

Doctoral Thesis at the Medical University of Vienna for obtaining the
Degree 'Doctor of Philosophy - PhD'

**Osteopontin in obesity-associated adipose
tissue inflammation and insulin resistance and
its interaction with monocyte chemoattractant
protein-1**

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1. ABSTRACT

1.1 Abstract – English

Obesity is associated with a state of chronic low-grade inflammation mediated by immune cells that are primarily located to the adipose tissue. The chronic inflammatory response appears to underlie obesity-induced metabolic deterioration including insulin resistance and type 2 diabetes. Osteopontin (OPN) and monocyte chemoattractant protein-1 (MCP-1) are inflammatory cytokines; the expression of both is strongly upregulated in adipose tissue upon obesity.

Here I studied OPN effects on obesity-induced adipose tissue inflammation and insulin resistance in genetic OPN deficiency and by antibody-mediated OPN neutralization. Next I generated OPN and MCP-1 double knockout (DoKo) mice to evaluate possible interaction of both cytokines with respect to obesity-associated metabolic and inflammatory alterations.

After feeding high-fat diet to induce obesity, mice lacking OPN gene displayed markedly improved insulin sensitivity compared with their wild-type littermates. Genetic OPN deficiency only moderately reduced obesity-induced adipose tissue inflammation and did not significantly affect macrophage accumulation. Targeting OPN action by a neutralizing antibody for five days significantly improved insulin sensitivity in diet-induced obese mice. Anti-OPN treatment attenuated adipose tissue macrophage infiltration and inflammatory gene expression and significantly reduced deleterious signal transduction related to insulin resistance. Notably, combined deletion of OPN and MCP-1 in DoKo mice led to augmented diet-induced obesity and insulin resistance compared to control mice.

These findings demonstrate that OPN is critically involved in obesity-associated adipose tissue inflammation and insulin resistance. Unexpectedly, OPN and MCP-1 double deficiency does not improve but even worsen the obesity-related metabolic perturbation indicating antagonistic effects. Taken together, targeting OPN action could provide a novel approach for the treatment of obesity-associated metabolic disorders.

1.2 Kurfassung – Deutsch

Adipositas geht mit einer chronischen subklinischen Entzündung einher, die ihren Ursprung im Fettgewebe nimmt. Diese chronisch entzündlichen Prozesse scheinen die Grundlage für Adipositas-assoziierte metabolische Veränderungen wie Insulinresistenz und somit auch für Diabetes mellitus Typ 2 zu sein. Osteopontin (OPN) und Monocyte Chemoattractant Protein-1 (MCP-1) sind inflammatorische Zytokine, deren Expression im Fettgewebe durch Adipositas deutlich hochreguliert wird.

In dieser Studie untersuchte ich Effekte von OPN auf die Adipositas-assoziierte Fettgewebsentzündung und Insulinresistenz bei genetischer OPN Defizienz und anhand von Antikörper-medierter OPN Neutralisation. Weiteres generierte ich OPN und MCP-1 Doppel Knockout (DoKo) Mäuse, um mögliche Wechselwirkungen zwischen den beiden Entzündungsmediatoren hinsichtlich Adipositas-induzierter metabolischer und inflammatorischer Veränderungen zu studieren.

OPN Knockout Mäuse, in denen mit einer fettreichen Diät Adipositas induziert wurde, zeigten gegenüber den Wildtyp-Mäusen eine deutlich verbesserte Insulinsensitivität, wobei die Fettgewebsentzündung nur moderat und die Makrophagenanzahl im Fettgewebe nicht signifikant vermindert waren. Die Neutralisation der OPN Aktivität mittels eines Antikörpers verbesserte innerhalb von fünf Tagen signifikant die Insulinsensitivität in adipösen Mäusen. Die Anti-OPN Behandlung verringerte die Makrophageninfiltration sowie die inflammatorische Genexpression und Signaltransduktion im Fettgewebe. Bemerkenswerterweise führte die kombinierte Defizienz von OPN und MCP-1 in den DoKo Mäusen zu einer Verstärkung der Diät-induzierten Adipositas und Insulinresistenz im Vergleich zu Kontrollmäusen.

Diese Daten zeigen, dass OPN ein kritischer Mediator der Adipositas-assoziierten Fettgewebsentzündung und Insulinresistenz ist. Wider Erwarten verbessert das gemeinsame Fehlen von OPN und MCP-1 die Stoffwechsellage bei Adipositas nicht, sondern führt sogar zu einer Aggravierung der Adipositas-induzierten metabolischen Veränderungen. Zusammenfassend könnte die Blockade von OPN Effekten eine neue therapeutische Strategie zur Behandlung der Insulinresistenz und des Diabetes mellitus Typ 2 darstellen

2. INTRODUCTION

2.1 Obesity and Epidemiology

Obesity is increasing at an alarming rate worldwide and is a fundamental risk factor for type 2 diabetes, cardiovascular disease and the metabolic syndrome (1). According to WHO estimations, globally more than one billion adults are overweight, 300 million of whom are obese.

Mammals have evolutionarily evolved mechanisms to store energy in order to guarantee survival in periods of drought and famine. However, the long-term storage of excessive amounts of nutrients can have negative impact on health. Weight gain and obesity are in favor to occur when food is energy dense because of high proportions of simple carbohydrates and saturated fats, such as is common in developed Western societies (2). In parallel with nutrient overload physical activity in industrialized countries has been declining over decades due to passive leisure activities, less physically demanding work and technological advances.

The metabolic syndrome and type 2 diabetes confer a high risk for cardiovascular morbidity and mortality. Many studies have reported an association between obesity and cardiovascular risk factors such as hypertension and dyslipidemia. The Multi-Ethnic Study of Atherosclerosis (MESA) revealed that obesity is also linked to subclinical cardiovascular disease independent of other classical cardiovascular risk factors. E.g. coronary artery calcium deposition and carotid artery intima-media-thickness are higher in obese populations than in lean controls irrespective of the ethnic origin (3).

The fiscal importance of the obesity epidemic is enormous since most patients need medical care throughout their lives. Hence, elucidating underlying pathophysiologic mechanisms and the development of novel therapeutic approaches targeting obesity and associated metabolic disorders are main issues for medical research and pharmaceutical industry.

2.2 Obesity and Inflammation: Evolutionary Considerations

Inflammation is described as the principal response of the body invoked to deal with injuries, the hallmarks of which include swelling, redness, pain and fever. A short-term adaptive inflammatory response is a crucial component of tissue repair and involves integration of many complex signals in distinct cells and organs.

However, the long-term consequences of prolonged inflammation are almost never beneficial. This certainly seems to be the case in metabolic diseases (4). But why are metabolic disorders often linked to inflammation? The functional units that control key metabolic and immune reactions in mammals have evolved from common ancestral structures. One such structure is the *Drosophila* fat body, which contains the mammalian homologues of the liver, the haematopoietic and immune system (5). Interestingly, this site is also recognized as the equivalent of mammalian adipose tissue (6). The *Drosophila*'s fat body functions in sensing energy and nutrient availability, and coordinates the appropriate metabolic and survival responses (5). Although in mammals distinct functional units and higher organs have formed they carry with them a phylogenetic heritage. Hence, a scenario is plausible where overlapping pathways regulate both metabolic and immune functions through common key regulatory molecules (4).

2.3 Obesity and Adipose Tissue Inflammation

The adipose tissue in mammals consists of 2 types: white and brown adipose tissue. Both share many metabolic characteristics but, whereas white adipose tissue mainly stores excess energy for subsequent needs, brown adipose tissue functions as an energy-dissipating organ. The white adipose tissue consists of subcutaneous and visceral depots. The visceral depot has been receiving most attention because it is considered to be more metabolically active, and because its released factors can be delivered to the portal venous system and, thus, can directly impact liver metabolism (7).

Epidemiologic evidence for a relationship between obesity and inflammation has existed for years, although these findings were not appreciated in terms of the pathophysiologic conditions associated with obesity. Obesity has been known to be associated with signs of chronic low-grade inflammation such as elevated serum concentrations of C-reactive protein (CRP) and inflammatory cytokines (8; 9). Though subclinical, this inflammatory state represents a crucial link between obesity and insulin resistance (2) and predisposes to cardiovascular disease in obese patients (10). The systemic inflammatory response primarily originates from white adipose tissue (6) which produces a variety of inflammatory proteins such as interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , monocyte chemoattractant protein-1 (MCP-1), and CRP (11; 12). Within obese adipose tissue, inflammatory

molecules are predominantly produced by nonfat cells such as macrophages (13; 14). Only very recently it has been discovered that genetic and diet-induced obesity causes an infiltration of adipose tissue with macrophages that most probably underlies the systemic inflammatory state (13; 15). Adipose tissue infiltrating macrophages are bone marrow-derived and an important source of inflammatory cytokines that interfere with adipocyte function. Since adipocyte function is crucial for whole body insulin sensitivity, adipose tissue macrophages are considered as a critical factor for the development of obesity-induced insulin resistance and type 2 diabetes (4; 16). The number of macrophages present in white adipose tissue strongly correlates with obesity and adipocyte size in both human subjects and mice. However, the molecular mechanisms underlying the obesity-induced adipose tissue inflammation are still enigmatic. Several adipokines, i.e. cytokines secreted from cells located in adipose tissue including macrophages, were proposed to be crucially involved, but a causal mechanism of specific molecules mediating adipose tissue infiltration with macrophages and subsequent deterioration of insulin sensitivity in obesity has not yet been unequivocally defined.

2.4 Endoplasmic Reticulum Stress – Initiator of Adipose Tissue Inflammation?

The primary cause for obesity-induced inflammation is not yet fully understood. A potential source for the initiation of inflammation in obesity is endoplasmic reticulum (ER) stress. Overnutrition and obesity cause ER stress in liver and adipose tissue due to excess lipid accumulation and disturbed energy metabolism (17). ER stress activates a stress response signaling network called the unfolded protein response (UPR) that drives protective but also apoptotic and inflammatory reactions. Transmembrane proteins including the protein kinase/endoribonuclease IRE1 get activated upon ER stress. IRE1 initiates non-spliceosomal splicing of the mRNA for the transcription factor X-box binding protein 1 (XBP-1) that controls protective responses to ER stress. IRE1 also induces an inflammatory signaling cascade by activating I-kappa-B kinase (IKK), the mitogen-activated protein kinase (MAPK) p38 and c-Jun N-terminale kinase (JNK), and finally the major inflammatory transcription factor nuclear factor kappa B (NF- κ B). Consequently, obesity-induced ER stress leads to insulin receptor substrate-1 (IRS-1) serine phosphorylation and thereby inhibits insulin signaling (18). Reports that protective responses to ER stress are partially blunted in mice lacking one allele of

the XBP-1 gene support the proposed concept of ER stress regulation. Interestingly, heterozygous XBP-1^{+/-} mice develop severe insulin resistance even when on a genetic background that is less susceptible to obesity (17). Vice versa, administration of chemical chaperones that are known to reduce ER stress can restore insulin sensitivity in obese mice (19). Furthermore, ER stress has been shown to downregulate expression of the insulin sensitivity marker glucose transporter type 4 (GLUT4) in adipocytes (20). Taken together, ER stress directly affects insulin signal transduction in insulin target cells, e.g., by inducing inflammatory signaling and thereby contributes to insulin resistance. ER might be a site for the sensing of metabolic stress and the translation of that stress into inflammatory signaling and responses. In fact, the ER can be considered as an essential site of integration between nutrient and pathogen responses since it is very sensitive to glucose and energy availability, lipids and pathogens. Increased obesity leads to an environment that further challenges ER function and capacity owing to architectural constraints that limit ER expansion. Hence, obesity provides many conditions that could lead to ER stress (6).

2.5 Adipose Tissue Macrophages

2.5.1 The Role of Macrophages in Adipose Tissue Inflammation

The adipose tissue is nowadays regarded as an endocrine organ that produces large amounts of inflammatory cytokines and chemokines amongst others. Mediators derived from the adipose tissue are called adipokines including Leptin, TNF- α , IL-1 β , IL-6, and MCP-1. Notably, the main source of inflammatory mediators within murine and human adipose tissue are macrophages (14), although other cell types, including adipocytes, preadipocytes, vascular endothelial cells, T-lymphocytes, and the mesothelium may contribute. A major advance in the understanding of obesity-induced inflammation was the finding that the augmented inflammatory adipokine production in obesity is associated with an increased abundance of macrophages in adipose tissue of obese mice (13). Numerous clinical studies have confirmed a correlation between body mass index (BMI) and adipose tissue macrophage numbers in humans. In particular, the visceral adipose tissue that is metabolically most relevant is enriched with macrophages under obese conditions (21). Adipose tissue macrophages probably interfere with adipocyte function by secreting inflammatory cytokines. Since adipocyte function is strongly associated

with systemic insulin sensitivity the local action of macrophages and their secreted molecules could enhance systemic insulin resistance (18). Hence, adipose tissue macrophages are assumed to critically contribute to obesity-induced inflammation and to the pathogenesis of type 2 diabetes.

2.5.2 Macrophage Recruitment to Adipose Tissue

One of the most important questions in the understanding of obesity-induced adipose tissue inflammation is what triggers macrophage recruitment into the adipose tissue. Obesity promotes necrosis-like adipocyte cell death most probably due to detrimental effects of adipocyte hypertrophy as it occurs during the enlargement of the fat depots. Dead adipocytes are frequently found to be surrounded by macrophages building so-called “crown-like structures”. Those macrophages accumulating in immediate vicinity of dead adipocytes are supposed to scavenge cell debris and free lipid droplets (22). Crown-like structures can be observed in subcutaneous as well as in visceral adipose tissue of obese patients, although less frequently as in rodent models of obesity (21; 22). However, the signal by which adipocyte death leads to increased adipose tissue macrophage recruitment is not known. The presence of necrotic-like adipocytes and crown-like structures in non-obese hormone-sensitive lipase mutant mice (22) suggests that metabolic alterations probably involving endoplasmic reticulum stress provoke chemokine production by stressed adipocytes. Chemokines such as MCP-1 released by adipose tissue resident cells are capable to navigate immune cells into the adipose tissue at sites of disturbed homeostasis. However, the particular chemotatic signals by which adipocyte death leads to increased adipose tissue macrophage recruitment warrant further investigations and alternative mechanisms are still elusive.

2.5.3 Phenotype and Activation of Adipose Tissue Macrophages

Similar to the Th1/Th2 concept of T-cell activation, a concept of M1/M2 polarization has recently been suggested for macrophages. Depending on the stimuli such as cytokines and microbial products, macrophages polarize into specialized cell types and exert unique functional properties (23). “Classical” macrophage activation occurs upon stimulation with interferon-gamma (IFN- γ) alone or in combination with lipopolysaccharide (LPS). Classically activated macrophages produce inflammatory cytokines (e.g., IL-1, IL-6, TNF- α), reactive oxygen species such as nitric oxide (NO)

upon inducible nitric oxide synthase (iNOS) activity, and are capable of inducing Th1-polarized T-cell responses. These “classical” macrophages are referred to as M1 type in contrast to “alternatively activated” M2 macrophages. M2 have been described to be primarily induced by IL-4 and IL-13 (23). M2 macrophages appear to play a role in tissue repair and parasite defense but exert poor anti-bacterial killing activity. M2 are of a predominantly anti-inflammatory phenotype as they counterbalance inflammatory properties of M1 macrophages by release of IL-10, IL-1 receptor antagonist (IL-1Ra) and transforming growth factor beta (TGF- β) (24-26).

Characterization of human adipose tissue macrophages revealed that their phenotype predominantly resembles the alternatively activated M2 type since they express typical M2 surface markers like the mannose receptor CD206, scavenger receptors, and distinct integrins (27). Also murine adipose tissue macrophages express M2-associated genes such as *Ym1*, *arginase* and *Il10* (28). The factors driving alternative macrophage differentiation in adipose tissue are still elusive. The marked expression of scavenger receptors and the mannose receptor, together with high endocytic activity, implicates a role of adipose tissue macrophages in uptake of lipids and lipoproteins, apoptotic cells, and glycoproteins and hence supports the concept of obesity-induced recruitment of macrophages into adipose tissue due to an increased abundance of necrotic-like adipocytes that need to be removed (18). In accordance with their M2 phenotype adipose tissue macrophages produce anti-inflammatory cytokines such as IL-10 and the IL-1Ra. On the other hand, adipose tissue macrophages are capable of secreting large amounts of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β underlining the role of macrophages in obesity-induced adipocyte dysfunction and metabolic disorders (21). Accordingly, high-fat diet feeding in mice causes a shift in cytokine expression of adipose tissue macrophages from M2- to M1-like patterns. Gene Expression of IL-10, *Ym1*, and *arginase* appeared to be downregulated in adipose tissue macrophages of obese mice while TNF- α and *Nos2* (iNOS) were upregulated (28). In contrast, weight reduction induces an increase of M2-like macrophages in adipose tissue of obese patients (29). Adipose tissue macrophages may be activated by factors released by adipocytes. However, the particular mechanisms how high-fat diet and/or weight gain influence the M1/M2 polarization and cytokine expression in adipose tissue macrophages is still unclear. Hence, identification of factors that attract macrophages

to the adipose tissue and determine their pro- or anti-inflammatory phenotype is one of the most intriguing current issues of obesity research.

2.6 Adipokines

Adipose tissue is no longer considered to be an inert tissue serving solely as an energy store, but is emerging as an important organ in the regulation of many pathological processes. The adipose tissue is releasing various soluble factors termed as adipokines or adipocytokines. Some adipokines such as adiponectin or leptin are exclusively produced by the adipose tissue others like TNF- α or IL-6 can be secreted by diverse cell types in different organs. Adipokines were found to influence lipid and glucose metabolism, not only in adipose tissue, but also in liver and skeletal muscle. They are involved in appetite regulation and in local as well as systemic inflammatory processes. Hence, the adipose tissue is now recognized as an endocrine organ with autocrine regulation mediated by adipokines.

2.6.1 Osteopontin (OPN)

Osteopontin (gene *Spp1*), also named secreted phosphoprotein-1 and sialoprotein-1, is a multifunctional protein expressed in activated macrophages and T cells, osteoclasts, hepatocytes, smooth muscle, endothelial, and epithelial cells (30; 31). OPN was originally classified as a T helper type 1 (Th1) cytokine that is involved in physiological and pathological mineralization in bone and kidney, cell survival, inflammation, and tumor biology (30; 32). OPN induces the expression of a variety of proinflammatory cytokines and chemokines in peripheral blood mononuclear cells (33). Moreover, it functions in cell migration, particularly of monocytes/macrophages (31), and stimulates expression of matrix metalloproteases to induce matrix degradation and facilitate cell motility (34). Notably, OPN plays a role in various inflammatory disorders, such as rheumatoid arthritis (35) and atherosclerosis (36), in diabetic macro- and microvascular diseases (37) and hepatic inflammation (38). Hepatic OPN expression is upregulated in obesity (39) and in various models of liver injury where OPN is localized to macrophages and Kupffer cells (40; 41). Furthermore, OPN is involved in the pathogenesis of non-alcoholic fatty liver disease (NAFLD), which is strongly associated with visceral obesity (39; 42; 43).

As reported recently, OPN gene expression is extensively upregulated upon obesity in human and murine adipose tissue (44-46). While OPN plasma

concentrations are elevated in morbidly obese patients, data are inconsistent in different murine models of obesity (44-47).

2.6.2. Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 (gene: *Ccl2*) is a chemokine and a member of the small inducible cytokine family, which plays a role in the recruitment of monocytes and T lymphocytes to sites of injury and infection (48). It is expressed by a number of cell types including skeletal muscle, smooth muscle and endothelial cells, macrophage but also adipocytes (49; 50). Via binding to its main receptor chemokine CC motif receptor 2 (CCR2) MCP-1 directs monocytes and macrophage precursors to sites of inflammatory lesions such as atherosclerotic plaques (51; 52). MCP-1 has been shown to trigger firm adhesion of monocytes to vascular endothelium under flow conditions (53). Indeed, MCP-1 expression is crucially implicated in monocyte extravasation through vascular endothelium (54). Hence, it is strongly involved in the pathogenesis of atherosclerosis by promoting directed migration of immune cells.

Augmented MCP-1 concentrations are found in synovial fluids of patients suffering from rheumatoid arthritis as well as in the bronchial epithelium of idiopathic pulmonary fibrosis and in tuberculosis effusions. MCP-1 is also significantly elevated during inflammatory skin diseases, in acute hepatitis or fulminant hepatic failure, and during experimental autoimmune encephalomyelitis (55). Accumulating evidence links MCP-1 to the pathogenesis of obesity-induced inflammation.

Gene expression of MCP-1 as well as of the chemokine receptor CCR2 is increased in the adipose tissue of obese humans and mice. Notably, MCP-1 expression has been found to be higher in visceral adipose tissue than in subcutaneous tissue and is closely related to the number of residing macrophages. Plasma concentrations of MCP-1 are significantly elevated in obese and diabetic patients suggesting a pathophysiologic implication in systemic insulin resistance (56; 57). *In vitro* data showing that treatment with MCP-1 impairs glucose uptake in 3T3-L1 adipocytes support this concept (58). However, recent studies in diet-induced obese MCP-1 deficient mice emerged conflicting results in terms of adipose tissue macrophage accumulation and insulin resistance (56; 59). Taken together, some data point towards a pathophysiologic role of MCP-1 in obesity-associated inflammatory alterations including macrophage recruitment while other reports do not

support this hypothesis. Therefore, the precise impact of MCP-1 in obesity remains to be determined.

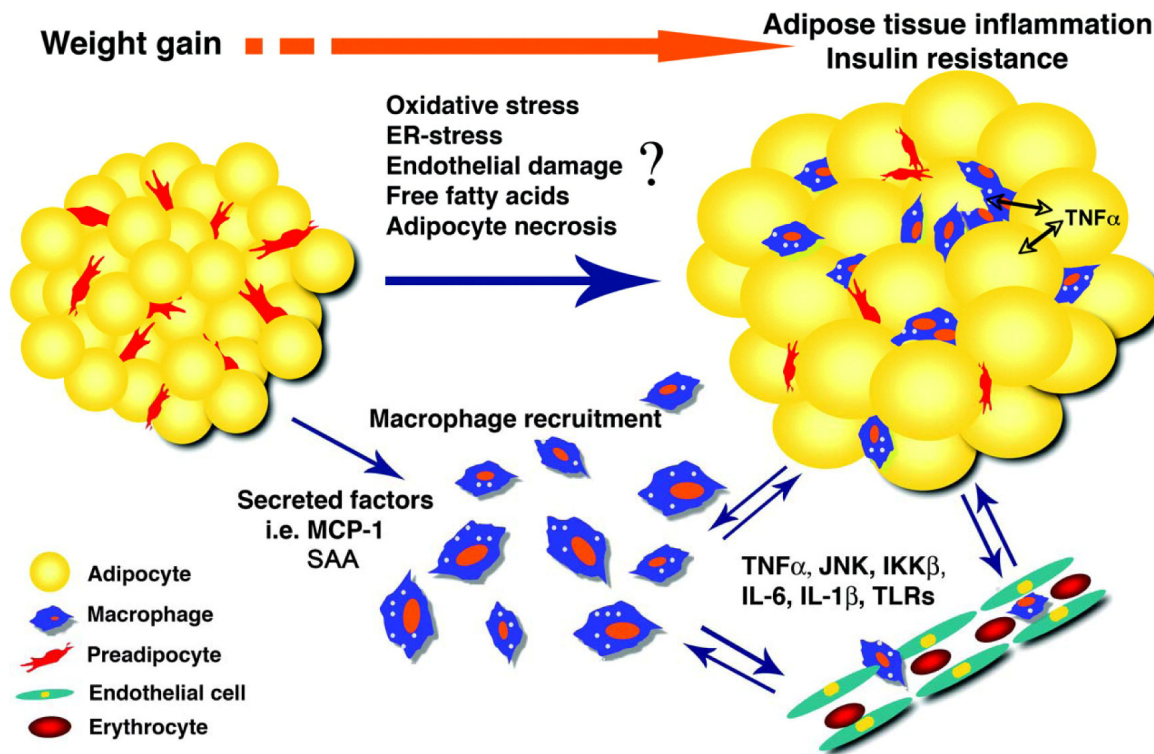


Figure 1: Schematic representation of obesity-induced adipose tissue inflammation. Weight gain is associated with enlargement of the adipose tissue which seems to interfere with adipocyte physiology. Adapted from Gustafson B. et al., Ref. 7

2.7 Insulin Resistance

Insulin resistance is determined as impaired responsiveness of cells to insulin in its target organs, i.e. adipose tissue, liver, and muscle. Insulin regulates glucose uptake and circulating free fatty acid concentrations. In adipose tissue, insulin decreases lipolysis thereby reducing free fatty acids efflux from adipocytes; in liver, insulin inhibits gluconeogenesis by reducing key enzyme activities; and in skeletal muscle insulin predominantly induces glucose uptake by stimulating the translocation of the GLUT4 glucose transporter to the plasma membrane. Insulin resistance leads to increased circulating free fatty acids concentrations and ectopic fat accumulation that impede insulin-mediated glucose uptake in skeletal muscle and elevate glucose production in liver (18).

By binding to its receptor insulin induces complex signaling cascades. In brief, insulin receptor-mediated tyrosine phosphorylation of insulin receptor substrates (IRS) leads to activation of two major pathways. The phosphatidylinositol 3-kinase (PI3K)-AKT pathway is largely responsible for insulin action on glucose uptake and suppression of gluconeogenesis. It results in GLUT4 translocation from its intracellular pool to the plasma membrane and glucose transport into the cell. The second pathway is the MAPK pathway that regulates gene expression and additionally interacts with the PI3K-AKT pathway to control cell growth and differentiation (60).

In the case of insulin resistance insulin signaling is negatively regulated as it occurs during phosphorylation of certain serine residues on IRS. This is mainly triggered by free fatty acid and inflammatory cytokine action. Serine kinases that phosphorylate IRS and thus hinder proper insulin signal transduction are IKK, JNK and other MAP kinases. The named serine kinases of IRS are also typical mediators of inflammatory signaling pathways, thus, providing an inhibitory crosstalk between inflammatory and insulin signaling. Other important molecular mediators that link inflammatory pathways to inhibition of insulin signaling are suppressor of cytokine signaling (SOCS) 1 and 3 as well as nitric oxide (NO). SOCS proteins are upregulated during inflammation, e.g. by interleukin-6, and induce ubiquitinylation and degradation of IRS proteins and thereby impede insulin signal transduction (18).

As mentioned above accumulating evidence has emerged that obesity is strongly associated with inflammation and is thereby involved in the development of insulin resistance. The chronic low-grade inflammation occurring in obese patients is determined by increased plasma levels of C-reactive protein, inflammatory cytokines such as TNF- α , IL-6, IL-1 β , IL-8, as well as the multifunctional protein leptin and free fatty acids. Almost all of those inflammatory markers were proven to alter insulin signal transduction *in vitro* and *in vivo* (2).

Taken together, insulin resistance in obesity is a reflection of long-term nutrient excess and is manifested through complex, heterogeneous mechanisms that can involve increased fatty acid flux, nutrient overload, ER stress, secretion of adipocyte-derived cytokines, and chronic tissue inflammation.

3. AIM OF THE STUDY

Obesity is associated with a state of chronic low-grade inflammation mediated by accumulating macrophages in the adipose tissue. The chronic inflammatory activity in adipose tissue appears to underlie obesity-induced metabolic deterioration including insulin resistance and type 2 diabetes. OPN and MCP-1 are inflammatory cytokines; the expression of both is strongly upregulated in the adipose tissue upon obesity. Determining the role of OPN and its interaction with MCP-1 in adipose tissue inflammation could contribute to the development of innovative strategies for treatment of obesity-induced complications including metabolic disorders. It is the aim of this thesis to elucidate the role of OPN in obesity-associated adipose tissue inflammation and insulin resistance and its interference with MCP-1.

4. RESEARCH DESIGN AND METHODS

4.1 Animals and diets

C57BL/6J wild type (WT), B6.Cg-Spp1^{tm1Bih}/J (OPN knockout; Spp1^{-/-} here referred to as OPN^{-/-}) and B6.129S4-Ccl2^{tm1Rol}/J (MCP-1 knockout, Ccl2^{-/-} here referred to as MCP-1^{-/-}) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). At 7 weeks of age male littermates were placed for 24 weeks on high-fat diet (HF, 60kcal% fat, D12492, Research Diets Inc., New Brunswick, NJ, USA) and low-fat diet (10kcal% fat, D12450B, Research Diets Inc.) or normal chow (both referred to as LF) to induce obesity and to serve as lean controls, respectively. All mice were housed in specific pathogen-free facility that maintained 12-hour light/dark cycle. Mice had free access to food and water and food intake was monitored. Blood was drawn after 3 hr fasting immediately before mice were sacrificed. Gonadal white adipose tissue (GWAT) pads were collected. The protocol was approved by the local ethics committee for animal studies and the Federal Ministry for Science and Research and followed the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

4.2 Experimental Setting

We studied the role of OPN in obesity-induced adipose tissue inflammation and insulin resistance in OPN knockout (OPN^{-/-}) mice and in wild-type (WT) mice that were treated with an OPN neutralizing antibody. Possible interference between OPN and MCP-1 action in obesity-associated adipose tissue inflammation and insulin resistance was investigated in OPN and MCP-1 double knockout (DoKo) mice.

Studies in OPN^{-/-} mice:

genotype	diet	sample size	function
WT	LF	10	Lean control lacking adipose tissue inflammation
WT	HF	10	Control for obesity-induced adipose tissue inflammation and insulin resistance

OPN-/-	LF	10	Lean control lacking adipose tissue inflammation
OPN-/-	HF	10	Role of OPN in obesity-induced adipose tissue inflammation and insulin resistance

OPN neutralization studies:

genotype and diet	sample size	intervention	function
WT HF	10	Anti-OPN antibody	OPN neutralization in obesity-induced adipose tissue inflammation and insulin resistance
WT HF	10	Control antibody	Untreated control for obesity-induced adipose tissue inflammation and insulin resistance
WT LF	5	Anti-OPN antibody	Lean control for OPN neutralization lacking adipose tissue inflammation
WT LF	5	Control antibody	Lean untreated control lacking adipose tissue inflammation

Studies in DoKo mice:

genotype	diet	sample size	function
WT	LF	10	Lean WT control lacking adipose tissue inflammation
WT	HF	10	Obese WT control for obesity-induced adipose tissue inflammation and insulin resistance
OPN-/-	LF	10	OPN deficiency in lean mice lacking adipose tissue inflammation
OPN-/-	HF	10	OPN deficiency in obesity-induced adipose tissue inflammation and insulin resistance
MCP-1-/-	LF	10	MCP-1 deficiency in lean mice lacking adipose tissue inflammation
MCP-1-/-	HF	10	MCP-1 deficiency in obesity-induced adipose tissue inflammation and insulin resistance

DOKO	LF	10	Combined OPN and MCP-1 deficiency in lean mice lacking adipose tissue inflammation
DOKO	HF	10	Interference of OPN and MCP-1 in obesity-induced adipose tissue inflammation and insulin resistance

4.3 Generation of DoKo mice

To generate DoKo mice, OPN^{-/-} and MCP-1^{-/-} single knockouts were intercrossed to receive double heterozygous offspring in the F1 generation. Double heterozygous littermates from F1 were crossed to generate DoKo mice in the next generation (F2). Animals obtained from F2 were genotyped by genomic PCR to identify DoKo mice.

4.4 Genotyping

At the age of two weeks tail tips (approx. 3mm) of mice obtained in the F2 generation were cut and immediately frozen on dry ice. Following proteinase K digestion total DNA was purified using commercially available DNeasy Blood & Tissue Kit® (Qiagen, Hilden, Germany). DNA concentrations were measured by NanoDrop® spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

Genomic PCR was performed using equal amounts of DNA, Taq polymerase (Roche, Basel, Switzerland) and the following set of primers (provided by Charles River). For OPN gene: wild-type forward 3'-CCATACAGGAAAGAGAGACC-5'; mutant forward 3'-AACTGTTTTGCTTGCATGCG-5'; common reverse 3'-CGTCCTGTAAGTCTGCAGAA-5'. For MCP-1 gene: wild-type forward 3'-ACAGCTTCTTTGGGACACC-5'; mutant forward 3'-CCTTCTATCGCCTTCTTGACG-5'; common reverse 3'-GGAGCATCCACGTGTTGGC-5'. Size of PCR products was assessed by agarose gel electrophoresis and ethidium bromide staining.

4.5 Antibody treatment

Mice were treated with a neutralizing anti-mouse OPN IgG (50 µg/mouse in phosphate buffered saline) or preimmune goat IgG for three times during five days by tail-vein injection. Osteopontin specific IgG (R&D Systems, Minneapolis, MN, USA) was produced in goats by immunizing with NSO-derived, recombinant mouse osteopontin. Mice were killed two days after last antibody application.

4.6 Metabolic measurements

Plasma glucose, cholesterol, triglyceride, and free fatty acid concentrations were measured in EDTA plasma by an automated analyzer (Falcor 350, A.Menarini Diagnostics, Florence, Italy). We used commercially available ELISA kits to determine plasma insulin (Mercodia AB, Uppsala, Sweden), IL-6, TNF- α , leptin, adiponectin, osteopontin (all R&D Systems), serum amyloid P (SAP) and high sensitivity C-reactive protein concentrations (hsCRP; both Alpco Diagnostics, Windham, NH, USA). We calculated homeostasis model assessment of insulin resistance (HOMA-IR) as an index for insulin resistance (61). Insulin sensitivity was assessed by insulin tolerance test (ITT) after a three hour fasting period. Blood glucose concentrations were measured before and 30, 60, 90 and 120 minutes after an intraperitoneal injection of recombinant human insulin (Actrapid[®], Novo Nordisk A/S, Bagsværd, Denmark; 0.75 U/kg body weight for HF and 0.25 U/kg for LF, respectively).

4.7 Indirect calorimetry

Indirect calorimetry was performed for 72h using an open-circuit, indirect calorimetry system including spontaneous activity by beam breaking (Oxylet, Panlab-Bioseb, Chaville, France). WT, OPN^{-/-}, MCP-1^{-/-} and DoKo mice on HF were analyzed for oxygen consumption (VO₂), carbon dioxide production (VCO₂), energy expenditure [calculated according to the following formula: $1.44 \times \text{VO}_2 \times (3.815 \times 1.232 \times \text{respiratory quotient (RQ)})$] and spontaneous activity. Activities of the mice were monitored by an infrared photocell beam interruption method. Mice were allowed to adapt for 24hrs to the new environment before indirect calorimetric measurements were performed. Food and water intake were continuously monitored. Data were analyzed by Metabolism 2.0 software (Panlab-Bioseb).

4.8 Immunofluorescence, immunohistochemistry, tunel staining, and flow cytometry

Frozen sections were prepared from murine GWAT. Sections were stained with rat anti-mouse F4/80 and Mac-2 IgG antibodies (Serotec, Oxford, UK and Cedarlane, Burlington, Ontario, Canada, respectively). Primary antibodies were detected with AlexaFluor 488 or AlexaFluor 594 goat anti-rat IgG antibodies

(Molecular Probes, Eugene, OR, USA). Nuclei were visualized by DAPI staining. Slides were mounted in Vectashield® (Vector Laboratories Inc., Burlingame, CA, USA) and examined under a fluorescence microscope (Leica, Wetzlar, Germany). Macrophage infiltration in adipose tissue was quantified by calculating the ratio of F4/80 and Mac-2 positive cells to total nuclei as described previously (62). Apoptotic cells were stained on frozen sections using the Fluorescein In Situ Cell Detection Kit from Roche, according to manufacturer's instruction, in parallel with double-staining for F4/80 and Mac-2, respectively, as described above.

For paraffin sections, GWAT were fixed with neutral buffered 4% paraformaldehyde and were paraffin-embedded. After dewaxation and rehydration immunohistochemical staining for Mac-2 (Serotec) was performed on adipose tissue sections using the ABC kit (Vector Laboratories) according to the manufacturer's recommendations. As a negative control, staining was performed on selected sections with isotype control. Samples were analyzed with standard light microscopy. Stromal vascular cells (SVC) of GWAT were isolated by collagenase digestion and centrifugation to remove adipocytes as described (44). Briefly, murine GWAT was cut into small pieces washed in PBS, and 0.5 g tissue/ml was digested with 0.03 mg/ml Liberase Blendzyme 3 (Roche) and 50 U/ml DNase I (Sigma, St. Louis, MO, USA) in RPMI-1640 (Invitrogen) for 60 minutes at 37°C. Digested tissues were passed through 200 µm mesh filters. After centrifugation at 1000 x g for 10 min at 4°C floating cells were removed. The pellets comprised the SVC fraction. Red blood cells were lysed in haemolysis buffer and remaining cells passed through a 70 µm mesh filter. SVC were subjected to flow-cytometry using directly fluorochrome-labeled antibodies against F4/80 (Serotec), and CD11c (BD Biosciences, San Jose, CA, USA).

4.9 Immunoblotting

Phosphorylation of c-Jun NH₂-terminal kinase (JNK) was determined essentially as described (62). Briefly, GWAT was homogenized and lysed on ice for 30min in tris-buffered saline, pH 7.4, containing 1% Triton X-100 (Pierce) and phosphatase and protease inhibitors. The tissue extract was cleared from fat, nuclei and debris by centrifugation. Identical amounts of protein were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Hybond ECL, Little Chalfont, Amersham, UK). Phosphorylated JNK (Thr-183, Tyr-185) and total JNK were

analyzed using respective mouse polyclonal antibodies (Cell Signaling) followed by a horseradish peroxidase-labelled secondary antibody (Accurate, Westbury, NY, USA). Chemiluminescence was generated by BM chemiluminescence substrate (Roche) and quantified on a Lumi-Imager (Roche).

4.10 Reverse transcription and gene expression

Parts of GWAT were immediately snap-frozen in liquid nitrogen for RNA isolation. Adipose tissue was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA was isolated according to manufacturer's protocol. One microgram of total RNA was treated with DNase I and reverse transcribed into cDNA using Superscript II and random hexamer primers (all Invitrogen). Gene expression normalized to 18S rRNA and Ubiquitin C, respectively, was analyzed by quantitative real-time RT-PCR on an ABI Prism 7000 cyclser using commercial Assays-on-Demand kits (all Applied Biosystems, Foster City, CA). Alternatively, the expression of following murine genes were quantified by use of self-designed primer pairs and iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA): *Tnf* (5'-CCAGACCCTCACACTCAGATCA-3') forward, (5'-TGGTATGAGATAGCAAATCGGCT-3') reverse; *Ccl2* (5'-AGGTCCCTGTCATGCTTCTGG-3') forward, (5'-CTGCTGCTGGTGATCCTCTTG-3') reverse; *Il10* (5'-CTGCTCTTACTGACTGGCATGAG-3') forward, (5'-CGCAGCTCTAGGAGCATGTG-3') reverse; *AdipoQ* (5'-GTCATGCCGAAGATGACGTTACT-3') forward, (5'-TCACCCTTAGGACCAAGAAGAC -3') reverse; *Il6* (5'-CTGCAAGAGACTTCCATCCAGTT-3') forward, (5'-GAAGTAGGGAAGGCCGTGG-3') reverse.

4.11 Statistics

All data are given as means \pm SE. Comparisons were assessed by unpaired 2-tail Student's *t*-test. For comparisons between DoKo mice and respective controls univariate ANOVA was calculated followed by Post Hoc Dunnett's test. A P-value of 0.05 or less was considered statistically significant.

5. RESULTS

5.1 Studies in OPN^{-/-} mice

5.1.1 OPN deficiency in diet-induced obesity

To assess the role of OPN in the development of diet-induced obesity, adipose tissue inflammation and insulin resistance, male OPN^{-/-} and WT mice were fed either LF or HF for 24 weeks. There was no difference in weight gain between WT and OPN^{-/-} on HF reaching 50.8 ± 0.6 g and 50.6 ± 0.9 g, respectively (Fig. 2A). Feed efficiency, as determined by weight gain/food consumption did not differ between both genotypes (Fig 2B). GWAT weight of HF-fed animals was significantly higher in OPN^{-/-} versus WT mice (Fig 2C). Thus, OPN deficiency did not interfere with weight gain by HF feeding. Plasma concentrations of the metabolic parameters including cholesterol, triglycerides and free fatty acids did not differ significantly between the two genotypes, except for cholesterol that was reduced in OPN^{-/-} compared to WT both on LF (Table 1).

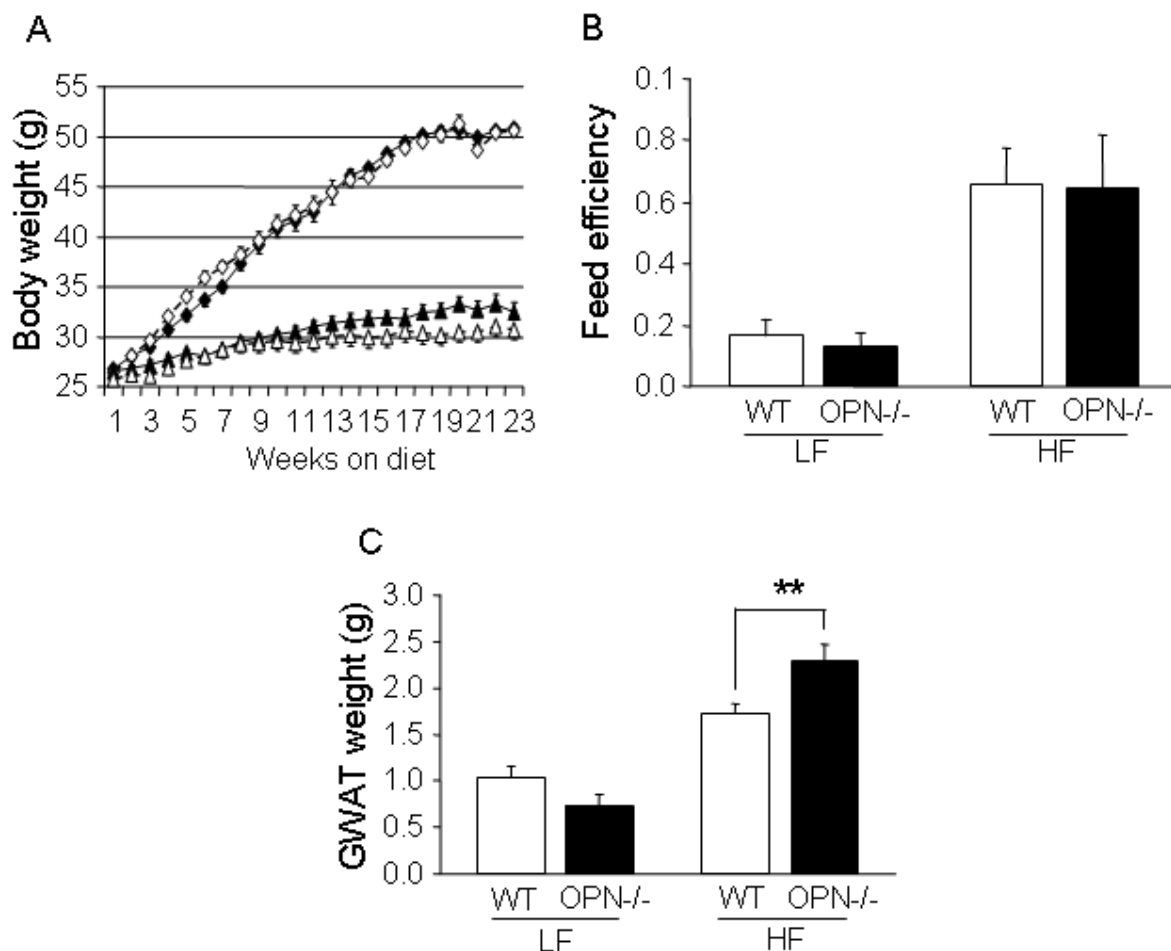


Figure 2: Body weight, feed efficiency and GWAT weight of WT and OPN^{-/-} mice. WT and OPN^{-/-} mice (n = 10 per group) were fed a LF or HF for 24 weeks. **(A)** Weight gain was monitored in WT (black symbols) and OPN^{-/-} (white symbols) mice on LF (triangles) or HF (squares) and did not differ between the two genotypes when on the same diet. **(B)** Feed efficiency (grams weight gain/grams food consumption) of WT and OPN^{-/-} mice. **(C)** GWAT weight of WT and OPN^{-/-} mice. Data are expressed as mean \pm SEM. **P < 0.01.

Plasma measurements

Parameter	WT LF	OPN ^{-/-} LF	WT HF	OPN ^{-/-} HF
Cholesterol (mg/dl)	104.9 \pm 3.8	70.4 \pm 5.3***	142.2 \pm 7.6	133.7 \pm 8.0
Triglycerides (mg/dl)	39.5 \pm 3.9	42.7 \pm 3.2	42.6 \pm 3.0	39.0 \pm 1.6
Free fatty acids (μ mol/l)	694.0 \pm 20.7	626.9 \pm 79.4	647.8 \pm 34.6	688.8 \pm 50.4
IL-6 (pg/ml)	n.d.	n.d.	5.8 \pm 1.1	8.2 \pm 1.3
MCP-1 (pg/ml)	n.d.	n.d.	93.2 \pm 6.4	84.6 \pm 5.8

Table 1: WT and OPN^{-/-} were fed a LF or HF for 24 weeks. Blood samples were obtained after a three hour fasting period and analyzed for indicated plasma parameters (n = 10 per group). Data are expressed as mean \pm SEM. ***P < 0.001. n.d. = not determined.

5.1.2 Improved insulin sensitivity in OPN deficient mice

To determine whether OPN deficiency affects insulin resistance in obesity, parameters of glucose metabolism were analyzed and insulin tolerance test (ITT) was performed in OPN^{-/-} and WT mice with HF-induced obesity. Whereas fasting plasma glucose concentrations were comparable in OPN^{-/-} and WT mice on HF, OPN^{-/-} mice on LF showed reduced plasma glucose compared to WT animals (Fig. 3A). Fasting plasma insulin concentrations were significantly decreased in HF-fed OPN^{-/-} versus WT mice, while plasma insulin was not different between the two genotypes on LF (Fig. 3B). Accordingly, HOMA-IR was significantly lower in OPN^{-/-}

compared to WT mice (Fig. 3C) indicating enhanced insulin sensitivity. This observation was further confirmed by ITT that revealed significant improvement in obese OPN^{-/-} compared to WT mice on HF (Fig. 3D). Taken together, these data show that genetic OPN deficiency ameliorates insulin sensitivity in obese mice.

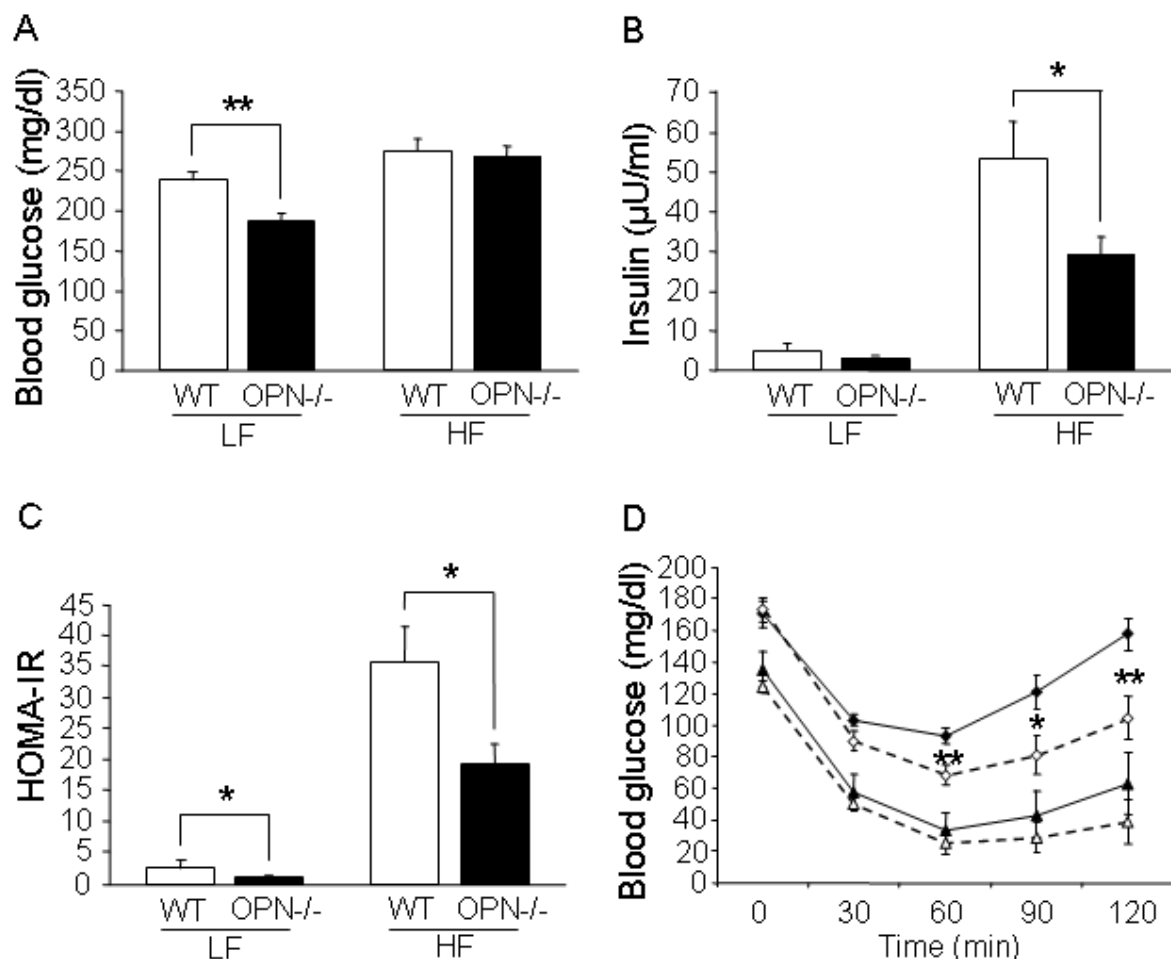


Figure 3: Insulin sensitivity in lean and obese WT and OPN^{-/-} mice. WT and OPN^{-/-} mice (n = 10 per group) were fed LF or HF for 24 weeks prior to metabolic characterisation. **(A)** Fasting plasma glucose concentrations; **(B)** fasting plasma insulin concentrations; **(C)** HOMA-IR. **(D)** An insulin tolerance test was performed in WT (solid lines) and OPN^{-/-} (dashed lines) after feeding a LF (triangles) or HF (squares). Blood glucose was measured following an intraperitoneal injection of insulin (0.75 U/kg body weight for HF and 0.3 U/kg body weight for LF). *P < 0.05, **P < 0.01

5.1.3 Genetic OPN deficiency moderately reduces adipose tissue inflammation but does not affect macrophage infiltration

Since chronic low-grade inflammation in adipose tissue including macrophage accumulation has repeatedly been suggested to cause insulin resistance I was interested whether OPN deficiency affects adipose inflammation. Gene expression of inflammatory proteins in GWAT known to be upregulated in obesity such as TNF- α (*Tnf*), MCP-1 (*Ccl2*) and iNOS (*Nos2*) was dramatically upregulated upon HF irrespective of genotype (Fig. 4). Notably, expression of inflammatory genes tended to be decreased by approximately 40% in OPN^{-/-} compared to WT both on HF, reaching borderline significance.

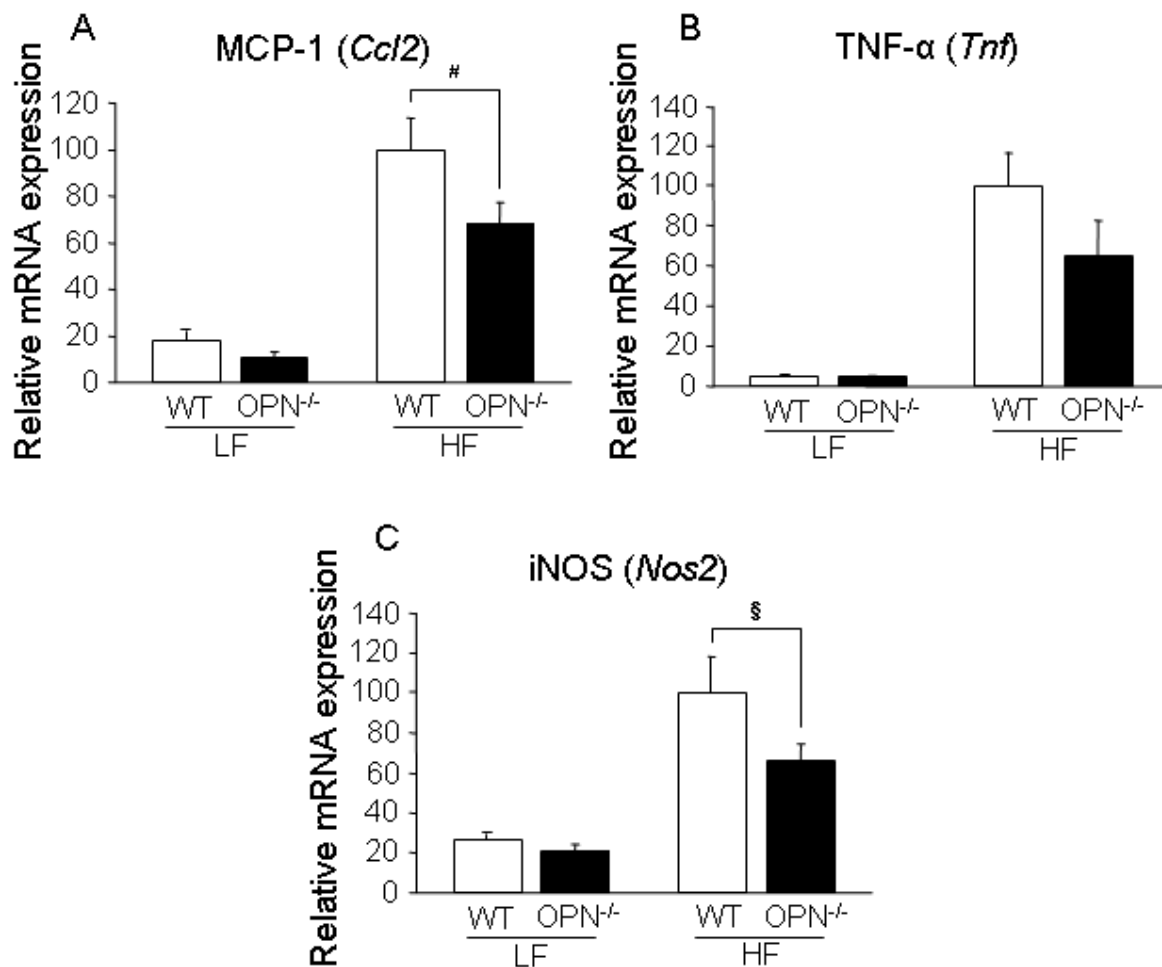


Figure 4: Adipose tissue inflammation in lean and obese WT and OPN^{-/-} mice. WT and OPN^{-/-} mice (n = 10 per group) were fed LF or HF for 24 weeks. mRNA expression of MCP-1 (A), TNF- α (B) and iNOS (C) was analyzed in GWAT. #P = 0.07, \$P = 0.09.

Obesity-induced macrophage infiltration in GWAT was analyzed by gene expression, immunofluorescence and flow cytometry. Gene expression of the macrophage marker F4/80 (*Emr-1*) was considerably higher in HF compared to LF in both genotypes, but differences between WT and OPN^{-/-} mice on HF were not observed (Fig 5A). Accordingly, macrophage accumulation as assessed by F4/80 staining of adipose sections was unaltered between both genotypes on the respective diet (Fig. 5B,C). In addition, FACS analysis of GWAT stromal vascular cells (SVC) revealed similar counts for the suggested high-fat diet-activated type of macrophages (F4/80⁺CD11c⁺) (28) irrespective of OPN deficiency (Fig. 5D). Hence, adipose tissue infiltration by macrophages was not affected by OPN deficiency.

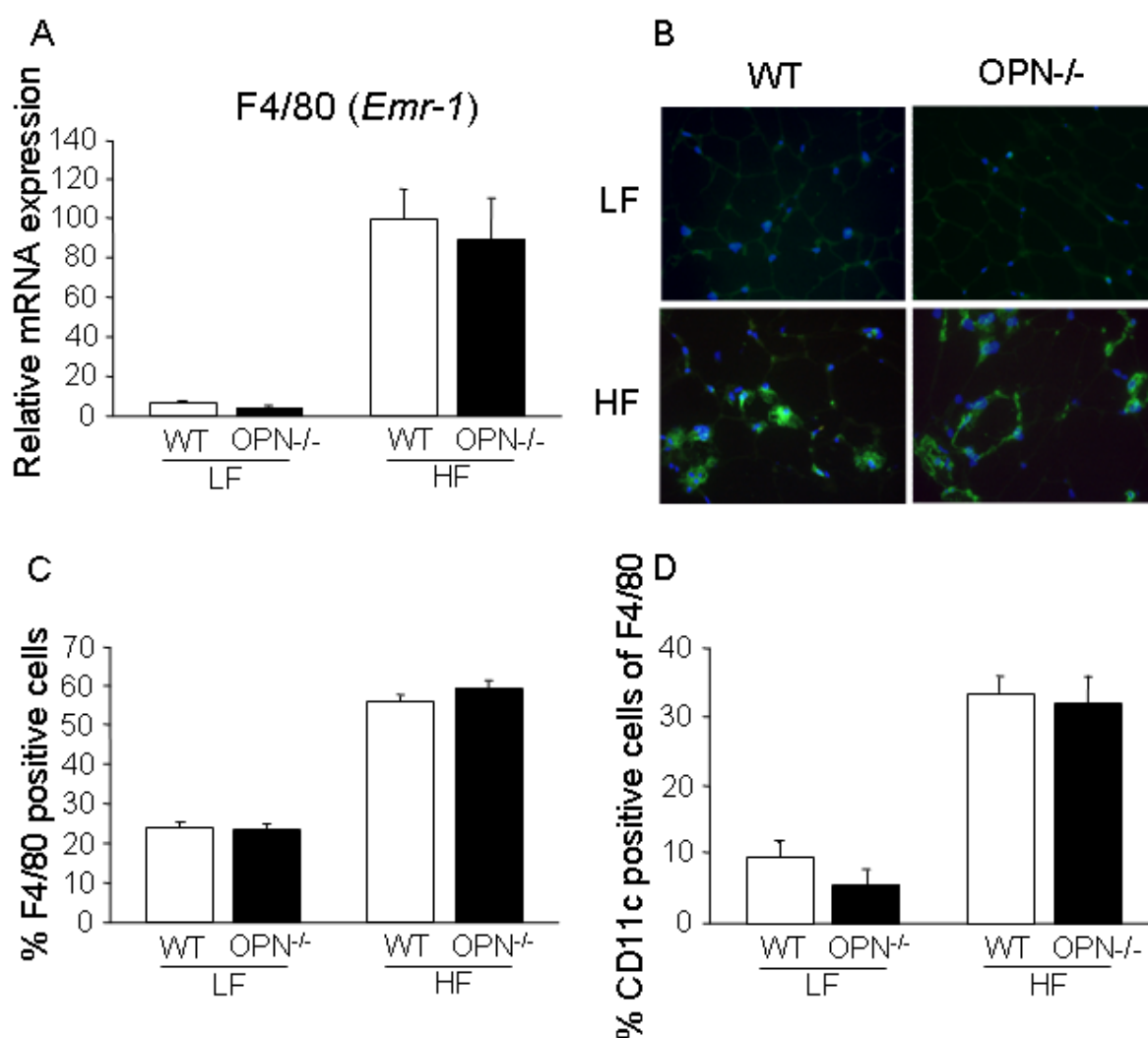


Figure 5: Adipose macrophage accumulation in lean and obese WT and OPN^{-/-} mice. WT and OPN^{-/-} mice (n = 10 per group) were fed LF or HF for 24 weeks. **(A)** mRNA expression of the macrophage marker F4/80 was analyzed in GWAT. **(B-C)**

Adipose tissue macrophage accumulation was determined by immunofluorescence analysis of GWAT isolated from WT and OPN^{-/-} mice. **(B)** Representative pictures are given. **(C)** Macrophages were counted as F4/80+ cells relative to total number of cells. **(D)** Flow cytometric quantitation of SVC isolated from GWAT of WT and OPN^{-/-} mice. SVC of WT and OPN^{-/-} were stained for F4/80 and the macrophage activation marker CD11c and analyzed by flow cytometry.

Next I analyzed the functional status of c-Jun NH2-terminal kinase 1 and 2 (JNK1 and JNK2), which are activated by phosphorylation and critically involved in the deterioration of insulin sensitivity. JNK 1 and 2 phosphorylation in GWAT protein extracts did not differ between OPN^{-/-} and WT mice (Fig. 6).

To characterize systemic inflammatory parameters plasma concentrations of IL-6 and MCP-1 were measured, both of which were unaltered between OPN^{-/-} and WT mice on HF (Table 1).

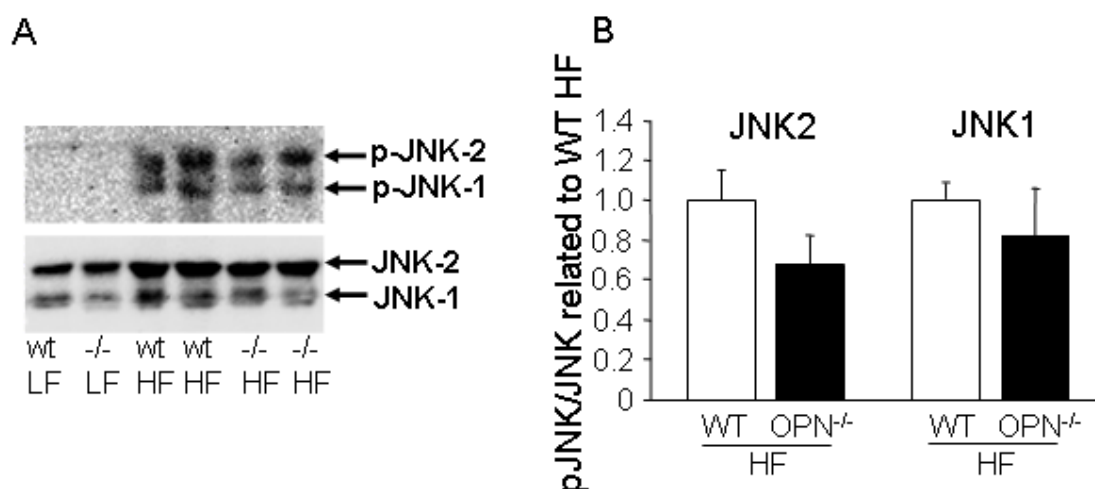


Figure 6: Inflammatory signaling in obese WT and OPN^{-/-} mice. JNK1 and JNK2 phosphorylation in GWAT of WT and OPN^{-/-} mice was analyzed. **(A)** A representative immunoblot is given. **(B)** The diagram shows means of the chemoluminescence intensity ratios from phosphorylated vs. total JNK protein related to WT mice.

5.2. OPN neutralization studies

5.2.1 OPN neutralization in diet-induced obesity

Male C57BL/6J mice were fed a HF or LF for 24 weeks to induce obesity and insulin resistance or to serve as lean controls, respectively. Mice from each group were then intravenously treated with a neutralizing anti-mouse OPN antibody or control IgG for three times during five days. Specificity of the antibody was tested by western blot detecting OPN protein only in plasma of WT but not of OPN^{-/-}-mice (Fig. 7).

Animals in the anti-OPN and control groups were of comparable body weight before and after treatment. GWAT pad weight did not differ between antibody-treated and control mice on the respective diet (Table 2).

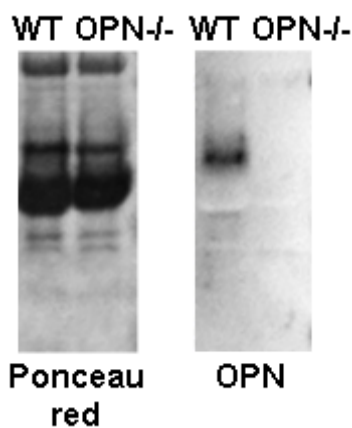


Figure 7: Immunoblot analysis of OPN in plasma. Plasma of WT and OPN^{-/-} was analyzed for OPN protein to test antibody specificity. A representative immunoblot and the respective Ponceau red control are given.

Body characteristics

Parameter	Control LF	Anti-OPN LF	Control HF	Anti-OPN HF
Body weight before (g)	33.0 ± 0.8	32.6 ± 0.5	51.8 ± 1.8	51.5 ± 0.7
Body weight after (g)	32.2 ± 0.4	32.0 ± 0.6	50.1 ± 1.7	50.4 ± 0.7
Fat pad weight (g)	0.27 ± 0.06	0.25 ± 0.06	1.83 ± 0.23	1.62 ± 0.11

Table 2: After feeding LF (n = 5) or HF (n = 10) for 24 weeks mice were treated with OPN neutralizing IgG or control IgG. Body weight was determined before and after

treatment. GWAT pad weight was measured immediately after sacrifice. Data are expressed as mean \pm SEM.

5.2.2 Diet-induced insulin resistance is reversed by antibody-mediated OPN neutralization

I first investigated whether insulin resistance in obese mice is ameliorated by systemic neutralization of OPN action. Strikingly, treatment with OPN neutralizing antibody markedly improved insulin sensitivity in obese mice as shown by significantly reduced blood glucose concentrations at 60, 90 and 120 minutes of an insulin tolerance test (Fig. 8A) and a declined area under the curve (Fig. 8C). In addition, insulin resistance as estimated by HOMA-IR was significantly lower after anti-OPN treatment (Fig. 8D). Insulin sensitivity was unaltered in mice on LF irrespective of anti-OPN treatment (Fig. 8B). Taken together these data strongly indicate enhanced insulin sensitivity in obese mice upon OPN neutralization. Plasma concentrations of glucose, cholesterol, triglycerides, free fatty acids, adiponectin, leptin, TNF- α and IL-6 did not significantly differ between groups (Table 3).

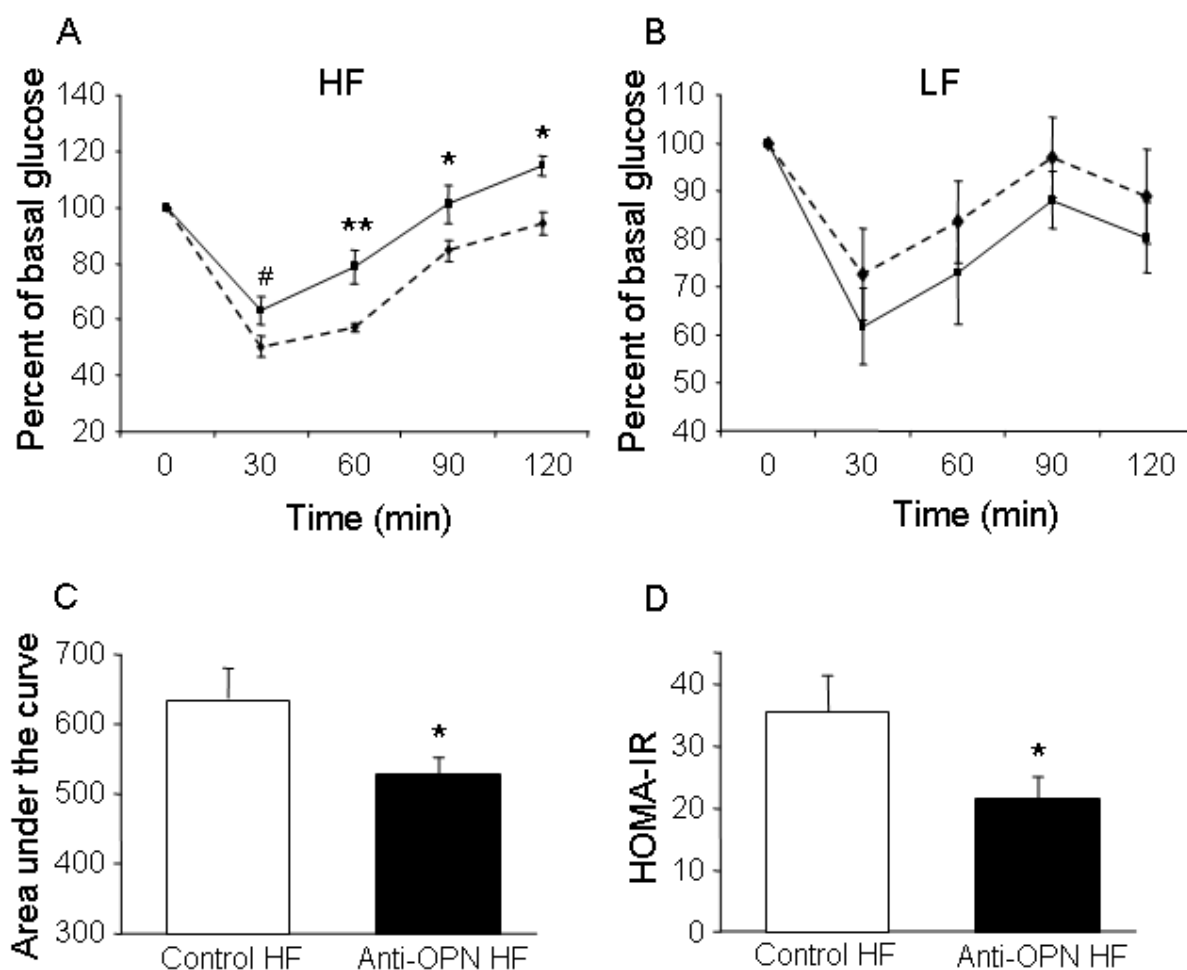


Figure 8: Insulin sensitivity is improved by OPN neutralization. Mice were fed HF to induce obesity or LF, respectively, for 24 weeks and were treated intravenously with an OPN neutralizing (Anti-OPN) or control antibody three times during five days at the end of the feeding period. An ITT was performed in lean and obese OPN antibody (dashed lines) and control antibody-treated mice (solid lines) one day after the last antibody application (n = 5 per group for LF and n = 8 per group for HF). **(A-B)** Percent of basal glucose during ITT in mice on HF (A) and LF (B). **(C)** Area under the curve. **(D)** HOMA-IR was calculated. *P ≤ 0.05, **P ≤ 0.01, #P = 0.06

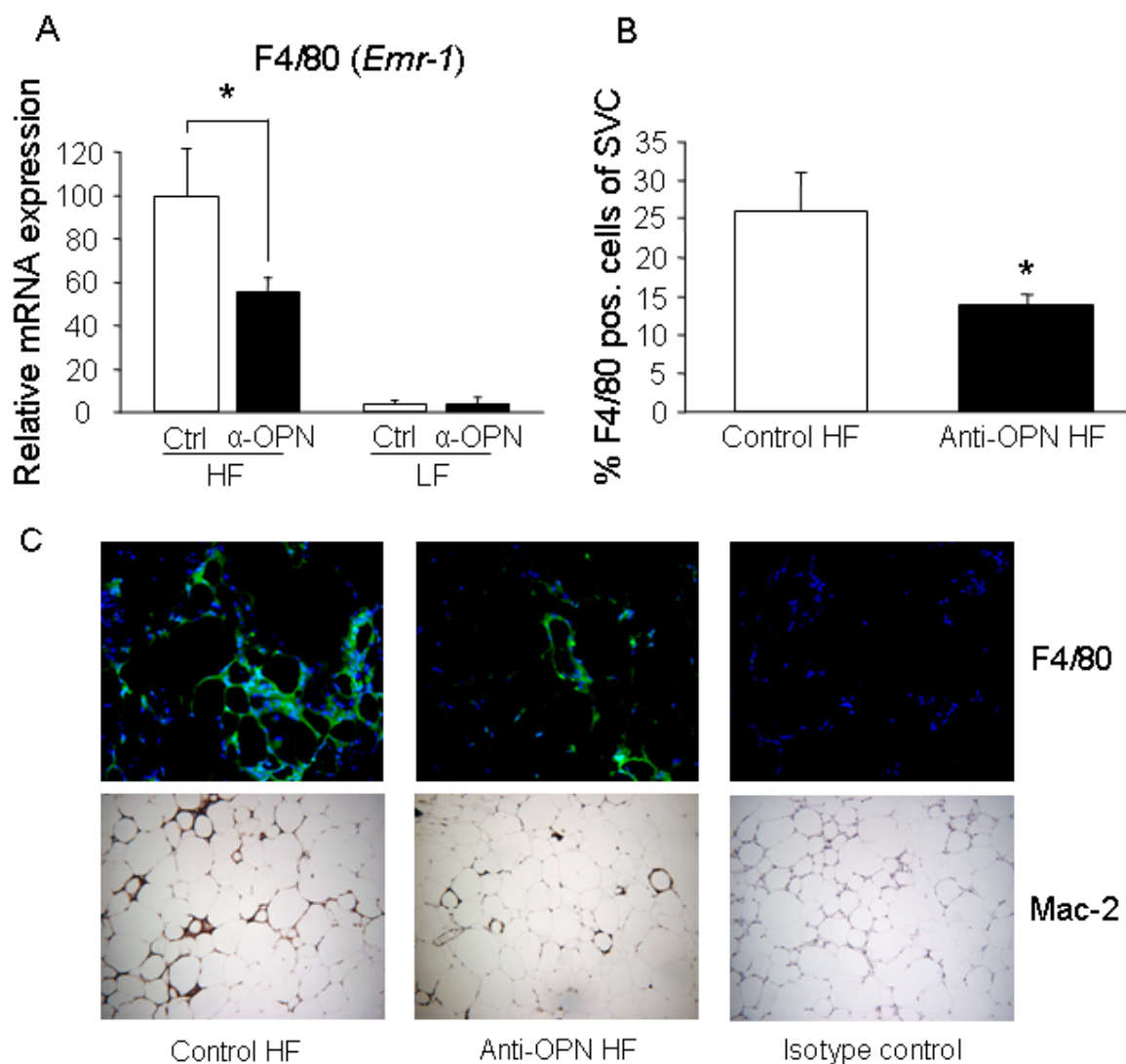
Plasma measurements

Parameter	Control LF	Anti-OPN LF	Control HF	Anti-OPN HF
Glucose (mg/dl)	175 ± 5.3	170.7 ± 11.7	332.9 ± 18.5	304.0 ± 23.7
Insulin (μU/ml)	2.46 ± 1.8	1.7 ± 0.7	48.4 ± 9.6	30.5 ± 5.3
Cholesterol (mg/dl)	1.1 ± 0.8	0.7 ± 0.3	98.1 ± 5.5	96.0 ± 7.8
Triglycerides (mg/dl)	60.2 ± 4.4	50.2 ± 3.0	62.6 ± 11.5	42.4 ± 1.9#
Free fatty acids (μmol/l)	162.6 ± 12.5	131.6 ± 15.4	309.7 ± 39.7	282.3 ± 17.9
Adiponectin (μg/ml)	n.d.	n.d.	38.8 ± 9.1	41.9 ± 10.6
Leptin (ng/ml)	n.d.	n.d.	43.3 ± 4.8	38.0 ± 3.9
IL-6 (pg/ml)	n.d.	n.d.	17.2 ± 6.2	12.2 ± 3.2
TNF-α (pg/ml)	n.d.	n.d.	3.1 ± 0.5	4.1 ± 2.8
Serum amyloid P (ng/ml)	36.5 ± 6.4	29.6 ± 5.8	86.0 ± 24.4	30.9 ± 8.1*
OPN (ng/ml)	180.5 ± 23.1	156.7 ± 13.0	180.5 ± 22.7	171.3 ± 11.9

Table 3: After feeding a LF or HF for 24 weeks mice were treated with OPN neutralizing IgG or control IgG. Blood samples were obtained after a three hour fasting period and analyzed for depicted plasma parameters (n = 5 – 10 per group). Data are expressed as mean ± SEM. *P ≤ 0.05, #P = 0.09 compared to Control HF.

5.2.3 OPN neutralization inhibits macrophage accumulation in obese adipose tissue

In order to investigate potential mechanisms underlying improved insulin sensitivity following OPN neutralization, I went on to examine adipose tissue inflammation and macrophage accumulation. mRNA expression of the macrophage marker F4/80, encoded by the *Emr-1* gene, was strikingly increased in obese GWAT (Fig. 9A). However, F4/80 gene expression was significantly downregulated upon OPN neutralization compared to control antibody treatment in HF mice, while F4/80 was unaffected between LF groups (Fig. 9A). In addition, the percentage of F4/80+ cells in the SVC fraction was markedly reduced as determined by FACS analysis (Fig. 9B). Accordingly, the number of macrophages in GWAT as determined by immunofluorescence (F4/80+) and immunohistochemistry (Mac-2) was significantly lower in obese antibody-treated compared to the HF control mice (Fig. 9C,D) but did not differ between lean mice (not shown).



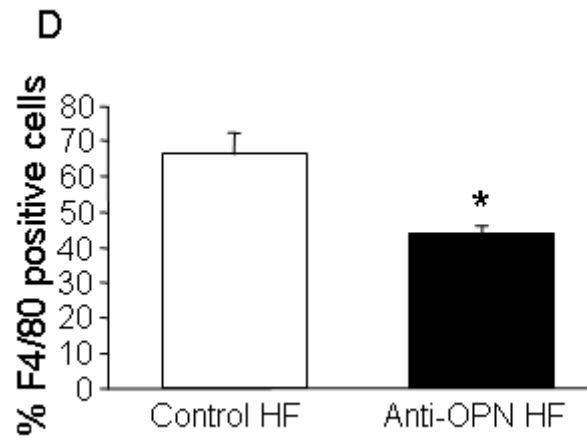


Figure 9: Adipose tissue macrophage accumulation is reduced by OPN neutralization. Adipose tissue macrophage accumulation is reduced by OPN neutralization. Obese HF- and lean LF-fed mice were treated with OPN neutralizing (Anti-OPN) or control antibody (n = 10 per group for HF and n = 5 per group for LF). **(A)** mRNA expression of the macrophage marker F4/80 (encoded by *Emr1* gene) was analyzed in GWAT by real-time RT-PCR. **(B)** Percentage of macrophages (F4/80-positive cells) in the SVC fraction of GWAT as determined by flow cytometry. **(C)** Adipose tissue macrophage accumulation was determined by immunofluorescence of F4/80+ cells (upper row) and immunohistochemical staining of Mac-2+ cells (bottom row) in GWAT isolated from HF-fed mice after anti-OPN or control antibody treatment. Representative pictures are given in 40-fold magnification. **(D)** Adipose tissue macrophages as detected by F4/80 positivity in tissue sections were counted as F4/80+ cells relative to total number of cells. (α -OPN = Anti-OPN, Ctrl = Control).

5.2.4 OPN neutralization promotes adipose macrophage apoptosis

Given the rapid reduction of adipose tissue macrophage numbers after OPN neutralization and a potential anti-apoptotic role of OPN in macrophages (63), I hypothesized that enhanced apoptosis in anti-OPN-treated mice could contribute to the disappearance of adipose tissue macrophages. TUNEL staining of GWAT sections revealed that the proportion of apoptotic F4/80+ cells was significantly increased by 2.16 ± 0.30 -fold in obese OPN antibody-treated compared to control-treated mice (Fig. 10). The abundance of apoptotic non-macrophages was generally low (< 15% of apoptotic cells) and did not differ between the antibody-treated and the control group on HF (data not shown). Hence, OPN neutralization in obese mice

reduces adipose tissue macrophage numbers at least in part by promoting adipose tissue macrophage apoptosis.

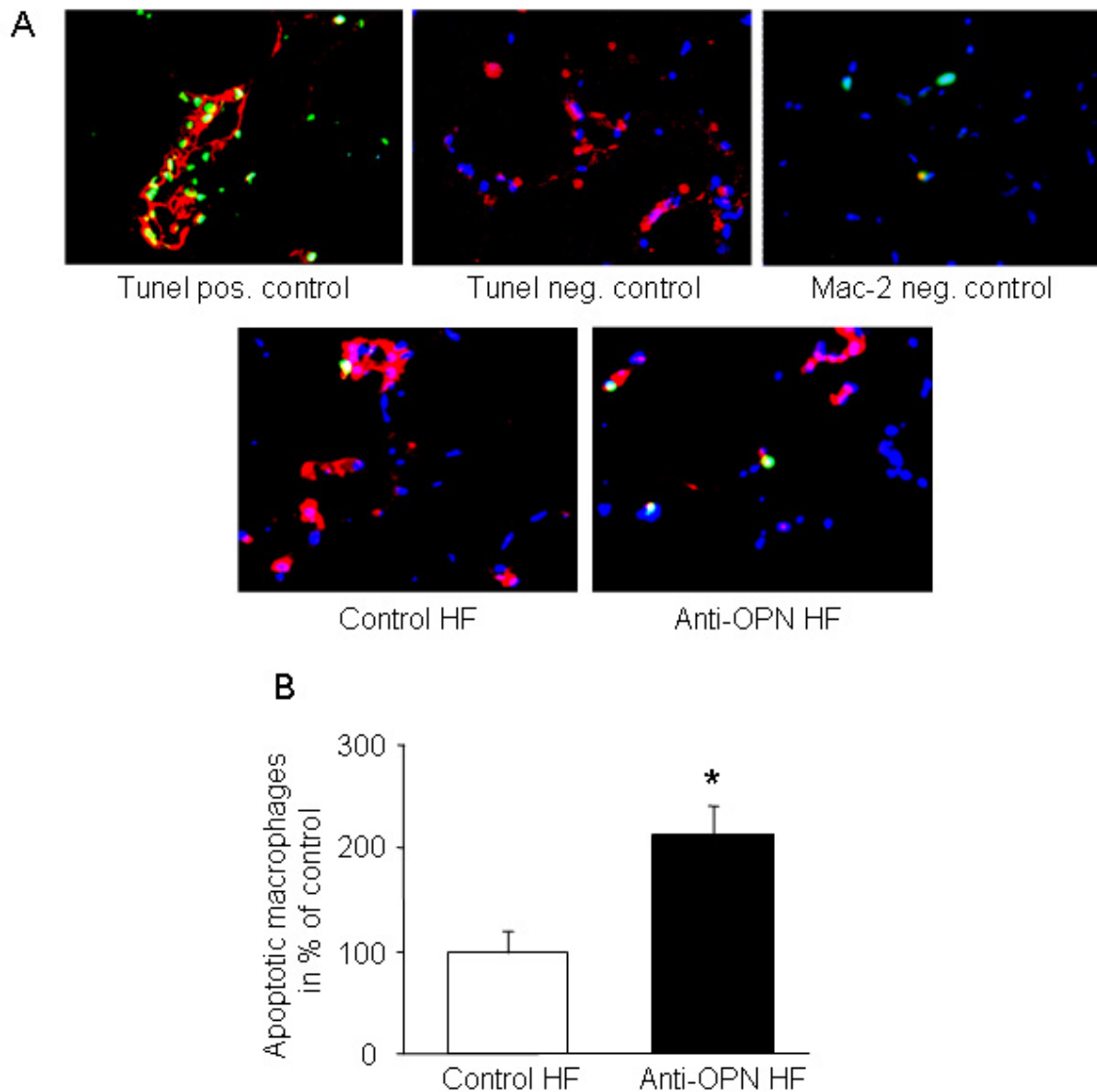


Figure 10: Adipose tissue macrophage apoptosis is enhanced in anti-OPN treated mice (A) Apoptotic cells were determined by tunel staining (green), macrophages were stained red by immunofluorescence using anti-F4/80 monoclonal antibody on frozen sections. Representative pictures are given. **(B)** Quantification of apoptotic macrophages (Tunel and F4/80 double-positive cells per F4/80-positive cells). * $P \leq 0.05$.

5.2.5 OPN neutralization attenuates obesity-induced adipose tissue inflammation

Immunoblot quantification of OPN protein revealed decreased OPN content in obese GWAT after OPN neutralization, even though not statistically significant (Fig. 11). However, OPN plasma concentrations were similar in antibody-treated and control mice and did not differ between lean and obese two days after the last antibody application (Table 3). Systemic concentrations of the inflammation marker serum amyloid P (SAP) were markedly elevated in obese control mice but returned to lean levels upon OPN neutralization (Table 3). In order to investigate potential effects of OPN neutralization on inflammatory signaling related to impaired insulin sensitivity in obese mice, I analyzed activation of JNK by determining phosphorylation of JNK1 and JNK2 in GWAT (64). Notably, anti-OPN treatment abolished JNK phosphorylation in obese (Fig. 12A,B) but not in lean mice (Fig. 12C,D). To further determine effects of OPN neutralization on adipose tissue inflammation, gene expression of the adipokines IL-6, TNF- α , MCP-1 was analyzed in GWAT. Notably, IL-6 gene expression in obese mice was markedly decreased upon anti-OPN treatment while TNF- α , MCP-1 were not significantly reduced (Fig. 12E-G). However, adiponectin mRNA expression was similar in all groups irrespective of diet and antibody treatment (Fig. 12H). Taken together these data strongly suggest that OPN neutralization effectively decreases deleterious inflammatory alterations in adipose tissue of obese mice.

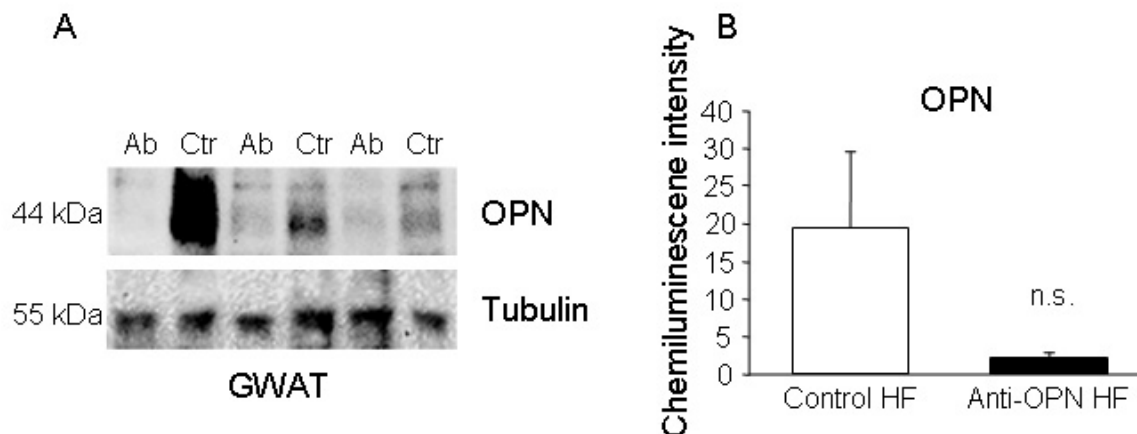


Figure 11: OPN protein expression in adipose tissue after anti-OPN treatment. GWAT of HF-fed anti-OPN (Ab) and control antibody-treated mice (Ctr) was analyzed for OPN protein. **(A)** A representative immunoblot is given together with a loading control (tubulin). **(B)** Quantification of OPN protein in GWAT. The diagram shows means of the chemiluminescence intensity. n.s. = not statistically significant.

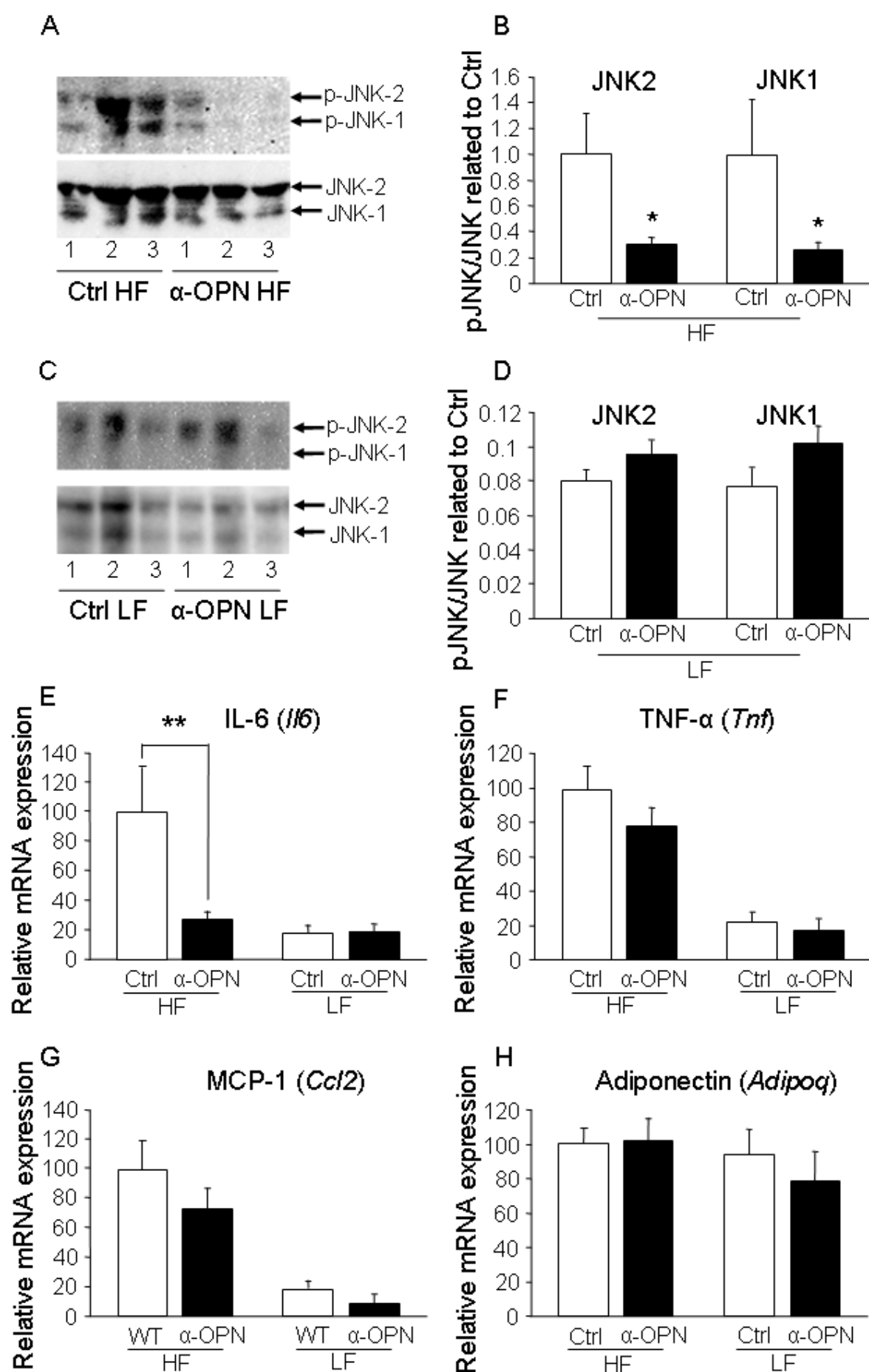


Figure 12: Adipose tissue inflammatory signaling and cytokine expression is attenuated by OPN neutralization in obese mice. Obese HF- and lean LF-fed mice were treated with OPN neutralizing (Anti-OPN) or control antibody (n = 10 per group for HF and n = 5 per group for LF). **(A-D)** Immunoblot analysis and quantification of JNK1 and JNK2 phosphorylation in GWAT. Representative blots are given for obese (A) and lean (C) adipose tissue. The diagrams show means of the chemiluminescence intensity ratios from phosphorylated vs. total JNK protein for obese (B) and lean (D) anti-OPN and control-treated mice. **(E-H)** mRNA expression of the inflammatory genes for IL-6 (*Il6*; E), TNF- α (*Tnf*; F), MCP-1 (*Ccl2*; G) and of adiponectin (*Adipoq*, H) was analyzed in GWAT. *P \leq 0.05, **P \leq 0.01

5.3 Studies in DoKo mice

5.3.1 OPN and MCP-1 double deficiency in diet-induced obesity

To assess the role of combined OPN and MCP-1 deletion in diet-induced obesity I generated OPN^{-/-}MCP-1^{-/-} double knockout (DoKo) mice. OPN^{-/-} and MCP-1^{-/-} single knockouts were intercrossed to receive double heterozygous offspring in the F1 generation. Heterozygous littermates from F1 were crossed to generate DoKo mice in the next generation (F2). Approximately 300 pups were obtained in F2, all of which were genotyped by genomic PCR. One mouse out of fifteen was double deficient for OPN and MCP-1 which closely reflects Mendelian inheritance distribution of 1:16. Hence, I could identify 20 DoKo mice, males and females equally distributed.

DoKo, OPN^{-/-}, MCP-1^{-/-} and WT mice were fed either LF or HF for 20 weeks. There was no difference in body weight between genotypes on LF (DoKo: 30.1 \pm 0.8g, OPN^{-/-}: 31.1 \pm 0.6g, MCP-1^{-/-}: 31.5 \pm 0.9g and WT: 30.9 \pm 1.0g). Unexpectedly, DoKo mice on HF gained significantly more weight than all other genotypes (Fig. 13A). Feed efficiency, as determined by weight gain/food consumption did not differ between obese mice (Fig. 13B). In order to test whether altered metabolic rate accounted for the differences in body weight indirect calorimetric measurements were performed. After 18 weeks of HF-feeding mice of each genotype were monitored in metabolic cages for 72h. Assessment of energy expenditure as well as spontaneous activity did not reveal any alteration in metabolic rates (Fig 13 C,D).

Taken together, combined deletion of OPN and MCP-1 markedly augmented diet-induced obesity but significant alterations in food intake or energy expenditure could not be found.

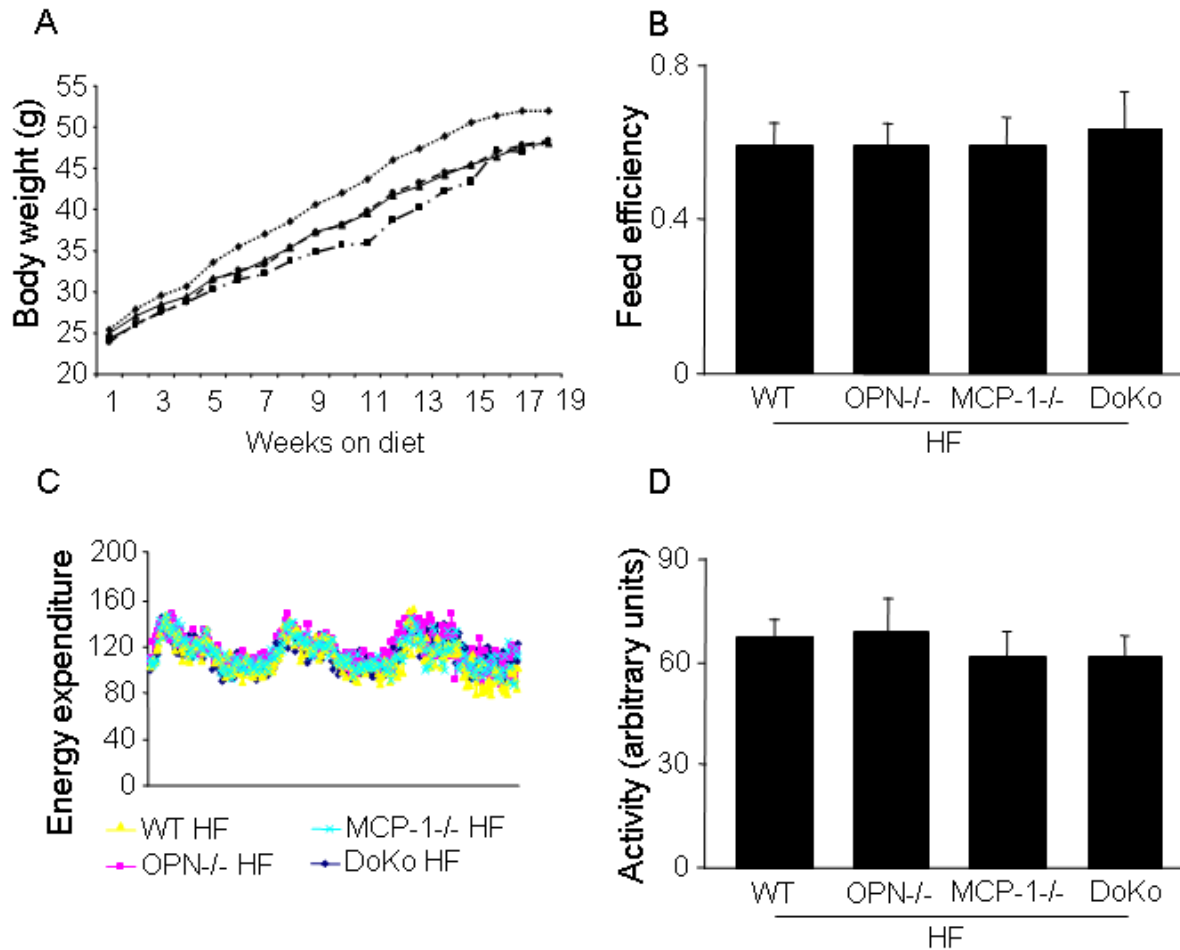


Figure 13: Combined OPN and MCP-1 deletion augments diet-induced obesity.

WT, OPN-/-, MCP-1-/- and DoKo mice (n = 10 per group) were fed a LF or HF for 20 weeks. **(A)** Weight gain was monitored in WT (solid lines), OPN-/- (dashed and dotted lines), MCP-1-/- (dashed lines) and DoKo (dotted lines) mice on HF and was significantly higher in DoKo mice. **(B)** Feed efficiency (grams weight gain/grams food consumption) of WT, OPN-/-, MCP-1-/- and DoKo mice on HF. **(C-D)** Indirect calorimetry in WT, OPN-/-, MCP-1-/- and DoKo mice after 18 weeks of HF-feeding. **(C)** Energy expenditure was calculated from oxygen consumption and respiratory quotient. **(D)** Activity was measured as number of infrared beams broken with time.

5.3.2 Obesity-induced insulin resistance is enhanced in DoKo mice

To determine whether OPN and MCP-1 double deficiency affects insulin resistance in obesity, I analyzed parameters of glucose metabolism and performed ITT in lean and obese WT, OPN^{-/-}, MCP-1^{-/-} and DoKo mice. Whereas insulin tolerance was comparable in all genotypes on LF, glucose clearance following intraperitoneal insulin injection was significantly retarded in obese DoKo mice compared to controls indicating enhanced insulin resistance (Fig. 14). Fasting plasma glucose concentrations were similar in mice on HF while insulin concentrations and HOMA-IR were significantly reduced in OPN^{-/-} mice compared to other genotypes, confirming results from our initial studies in OPN knockout mice. Free fatty acids were unchanged in obese mice of different genotypes whereas plasma triglycerides appeared to be significantly decreased in OPN^{-/-} compared to MCP-1^{-/-} and DoKo all on HF. Systemic levels of adiponectin, leptin and inflammatory IL-6 and MCP-1 did not differ between obese groups (Table 1). Altogether, OPN and MCP-1 double deficiency did not improve but even worsened obesity-induced insulin resistance, concordant with augmented body weight.

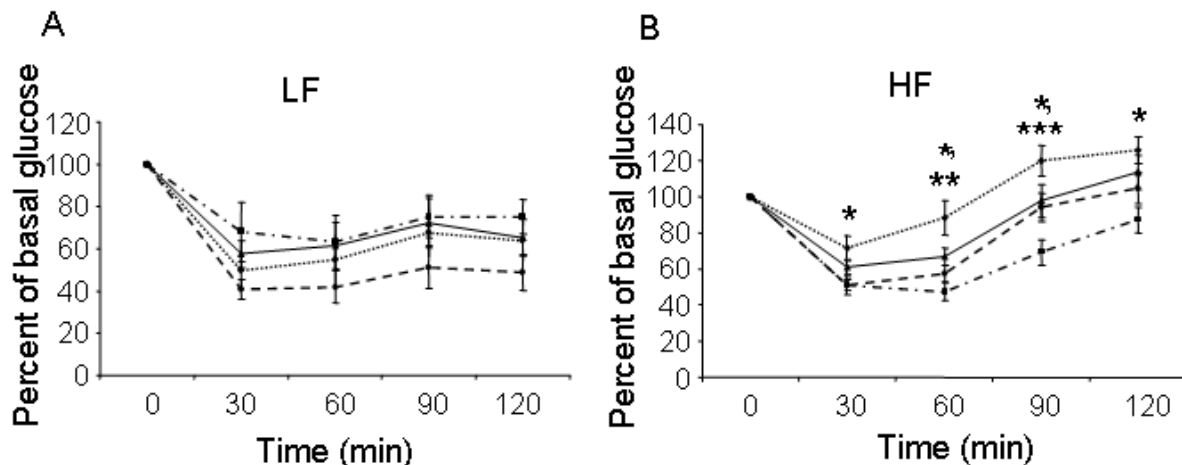


Figure 14: Perturbed insulin tolerance in obese DoKo mice. An insulin tolerance test was performed in WT (solid lines), OPN^{-/-} (dashed and dotted lines), MCP-1^{-/-} (dashed lines) and DoKo (dotted lines) mice after feeding a LF or HF. Blood glucose was measured following an intraperitoneal injection of insulin (0.75 U/kg body weight for HF and 0.3 U/kg body weight for LF). Glucose concentrations of DoKo mice were statistically compared to all other genotypes: *P at 30min < 0.05 vs. OPN^{-/-} and MCP-1^{-/-}; *P at 60min < 0.05 vs. WT and MCP-1^{-/-}, **P at 60min < 0.01 vs. OPN^{-/-};

*P at 90min < 0.05 vs MCP-1^{-/-}, ***P at 90min < 0.001 vs. OPN^{-/-}; *P at 120min < 0.05 vs. OPN^{-/-}.

Plasma measurements

Parameter	WT HF	OPN ^{-/-} HF	MCP-1 ^{-/-} HF	DoKo HF
Glucose (mg/dl)	245.3 ± 17.3	278.9 ± 14.1	260.8 ± 11.5	240.7 ± 18.2
Insulin (μU/ml)	61.9 ± 9.1	18.6 ± 3.3**	52.7 ± 11.6	41.6 ± 5.7
HOMA-IR	39.5 ± 6.9	13.3 ± 2.3**	29.5 ± 10.2	41.6 ± 5.7
Triglycerides (mg/dl)	36.2 ± 3.1	33.3 ± 1.0*	37.8 ± 2.1	39.5 ± 2.4
Free fatty acids (μmol/l)	224.5 ± 13.2	271.3 ± 27.0	223.3 ± 16.9	296.9 ± 36.7
Adiponectin (μg/ml)	56.2 ± 4.0	49.3 ± 4.8	48.3 ± 6.8	54.2 ± 5.0
Leptin (ng/ml)	86.8 ± 8.9	95.4 ± 13.6	79.4 ± 7.9	78.2 ± 6.9
IL-6 (pg/ml)	14.2 ± 4.7	20.6 ± 9.1	19.0 ± 8.6	16.5 ± 5.0
MCP-1 (pg/ml)	82.8 ± 17.3	67.6 ± 8.1		

Table 4: WT, OPN^{-/-}, MCP-1^{-/-} and DoKo mice (n = 10 per group) were fed HF for 20 weeks. Blood samples were obtained after a three hour fasting period and analyzed for indicated plasma parameters (n = 10 per group). Data are expressed as mean ± SEM. **P (Insulin) < 0.01 vs. WT, MCP-1^{-/-} and DoKo; **P (HOMA-IR) < 0.01 vs. WT, MCP-1^{-/-} and DoKo; *P (Triglycerides) < 0.05 vs. MCP-1^{-/-} and DoKo.

5.3.3 OPN and MCP-1 double deficiency does not affect adipose tissue inflammation

Adipose tissue inflammation was assessed by gene expression analyses of inflammatory mediators known to be upregulated upon obesity. I studied mRNA expression of the macrophage markers CD68 and CD11c in adipose tissue of lean and obese WT, OPN^{-/-}, MCP-1^{-/-} and DoKo mice. I did not detect any differences between genotypes on the respective diet. Next, gene expression of inflammatory IL-6 and TNF-α was determined without observing any alterations between different groups of mice (Fig. 15). Hence, combined deletion of OPN and MCP-1 does not affect obesity-induced adipose tissue inflammation.

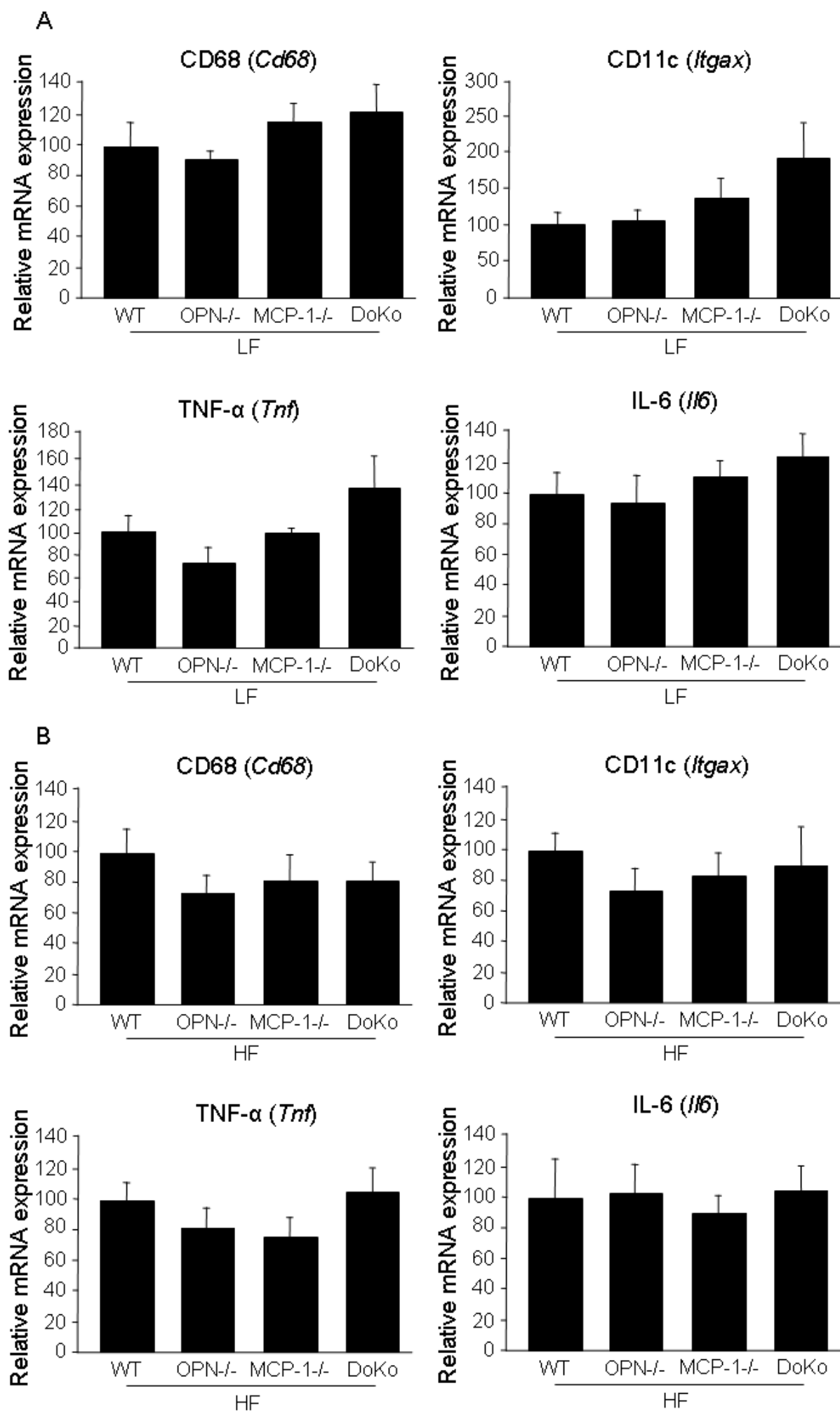


Figure 15: Adipose tissue inflammation in DoKo mice. WT, OPN^{-/-}, MCP-1^{-/-} and DoKo mice (n = 10 per group) were fed a LF or HF for 20 weeks. mRNA expression of the given inflammatory genes was analyzed in GWAT of **(A)** lean and **(B)** obese mice.

6. DISCUSSION

The increasing prevalence of obesity demands novel preventive and therapeutic approaches to treat obesity-associated complications particularly insulin resistance that leads to type 2 diabetes and promotes cardiovascular disease. We and others have recently reported that OPN and MCP-1 expression is considerably upregulated in human obesity as well as mouse models of genetic and diet-induced obesity (12; 44; 45; 56). MCP-1 was initially shown to critically contribute to obesity-induced adipose tissue inflammation and insulin resistance (56). However, during the experimental work of this thesis conflicting results regarding the role of MCP-1 in obesity-associated adipose tissue macrophage accumulation and metabolic deterioration were published. Here I show that not only genetic OPN deficiency but also antibody targeting of OPN markedly improves insulin sensitivity in murine diet-induced obesity. Unexpectedly, combined deletion of OPN and MCP-1 leads to an increase in diet-induced obesity and subsequently reverses beneficial effects of single OPN deletion resulting in impaired insulin sensitivity.

Obesity-associated insulin resistance was significantly reduced in obese mice irrespective of whether OPN was eliminated by genetic knockout or antibody-mediated neutralization (Figs. 2 and 8). The genetic approach shows that obesity-induced metabolic alterations are improved if OPN is already absent during development of fat depots. Short-term neutralization of OPN demonstrates that inflammation and insulin resistance is attenuated when obesity has already been established. In addition, these results indicate that negative OPN effects on glucose metabolism are obesity-dependent because neither genetic OPN deficiency nor OPN neutralization did alter insulin sensitivity and adipose tissue inflammation in lean mice. However, not all of the inflammatory parameters tested concurred in both experimental models. Whereas macrophage accumulation in adipose tissue differed significantly between antibody-treated and control mice, it was comparable in obese OPN^{-/-} mice and the respective WT controls (Figs. 5 and 9). Inflammatory gene expression in GWAT was only moderately decreased in HF-fed OPN^{-/-} compared to WT (Fig. 4), and the activation of the inflammatory proteins JNK 1 and 2 was similar in both genotypes (Fig. 6), other than in anti-OPN treated WT mice where inflammatory gene expression and JNK activation in GWAT were considerably downregulated (Fig. 12). Apparently, macrophage accumulation and inflammatory

processes occurring in adipose tissue upon diet-induced obesity are reversed by short-term neutralization of OPN but not by genetic deficiency. Therefore, it could be speculated that unidentified escape mechanisms emerge in OPN^{-/-} mice during the development of diet-induced obesity that restore macrophage migration and adipose tissue inflammation despite lack of OPN. However, during the preparation of this thesis an article was published, demonstrating that genetic OPN deficiency is able to attenuate adipose tissue macrophage infiltration and insulin resistance in a model of murine diet-induced obesity (46). One noticeable difference between the present study and the study by Nomiya et al. was the genetic background of the mice. While we used OPN^{-/-} mice on a C57BL/6J background, mice used in Ref. 46 were on a Black Swiss background. Whether and how the differences in the genetic background in these particular studies can impact obesity-induced adipose tissue alterations remains unsolved.

Obesity-induced insulin resistance is associated with macrophage accumulation in adipose tissue (13). OPN is involved in macrophage migration and macrophage-driven inflammatory disorders (31; 35; 36). Treatment with anti-OPN antibody for only 5 days significantly decreased adipose tissue macrophage numbers in obese mice (Fig. 9). Similarly, blocking of the chemokine receptor CCR2 for 9 days resulted in a significant reduction of adipose tissue macrophage accumulation (12). Thus, macrophage turnover appears to be rather high in obese murine adipose tissue.

OPN has previously been shown to be a survival factor for macrophages in HIV-induced brain disease (63) and for T-cells in an arthritis model (65). Here I show that the rapid reduction of adipose tissue macrophages by OPN neutralization was accompanied by a significantly increased number of apoptotic macrophages (Fig. 10), indicating that macrophage apoptosis contributes to reduced macrophage abundance in anti-OPN treated mice. Aside from enhanced macrophage apoptosis, disturbed cell migration could contribute to the reduction of macrophages in adipose tissue of anti-OPN treated mice, since OPN is well-known to be critically involved in migration of monocytes and macrophages (31; 46). Hence, the marked obesity-induced upregulation of OPN expression in adipose tissue (44) could promote macrophage accumulation not only by stimulating cell migration (66) but also by preventing macrophage apoptosis.

Since reduced OPN plasma concentrations after anti-OPN treatment were not observed (Table 3) I assume functional neutralization of OPN to underlie the decrease in adipose tissue macrophage numbers (67; 68). Since macrophages are the major source of OPN in adipose tissue (44), the somewhat reduced tissue OPN content following OPN neutralization (Fig. 11) is probably due to diminished local production.

HF causes activation of the JNK pathway that leads to insulin resistance by serine phosphorylation of IRS proteins (6; 64; 69). OPN neutralization inhibited inflammatory signaling by negatively regulating JNK 1 and JNK 2 phosphorylation in obese adipose tissue (Fig. 12). In contrast to decreased GWAT expression of IL-6, a potent mediator between obesity-induced adipose tissue inflammation and insulin resistance (70), TNF- α and MCP-1 expression remained unaffected (Fig. 12). These data are in accordance with a recent publication showing that adipose tissue specific deletion of JNK1 significantly reduced obesity-induced insulin resistance and IL-6 expression in adipose tissue while TNF- α remained unaltered (71). Taken together, these results emphasize abolished JNK activation to be a crucial mechanism for anti-inflammatory effects of OPN neutralization in adipose tissue.

MCP-1 is important for recruiting monocytes that represent macrophage precursors into atherosclerotic tissue and other chronic inflammatory lesions (55; 72). A central role in inducing insulin resistance in adipocytes and skeletal muscle cells has been proposed. MCP-1 interacts with its receptor, CCR2 on the surface of monocytes to initiate signaling, which ultimately leads to cell adhesion and tissue infiltration. In mice and humans, MCP-1 production increases in plasma and adipose tissue in both diet-induced and genetic forms of obesity. These data suggest that monocytes are recruited to and infiltrate expanding adipose tissue, where they differentiate into macrophages (73). Mice deficient for the MCP-1 receptor CCR2 showed reduced macrophage content and improved insulin sensitivity after HF-feeding compared to wild-type animals (12). Studies in MCP-1 knockout mice and mice overexpressing MCP-1 support a role of this chemokine in attracting macrophages to adipose tissue and reduced insulin sensitivity in HF-induced obesity (56), but these data have recently been combated by others (59; 73). Those contradicting studies have emerged during the preparation of this thesis and showed that macrophages accumulate in adipose tissue in response to diet-induced obesity even in the absence of MCP-1. Inouye et al. as well as Kirk et al. detected similar

numbers of macrophages in adipose tissue of MCP-1^{-/-} and control mice fed a HF. They also found that MCP-1^{-/-} mice on that diet were insulin resistant, suggesting that MCP-1 does not account for obesity-induced macrophage recruitment in adipose tissue and related metabolic effects (59; 73). These data are in accordance with results of this thesis that did neither reveal a difference in adipose tissue inflammation and macrophage accumulation nor in metabolic alterations between HF-fed MCP-1^{-/-} and WT mice (Fig. 14 and 15, and Table 1). Therefore, a pathogenic role of MCP-1 in obesity-induced inflammation and metabolic deterioration remains speculative. However, in addition to MCP-1 numerous other chemokines such as MCP-2, -3, and -4 can bind to and subsequently activate CCR2 and, hence, could promote macrophage migration into the adipose tissue. Indeed, gene expression of almost all of the named chemotactic factors is upregulated in the adipose tissue of obese subjects. In addition to CCR2, adipose tissue expression of some other chemokine receptors including CCR1, CCR3 and CCR5 is increased in obesity (74). Their precise role in obesity-associated macrophage recruitment to adipose tissue has not been studied yet.

Since previous reports (12; 56) and own data (Figs. 3 – 10) strongly suggested a pathogenic role for OPN and MCP-1 in obesity-associated adipose tissue macrophage infiltration and insulin resistance I sought to investigate a possible interference between both cytokines in adipose tissue inflammation and metabolic alterations. Unexpectedly, studies in DoKo mice revealed that combined deletion of OPN and MCP-1 augments rather than reduces diet-induced obesity compared to single knockout and WT controls (Fig.13). Neither OPN nor MCP-1 is known to impact food intake or energy expenditure. Nevertheless I tested whether OPN and MCP-1 double deficiency did affect feed efficiency or metabolic rate and could not detect any difference by indirect calorimetry (Fig. 13). Hence, altered feed efficiency and disturbed substrate utilization are unlikely to account for increased body weight in DoKo mice on HF.

Adipose tissue macrophage accumulation as assessed by gene expression of highly specific macrophage markers CD68 and CD11c did not differ between genotypes (Fig. 15), suggesting that OPN and MCP-1 double deficiency does not impact obesity-associated macrophage recruitment to adipose tissue.

Characterization of glucose metabolism showed markedly enhanced insulin resistance in obese DoKo mice compared to controls (Fig. 14), indicating that

combined deletion of OPN and MCP-1 does not improve but even worsen obesity-related metabolic perturbation. The reason why beneficial effects of OPN deficiency (Fig. 3 and Ref. 46) are reversed by additional MCP-1 deficiency is elusive but it is most probable that increased body weight in DoKo mice could account for enhanced insulin resistance. Moreover, it could be speculated that genetic knockout of two inflammatory cytokines that are involved in the regulation of many immune processes initiates unknown antagonistic mechanisms that disturb metabolic balance. Notably, tissues and organs other than adipose could contribute to the antagonistic effect such as the central nervous system.

The proposed thesis revealed a pathogenic role of OPN in obesity-associated insulin resistance by showing that genetic OPN deficiency as well as systemic OPN neutralization improved insulin sensitivity in obesity. OPN neutralization interferes with obesity-associated macrophage accumulation and inflammatory signaling in adipose tissue. Surprisingly, combined deletion of OPN and MCP-1 increased diet-induced obesity and insulin resistance suggesting that concurrent loss of both inflammatory cytokines perturbs metabolic balance. In conclusion, targeting OPN action in vivo could improve metabolic regulation and cardiovascular risk in obese patients.

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8. LIST OF ABBREVIATIONS

BMI	body mass index
Ccl2	chemokine (C–C motif) ligand 2
CCR2	C–C chemokine receptor type 2
DoKo	OPN and MCP-1 double knockout
ER	endoplasmic reticulum
GLUT4	glucose transporter type 4
GWAT	gonadal white adipose tissue
HE	hematoxylin and eosin
HF	high-fat diet
HOMA-IR	homeostasis model assessment of insulin resistance
hsCRP	high sensitivity C-reactive protein
IKK	I-kappa-B kinase
IL-	interleukin-
IFN- γ	interferon-gamma
iNOS	inducible nitric oxide synthase
IRE1	protein kinase/endoribonuclease 1
IRS	insulin receptor substrate
ITT	insulin tolerance test
JNK	c-Jun N-terminale kinase
LF	low-fat diet;
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
NAFLD	non-alcoholic fatty liver disease
NF- κ B	nuclear factor kappa B
NO	nitric oxide
OPN	osteopontin
PI3K	phosphatidylinositol 3-kinase
SAP	serum amyloid P
SEM	standard error of the mean
SOCS	suppressor of cytokine signaling
Spp1	secreted phosphoprotein 1

STAT3	signal transducer and activator of transcription 3
SVC	stromal vascular cells
Th1	T helper type 1
TNF- α	tumor necrosis factor alpha
UPR	unfolded protein response
WT	wild-type
WHO	World Health Organization
XBP-1	X-box binding protein 1

9. PUBLICATIONS BASED ON THE THESIS

Kiefer FW, Zeyda M, Todoric J, Huber J, Geyeregger R, Weichhart T, Aszmann O, Ludvik B, Silberhumer GR, Prager G, Stulnig TM. ***Osteopontin expression in human and murine obesity: extensive local upregulation in adipose tissue but minimal systemic alterations.*** Endocrinology 2008; 149:1350-1357

Kiefer FW, Zeyda M, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Säemann MD, Geyeregger R, Schleder M, Kenner L, Stulnig TM. ***Neutralization of osteopontin inhibits obesity-induced inflammation and insulin resistance.*** Diabetes published online on January 27, 2010. doi:10.2337/db09-0404

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12. AUTHORS CURRICULIM VITAE

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Education

2008-present: Residency in Internal Medicine at the Clinical Division of Endocrinology and Metabolism, Department of Medicine III, General Hospital of Vienna, Austria

2006-2010: PhD studies in the program Cell Communication in Health and Disease at the Medical University of Vienna, Austria (12 participants were elected among over 200 international applicants for this self-contained program)

2000-2006: Studies in Human Medicine at the Medical University of Vienna, Austria and the Medical Faculty of the University of Heidelberg, Germany

2006: Graduation to Doctor of Human Medicine

2005: Studies in English at the Department of English and American Studies, University of Vienna, Austria

1991-1999: Secondary school in Laa/Thaya, Austria with emphasis on foreign languages. Final examination with excellent success

Research experience

08/2008: Training course for euglycemic-hyperinsulinemic clamp tests in mice at the German Mouse Clinic, Helmholtz Center Munich, Germany

2006 - present: PhD studies focused on obesity-induced adipose tissue inflammation and insulin resistance at the Clinical Division of Endocrinology and Metabolism, Department of Internal Medicine III, Medical University of Vienna
Groupleader: Prof. Thomas M. Stulnig

09/2004: Research project participation at the Biomedical Center Lund, Sweden, focussed on atherosclerosis
Groupleader: Prof. Anna Hultgårdh-Nilsson

Experimental skills

Extensive experiences in mouse handling (insulin and glucose tolerance test, cardiac puncture and intravenous injections, organ preparation), indirect calorimetry, genotyping by genomic PCR, RNA isolation, cDNA synthesis, quantitative real-time RT-PCR, immunohistochemistry and fluorescence microscopy, protein analysis (immunoprecipitation, western blot), ELISA, plasmid preparation, statistical analyses, manuscript preparation

Language skills

German (mother tongue)

English (fluent)

French (advanced)

Teaching

2009:	Tutor in PhD – Propedeutics of the PhD program of the Medical University of Vienna
Since 2008:	Mentoring of diploma students
2008:	Tutor in Physical Examination as part of the Medical Curriculum Vienna, Medical University of Vienna
2006-2007:	Tutor in problem-based learning, Core Unit for Medical Education, Medical University of Vienna
2003-2006:	Tutor at the Institute of Physiology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna

Awards and scholarships

2009:	1 st Abstract Prize of the Austrian Society for Endocrinology and Metabolism
2009:	Travel grant of the European Association for the Study of Obesity (EASO)
2009:	CCHD award dedicated to the three best PhD-students of the year 2008
2008:	1 st Prize for best project presentation at the 5 th International Conference of Postgraduate Medical Students, Hradec Králové, Czech Republic

2008:	1 st Abstract Prize of the Austrian Diabetes Association at the 36 th Annual Meeting of the Austrian Diabetes Association, Baden, Austria
2008:	Research Award of the ERSTE Bank, Austria
2008:	Travel Award of the Austrian Obesity Association (ASO) for best oral presentation at the 9 th Annual Meeting of the ASO, Seggau, Austria
2008:	1 st Poster Prize of the 4 th PhD-Symposium of the Medical University of Vienna
2008:	Travel Award of the Austrian Society of Allergology and Immunology
2008:	Travel Award “International Communication” by the Austrian Research Association
2006:	Siegfried Ludwig-Fond scholarship for postgraduate studies
2005:	Sokrates/ERASMUS mobility scholarship
2005:	NÖ-Top-Stipend for studies abroad of the Lower Austrian State Academy
2004 and 2005:	Student scholarship for outstanding success of the Medical University of Vienna
2003 and 2004:	Siegfried Ludwig-Fond scholarship for undergraduate studies

Memberships

Since 2008:	Alumni Club Medical University of Vienna, Austria
Since 2008:	Austrian Obesity Association (ASO)
Since 2008:	Austrian Society of Allergology and Immunology
2007-2008:	President of the Young Scientist Association (YSA) of the Medical University of Vienna
2006-2007:	Vice president of the Young Scientist Association (YSA) of the Medical University of Vienna
Since 2006:	Alumni Club University Heidelberg, Germany

Congress organization

02/2009	<i>2nd International Workshop on Cell Communication in Health and Disease</i> at the Medical University of Vienna
10/2008	<i>Methods Workshop</i> by the Young Scientist Association of the Medical University of Vienna
05/2008	<i>4th PhD-Symposium</i> of the Medical University of Vienna with Renée Schröder, Aurelio Telemann and Jonathan Kagan
02/2008	<i>1st International Workshop on Cell Communication in Health and Disease</i> at the Medical University of Vienna
12/2007	<i>Publication Symposium</i> at the Medical University of Vienna with Jennifer Bell (The Journal of Experimental Medicine) Robert Schwartz (The New England Journal of Medicine) and Walter Hörl (The European Journal of Clinical Investigations)
06/2007	<i>3rd PhD-Symposium</i> of the Medical University of Vienna with Carl Djerassi, Josef Penninger and Lukas Pelkmanns
03/2007	<i>HIV-Symposium</i> at the Medical University of Vienna with Robert C. Gallo

Peer Review Publications

Kiefer FW, Zeyda M, Todoric J, Huber J, Geyeregger R, Weichhart T, Aszmann O, Ludvik B, Silberhumer GR, Prager G, Stulnig TM. ***Osteopontin expression in human and murine obesity: extensive local upregulation in adipose tissue but minimal systemic alterations.*** Endocrinology 2008; 149:1350-1357

Huber J, **Kiefer FW**, Zeyda M, Ludvik B, Silberhumer GR, Prager G, Zlabinger GJ, Stulnig TM. ***CC chemokine and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity.*** J Clin Endocrinol and Metab. 2008; 98(8):3215-21.

Geyeregger R, Shehata M, Zeyda M, Kiefer FW, Stuhlmeier KM, Zlabinger GH, Porpaczy E, Jäger U, Stulnig TM. ***Liver X receptors interfere with cytokine-induced proliferation and cell survival in normal and leukemic lymphocytes.*** J Leukoc Biol. 2009; 86(5):1039-48.

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Patsch JM, Kiefer FW, Varga P, Rauner M, Stupphann D, Resch H, Zysset PK, Stulnig TM, Pietschmann P. ***Increased bone resorption and impaired bone architecture in a mouse model of high-fat diet-induced obesity.*** Metabolism 2010, In press

Review Articles

Kiefer FW, Stulnig TM. **Ein Hormon stellt sich vor: Osteopontin.** Journal für klinische Endokrinologie und Stoffwechsel 2009, 2: 26-27

Printed Abstracts (selected)

Kiefer FW, Zeyda M, Neschen S, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM

Osteopontin: Mediator of obesity-associated hepatic steatosis and insulin resistance. The Middle European Journal of Medicine 2009; 121(21-22):S17, Suppl. 4

(Accepted for oral presentation at the 37th Annual Meeting of the Austrian Diabetes Association, November 19 – 21, Salzburg, Austria)

Vila G, Kiefer FW, Reiter MH, Stulnig TM, Luger A

Characterization of serum- and glucocorticoid-inducible kinase 1 in adipose tissue. The Middle European Journal of Medicine 2009; 121(21-22):S28, Suppl. 4

Kiefer FW, Zeyda M, Neschen S, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM

Osteopontin deficiency prevents obesity-associated hepatic steatosis and insulin resistance. Diabetologia 2009, 52:S33, Suppl. 1

(Accepted for oral presentation at the 45th Annual Meeting of the EASD, September 29 – October 2, Vienna, Austria)

Kiefer FW, Zeyda M, Neschen S, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM

Osteopontin is a mediator of obesity-associated hepatic steatosis and insulin resistance. Diabetes 2009, 58:A437, Suppl. 1

(Accepted for poster presentation at the 69th Scientific Sessions of the American Diabetes Association, June 5 – 9, 2009, New Orleans, LA, USA)

Zeyda M, Gollinger K, Kriehuber E., Kiefer FW, Neuhofer A, Stulnig TM

Three Functionally Distinct Populations of Adipose Tissue Macrophages Defined by the Mannose Receptor and CD11c in Obese Mice. Diabetes 2009, 58:A452, Suppl. 1

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Zeyda M, Stulnig TM

Targeting osteopontin improves obesity-induced inflammation and insulin resistance. Obesity Facts 2009, 2:15 Suppl. 2

(Accepted for oral presentation at the 17th European Congress on Obesity, May 6 – 9, 2009, Amsterdam, Netherlands)

Kiefer FW, Gollinger K, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM

Neutralization of osteopontin inhibits obesity-induced adipose tissue inflammation and insulin resistance. The Middle European Journal of Medicine 2008, 120(19-20):S11, Suppl.5

(Accepted for oral presentation at the 36th Annual Meeting of the Austrian Diabetes Association, November 20 – 22, Baden, Austria)

Binder NB, Kiefer FW, Stulnig TM, Smolen JS, Redlich K

Mcp-1(-/-) mice are osteopetrotic. Arthritis and Rheumatism 2008, 58(9):S652, Suppl. S

Kiefer FW, Todoric J, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM

Osteopontin enhances insulin resistance in diet-induced obesity

Journal für Klinische Endokrinologie und Stoffwechsel 2008, 1:8,

(Accepted for oral presentation at 13th Annual Meeting of the Austrian Society for Endocrinology and Metabolism, May 15 – 17, 2008, St. Wolfgang, Austria)

Zeyda M, Kiefer FW, Geyeregger R, Weichhart T, Huber J, Stulnig TM.

Osteopontin links obesity-induced inflammation with insulin resistance. The Middle European Journal of Medicine 2008, 120(19-20):S99, Suppl.1

(Accepted for poster presentation at Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), Sept. 3 – 6, 2008, Vienna, Austria)

Stulnig TM, Kiefer FW, Todoric J, Gollinger K, Weichhart T, Geyeregger R, Zeyda M.

Short-term high-fat feeding induces insulin resistance that is independent of adipose tissue macrophage infiltration and prevented by osteopontin deficiency. Diabetologia 2008, 51:S295, Suppl.1

(Accepted for poster presentation at the 44th Annual Meeting of the European Association of the Study of Diabetes EASD, Sept 8 -11, 2008 Rome, Italy).

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

Osteopontin deficiency and neutralization ameliorate obesity-induced insulin resistance. Diabetes 2008, 57:A487, Suppl.1

(Accepted for poster presentation at 68th Scientific Sessions of the American Diabetes Association, June 5 – 10, 2008, San Francisco, CA, USA)

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

Short-term high-fat diet induced insulin resistance in mice is independent of adipose tissue macrophage infiltration and prevented by osteopontin deficiency. Diabetes 2008, 57:A743, Suppl.1

Kiefer FW, Todoric J, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

Absence of osteopontin reduces insulin resistance in murine diet-induced obesity. Diabetologie und Stoffwechsel 2008, 3:S72, Suppl.

(Accepted for oral presentation at 43th Annual Meeting of the German Diabetes Society, April 30 – May 03, 2008, Munich, Germany)

Patsch JM, Kiefer F, Rauner M, Stupphann D, Resch H, Stulnig T, Pietschmann P.

Diet-induced hyperinsulinaemia negatively affects bone in male C57/BL mice.

Bone 2008, 42:S62-S63, Suppl.1

Binder NB, Kiefer F, Stulnig T, Smolen JS, Redlich K

MCP-1 deficient mice are osteopetrotic. Annals of the Rheumatic Diseases 2008, 67:A39, Suppl.1

Kiefer FW, Todoric J, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

Osteopontin deficiency improves insulin sensitivity in diet-induced obesity.

The Middle European Journal of Medicine 2007, 119:12, Suppl.4

(Accepted for poster presentation at 35th Annual Meeting of the Austrian Diabetes Association, Nov. 29 – Dec. 01, 2007, Innsbruck, Austria)

Huber J, Kiefer F, Zeyda M, Ludvik B, Silberhumer GR, Prager G, Stulnig TM.

Expression of CC chemokines and CC chemokine receptors in adipose tissue is increased during obesity. The Middle European Journal of Medicine 2007, 119:10, Suppl.4

(Accepted for poster presentation at 35th Annual Meeting of the Austrian Diabetes Association, Nov. 29 – Dec. 01, 2007, Innsbruck, Austria)

Huber J, Kiefer F, Zeyda M, Prager G, Stulnig TM.

Obesity is associated with CC chemokine and CC chemokine receptor expression in human visceral and subcutaneous adipose tissue. Diabetes 2007, 56:A468, Suppl.1

(Accepted for poster presentation at 67th Annual Meeting of the American Diabetes Association ADA, Chicago IL, June 22 – 26, 2007).

Huber J, Kiefer F, Zeyda M, Ludvik B, Silberhumer G, Prager G, Stulnig TM.

CCL2 and CCL3 chemokine gene expression and their receptors in human visceral and subcutaneous adipose tissue is associated with obesity and insulin resistance. Experimental and Clinical Endocrinology & Diabetes 2007, 115:S72, Suppl.1

Invited talks

Mouse models of obesity and type 2 diabetes

Session: Seminar Basic Science

37th Annual Meeting of the Austrian Diabetes Association, November 19 – 21, 2009, Salzburg, Austria

Novel cyto- and adipokines

Session: Obesity and Macrovascular Risk

10th Annual Meeting of Austrian Obesity Association, October 30 – 31, 2009, Seggau, Austria

Osteopontin in obesity-induced insulin resistance

Lecture at the Invited Speaker Seminar of the Ludwig Boltzmann Institute for Cancer Research (LBI-CR), Dec. 15th, 2008, Vienna, Austria

Oral presentations

Osteopontin: mediator of obesity-associated hepatic steatosis and insulin resistance

Kiefer FW, Zeyda M, Neschen S, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM

37th Annual Meeting of the Austrian Diabetes Association, November 19 – 21, 2009, Salzburg, Austria

Osteopontin deficiency prevents obesity-associated hepatic steatosis and insulin resistance

Kiefer FW, Zeyda M, Neschen S, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM

45th Annual Meeting of the European Association for the Study of Diabetes, September 30 – October 2, 2009, Vienna, Austria

Systemic osteopontin neutralization inhibits obesity-induced insulin resistance

Kiefer FW, Zeyda M, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM

44th Annual Meeting of the German Diabetes Society, May 20 – 23, 2009, Leipzig, Germany

Obesity-induced insulin resistance is reversible by systemic osteopontin neutralization

Kiefer FW, Gollinger K, Pfau B, Weichhart T, Zeyda M, Stulnig TM

14th Annual Meeting of the Austrian Society for Endocrinology and Metabolism, May 7 – 9, 2009, St. Wolfgang, Austria

Targeting osteopontin improves obesity-induced inflammation and insulin resistance

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Zeyda M, Stulnig TM

17th European Congress on Obesity (ECO), May 6 – 9, 2009, Amsterdam, Netherlands

Osteopontin – a mediator of obesity-induced adipose tissue inflammation and insulin resistance

Kiefer FW, Zeyda M, Gollinger K, Todoric J, Weichhart T, Huber J, Geyeregger R, Stulnig TM.

5th International Conference of Postgraduate Medical Students (Participation as representative of the Medical University of Vienna); Nov. 27 – 29, 2008, Hradec Králové, Czech Republic

Neutralization of osteopontin inhibits obesity-induced adipose tissue inflammation and insulin resistance

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.
36th Annual Meeting of the Austrian Diabetes Association, Nov. 20 – 22, 2008,
Baden, Austria

Osteopontin – a mediator of obesity-induced insulin resistance

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.
9th Annual Meeting of Austrian Obesity Association, Oct. 24 – 26, 2008, Seggau,
Austria

The role of osteopontin in obesity-induced adipose tissue inflammation and insulin resistance

Kiefer FW, Todoric J, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.
43th Annual Meeting of the German Diabetes Society, April 30 – May 03, 2008,
Munich, Germany

Poster presentations

Osteopontin promotes obesity-associated hepatic steatosis and insulin resistance

Kiefer FW, Zeyda M, Neschen S, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM.
5th PhD-Symposium of the Medical University of Vienna, June 17 – 19, 2009,
Vienna, Austria

Osteopontin is a mediator of obesity-associated hepatic steatosis and insulin resistance

Kiefer FW, Zeyda M, Neschen S, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Kenner L, Stulnig TM
69th Scientific Sessions of the American Diabetes Association, June 5 – 9, 2009,
New Orleans, LA, USA

Osteopontin links obesity-induced inflammation with insulin resistance

Zeyda M, Kiefer FW, Geyeregger R, Weichhart T, Huber J, Stulnig TM.

Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), Sept. 3 – 6, 2008, Vienna, Austria

Osteopontin deficiency and neutralization ameliorate obesity-induced insulin resistance

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

68th Scientific Sessions of the American Diabetes Association, June 6 – 10, 2008, San Francisco, CA, USA

Genetic osteopontin deficiency and antibody-mediated neutralization improve obesity-induced Insulin resistance

Kiefer FW, Todoric J, Gollinger K, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

4th PhD-Symposium of the Medical University of Vienna, May 28 – 29, 2008, Vienna, Austria

Osteopontin deficiency improves insulin sensitivity in diet-induced obesity

Kiefer FW, Todoric J, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

35th Annual Meeting of the Austrian Diabetes Association, Nov. 29 – Dec. 01, 2007, Innsbruck, Austria

Osteopontin deficiency improves insulin sensitivity in murine diet-induced obesity

Kiefer FW, Todoric J, Weichhart T, Geyeregger R, Zeyda M, Stulnig TM.

3rd PhD-Symposium of the Medical University of Vienna, June 21 – 22, 2007, Vienna, Austria