# CYTOKINES IN METABOLIC FUNCTIONS

**Anna Benrick**

UNIVERSITY OF GOTHENBURG

Section of Endocrinology
Institute of Neuroscience and Physiology
The Sahlgrenska Academy at University of Gothenburg
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# ABSTRACT

During infections, circulating cytokines are largely produced by immune cells. In healthy obese individuals, large parts of these circulating cytokines are produced in adipose tissue, for instance by macrophages that have accumulated there. The aim of this thesis was to investigate the role of cytokines, in particular interleukin-6 (IL-6), IL-1β and leukemia inhibitory factor (LIF), in the regulation of metabolism and body fat mass. Furthermore, we also wanted to examine the role of the IL-6 signal transducer (IL6ST)/gp130 receptor signalling.

We have previously shown that IL-6 depleted (IL-6 -/-) mice develop late-onset obesity and we have now found a similar effect on IL-1 depletion. We have used IL-1 receptor type I depleted (IL-1RI -/-) mice to study the role of endogenous IL-1 on obesity, as measured by DEXA. The obesity in IL-1RI -/- was accompanied by decreased insulin and leptin sensitivity. Spontaneous locomotor activity and fat utilization, as measured in metabolic cages, were decreased in pre-obese IL-1RI -/- animals. At the hypothalamic level, deficiency of endogenous IL-1 activity in knockout mice was associated with enhanced expression of the obesity promoting peptides NPY and MCH, and decreased expression of the obesity suppressing peptide orexin. In IL-6 -/- mice, the expression of corticotrophin releasing hormone, a known stimulator of energy expenditure and the sympathetic nerve system, was decreased, as shown by RT-PCR. Moreover, endogenous IL-6 and IL-1β seemed to affect each others' expression in the hypothalamus. Therefore, IL-6 and IL-1 may interact in the CNS, presumably in the hypothalamus, to suppress fat mass, possibly by increasing energy expenditure and maybe especially fat burning.

LIF is a member of the IL-6 receptor family, which shares the IL6ST/gp130, and has been reported to decrease obesity. We found that systemic LIF treatment could reduce white and brown fat depots in ovariectomized mice, suggesting that LIF can reduce obesity independently of estrogen signalling.

Obesity and inflammation are key components in the development of atherosclerosis and myocardial infarction. We identified an association between an IL6ST/gp130 polymorphism in amino acid 148 (Gly/Arg) and risk of myocardial infarction in a hypertensive population. *In vitro* studies showed decreased proliferation and lower STAT-3 phosphorylation in cells transfected with gp130 148Arg compared to gp130 148Gly. Structural modelling suggested changes in the stability and functional properties of the gp130 148Arg molecule.

The present results suggest that the cytokines IL-6, IL-1 and LIF are involved in the regulation of body fat mass and energy expenditure. The effects of IL-6 and IL-1 may be exerted at the CNS level and involve altered expression of hypothalamic peptides regulating fat mass and energy expenditure. This can constitute a possible mechanism contributing to the mature-onset obesity in IL-6 -/- and IL-1RI -/- mice. LIF may suppress obesity via estrogen independent effects in the periphery. In human subjects, the 148th amino acid arginine of the gp130 receptor is associated with decreased risk of myocardial infarction, possibly due to an impaired responsiveness to cytokines in the IL-6 receptor family.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals in the text:

**I. Mature-onset obesity in interleukin-1 receptor I (IL-1RI) knockout mice**
M Garcia, I Wernstedt, A Berndtsson, M Enge, M Bell, O Hultgren, M Horn, B Ahrén, S Enerback, C Ohlsson, V Wallenius, J-O Jansson
*Diabetes, 55:1205-1213, 2006*

**II. Deficiency of interleukin-6 (IL-6) or IL-1 action influences hypothalamic fat regulating peptides**
A Benrick, L Strandberg, E Schele, S Pinnock, E Egecioglu, I Wernstedt, M Enge, S Dickson, J-O Jansson
*Manuscript*

**III. Leukemia-Inhibitory Factor reduces body fat mass in ovariectomized mice**
J-O Jansson, S Moverare-Skritic, A Berndtsson, I Wernstedt, H Carlsten, C Ohlsson
*European Journal of Endocrinology, 154, 349-354, 2006*

**IV. A non-conservative polymorphism in the IL-6 signal transducer (IL6ST)/gp130 is associated with myocardial infarction in a hypertensive population**
A Benrick, P Jirholt, I Wernstedt, M Gustafsson, J Scheller, AL Eriksson, J Borén, T Hedner, C Ohlsson, T Härd, S Rose-John, J-O Jansson
*Regulatory Peptides, 146, 189–196, 2008*

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# ABBREVIATIONS

| | |
|---|---|
| AgRP | agouti-related protein |
| ARC | arcuate nucleus |
| BAT | brown adipose tissue |
| CNS | central nervous system |
| CNTF | ciliary neurotrophic factor |
| CRH | corticotrophin releasing hormone |
| CVD | cardiovascular disease |
| DMN | dorsomedial nucleus |
| HPA | hypothalamic-pituitary-adrenal |
| ICV | intracerebroventricular |
| IL-1 | interleukin-1 |
| IL-1Ra | interleukin-1 receptor antagonist |
| IL-6 | interleukin-6 |
| IL-6Rα | interleukin-6 receptor α |
| IL6ST | interleukin-6 signal transducer |
| i.p. | intraperitoneally |
| i.v. | intravenously |
| LHA | lateral hypothalamic area |
| LIF | leukemia inhibitory factor |
| LIFR | leukemia inhibitory factor receptor |
| LPS | lipopolysacharide |
| MCH | melanocyte concentrating hormone |
| NPY | neuropeptide Y |
| NTS | nucleus of the solitary tract |
| OVX | ovariectomized |
| POMC | pro-opiomelanocortin |
| PVN | paraventricular nucleus |
| RER | respiratory exchange ratio |
| SNP | single nucleotide polymorphism |
| SNS | sympathetic nerve system |
| TNF-α | tumour necrosis factor-α |
| UCP | uncoupling protein |
| VMN | ventromedial nucleus |
| -/- | depletion |

Abbreviations occurring only once or twice in the text are generally spelt out where they occur.

# INTRODUCTION

## Metabolic Regulation

The history of the scientific study of metabolism stems from 1614 when Italian Santorio described how he weighed himself before and after eating, sleeping, working, fasting, drinking, and excreting. He found that most of the food he took in was lost through what he called insensible perspiration [1].

Metabolism is the chemical reactions that occur in the body to convert the food we eat into energy. Since energy can not be created or destroyed, according to the first law of thermodynamics, it has to be used or stored within the body. The metabolic processes in the body do not happen at random, but are tightly regulated to enable use of the energy in ingested food in the most efficient way. This is especially important as energy intake usually is not matched with energy demanding activities. Therefore, the ability to store energy that can be released when needed is crucial for survival. The metabolic regulation depends mainly on endocrine and neuronal systems derived from the central nervous system (CNS) and periphery, to maintain homeostasis. These systems sense the energy balance and regulate the storage and release of energy [2]. In the end the metabolic regulation occurs at a molecular level by modulations of enzyme activity. A striking feature of metabolism is the similarity of the basic metabolic pathways between vastly different species, a result of early appearance in evolutionary history, and the high efficiency of these pathways.

## Obesity

### Definition and Consequences

Obesity was once considered a symbol of wealth and social status, but in modern Western culture were food supply mostly is secured, the obese body shape is widely regarded as unattractive and obesity is often seen as a sign of lower socio-economic status [3]. However, obesity should not be considered only as a cosmetic problem. In clinical practice, obesity is viewed as a serious public health problem, with reduction in life expectancy. In fact, obesity is a major risk factor for cardiovascular disease (CVD), e.g. atherosclerosis, hypertension, stroke, and myocardial infarction, and type-2 diabetes, sleep apnea, depression and certain cancers [3, 4].

The definition of obesity varies, but clinically it may be regarded as a chronic condition in which body fat is increased to a point where it is a health hazard.

### ”The Thrifty Genotype”-Hypothesis

It is widely accepted that obesity is the result of interaction between genes and the surrounding environment. In 1962 James Neel suggested that the human population carries a genetic predisposition to store body fat, because, during the first 99% of *Homo sapiens* life on earth, when we lived in hunter and gatherer cultures, there was often either feast or famine. During periods of famine selection would favour individuals that had been more successful in body fat deposition, i.e. individuals with thrifty genes [5]. In the modern world, where food is available in abundance, we are programmed to deposit fat in preparation for a famine that never comes. Therefore, we see an increase in the frequency of obesity as more and more people have come to enjoy the blessings of civilization. Still, if thrifty genes had a selective advantage, why did not the 35-40% of the population that has stayed lean inherit these genes?

Recently, the thrifty genotype hypothesis has been questioned, mainly because famines are rare and infrequent phenomena that are insufficient for thrifty genes to propagate [6]. Instead, it was suggested that the absence of human predation around two million years ago, led to changes in body fat mass due to random mutations rather than direct selection. Under predation pressure, the risk of starvation and poor immune response keeps body masses up while the risk of being killed by a predator keeps body masses down, resulting in small variations in body fat mass [6]. Consequently, the absence of predation removes the upper limit of body mass, allowing a drift upwards resulting in obesity, while there still is a strong disease-related selection against too low body masses. Moreover, since the upward drift is presumed to happen at random, this can explain why many individuals are still normal weigh.

Whatever theory that is put forward to explain body weight and fat regulation, it follows the first law of thermodynamics; that changes in body fat can not occur unless there is a difference between energy intake and energy expenditure.

## CNS Regulation of Energy Balance

### CNS Control of Body Weight

Many adult people maintain a relatively stable body weight throughout life and human and animal studies show that individuals that are forced to

over eat during shorter periods return to their initial body weight when allowed to eat only as much as they please [7]. This suggests that each individual has its own set-point and that a change in body weight is counteracted by regulatory processes of energy homeostasis. Moreover, despite great variations in daily calorie intake and physical activity, the total energy intake tends to match energy expenditure over time (Figure 1).

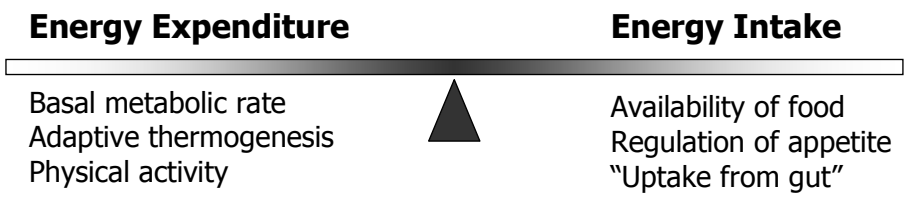


**Figure 1. Energy balance and its components**
The first law of thermodynamics states that energy can neither be created nor be destroyed. Thus, if the energy intake exceeds the energy expenditure, the remaining energy will be stored. Both energy intake and energy expenditure are regulated by biological control mechanisms, which aim to achieve long-term energy balance and a stable body weight. Adapted from Spiegelman et. al.[8].

Energy homeostasis and body weight is maintained relatively constant by nutrient signalling from the periphery to the CNS and back. This requires input from endocrine and neural signals, produced in proportion to body fat content, informing the CNS of the current energy status of the body.

Adiposity signals like leptin and insulin interact with pathways in the hypothalamus, stimulating satiety and energy expenditure. Afferent signals from the liver and the gastrointestinal tract are transmitted through the vagus nerve and sympathetic nerve fibres to the nucleus of the solitary tract (NTS), where they are integrated with hypothalamic input [2, 9]. Moreover, leptin action in the arcuate nucleus (ARC) has been shown to regulate brainstem response to satiety signals in the NTS, via connections between the hypothalamus and brainstem [10] (Figure 2).

## Hypothalamic Regulation of Energy Intake and Expenditure

Electrical stimulation and lesion studies in the 1940´s and 50´s showed that there is a satiety centre in the hypothalamus (now known to include ventromedial nucleus (VMN) and the ARC), and that these parts of the hypothalamus send inhibitory projections to the hunger centre in the lateral hypothalamic area (LHA) [11]. Two orexigenic peptides have been found in the LHA, melanocyte concentrating hormone (MCH) [12] and orexin [13], supporting previous findings that this is a hunger centre. Later

it also became clear that the hypothalamus is important for the regulation of energy expenditure [11].

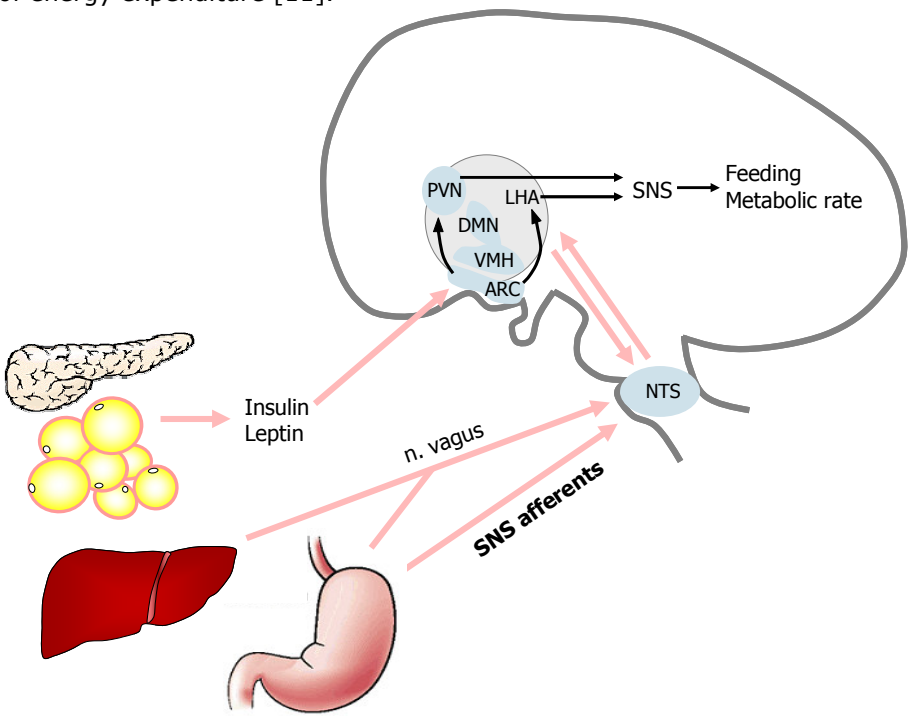


**Figure 2. Adiposity signals to the ARC interact with central autonomic circuits to regulate body weight**
Adiposity signals, such as leptin and insulin are transported with the blood to the brain where they interact with the arcuate nucleus (ARC), which in turn project to the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) within the hypothalamus. Afferent satiety signals from the gastrointestinal tract and the liver travel via the vagus nerve and sympathetic nerves to neurons in the nucleus of the solitary tract (NTS) in the brainstem. There is reciprocal interaction between the brainstem and the hypothalamus. The hypothalamus regulates anabolic and catabolic pathways to affect satiety signals and energy expenditure mediated by efferents in the sympathetic nervous system (SNS). Adapted from Schwarts et. al. [2].

Today the regulation of food intake and energy expenditure is thought to be organized in neuronal networks, projecting between different structures of the brain. The ventromedial part of hypothalamus, consisting of the VMN and ARC, serves as a central integrator of numerous peripheral afferent signals. Through neurotransmitter systems located within the paraventricular nucleus (PVN) and the LHA, these afferent signals are transduced into efferent signals, which control food intake and metabolic rate. The dorsomedial nucleus (DMN) has extensive connections with the

ventromedial part of hypothalamus and LHA and DMN integrate and process information from these nuclei. Areas in PVN and LHA are richly supplied by axons from the ARC, and neurons of the PVN and LHA, in turn, project to the ARC, creating a bidirectional communication between ARC and the second order neurons of PVN and LHA (Figure 3).

Numerous neuronal pathways implicated in energy balance regulation converge in the PVN, the main site of secretion of corticotrophin releasing hormone (CRH) and thyrotrophin releasing hormone. For instance, nutritional signals to the hypothalamic-pituitary-adrenal (HPA) axis are integrated in the PVN [9]. A great deal has been learned about the hypothalamic control of appetite and energy balance following the discovery of leptin in 1994, based on the fact that these hypothalamic systems are downstream targets of leptin action [14].

## Leptin

Leptin is a circulating satiety peptide produced by white adipose tissue that mediate information about energy stores to the ARC and VMN. Centrally, it interacts with key anorexigenic and orexigenic systems to reduce food intake and activate the sympathetic nervous system (SNS) [15] (Figure 3). The complete absence of leptin leads to a syndrome of intense hyperphagia and morbid obesity in humans and rodents [16, 17] which can be reversed by recombinant leptin treatment [18, 19].

An obvious question is why leptin has proved disappointing for the treatment of regular obesity despite the fact that the central regulation of fat mass is so dependent on leptin? Obese subjects generally already have high levels of serum leptin and can be considered to be leptin resistant. One possible reason for this is an impaired transport of leptin into the CNS, maybe simply due to overloaded transport capacity in the presence of high leptin levels [20-22]. However, resistance could also occur at the neuron level, possibly mediated by excessive suppressor of cytokine signaling-3 (SOCS-3) activity in leptin-responsive cells [23], a state seen in mice with obesity induced by high fat diet, which show impaired response to ICV injected leptin [24]. It is possible that the principal purpose of leptin was not the prevention of obesity, but to be a signal giving the brain information about prolonged fasting and loss of body fat [8, 25]. Thus, leptin was maybe never designed to act at the plasma level that is now seen in our over-nourished western society. However, it seems like each individual has a personal leptin threshold for sensing nutrient status and that this threshold can be shifted upwards in obesity [26]. If the level of adiposity and leptin serum levels drop below this value, the ventromedial hypothalamus senses starvation. Consequently, in the leptin deficient state appetite and energy storage are promoted and energy

expenditure is reduced [9, 27]. Obese persons may manifest the starvation response at a higher level of leptin, which counteract weight loss and reduce quality of life.

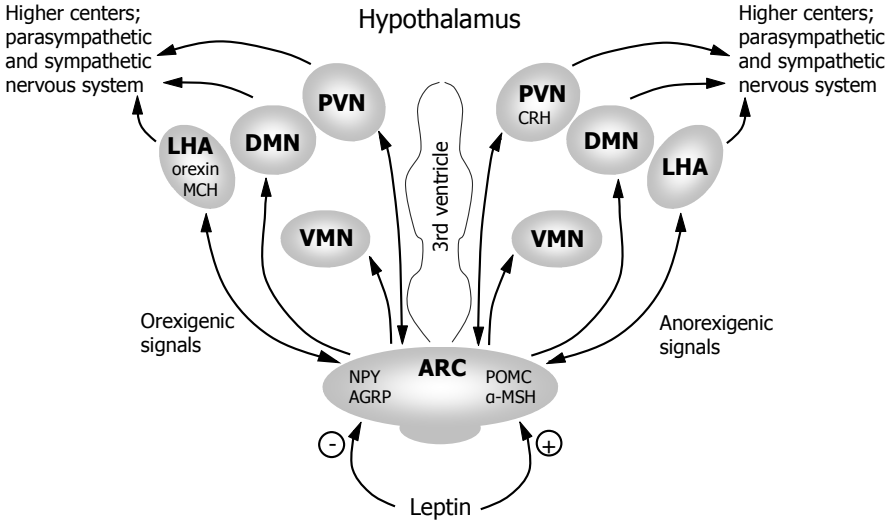


**Figure 3. Schematic presentation of the hypothalamus**
Cross section of the hypothalamus showing important areas for food consumption and energy expenditure regulation. Orexigenic and anorexigenic signals are depicted to the left and the right of the third ventricle, respectively. Leptin up-regulates anorexigenic signals and down-regulates orexigenic signals. Arcuate nucleus (ARC), ventromedial nucleus (VMN), lateral hypothalamic area (LHA), paraventricular nucleus (PVN), agouti-related protein (AgRP), alpha-melanocyte-stimulating hormone (α-MSH), corticotrophin-releasing hormone (CRH), melanin-concentrating hormone (MCH), neuropeptide Y (NPY), pro-opiomelanocortin (POMC). Adapted from Crowley et. al. [28]

## The Arcuate Nucleus

Although the brain is protected against circulating toxins by the blood-brain-barrier, there is a lack of blood-brain-barrier in the median eminence area, located in the mediobasal hypothalamus. The median eminence represents a particularly important region, as it is a small area where the interplay between peripheral organs and the brain takes place. The ARC directly overlies the median eminence and some arcuate neurons are, by having specific receptors for blood-borne hormones and nutrients such as leptin and insulin, able to sense the metabolic state of the organism [2].

The first order neurons in the ARC receive first hand information regarding nutritional status. Neuropeptide Y (NPY) and agouti-related protein (AgRP)

are co-expressed in the medial ARC and regulate MCH and orexin neurons in the LHA as well as thyrotrophin releasing hormone and CRH in the PVN [2, 29]. NPY and AgRP stimulate feeding and decrease energy expenditure. In contrast, pro-opiomelanocortin (POMC) and cocaine-amphetamine regulated transcript (CART), also produced in the ARC, decrease feeding and increase energy expenditure. Leptin directly inhibits expression of NPY and directly stimulates POMC and CART, while it indirectly inhibits MCH and orexin [2, 29] (Figure 3). As lesions of the ventromedial hypothalamus result in obesity, it seems that obesity suppressing POMC and CART containing neurons are dominant, and the NPY- and AgRP-containing neurons mainly modulate the action of the former neurons. The action of peripheral signals on these neurons triggers a cascade of neuronal events in higher CNS centres, resulting in autonomic effectors that regulate energy intake and expenditure.

## Cytokines in Inflammation, Metabolic Regulation and CVD

### Cytokines

Cytokines are a group of smaller water-soluble proteins and peptides that are used in intracellular communication through receptor-ligand interaction and can have effects on both nearby cells or throughout the organism. Cytokines have been variously named as lymphokines, interleukins and chemokines, based on their presumed function, cell of secretion or target of action. Since cytokines are characterized by considerable redundancy and pleiotropism, and have physiological actions far beyond those originally discovered, such distinctions may be out-of-date.

Cytokines are produced by a wide variety of cell types (e.g. haemopoietic and glia cells, hepatocytes, adipocytes, myocytes and maybe also neurons). Circulating cytokines are predominantly produced by cells of the innate immune system (i.e. monocytes and macrophages) [30], and are involved in a variety of immunological, inflammatory and infectious diseases.

Some cytokines can elicit reverse effects depending on the *in vivo* environmental circumstances [31]. Therefore it is important to take into account where, i.e. central or peripheral effects, and under which conditions, e.g. exercise, infection, obesity or normal state, cytokine effects are being investigated. However, not all their functions are limited to the immune system, as they are also involved in several developmental processes during embryogenesis, reproduction and cardiovascular function, and more recently they have been shown to be important in the regulation of metabolic functions [32-35] (Figure 4).

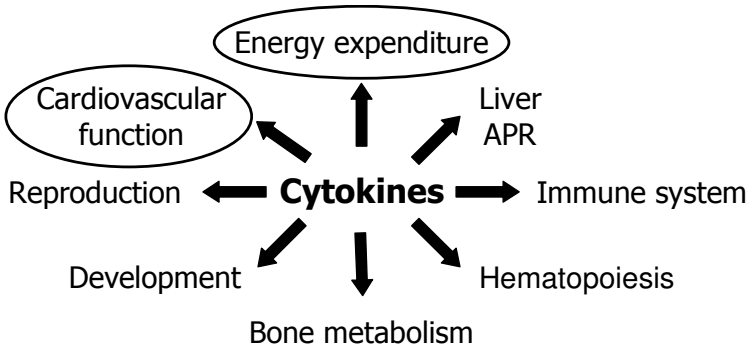


**Figure 4. The pleiotropy of cytokines**
Cytokines can modulate various biological responses e.g. proliferation, survival and apoptosis in several organs. Acute phase response (APR)

## Metabolic Regulation of Cytokines in Sickness

It is well known that the immune system is energy consuming and maintenance of immune functions has been estimated to account for as much as 15% of the daily energy expenditure in healthy individuals [36] and even more during infection. When the immune system is fighting pathogens, a special group of cytokines (chemokines) signal immune cells such as T-cells and macrophages to travel to the site of infection. Once the cells are there, cytokines activate them and stimulate production of even more cytokines [37]. Many metabolic processes respond directly or indirectly to proinflammatory cytokines to ensure an adequate supply of nutrients for proliferation of lymphocytes and macrophages, antibody production and hepatic synthesis of acute phase proteins. The prevailing hypothesis is that during periods of immune challenge, cytokines direct nutrients away from tissue growth and other non-immune functions in support of the immune defence [38]. Consequently, infection causes major alteration in the metabolism. In this state glucose uptake by peripheral tissue is reduced and cytokines block the suppression of hepatic gluconeogenesis by insulin. Since energy intake is typically reduced in sickness, fatty acid oxidation is increased to provide energy and protein degradation is increased to supply amino acids for production of acute phase proteins [38].

To date, the knowledge of fuel utilization by immune cells is limited and available data is mostly based on *in vitro* studies. However, the collected picture suggests that immune cells use glucose as their main fuel and this is facilitated, in part, by increased expression of glucose transporters [39, 40]. In addition, immune cells express the insulin receptor and respond to insulin [40]. Furthermore, glutamine seems to be essential for optimal

immune cell function and is highly used both as a primary fuel and as a carbon and nitrogen donor for nucleotide precursor synthesis [41]. Although fatty acids are used as fuel, but at a lower rate, their oxidation does not appear to be crucial for immune cell function [39].

Proinflammatory cytokines do not only affect the metabolism of specific nutrients, but also act in the brain to induce fever and psychological and behavioural changes, called sickness behaviour, that influence whole-body energy balance. By preventing metabolically expensive non-immune activities (e.g. foraging, social interaction) and favouring those that decrease heat loss and increase heat production (e.g. rest and shivering), infection is associated with fever and anorexia [37]. This increase in metabolic rate together with a reduction in appetite may cause a negative energy balance, loss of lean body mass and consequently cachexia [42].

Systemic cytokines can cause sickness behaviour by inducing expression of cytokines in the brain via one or two of the following routes; blood-borne cytokines can enter the brain directly via transport across the blood-brain barrier [21]. Alternatively endogenous cytokines are produced and released in the brain in response to peripheral cytokines, possibly via the vagus nerve [42]. The exact mechanisms behind the induction of these responses are not known, but cytokines are no doubt involved in the regulation of fever and sickness behaviour [37, 42].

## Metabolic Regulation of Cytokines in Health

Adipose tissue was previously regarded as a rather passive tissue, which primary role was to store and release fat in response to the body's energy needs. However, mature adipocytes account for only half of the total cell numbers in adipose tissue, where they share the space with fibroblasts, endothelial cells, pre-adipocytes and macrophages. Nowadays, adipose tissue is considered a highly active endocrine organ secreting a range of biologically active substances. These proteins, synthesized by and released from the adipocytes and/or adipose tissue, are termed adipokines and are often cytokines, e.g. tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), leukemia inhibitory factor (LIF), interleukin-1β (IL-1β) and interleukin-1 receptor antagonist (IL-1Ra) [43-46].

Adipokines are involved in a wide range of physiological and metabolic processes e.g. lipid metabolism, glucose homeostasis, inflammation and energy balance [43]. The expression and release of inflammatory cytokines are increased in adipose tissue with obesity and increased circulating levels of these proteins have been suggested to be important for the development of obesity related disease, in particular insulin resistance and CVD [47, 48]. The most famous adipokine is leptin and the

primary finding is that leptin decreases appetite and increases energy expenditure via effects exerted on the hypothalamus. However, similarly to other cytokines (Figure 4), leptin is involved in many biological processes and is also involved in metabolism and immune response. Leptin has direct effects on adipose tissue by inhibiting lipogenesis and stimulating lipolysis [49]. Leptin synthesis is increased during infection when high circulating levels of leptin no longer correlate with body fat mass and possibly also contribute to anorexia and weight loss [25].

In contrast to leptin, IL-6 and members of the IL-1 family were initially identified as regulators of immune response. In healthy individuals, a large part of circulating IL-6 and IL-1Ra is produced in adipose tissue e.g. by adipocytes and macrophages. The role of cytokines, e.g. IL-6, IL-1 and LIF, in healthy animals and humans is not well known but has been suggested to be of little importance, partly because circulating levels often are low in the absence of infection or severe stress. However, if a metabolically active cytokine like leptin also is involved in regulation of the immune system it is not unlikely that other cytokines play a role in metabolic regulation. Furthermore, an increasing number of polymorphism studies show that genetic variations in the IL-6 and IL-1 system genes are associated with both changed activity and expression of these genes, as well as alterations in body fat mass [50-55]. These effects might be exerted at the level of the CNS, as IL-6 and IL-1 knockout mice develop mature-onset obesity [32, 34]. Moreover, centrally administered IL-6 and LIF can decrease body fat without causing acute-phase reaction [56, 57] and a single ICV injection of IL-6 or IL-1 increases energy expenditure [34, 58].

## Cytokines in Inflammation and CVD

Obesity is associated with an increased risk of CVD [3] and is linked to a state of chronic inflammation [59], as overweight and obese subjects have elevated serum levels of C-reactive protein, IL-6 and TNF-α, all known markers of inflammation. Inflammation is a key component in the development of atherosclerosis and systemic inflammation is often associated with increased risk of cardiovascular events, i.e. myocardial infarction, stroke and peripheral artery disease. It has been hypothesized that proinflammatory cytokines from adipose tissue contribute to chronic inflammation and thereby also to CVD.

IL-6 is a powerful inducer of the acute phase response [60], and the acute phase reactant C-reactive protein is an important predictor and strong risk factor for CVD [61]. Other proinflammatory cytokines, like IL-1β and TNF-α, are also able to regulate the acute phase response. However, animal studies indicate that the role of IL-6 is the primary one since only

IL-6 -/- mice show an impaired acute phase response, while IL-1β and TNF-α knockout animals show normal responses [60]. Furthermore, *in vitro* studies suggest that IL-6 is expressed to a greater extent in visceral than subcutaneous fat [62]. This fits well with the association between visceral fat mass and risk of CVD. Moreover, the IL-6 signal transducer (IL6ST)/gp130 is a key component in the inflammatory signal pathway. Recently we (Paper IV) and others [63] have provided evidence that variations in gp130 activity can influence the risk of atherosclerosis, coronary artery disease and myocardial infarction.

## Receptors and Signal Transduction

The effect of a particular cytokine on a given cell depends on the levels of the cytokine, the abundance of the complementary receptor on the cell surface, and downstream signals activated by receptor binding. Each cytokine binds to a specific cell-surface receptor. Subsequent cascades of intracellular signalling then alter cell functions. Interestingly, cytokines are characterized by considerable "redundancy", in that many cytokines appear to share receptor subunits and exert similar functions [64-66].

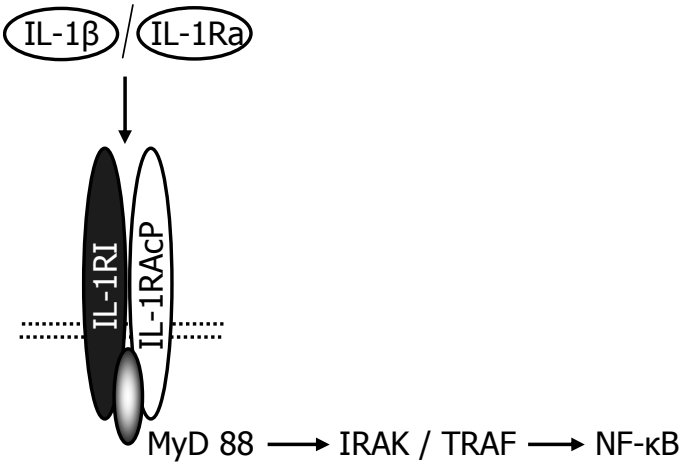


**Figure 5. IL-1 signalling**
IL-1β and the members of IL-1 family of ligands: IL-1α/β and IL-1 receptor antagonist (IL-1Ra) act through a heterodimer consisting of the IL-1 type I receptor (IL-1RI) and IL-1 accessory protein (IL-1AcP). Binding of agonist induces the recruitment of MyD 88 and initiates the activation of IRAK/TRAF pathway leading to nuclear factor-kappaB (NF-κB) activation.

The IL-1 family consists of IL-1β, IL-1α and the endogenous receptor antagonist IL-1Ra which all bind to the same receptors; the type I and type II IL-1 receptors. The heterodimeric IL-1RI complex is composed of

the IL-1RI and the IL-1 accessory protein (IL-1AcP) and is responsible for signal transduction, while the IL-1RII acts as a "decoy" receptor unable to activate the signalling cascade [67]. Binding of IL-1 drives dimerization of the IL-1RI with its accessory protein, followed by recruitment and phosphorylation of the IL-1 receptor-associated kinase (IRAK) via the docking molecule MyD88, leading to nuclear factor-kappaB (NF-κB) activation [68] (Figure 5).

IL1Ra acts as a negative regulator of IL-1β and IL-1α actions. IL-1Ra -/- mice (excess IL-1 signalling) are lean and resistant to diet induced obesity, and have increased energy expenditure and low serum insulin levels without changes in food intake [69, 70]. Conversely, IL-1Ra, which is produced by the white adipose tissues, is up-regulated in the serum of obese humans and experimental animals [44, 71].

The IL-6 cytokine family consisting of IL-6, IL-11, oncostatin M, LIF, ciliary neutrophic factor (CNTF) and cardiotrophin-1, shares the gp130 signal transducer and signal through gp130 and a ligand specific receptor [30]. Although there is some crosstalk among the gp130 cytokines, the signalling pathway is not common to all family members. The gp130 is ubiquitously expressed across all cell types but it can not transduce signals without the ligand specific receptor which is expressed more selectively. The LIF receptor (LIFR) is ligand specific but is also present in the receptor complexes for oncostatin M, CNTF and cardiotrophin-1. In contrast to gp130 and LIFR, the cytoplasmic domain of IL-6 receptor α (IL-6Rα) is not necessary for signal transduction and the IL-6Rα exists in a soluble form (sIL-6Rα) that acts in an agonistic manner called transsignaling, allowing stimulation of cells only expressing gp130 [72].

However, the scenario is more complex since gp130 and the LIFR also exist in a soluble form, which act in an antagonistic way by binding to circulating IL-6 and LIF respectively and thereby limit transsignaling and unspecific endocrine effect of IL-6-family-cytokines [73, 74]. The gp130 receptor shares a large degree of homology with the long form of the leptin receptor, as both activate the JAK-STAT (janus kinase – signal transducer and activator of transcription) signalling pathway [64], which subsequently activates pathways important in energy balance, i.e. mTOR (mammalian target of rapamycin) [75] and PI3K (phosphatidylinositide 3-kinase) [76, 77]. Furthermore, phosphorylated STAT-3 induces suppressors of cytokine signalling (SOCS) proteins, which lead to inhibition of cytokines by dephosphorylation of components in the signalling transduction cascade [78, 79]. Stimulation of gp130 cytokines can also lead to activation of the MAPK (mitogen-activated protein kinase) signalling pathway [80, 81] (Figure 6).

Hyper-IL-6 is a highly active designer cytokine consisting of IL-6 and sIL-6Rα [82]. The fusion protein Hyper-IL-6 is 100-1000 times more active than the separate proteins IL-6 and sIL-6R. Hyper-IL-6, which has been used in Paper IV, can be used to stimulate several types of target cells, which are not stimulated by IL-6 alone, as they do not express the membrane bound IL-6Rα.

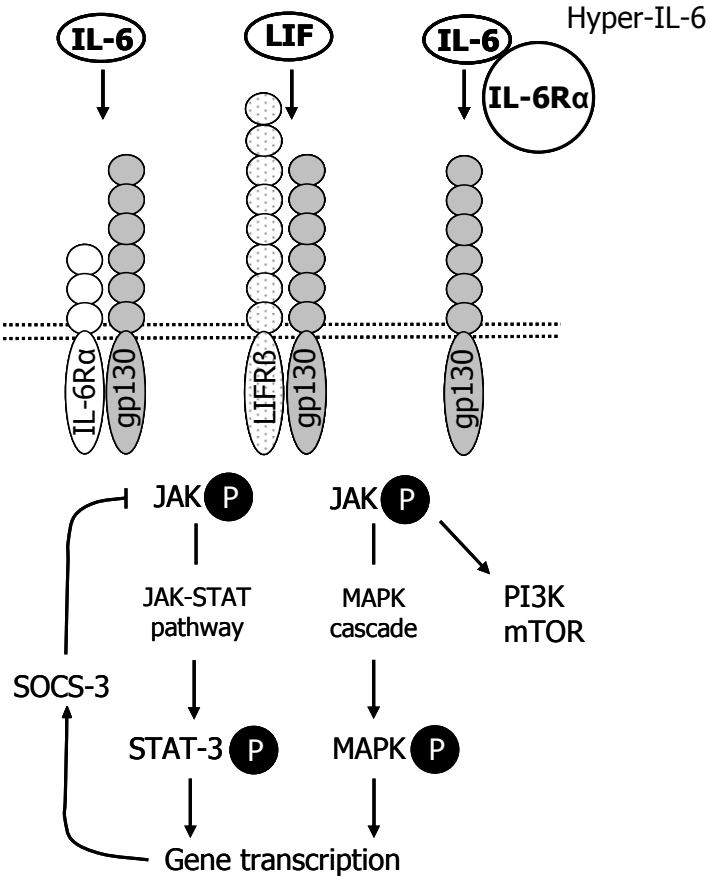


**Figure 6. IL-6 family signalling**
The IL-6 cytokine family shares the gp130 signal transducer and signal through gp130 and a ligand specific receptor. The IL-6/IL-6Rα fusion-protein Hyper-IL-6 can activate all cells with gp130, in the absence of the ligand specific receptor. Binding of agonist induces phosphorylation of JAK and initiates the activation of JAK/STAT or JAK/MAPK pathway. Subsequent SOCS-3 stimulation leads to inhibition of the cytokine signalling transduction cascade.

## CNS Expression of Cytokines and Receptors

Cytokines and cytokine receptors have been found in the CNS and are expressed both under pathophysiological and normal conditions. In the CNS it has been reported that IL-6, IL-1 and LIF are synthesized mainly by microglia cells and also from astrocytes, and that their expressions increase dramatically during acute phase response and brain injury [83]. However, as indicated below there are also data indicating that these cytokines can be produced centrally by neurons.

IL-1, and other cytokines, act on the brain via two pathways; one neuronal route represented by the primary afferents neurons that innervate the body site where the infection takes place and one humoral pathway. The first involves the production of IL-1 by microglia cells in the choroid plexus, and the latter the effects by circulating IL-1 on the brain at the circumventricular organs followed by diffusion of IL-1 to brain cells expressing IL-1 receptors. IL-1RI is diffusely spread across the brain, with the highest level of neuronal expression in the hypothalamus and hippocampus and in non-neuronal cells in the choroid plexus [68]. The ARC contains cells that express low levels of IL-1RI [84], and hypothalamic expression of IL-1RI is activated by central injections of IL-1β or lipopolysaccaride (LPS) [85, 86].

IL-1β and IL-1Ra in the hypothalamus are induced by IL-1β or LPS administrated to the brain [85, 86]. IL-1β is expressed in the dorsal hypothalamus and pituitary. IL-1Ra on the other hand, is markedly induced in the hippocampus and cortex and to a lesser extent in the hypothalamus after peripheral LPS administration. The LPS induced IL-1Ra expression may stand for an attempt to balance IL-1RI activity after an injury or infection. The IL-1RAcP is expressed at a high level in the hypothalamus and all other regions tested, and is much more abundant in the brain than at the periphery [68].

IL-6 and IL-6Rα mRNA have been reported to be expressed in the hippocampus, VMN and DMN of the hypothalamus [87] and IL-6 is rapidly stimulated by LPS injections that generate IL-6 transcription in the choroid plexus and median eminence [88]. IL-6Rα is also expressed in the parvocellular neurons in PVN and in median eminence [88]. Using immunohistochemistry, our studies show that IL-6 and IL-6Rα is expressed in the ARC, PVN, anterior hypothalamic area and supraoptic nucleus in untreated mice. Conversely gp130 is ubiquitously distributed throughout the brain and sgp130 is expressed at high levels in the cerebrospinal fluid; 100 times more than sIL-6Rα and 100.000 times more than IL-6 [89].

Less is known about the distribution of LIF and LIFR in the brain; however some measurements indicate that LIF and its receptor are expressed in the hypothalamus and pituitary [90, 91].

In summary, IL-6, IL-1 and LIF are all expressed in the hypothalamus and we hypothesize that this is the central site of action for the metabolic effects induced by these cytokines.

# AIMS

## General Aims

The basis of this thesis was the previously reported mature-onset obesity in IL-6 -/- mice. In the light of this we wanted to investigate whether other cytokines, such as IL-1β and LIF, are involved in the regulation of metabolism and body fat mass. Secondly we aimed to identify possible hypothalamic mechanisms by which IL-6 and IL-1 exert their effects on metabolism and obesity. Finally, we investigated whether genetic variations in cytokine signalling pathways are important for the cardiovascular consequences of metabolic diseases.

## Specific Aims

The specific aims for each individual paper were:

**Paper I:** To investigate the effects of endogenous IL-1 on body fat mass and metabolism.

**Paper II:** To identify possible hypothalamic mechanisms by which IL-6 and IL-1 exerts its effects on metabolism and obesity.

**Paper III:** To investigate the effect of LIF, a well known stimulator of the IL-6 signal transducer, on body fat mass.

**Paper IV:** To study the association between genetic variants of the IL-6 signal transducer and the risk of myocardial infarction, a disease coupled to obesity and metabolic disturbances.

# METHODOLOGICAL CONSIDERATIONS

## Genetically Modified Mice

Rodents are a good model for studying basic physiological functions of mammals and have proved to be powerful models for understanding obesity in humans. Mice, the most commonly used vertebrate species, are widely considered to be an acceptable model of inherited human disease and share 99% of their genes with humans. The reasons for the popularity of the mouse are convenience of breeding and, especially, the introduction of genetic engineering technology. Genetically modified mice have been used successfully to discover genes and pathways that can regulate body weight and body fat. C57BL/6 is a common inbred mouse strain that is widely used as "genetic background" for genetically modified mice. It is particularly appreciated in the obesity and diabetes research area as it develops obesity and insulin resistance when fed a high fat diet [92].

Because of the multitude and complexity of the disturbances in energy homeostasis that are associated with obesity, it has been difficult to determine which abnormalities that are causative versus less important phenomena in this metabolic state. Moreover, obesity is a disorder that in most cases depends on several factors, such as environmental and genetic factors. Therefore, the degree of obesity of a knockout mouse is not easy to predict since the outcome depend on factors like diet, litter size, handling, room temperature, pathogens and maternal factors. One also has to keep in mind that, because of the redundancy and compensation of the regulatory machinery, the interpretation of targeted gene mutation is sometimes not straightforward in unravelling the physiology. Modifying the synthesis of a particular gene at all sites and developmental stages may be a relatively crude way of investigating its functions. Inducible and tissue specific knockout animals aimed at depleting a specific gene product in a certain tissue and age could lead to a better understanding of the system. However, despite these limitations, observations of mice with global gene knockouts have shed new light on the understanding of energy homeostasis equation, two examples being the study of IL-6 and IL-1RI knockout mice.

## Administration Routes

The choice of administration routes was dependent on the aim of the study. In Paper II permanent guide cannulae connected to osmotic pumps (Alzet Mini-Osmotic Pump Model 2002, Durect, Cupertino, CA) were implanted into the brain to facilitate continuous intracerebroventricular (ICV) administration in rats. Stereotactic coordinates (0.6 mm posterior to bregma, 1.4 mm lateral to midline and 4.0 mm below the outer surface of

the skull) were chosen so that the administrated recombinant rat IL-6 would reach the lateral ventricle. Guide cannulae were held in position by dental cement attached to three stainless steel screws driven into the skull. The ICV route is a very good way of giving bioactive substances directly into the CNS and thereby bypassing the blood brain barrier. The injected substance diffuses within the ventricles and can easily affect sites close to the ventricle, such as the PVN and ARC of the hypothalamus. It should be kept in mind that when a substance is injected ICV several brain sites will most probably be affected, as the cerebrospinal fluid flows from the lateral to the $4^{th}$ ventricle and then into the brainstem.

In Paper I and III, mice were treated with daily i.p. injections, which is a fairly quick way to administrate a relatively large volume. I.p. injections are easy to perform but should be placed in the lower part of the peritoneum to avoid penetration of the intestines, which might cause an infection and certainly will limit the desired biologic effect of the administrated drug. In Paper I mice were given i.v. injections of glucose. The i.v. injections mostly provide a very fast uptake to most non-SNS tissues and thereby rapid biological effects, as the substance is administrated directly into the blood stream. A disadvantage might be that much smaller volumes can be injected compared to i.p. injections. Furthermore, the animal has to be restrained or sedated, and warmed before injection into the tail vein. This is more stressful for the animal and the anaesthesia will slow down the metabolism.

## Indirect Calorimetry in Metabolic Cages

Direct calorimetry is based on the assumption that all cellular metabolic events ultimately result in heat. Accurate measurement of heat production would then give information about the metabolic rate (i.e. direct calorimetry). However, in reality heat production is difficult to measure precisely and therefore indirect calorimetry has become the most commonly used method to measure metabolic rate. This method is based on measurements of oxygen consumption, assumed to originate from oxidation of nutrients and has proven to be a highly accurate estimate of energy expenditure. An animal inhales ambient air that has a constant composition and the changes in oxygen and carbon dioxide percentage in expired compared with inspired air reflect the ongoing metabolic processes.

We have the opportunity to measure oxygen consumption ($VO_2$), carbon dioxide production ($VCO_2$) and locomotor activity by two different indirect open-circuit calorimetry systems. One Oxymax® system from Columbus instruments (Columbus, OH,USA) and one INCA® metabolic system from SOMEDIC (Somedic sales, Hörby, Sweden) with an integrated telemetry

receiver system from Minimitter. The Oxymax® system was used in Paper I, while the INCA® system has been used in Figure 7 in the Summary and in a paper not included in this thesis [93]. The mice were conscious and unrestrained during the measuring periods in the chambers and had free access to food and water. Measurements in the Oxymax® system can be performed at room temperature and at thermoneutrality (~30ºC) while the ambient temperature can be set anywhere between 4-30ºC in the INCA® system. Basal metabolism was assessed in the thermoneutral zone, which is the temperature (~30°C) at which the mice do not need to use energy to actively maintain its body temperature.

An air sample was withdrawn from each cage every 2$^{nd}$ minutes, and $O_2$ and $CO_2$ content were measured. These values were used to calculate $VO_2$ and $VCO_2$. The respiratory exchange ratio (RER) is the ratio of carbon dioxide output ($VCO_2$) to oxygen uptake ($VO_2$), that is: RER= $VCO_2/VO_2$. The RER value will differ depending on the metabolic state. Since 6 units of $CO_2$ are produced and 6 units of $O_2$ are consumed when carbohydrates are oxidized, RER is equal to 1, whereas RER will fall to around 0.7 when fat are being used exclusively for metabolism, as about 16 units of $CO_2$ are produced and 23 units of $O_2$ are consumed.

The Oxymax® system is equipped with infrared sensors to detect the activity of the animals, and therefore the cages contained no bedding or nesting material. The cages were considerably smaller compared to the home cages that the mice were used to, which makes locomotor activity data difficult to interpret. The small cages may also affect the behaviour of the mice. The INCA® system can measure body core temperature, activity and heart rate by telemetry (E-Mitters, Mini-Mitter, Oregon, US). An advantage of biotelemetry is the possibility to obtain physiological measurements with high resolution from freely moving animals, without introducing stress by handling. A potential disadvantage is that it is an invasive technique that requires surgery, but the risk of adverse effects on behaviour and physiological functions can be decreased by increasing body-to-transmitter size ratio. INCA® cages are relatively large and contain bedding and nesting material, which improve locomotor data.

Correcting oxygen consumption for the metabolically active mass of the animal is important when comparing data from animals of different body weight and surface/volume ratio. There are several ways of scaling the oxygen consumption but none of them are perfect. A widely used formula is based on the finding that metabolic rate correlates to the body weight raised to the power of 0.75 [94]. Indirect calorimetry measurements carried out in animals that are not eating or moving, and are kept in the thermoneutral zone show the basal metabolic rate of the mouse. This is a measure of all body processes that require energy and are needed for the

survival of the animal. Moreover, at thermoneutrality the metabolism will correlate directly with the metabolically active body mass (see below). If measurements of the metabolic rate are carried out at temperatures below the thermoneutral zone, a mouse with higher body weight and increased fat mass has, because of better insulation and lower body surface/volume ratio, a decreased need of energy expenditure to defend body temperature than has a smaller mouse.

Moreover, the proportion of metabolically active tissue varies between individuals. Thus, oxygen consumption per gram body weight may falsely give too low a value in an obese mouse compared with a lean mouse. One way of correcting for differences in body composition is to relate the metabolic rate to lean body mass instead of total body mass. However, this method assumes that the energy expenditure of the adipose tissue can be ignored. Although the metabolic rate of adipose tissue is low, it is definitively not zero. Therefore, the metabolic rate expressed per lean body mass of a very obese animal might falsely be too high. One way to avoid scaling problems is to try to investigate the metabolic rate in mice of equal body weight and body composition, i.e. before the onset of obesity if studying obese phenotypes.

## Glucose and Insulin Tolerance Test

The glucose reducing effect of insulin treatment was assessed in awake animals. Blood was withdrawn from the tail to determine fasting blood glucose before a load of human insulin was administered i.p. (1U/kg body weight). Further samples were collected 15, 30, and 60 minutes after the insulin challenge. Blood glucose levels were determined by an ABL 700 series analyzer (Radiometer, Denmark) or a blood glucose meter (Accu-Chek, Roche, Germany). The insulin injection can sometimes cause the blood glucose to drop so low that the mice show signs of hypoglycaemic shock such as convulsions or coma. This may occur if blood glucose goes below 2mmol/L. Animals were observed throughout the test and hypoglycaemic individuals were rescued with oral administration or subcutaneous injections of glucose solution. To lower the risk of hypoglycaemia, the insulin dose was later decreased to 0.5U/kg body weight in other experiment. This regimen still caused a robust decrease in blood glucose.

Glucose clearance after a bolus dose of D-glucose was measured in fasted anesthetized mice. Blood samples were taken from the retrobulbar capillary plexus 1, 5, 10, 20, 30, and 50 minutes after an i.v injection of D-glucose (1g/kg; 10µl/g body weight) into the tail vein. Glucose levels were analyzed immediately in blood samples, while plasma samples were collected for insulin measurements by ELISA. The injected glucose

concentration must be high to keep the injection volume down in obese animals; otherwise the large volume may cause heart failure and death. Another possible protocol includes i.p. administration of glucose (1g/kg) and collection of blood samples from the tail after 30, 60, 90 and 120 minutes. This setup worked well in our hands for both normal and over weight mice, with only small deviations within groups.

## DEXA

Dual energy X-ray absorptiometry (DEXA) is a non-invasive method that can be used for determining body fat mass. This technique enabled us to measure body composition in sedated animals. During measurement, the whole animal was exposed to a small beam of both high and low-energy X-rays. The absorbance of X-ray energy is different in tissues with different densities, and the amount of radiation absorbed when each X-ray is passed through the body was measured. The ratio of energy attenuation in the luminescent panel separated bone, lean mass and fat tissue. A quality control phantom mouse was used for the calibration carried out before imaging. Body fat (g) and (%), lean body mass (g) and total bone mineral density ($g/cm^2$) and bone mineral content (g) were determined by densiometry using a PIXImus imager (GE Lunar, Madison, WI, USA) or a Norland pDEXA Sabre (Fort Atkinsson, WI, USA). Fat mass and lean body mass were calculated using the PIXImus software (version 2.00) or the Sabre Research software (version 3.9.2) together with image analysis using the Scion Image software (Scion Corp., MD, USA).

The inter-assay coefficient of variation for the Norland pDEXA measurements of percent fat area was increased with decreasing fat mass from 1.0% for mice with 30% fat area to 17.6% for mice with 1.4% fat area [95]. The large variation for mice with a small fat area indicates that the sensitivity of the assay is decreased in very lean mice and that the lower sensitivity limit for the Norland pDEXA image procedure is around 3% fat area. To my notion, available literature does not contain any higher sensitivity limit for very obese animals but one may speculate that the variation also increase with an increasing fat mass reaching 45-55%. This should be kept in mind when investigating very obese animal models.

The values of fat mass obtained by PIXImus showed a low coefficient of variation (~2%) and close correlation with chemically extracted fat mass [96]. Although, the fat mass weight tended to be overestimated [96], but if interested in comparing differences in fat mass between groups and not absolute values, this is acceptable as long as the coefficient of variation is kept low. And, because of the close relationship for fat and lean mass obtained by the two methods, prediction equations can be used to more accurately predict body composition.

One advantage with the PIXImus is that it is relatively fast. Therefore, before and after treatment scans for several animals could be collected and average values calculated for different groups.

Magnetic resonance imaging is another imaging technique and is based on signals from hydrogen nuclei in fat and water in strong uniform magnetic field. This technique was used to quantify fat mass in Paper I. The computer software produces illustrative 3-dimensional pictures that show certain body fat depots e.g. visceral and subcutaneous fat but the technique is time consuming and laborious.

## Analysis of mRNA

### Real Time Quantitative PCR Analysis

PCR is a very sensitive method to detect specifically amplified gene products. The advantages of using quantitative real time-PCR for measuring mRNA are that a very small amount of RNA is needed for each analysis and that the method has a high throughput. Real time-PCR analysis makes it possible to quantify specific mRNAs over time by combining the 5´nuclease activity of a DNA polymerase and a fluorescent probe that span an exon-exon boundary for a specific gene, or a fluorescent dye binding to double stranded DNA. The increase in fluorescence intensity is proportional to the amount of amplicon produced.

Real time-PCR analysis was used to explore the expression of hypothalamic genes of interest. Total RNA was isolated from hypothalamus as described in Paper II. In brief: the tissues were homogenized in QIAzol Lysis Reagent using a TissueLyser (Qiagen, Sweden) and RNA was isolated by a colon kit. cDNA synthesis was performed by iScript™ cDNA synthesis kit (BioRad, USA). The RT-PCR was performed on an Mx3005P™ Real-Time PCR System (Stratagene, USA). Transcripts were detected using probes labelled with FAM in the 5' end or alternatively, the transcripts were detected using the SYBR® Green detection system. Melting curves were performed to verify the PCR products.

Using fluorescent reporter probes is the most accurate and most reliable of the methods, but also the most expensive. Therefore, SYBR® Green was mainly used for detection. One has to keep in mind that double stranded DNA dyes such as SYBR® Green will bind to all double stranded DNA PCR products, including non-specific PCR products like primer dimers. This can potentially interfere with or completely prevent accurate quantification of the intended target sequence. However, the problem can be reduced by evaluation of primers by amplicon size and PCR efficiencies. All samples were run in duplicates and normalized by using mouse acidic ribosomal

phosphoprotein PO (m36B4) as an endogenous control. The relative expression levels were estimated using the comparative threshold cycle method.

## In situ Hybridization

*In situ* hybridization is a technique that uses a labelled complementary RNA strand (i.e. probe) to localize a specific mRNA sequence in a section of tissue. mRNA levels were measured with *in situ* hybridization as described in Paper II. In brief, brains were sectioned, transferred to poly-lysine coated slides and fixed with 4% paraformaldehyde (Sigma, Dorset, UK). Oligonucleotide probes complementary to the mRNA of the peptide under study were labelled with $^{35}S$, slides were incubated with the labelled probe overnight and then thoroughly washed and air-dried before exposure to X-ray film (Amersham, Buckinghamshire UK). The films were developed after 3-5 days, depending on the peptide, and the optical densities were measured and compared against a $C^{14}$-labelled standard of known radioactivity (Amersham, Buckinghamshire, UK).

The two different ways of measuring mRNA used in this thesis work are both common techniques that have slightly different advantages. The real time-PCR technique is very sensitive and can detect very small differences in expression levels. Furthermore, several genes can be analyzed rather quickly, and from a small tissue sample. However, the *in situ* hybridization technique has one big advantage compared to the real time-PCR in that one can determine the exact location and at the same time get a relatively good quantification of the mRNA expression of discrete cell groups. Nevertheless, this method is time consuming and only a handful of genes can be analyzed du to limited numbers of brain sections.

## Statistical Analysis

All analysis were performed using the SPSS statistical software (SPSS, Chicago, IL, USA). All values were calculated and presented as average ± standard error of the mean (sem), except from when standard deviation values were more informative. Comparisons were performed with Student's t-test followed by Bonferroni's correction when differences between more than two experimental groups were analyzed. In some experiments one-way ANOVA for repeated measurements was used. Data derived from the same animal at several times was analyzed with ANOVA for repeated measurements to evaluate differences between experimental groups. P values $< 0.05$ were considered significant. When appropriate, values were normalized with logarithmic transformation, using $\log_2$ values.

# SUMMARY OF RESULTS AND DISCUSSION

## Paper I-III: Cytokines Affecting Body Fat Mass

### IL-1RI -/- Mice Develop Mature-Onset Obesity, Paper I

Wild-type and IL-1RI -/- mice were not different in body weight until five months of age, when IL-1RI -/- male and female animals started to deviate from wild-type mice. At nine months of age IL-1RI -/- mice were 20% heavier than wild-type controls. The increased body weight was associated with increased body fat mass and increased crown-rump length. Even though the IL-1RI animals were longer, they still had an increased body mass index (BMI), calculated as the square of body weight divided with the square of crown-rump length, indicating that the increased body weight was associated with obesity and not only growth. The lean body mass in relation to body weight was lower in IL-1RI -/- mice compared to wild-type controls but the lean body mass in grams was instead increased in IL-1RI -/-. Serum analyses of leptin and insulin-like growth factor-1 (IGF-1) confirmed these finding as increased serum levels of leptin have been shown to correlate with fat mass and IGF-1 is an important growth factor. It seems likely that the increased IGF-1 levels play a role in the increased absolute lean body mass and longitudinal growth observed in IL-1RI -/- mice. Moreover, it can not be ruled out that the increase in IGF-1 also contributes to the development of obesity [97].

IL-1 seems to possess a tonic fat-suppressing effect in healthy animals, as lack of IL-1 activity due to depletion of the IL-1RI, caused an increase in fat mass in mice. Additionally, excess IL-1 activity due to IL-1Ra -/- in mice caused leanness and resistance to diet induced obesity [69, 70]. These studies demonstrate a clear role for IL-1 in body fat regulation. Furthermore, the result from this and other studies indicate that four different cytokines; IL-1, IL-6, IL-18 and GM-CSF all suppress fat mass, probably via CNS effects [35, 57, 70, 98, 99]. Moreover, knockout of IL-1RI, IL-6, IL-18 and GM-CSF all result in mature-onset obesity, between five and six months of age, indicating that the loss of one of these genes is not severe enough to alter fat mass in young mice [34, 35, 98, 99]. However, IL-6 -/- IL-1 -/- double knockout mice become obese as early as 13 weeks of age [32]. One may speculate that there is a redundancy between cytokines, so that loss of one of them is compensated by others in young animals. However, at older age the loss of one cytokine may results in obesity only together with an age-related factor.

## IL-1RI -/- Mice had Decreased Insulin and Leptin Sensitivity, Paper I

The obesity in older IL-1RI -/- mice was accompanied by a disturbed glucose metabolism. Basal serum insulin levels were elevated in IL-1RI -/- animals and an insulin tolerance test showed that these animals, after an acute injection of insulin, had a reduced stimulation of glucose disposal, an indication of decreased insulin sensitivity. However, normal basal glucose levels were still seen in these mice compared with wild-type mice. Glucose uptake was studied after a bolus dose of glucose i.v.. Plasma glucose concentrations were then higher in IL-1RI -/- 50-70 minutes after injection, demonstrating a mild impairment of plasma glucose elimination. Given that obesity can cause insulin resistance, it is not surprising that the obesity in IL-1RI -/- mice is accompanied by decreased insulin sensitivity and glucose uptake. In line with this, lean IL-1Ra -/- mice had increased insulin sensitivity [69, 70].

In contrast to the arguments brought forward above, there are also reasons to believe that lack of IL-1 activity in IL-1RI -/- mice would increase insulin sensitivity. Inflammation in general is associated with decreased insulin sensitivity [100], suggesting that these animals lacking an important proinflammatory cytokine may have increased insulin sensitivity. Furthermore, depletion of IL-6 is associated with increased insulin sensitivity in young mice, but this is then converted into insulin resistance in older animals (unpublished results). Figure 4B in Paper I, indicates that this may also be true for IL-1RI -/- animals, as there was a tendency for decreased basal serum insulin levels in four-month-old mice. A putative positive effect caused by of lack of IL-1 and IL-6 on insulin sensitivity might be masked by the influence of obesity in older animals. Although it can not be excluded that IL-1 can induce decreased insulin sensitivity at high pathological levels in conjunction with other cytokines, available data indicate that lower doses of IL-1 can increase insulin sensitivity *in vivo* [101]. Therefore, IL-1 effects on insulin actions may be dose-dependent as well as age-dependent.

It has been suggested that leptin resistance is a contributing factor to the development of obesity [102], and IL-1 has been suggested to mediate leptin effects in the CNS [103-105]. Therefore, we studied the effect of leptin treatment on body weight and food intake in wild-type and IL-1RI -/- mice. When given to younger animals, only wild-type mice responded to the intermediate dose (25µg/mouse) of leptin, by decreased food intake and body weight. Both IL-1RI -/- and wild-type mice responded to the higher dose of leptin (120µg/mouse). However, there were no effects of the higher dose of leptin when given to older obese IL-1RI -/- mice while this dose had effects on body weight and food intake in age-matched wild-type animals. Lack of IL-1RI activity in young

IL-1RI -/- mice may cause leptin resistance that precedes the development of obesity in older animals. This is in line with previous results demonstrating that IL-1 activity is important in mediating leptin effects [103] and that leptin treatment increases hypothalamic levels of IL-1 [103, 105]. Taken together, these results suggest that the decreased leptin sensitivity seen in young mice with defective IL-1 signalling may contribute to the development of mature-onset obesity.

## IL-1RI -/- Mice had Higher RER and Lower Locomotor Activity, Paper I

When total energy expenditure ($O_2$ consumption, ml/min) was measured by indirect calorimetry in young IL-1RI -/- and wild-type mice, no significant difference was discovered. However, the RER was higher in IL-1RI -/- during the first six hours during the dark phase. This finding indicates that the IL-1RI -/- mice had increased carbohydrate utilization rate rather than oxidation of fat as an energy source during this period. Similar observations have been made in IL-6 -/- mice [93, 106]. Acute central injections of IL-1 or IL-6 increase energy expenditure without any effect on RER [34, 107], while continuously ICV administration of IL-6 during one week seems to decrease RER in rats (Figure 7, unpublished results). Suggesting that chronic IL-6 treatment can increase lipid utilization, this is in line with several studies that show an increased fatty acid oxidation *in vivo* after infusion of IL-6 in humans [108-110]. One may speculate that this decrease in RER would also be seen after central chronic IL-1 treatment. However, to my notion, this hypothesis has neither been confirmed nor rejected in the literature, and data from IL-1Ra -/- mice show a circadian pattern with increased RER during night time and decreased RER during day time [69].

Younger IL-1RI -/- mice showed a decreased locomotor activity during the first six hours of the night, i.e. at the time when they also had higher RER and lower fat burning. On the other hand there was no difference in locomotor activity in comparison with wild-type mice, during other parts of the 23-hour period measured. This is in line with the decreased locomotor activity reported during early night in IL-1β -/- mice [111]. Differences in spontaneous locomotor activity or non-exercise activity thermogenesis, such as fidgeting, have been discussed in relation to human and rodent obesity [7, 112, 113] and both high RER, and low spontaneous activity are predictors of obesity in Pima Indians [114, 115].

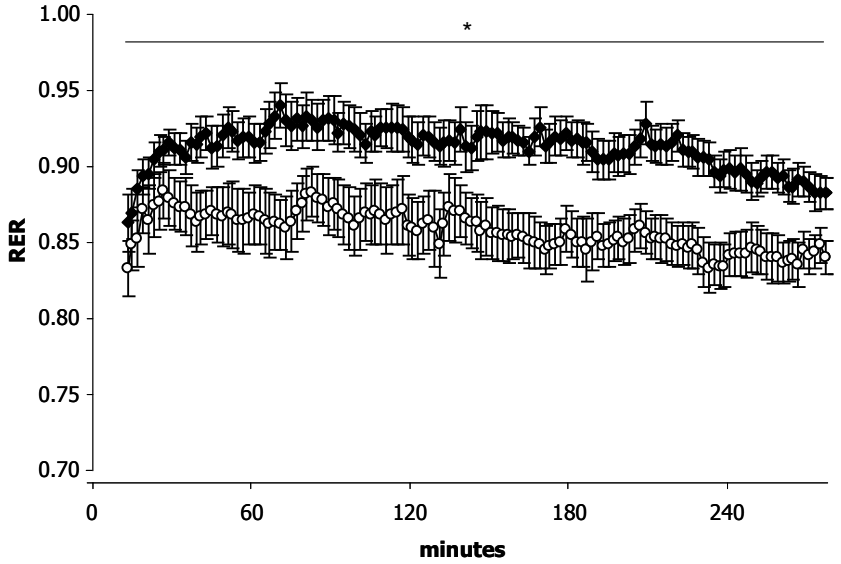


**Figure 7. IL-6 treatment ICV decrease RER**
Decreased RER ($VO_2/VCO_2$) in rats continuously treated with IL-6 ICV for one week, via osmotic pumps. Vehicle (♦) and rat recombinant IL-6, 250ng/day (○). * $p < 0.05$, calculated with one-way ANOVA for repeated measurements.

## IL-1RI -/- Mice had Increased NPY and Decreased Orexin Levels, Paper II

The expression of the orexigenic peptides NPY and MCH were increased in older IL-1RI -/- animals. In theory, this increase in orexigenic peptides could increase the food intake in these animals. However, the food intake data are difficult to interpret, as older IL-1RI -/- mice ingested more food per day than wild-type animals in absolute amounts, while the food intake in relation to body weight was not altered (Paper I). IL-1 may be important for the anti-obesity effect of leptin, as IL-1RI -/- mice are partly leptin resistant (Paper I) [103], and leptin treatment induces IL-1ß release from microglia cells *in vitro* [116]. However, it remains to be investigated whether the enhanced levels of NPY and MCH expression in the present study are due to deceased capacity of leptin to suppress the production of these peptides in IL-1RI -/- mice.

Obesity prone young IL-1RI -/- mice showed a marked decrease in orexin, while there was no difference between older animals. It is unclear whether this decrease in orexin expression is of importance for the obesity observed in older in IL-1RI -/- animals. Orexin was named due to its stimulation of food intake, and orexin neurons are found in the lateral hypothalamus, the classic "feeding" centre of the brain [117]. Apart from

stimulating appetite, orexin causes arousal instead of sleep, and enhances the activity of the SNS, body temperature, locomotor activity and metabolic rate [117, 118].

The promotion of energy expenditure by endogenous orexin seems to be more pronounced than the effect on food intake. Also the overall effect of endogenous orexin seems to be to decrease obesity, as genetic depletion of the orexin gene or of orexin producing neurons, results in an obese phenotype [119, 120]. Therefore, endogenous orexin may suppress obesity by stimulating energy expenditure more than energy intake. As mentioned above, young IL-1RI -/- mice have decreased locomotor activity (Paper I), but it remains to be investigated whether decreased orexin expression could mediate these effects. Orexin axons show a widespread projection to many different brain areas, and one of the densest projections of orexin neurons are found in the PVN [117]. Moreover, direct injections of orexin into the PVN have been shown to increase spontaneous physical activity (SPA), independently of feeding behaviour [121], while loss of orexin activity results in hypophagic animals that are much less active [120]. However, feeding with high fat diet has been reported to stimulate orexin expression in the perifornical lateral hypothalamus [122, 123] and to decrease SPA [122]. It has been suggested both that fat-induced increase in orexin expression could cause more craving for high fat diet via effect on the reward systems, and that it could thereby increase the risk of obesity [122, 124]. To sum up, the role of orexin in body fat regulation seems to be complex and not thoroughly understood.

We found no change in hypothalamic POMC expression in IL-1RI -/- mice, although IL-1 may stimulate POMC expression [125]. The reason for this discrepancy is unknown but could be due to the fact that only a subset of ARC POMC neurons are regulated by IL-1β [125], and that measurement of POMC expression in the whole hypothalamus may not detect alterations in subgroups of POMC neurons. In addition, CRH expression levels were not altered in IL-1RI -/- mice even though IL-1 has been shown to stimulate the HPA axis and activate parvocellular neurons in the PVN [126-128]. However, the redundancy between different cytokine actions might cover for the loss of IL-1 signalling, i.e. IL-6 may induce sufficient CRH expression in IL-1RI -/- mice.

## IL-6 -/- Mice had Decreased CRH Levels, Paper II

The levels of CRH mRNA in the hypothalamus were decreased by 40% in older IL-6 -/- mice, suggesting that endogenous IL-6 is a physiologic regulator of hypothalamic CRH expression. These results were supported by the finding that CRH mRNA levels in the hypothalamus were enhanced

by 50% after chronic ICV treatment with IL-6 to rats, indicating that IL-6 stimulates CRH expression via an effect at the CNS level. This is in line with the solid association between IL-6 and CRH expression after induction of inflammation [129-131], which has now been extended to healthy animals. The decreased CRH expression could partly explain the decreased activity of the SNS and subsequent obesity in healthy IL-6 -/- mice [34, 93, 106], as low CRH levels have been associated with decreased activation of SNS and decreased energy expenditure. This, in turn, could lead to obesity over time [132]. It has been reported that chronic central IL-6 exposure is associated with increased uncoupling protein (UCP)-1 expression in brown adipose tissue (BAT) and that this effect is dependent on the SNS [133]. Based on these findings, we hypothesize that endogenous IL-6 stimulates energy expenditure and thermogenesis, and decreases obesity via enhanced expression of CRH in the PVN and subsequent activation of the SNS.

Leptin plays an important role in the regulation of energy balance-stimulated thermogenesis [14, 18]. Leptin induces expression of CRH mRNA in the PVN [134] and activates sympathetic nerves to increase UCP-1 activation in BAT. Moreover, it has been shown that co-infusion of a CRH antagonist can block the effects of leptin on feeding, adiposity and UCP-1 expression [135, 136], indicating that CRH neurons may be an important mediator of these effects of leptin. In this study, despite the fact that they have been shown to have high leptin levels at this age [34], older IL-6 -/- mice had decreased CRH expression, suggesting that central IL-6 is at least as important as leptin in stimulating CRH expression.

## Altered Cytokine Expression in IL-6 -/- and IL-1RI -/- Mice, Paper II

The hypothalamic expression of IL-1ß was increased in IL-6 -/- and in IL1RI -/- mice. In contrast, the hypothalamic expression of IL-6 was decreased in IL-1R1 -/- animals. These results are in line with the indications that IL-1 and IL-6 interact to suppress fat [32-34] and that this effect is exerted at the hypothalamic level [34, 57, 58, 103]. The decreased IL-6 expression in IL-1RI -/- mice indicates that endogenous IL-1 can stimulate hypothalamic IL-6 production in healthy mice. In sick mice, IL-6 has been shown to be necessary for fever response induced by IL-1ß [137]. The up-regulated IL-1ß expression in IL-6 -/- mice could possibly reflect a negative feedback inhibition of hypothalamic IL-1 production by IL-6 under baseline conditions, as suggested previously to be the case during pathologic conditions [138].

Our results also indicated that IL-6 and IL-1 could inhibit their own expression at the hypothalamic level, as lack of biological activity exerted by either of these genes increased the mRNA expression of the same

gene. This is in contrast to previous results that indicate that IL-6 and IL-1 stimulate their own production [138]. One reason could be that we study basal homeostatic conditions, which may differ from feed forward regulation during pathologic conditions, such as inflammation. Alternatively, the hypothalamus may differ from other parts of the body.

Taken together these results strengthen our view regarding an interaction between IL-6 and IL-1 at the hypothalamic level during baseline conditions, e.g. in suppression of body fat (Figure 8).

## Altered Cytokine Expression during Fast, Paper II

Hypothalamic IL-1β was decreased in wild-type mice after fast, while CRH and TNF-α levels were increased after fast. The decreased hypothalamic IL-1ß expression is likely, at least partly, to be explained by decreased serum leptin levels in conjunction to fast, as leptin has been reported to stimulate hypothalamic IL-1ß production [103, 116, 139, 140]. The decreases in IL-1ß could be of importance for the decreased immune response and/or energy expenditure that is seen in response to fasting [103]. However, contradictory results have been reported in fasted rats, where one study shows elevated [86] and another decreased [105] hypothalamic IL-1ß levels. It remains to be further evaluated whether IL-1 is consistently decreased after long term fast in these different species.

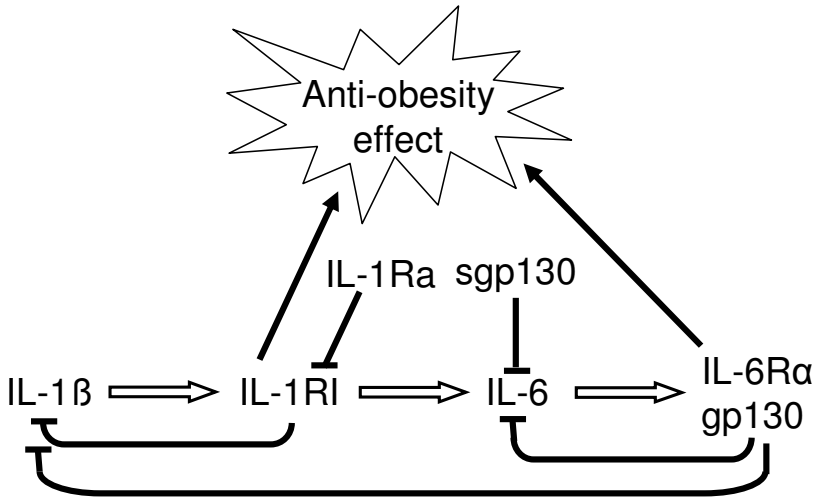


**Figure 8. Anti-obesity effect mediated by IL-1 and IL-6**
IL-6 expression and secretion is induced by IL-1β. There is also evidence that IL-6 in turn regulates the release of IL-1. The effect of IL-1β and IL-6 can be inhibited by the endogenous antagonist IL-1Ra and soluble gp130, respectively.

CRH is assumed to stimulate SNS and energy expenditure [141, 142], and so the increased CRH levels could not easily explain the suppression of energy expenditure that are seen during fast. Regarding SNS activity after fasting, there is a discrepancy in the literature [143, 144]. The finding that CRH is elevated in wild-type animals after 18 hours fast could be of importance for the increase in ACTH and corticosterone levels observed during this state [143, 145]. In line with this assumption, a diminished HPA axis response is observed in CRH -/- animals after fast [145]. Still, it is well known that the HPA axis and the SNS are regulated by different groups of CRH containing neurons in the PVN [138] and we have not yet been able to determine which of the parvocellular neurons that express the elevated CRH levels.

## LIF Treatment Reduced Body Fat Mass, Paper III

There is an increasing body of data indicating that cytokines play a role in body fat regulation. Therefore, we investigated whether an additional cytokine of the IL-6-receptor family, LIF, possessed anti-obesity effects in an obese ovariectomized (OVX) mouse model. OVX mice are known to gain body fat and we observed a significant increase in body weight and fat mass (~30%) as well as a three-fold increase in leptin levels after OVX.

It is well recognised that the exposure to estrogens decreases fat mass, especially visceral fat [146-149]. Estrogen replacement therapy has been shown to enhance the expression of LIFR [150]. Therefore, we hypothesised that LIF and estradiol may interact in a way where estradiol is necessary for the effects of LIF. However, we found that LIF treatment to OVX mice caused a significant reduction in the weight of white fat depots and serum leptin levels, suggesting that estrogen signalling is not required for this effect.

LIF is thought to play a role in the cachexia syndrome by the inhibition of adipocyte lipoprotein lipase activity. [83, 151]. In contrast, LIF does not decrease lipoprotein lipase activity in skeletal or cardiac muscle [151]. In rats, systemic LIF administration increases hepatic triglyceride secretion by stimulating both lipolysis and de novo synthesis of fatty acids [152] (Figure 9). Taken together, these data indicate that LIF-induced effects on suppression of body fat include actions on fat metabolism and that these actions in mice are not dependent on endogenous ovary-derived estrogens.

The dose of LIF used in our experiment also caused a significant decrease in BAT. However, the reduction in brown fat mass was not accompanied by altered UCP-1 or -3 protein expression. UCP-1 is used to generate heat by

non-shivering thermogenesis and is restricted to brown fat while UCP-3 is mainly expressed in skeletal muscle. The primary function of UCP-3 is not the regulation of energy metabolism but nevertheless includes involvement in the regulation of mitochondrial fatty acid transport and regulation of glucose metabolism [153]. UCP-1 protein expression is an index of thermogenesis in BAT [154], and hence the unchanged UCP-1 protein levels suggest that the weight reducing effects of LIF did not involve increased energy expenditure through non-shivering thermogenesis.

The finding that UCP-3 levels were unchanged supports the assumption that LIF does not influence energy metabolism in BAT. These data are supported by the finding that central administration of an adeno-associated viral vector encoding LIF did not affect UCP-1 protein expression [56]. However, LIF has been reported to decrease food intake when given ICV [56, 155], suggesting that LIF induces its weight reducing effect mainly through reduced appetite, possibly via a central effects. Yet, the literature studied does not hold any information about food intake after subcutaneous or i.p. injections of LIF [156, 157], leaving the question open whether peripheral LIF treatment can induce anorexia in a similar way as peripheral IL-1β injection [158]. It is a weakness that we did not measure food intake throughout the study. It would have been interesting to see whether LIF treatment could reverse the increase in food intake that has been reported after OVX in mice [159], and whether or not peripheral LIF treatment has the potential to induce anorexia.

OVX mice had decreased uterus weight and trabecular bone mineral density, and increased thymus weight. LIF treatment reversed the decrease in thymus weight but had no effect on uterus weight or bone mineral density. It is well known that OVX increases thymus weigh and that estrogen replacement therapy can reverse this effect [160-162].

Since estradiol enhances LIFR expression [150] it is possible that estrogen-mediated stimulation of LIF responsiveness also mediates this effect of endogenous estrogens. However, this well established thymus reducing effect of LIF is not dependent on endogenous estrogens [156, 157, 163]. Another possibility is that LIF indirectly suppresses thymus weight by decreasing body fat and leptin secretion. Thymus atrophy is a prominent feature of malnutrition, and starvation of mice significantly reduced thymocyte number [164]. These starvation-induced thymus changes were prevented by administration of exogenous leptin [164], indicating that circulating leptin levels have direct effect on thymus weight.

LIF treatment did not reverse the drastic decrease in uterine weight or bone mineral density after OVX. Therefore, these effects by endogenous

estrogens are likely to be independent of LIF. Another possibility, however, could be a tissue specific lack of LIFR in bone and uteri after OVX, due to a pronounced dependence of endogenous estrogens for LIFR expression in these organs [150].

The finding that LIF suppresses fat mass in OVX mice is in line with previous results that show that systemic LIF treatment to monkeys [156, 165] and mice [157] and central LIF gene therapy in rats [56] all decrease body weight. This may be a part of a generalized effect of cytokines acting through the IL6ST/gp130.

Recent research has focused on the role that gp130 receptor ligands may play as potential targets of obesity. We have shown that IL-6 itself selectively decreases fat mass, possibly via a central effect that increases energy expenditure [34, 57]. Another IL-6 family member, CNTF, mimics the biological actions of leptin by acting on the same genes in the ARC, resulting in decreased food intake and weight loss [166]. The anorexigenic and anti-obesity effects of this factor in the CNS seem to be independent of the leptin system and appear to be effective in obesity [166, 167], although this condition is often accompanied by leptin resistance (see Introduction, leptin section). CNTF and IL-6 also act on metabolic signalling pathways in peripheral tissue by the activation of AMPK [168-170] and increased fat oxidation [109, 110, 170], which is of primary importance to the regulation of body weight and insulin resistance. However, the major anti-obesity effect of these gp130 ligands may be exerted via the brain [56, 57, 167].

LIF and the LIFR in mice are expressed in the pituitary and in the PVN, DMN and ARC [90, 91] the sites of production, release and actions of neuropeptides implicated in the hypothalamic control of energy intake and expenditure. One may speculate that LIF could act on anorexigenic neuropeptides in the PVN and ARC, i.e. POMC and CRH, as ICV injections of LIF decreased food intake [56, 157]. During inflammation, cytokines that stimulate corticotroph POMC expression and ACTH secretion, e.g. LIF, are produced both peripherally and within the hypothalamus and pituitary [83, 171]. POMC expression in the pituitary leads to the release of ACTH and a stimulated HPA axis [83, 172]. The HPA axis is known to influence the energy homeostasis, and may contribute to the fat suppressing actions of LIF (Figure 9).

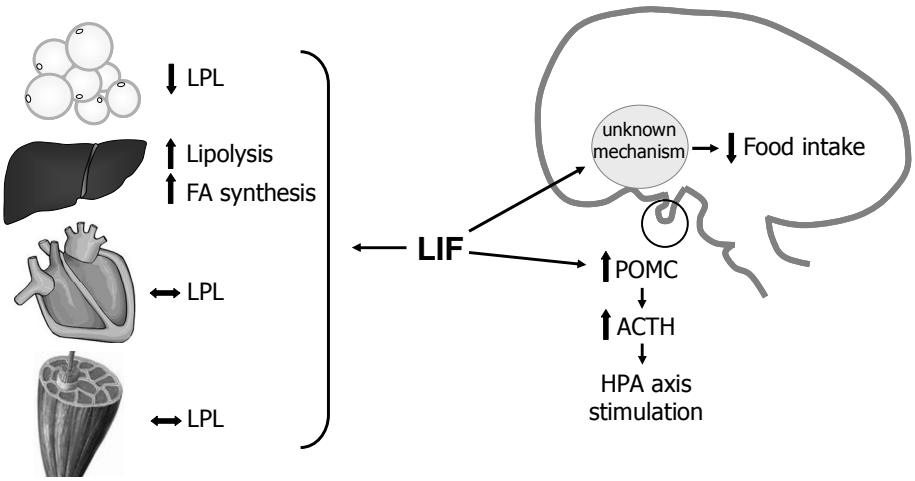


**Figure 9. Working hypothesis regarding central and peripheral effects mediating LIF-induced fat suppressing actions.**
Net effect after peripheral LIF stimulation may lead to increased fat utilization, which is in line with other cytokines, e.g. IL-6, that have also been shown to stimulate fat oxidation. Central actions of LIF involve HPA axis activation and possibly decreased food intake due to POMC expression in the hypothalamus. Lipoprotein lipase (LPL), fatty acid (FA)

In conclusion, LIF has been shown to exert anti-obesity effects in various animal models [56, 156, 157], and we can add to the body of knowledge that such an effect can also be seen in obese mice in the absence of ovarian estrogens.

## Paper IV: Cytokine Signalling affecting Myocardial Infarction

### An IL6ST/gp130 SNP was Associated with Myocardial Infarction

It has become clear that inflammation is a key component in the development of atherosclerosis and myocardial infarction, as suggested in the so called inflammation hypothesis [173, 174]. Although it is established that there is a link between inflammation and cardiovascular risk, with the latter largely being genetically determined, there are few genetic variants of the immune system that have been linked to cardiovascular disease including myocardial infarction [175]. For that reason we investigated the association between an IL6ST/gp130 polymorphism, function and risk of myocardial infarction.

In a prospectively followed hypertensive cohort we found that the 148Arginine (Arg) variant of the gp130 receptor was associated with decreased risk of myocardial infarction. Patients and controls were matched for sex, age and smoking status and the significantly decreased odds ratio for getting a myocardial infarction when carrying the Arg+ allele was still valid after adjustment for all significant risk factors for myocardial infarction in this study (Figure 10). This was in support of the gp130 Glycine(Gly)148Arg single nucleotide polymorphism (SNP) as an independent risk factor for myocardial infarction, together with leptin and high density lipoprotein (HDL), which are both associated with obesity.

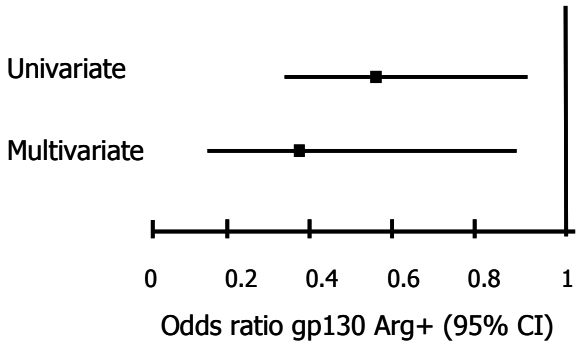


**Figure 10. Increased OR for myocardial infarction in Arg+ individuals**
Odds ratio (OR) and 95% confidence interval (CI) for getting myocardial infarction in arginine carrying (Arg+) individuals compared to non-carriers. Both univariate analysis (p=0.02) and multivariate analysis (p=0.02), with adjustment for all significant risk factors for myocardial infarction in this study, are shown.

## An IL6ST/gp130 SNP Showed Lower Proliferation Rate and STAT-3-P

Cells from a cell line that lacks endogenous gp130 were stably transfected with the two gp130 SNP variants. We found no significant difference in cell surface expression of gp130 between the two 148Gly and 148Arg variants. When the cell lines were stimulated with Hyper-IL-6, a factor consisting of coupled IL-6 and soluble IL-6Ralpha [82], the 148Arg variant proliferated less in response to gp130 stimulation as compared to cells transfected with gp130 148Gly. However, there was no difference in proliferation rate when cells were stimulated by IL-3.

The receptors for IL-3 are endogenously present on the used cell line, and the signalling pathways for IL-3 are separate from those for Hyper-IL-6. The similar effects exerted by IL-3 in the two cell variants therefore indicate that the decreased proliferation after Hyper-IL-6 stimulation in the 148Arg cell line specifically is due to impaired binding to, or signalling via the gp130 receptor. Furthermore, the time dependent stimulation of

STAT-3 phosphorylation by Hyper-IL-6 was consistently lower in cells expressing the 148Arg gp130 variant, while there was no difference in the levels of non-phosphorylated STAT-3 between the 148Gly and 148Arg cell lines.

These results support that the 148Arg variant is associated with decreased biological activity. This assumption is further supported by structural modelling, that pointed towards a change in stability and functional properties in the gp130/IL-6Rα complex after substitution of a glycine to an arginine in position 148. Still, any conclusion about the mechanistic effects of the Gly148 to Arg148 substitution requires further experiments, for instance to monitor any differences in subunit assembly or gp130 binding affinity for the different IL-6 family ligands. However, it could be postulated that the decreased risk of myocardial infarction in people carrying the Arg+ allele is partly due to decreased gp130 activation.

A recent study showed that hepatocyte-specific gp130 -/- mice on an atherosclerosis-prone genetic background, apolipoprotein E (apoE) -/- mice, had less aortic atherosclerosis with a reduced macrophage-positive plaque area, compared to mice with apoE -/- only [63]. Such organ specific gp130 -/- models are needed to study gp130 deficiency in relation to acute phase response, as complete lack of gp130 results in death *in utero* [176]. These hepatocyte specific gp130 -/- animals have a significantly reduced acute phase response after feeding with a high fat diet, and isolated macrophages showed reduced migration after stimulation [63]. Since macrophage migration is involved in the formation of foam cells that are found in atherosclerotic plaques, the decreased migration can possibly mediate the decreased plaque area seen in these animals.

Collectively, these findings show that the acute phase response plays an important role in the formation of atherosclerosis and that the development of atherosclerotic lesions can be reduced by an impaired gp130 signal transduction.

Although the exact mechanism for the results observed in Paper IV is unknown, these data are clearly in line with the inflammation hypothesis for CVD. Gp130 is the common signal transducer for the IL-6 cytokine family [66] and gp130 activation is regarded as a key event in inflammation. It is widely expressed in different cell types, but it may be noted that gp130 is expressed in both endothelial cells and smooth muscle cells of coronary arteries [177].

The fact that gp130 has a key role in mediating proinflammatory effects makes it a logical regulator of inflammation that may be altered by genetic

variations. However, it can not be excluded that the degree of gp130 activation could affect the risk of myocardial infarction via non-inflammatory routes. There are indications that gp130 is essential for the hypertrophic response to pressure overload and for the inhibition of apoptosis in myocytes [178-180]. Moreover, cardiotrophin-1, a cytokine acting via gp130, has been reported to stimulate production in endothelial cells of endothelin-1 [181]. This factor, in turn, can induce vasoconstriction and proliferation of smooth muscle cells, which can increase the risk of myocardial infarction [182]. Moreover, proliferation of smooth muscle cells might also be promoted by the gp130 activation seen in these cells [177].

The risk of atherosclerosis and cardiac disease is partly genetically determined, by multiple genomic loci interacting with the environment [183]. Some of these genetic variations may modulate the inflammatory response and consequently alter susceptibility to CVD. In spite of this, there are a limited number of immune related polymorphisms that have been reported to affect the risk of myocardial infarction. Two examples are the Toll-like receptor 4 Asp199Gly polymorphism [184] and the IL-6 promoter -174G/C SNP, which have been associated with risk of CVD. However, polymorphism studies should always be interpreted with caution, as they only show associations, and not proof, of cause-effect relations. For instance, the association seen in this paper could be due to linkage disequilibrium between the Gly148Arg polymorphism and another polymorphism that is affecting the risk of myocardial infarction.

The linkage of a number of polymorphisms in the IL6ST/gp130 gene, including the Gly148Arg SNP, were further investigated by Luchtefeld et. al., and the haplotype analysis showed that nine of these SNPs, including Gly148Arg, fall into one block with strong linkage disequilibrium. One SNP was significantly associated with cardiovascular artery disease, while the Gly148Arg polymorphism showed a trend for association with cardiovascular artery disease [63]. Even though it has not been fully evaluated which polymorphisms and which alleles that are associated with increased and decreased risk of CVD, these data provides critical evidence that gp130 plays an important role in human CVD.

In conclusion, the Gly148Arg polymorphism of the IL6ST/gp130 subunit of the IL-6 receptor family was associated with decreased risk of myocardial infarction, also when several conventional risk factors were taken into account. The relevance of this clinical finding was supported by the results from cell studies and by studies on hepatocyte specific gp130 -/- apoE -/- double knockout mice. This finding may add to a still limited but growing list of immune related polymorphisms that could provide molecular genetic explanations for the inter-individual variations in susceptibility to CVD.

# CONCLUDING REMARKS

During recent years, it has become clear that obesity is linked to actions of the immune system. Leptin is secreted by adipocytes and decreases appetite and increases energy expenditure via effects on the hypothalamus. However, there is now evidence that leptin plays a role also in the regulation of the immune system and the metabolism during infection. Conversely, we believe that cytokines, in addition to regulating the immune system, can be important for the regulation of metabolism during health. This thesis has focused on the metabolic effects of three such cytokines, IL-1, IL-6 and LIF. These cytokines are expressed in diverse tissues, acting on short distances, i.e. in auto- and paracrine signalling, but are also released into the blood stream, which enables endocrine effects.

Depletion of IL-6 or IL-1RI in knockout mice results in mature-onset obesity, indicating that IL-1 and IL-6 activation possesses a tonic fat-suppressing effect in healthy animals. IL-6, IL-1 and their receptors and the endogenous IL-1Ra are all expressed in the CNS, including the hypothalamus, and the metabolic effects of IL-6 are more pronounced when it is administrated centrally, as opposed to peripherally, highlighting the importance of central actions of IL-6. The central effects of IL-1 and IL-6 include responses known to be mediated via the SNS, e.g. increased energy expenditure, increased heart rate and increased blood pressure. It is not yet known where in the CNS IL-6 and IL-1 exert their effects, but there is evidence that IL-6 increases the production of CRH and IL-6 -/- mice have decreased levels of CRH in the hypothalamus. CRH is an important stimulator of the HPA axis and SNS-outflow during stress. Therefore, the results above are in line with the finding that stress-induced SNS-outflow seems blunted in IL-6 -/- mice.

IL-1RI -/- mice have decreased expression of the obesity suppressing peptide orexin at young age and enhanced expression of the obesity promoting peptides NPY and MCH at older age. This can constitute a possible mechanism contributing to the mature-onset obesity.

The obesity in IL-1RI -/- mice was accompanied by decreased insulin and leptin sensitivity. In humans, circulating IL-6 is increased in obesity and suggested to aggravate insulin resistance. Although it can not be excluded that IL-1 and IL-6 can decrease insulin sensitivity at high pathological levels in conjunction with other cytokines, available data indicate that lower doses of IL-1 and IL-6 can increase insulin sensitivity *in vivo*, possibly by decreasing obesity. Therefore, an assumed positive effect of lack of IL-1 and IL-6 on insulin sensitivity might be masked by the influence by obesity in older animals.

Inflammation is a key component in the development of atherosclerosis and systemic inflammation is often associated with increased risk of cardiovascular events, i.e. myocardial infarction, stroke and peripheral artery disease. In addition, obesity is associated with increased risk of CVD and is linked to a state of chronic inflammation, as overweight and obese subjects have elevated serum levels of inflammation markers. Moreover, it has been hypothesized that proinflammatory cytokines from adipose tissue contribute to the chronic inflammation and thereby also to CVD.

In a prospectively followed hypertensive cohort we found an association between gp130, a key component in the inflammatory signal pathway, and myocardial infarction. The less common 148Arg variant of the gp130 receptor was associated with decreased risk of myocardial infarction. Cell signalling studies indicated that the 148Arg variant is a less potent inducer of cell signalling in response to IL-6, suggesting that the acute phase response plays an important role in the formation of atherosclerosis, and that the development of atherosclerotic lesions can be reduced by an impaired gp130 signal transduction. We, and others, have provided evidence that variations in gp130 activity can influence the risk of atherosclerosis, coronary artery disease and myocardial infarction.

An additional cytokine, LIF, also have metabolic effects, seen as decreased fat mass. It seems fascinating that several immune factors also seem to have body fat regulating properties in healthy individuals, with no evident immune activation. Like other biological mediators, the effects of IL-1, IL-6 and LIF depend probably on the concentration, the site of action and the presence of interacting factors. IL-1, IL-6 and LIF have been shown to be involved in the regulation of energy intake and expenditure, affecting body fat mass, and these effects in the long run may protect against weight gain, obesity and metabolic disturbances. In addition, variations in cytokine signalling via gp130 can influence the risk of cardiovascular disease. Therefore, we hypothesize that cytokines and immune regulating factors can provide new drug targets for obesity, and that these new targets may act on metabolic disturbances from a different angle.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Denna avhandling visar att fetma är kopplat till den del av immunförsvaret som kallas det medfödda immunsystemet. Det medfödda immunförsvaret är kroppens första försvarslinje som snabbt kan producera immunstimulerande ämnen, så kallade cytokiner, som svar på en infektion. I denna avhandling har tre av dessa cytokiner studerats mer ingående; interleukin-1 (IL-1), interleukin-6 (IL-6) och leukemi-inhiberande faktor (LIF).

Vi har tidigare funnit att om man slår ut delar av det medfödda immunsystemet (den immunstimulerande cytokinen IL-6) hos möss blir dessa fetare än vanliga möss. Vi har nu studerat en annan musmodell som saknar receptorn för IL-1. Eftersom IL-1 binder till IL1-receptorn för att utöva biologiska effekter, är IL-1-aktiviteten utslagen i möss utan IL-1-receptor. Vi har nu funnit att också dessa möss utvecklar fetma.

Insulin frisätts för att hjälpa kroppen att ta upp sockret i blodet efter måltider. De feta mössen som saknar receptorn för IL-1 var mindre känsliga för insulin. Minskad insulinkänslighet anses vara ett förstadium till sjukdomen diabetes med högt blodsocker, vilken ofta ses i samband med fetma. Avsaknad av IL-1-aktivitet ledde även till en minskad fettförbränning och en minskad fysisk aktivitet, faktorer som tros spela roll för utvecklingen av fetma. När vi undersökte hypotalamus, den del av hjärnan som reglerar födointag och ämnesomsättning, visade det sig att möss utan IL-1-receptorer hade påverkade nivåer av signalsubstanser som tidigare visats reglera födointag och kroppsfett. IL-1 och IL-6 påverkade också varandras nivåer i hypotalamus. Därför drar vi slutsatsen att IL-1 och IL-6 påverkar risken för fetma genom att samverka i det område i hjärnan som styr födointag och ämnesomsättning. Det verkar troligt att IL-1 och IL-6 minskar fettmassan genom att öka ämnesomsättningen, kanske särskilt fettförbränningen.

Behandling med cytokinen LIF visade sig kunna minska fettmassan hos en musmodell som efterliknar kvinnor i klimakteriet. Dessa möss har mycket låga östrogennivåer och tenderar, på grund av att de fått äggstockarna bortopererade, att gå upp i vikt. LIF verkar alltså kunna minska fettmassan oberoende av östrogen. Mekanismen bakom den minskade fettmassan är okänd men möjligen skulle LIF kunna verka genom att öka fettförbränningen och minska födointaget. Detta genom att på samma sätt som IL-1 och IL-6 påverka hypotalamus, den del av hjärnan som reglerar energiintag och ämnesomsättning.

Länge trodde man att fettvävnaden endast var en lagringsdepå för fett (lipider), men modern forskning har visat att fettväven även insöndrar en

mängd signalämnen, bland annat cytokiner, till blodet. Av dessa är många inblandade i energibalansen, inklusive IL-1, IL-6 och LIF. Via transport med blodet kan de nå hjärnan och där utöva effekter på kroppens totala fettmassa.

De senaste åren har man funnit att fetma på olika sätt är sammankopplat med vårt immunförsvar. Bland annat har man upptäckt att fetma orsakar en kronisk, så kallad låggradig inflammation i fettväven, vilket leder till insöndring av inflammatoriska komponenter i blodet. Dessa komponenter kan i sin tur påverka inflammatoriska sjukdomsförlopp som till exempel åderförfettning, som kan ger upphov till hjärtinfarkt och stroke.

Vi har undersökt sambandet mellan en receptor för IL-6 och LIF och hjärtinfarkt. I studien ingick nästan 1500 män och kvinnor i övre medelåldern. De som hade en medfödd svagare variant av denna receptor hade i genomsnitt 50% lägre risk att drabbas av hjärtinfarkt än de med den mer vanligt förekommande receptorformen. Den minskade risken för hjärtinfarkt skulle kunna bero på att dessa individer inte är lika känsliga för cytokiner som IL-6 och LIF då receptorn har nedsatt funktion. För en person som är i riskzonen att drabbas av en hjärtinfarkt, och som kanske har flera riskfaktorer för hjärt-kärlsjukdom, kan detta vara av betydelse för den totala riskbilden. Studier i möss bekräftar att denna receptor kan vara inblandat i utvecklingen av hjärt-kärlsjukdom.

Fetma innebär ett akut globalt hälsoproblem, som ökar risken för åldersdiabetes, hjärt-kärlsjukdom och tidig död. Den ökade fetman hotar därmed det allmänna hälsotillståndet världen över, samtidigt som dagens läkemedel mot fetma är bristfälliga. Denna avhandling visar att cytokinerna IL-1, IL-6 och LIF är delaktiga i regleringen av energiomsättningen och mängden kroppsfett och att IL-1 och IL-6 delvis utövar sina effekter via hjärnan. Att immunreglerande faktorer nu visats delta i reglering av energiförbrukning och kroppsfett kan leda till nya läkemedel som kan angripa fetma på ett helt nytt sätt.

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