worth conducting and what to focus on if such a project was initiated. A pilot study also gives one an opportunity to practice, fine-tune and validate the methods that will be used in the full-scale study (Ruxton & Colegrave 2006).

A novel aspect in our study design was to analyse global gene expression during fetal development and early post-natal life. Apparently, this has not been reported previously, or at least not in a similar context as in the present study.

Since this was a pilot study, the groups were kept small and the follow-up period of the pups lasted only until one month of age. However, these novel preliminary results were interesting.

### *6.3.2 The choice of the animal model*

The choice of species is an important aspect in animal studies. Humans and rodents resemble each other in many ways with similar organs, genes and biological pathways. However, some major differences exist as well. One of the most evident is that rodents are born with an underdeveloped brain and endocrine system that continues to mature significantly during the weaning period (Vuguin 2007). Therefore, it is obvious that results from animal studies may not directly be applicable to humans.

The rat was considered an appropriate animal model for this pilot study since the sample sizes would be large enough to permit the analysis of several biomarkers. Another factor to be evaluated was whether to use an outbred strain or an isogenic one. The genetic and phenotypic variability is greater within an outbred strain when compared to an isogenic strain. This means that more animals are needed to achieve statistically reliable results. However, when comparing the variation between isogenic strains, it is usually greater than within an outbred strain and therefore the results obtained using one isogenic strain may not be generalised to other strains or species. Therefore, the use of the outbred Sprague-Dawley rat was considered to be the better option.

One drawback in using the Sprague-Dawley rat is that it is believed that the end-point of our interest, atherosclerosis, is difficult to detect in this strain. Conventional rodents do not easily develop atherosclerotic plaques even when fed a high-fat diet. In the pilot study, this was not a significant problem since the pups were followed up only until one month of age and atherosclerosis would probably not be detected at that time in any rat strain. With a longer follow-up period, presumably metabolic disturbances like insulin resistance and accumulation of excess adipose tissue can be detected in this model. Hence, the risk factors that predispose to atherosclerosis could be studied even if the end-point itself might not be measurable.

The small number of animals might weaken the credibility of the results of our study. However, laboratory rats, even if an outbred strain is used, are likely to be much more homogenous than freely-living animals or humans. Therefore, the variability between animals in our study may be considered small enough to

obtain reliable results. Nonetheless, the results require further verification with a larger study group.

### 6.3.3 The effect of undernutrition on gene expression

A relatively small number of differentially expressed genes in the fetuses and pups were detected. Many of the genes were involved in developmentally fundamental processes, like cell growth, adhesion and communication and hence, participate in several crucial processes. Therefore, one could speculate that the changes in the expression of such genes may have profound effects on organ development. On the other hand, major and extensive changes are not likely to occur in the fetal stage since they would probably result in spontaneous abortion of the damaged fetus.

In the 1 day old pups, several genes encoding pancreatic digestion enzymes were affected by the energy restriction. These produce proteins that are all secreted from the exocrine pancreas. Previous studies have shown that the development of the endocrine pancreas is severely affected by fetal undernutrition in rats, leading to insulin resistance in later life (reviewed by Fowden & Hill 2001). The endocrine pancreas was not examined in the present study but interestingly, our results suggest that also the exocrine pancreas may be affected by fetal undernutrition. The long-term effects of a possible pancreatic dysfunction remain to be investigated. Given the fundamental role of the pancreas in glucose and insulin metabolism as well as in the digestion of nutrients, significant perturbations might be expected. In humans, a reduced secretion capacity of the exocrine pancreas is associated with diabetes (Hardt *et al.* 2000).

The milk composition of the dams was also analysed to see whether the observed gene expression changes could be explained by alterations in milk quality. The major components of milk, protein and fat, were determined but no statistically significant differences between the feeding groups could be detected. This suggests that the gene expression changes may be related to the function of the exocrine pancreas itself and were not induced by milk quality.

If the gene expression in specific organs or tissues had been analysed, perhaps a completely different set of genes that were differentially expressed between the groups would have been observed. In our study design, these subtle differences may have been overwhelmed by the expression changes in the "fundamental" genes that are likely to be operating in the development of several organs.

### 6.3.4 The observed changes in peptide hormones may predispose to insulin resistance

The peptide hormones adiponectin and resistin are associated with obesity and insulin resistance. These peptides were affected by fetal undernutrition in our study, since we detected higher resistin and lower adiponectin plasma concentrations in the pups from the undernourished dams. These kinds of changes may predispose the pups to insulin resistance and T2D in later life. This conjecture is based on previous studies that have reported elevated resistin levels in animal models of insulin resistance (reviewed by Rea & Donnelly 2004) and that administration of resistin to healthy animals impairs insulin action (Steppan *et al.* 2001). In addition, the down-regulation of adiponectin concentrations in the undernourished pups could also contribute to the development of insulin resistance since it has been demonstrated in both humans (Matsuzawa *et al.* 2004) and mice (Combs *et al.* 2003) that low adiponectin levels are associated with insulin resistance.

However, in our study, at 1 month of age, no changes in fasting insulin concentrations were seen between the groups though one drawback in this study was the lack of any blood glucose measurements. Therefore, no evidence of impaired insulin action or insulin resistance could be demonstrated in this pilot study. It is possible that a longer follow-up period would be required before the manifestation of any signs of altered glucose/insulin metabolism would be apparent.

## 6.4 Clinical relevance of the studies

There is a continuing quest for new biomarkers and susceptibility alleles for atherosclerosis. Positive findings are, of course, interesting as such, from the perspective of basic research. However, in most cases the ultimate goal is to find markers for screening, diagnosis and prognosis which will be beneficial in clinical use. These could assist in the prevention of disease (screening biomarkers), verifying diagnosis (diagnostic biomarkers) and predicting the disease progression and assist in planning intervention strategies in individuals with overt disease (prognostic biomarkers) (Gerszten & Wang 2008).

An important question when considering the usefulness of new screening biomarkers or genetic variants in clinical management of atherosclerosis is what

additional value they provide when compared to the measurement of traditional risk factors, such as plasma lipoprotein levels.

Some experts have proposed that genetic testing for CVD is not ready for clinical use at present. It can be argued, for example, that genotyping the *APOE* genotype might not be worthwhile, since the *APOE* variants exert the majority of their effects on CVD risk through plasma lipid levels and hence, including lipid levels in the risk algorithm will remove much (or even all) of the information that would be obtained by determining an individual's *APOE* genotype (Humphries *et al.* 2004).

However, the genetic variants may affect an individual's response to drugs. Considering the large number of patients receiving different drugs for the prevention and treatment of CVD, even small a variation may be significant when evaluating the efficacy and safety at the population level (Arnett *et al.* 2007). Perhaps most importantly, genetic studies may help clarify the biological mechanisms that are involved in disease development and progression.

The clinical relevance of these results is not unambiguous. Rather, they warrant further studies. Our primary aim is to conduct a larger-scale rat study to elucidate the developmental origins of atherosclerosis. The pilot study already provided some hints of what kind of impact could be seen in fetally undernourished offspring after a longer follow up period. In addition, various novel factors will need be investigated.

To summarise, the observed associations provide important clues for the planning of future studies.

# 7 Conclusions

The purpose of this study was to explore both traditional as well as potential novel risk factors involved in the pathogenesis of atherosclerosis. The focus was upon the GH/IGF-I axis, obesity, and the developmental origins of atherosclerosis. The main findings of the present study are as follows:

1. Low IGF-I concentrations were associated with several risk factors for atherosclerosis. However, a positive association between IGF-I concentrations and carotid artery IMT was also observed. The results suggest that IGF-I could be involved in the development of atherosclerosis, but the association appears to be influenced by gender, since no such positive association was observed in men. To our knowledge, this has not been reported earlier, perhaps because it has not been studied as such.
2. The hypocaloric diet and weight loss resulted in a global decrease in hepatic gene expression. This may be related to a diminished metabolic rate as a compensatory mechanism as the body to strives to resist weight loss. Some of the observed gene expression changes could plausibly be connected to a reduction in oxidative stress and improved insulin sensitivity in the diet intervention group. In addition, in the present study several genes not previously linked to obesity and weight loss were down-regulated and one could argue that many of these could be worthwhile studying in more detail.
3. Maternal energy-restriction during gestation resulted in elevated serum total cholesterol levels, elevated serum resistin concentrations and lower serum adiponectin concentrations in 1 month old rat offspring when compared to the control group. The observed changes in the obesity-related peptide hormones may predispose the offspring to insulin resistance in later life. This, along with the elevated cholesterol levels, suggest that undernutrition during fetal development can induce unfavourable changes that may be related to the development of CVD.
4. Energy-restriction during gestation affected a surprisingly small number of genes in 13 and 17 day old rat fetuses and 1 day old rat pups, probably because fetal development is strictly controlled and no major changes can occur without the termination of gestation. The expressions of many pancreatic digestion enzymes were affected in the 1 day old pups but no major differences in milk composition between the feeding groups could be demonstrated. Therefore, the results may reflect significant alterations in

pancreas function that are a consequence of fetal undernutrition. The consequences for disease development remain to be investigated, but given the fundamental role of the pancreas in insulin and glucose metabolism as well as digestion, some important changes could be expected.

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# Original articles

I Hietaniemi M, Pöykkö SM, Ukkola O, Päivänsalo M & Kesäniemi YA (2005) IGF-I concentrations are positively associated with carotid artery atherosclerosis in women. Ann Med 37(5): 373–82.

II Hietaniemi M, Jokela M, Rantala M, Ukkola O, Vuoristo JT, Ilves M, Rysä J & Kesäniemi YA (2009) The effect of a short-term hypocaloric diet on liver gene expression and metabolic risk factors in obese women. Nutr Metab Cardiovasc Dis. 19(3):177–83.

III Hietaniemi M, Malo E, Jokela M, Santaniemi M, Ukkola O & Kesäniemi YA (2009) The effect of energy restriction during pregnancy on obesity-related peptide hormones in rat offspring. Peptides 30: 705–709.

IV Hietaniemi M, Santaniemi M, Malo E, Ukkola O, Kesäniemi YA & Jokela M. Gene expression profiles in fetal and neonatal rat offspring of energy-restiricted rat dams. Manuscript.

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# ACTA UNIVERSITATIS OULUENSIS



ISBN 978-951-42-9128-9 (Paperback)
ISBN 978-951-42-9129-6 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)

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