Interactions between Nutrition, Obesity and the Immune System

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Till min familj

ABSTRACT

There are several links between body fat and the immune system. For example, mice lacking activity of the pro-inflammatory interleukin-(IL)-1 and IL-6 develop obesity. Conversely, obesity is associated with adipose tissue inflammation and increased risk of infection. The aims of this thesis were to investigate (1) the effect of Western diet on *Staphylococcus aureus* (*S. aureus*)–induced mortality in mice; (2) if dietary fat composition affects mortality in *S. aureus* inoculated mice; and if IL-6 and IL-1 system gene polymorphisms, associated with expression, are associated with fat mass in (3) young and (4) elderly men.

The *S. aureus*-induced mortality was investigate in mice fed a lard-based high-fat diet (HFD) rich in saturated and monounsaturated fatty acids (HFD/S) or a low fat diet (LFD). After 8 weeks on these diets, the mice were intravenously inoculated with *S. aureus*. The obese HFD/S-fed mice had increased *S. aureus*-induced mortality compared with the lean LFD-fed mice. The HFD/S-fed mice showed signs of immune suppression as evident by increased bacterial load and decreased capacity to phagocytose bacteria. We then added a group of mice fed a HFD rich in polyunsaturated fatty acids (HFD/P) from fish. The HFD/P-fed mice displayed a degree obesity and glucose intolerance that was milder than in the HFD/S-fed mice, but higher than in LFD mice. However, the *S. aureus*-induced mortality and the bacterial load of HFD/P-fed mice were comparable with that of LFD-fed mice, and markedly lower than that of mice fed HFD/S.

Gene polymorphisms were investigated in two well-characterized population-based cohorts of young and elderly Swedish men. In young but not elderly men, we found that carriers of the T variant of the +3953 C>T *IL1B* polymorphism had lower total fat mass, compared with CC carriers. In elderly but not young men, the *IL1B* -31T>C polymorphism was associated with total fat mass. In young but not elderly men, we found that *IL-1RN**2 carriers, with two repeats of the *IL1RN* 86 base pair variable number tandem repeat polymorphism, had increased total fat mass. Also, *IL1RN**2 was associated with increased IL-1Ra production *in vitro* and enhanced serum IL-1Ra *in vivo*. We also confirmed

earlier findings that the C variant of the -174G>C *IL6* is associated with obesity in elderly men.

Thus, the present results indicate the *S. aureus*-induced mortality is associated with dietary fat consisting of saturated and monounsaturated fatty acids, but not polyunsaturated fatty acids. We also show that polymorphisms in the *IL1B*, *IL1RN*, and *IL6* genes are associated with obesity. In conclusion, this thesis emphasize that there are reciprocal interactions between the immune system on one hand and obesity and nutrition on the other.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Det finns flera kopplingar mellan risken för fetma och immunförsvaret. Man har t.ex. funnit att möss, där delar av immunförsvaret slagits ut, blir fetare än vanliga möss. Detta gäller bl.a. då man slår ut effekterna av de immunstimulerande substanserna interleukin-6 (IL-6) och interleukin-1 (IL-1). Kroppsfett kan också omvänt påverka immunförsvaret. Fetma är nämligen förknippat med inflammation, dvs. en slags aktivering av immunförsvaret i avsaknad av infektion. Fetma är paradoxalt också kopplad till ökad infektionsrisk. *Staphylococcus aureus* (*S. aureus*) är varbakterier som ofta är motståndskraftiga mot antibiotika och de är en vanlig orsak till död i blodförgiftning hos människa. Syftet med denna avhandling var att undersöka om dödligheten hos möss som är infekterade med *S. aureus* påverkas av (1) västerländsk högfettdiet eller (2) matens fettsyrefördelning mellan mättat och fleromättat fett. Dessutom ville vi utreda om vanligt förekommande förändringar i generna för immunreglerarna IL-6 och IL-1 är kopplade till mängden kroppsfett hos (3) unga och (4) äldre män.

Den *S. aureus*-inducerade dödligheten undersöktes hos möss som fick en späckbaserad diet med högt fettinnehåll, mestadels innehållande mättade och enkelomättade fetter, eller en lågfettdiet. Efter 8 veckor på dessa dieter hade mössen som fått högfettdiet blivit feta och mössen infekterades intravenöst med *S. aureus*. Vi fann att mössen som ätit mättad högfettdiet hade en ökad dödlighet till följd av infektionen. De hade fler bakterier och tecken på en sänkt immunaktivitet så som minskad förmåga hos vissa vita blodkroppar att äta upp bakterier, via så kallad fagoytos. I nästa experiment undersöktes ytterligare en grupp möss som fick äta högfettdiet, men denna diet innehöll fiskolja som innehåller en stor andel fleromättade fetter istället för späck och mättade fettsyror. Jämfört med mössen i lågfettgruppen, blev mössen som fått fleromättat fett fetare och de hade också sämre blodsockerkontroll, men de klarade sig bättre i dessa avseenden än mössen som fått mättat fett. Trots detta var dödligheten och bakteriehalten hos möss som fått fleromättad fiskdiet lika låga som i lågfettgruppen och betydligt lägre än hos mössen som fått mättade fetter i späckdieten.

Genförändringarna undersöktes i två välkarakteriserade studiegrupper där svenska unga och äldre män slumpmässigt valts ut från befolkningen. Fyra olika genvariationer i tre gener (*IL1B*, *IL1RN* och *IL6*) undersöktes. Man har sedan tidigare sett att dessa genvariationer är kopplade till förändringar i hur mycket protein eller äggviteämne som bildas med genen som mall. Vi fann att den starkare, men ovanligare, varianten (kallad T) av en genvariation (+3953 C>T) i genen för IL-1 var associerad med lite kroppsfett hos unga, men inte äldre, män. I samma gen såg vi att en annan genvariant (-31 T>C) var associerad med kroppsfett hos de äldre, men inte yngre, männen. Det finns en kroppsegen hämmare till IL-1 som heter IL-1Ra. I genen för IL-1Ra finns en variant som kallas 86 bp *IL1RN**2 och denna har setts ge ökad genaktivitet. Hos unga, men inte äldre, män var denna variant kopplad till ökat kroppsfett och ökad halt av IL-1Ra i blodet. Hos de äldre männen var den starka genvarianten (G) av IL6 -174 G>C, som ger mer IL-6 protein, kopplad till mindre kroppsfett.

Sammanfattningsvis tyder resultaten på att förmågan att bekämpa en bakterieorsakad blodförgiftning påverkas av kosten. Man har större chanser att överleva om man ätit diet med liten fetthalt eller med mycket fleromättat fett från fet fisk, jämfört med om man ätit mättat fett från späck. Vi visar också att flera varianter i immunreglerade gener som ger stärkt immunförsvar också ger mindre fetma. Denna avhandling understryker därför att det finns flera olika sorters kopplingar mellan immunförsvaret å ena sidan och fetma och nutrition å andra sidan.

LIST OF PUBLICATIONS

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

I **Mice chronically fed high-fat diet have increased mortality and disturbed immune response in sepsis**
Strandberg L, Verdrengh M, Enge E, Andersson N, Amu S, Önnheim K, Benrick A, Brisslert M, Bylund J, Bokarewa M, Nilsson S, Jansson JO
PLoS ONE 2009 Oct;4(10):e7605

II **Septic mortality is lower in mice fed a diet rich in polyunsaturated compared with saturated fatty acids**
Strandberg L, Benrick A, Andersson N, Nilsson S, Jansson JO
Manuscript

III **Interleukin-1 system gene polymorphisms are associated with fat mass in young men**
Strandberg L*, Lorentzon M*, Hellqvist A, Nilsson S, Wallenius V, Ohlsson C, Jansson JO
J Clin Endocrinol Metab 2006 Jul;91(7):2749-2754

IV ***IL6* and *IL1B* polymorphisms are associated with fat mass in older men: the MrOS Study Sweden**
Strandberg L*, Mellstrom D,* Ljunggren O, Grundberg E, Karlsson MK, Holmberg AH, Orwoll ES, Eriksson AL, Svedberg J, Bengtsson M, Ohlsson C, Jansson JO
Obesity (Silver Spring) 2008 Mar;16(3):710-713

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CONTENTS

ABBREVIATIONS

A	adenine
bp	base pair
BMI	body mass index
C	cytosine
CARS	compensatory anti-inflammatory response syndrome
Ccl	chemokine (C-C motif) ligand
Ccr	chemokine (C-C motif) receptor
cDNA	complementary DNA
CFU	colony forming unit
CNTF	ciliary neurotrophic factor
CNV	copy number variation
DNA	deoxyribonucleic acid
DXA	dual-energy X-ray absorptiometry
G	guanine
GM-CSF	granulocyte macrophage colony-stimulating factor
GOOD	Gothenburg Osteoporosis and Obesity Determinants
GWA	genome-wide association
HFD	high-fat diet
HFD/S	HFD rich in saturated and monounsaturated fatty acids
HFD/P	HFD rich in polyunsaturated fatty acids
HLA	human leukocyte antigen (in humans the same as MHC)
Hmox1	Heme oxygenase 1
IL	interleukin
IL-1RI	IL-1 receptor I
IL-1Ra	IL-1 receptor antagonist
LD	linkage disequilibrium
LDA	low density arrays
LPS	lipopolysaccharide
MHC	histo-compatibility complex
mRNA	messenger RNA
MrOS	Osteoporotic Fractures in Men
MUFA	monounsaturated fatty acids
NF-κB	nuclear factor-κB
PCR	polymerase chain reaction
PMA	phorbol myristate acetate
PPAR	peroxisome proliferator-activated receptor
Ptprc	protein tyrosine phosphatase receptor type C

PUFA	polyunsaturated fatty acids
RNA	ribonucleic acid
ROS	reactive oxygen species
S. aureus	*Staphylococcus aureus*
SFA	saturated fatty acids
SIRS	systemic inflammatory response syndrome
SNP	single nucleotide polymorphism
T	thymine
TNF-α	tumor necrosis factor-α
VNTR	variable number tandem repeat

Gene and protein nomenclature clarification
Gene symbols are their three or more italic letters. For humans all letters are uppercase but only the first for mice. Many protein abbreviations are written like the gene symbols except for the not italicized letters. However for long known proteins these may differ from the gene symbol.

INTRODUCTION

The immune system

The immune system is divided into an innate or native and an adaptive part. The innate immunity is the oldest form of immune defense and it is present in all multi-cellular organisms while adaptive immunity only is present in vertebrates. The innate immunity responds quickly to infections but in the same way every time it encounters a certain pathogen. In contrast, adaptive immunity takes a few days to activate, but it has a memory and therefore responds quicker and better the second time an individual is exposed to a pathogen. The innate immunity responds to common structures of pathogens, such as bacterial cell wall components and bacterial or viral deoxyribonucleic acid (DNA). Typically, these components are essential for the survival of the pathogen. The innate immunity consists of barriers like the skin and mucosa, and also antimicrobial compounds in the mucosa, immune cells such as phagocytic cells (macrophages and neutrophils) and natural killer cells, proteins called cytokines produced by these cells, and blood borne proteins called complement factors (1).

The adaptive immunity is very specific and can differentiate between substances even if their structure is similar. The cells in the adaptive immune system are called lymphocytes and the major types are the B and T cells. These cells are involved in antibody production, memory, destruction of infected cells, and control of inflammatory response (1).

Immune cells

Neutrophilic granulocytes

Neutrophils have a segmented nucleus (they are therefore also called polymorphonuclear cells) and their cytoplasm is filled with different kinds of granule. These contain for example lactoferrin, nicotinamide adenine dinucleotide phosphate-(NADPH)-oxidase, and bactericidal components. Lactoferrin can inhibit bacterial growth by binding of iron while NADPH-oxidase produces reactive oxygen species (ROS) that are toxic to microorganisms and tissues. Neutrophils are efficient phagocytes and the

phagocytosed bacterium is contained within the cell in a phagosome. The bacterium is then killed when the contents of the granule is emptied into the phagosome. Extracellular bacteria can also be killed by emptying the granule into the surrounding tissue, but this will also cause tissue damage. Neutrophils are short-lived cells that circulate in blood for less than a day. During inflammation endothelial cells produce chemokines, among others interleukin-8 (IL-8) that specifically attracts neutrophils. This leads to accumulation of neutrophils at sites of inflammation, long before monocytes and lymphocytes (1, 2).

Monocytes/macrophages

Monocytes do not have a segmented nucleus, and are together with lymphocytes, called mononuclear cells. The monocytes circulate in the blood for around one day and then enter tissues were they mature into macrophages. Like neutrophils, macrophages are phagocytes and they are also able to produce some ROS. Monocytes are the major producer of cytokines and secrete many different ones, both pro- and anti-inflammatory. These cells can also produce other inflammatory molecules like leukotrienes and prostaglandins that are derived from fatty acids. These factors help in recruiting and activating other immune cells. The macrophages also help in clearing the infection by phagocytosing dead cells, e.g. apoptotic neutrophils (1, 2).

B cells

B cells mature in the bone marrow and then circulate between blood and secondary lymph organs, i.e. lymph nodes and lymph vessels. Each B cells can recognize one unique structure (antigen). The antigen is recognized when specific antibodies on the cell surface binds its antigen, which activates the B cell and then lead to clonal expansion (proliferation) of the B cells. These can then mature into plasma cells that secrete large amounts of its specific antibody. Antibodies help to kill microbes for example by attachment to microbes and thereby marking them for phagocytosis. Some of the activated B cells instead mature into memory cells. They are long-lived and can respond quickly when they encounter their specific antigen again (1, 2).

T cells

Immature T cells leave the bone marrow to mature in the thymus ("T" stands for thymus-derived). Like B cells they only recognize one specific antigen with their T cell receptor. In order for T cells to recognize its antigen it has to be presented on a certain molecule called the major histo-compatibility complex (MHC). MHC class I molecules are found on all cells except red blood cells. The MHC class II molecules are found on immune cells that reside in tissues, i.e. dendritic cells and macrophages. T cells can be divided into three different types: T helper cells, T regulatory cells, and T cytotoxic cells. The two former cell types are so called CD4 positive and they recognize antigens presented on MHC class II molecules. The cytotoxic T cell is CD8 positive and recognizes antigens presented on MHC class I molecules. After antigen recognition T cells become activated, proliferate into effector or memory cells. The effector T helper cells activate macrophages and B cells by expression of membrane molecules and secretion of cytokines. There are different kinds of T helper cells (e.g. Th1 and Th2) and they are defined by their secreted cytokines (discussed below). Briefly, Th1 cells produce interferon-γ and stimulate microbicidal functions in phagocytes. Th2 cells produce e.g. IL-10, IL-4, and IL-5 and these suppress macrophages, promote defense against helminthes and are important for allergic responses. The effector cytotoxic T cells can kill viral infected and tumor cells. T regulatory cells suppress T cell response and thus induce tolerance to its specific antigen (1, 2).

Cytokines

Cytokines are small soluble molecules that mediate intercellular communications in the immune system (1). They are produced by immune cells, but also by other cells like endothelial and epithelial cells, and by adipocytes and myocytes (1, 3, 4). Cytokines have numerous and often redundant functions, such as chemotaxis, immune modulation, and hematopoiesis (1).

Interleukin-1 (IL-1) system

The principal sources of IL-1 are macrophages and endothelial cells. It is a pro-inflammatory cytokine that was first shown to induce fever, but it is now also known to cause induction of neutrophilia, endothelial cell activation, increased IL-6 levels, anorexia, and increased acute-phase protein synthesis etc. The IL-1 system has several components, including two agonists, IL-1β and IL-1α. Biological effects are exerted via the IL-1 receptor I (IL-1RI). IL-1 actions can

be inhibited by competitive binding of the endogenous antagonist IL-1 receptor antagonist (IL-1Ra) to IL-1RI or by the binding of IL-1 to a second type of IL-1 receptor, IL-1RII. This is a decoy receptor which prevents IL-1 from IL-1RI binding. A delicate balance between IL-1 and IL-1Ra is of importance for regulation of immune function. Disturbances of the IL-1 system have been implied to be involved in arthritis, kidney and liver disease and the damage of insulin producing pancreatic cells during development of type-1 diabetes and also type-2 diabetes (5-7). IL-1 has also been shown have metabolic functions and for example affect fat mass. This will be discussed more in the sections "Cytokine deficiency and obesity" and "Summary of results and discussion".

Interleukin-6 (IL-6)

IL-6 is produced by macrophages, endothelial cells, T cells etc. and the synthesis is induced by microbes, IL-1, and tumor necrosis factor-α (TNF-α). IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory functions. Some of the pro-inflammatory functions include stimulation of acute phase reactants in the liver during infection and proliferation of antibody-producing B cells (1). The anti-inflammatory properties of IL-6 include inhibited production of pro-inflammatory cytokines such as IL-1, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and interferon-γ, while it increases the synthesis of the anti-inflammatory glucocorticoids, IL-1Ra, and soluble TNF receptor (8-10). The anti-inflammatory properties of IL-6 seem important for controlling local and systemic acute inflammatory responses (11). IL-6 is also released from muscle during exercise and this probably mediates at least some of the beneficial effects of exercise (12).

Interleukin-10 (IL-10)

IL-10 is produced by various cells such as T-helper cells (Th2), regulatory T cells monocytes, macrophages, and B cells. IL-10 is a potent anti-inflammatory cytokine and the major effects seem to be exerted on dendritic cells and macrophages. This leads to decreased capacity to present antigens with MHC class II molecules, which in turn leads to inhibition of T cell activation. In addition, IL-10 is a powerful inhibitor of the production of most cytokines in macrophages and dendritic cells, the exception being IL-1Ra which is up-regulated. IL-10 has been used in several clinical studies to treat inflammatory diseases. The results are so far inconclusive but may improve with better ways of administration (13).

Tumor necrosis factor-a (TNF-a)

The major source for this pro-inflammatory cytokine is macrophages, but it can also be produced in T cells. TNF-α synthesis is strongly induced by the cell wall component lipopolysaccharide (LPS) from gram-negative bacteria. Like IL-1, TNF-α can activate endothelial cells in order to attract neutrophils and monocytes. In addition, TNF-α stimulates synthesis of acute-phase proteins from the liver and induce fever by acting in the hypothalamus, albeit less so than IL-6 and IL-1, respectively. Chronically elevated TNF-α levels suppress appetite and leads to wasting of muscle and fat, so called cachexia (1). Several different TNF-α inhibitors are used in the clinic for inflammatory diseases such as rheumatoid arthritis, psoriasis, and ulcerous colitis (14).

Sepsis

Definition

Sepsis is defined as the systemic inflammatory response to a confirmed or suspected infection. The symptoms include body temperature under 36 or over 38°C, increased heart and respiratory rate, and altered white blood cell counts. The same symptoms can occur without infection and the disease is then called systemic inflammatory response syndrome (SIRS). Sepsis can progress into severe sepsis, which is associated with organ dysfunction, hypoperfusion, or hypotension. Further progression can lead to septic shock, which is defined as "sepsis-induced hypotension, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction" (15, 16).

Epidemiology

The incidence of sepsis and severe sepsis is increasing (17-20) and although the mortality rate has decreased, the total number of deaths is increasing (17, 19). In the United States septic mortality is the tenth most common reason for overall death (21) and the mortality rate in 1995-2000 was 18%. Severe sepsis and septic shock have even higher mortality rates of about, 30-50% (18, 19, 22, 23). In the United States, gram-positive bacteria and fungi have increased as a cause of sepsis, and gram-positive bacteria are now a more common cause of sepsis

than gram-negative bacteria (17). *Staphylococcus aureus* (*S. aureus*) is the most common cause of gram-positive sepsis (22, 24).

Etiology

Probable causes of the increased incidence of sepsis are the increase in invasive surgical procedures, use of immunosuppressive treatments, chemotherapy, transplantations, HIV, and microbial resistance (17). There are also several known genetic variants that have been associated with increased risk of sepsis and septic death (25).

Treatments and progression of the disease

There are few treatments for sepsis, and they include "early, goal-directed therapy" which means that measures are taken to avoid imbalance between oxygen delivery and oxygen demand as this may lead to hypoxia and shock (26), lung-protective ventilation and broad spectrum antibiotics (27). The mechanisms in sepsis are poorly understood, but sepsis has been thought of as a hyperinflammatory disease and many clinical trials have aimed at decreasing this hyperinflammation by using immune suppression, for example TNF-α inhibition and corticosteroids (28). Although many of these treatments have been successful in animal models, the results in clinical studies have often been discouraging (27, 29), and a meta-analysis even showed that anti-TNF-α treatment may be harmful (30). Possibly the only exception to the lack of effect is the anti-inflammatory and anti-coagulant activated protein C, which has been found to decrease mortality somewhat in patients with severe sepsis (22). It is now thought that sepsis induces an initial hyperinflammatory phase that eventually progress into a phase of immune suppression (28), also termed compensatory anti-inflammatory response syndrome (CARS) (31). However, it has also been postulated that SIRS and CARS instead occur simultaneously and that SIRS dominate in the inflamed tissue whereas CARS occurs in the systemic circulation (32).

The immune suppression during sepsis seems to affect parts of both the innate and the adaptive immune systems, but there are also aspects of the immune system that do not seem to be inhibited. For instance, apoptosis is increased in lymphocytes (33, 34), but decreased in neutrophils (35). Lymphocyte apoptosis seems important in sepsis, as inhibited apoptosis through administration of caspase inhibitors or overexpression of *Bcl2* reduces organ injury and death, at least in animal studies (36-38).

Pro-inflammatory cytokine production in LPS-stimulated neutrophils (39) and monocytes (40-42) from septic patients is lower than in controls, but this may depend on the used stimuli and time of measurement (39, 43). The anti-inflammatory cytokine IL-10 has been shown to be increased in serum (41, 44) and after LPS stimulation of monocytes from patients with sepsis (42). In addition, high serum levels of IL-10 have been associated with increased mortality in sepsis (45). IL-10 may have an important role in sepsis by decreasing the number of human leukocyte antigen (HLA)-DR surface receptors, which are essential for antigen-presentation to T helper cells (44, 46). Low HLA-DR expression on monocytes have been associated with decreased survival in sepsis or septic shock in some (45, 47, 48) but not all (49) studies. A slow recovery of HLA-DR expression is also associated with increased risk of acquiring secondary infections (49).

During the suppressive state, it seems logical to try immune stimulating therapy, but there are few examples of beneficial effects by such treatment. In a small study of septic patients with reduced HLA-DR expression, treatment with the monocyte activator interferon-γ lead to increased HLA-DR expression (50). Increased survival in the treatment group was reported, but with only 9 treated patients this needs further evaluation (50). Also, GM-CSF has been found to reduce the time of mechanical ventilation in a small, but double-blind, randomized, placebo-controlled trial (51).

Obesity

Definition and epidemiology

A body mass index (BMI; kg/m^2) over or equal to 30 kg/m^2 is defined as obesity and over or equal to 25 kg/m^2 as overweight (52). The prevalence of obesity has become epidemic and in year 2005, 74% of Americans were overweight and 39% were obese (53). In Sweden the prevalence in 2005 of overweight and obesity was 50% and 11%, respectively (53).

Etiology

The most convincing factors to promote development of obesity are a sedentary lifestyle, and high intake of energy-dense and micronutrient-poor foods, such as diets rich in fat or sugar (54). In addition to environmental factors, heritage has

also been found to play a role. Studies of monozygotic twins have shown that genetic factors may determine around 70% of adiposity, while adoption studies show figures of 30% or less (55). Even though the exact degree of genetic influence can be debated, it is clear that it is of importance. However, only in rare cases obesity is caused by a genetic variant in one gene. Instead, several gene variants are thought to interact, and obesity is said to be a polygenetic disease. By now, over 200 genes have been implicated in murine obesity and more than 100 human genes have been found to be associated with obesity (56).

Co-morbidities

Obesity is associated with several co-morbidities, for example type-2 diabetes, different cancers, cardiovascular disease like atherosclerosis, and asthma (57). Especially individuals with abdominal obesity are at risk. In particular, it is the visceral part of the abdominal fat that is most dangerous and high levels of visceral fat is more common in men than in women (58).

Obesity and inflammation/immunity

Inflammation in adipose tissue in obesity

A cause for some of the co-morbidities, such as type-2 diabetes and atherosclerosis, is believed to be the chronic inflammation starting in the adipose tissue (59, 60). Inflammation is evident as obesity has been shown to up-regulate adipose tissue production of mostly pro- but also anti-inflammatory cytokines (3, 59, 61-67). The trigger for this inflammation is unknown but may involve hypoxia (68) and hypoxia-induced fibrosis (69), adipose tissue cell death (70), adipocyte stress (71), and adipocyte production of chemokines (72).

Immune cell infiltration in adipose tissue

The events described above, may in turn attract inflammatory cells to the adipose tissue. Macrophages were the first immune cells to be found in adipose tissue (66, 67), but more recently neutrophils (73), B cells (74), T cells (74-76), and mast cells (77) have also been identified. Especially the macrophages, together with adipocytes, are believed to produce many of the cytokines released from the adipose tissue into the blood circulation (3, 59, 61-67).

Neutrophils have been shown to migrate into adipose tissue just a couple of days after initiation of high-fat feeding, but the infiltration seems transient (73). B cells were increased three weeks after high-fat diet (HFD), followed by T cells (78). $CD8^+$ T cells, so called cytotoxic T cells, accumulate in epididymal adipose tissue after 2 weeks on high-fat feeding to mice, and the expression of the CD8 gene (*CD8A*) messenger RNA (mRNA) is increased in obese humans, suggesting accumulation of $CD8^+$ T cells also in humans. These cytotoxic T cells seem to be essential for macrophage recruitment by release of chemokines and for sustainment of inflammation. Together with the adipose tissue, $CD8^+$ T cells induce differentiation of monocytes into macrophages. Deficiency of $CD8^+$ T cells improves insulin sensitivity and glucose tolerance, without affecting obesity (79). In contrast to $CD8^+$ T cells, $CD4^+$ T cells and T regulatory cells are reduced in murine obesity and similar data have also been found in humans (79-81). In Rag-1 null mice, which lack T cells, HFD induces a larger weight gain and insulin resistance than in wildtypes on HFD. Replacing $CD4^+$ T cells, in particular the anti-inflammatory Th2 subset, in Rag-1 null mice lowers body weight and improves insulin sensitivity. Immunotherapy that increases the T regulatory cells also improves insulin sensitivity in mice (80). The recently discovered accumulation of mast cells in fat of obese humans and mice seem to have a pro-obesity effect, possibly through increased angiogenesis (77).

After the initial T cell infiltration, macrophages accumulate in obese adipose tissue (79, 82) and the adipocyte size is positively correlated with macrophage concentration in both humans and mice (66). Macrophages often aggregate to form “crown-like structures” surrounding individual adipocytes. The surrounded adipocytes are dead, probably by necrosis or a necrosis-like process, and the adipocyte death is positively associated with increasing adipocyte size (70). The reason why large adipocytes die is not completely understood, but it is known that growth of adipose tissue is accompanied by hypoxia (68). Hypoxia is associated with up-regulation of hypoxia-inducible factor 1α, which in obesity does not seem to induce angiogenesis but instead fibrosis and possibly increased adipocyte stress and subsequent macrophage infiltration (69).

Like T cells, macrophages seem to alter insulin resistance, in this case to the worse. Mouse models were macrophage accumulation in adipose tissue is limited due to knockout of either the monocyte attractant chemokine (C-C motif) ligand 2 (*Ccl2*) or its receptor chemokine (C-C motif) receptor 2 (*Ccr2*) have improved insulin sensitivity (63, 83). Although obesity increases the macrophage content of adipose tissue, there are still macrophages in lean mice

and humans (66, 70), but these seem different from those that accumulate during obesity (84). Macrophages resident in lean mice have features of so called M2 or "alternatively activated" macrophages and these are generally anti-inflammatory and involved in for example tissue repair. The infiltrating macrophages on the other hand, mature into M1 or "classically activated" macrophages that instead are pro-inflammatory. *Ccr2* deficient mice have small amounts of resident macrophages in their adipose tissue and they seem to be of the M2 activated sort. Lumeng *et al*. therefore suggested that circulatory $Ccr2^{+}$ monocytes are recruited to fat by the increased Ccl2 production from expanding adipocytes, followed by M1-polarization and promotion of insulin resistance (84). Winer *et al*. recently suggested that increased ratio of Th1 versus T regulatory cells and Th1 versus Th2 cells, respectively, may lead to the transition of anti-inflammatory M2 macrophages into the pro-inflammatory M1 type (80).

Inflammation in non-fat tissue in obesity

In addition to the inflammation in adipose tissue, obesity-induced lipid accumulation in the liver leads to hepatic inflammation. The inflammation is induced through activation of the immune-stimulatory transcription factor nuclear factor-κB (NF-κB) and downstream cytokine production. This causes insulin resistance both locally in liver and systemically (85). Moreover, chronic HFD activates NF-κB also in the hypothalamus. The activation is at least in part due to elevated endoplasmic reticulum stress in the hypothalamus (86). Thus, this indicates that there is inflammation outside the adipose tissue in obesity.

Cytokine deficiency and obesity

In addition to the findings that obesity leads to inflammation/immune system activation, there are also data showing that the immune system can affect obesity. In particular, deficiency of several genes coding for innate immune factors, such as IL-6, GM-CSF, IL-1RI, and IL-18, leads to mature-onset obesity in mice (87-90). Moreover, combined IL-6 and IL-1 deficiency causes early-onset obesity in mice (91) indicating that there is some overlap in the functions of IL-6 and IL-1. Conversely, mice with enhanced IL-1 activity due to IL-1Ra gene knockout are lean and resistant to diet-induced obesity (92).

The obesity suppressing effect of IL-6 appears to be due to its ability to increase energy expenditure (87, 93). This effect is probably mediated in the brain since acute intracerebroventricular, but not peripheral, IL-6 injection increased energy

expenditure in rats (87). The hypothalamus might be the site of action in the brain since an altered expression of peptides that regulate energy balance have been found in IL-6 deficient mice. Also, the receptor for IL-6, IL-6Rα, is expressed in the hypothalamus and it is co-expressed in cells expressing energy balance regulating peptides, for instance in the paraventricular nucleus (94).

The mechanism whereby IL-1 mediates anti-obesity effects may be partly through leptin. It has been shown that leptin injection specifically increases the hypothalamic levels of IL-1β, and leptin-induced hypophagia is not observed in IL-1RI deficient mice (89, 95). Further support for this is the inhibition by IL-1Ra administration on leptin-induced hypophagia (95). It is possible that IL-1Ra deficient mice increase energy expenditure by facilitating leptin signaling due to their excessive IL-1 activity (96). IL-1 may also have peripheral effects. For example, the decreased lipid accumulation in IL-1Ra deficient mice was suggested to be due to their lower insulin levels, which in turn may cause their observed decrease in lipase activity (92). Similarly, decreased lipoprotein lipase expression was found in another study on IL-1Ra deficient mice (96). This is also in accordance with the literature as IL-1 inhibits expression and activity of lipoprotein lipase *in vitro* (97). Another peripheral effect by IL-1 seems to be decreased adipocyte differentiation (96)

Furthermore, deficiency of certain non-cytokine immune molecules can also cause obesity in mice. One example is leukocyte adhesion molecules such as inter-cellular adhesion molecule 1 (ICAM-1) and Mac-1 deficiency that leads to mature-onset obesity (98).

The data described above indicate that several pro-inflammatory cytokines may decrease obesity via effects at the hypothalamus. In contrast, there are some recent findings indicating that certain factors related to immune stimulation and inflammation may instead promote obesity. These include endoplasmic reticulum stress and stimulation of NF-κB, toll like receptor-4, and MyD88 (86, 99, 100). The reason for these seemingly contradictory findings needs investigation in the future.

Infection and obesity

It is well established that obesity is associated with chronic inflammation, i.e. immune stimulation (59, 66, 67). Considerably less is known about how this condition may influence the main task of the immune system, to combat infections. Clinical findings indicate that obesity is associated with increased

susceptibility to infections. The various infections include nosocomial, surgical, odontogenic, respiratory, bacteremia etc. (101-104). However, this association could be due to multiple factors, e.g. longer surgery and hospitalization time of obese patients, with increased risk for nosocomial infections (101). Alternatively, obesity could be secondary to immune defects. As discussed above, absence of activity of several immune stimulators such as IL-6, GM-CSF, IL-1, and IL-18, leads to obesity (87-91, 105). Conversely, ingestion of energy dense food and obesity may suppress the immune response. A possible link is that obesity-induced insulin resistance suppresses the immune system, but the data are not conclusive. In critically ill patients, including many with sepsis, treatment with insulin to normalize blood glucose levels was initially reported to improve survival (106, 107). However, this was not confirmed in recent meta-analyses (108, 109). To sum up, in clinical materials it is very difficult to clarify the possible causality, as well as cellular and molecular links, between obesity and potentially defective immune functions. Hence, systematic studies in experimental animals could be of value to clarify these issues.

Dietary fatty acids

Fat, or triglycerides, are made of one glycerol molecule and three fatty acids. Fatty acids have a hydrophilic acid part and a hydrophobic carbon chain. The carbon chain can differ in length and degree of saturation. Saturated fatty acids (SFAs) contain no double bonds. Therefore, its carbon chain is composed of $–CH_2–$ units, i.e. fully saturated with hydrogen atoms (Fig. 1).

Figure 1. Molecular structure of palmitic acid, a common SFA with a 16 carbon chain.

Monounsaturated fatty acids (MUFAs) contain one double bond and poly-unsaturated fatty acids (PUFAs) contain two or more double bonds (Fig. 2). The kind of fat that we eat most in the Western World is saturated and monounsaturated. The former can be found mostly in dairy products and in palm and coconut oil, while the latter is found in nuts, avocado and olive oil. Meat contains a fairly equal mixture of SFA and MUFA. PUFA are found in large amounts in fat fish, like mackerel and salmon, and also in plant products such as linseed, canola, and soybean oil. PUFA can be further defined as n-3 (or omega-3) or n-6 (or omega-6) fatty acids, meaning that they have their first double bond at the third or sixth carbon, respectively, counted from the end of the carbon chain. Fish oil contains high amounts of the long-chain n-3 PUFAs eicosapentaenoic and docosahexaenoic acid, while plants contain shorter n-3 PUFA, e.g. α-linolenic acid (110).

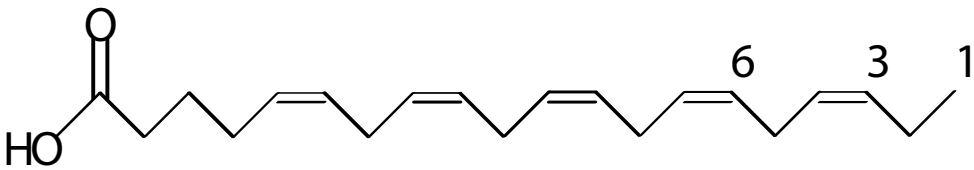

Figure 2. Molecular structure of eicosapentaenoic acid, a n-3 PUFA with a 20 carbon chain containing 5 double bounds starting from the third carbon from the right.

In the 1950's, it was first recognized that dietary fat could contribute to cardiovascular disease in the Western World (111). However, Greenland Eskimos were known to eat large amounts of fat but still had a very low incidence of cardiovascular disease. In the 1970'ies Bang and Dyerberg found that the Eskimos had low levels of cholesterol and lipoproteins, except for the high-density lipoproteins that now is known as "the good cholesterol". The high intake of marine PUFA in Eskimos compared with Danes led Bang and Dyerberg to suggest that quality of dietary fat rather than quantity is important for development of cardiovascular disease (112, 113). The beneficial effect of long-chain n-3 PUFA on cardiovascular disease has since been demonstrated in several randomized trials and in many epidemiologic studies of fish consumption (114, 115). A few years ago Omacor capsules, containing ethyl esters of long-chain n-3 PUFAs, was approved by the United States Food and

Drug Administration for treatment of severe hypertriglyceridemia. Fish oil has also been shown to have some beneficial effect for inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (116, 117).

Long-chain n-3 PUFAs are thought to promote beneficial effects on metabolic functions via their anti-inflammatory properties. Immune cells normally contain large amount of the n-6 PUFA arachidonic acid. This fatty acid can be converted into eicosanoids such as inflammatory and thrombotic prostaglandins, leukotrienes, and thromboxanes. Increased dietary intake of long-chain n-3 PUFA leads to incorporation into phospholipids of cell membranes and they then replace some of the arachidonic acid. Long-chain n-3 PUFAs thus inhibit eicosanoid production from arachidonic acid. In addition, long-chain n-3 PUFAs can be used for eicosanoid synthesis, but these are often less inflammatory and thrombotic than those synthesized from arachidonic acid (118, 119). However, lately it has been found that eicosanoids from arachidonic acid also have some anti-inflammatory effects (119). This is consistent with the findings that also n-6 PUFA has shown some protective effects on cardiovascular disease (120). The long-chain n-3 PUFAs from fish can modulate inflammation by altering production of cytokines (119, 121). This effect appears to be due to modulation of the activity of transcription factors, such as peroxisome proliferator-activated receptors (PPARs) and NF-κB, which in turn of course alters gene expression (119, 122). In contrast, SFA have inflammatory effects through e.g. induction of endoplasmatic reticulum stress (123, 124) and activation of toll-like receptors to increase cytokine production (125, 126).

Long-chain n-3 PUFA also seem to have metabolic effects that differ from SFA. For example it has been shown that mice fed long-chain n-3 PUFA compared with SFA-fed mice have lower body weight and fat mass (127-131). This may be explained by the decreased lipogensis and increased lipid oxidation seen in PUFA-fed mice (132, 133). Long-chain n-3 PUFA have also been shown to increase brown adipose tissue thermogenesis and uncoupling protein 2 expression in hepatocytes and this may lead to increased energy expenditure (134, 135).

Genetics

In the mid 1800's Mendel found that some characteristics of plants were inherited by the next generation according to certain laws. About 100 years later in the mid 1900's DNA was found to contain the information necessary for inheritance. DNA consists of two long polymers of nucleotides and it is formed as a double-helix. The nucleotides are made from three components: a nitrogenous base, a sugar unit and a phosphate group. To form a helix the two polymers has to be bound to each other and these bonds are formed between bases on the different strands. DNA contains four different bases and they are called adenine (A), thymine (T), cytosine (C), and guanine (G). Bonds are only formed between A and T, and between C and G. The sequence of these bases is what makes the genetic code (136).

DNA makes up the genetic material called the genome in all living organisms and some viruses. The genome is divided into chromosomes and in humans there are 23 pairs. These consist of 22 pairs of autosomal chromosomes and one pair of sex chromosomes, of which women have two X-chromosomes while men have one X- and one Y-chromosome. One of the chromosomes in each pair is inherited from the mother and the other from the father (136).

The chromosomes contain many genes that code for proteins. To produce a protein, the gene needs to be transcribed into ribonucleic acid (RNA). RNA is similar to DNA but it is single stranded and composed of another kind of sugar, and instead of the base T, RNA has a uracil base (U). Transcription can start when a RNA polymerase binds to a specific DNA sequence, the promoter, which lies upstream of the gene. After the RNA is transcribed, it is spliced so that coding regions (exons) are fused together while non-coding regions (introns) are removed. The RNA is then translated into an amino acid chain, a protein. This is done by an enzyme that reads three bases (a codon) of the RNA strand at a time, the codon corresponds to an amino acid, and as the enzyme reads on more amino acids are connected to the first, eventually making a protein (136).

Genetic variation

Mutations of DNA have lead to a genetic variation between humans in about 0.5% of the genome (137). A polymorphism is a genetic variation at a particular site of the genome that occurs in at least 1% of the population (138). The different genetic variants at a site are called alleles. There are several different

types of polymorphisms including copy number variations (CNVs), variable number tandem repeat (VNTR), and single nucleotide polymorphisms (SNPs). CNVs are relatively large DNA segments that are present in variable numbers produced by insertions or deletions (139). For VNTRs the repetitive DNA sequence is shorter than for CNVs and the repetitions are directly adjacent to each other. The most common polymorphism is the SNP, were only one nucleotide/base is substituted. It may also be deleted or inserted, but that is much less common. SNPs can be found all over the genome, in exons, introns, promoter regions, or between genes. SNPs in exons can be characterized further into synonymous or non-synonymous SNPs. The latter leads to a changed amino acid, whereas the synonymous does not. It is possible to change a nucleotide without changing the amino acid, as there are 64 possible codons but only 20 amino acids. An amino acid change can affect the protein stability, ligand binding, and posttranscriptional modifications (140), while SNPs in the promoter region can affect transcription (141). Synonymous or intronic SNPs can affect splicing and/or mRNA stability (142).

Most SNPs have two different alleles, for example A and G. A person who has two A (one from the mother and one from the father) or two G alleles are said to be homozygous, while a person who has different alleles, in this case A and G, are called heterozygous. SNPs that are close to each other on the chromosome are often inherited together, they are in so called linkage disequilibrium (LD). This is due to the fact that a recombination between the SNPs is less likely if the distance between them is short (138). Recombination is the exchange of genetic material between two chromosomes of the same sort during the formation of sperm or egg cells. As an example, if you have two close SNPs where SNP 1 is A/G and SNP 2 C/G, there will be only two possible allele combinations or so called haplotypes in the absence of recombination. Say that the SNP 1 A allele always is inherited together with the SNP 2 G. The two possible haplotypes are then AG or GC. This makes it only necessary to determine one of the SNPs in order to figure out the other one. If the two SNPs are far apart there is a larger chance of recombination between the SNPs, and they are therefore less likely to be inherited together. When recombination has occurred between two SNPs three haplotypes exists. The degree of co-inheritance is determined by LD and common measures are D' and r^2.When D'= 1 or r^2= 1 the loci are said to be in perfect LD and no recombination has occured between the SNPs (143).

AIMS OF THE THESIS

The overall aims of this thesis were to investigate interactions between obesity, nutrition and the immune system in both mice and men.

Specific aims

Paper I — To study the effect of Western diet on mortality induced by intravenous *S. aureus* inoculation and the immune functions before and after bacterial inoculation

Paper II — To investigate if dietary fat composition affects the mortality following *S. aureus* inoculation of mice

Paper III — To investigate if common polymorphisms of the IL-1 system, associated with IL-1 activity, are associated with fat mass in young men

Paper IV — To investigate if common polymorphisms of *IL6* and the *IL1*-system are associated with fat mass in elderly men

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in the Material and Methods sections of the individual papers, while more general comments are presented below.

Animal model

Mice

Consumption of calorie dense diets that have high fat or sugar content have been found to promote obesity in humans (54). In mice, fat content seems to be a major promoter of obesity (144) and therefore high-fat feeding is a commonly used model to induce obesity in mice. We have fed HFD chronically to the inbred mouse strain C57BL/6. The C57BL/6 mice on HFD share many features of obese humans, as they develop not only obesity but also hyperglycemia, hyperinsulinemia, hypertension and abdominal obesity (144-146, 147.). Obesity in mice is not only caused by diet but, like in humans, it is also dependent on multiple genetic factors (56, 148-150).

Another model of obesity is the leptin-deficient Ob/Ob mouse which develop severe obesity from a young age, even when on low-fat normal chow (151). Although widely used, the Ob/Ob mice have the drawback that leptin-deficiency is extremely rare in humans; only a handful of people have so far been diagnosed with this deficiency.

Diet

Mice were fed low-fat diet (LFD), HFD rich in SFA and MUFA (HFD/S) or HFD rich in PUFA (HFD/P) for eight weeks. In earlier studies included in Paper I, we used LFD R36 (Lactamin AB, Stockholm, Sweden) and HFD D12309 (Research Diets, New Brunswick, NJ). These two diets vary a lot not only in fat content, but also in the source of macronutrients and amount of micronutrients. In order to limit the difference between the HFD and LFD, we started to use LFD D12450B (Research Diets) and HFD D12492 (Research Diets), that mainly differ in the fat content. The main fat source in the latter diet was lard, as opposed the HFD/P where 69% of the lard was replaced by menhaden fish oil.

Menhaden fish oil was chosen as it has a high content of PUFA. Most importantly the long-chain n-3 PUFA content is high and the n-6 levels are low, making this diet rather healthy albeit the high fat content. A larger portion of lard was not replaced with menhaden oil, because of practical problems to then keep the diet in a pelleted form.

Sepsis model

Mice were inoculated by an intravenous injection in the tail vein, with 0.2 ml of *S. aureus* LS-1 solution containing 5×10^7 colony forming units (CFU), as previously described (152). The intravascular administration of bacteria has been regarded as a clinically irrelevant model of sepsis, as the mice usually are given high doses of bacteria ($>10^9$ CFU/kg). This produces intoxication and high immediate mortality, while sepsis progresses over days or weeks (153). Although we used intravenous administration we did not use a very high dose of bacteria (approximately $1\text{-}2\times10^8$ CFU/kg) and the first deaths usually occurred after a couple of days, and continued until the end of 2.5 week study period. We therefore believe this is a relevant model of clinical sepsis.

Human cohorts

Studies of mice have shown that both the IL-6 and the IL-1 systems can be of importance for obesity development (87, 89, 91, 92). In addition, earlier SNP studies showed an association between BMI and polymorphisms within the IL-6 and IL-1 system (165, 219, 232, 233). As BMI is a fairly inaccurate measure of obesity (154), we undertook studies to confirm that IL-1 and IL-6 related genes are associated with obesity in two large well characterized cohorts were data on body fat, as measured accurately by dual-energy X-ray absorptiometry (DXA), was available.

The GOOD study

The population-based Gothenburg Osteoporosis and Obesity Determinants (GOOD) study was initiated to determine environmental and genetic factors involved in the regulation of bone and fat mass. Study subjects were randomly identified using national population registers, contacted by telephone, and asked to participate in the studies. A total of 1068 men (Table 1), from the greater Gothenburg area in Sweden were included. The subjects had to be more than 18

and less than 20 years of age and willing to participate in the study. There were no other exclusion criteria and almost half (49%) of the study candidates agreed to participate and were enrolled. The majority of subjects were white. Informed consent was obtained from all study participants (155).

Height and weight were measured using standard equipment. A standardized questionnaire was used to collect information about amount of present physical activity (hours per week). Body composition was determined by DXA. Blood was collected and white blood cells were isolated from a subgroup of 148 individuals (155) (Paper III). Missing data are due to unsuccessful genotyping and/or phenotyping.

Table 1 Characteristics of the men in the GOOD and MrOS studies

	Cohort	
Variable	**GOOD**	**MrOS**
Number of subjects	1068	3014
Age (years)	18.9 ± 0.6	75.4 ± 3.2
Total fat (kg)	13.4 ± 8.0	22.0 ± 7.7
Total lean tissue (kg)	57.4 ± 6.2	55.5 ± 6.8
BMI (kg/m^2)	22.4 ± 3.2	26.4 ± 3.6
Leptin (ng/ml)	7.7 ± 8.5	21.8 ± 20.1
Trunk fat (kg)	6.8 ± 4.3	12.8 ± 5.1
Arm fat (kg)	0.56 ± 0.41	1.1 ± 0.42
Leg fat (kg)	2.5 ± 1.4	3.1 ± 1.0

Data are mean ± SD. Adapted from (156).

The MrOS study

The Osteoporotic Fractures in Men (MrOS) study is an international multicenter study including elderly men in Sweden (3014) (Table 1), Hong Kong (≈2000), and the United States (≈6000). We investigated the population-based Swedish part of the study where men, aged 69-81 years, were recruited at three academic medical centers: Sahlgrenska Academy in Gothenburg (n=1010), Malmö University Hospital (n=1005), and Uppsala University Hospital (n=999). The participation rate was 45%. Study subjects were randomly identified using national population registers, contacted, and asked to participate. To be eligible for the study, the subjects had to be able to walk without aids, and they were not allowed to have bilateral hip prosthesis. There were no other exclusion criteria. Informed consent was obtained from all study participants (157).

Height and weight were measured using standard equipment. A standardized questionnaire was used to collect information about amount of physical activity, and smoking. Physical activity was the subject's average total daily walking distance, including both walking as a means of exercise and leisure, and as a means of outdoor transportation in activities of daily life. Blood was collected and body composition was determined by DXA (157). Missing data are due to unsuccessful genotyping and/or phenotyping.

Both these studies are unique in that they are large, population-based studies, carefully phenotyped, and within a narrow or fairly narrow age range, including one gender and an ethnically rather homogenous group. All these factors contribute to low variability and increased power of the studies.

Assessment of body composition and obesity

Dual-energy X-ray absorptiometry (DXA)

For small animals like mice, the traditional way to analyze body composition has been carcass analysis using chemical extraction techniques, which made it necessary to kill the animals. Now DXA is a widely used non-invasive technique that allows repeated investigations of bone and body composition in humans as well as in animals. Apart from being non-invasive, the DXA is also fast and uses low doses of radiation, compared to for instance a chest X-ray.

The X-rays emitted from the DXA is divided by a filter into high- or low energy photons. When they pass through the body, some of the photons are absorbed by the tissue and the amount of unabsorbed photons is detected by sensors. The absorbance is different in tissues of different densities, with bone being the tissue with the highest density and absorbance. The use of two energies allows bone mineral to be separated from soft tissue, and also soft tissue to be divided into fat and bone free lean tissue (158).

Human DXA machines can not measure small animals like mice and therefore special DXAs for small animals have been developed (e.g. PIXImus2, Lunar GE Medical systems, Madison, WI). As older obese mice are hard to completely fit into the scanned area, the head was omitted from DXA analysis in Paper I and II.

Body mass index (BMI)

BMI is a simple and inexpensive measure and it is therefore often used to assess obesity. Unfortunately it is not a very good measurement of obesity. BMI gives no information about a person's body composition; it only relates body weight to height. The weight can be dominated by fat or by muscle, but it is the excess fat that leads to co-morbidities. In addition to the large variation between individuals, there are also sex, age, and ethnic variations. Females have higher fat content than men, and older people have higher fat content than younger, at the same BMI. Asians, and especially Indians, carry more fat than Caucasians at a given BMI. In contrast, most Africans carry more muscle at the same BMI (154).

For these reasons, fat mass, as determined by DXA, has been used as the primary measurement of obesity. However, BMI can be useful in monitoring changes over time or regional differences within a country.

Genotyping

There are several possible methods to perform genotyping, i.e. determine a person's nucleotide sequence for a specific polymorphism. The method of choice depends on the type of polymorphism, how many polymorphisms you want to determine, and of course the price. There has been a fast development in this area and it is now possible to perform so called genome-wide association (GWA) studies. This means that the entire genome can be scanned and that hundreds of thousands or millions of SNPs can be simultaneously genotyped in one sample. This also means that you can design an experiment without any prior hypothesis about which SNP or gene that is associated with a certain phenotype, e.g. a disease. Due to the huge number of investigated SNPs and the thereby following mass significance problem, only highly significant associations can be considered as "true" and it is also important to confirm the finding in an additional cohort (159). So far, the GWA studies have found associations between several diseases and genes. These include age-related blindness and *CFH*, obesity and e.g. *FTO* and *MC4R*, typ-2 diabetes and e.g. *TCF7L2* and *CDKAL1*, and Crohn's disease and *NOD2* (160). In the future GWA studies may also contribute to studies of disease pathways and personalized treatment and pharmaceuticals.

During the time when the studies in Paper III and IV were conducted, GWA studies were not possible to conduct. The studies of Paper III and IV are instead hypothesis based and investigated a few SNPs in relation to obesity. The hypothesis was based on animal studies showing obesity in IL-6 and IL-1RI deficient mice and previous human SNP studies showing a functionality or possible functionality of the chosen SNPs (87, 89, 141, 161-165).

In Paper III and IV, DNA for genotyping was prepared from blood using commercially available kits. To explain the different methods of genotyping I first have to explain the process of DNA amplification, the polymerase chain reaction (PCR) (166).

Polymerase chain reaction (PCR)

DNA amplification is necessary since the starting amount of DNA is very small, while we need many copies for a certain analysis. Another reason for using PCR is that we are usually only interested in a specific DNA region, the target DNA. PCR amplification is started by heating the DNA sample to 95°C; this separates, or denatures, the double-stranded DNA into two single strands called templates. By lowering the temperature to 60°, it is possible for a short single-stranded DNA molecule, or primer, to bind (anneal) the single-stranded template DNA as they are complimentary. Two primers are used that can bind each of the template DNA strands some 100 to 1000 bases apart from each other. The sample is then heated to 72°C and this activates an elongation enzyme called polymerase. The polymerase binds the double-stranded primer-template hybrid and then starts elongating the primer by subsequently adding nucleotides that are complementary to the template. The polymerase stops elongating when it comes to the end of the template DNA. By now there has been a doubling in the amount of the target DNA region. This cycle is then repeated some 40 times, resulting in a trillion-fold increase of target DNA (166).

SNP genotyping

TaqMan

This is a quick and simple technique for SNP genotyping. In Paper IV, this method was used for genotyping of the following SNPs: rs1143634[1] (*IL1B* +3953 C>T), rs1143627 (*IL1B* -31 T>C), rs419598 (*IL1RN* +2018 T>C), and rs1800795 (*IL6* -174 G>C).

There are two major steps in this method. During the first, PCR is used to amplify a target region of DNA containing the SNP of interest (Fig. 3). Included in the reaction mixture are also two allele-specific probes that are used to discriminate between the two possible alleles, for example A and C. A probe is a single stranded DNA molecule, like the primers, but shorter and they have a reporter dye – one for each probe (VIC or FAM) at one end and a quencher at the other end. For example, the probe specific for A contains the VIC dye and the C-specific probe contains FAM. During the annealing phase, the probes anneals to the DNA if its target allele is present. When elongation starts, the polymerase will elongate from the primer and when it encounters the probe it will degrade it, parting the dye from the quencher. When the dye is no longer close to the quencher it starts fluorescing. For every PCR cycle the fluorescence is increasing and during the second step, the amount of fluorescence is detected by a sensor that can discriminate between the fluorescence coming from VIC or FAM. The results are then plotted on an X-Y graph and a person homozygous for A will only have fluorescence coming from VIC and this gives a high value on the X-axis, but low values on the Y-axis. A person homozygous for C will instead have high FAM fluorescence and high values on the Y-axis and low on the X-axis. A heterozygous person will have medium levels of FAM and VIC, placing them medium high on both the X- and Y-axis (167).

[1] rs stands for reference and a rs number is a unique number so that all SNPs can be precisely identified.

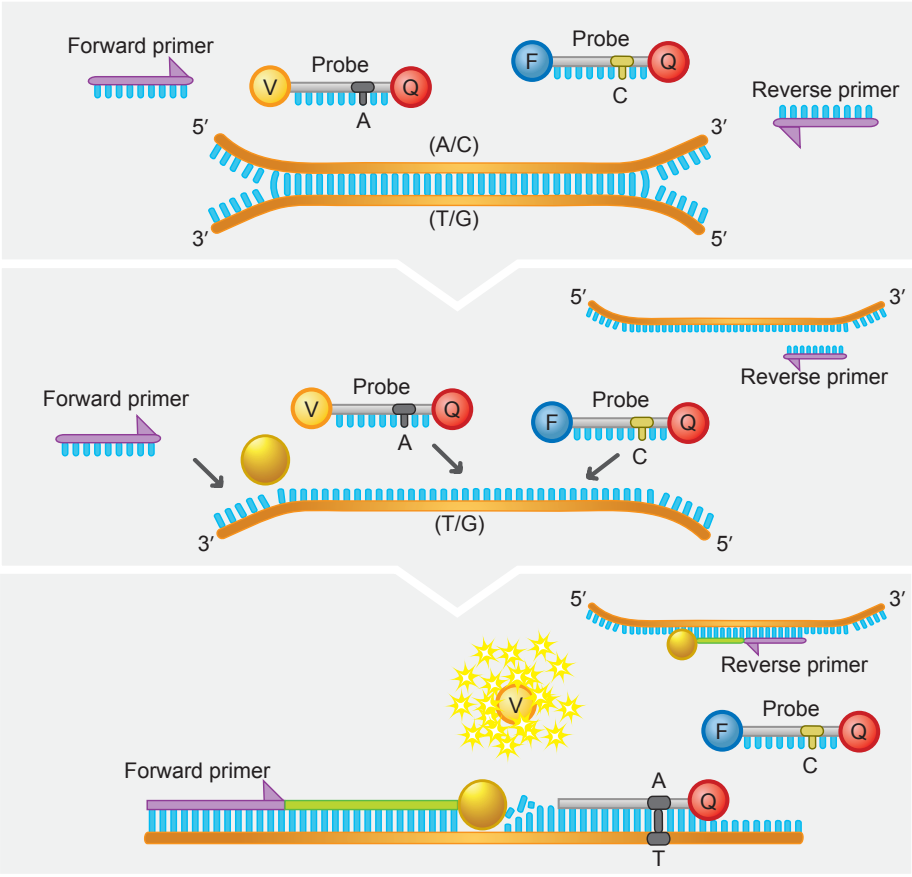

Figure 3. Schematic picture of TaqMan genotyping. The upper panel depicts the reaction components. The middle panel shows the denatured template and the annealing of primes and probe. In the bottom panel the matching probe has annealed to the template DNA and the polymerization leads to the fluorescent signal. Abbreviations: V, VIC; F, FAM; Q, quencher.

Sequenom

The MassARRAY genotyping method from Sequenom (San Diego, CA, USA) combines PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) techniques. The first step is amplification of the DNA containing the SNP. Thereafter, the unincorporated nucleotides are dephosphorylated, which means that they can no longer be used for further DNA amplification. During the next step a new primer anneals adjacent to the SNP (Fig. 4). Present in this reaction are also three modified nucleotides that lacks an oxygen group on their sugar unit (called

dideoxynucleotides, e.g. ddA, ddC, and ddT), and one unmodified deoxynucleotide (e.g. dG). The polymerase will then try to elongate the primer. If the SNP is a T to C SNP and the primer has bound next to the T allele it will incorporate a modified A nucleotide. The polymerase is then unable to incorporate the next nucleotide as it cannot connect it to the modified A nucleotide. The product in this case is then the primer plus the A nucleotide. When elongating the primer next to the C allele, the polymerase can incorporate the complimentary G and also the following nucleotide as the G nucleotide is unmodified. In this case, the product is the primer plus two nucleotides. The elongation products are then separated by MALDI-TOF MS by first vaporizing and then ionizing the sample. Thereafter an electrical field is applied, making the elongation products fly to a detector. Larger products, like the product with two elongated nucleotides, will have longer time of flight than smaller ones. This makes it possible to separate the two possible products and hence determine the genotype (168).

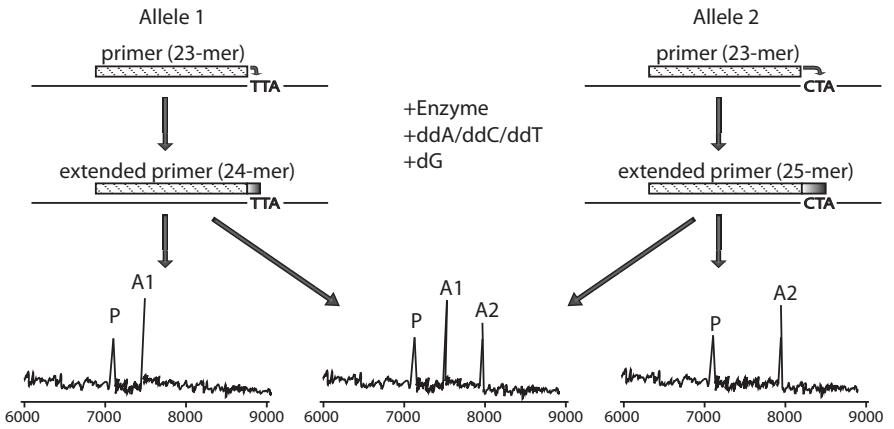

Figure 4. Schematic figure of the MassARRAY method. The bottom part represents the different mass spectra obtained. The spectra to the left belongs to an individual homozygous for allele 1 (TT), the middle spectra belongs to a heterozygous individual, and the right spectra belongs to an individual homozygous for allele 2 (CC). Adapted from (168). Abbreviations: P, primer; A1, allele 1; A2, allele 2.

This method was used in Paper III to genotype the rs1143634 (*IL1B* +3953 C>T), rs1143627 (*IL1B* -31 T>C) SNPs. The advantage with this technique is the possibility to multiplex, i.e. analyze several SNPs in one sample, making it cost efficient when measuring many SNPs simultaneously.

Variable number repeat (VNTR) genotyping

As the *IL1RN* 86 base pair (bp) VNTR is not a SNP, the previously described methods are not possible to use. The *IL1RN* 86 VNTR is 86 bp long and the number of repeats for this sequence is between 2 and 6. In brief, this method uses fluorescently labeled primers to amplify the polymorphic DNA sequence. This generates fluorescent PCR products that have different size depending on the number of repeats. The PCR products are then analyzed on a capillary machine where the smaller products wander trough the capillary faster than the larger ones. The size of the products is determined by comparing with a size standard consisting of DNA fragments with known and different sizes that are fluorescently labeled with a different color than the primer.

This VNTR polymorphism is in complete LD with the +2018 T>C intronic SNP and due to easier genotyping the latter was used for Paper IV.

mRNA expression

Gene expression is often determined by measuring mRNA levels since this is more easily done than quantifying protein levels. However, one must keep in mind that mRNA expression is not always in close relation to protein levels, and after all it is the proteins that affect cellular processes etc. mRNA expression can be determined by a sensitive method with high trough-put called real-time reverse transcriptase PCR. It is based on PCR, but since the polymerase used for PCR only uses DNA templates, the mRNA first has to be transformed into complementary DNA (cDNA). This is done by the enzyme reverse transcriptase and PCR is then used to amplify a gene-specific region. If the amount of amplified DNA was to be analyzed after e.g. 40 cycles it would not be proportional to the amount of starting DNA. This is due to the fact that in a sample with high starting levels the reaction components will be used up before the end of the 40 cycles. The amount of DNA must therefore be analyzed after every cycle. This is done by adding a fluorescent probe or a dye, which fluoresce when bound to double-stranded DNA, to the reaction mixture. The probe works in the same way as for TaqMan SNP genotyping, so the report dye starts fluorescing when it is separated by the polymerase from its quencher. SYBR Green I is a dye that fluoresce when binding double-stranded DNA. For both these options, the amount of amplified DNA is proportional to the fluorescence intensity. A disadvantage with SYBR Green is that it will bind all

double-stranded DNA, while probe detection is much more specific but also a lot more expensive. However, by using SYBR Green it is possible to verify that only one specific PCR product has been generated. Due to the exponential nature of PCR, small pipetting errors can substantially affect the results. This is minimized by normalizing the mRNA expression of the target gene with a reference gene that should have constant expression (169). This technique was used in Paper I to measure expression 5 genes: *Il1rn*, *Il10*, *Il6*, *Il1b*, and *Tnf*, encoding IL-1Ra, IL-10, IL-6, IL-1β, and TNF-α, respectively.

Recently, highly multiplexed assays for real-time PCR, so called low density arrays (LDA), can be purchased. The same principles apply but it is now possible to analyze mRNA levels for 384 genes simultaneously. In Paper II a LDA card analyzing 96 genes was used to screen for possible immune effects of different diets.

SUMMARY OF RESULTS AND DISCUSSION

Paper I-II: Dietary fat affecting *S. aureus*-induced mortality

For clarity I will here refer to the HFD used in Paper I as HFD/S as it is rich in SFA and MUFA like the diet referred to as HFD/S in Paper II. Essentially, it is the same diet.

Increased mortality in mice chronically fed HFD/S but not HFD/P

Mice that were fed HFD/S chronically (for 8 weeks) had increased *S. aureus*-induced mortality, while acute administration of HFD/S at the time of bacterial inoculation did not increase mortality. In contrast, chronically HFD/P-fed mice did not have increased mortality compared with mice fed LFD. In addition we found increased mortality in genetically obese Ob/Ob mice fed a LFD compared with wild type mice fed LFD.

So what is causing the increased mortality in mice fed HFD/S? Is it the obesity, the obesity-associated inflammation, the insulin resistance/glucose intolerance, or the diet? Some of the possibilities are summed up in Fig. 5. The finding that long-term but not short-time dietary HFD/S as well as the Ob/Ob genotype is associated with increased mortality and obesity, may support a link between sepsis mortality and obesity. On the other hand, it is possible that the reason for increased mortality differ between Ob/Ob and HFD/S-fed mice. Ob/Ob mice have some immune defects, e.g. suppression of T cells (170, 171), and we do not know if this is also the case in HFD-fed mice in this study.

Another possible cause for the increased mortality in HFD/S-fed mice may be glucose intolerance/hyperglycemia. It has been shown that critically ill patients, who often develop hyperglycemia, have reduced mortality after intensive insulin therapy that normalizes blood glucose levels (106, 107). However, these results have been questioned lately (108, 109). Furthermore, compared with LFD-fed mice the HFD/P-fed mice had increased basal glucose levels and decreased clearance after a glucose load, and yet their mortality did not differ from that of mice fed LFD. This makes hyperglycemia a less likely cause of mortality in HFD/S-fed mice.

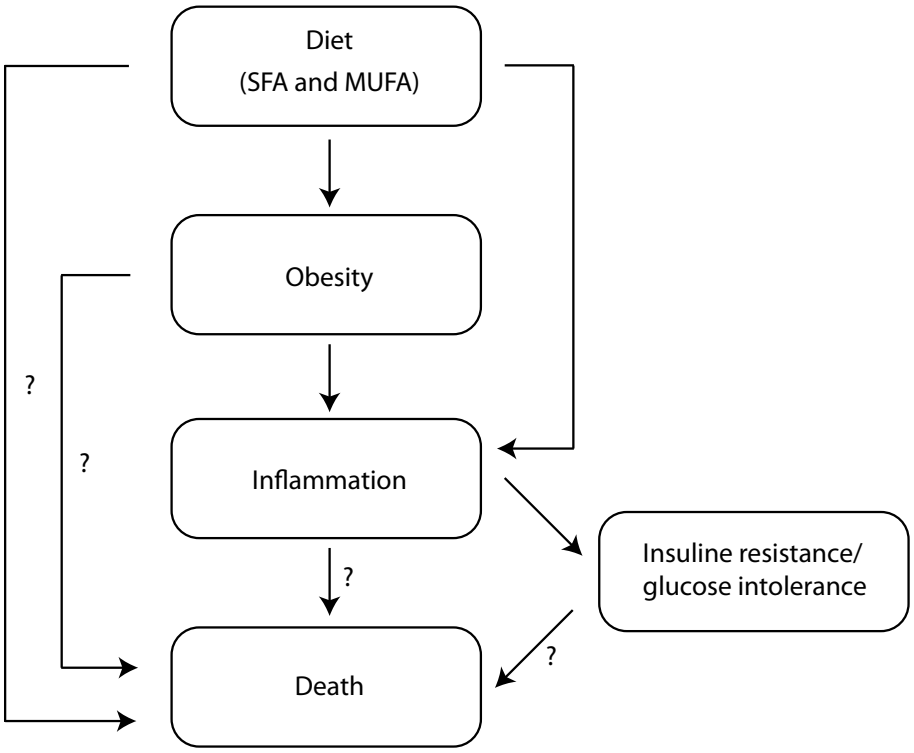

Figure 5. Schematic depiction of possible ways by which SFA and MUFA may increase septic mortality; either directly or indirectly via effects on obesity, inflammation or insulin resistance/glucose intolerance.

A third possibility for the increased mortality in HFD/S-fed mice may be a more direct dietary effect. The HFD/P-fed mice were leaner than the HFD/S-fed mice, but still more obese than LFD-fed mice. If obesity was the cause for mortality, a semi-high mortality would be expected for the HFD/P-fed mice. Instead, we found comparable mortality in LFD- and HFD/P-fed mice. Due to the large difference in mortality between the obese HFD/S- and HFD/P-fed mice, I think that a direct effect of the diet, independent of obesity and diabetes, is the foremost likely factor to explain the increased mortality in HFD/S- compared with HFD/P-fed mice. Our results are in line with some studies, but not all, showing decreased mortality to infections after long-chain n-3 PUFA feeding (Table 2) (172-177). These discrepancies may be due to differences in mouse strain, diet composition, duration of feeding, control diet, vitamin

supplementations, and route of infection. Some discrepancy can however be expected due to the fact that many different types of pathogens have been investigated and these require different kinds of immune defense. Fish oil feeding was extensively investigated during various bacterial infections, and was shown to increase survival in half of the studies (Table 2) (172-175). Although many types of bacteria have been investigated, few of them have been studied repeatedly. From these data it seems rather conclusive, however, that the capacity to combat *Klebsiella pneumonia* infections is positively affected (172, 175, 178). Only one previous study has investigated the effect of fish oil on *S. aureus* infection and like in Paper II they found an increase in survival (179). In addition, fish oil appears to conclusively increase survival to protozoans (Table 2) (176, 178, 180). Viral infections have been investigated less extensively and there is no consensus of whether fish oil is beneficial for survival or not (Table 2) (174, 177).

Table 2 Survival to infections in animals fed fish oil[1]

Pathogen class	Increased	Decreased	Not changed
Total	11	5	6
Bacteria	7	4	4
Gram-positive	2	1	1
Gram-negative	5	2	3
Virus	1	1	2
Protozoa	3	-	-

[1]Table generated from references: (172-177)

Increased bacterial load in HFD/S- but not HFD/P-fed mice

Exactly how diet affects the immune system to induce mortality is not known. However, immune suppression, at least at some time points and in some parts of the immune system, seems plausible. This is based on the finding that the bacterial load was largely increased in the kidneys 5-7 days after inoculation in the HFD/S- compared with the LFD-fed mice (Paper I). In addition, the HFD/P-fed mice did not have increased bacterial load, but instead the levels were comparable to those in LFD-fed mice, providing an association between bacterial load and mortality. This is in line with the assumption that the high mortality in mice fed HFD/S is due to suppressed immune function. Conversely,

the low mortality in mice fed HFD/P could be due to enhanced immune function. The reason for the HFD/S-fed mice inability to clear the bacteria and thereby survive the infection is unknown, but we observed several possible mechanisms, as discussed below.

Adipose tissue inflammation and serum cytokine levels

It is well known that obesity induced by HFD (generally high in SFA and MUFA, rather than PUFA), as well as obesity in Ob/Ob mice on LFD, is associated with macrophage infiltration, inflammation and cytokine production in adipose tissue (3, 59, 61, 64-67). Similarly, we found increased levels of macrophages in the adipose tissue of HFD/S-fed mice, as determined by mRNA expression of the macrophage markers *Emr1* and *Cd68*. These results were confirmed by immunohistochemistry which showed higher numbers of crown-like structures, i.e. adipocytes encircled by macrophages, and aggregates of macrophages in the adipose tissue of HFD/S-fed mice (data not shown).

Consistent with the increased macrophage infiltration, uninfected HFD/S-fed mice had higher mRNA expression of the anti-inflammatory cytokines *Il1rn* and *Il10* in gonadal fat (Paper I and II). The pro-inflammatory *Il1b* and *Tnf*, in addition to the pro- and anti-inflammatory *Il6,* were increased in HFD/S-fed mice in Paper I, but not in Paper II. This may be due to the smaller weight difference, between LFD- and HFD/S-fed mice in Paper II compared with Paper I (10.0 g vs. 14.1 g, respectively). This presumably means that the fat weight difference was smaller and hence there was a smaller difference in the degree of adipose tissue inflammation. In addition, the number of mice was smaller in Paper II.

When infected, the HFD/S-fed mice still had higher expression of the anti-inflammatory cytokines *Il1rn* and *Il1b* in gonadal fat. Among the pro-inflammatory cytokines the *Il1b* expression was increased, while there was no difference for *Il6* and *Il1b*.

Serum levels of the measured cytokines were in many cases difficult to detect in uninfected mice, but they rose during infection. For uninfected mice the only difference was the increased levels of IL-1Ra in HFD/S- and HFD/P-fed mice compared with LFD-fed mice. During infection there were an increase in IL-1Ra, IL-10, and IL-1β, and a tendency for increased TNF-α and IL-6 levels in HFD/S-compared with LFD-fed mice.

In summary, we found increased macrophage infiltration and consistently higher serum levels and mRNA expression of anti-inflammatory cytokines in HFD/S-fed mice, and this difference was seen both in uninfected and infected mice. This may be important for survival as IL-1Ra treatment before inoculation enhances mortality in experimental sepsis (181) and IL-10 neutralization has been shown to enhance survival to sepsis (182). Indeed, mortality in clinical sepsis often occurs days to weeks after the first clinical event and is associated with a shift toward an immunosuppressive state (28, 153).

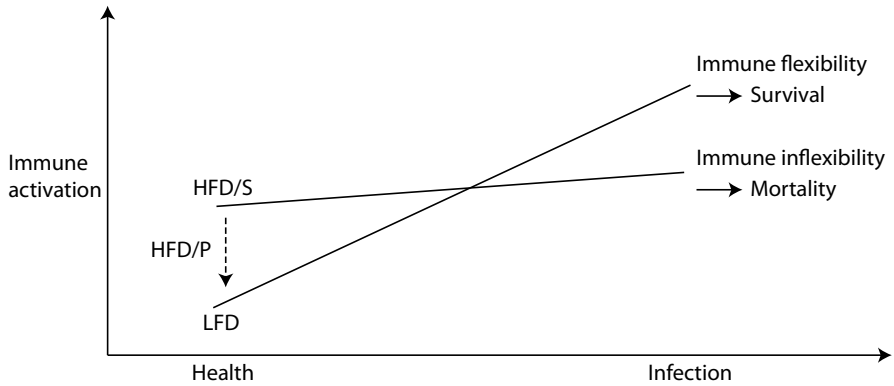

Figure 6. Schematic view of how HFD/S-fed mice may have increased immune activity in the absence of infection, but decreased immune activity in the presence of an infection. This lack of dynamic effect in mice fed SFA and MUFA may be regarded as immunologic inflexibility.

Chronic adipose tissue inflammation, and perhaps in particular the high anti-inflammatory cytokine expression, may prevent the immune system from proper activation during infection. This is in line with previous findings that the autoimmune diseases rheumatoid arthritis and systemic lupus erythematosus are associated with increased risk of acquiring infections. This finding seems to be independent of immunosuppressive treatments given to the patients (183, 184). One may thus speculate that chronic inflammation leads to immunological inflexibility (Fig. 6). Inflexibility may be an important concept not only for immune functions but also for metabolic functions. Indeed, there is a phenomenon termed metabolic inflexibility. This means, for example, that the body does not switch from fat to glucose oxidation when glucose levels are high

and this has been associated with diabetes (185). We here speculate that in conditions with immunological inflexibility, the immune system is both unable to completely turn off in the absence of an infection, and to turn on at maximal efficiency during an infection (Fig. 6).

The adipose tissue inflammation may be induced by HFD/S-associated obesity or possibly by the pro-inflammatory fatty acids in the HFD/S. In the latter case, the anti-inflammatory n-3 fatty acids in the HFD/P may instead prevent or diminish adipose tissue inflammation (186). This effect of HFD/P may in turn lead to immunologically flexible mice that survive *S. aureus* infection. The HFD/P-fed mice do survive the infection, but our LDA immune cards show that mRNA expression in gonadal fat mostly does not differ substantially from HFD/S-fed mice, as discussed below (Paper II).

Due to the increased macrophage infiltration into adipose tissue of HFD/S-fed mice, we were not surprised to find increased expression of the monocyte attracting chemokine *Ccl2*. This is also in line with an earlier study (63) showing that *Ccl2* deficiency decrease macrophage infiltration. However, the HFD/P-fed mice did not have increased *Ccl2* expression but they still had increased macrophage infiltration, as determined by mRNA expression of macrophage marker *Cd68* and immunohistochemistry (data not shown). These data are in contrast to a study by Todoric *et al*. who showed that long-chain n-3 PUFA rich HFD decreased macrophage infiltration compared with a saturated HFD. The reason for this discrepancy may be due to the different genetic background of the mice, as we used wildtype C57BL/6 mice and not the genetically obese leptin receptor deficient db/db mice used by Todoric and coworkers (186). In addition, the diets also differ, as our PUFA rich diet contained more SFA and MUFA and n-3 PUFA, and less n-6 PUFA (186, 187). The diet used by Todoric and coworkers was also supplemented by a large amount of α-tocopherol (the active form of vitamin E), that in it self can have immunomodulatory effects (186, 188).

The chemokine *Ccl3* (also known as macrophage inflammatory protein-1 alpha) has been reported to be positively associated with obesity (189) and in Paper II it was also up-regulated in HFD/S-fed mice, and even more so in HFD/P-compared with LFD-fed mice. Despite the ability of Ccl3 to induce chemotaxis in e.g. neutrophils, monocytes and lymphocytes (190-192), we did not detect any difference in cell type markers, beside the already mentioned *Cd68* macrophage marker.

Like in uninfected HFD/S-fed mice, the *Il1rn* mRNA expression and IL-1Ra serum levels were higher in HFD/P- than in LFD-fed mice. However, HFD/P-fed mice did not differ from LFD-fed mice with regard to *Il10*, *Il6* or *Tnf* mRNA expression, although the *Il1b* expression was somewhat increased. Heme oxygenase 1 (*Hmox1*) and the leukocyte cell surface molecule protein tyrosine phosphatase receptor type C (*Ptprc;* also called Cd45 and common leukocyte antigen), were similarly up-regulated in both HFD/S- and HFD/P-fed mice compared with LFD-fed mice. *Hmox1* can protect against oxidative stress and its expression has been found to be up-regulated in obese individuals, but presumably not enough to protect from insulin resistance (193). *Ptprc* is widely expressed on hematopoietic cells and perhaps the increased expression just mirrors the increased macrophage infiltration in the HFD/S- and HFD/P-fed mice. Indeed, the expression of *Ptprc* and the macrophage marker *Cd68* was highly correlated ($r^2 = 0.78$, $P = 0.0001$).

Due to the few differences between HFD/S- and HFD/P-fed mice, adipose tissue inflammation may not play that big a role in causing *S. aureus*-induced mortality. However, HFD does not only produce inflammation in adipose tissue, but also in liver and hypothalamus (85, 86) and thus it cannot be excluded that HFD/S-induced inflammation in other organs or systemically could be of importance for mortality.

Changed proportion of blood monocytes and function in HFD/S-fed mice

FACS analysis showed a 60% increase in the blood proportion of monocytes in uninfected HFD/S-fed mice compared with LFD-fed mice, but after bacterial inoculation there was instead a 70% decrease. Although the HFD/S-fed mice had more monocytes before infection, they may still be less prepared to fight invading bacteria as they had a lower proportion of phagocytosing monocytes than LFD-fed mice. However, a measure of ingested number of bacteria per monocyte did not differ between groups. Phagocytic activity in infected mice could not be determined due to small samples and few monocytes. At present, it is unclear to what extent decreased macrophage function contributes to decreased immune response to *S. aureus* in HFD/S-fed mice. Nonetheless, monocytes are important for defense against *S. aureus* as depletion leads to increased *S. aureus*-induced mortality (194). On the other hand, it is possible that the increased monocytes proportion may compensate for the decreased function.

Neutrophilic dysfunction in HFD/S-fed mice

There was a decrease in the percentage of neutrophilic granulocytes in the blood of uninfected HFD/S-fed mice compared with LFD-fed mice, but 5–7 days after bacterial inoculation there was instead an increase. This increase may be compensatory, since neutrophilic depletion prior to *S. aureus* infection leads to a rebound effect 6 days after bacterial inoculation (152). The earlier described sepsis-induced reduction of neutrophil apoptosis (35) may also be differently affected by diets.

Granulocytes were tested for phagocytic activity both in uninfected mice and 5–7 days after bacterial inoculation. In the uninfected HFD/S-fed mice the proportion of granulocytes that were able to phagocytose staphylococci was decreased by 40%, compared with LFD-fed mice. Furthermore, granulocytes from uninfected HFD/S-fed mice ingested fewer bacteria per granulocyte in uninfected HFD/S-fed mice. However, 5–7 days after bacterial inoculation there was no difference between groups.

These signs of decreased granulocyte/neutrophil efficacy in HFD/S-fed mice presumably contribute to the mortality in these mice. Certainly, neutrophilic dysfunction during other circumstances can contribute to increased bacterial growth and increased mortality (195-197), and reasonably this can happen also in HFD/S-fed mice. Neutrophils are often first to migrate into tissues in response to invading pathogens and to attack bacteria in the blood circulation (198, 199). Furthermore, neutrophilic depletion in mice leads to severely increased mortality in *S. aureus*-induced sepsis (152). In addition, most patients with congenital neutropenia, also known as Kostmann syndrome, die from bacterial infections in early childhood, unless properly treated (200). These findings emphasize the importance of neutrophils in the early clearance of bacteria. Interestingly, there are indications that neutrophils are of importance not only to overcome an infection, but also for the subsequent resolution of the inflammation (201).

Decreased ROS production in HFD/S fed mice

Neutrophilic granulocytes and macrophages are both capable of ROS production that contributes to microbial killing. We measured the capacity of peritoneal lavage cells, containing neutrophils, macrophages and lymphocytes, to secrete ROS 5–7 days after *S. aureus* inoculation. Unstimulated lavage cells from HFD/S-fed mice produced only 7% of the ROS produced in lavage cells

from LFD-fed mice. In contrast, the maximal capacity of ROS production, as measured after phorbol myristate acetate (PMA) stimulation, did not differ between groups. To find out if the ability to produce ROS was altered already before entering the blood circulation we isolated granulocytes from bone marrow. The granulocytes were then stimulated with D-peptide, live opsonized *S. aureus*, or PMA. No differences in ROS production were detected between HFD/S- and LFD-fed mice. Besides a decreased capacity to kill bacteria, a decreased ROS production could lead to slower resolution as ROS induce apoptosis in neutrophils, which is a prerequisite for resolution (202).

Speculations on the immune altering effects of HFD/P

Our results indicate that the detrimental effect on mortality by long-term HFD/S exposure is specific for dietary fat consisting of SFA and MUFA, rather than obesity. The mechanism(s) whereby HFD/P prevents mortality is unknown, but its protective role is presumably due to its high content of long-chain n-3 PUFAs and possibly through their anti-inflammatory effects. For example, n-3 PUFA has been shown to decrease the production of adhesion molecules, interleukins, prostaglandins and leukotrienes (119, 121, 203). Another mechanism for the preventive functions of HFD/P could be through the antithrombotic effects of long-chain n-3 PUFAs (204). Prevention of thrombosis and blood clotting can be crucial for survival of sepsis, given that the only drug approved by the U.S Food and Drug Administration for treatment of sepsis in humans, so far, is the coagulation inhibitor activated protein C.

In addition, n-3 PUFA also increases the production of the anti-inflammatory adipokine adiponectin (186, 205). This is interestingly as circulating adiponectin levels decrease with increasing fat mass and also during polymicrobial sepsis (206, 207). Moreover, adiponectin deficient mice have severely increased mortality during polymicrobial sepsis, while the adiponectin-inducing drug roziglitazone increased survival in wild type mice (207). Thus, there is a possibility that adiponectin may play a role for the decreased mortality in HFD/P-fed mice compared with HFD/P-fed mice.

Paper III-IV: Immune SNPs affecting body fat

Studies of mice have shown that IL-6 and IL-1RI deficiency leads to obesity (87, 89). We therefore wanted to know if polymorphisms in the IL-6 and IL-1 system affect obesity in humans. This was investigated in young men of the GOOD study (Paper III) and in elderly men of the MrOS study (Paper IV).

Polymorphisms in the IL-1 system and obesity

The IL1B -31 T>C SNP was associated with obesity in elderly men

The *IL1B* -31 T>C SNP is found in the promoter; more precisely in the TATA box were transcription factors bind to initiate transcription. It is therefore reasonable that a SNP changing the first T to a C could be of major importance for gene expression as it may interfere with DNA-protein binding and subsequent transcription (208). Indeed, the T allele has been shown to induce more formation of DNA-protein (presumably transcription factors) complex compared to the C allele (164), and also to increase transcription *in vitro* (209-212). Therefore we were somewhat surprised to not find an association between this SNP and obesity in the young men, although we found an association in the elderly men. However, it is unclear which allele that caused this association, as the heterozygous individuals had lower fat mass than the homozygous groups. We tried to find the proper genetic model for this SNP, and observed that a dominant model for the C-allele, i.e. C+ versus TT individuals, was associated with obesity. As discussed in Paper IV, this may be coincidental as exclusion of the CC group produced a higher slope for TT versus TC than for the dominant C-model. This may indicate that the C-allele is associated with decreased fat mass after all. Data from a Japanese study may support this as both men and women of the TT genotype had increased measures of obesity (213).

The discrepancies between the studies of young and elderly men and the finding that heterozygotes had the lowest fat mass in the elderly men may be due to complex interactions with neighboring SNPs, such as the *IL1B* -511 C>T SNP (209). It has been reported that in combination with certain SNPs the T allele does not lead to increased expression or IL-1 protein secretion, but rather the opposite (209, 214). To complicate matters further, transcription at the C allele could be differentially regulated as an additional DNA-protein complex has been reported to form when allowing nuclear proteins to bind to the C compared with the T allele (164, 209, 212).

The IL1B +3953 C>T SNP was associated with obesity in young men

This SNP is situated 3953 bp downstream of the promoter in exon 5 and it is synonymous. The T allele has previously been shown to increase *IL1B* mRNA (215) expression and to produce higher levels of IL-1β *in vitro* (161, 216), although not in all studies (214, 217). In line with mouse studies showing a connection with IL-1 activity and leanness, we found an association between carriers of the T allele and decreased fat mass. The T carriers also had decreased BMI, serum leptin, and the regional measures of trunk, arm and leg fat. However, these findings could not be replicated in the elderly men.

In line with our finding in younger men, a Brazilian study of both men and women with a wide age-range (18-92 years of age) showed that CC individuals more often than T carriers were overweight and obese (218). Similarly, two small studies of less than 300 Korean women have shown a tendency towards decreased obesity or overweight in T carries, although not significantly (219, 220). In contrast, the T allele was associated with increased waist circumference in an Australian coronary heart disease population (221). This was a non-population based study, it included both women, and the age span was 26-60 years of age (221), making this study very different from the GOOD study. One additional SNP was genotyped in the follow up study, but it was not associated with obesity (156).

The IL1RN 86 bp VNTR was associated with obesity and serum IL-1Ra in young men

The *IL1RN* gene has an 86 bp VNTR in intron 2. The number of repeats vary from two to six, with four repeats being the most common (called the *IL1RN**1 allele) and two repeats being the second most common allele (called *IL1RN**2) (222). As mentioned in the methodological considerations part, this polymorphism is in complete LD with the synonymous +2018 T>C SNP in exon 3 (223). The *IL1RN**2 allele was associated with increased production of IL-1Ra in monocytes (224) and increased plasma levels in healthy blood donors (162). In line with these data, we found that the *IL1RN**2 allele was additively associated with increased IL-1Ra serum levels in the GOOD study, and it was also associated with LPS-stimulated IL-1Ra production in mononuclear cells from blood (Paper III).

In line with the decreased obesity seen in IL-1Ra deficiency mice (96), we found that carriers for the high IL-1Ra producing *IL1RN**2 allele was associated

with increased fat mass, but not BMI, in the GOOD study of younger men (Paper III). In contrast, this finding was not observed in the elderly men of the MrOS study (Paper IV). Few studies have investigated the *IL1RN* VNTR polymorphism in association with obesity or BMI. Prior to Paper III, there was a non-significant association between BMI and the *IL1RN**2 allele in a small study of Korean women. More recently, *IL1RN**2 was associated with obesity in small study of Caucasian Italian women (225).

Other polymorphisms in the *IL1RN* gene have now also been studied in relation to obesity and the T allele of rs4251961 was associated with increased BMI in a study by Rafiq *et al*. (226). The T allele of this SNP was also associated with increased serum levels of IL-1Ra, as was the T allele of rs579543 SNP (in LD with the 86 bp VNTR). Similarly to Paper III, the latter SNP did not show an association with BMI (226). It would have been interesting to know if rs579543 still was associated with fat mass as analyzed by DXA, like the VTNR in Paper III. However, unfortunately Rafiq *et al.* did not use this method.

To follow up on Paper III and IV, our group has systematically investigated SNPs in the *IL1RN* gene in the GOOD study. We genotyped 15 *IL1RN* SNPs and found a significant association between fat mass and a C>T SNP (rs4252041) in the 3' untranslated region besides the +2018 SNP. This SNP was also part of a haplotype containing five SNPs that showed an association with fat mass (156). In contrast to the +2018 SNP (156) and 86 bp VNTR (Paper III), the association between rs4252041 and fat mass could be confirmed in the MrOS study (156). In the follow-up paper, we found no association between rs4252041 and serum IL-1Ra, showing that an effect on serum IL-1Ra is not necessary for a SNP in the *IL1RN* gene to be associated with obesity.

How can IL-1 system polymorphisms affect obesity?

The general mechanism whereby the studied IL-1 system polymorphisms are thought to affect obesity is by altered protein production. Several mechanisms have been proposed for the anti-obesity effects of IL-1β. IL-1β can act centrally and has been shown to mediate, at least in part, the hypothalamic actions of leptin such as decreased food intake (95). Excess IL-1, like excess leptin, has also been shown to increase energy expenditure (96). Moreover, IL-1β is expressed in the hypothalamus, and the levels are enhanced by leptin and reduced by fasting (95, 227). In humans, it has been shown by Meier *et al*. and in Paper III that the plasma/serum levels of IL-1Ra is increased in obese individuals. Thus Meier *et al*. suggested that decreased IL-1 activity due to

elevated IL-1Ra production could contribute to leptin resistance in obese individuals (228). Suggested anti-obesity actions of IL-1 outside the brain include inhibited adipocyte differentiation, decreased adipocyte lipid accumulation, possibly due to increased lipolysis and decreased lipoprotein lipase activity, and increased leptin secretion from preadipocytes (92, 96, 97, 229).

To my knowledge, none of the possibly anti-obesity effects of IL-1 have been investigated for associations with SNPs in the IL-1 system.

IL-6 polymorphism and obesity

The IL6 SNP -174 G>C was associated with obesity in elderly men

The IL6 -174 G>C SNP (rs1800795) is found in the promoter, 174 bp upstream of transcription start. Commonly occurring haplotypes containing the G allele has been found to be stronger enhancer of transcription than C containing haplotypes (141, 163, 230, 231). In line with the obesity development in IL-6 deficient mice, we found an association between the transcription inducing G allele and decreased fat mass in elderly men in the MrOS study, but not in younger men in the GOOD study. The finding in the elderly men is supported by several studies (165, 232, 233), but not all (234-236), showing an association with the G allele and decreased BMI. More recently two meta-analysis of over 24,000 individuals have failed to find an association between BMI and the -174 G>C SNP (237, 238). However this does not necessarily mean that there is no association between this SNP and fat mass as BMI is a rather poor measure of obesity (154).

Just recently, we have submitted an extensive investigation of SNPs in the IL-6 system. For the younger men we found an association between fat mass and a recently identified G>A SNP (rs10242595) just downstream of the IL6 gene. This finding was close to significant in the MrOS Sweden study and significant in the Caucasian individuals of the MrOS US study. Combining the three studies produced a highly significant association. Another IL6 SNP that has been associated with obesity is the promoter polymorphism rs2069827 (Andersson, N. *et al.*, submitted). This SNP, and some *IL6* gene haplotypes, was associated with BMI and waist circumference in a study of two cohorts of about 3000 men and women (237). However, a SNP in strong LD with the rs2069827

SNP was not associated with fat mass in the young men of the GOOD study (Andersson, N. *et al.*, submitted).

How can IL-6 polymorphisms affect obesity?

Similarly to the investigated IL-1 system polymorphisms, the *IL6* -174 G>C rs1800795 SNP is thought to affect obesity via altered protein production. There have been conflicting reports whether G carriers have decreased or increased IL-6 serum levels, and in a meta-analysis of over 5500 individuals there was no association between the SNP and circulating IL-6 (238). However, the effect of IL-6 on obesity may be mediated locally rather than systemically. In particular, IL-6 seems to be locally produced in the brain and the levels are negatively correlated with body fat, but uncorrelated with serum levels of IL-6 (239, 240). Moreover, chronic intracerebroventricular IL-6 treatment decreases body fat, while there is no effect of a similar IL-6 dose given intraperitoneally (87, 241, 242). IL-6 is presumably acting at the hypothalamic body fat regulating circuits to increase energy expenditure (87, 241, 243, 244). This is in line with the increased energy expenditure observed in *IL6* -174 G carriers, while IL-6 deficient mice have decreased energy expenditure (93, 245). The effect on energy expenditure may be due to sympathetic induction of uncoupling protein 1 in brown adipose tissue to increase thermogenesis in rodents (242). Although the central effects of IL-6 seems most robust, peripheral effects on body weight and fat mass have been found in mice with IL-6 secreting tumors and in primates peripherally treated with IL-6 (246, 247). In addition, peripheral IL-6 treatment to humans has been shown to induce lipolysis and fat oxidation (248). Finally, expression of IL-6 in skeletal muscle has been shown to cause a decrease in body fat mass, in addition to liver inflammation and hyperinsulinemia in mice (249).

Discrepancy between the GOOD and MrOS study

For the investigated SNPs there was a discrepancy between the GOOD and the MrOS study. This can be due to various reasons; one of these is of course that the first finding was a coincidence. Alternatively, a SNP may only be of importance at a certain age, which could result in discrepancies between the results in the young men of Paper III and the older men in Paper IV. In line with this, obesity of IL-6 and IL-1RI knockout mice was only seen at certain ages (87, 89). Another reason may be interference from small subpopulations of non-Caucasian individuals. For example, the *IL6* -174 C allele is non-existent or

extremely rare in all but Caucasians. A person that is non-Caucasian and obese will therefore diminish the chances to find a true association between the C variant and obesity, as he will have the G variant. Recently, algorithms have been developed to determine ethnicity in GWA studies, and this will diminish the risk of such confounding factors in the future.

The MrOS study had the exclusion criteria that the participants had to be able to walk without aid and they were not allowed to have bilateral hip prosthesis. This could potentially result in a slight biased selection of study participants compared with the male general population in this age range in the Gothenburg area. The GOOD cohort may be more representative of the general young male population in the Gothenburg area with regard to height, weight, and BMI (155). Furthermore, genetic variations may interact with environmental factors to modulate the risk of obesity. Indeed, a G/A SNP 6054 bp downstream of the promoter in the *IL1B* gene is associated with the level of PUFA in cell membranes, which in turn is correlated with dietary PUFA intake. This SNP is also associated with the metabolic syndrome among individuals with low PUFA content (250). Intake of different types of fat has also been shown to interact with a *TNF* polymorphism and disease activity in Crohn's disease and obesity (251, 252). Genes other than cytokine genes seems to interact with dietary fatty acid intake as SNPs in the leptin and PPAR-γ gene have been found to increase the obesity risk with increasing intake of n-6 fatty acids (251). Carbohydrate intake may also interact with polymorphisms to increase the risk of obesity (253). A study from the Swedish National Food Administration shows that there is a difference in food intake between younger and elderly people. The elderly have higher intake of fish, but lower consumption of French fries, compared with younger individuals (254). Thus it is possible that some of the differences between the GOOD and MrOS study may be due to different diets in young and elderly individuals. However, a problem with studies on gene environment interactions is the large number of possible combinations between environmental conditions and SNPs that may cause a mass significance problem. Discrepancies may also be influenced by the finding that SNPs in one of the closely situated IL-1 system genes can influence gene expression in nearby genes. For example, the *IL1RN**2 allele has been associated with decreased IL-1α, but also increased IL-1β, production (224, 255).

In summary, none of our findings in Paper III could be replicated in Paper IV. However, even if we were not able to replicate any individual polymorphisms, we did succeed in replicating the finding that polymorphisms in general in the

IL1B, *IL1RN*, and *IL6* genes, are associated with obesity in Paper III, IV and also in two follow-up papers (156)(Andersson, N. *et al.*, submitted). This is also in line with murine gene knockout and human gene transcriptional studies.

CONCLUDING REMARKS

This thesis has a wide perspective on immunology and obesity/nutrition, an area that has expanded vastly during the latter years. Not long ago adipose tissue was considered to be a boring lump of fat with the one and only function to store fat. By know we know that adipose tissue secretes many substances like cytokines, leptin, adiponectin, and plasminogen activator inhibitor-1 (256). Regulation of cytokine levels seems important not only in disease, but also in health. For example, our genetic studies show that polymorphisms in the IL-1 system and in the IL-6 gene are associated with obesity in men. There are data relating these polymorphisms to altered cytokine production. However, it is still unclear weather these are the "true" and functional polymorphisms or if they are just linked to the functional ones. To control the inflammation in rheumatoid arthritis, cytokine inhibitors are now being used. These cytokine inhibitors include a monoclonal IL-6 antibody (tocilizumab) and recombinant IL-1Ra (anakinra) (14). The former have metabolic side effects like increased cholesterol levels (14), which could be expected from mice studies (257). However, there is no apparent effect on weight gain, possibly due to an inability of the antibody to cross the blood-brain barrier and inhibit IL-6 in the central nervous system. On the other hand, recombinant IL-1Ra has positive effects on β-cell function in type-2 diabetes (7, 258). This may seem opposed to the glucose intolerance and insulin insensitivity in the IL-1RI deficient mice (89). However, the obesity in these mice probably overrides the positive effect that lack of IL-1 activity would have on β-cell function. Not surprisingly, side effects common to both anakinra and tocilizumab are different kinds of infections (14).

Due to safety problems and the probable need for intracerebroventricular administration of IL-6 and IL-1, these cytokines will most likely never become drugs against obesity. However, treatment with the IL-6 related cytokine ciliary neurotrophic factor (CNTF) decreases obesity in mice and humans (259, 260). Moreover, there is a study indicating that high levels of CNTF in the arcuate nucleus is associated with leanness in rats, suggesting a role for endogenous CNTF (261). In humans a recombinant and somewhat modified form of CNTF was used in Phase 3 studies, but unfortunately there was nausea and

neutralization of the beneficial anti-obesity effect of the compound, probably due to antibody development. It is unclear if this could be avoided with other CNTF-like molecules.

Conversely to the obesity-modulating effects of the immune system, diet can also modulate the immune system. We found that obese HFD/S-fed mice hade increased mortality compared with lean LFD-fed mice. On the other hand, mortality was comparable in obese HFD/P- and LFD-fed mice. A possible explanation for the difference between HFD/S- and HFD/P-fed mice may be a lesser chronic inflammation in HFD/P-fed mice, probably due to the anti-inflammatory effects of fish oil. This hypothesis needs verification, but regardless of the mechanism, the present results indicate that the detrimental effect of long-term HFD exposure is specific for dietary fat consisting of SFA and MUFA.

In light of the large dietary effect on mortality, we suggest that studies on experimental sepsis should be performed in models that are metabolically similar to the clinical situation, e.g. in animals fed similar diet as humans. This may accelerate the discoveries of new treatments for sepsis that are effective, not only in mice, but more importantly also in humans. Interestingly, dietary intake of long-chain n-3 PUFAs in combination with aspirin leads to the production of anti-inflammatory molecules called resolvins and protectins that promote resolution of inflammation (201).

In conclusion, this thesis emphasize that there are reciprocal interactions between the immune system on one hand and obesity and nutrition on the other.

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