MIIKA HEINONEN

Apelin, Orexin A and Ghrelin Levels in Obesity and the Metabolic Syndrome

Doctoral dissertation

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ABSTRACT

Obesity has become a world-wide epidemic and a major burden for health-care system in countries that have adopted a Western lifestyle. The metabolic syndrome (MetS) is a cluster of risk factors predisposing to complications of obesity, including hypertension, hypercholesterolemia and impaired glucose tolerance. Patients with MetS are at high risk for diabetes and cardiovascular diseases. Essential features of MetS are insulin resistance and low grade systemic inflammation. During the past decade, several peptides regulating food intake, insulin sensitivity, blood pressure and inflammation have been discovered from adipose tissue and the gut. The physiological significance of many of these compounds is unclear. This study was established to determine whether circulating levels of three adipose tissue and gut derived peptides are altered in obesity and MetS.

Apelin is a peptide detected in cardiovascular system, adipose tissue, gut, pancreas and hypothalamus. Administration of apelin in pharmacological doses affects food intake and potently stimulates heart rate and contraction in animals. In humans, peripheral administration of apelin causes a nitric oxide mediated arterial vasodilatation. Its expression in adipose tissue is up-regulated by inflammation and insulin. In the current study, plasma apelin level was increased in morbid obesity, yet the correlation to body adiposity during diet-induced weight loss was weaker than for the abundant adipokines leptin and adiponectin. Minor changes in apelin levels in response to a pronounced diet-induced weight loss in patients with MetS were related to arterial pressure and inflammation.

Orexin A (OXA) was discovered as a hypothalamic peptide regulating food intake, wakefulness and sleep. Subsequent studies revealed that orexin A and its receptors are expressed in various tissues outside the central nervous system (CNS) such as the gastrointestinal tract and pancreas, where it modulates gastrointestinal motility and secretion of bicarbonate and insulin. It has been detected also in blood, yet source and the physiological role of circulating OXA is unknown. In the present study, plasma OXA level was increased in morbid obesity and decreased in obese children with Prader-Willi syndrome.

Ghrelin is an orexigenic peptide secreted by stomach in response to low energy status. Plasma ghrelin level raises prior to and decreases after a meal, suggesting strong involvement in the regulation of food intake. The physiological significance of ghrelin in energy metabolism is controversial, since ghrelin-deficient and ghrelin receptor-deficient mice have normal growth rate and appetite. Ghrelin secretion may be regulated by postprandial signals and insulin, yet the current data is contradictory. In the present study, postprandial suppression of plasma ghrelin was impaired in patients with MetS independently of insulin. In addition, ghrelin increased in response to weight loss, but the increase was not sustained during prolonged weight reduction.

In conclusion, the present study demonstrated that circulating apelin, orexin A and ghrelin levels are altered in obesity. These results help to define the roles of these peptides in obesity and MetS.

National Library of Medicine Classification: QU 68, WD 210, WK 185, WK 820
Medical Subject Headings: APLN protein, human [Substance Name]; Blood Glucose; Body Mass Index; Energy Metabolism; Ghrelin; Insulin; Inter cellular Signaling Peptides and Proteins; Leptin; Metabolic Syndrome X; Neuropeptides; Obesity; Obesity, Morbid; orexins [Substance Name]; Peptide Hormones; Prader-Willi Syndrome; Weight Loss
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Miika Heinonen
ABBREVIATIONS

5-HT  Serotonin, 5-hydroxytryptamine
5-HT<sub>2C</sub>  Serotonin receptor 2C
ACN  Acetonitrile
ANOVA  Analysis of variance
APJ  Apelin receptor
APOE  Apolipoprotein E
ARC  Arcuate nucleus
BMI  Body-mass index
DPP4  Dipeptidyl peptidase-4
cAMP  Cyclic adenosine monophosphate
CCK  Cholecystokinin
CCK1R  Cholecystokinin receptor-1
CNS  Central nervous system
CREB  cAMP response element binding
CRP  C-reactive protein
CSF  Cerebrospinal fluid
CT  Computed tomography
CV%  Coefficient of variation
EC<sub>50</sub>  Half-maximal effective concentration
ELISA  Enzyme-linked immunoassay
ESI-MS/MS  Electrospray ionization tandem mass spectrometry
ENS  Enteric nervous system
FDA  Food and Drug Administration
GHS-R  Growth hormone secretagogue receptor
GI  Gastrointestinal
GLP-1  Glucagon-like peptide-1
HDL  High-density lipoprotein
HOMA-IR  Homeostasis model of insulin resistance
HU  Hounsfield unit
HWL  High weight loss
i.c.v.  Intracerebroventricular
IL-6  Interleukin-6
i.p.  Intraperitoneal
i.v.  Intravenous
IRS-1  Insulin-receptor substrate-1
KO  Knock-out
LDL  Low-density lipoprotein
LHA  Lateral hypothalamic area
LWL  Low weight loss
MAP  Mean arterial pressure
MetS  The metabolic syndrome
mRNA  Messenger ribonucleic acid
MWL  Medium weight loss
NCEP  National Cholesterol Education Program
NO  Nitric oxide
NPY  Neuropeptide Y
NT-proBNP  N-terminal prohormone brain natriuretic peptide
OX<sub>1</sub>  Orexin receptor-1
OX<sub>2</sub>  Orexin receptor-2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>OXA</td>
<td>Orexin A</td>
</tr>
<tr>
<td>OXB</td>
<td>Orexin B</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen inhibitor activator-1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<tr>
<td>PPO</td>
<td>Prepro-orexin</td>
</tr>
<tr>
<td>PWS</td>
<td>Prader-Willi syndrome</td>
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<tr>
<td>RIA</td>
<td>Radioimunoassay</td>
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<tr>
<td>RP-HPLC</td>
<td>Reverse-phase high-pressure liquid chromatography</td>
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<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SMOMS</td>
<td>Scandinavian multicenter study of obese subjects with the metabolic syndrome</td>
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<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis-factor alpha</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VLCND</td>
<td>Very-low-caloric diet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WHR</td>
<td>Waist-to-hip ratio</td>
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<tr>
<td>WM</td>
<td>Weight maintenance period</td>
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1. INTRODUCTION

Over the past few decades obesity has become a major burden for health world-wide. Its prevalence in developing countries that have adopted a Western lifestyle has tripled in 20 years. Today more than 1.1 billion adults world-wide are overweight (BMI > 25 kg/m$^2$) and 312 million of them are obese (BMI > 30 kg/m$^2$) (Hossain et al., 2007). In Finland, already 57% of adult men and 43% of adult women are overweight and 15% of men and 16% of women are obese (Helakorpi et al., 2008). In the United States, the percentage of overweight adults has increased from period 1988 - 1994 to 2003 - 2004 from 56% to 66% and the incidence of obesity has increased from 23% to 32% (Flegal et al., 2002; Ogden et al., 2006). Alarmingly, the prevalence of overweight among school-age children and teens in the United States has more than tripled (from 5% to 16%) in the last three decades and similar trends have been observed world-wide (Flegal et al., 2006). Obesity is a major cause for development of the metabolic syndrome (MetS), a state characterized by overweight, insulin resistance, hypertension and impaired lipid metabolism and body fat distribution. Individuals with MetS have marked risks for the development of type 2 diabetes and they possess high cardiovascular mortality (Reaven, 1988; Klein et al., 2002; Lakka et al., 2002). Due to these adverse consequences, obesity has been estimated to decrease life expectancy by 7 years at the age of 40 years (Peeters et al., 2003). The prevalence of MetS in Finland has varied 14 - 21% depending on the definition (Laaksonen et al., 2002), while in the United States the prevalence in 49 - 59 years old men has been estimated at 30% (Ford, 2005). In addition, obesity predisposes to the development of cancer, asthma, osteoarthritis, sleep apnea, pregnancy complications and depression leading to overall decrease in quality of life. The estimated costs of obesity-related diseases for the health care system in the European Union exceeded 32 billion euros in 2002 (Fry and Finley, 2005).

Excess energy intake and decreased energy consumption due to a sedentary Western lifestyle are the main contributors to the obesity epidemic (Stein and Colditz, 2004). Energy balance is regulated by a complex network of neurons in central and lateral hypothalamus. In obesity, these regulatory mechanisms fail to inhibit excess food intake and storage of energy. Hypothalamic neurons receive neuronal and neurohumoral feedback from peripheral tissues that are in direct contact with ingested nutrients. Anorexigenic and orexigenic peptides secreted from the gastrointestinal (GI) tract and pancreas regulate short-term food intake, while peptides from adipose tissue regulate long-term energy balance. The exact functions of many of these peptides are not known.
Rather than being a passive energy depot, adipose tissue has been shown to be an active endocrine organ producing various peptides regulating food intake, insulin resistance, blood pressure and inflammation. Currently, the functions of these potential peptides are not fully understood. In some patients, genetic defects are responsible for development of obesity. Prader-Willi syndrome (PWS) is a genetic disorder characterized by failure to thrive, early-onset hyperphagia and obesity, hypotonia, hypogonadism, growth hormone deficiency, respiratory distress and mental retardation (Goldstone, 2004). Without adequate dietary control, PWS leads to morbid obesity, type 2 diabetes and mortality in early adulthood. The endocrinological disturbances responsible for all these complications have not been elucidated. Thus, the treatment of PWS is currently difficult.

This thesis was initiated to assess the possible roles of three GI tract and adipose tissue derived peptides in obesity and MetS. Therefore, circulating levels of apelin, OXA and ghrelin were measured under different metabolic conditions in morbidly obese patients, patients with MetS and obese children with PWS.
2. REVIEW OF THE LITERATURE

2.1. The metabolic syndrome

2.1.1. Definition

Early description of MetS proposed by Reaven (1988) included obesity, insulin resistance, hypertension and dyslipidemia characterized by elevated triglycerides and low HDL concentrations. Since then, various definitions of MetS have existed (Reaven, 1988; Liese et al., 1998). The National Cholesterol Education Program (NCEP) expert panel (1999) and World Health Organization (WHO) (2001) have published their definitions to facilitate research and comparison between studies (Table 1). Using these definitions, patients with MetS have been shown to possess higher risk for the development of atherosclerosis, coronary artery disease and type 2 diabetes than individuals with simple obesity alone (Reaven, 1988; Klein et al., 2002; Lakka et al., 2002).

Table 1. The definitions of the MetS by modified WHO (1999) and NCEP ATP III (2001) criteria.

<table>
<thead>
<tr>
<th>WHO definition</th>
<th>NCEP ATP III definition</th>
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<tbody>
<tr>
<td>At least ONE of the following:</td>
<td>At least THREE of the following:</td>
</tr>
<tr>
<td>• Hyperinsulinemia (upper quartile of the non-diabetic population)</td>
<td>• Fasting plasma glucose ≥ 6.1 mmol/l</td>
</tr>
<tr>
<td>• Fasting plasma glucose ≥ 7.0 mmol/l</td>
<td>• Abdominal obesity*: waist circumference &gt; 102 cm in men and &gt; 88 cm in women</td>
</tr>
<tr>
<td>• A 2-hr glucose ≥ 7.8mmol/l</td>
<td>• HDL &lt; 1.0 mmol/l in men and &lt; 1.3 mmol/l in women</td>
</tr>
<tr>
<td>• any medication for diabetes mellitus</td>
<td>• Blood pressure ≥ 130/85 mmHg or on medication</td>
</tr>
<tr>
<td>And at least TWO of the following:</td>
<td></td>
</tr>
<tr>
<td>• Abdominal obesity</td>
<td></td>
</tr>
<tr>
<td>Definition 1: Men WHR ≥ 0.90 or BMI ≥ 30 and women WHR ≥ 0.85 or BMI ≥ 30</td>
<td></td>
</tr>
<tr>
<td>• Dyslipidemia</td>
<td></td>
</tr>
<tr>
<td>Serum triglycerides ≥ 1.70 mmol/l or HDL &lt; 0.9mmol/l in men and &lt; 1.1mmol/l in women</td>
<td></td>
</tr>
<tr>
<td>• Hypertension</td>
<td></td>
</tr>
<tr>
<td>Blood pressure ≥ 140/80 mmHg</td>
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</tbody>
</table>

*Some male patients can develop multiple metabolic risk factors when the waist circumference is only 94-102 cm. Such patients may have strong genetic contribution to insulin resistance and they should benefit from changes in life habits, similarly to men with categorical increases in waist circumference.

2.1.2. Pathophysiology of MetS

MetS has also been called “insulin resistance syndrome”, since several features of MetS such as hyperinsulinemia, glucose intolerance, hypertriglyceridemia and low HDL level and type 2 diabetes
may be accounted by resistance to the actions of insulin to carbohydrate and lipid metabolism (DeFronzo and Ferrannini, 1991; Dandona et al., 2005). Even though it has been long known that excess adipose tissue is often accompanied by insulin resistance (Reaven, 1988), the actual mechanisms causing the features of MetS are only partly understood.

The main depots of fat in humans are subcutaneous (SAT) and visceral adipose tissue (VAT). VAT has been considered the main culprit in MetS. VAT has been shown to secrete greater amounts of pro-inflammatory cytokines than SAT (Fain et al., 2004). In addition, VAT delivers released compounds directly to the portal vein contributing to hepatic insulin resistance. Recent studies have indicated that increased accumulation of fat in obesity leads to low-grade systemic inflammation. This association was clearly demonstrated by Hotamisligil et al. (1993), who showed that TNF-α mRNA was induced in adipose tissue in several rodent models of obesity and diabetes. Subsequent studies have confirmed that obesity and MetS are accompanied by increased levels of pro-inflammatory cytokines such as TNF-α, IL-6, CRP and PAI-1 and fibrinogen (Kern et al., 2001; Vozarova et al., 2001; Kressel et al., 2009). Although immune cells, fibroblasts, endothelial cells, and monocytes have traditionally been regarded as the major sources of circulating cytokines (Fried et al., 1998), a considerable proportion of circulating IL-6 is derived from the adipose tissue (Mohamed-Ali et al., 1997). In contrast, TNF-α originates from infiltrated macrophages and it may not be secreted by adipocytes in vitro. Adipose tissue is abundantly infiltrated by macrophages, which may be the source of inflammatory cytokines, but they can also modulate secretory activity of adipocytes (Xu et al., 2003).

A series of studies have revealed another likely mechanism leading to insulin resistance. It has been shown that TNF-α may induce serine phosphorylation of IRS-1, which in turn causes an inhibitory phosphorylation of insulin receptor. Thus, TNF-α could directly inhibit the downstream signal transduction of the insulin receptor (Hotamisligil et al., 1996). Consistently with this mechanistic data, neutralization of TNF-α in obese fa/fa rats causes a significant improvement in the peripheral insulin sensitivity (Hotamisligil et al., 1993). Also IL-6 induced insulin resistance in adipocytes in vitro by inducing inhibitory tyrosine phosphorylation of IRS-1 and by down-regulation of gene expression of several co-factors (Rotter et al., 2003). Thus, increased inflammation may directly interfere in insulin signal transduction, possibly leading to insulin resistance in tissues.

In addition, insulin itself is also an important anti-inflammatory regulator. Insulin has been shown to suppress proinflammatory transcription factors and downstream genes such as PAI-1 (Dandona et al., 2001; Aljada et al., 2002). Consistently, treatment of type 2 diabetes with insulin for 2 weeks causes a reduction in plasma CRP concentration (Takebayashi et al., 2004). Similarly, insulin treatment in acute severe hyperglycemia causes a rapid decrease in circulating inflammatory factors.
cytokines (Stentz et al., 2004). Thus, in insulin resistant state, insulin fails to suppress inflammatory drive leading to increased expression of pro-inflammatory mediators.

2.2. Adipokines and gastrointestinal peptides in obesity

2.2.1. Dysregulation of adipokines in obesity

In obesity, the size and number of adipocytes are increased and this is accompanied by changes in the gene expression profile in large adipocytes (Bluher et al., 2002). Adipose tissue is infiltrated with macrophages and the macrophage quantity has been correlated with measures of insulin resistance (Otto and Lane, 2005). In addition to stimulation of low-grade inflammation, the secretion of adipokines regulating food intake, insulin sensitivity, blood pressure and inflammation is altered in obesity. Leptin is 167-amino acid adipokine secreted largely by adipose tissue (Zhang et al., 1994). Leptin production is augmented in large adipocytes (Considine et al., 1996). The circulating level of leptin parallels adipose tissue mass and is therefore increased in states of obesity and overfeeding. Conversely, leptin levels decrease in starvation in rodents and humans. Impaired leptin signaling in genetically engineered animals induces massive hyperphagia and obesity, indicating that leptin is essential in the regulation of long-term food intake and energy expenditure (Friedman and Halaas, 1998). Most obese individuals become resistant to the satiety and weight-reducing effects of leptin. Thus, use of leptin as anti-obesity drug in humans is currently limited.

Adiponectin is a 30 kDa adipokine secreted exclusively from adipocytes (Scherer et al., 1995). Adiponectin circulates in several different isoforms with distinct biological functions. The insulin sensitizing functions are linked to the high-molecular weight isoform, while some effects have been attributed to hexamer and trimer isoforms (Wang et al., 2008). Adiponectin levels are decreased in obesity and insulin resistant states (Weyer et al., 2001). Low adiponectin levels have been linked to higher prevalence of diabetes, hypertension, atherosclerosis and endothelial dysfunction (Weyer et al., 2001; Kadowaki and Yamauchi, 2005; Chow et al., 2007). Genetically engineered mice lacking adiponectin have reduced insulin sensitivity (Maeda et al., 2002). Overexpression of adiponectin in ob/ob mice results in dramatic metabolic improvements, including reversal of diabetic phenotype, reduction of macrophage infiltration in adipose tissue and systemic inflammation (Kim et al., 2007).

In addition to leptin and adiponectin, variations in levels of adipose tissue-derived peptides including resistin, retinol-binding protein-4, visfatin, angiotensin II, acylation-stimulating protein, TNF-α, IL-6 and PAI-1 have been observed in obesity. The various functions of these potential peptides is beyond the scope of this thesis, and are discussed in detail in excellent reviews (Rajala and Scherer, 2003; Rasouli and Kern, 2008).
2.2.2. Role of gastrointestinal peptides in obesity

The GI tract and pancreas secrete multiple peptides and hormones that regulate food intake, glucose metabolism, GI motility and secretion. These compounds signal to the brain and may be essential in the regulation of food intake (Strader and Woods, 2005). Majority of the vagal fibers are afferent (Prechtl and Powley, 1990) underlining the importance of the direct neuronal gut-brain axis. The enteric nervous system (ENS) consists of myenteric and submucosal plexuses located between the muscle layers of the GI tract. Presence of food in the bowel activates epithelial enteroendocrine cells leading to secretion of various GI peptides such as insulin, glucagon, cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), peptide YY, pancreatic polypeptide, gastric inhibitory peptide and vasoactive intestinal peptide. These peptides may regulate whole body energy homeostasis, gut motility and secretion by binding their receptors on ENS neurons and secretory cells in the intestinal mucosa (Bray, 2000). Some of the GI peptides may also bind to their receptors on vagal afferent fibers that are widely dispersed throughout the gut and enter the brain via the blood stream (Schwartz, 2000).

A classical example of a GI peptide is CCK, which is secreted by the duodenal and jejunal mucosa in response to nutrients in the duodenum. CCK stimulates gallbladder contraction and bile and pancreatic secretion and inhibits gastric secretion. In addition, CCK binds to CCK1R receptors on the local vagus fibers decreasing gastric emptying and increasing satiety (Schwartz and Moran, 1994). CCK1R is also expressed in the hindbrain and hypothalamus indicating that circulating CCK may activate hypothalamic neurons directly. Rats genetically lacking functional CCK1R receptors become markedly hyperphagic and overweight (Funakoshi et al., 1995). However, fasting plasma CCK levels in obese subjects have been reported to be increased, rather than decreased (Baranowska et al., 2000). The satiating effect of acute intravenous (i.v.) infusion of exogenous CCK in obese subjects does not appear to differ from that observed in healthy lean subjects (Lieverse et al., 1995), suggesting that CCK could be a target molecule in treatment of obesity.

GLP-1 is another GI peptide that is post-translationally processed from preproglucagon. GLP-1 belongs to the incretin hormone group and is produced by L cells in the distal small intestine and colon in response to food intake. Post-prandial GLP-1 levels have been decreased in obesity in some (Verdich et al., 2001), but not all studies (Feinle et al., 2002). Although GLP-1 is rapidly degraded by DPP4 in plasma, peripheral GLP-1 infusion has been reported to cause a dose-dependent reduction in food intake in humans (Gutzwiller et al., 1999). In addition, the GLP-1 analog exenatide stimulates glucose-dependent insulin release and inhibits glucagon secretion (DeFronzo et
The diverse functions and release of incretins are beyond the scope of this thesis and they are discussed elsewhere (Karhunen et al., 2008; Vincent et al., 2008).

2.2.3. Apelin

2.2.3.1. Production of apelin

Apelin is a peptide discovered from bovine stomach extracts as an endogenous ligand for the orphan receptor APJ (Tatemoto et al., 1998). Apelin is a product of APLN gene and translated as a 77 amino-acid prepropeptide. The prepropeptide is subsequently cleaved to form several bioactive peptides denoted by their length, including apelin-12, -13, -16, -17, -19 and -36 (Figure 1). Studies using synthetic peptides have revealed that apelin-13 and -36 may be the most abundant and biologically active fragments (Tatemoto et al., 1998; Hosoya et al., 2000; Kawamata et al., 2001). Structural studies showed that APJ has 31% structural similarity with angiotensin receptor I (Murphy et al., 1991; O'Dowd et al., 1998). In addition, apelin-36 is degraded to apelin-13 by angiotensin-converting enzyme-related carboxypeptidase 2 (Vickers et al., 2002).

2.2.3.2. Apelin in regulation of cardiovascular functions

Several studies have linked apelin to the regulation of cardiovascular system. Apelin has been shown to potently stimulate heart rate and contraction in animals (Szokodi et al., 2002; Berry et al., 2004). However, variable results following central and peripheral administrations of apelin on blood pressure have been observed. Peripherally administered apelin-12 and [pGlu]-apelin-13 caused vasodilatation via a nitric oxide (NO) dependent mechanism in anesthetized and conscious rats (Tatemoto et al. 2001; Cheng et al., 2003; Mitra et al., 2006). In contrast, increases in MAP and heart rate were observed following intracerebroventricular (i.c.v.) injection of (Pyr)apelin-13, while the effects of peripheral injections were weak (Kagiyama et al., 2005). I.c.v. injection of pharmacological doses of apelin-13 in conscious rats showed no effect, while i.v. injections slightly decreased MAP and increased heart rate (Reaux et al., 2001). However, apelin KO mice do not have significant changes in blood pressure and heart rate and blockage of APJ does not affect blood pressure and heart rate in rats with portal hypertension (Ishida et al., 2004; Tiani et al., 2008). A recent study by Japp et al. (Japp et al., 2008) shows that apelin-36 and (Pyr)-apelin-13 cause NO-dependent arterial vasodilatation in human brachial arteries with no apparent effects on venous tone, heart rate or systemic blood pressure.
Apelin has been suggested to play a protective role in heart failure, since apelin ameliorates isopretanol-induced cardiac injury in rats. A simultaneous downregulation of endogenous apelin is observed (Jia et al., 2006). Plasma apelin levels are reduced also in patients with chronic heart failure (Chong et al., 2006) and in left ventricular dysfunction after ischemic heart disease (Foldes et al., 2003). In addition, plasma apelin is increased 9 months after cardiac resynchronization therapy together with left ventricular reverse remodeling, decreased NT-proBNP levels and improved ejection fraction supporting a protective role for apelin in cardiac dysfunction (Francia et al., 2007). Apelin KO mice develop cardiac overload and cardiac dysfunction with age suggesting that apelin may help to maintain the cardiac function during persistently elevated blood pressure (Kuba et al., 2007). However, tissue concentrations of apelin are increased and APJ is up-regulated in end-stage heart failure (Chen et al., 2003). In patients with idiopathic dilated cardiomyopathy of variable severity, but with similar ejection fractions and NT-proBNP levels, no differences in plasma apelin is observed (Miettinen et al., 2007). Similarly, apelin levels do not significantly predict the development of acute heart failure (van Kimmenade et al., 2006).

Apelin-APJ signaling has also been linked to the development of atherosclerosis. Hashimoto et al. (2007) found that APJ−/−APOE−/− mice fed with high-cholesterol diet have reduced lesion size compared with APJ+/−APOE−/− mice. Another study showed that apelin blocks angiotensin II induced
formation of atherosclerotic lesion areas and blood pressure in \text{APOE}^{+/-} mice (Chun et al., 2008). These findings indicate that apelin may participate in the regulation of cardiovascular functions.

\subsection*{2.2.3.3. Apelin in food and water intake}

Both apelin and APJ expression have been localized in the hypothalamus in the anterior pituitary and around the supraoptic and paraventricular nuclei suggesting involvement in hormone release and regulation of food and water intake (De Mota et al., 2000; O'Carroll et al., 2000; Reaux et al., 2001). Indeed, i.c.v. administration of apelin-13 decreases food intake in fed and starved rats (Sunter et al., 2003). A similar effect is observed with apelin-12 during nocturnal administration, while acute day-time i.c.v. injections increased food intake (O'Shea et al., 2003). A recent study by Valle et al. (2008) showed that i.c.v. injection of apelin-13 for more than 10 days increases food intake, locomotor activity and body temperature in mice. In contrast, intraperitoneal (i.p.) administration of apelin-13 for 10 days does not affect food intake, yet dose dependently inhibits body weight gain in rats (Higuchi et al. 2007). In addition, apelin-13 increases body temperature and expression of uncoupling protein-1 in brown adipose tissue, suggesting that apelin may also regulate body temperature. Hence, apelin may participate in the regulation of food intake in animals, but further studies are required to determine the exact mechanisms.

In the hypothalamus, apelin has been involved in the regulation of fluid homeostasis by inhibiting the electrical activity of vasopressin-releasing neurons (De Mota et al., 2004). However, studies regarding the effect of apelin on water intake have yielded variable results. Central and systemic injections of apelin increase water intake in water-depleted rats (Lee et al., 2000; Taheri et al., 2002), but an inhibitory effect on drinking has been found in rats deprived for water for 48 hours (Reaux et al., 2001). A recent study found no reliable effect on water intake after central or peripheral administrations of pharmacological doses of \text{[pGlu]}\text{apelin-13} (Mitra et al., 2006).

\subsection*{2.2.3.4. Apelin in peripheral tissues}

Outside the CNS, apelin mRNA has been detected in a wide range of tissues including vascular endothelial cells, stomach, kidney, lung, mammary gland and adipose tissue in rodents and humans (Tatemoto et al., 1998; Medhurst et al., 2003; Kleinz and Davenport, 2004; Wang et al., 2004). Similarly, APJ mRNA has been detected in multiple organs including lung, heart, adipose tissue, small intestine, colonic mucosa, ovaries, thyroid gland and hypothalamus (Edinger et al., 1998; Hosoya et al., 2000; O'Carroll et al., 2000).

Like many other peptides, apelin has been suggested to possess multiple physiological roles. Apelin expression is increased in SAT in response to a high-fat diet in rats (Garcia-Diaz et al., 2007)
and plasma apelin levels are elevated in high-fat fed mice (Boucher et al., 2005). Conversely, apelin expression was reduced in streptozotocin-induced diabetes and after fasting in mice. Since apelin is secreted into the medium in cultured adipocytes, the authors named apelin a novel adipokine (Boucher et al., 2005). In these mice, no difference in apelin expression in stromal-vascular and adipocyte fractions was observed. However, analysis of apelin mRNA levels in rats revealed a higher expression in stromal-vascular fractions than in adipocytes in subcutaneous and retroperitoneal fat pads. In addition, apelin expression is increased by high-fat diet in subcutaneous, but not retroperitoneal fat (Garcia-Diaz et al., 2007).

Apelin may also modulate glucose homeostasis and improve insulin sensitivity in animals. I.p. administration of apelin-13 decreases insulin levels and improves glucose tolerance in lean and obese rats (Higuchi et al. 2007). I.v. administration of apelin enhances glucose uptake in skeletal muscle and lowers glucose levels in mice (Dray et al., 2008). Instead, apelin-36 inhibits glucose-stimulated insulin secretion in mice (Sorhede Winzell et al., 2005).

Interestingly, apelin expression in mouse and human adipose tissue is upregulated by insulin and TNF-α, but not glucose (Boucher et al., 2005; Daviaud et al., 2006). Apelin partially suppresses cytokine production by mouse spleen cells suggesting that apelin may be involved in the regulation of inflammation (Habata et al., 1999). A recent study by Castan-Laurell et al. (2008) showed that adipose tissue apelin and APJ mRNA and plasma apelin peptide levels are decreased after 3 months of diet-induced weight loss in obese patients. A correlation between apelin, insulin and TNF-α were observed in a subgroup of individuals with the highest improvements in insulin sensitivity.

In the gut, apelin-13 and -36 stimulate gastric cell proliferation. Apelin-12, -13 and -19 induce CCK-release from murine enteroendocrine STC-1 cells (Kiehne et al., 2001; Wang et al., 2004). Apelin immunoreactivity has been detected in a vesicle-like structures in oxyntic cells in the rat stomach suggesting that apelin might function as a luminal CCK-releasing factor. Since CCK binds to CCK1R receptors on the local vagus fibers decreasing gastric emptying and increasing satiety, apelin could modulate post-prandial CCK signaling (Schwartz and Moran, 1994).

### 2.24. Orexin A

#### 2.24.1. Orexins

Orexins (or hypocretins) were discovered by two independently working groups as hypothalamic peptides with homology to GI peptide secretin. Orexin A increases food intake in rats (de Lecea et al., 1998; Sakurai et al., 1998). Orexin A (OXA; hypocretin 1) and orexin B (OXB; hypocretin 2) are 33- and 28-amino acid peptides originating from a single precursor produced by the prepro-orexin (PPO) gene. PPO is proteolytically cleaved and the cleavage products are
postranslationally processed (Lee et al., 1999). The actions of OXA and OXB are mediated via binding to closely related OX₁ and OX₂ receptors belonging to the family of G-protein coupled receptors (Sakurai et al., 1998). OXA selectively binds OX₁, while OXB binds both OX₁ and OX₂ with slightly lower affinities. OXA has been more active in the stimulation of food intake in rats, while functions of OXB are generally less well characterized (Sakurai et al., 1998; Haynes et al., 1999).

### 2.2.4.2. OXA in CNS

Initial studies showed that orexins are highly expressed in rat hypothalamic areas known to regulate food intake, the sleep-wake cycle and neuroendocrine functions. The highest PPO mRNA expression and OXA peptide level has been detected in the lateral hypothalamic area (LHA), yet orexin immunoreactivity has also been detected in the ventromedial hypothalamus and the perifornical, arcuate (ARC) and dorsal motor nuclei (de Lecea et al., 1998; Sakurai et al., 1998; Taheri et al., 1999). In rats, both orexin receptors are abundantly expressed in hypothalamic areas, including ARC, the paraventricular nucleus, the locus coeruleus and the dorsal raphe nucleus (Trivedi et al., 1998; Lu et al., 2000; Backberg et al., 2002). The locus coeruleus and raphe nucleus in the caudal brain stem are two centers known to regulate arousal, suggesting an involvement of orexins in the regulation of sleep-wake cycle (Kilduff and Peyron, 2000; Mignot, 2004). Indeed, familial canine narcolepsy in Labrador retrievers and Doberman pinchers is caused by a mutation in the OX₂ receptor implying a major role of this receptor in the sleep regulation (Lin et al., 1999). Genetic ablation of orexin neurons results in narcolepsy, hypophagia and obesity in mice (Hara et al., 2001). Orexigenic fibers from the hypothalamus spread to several brain areas, including the cerebral cortex, hippocampus, amygdala, thalamus, nucleus of solitary tract and locus coeruleus. Orexins may therefore be involved in various metabolic and behavioural processes linked to food intake, energy homeostasis and sleep (Nambu et al., 1999; Peyron et al., 1998).

Administration of OXA into brain ventricles acutely increases food intake in rats, while OXB has been less effective (Sakurai et al., 1998; Haynes et al., 1999). Subsequent studies have shown that OXA activates neurons in various hypothalamic areas linked to food intake such as ARC, paraventricular nucleus, ventromedial hypothalamus and nucleus of solitary tract (Date et al., 1999; Mullett et al., 2000). I.c.v. injection of anti-OXA antibodies blocked fasting stimulated feeding, while i.p. injections had no effect (Yamada et al., 2000). However, i.p. injection of the selective OX₁ receptor antagonist SB-334867 inhibited baseline feeding, weight gain and the feeding response elicited by i.c.v. injection of OXA (Rodgers et al., 2001).
Prolonged and continuous i.c.v. administration of OXA did not increase cumulative food intake, since increased light phase food intake was followed by reduced nocturnal feeding (Haynes et al., 1999; Yamanaka et al., 1999). Therefore, the result may be affected by circadian rhythms and a short-term rather than long-term effect is likely. An increase in food intake has been reported at doses varying 7 - 36 µg (Rodgers et al., 2002) and the lowest effective dose has been reported 0.25 µg (Smart and Jerman, 2002). The stimulatory effect of centrally administered OXA was similar to galanin and melanin-concentrating hormone, but substantially less than the effect seen with the most potent feeding stimulator of the currently known neuropeptides NPY (Edwards et al., 1999).

Orexin level has been shown to increase during low energy conditions and decrease when the energy level is high. Hypothalamic orexin and PPO mRNA increase with fasting and acute insulin-induced hypoglycaemia in rats (Sakurai et al., 1998; Cai et al., 1999; Karteris et al. 2005). Similarly, isolated orexin containing LHA neurons are activated by low glucose in vitro (Muroya et al., 2001). Both OX₁ and OX₂ productions are up-regulated by fasting in the rat hypothalamus (Karteris et al. 2005). In genetically obese ob/ob and db/db mice with high basal glucose levels PPO mRNA, OXA and OXB levels were decreased in LHA compared with controls (Yamamoto et al., 1999). In addition, hypothalamic PPO mRNA levels in obese Zucker fatty rats are decreased and weight gain further decreased PPO expression. However, after chronic food restriction accompanied by significant reductions in weight, glucose, insulin and leptin concentrations, no difference in hypothalamic PPO mRNA expression was observed (Cai et al., 1999).

As discussed above, the orexin neurons are strategically situated in the LHA and are activated by low energy status. In fact, orexin neurons in the LHA have been shown to regulate the essential components of the hypothalamic network regulating energy balance. NPY is the most potent feeding stimulator of the currently known neuropeptides and is produced in the ARC. NPY-containing neurons in the ARC are activated by low glucose levels (Beck et al., 1990), while POMC in the ARC is decreased (Brady et al., 1990; Steiner et al., 1994). NPY neurons in the ARC express OX₁ (Suzuki et al., 2002) and OXA neurons in LHA send orexin-containing axon terminals to NPY and POMC neurons in the ARC (Horvath et al., 1999). An elegant study by Muroya et al. (2004) showed that OXA may stimulate food intake by directly activating NPY neurons and suppressing POMC neurons in the ARC. The blockage of NPY receptors Y1 and Y5 by using specific antibodies suppressed OXA induced feeding, suggesting that NPY neurons are downstream of OXA neurons (Dube et al., 2000; Yamanaka et al., 2000).
2.2.4.3. OXA in the gut-brain axis

OXA has been detected in various peripheral tissues, including stomach, duodenum, ENS, pancreas, adrenal gland, lung, kidney, adipose tissue, spleen, testis and ovaries (Heinonen et al., 2008). Both orexins and orexin receptors have been located in both myenteric and submucosal plexuses in the mouse, rat, guinea pig and human (Table 2). OX1 is expressed in enteric neurons, while OX2 expression has been localized to endocrine cells (Naslund et al., 2002). OXA is colocalized with gastrin and OX1 has been detected in the gastric corpus in the rat (Ehrstrom et al., 2005b). The highest density of orexin immunoreactivity along the GI tract has been detected in the duodenum where nutrients first arrive from the stomach (Kirchgessner and Liu, 1999). In addition, OX1 and OX2 are expressed and immunoreactivity to orexin has been located in pancreatic islets. Also nerve fibers and paravascular nerve bundles associated with blood vessels showed orexin-immunoreactivity (Kirchgessner and Liu, 1999).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Method</th>
<th>PPO</th>
<th>OXA</th>
<th>OXB</th>
<th>OX1</th>
<th>OX2</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENS</td>
<td>IHC</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>human</td>
<td>Ehrstrom et al., 2005a</td>
</tr>
<tr>
<td>stomach</td>
<td>IHC</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>rat</td>
<td>Naslund et al., 2002</td>
</tr>
<tr>
<td>duodenum</td>
<td>IHC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>h, r, gp, m</td>
<td>Kirchgessner and Liu, 1999</td>
</tr>
<tr>
<td>small intestine</td>
<td>IHC</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>human</td>
<td>Nakabayashi et al., 2003</td>
</tr>
<tr>
<td>GI tract</td>
<td>IHC</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>human</td>
<td>Kirchgessner and Liu, 1999</td>
</tr>
<tr>
<td>Stomach</td>
<td>RT-PCR, IHC</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>rat</td>
<td>Kirchgessner and Liu, 1999</td>
</tr>
<tr>
<td>Pancreas</td>
<td>RT-PCR</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>rat</td>
<td>Kirchgessner and Liu, 1999</td>
</tr>
<tr>
<td>beta cells</td>
<td>IHC</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>rat, gp</td>
<td>Kirchgessner and Liu, 1999</td>
</tr>
<tr>
<td>nerve fibers</td>
<td>IHC</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>rat, gp</td>
<td>Kirchgessner and Liu, 1999</td>
</tr>
<tr>
<td>Vagus nerve</td>
<td>IHC</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>human</td>
<td>Burdyga et al., 2003</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>RT-PCR</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>rat</td>
<td>Johren et al., 2001</td>
</tr>
</tbody>
</table>

Like in the hypothalamus, OXA neurons in ENS are activated upon fasting as measured by immunoreactivity and CREB expression (Kirchgessner and Liu, 1999). Consistently, a decrease in plasma OXA levels has been observed after a meal (Ehrstrom et al., 2005b). However, hypoglycemia stimulated release of OXA from pancreatic islets (Ouedraogo et al. 2003) where
Orexin was found to be co-stored with insulin in secretory granules in pancreatic β-cells. Interestingly, orexins has been shown to modulate glucose homeostasis by affecting both insulin and glucagon release. In vivo, subcutaneous administration of OXA stimulates insulin release from β-cells in rats and both OXA and OXB stimulate insulin release in vitro perfused islets (Nowak et al., 2000; Nowak et al., 2005). These findings are in accordance with increased feeding, since increased insulin secretion followed by lower glucose levels stimulates feeding. A recent study by Göncz et al. (2008) showed that OXA inhibits glucagon secretion in perfused rat pancreas in situ and isolated pancreatic islets in vitro. In addition, orexin-immunoreactive neurons were shown to express leptin receptors indicating that OXA neurons in the gut may respond to the whole body energy status (Kirchgessner and Liu., 1999; Liu et al., 1999). Thus, orexins may play an important role in the regulation of glucose homeostasis.

Orexin has been detected in the circulation and its levels respond to changes to the metabolic state. However, the source of plasma OXA has not been elucidated. As discussed above, orexin neurons in the hypothalamus and ENS are activated in response to fasting. Consistently, fasting for 10 days significantly increased plasma OXA levels in normal weighted subjects from 29.9 ± 1.6 pg/ml to 47.9 ± 5.4 pg/ml (Komaki et al., 2001). Adam et al. (2002) found that OXA levels in individuals with BMI ranging 19.8 - 59 kg/m² were significantly lower in overweight and obese individuals with a negative correlation to BMI. However, the overall changes were minor. A negative correlation between BMI and OXA has also been described in obese women (Baranowska et al., 2005). In addition, weight loss in obese children was associated with increased plasma OXA immunoreactivity (33.3 ± 1.97 pg/ml vs. 51.7 ± 3.07 pg/ml; Bronsky et al., 2006). Slightly lower basal plasma OXA levels have been observed in patients with narcolepsy (20.8 ± 4.3 pg/ml) than in healthy control subjects (26.7 ± 3.2 pg/ml) (Higuchi et al., 2002). Deranged plasma OXA levels have also been detected in sleep-apnea disorders. OXA plasma levels were lower in untreated (9.4 ± 1.9 pg/ml) and treated patients with obstructive apnea-hypopnea syndrome (OSAS) (4.2 ± 1.5 pg/ml) than in healthy subjects (20.6 ± 4.5pg/ml) (Busquets et al., 2004). Arihara et al. (2001) measured basal plasma OXA concentrations of 1.94 ± 0.24 pmol/l (6.9 ± 0.9 pg/ml) in 17 healthy individuals.

OXA has been shown to modulate gut motility. I.v. infusion of OXA inhibited the gastric migrating motor complex in anesthetized rat. Inhibition was not affected by bilateral vagotomy suggesting a peripheral mechanism of action (Ehrstrom et al., 2003). Another study found that i.v. infusion of OXA alone had no effect on either acid secretion, plasma gastrin or gastric emptying, while OX₁ antagonist inhibited both gastric acid secretion and increased gastric retention of the liquid nutrient in rats (Ehrstrom et al., 2005b). Plasma OXA levels decreased after intake of the nutrient meal and infusion of the OX₁ antagonist. Only weak effects were seen on plasma glucose...
and insulin by OXA. In guinea pig ileum and rat duodenum, orexin is co-localized with vasoactive intestinal peptide P (VIP) and substance P in the submucous and myenteric plexuses (Kirchgessner and Liu., 1999; Naslund et al., 2002). VIP and substance P are GI peptides known to control gastric motility and secretion (Cooke, 1998; Ljung and Hellstrom, 1999). I.v. infusion of OXA produces a comparable inhibitory effect to VIP on migrating motor complex suggesting that orexin may modulate the relaxation in the peristaltic motility (Naslund et al., 2002). OXA was also colocalized with serotonin in enterochromaffin cells in rat duodenal mucosa (Kirchgessner et al., 1992; Naslund et al., 2002). Enterochromaffin cells appear to be sensory transducers responding to luminal stimuli by secreting 5-HT which in turn may directly modulate the signals of mucosal vagal afferent fibers (Kirchgessner et al., 1992). Central infusion of OXA into the brain ventricles or dorsal motor nucleus of the vagus stimulates pancreatic and gastric secretion and gastric contractility (Takahashi et al., 1999; Krowicki et al., 2002; Miyasaka et al., 2002). I.c.v. administration of OXA induces gastric acid secretion in rats, while peripheral infusion has no effect. The effect was abolished by vagotomy suggesting a central mechanism of action (Takahashi et al., 1999).

OXA has been shown to stimulate secretion of intestinal fluids. I.c.v. injection of OXA dose-dependently stimulated pancreatic fluid and protein output and this effect was abolished by pretreatment with the ganglion blocker hexamethonium and atropine. I.c.v. injection of OXB and i.v. injection of OXA had no effect. These results suggest a vagus nerve-dependent role for OXA in digestion (Miyasaka et al., 2002). However, OX₁ are detected in duodenal mucosa in rats where OXA invoked a dose-dependent stimulation of bicarbonate secretion. The stimulation is blocked by SB-334867 which is a partial agonist of OX₁, but not by atropine suggesting independence from vagal cholinergic pathways (Bengtsson et al., 2007). Dose-dependent stimulation of bicarbonate secretion is abolished by overnight fasting, suggesting that the effects of orexin A are modulated by energy status (Flemstrom et al. 2003). Consistently, an overnight fast suppresses OX₁ and OX₂ expression and OX₁ protein levels in the rat duodenal mucosa (Bengtsson et al., 2007).

Orexin A also stimulates CCK release via an OX₁ and Ca²⁺-dependent mechanism (Larsson et al., 2003). CCK is secreted by mucosal enteroendocrine cells in response to nutrients in the gut lumen. As discussed earlier CCK binds to CCK1R receptors on the local vagus fibers decreasing gastric emptying and increasing satiety (Schwartz and Moran, 1994). OXA modulates the vagal response to CCK via the CC1R receptor indicating that OXA may regulate gut-brain signaling by CCK (Burdyga et al., 2003).
2.2.5. Ghrelin

2.2.5.1. Characteristics of ghrelin

Ghrelin is an acylated 28-amino acid peptide that was isolated from rat stomach extracts as an endogenous ligand for GH secretagogue receptor 1a (GHS-R1a) (Kojima et al., 1999). The third serine residue of ghrelin is post-translationally esterified by octanoid acid, which is essential for its biological activity. Ghrelin is secreted mainly by A/X-cells in oxyntic glands in the stomach submucosa. Approximately 70% of the ghrelin is produced by stomach and the rest is mainly produced by the small intestine (Ariyasu et al., 2001; Jeon et al., 2004). Minor amounts of ghrelin have been detected in the lungs, pancreatic islets, adrenal cortex, kidney and brain (Kojima et al., 1999; Hosoda et al., 2000).

Administration of pharmacological doses of ghrelin potently increases food intake and weight gain in rodents (Tschop et al., 2000; Wren et al., 2001b; Murakami et al., 2002) and humans (Wren et al., 2001a). Conversely, administration of GHS-R1 antibodies inhibits energy intake, weight gain and gastric emptying in lean, obese and leptin-deficient mice (Asakawa et al., 2003). However, ghrelin-deficient and ghrelin receptor-deficient mice have a normal growth rate and appetite (Sun et al., 2003; Wortley et al., 2004). Ghrelin also potently increases GH secretion (Date et al., 2000; Wren et al., 2000). Ghrelin stimulates gastric motility, gastric acid secretion and pancreatic exocrine secretion suggesting that ghrelin prepares gut for effective transport and processing of food (Masuda et al., 2000; Asakawa et al., 2001; Miyasaka et al., 2002). Ghrelin concentrations in the circulation rise prior to and fall shortly after a meal suggesting involvement in the regulation of short-term food intake (Cummings et al., 2002b; Shiiya et al., 2002). Circulating ghrelin concentrations are decreased in obesity and in insulin resistant and diabetic patients (Poykko et al., 2003; Shiiya et al., 2002; Tschop et al., 2001). Ghrelin levels increase in response to weight loss induced by gastric banding (Hanusch-Enserer et al., 2004) and during a low-fat high-carbohydrate diet (Cummings et al., 2002b; Weigle et al., 2003). Consistently, ghrelin secretion is increased in anorexia and cachexia (Nagaya et al. 2001a; Otto et al. 2001). Gastric bypass suppresses ghrelin levels and abolishes postprandial ghrelin response (Cummings et al., 2002b). However, the increase of ghrelin after weight loss may be an acute adaptation to negative balance, since the increase is not sustained 1 year after weight loss (Garcia et al., 2006).

Ghrelin has been shown to cross the blood-brain barrier by nonsaturable transmembrane diffusion and to stimulate food intake by activating orexigenic NPY and Agouti-related peptide-containing neurons in the ARC and orexin-expressing neurons in the LHA (Kamegai et al., 2001; Banks et al., 2002; Toshinai et al., 2003). Moreover, ghrelin exerts its effect on orexin neurons

28
independently of NPY. In addition, NPY and orexin receptor antibodies together inhibited more than 80% of ghrelin-induced feeding (Toshinai et al., 2003). Chemical and surgical vagotomy abolished the effects of peripherally administered ghrelin on food intake and GH secretion in rats, suggesting that ghrelin functions via the vagus (Date et al., 2002). However, another study found that i.p. administered ghrelin stimulates food intake also in rats with subdiaphragmatic vagotomy, suggesting that ghrelin induced feeding may not require vagal afferents signals (Arnold et al. 2006).

In addition to its important roles in gastrointestinal tract and in the regulation of energy homeostasis, ghrelin may also regulate cardiovascular functions. I.v. administration of ghrelin causes a vasodilatation without changing heart rate in humans (Nagaya et al., 2001b). GHS-R1a is expressed in the myocardium and aorta in low levels in rats (Gnanapavan et al., 2002). Subsequent studies using [I\(^{125}\)]-Tyr4-desacyl-ghrelin have proposed the existence of a putative subtype of GHS-R1a in cardiomyocytes that mediates the anti-apoptotic effect of ghrelin in these cells (Baldanzi et al., 2002). Ghrelin may also have anti-inflammatory effects on vascular endothelial cells, since ghrelin inhibits basal and TNF-\(\alpha\) induced cytokine release in human umbilical endothelial cells (Li et al., 2004). Interestingly, ghrelin improves cardiac contractility and left ventricular function in chronic heart failure and reduces infarct size in isolated rat heart (Chang et al., 2004).

2.2.5.2. Regulation of ghrelin release

Signals regulating ghrelin secretion have been suggested to originate from intestinal post-absorptive events independently of gastric distention and vagal feedback (Cummings, 2006). Insulin changes inversely to ghrelin levels and it has been proposed that insulin rather than glucose regulates ghrelin secretion (Saad et al., 2002; Flanagan et al., 2003). Some studies have shown that insulin administration decreases plasma ghrelin concentrations (Saad et al., 2002; Flanagan et al., 2003; Leonetti et al., 2003), while in other studies the effect was not apparent (Spranger et al., 2003; Caixas et al., 2008).

Nutrients in the meal differently regulate postprandial secretion of ghrelin. A carbohydrate-rich meal induces a greater and more rapid suppression of postprandial ghrelin levels than protein and fat (Erdmann et al., 2003; Monteleone et al., 2003), while the suppression after high protein meal is prolonged compared with fat and carbohydrates (Foster-Schubert et al., 2008). High-fat diet has been stimulated gastric ghrelin mRNA expression (Doucet et al. 2004), while oral and i.v. administrations reduce circulating ghrelin levels in rats (Lee, 2002). In humans, continuous lipid infusion does not influence circulating ghrelin levels (Mohlig et al., 2002).

Both sympathetic and parasympathetic nervous systems have been suggested to affect ghrelin secretion. Stimulation of sympathetic nerves increased ghrelin levels in rats (Mundinger et al.,
2006), while the muscarinic receptor blocker atropine decreases ghrelin levels in fasting humans (Broglio et al., 2004; Maier et al., 2004). Elevation of ghrelin levels induced by food deprivation is prevented by subdiaphragmatic vagotomy in rats supporting the involvement of parasympathetic nervous system in ghrelin release (Williams et al., 2003b). Ghrelin secretion does not require luminal nutrients in the stomach and duodenum, yet postprandial insulin, intestinal osmolarity and the ENS may be involved (Murdolo et al., 2003; Williams et al., 2003a; Williams et al., 2003b).

2.3. Genetic obesity – Prader-Willi syndrome

Prader-Willi syndrome is a genetic disorder characterized by an infantile failure to thrive and hypotonia, hypogonadism, growth hormone deficiency, respiratory distress, mental retardation and early on-set extreme hyperphagia and obesity (Holm et al., 1993; Goldstone, 2004). PWS is rare condition with estimated incidence of 1 in 25 000 births in the United Kingdom (Whittington et al., 2001). The syndrome arises from the lack of expression of paternally inherited genes in chromosome locus 15q11-q13 either by genomic imprinting, uniparental disomy or deletion (Goldstone, 2004). The region 15q11-q13 contains an imprinting centre and deletion of this region abolishes the expression of paternally inherited genes (Yang et al., 1998). Imprinting of maternally inherited genes in the same locus results in the Angelman syndrome, a condition characterized by severe mental retardation, ataxia and absent speech (Clayton-Smith and Pembrey, 1992).

PWS is likely to arise from a disruption of several genes in the locus 15q11-q13 (Goldstone, 2004). SNURF-SNRPN is a complex locus that regulates imprinting and encoding Magel2, several proteins and small nucleolar RNAs (Nicholls and Knepper, 2001). Studies utilizing mouse models have revealed that candidate genes, necdin and melanoma antigen family L2 that are imprinted in locus 15q11-q13 and are not expressed in the developing brain of the mouse model of PWS. Functions of the genes located in 15q11-q13 in the development of PWS are complex and not completely understood (Goldstone, 2004).

Neuroendocrine and metabolic disturbances observed in PWS indicate abnormalities in the development of hypothalamus. Rapid onset of abnormal feeding behaviour between ages 1 - 6 years includes obsession with food, food stealing, reduced satiety and earlier return of hunger after eating (Goldstone, 2004). Without adequate dietary control, PWS leads to morbid obesity and type 2 diabetes and mortality below 35 years of age (Greenswag, 1987). Classical endocrinological alterations include growth hormone deficiency and elevated plasma ghrelin levels (Cummings et al., 2002a; Haqq et al., 2003). Elevated ghrelin level differs from other states of obesity such as leptin resistance and genetic leptin deficiency, where ghrelin levels are decreased (Tschop et al., 2001; Cummings et al., 2002a). Increased ghrelin levels might be caused by the decreased visceral fat and
relative hypoinsulinemia observed in PWS, but the exact mechanisms still remain to be confirmed (Goldstone et al., 2001).

In addition, PWS patients have been shown to display sleep disturbances, including excessive daytime sleepiness and disturbed rapid eye movement sleep (Manni et al., 2001; Nevsimalova et al., 2005). Sleep apnea is also frequent in PWS (Holm et al., 1993; Nevsimalova et al., 2005). Reduced CSF OXA levels have been observed in PWS patients in several studies (Dauvilliers et al., 2003; Arii et al., 2004; Nevsimalova et al., 2005). PWS patients also display an abnormal chemoreceptor response to hypoxia and hypercapnia (Arens et al., 1994; Gozal et al., 1994) and blunted arousal response to hypercapnia (Livingston et al., 1995), suggesting an altered control of ventilation. Interestingly, OXA has been shown to stimulate breathing via medullary and spinal pathways in rats (Young et al., 2005). Lack of OXA in pre-proorexin knock-out mice leads to decreased peripheral chemosensitivity to carbon dioxide during wakefulness and this effect may be partially restored by i.c.v. supplementation of OXA (Deng et al., 2007). In mice deficient in Magel2, one of four genes within the PWS region in chromosome 15q11-q13, the OXA peptide concentration in hypothalamus was 60% lower than in the wild-type. However, no reduction in number of hypothalamic OXA neurons was observed in post mortem analysis of PWS patients (Fronczek et al., 2005).

2.4. Treatment of obesity

2.4.1. Diet-induced weight loss

Weight loss is important in reducing the risk of MetS and type 2 diabetes and improving glucose homeostasis, dyslipidemia and blood pressure in these patients. Dietary interventions often lead to the recommended weight loss (5 - 10%) during 6 months, but the real challenge is long-term weight maintenance. Avenell et al. (2004) analyzed 12 balanced low-calorie diets with deficit of 600 kcal/day and found that the treatment group lost in average 5.31 kg more weight than the controls during 12-month follow-up. Balanced low-calorie diets decreased incidence of type 2 diabetes, and improved control of other risk factors, with weight loss sustained for 3 years. Another systematic review analyzing 16 studies showed that weight loss after 2-3 years was 3.5 ± 2.4 kg and after 4-7 years 3.6 ± 2.6 kg (Douketis et al., 2005).

The energy content of the diet may be decreased by modifying the proportions of nutrients. Dietary fat has high energy content and therefore low-fat diets have been widely applied to help patients to lose weight. A large meta-analysis of almost 50 000 postmenopausal women showed that ad libitum low-fat diet produces 2.2 kg weight loss during first year and maintained 0.4 kg lower weight than control women during an average of 7.5 years of follow-up. In addition, weight loss
was greatest among women in either group who decreased their percentage fat in energy intake (Howard et al., 2006). In another systematic review of 16 studies including 1910 patients, ad libitum low-fat diet results in 3.2 kg weight loss during 2-12 months. Low-fat diet was most beneficial for individuals with highest initial weight (Astrup et al., 2000).

The efficacy of low-carbohydrate diets with low glycemic index and overall lower glycemic load has also been studied. The glycemic index is based on comparison of insulin response of the chosen meal to a 50 g portion of white bread (Ludwig, 2002). Two randomized clinical trials comparing low-glycemic load (high-fiber) and a conventional diet failed to show any differences in weight, yet resulted in improvements in insulin resistance, triglycerides and resting energy expenditure (Pereira et al., 2004; Ebbeling et al., 2007). In the Finnish Diabetes Prevention study, dietary fat and fiber were significant predictors of sustained weight reduction and progression to type 2 diabetes in high-risk subjects (Lindstrom et al., 2006). Rye-bread containing high levels of fiber also decreases postprandial insulin response (Leinonen et al., 1999). Thus, high fiber content of the food may provide additional benefits independently of weight loss.

High-protein diets have also been shown to enhance weight maintenance. Individuals on ad libitum high-protein diet lost significantly more weight than individuals on medium-protein diet (9.4 kg vs. 5.9 kg). A high-protein diet was accompanied with 10% decrease in intra-abdominal fat depot, yet no differences in blood measures were detected. After 12 months, difference in weight was no longer significant and 50% of study participants were dropped out partly due to difficulties in maintaining the high-protein diet (Due et al., 2004). Protein has been accepted to be more satiating than isoenergetic ingestion of carbohydrates and fat (Westerterp-Plantenga., 2008). The mechanisms may involve induced thermogenesis, increased GLP-1 and CCK and prolonged suppression of postprandial ghrelin secretion (Latner and Schwartz, 1999; Blom et al., 2006; Lejeune et al., 2006).

A very-low-calorie diet (VLCD) is designed to contain energy levels between 200 and 800 kcal/day. Various VLCD diets have been utilized to achieve rapid weight loss. No significant differences between three VLCD diets containing 400-800 kcal/day were observed in obese women. Participants lost on average 17.8 ± 0.6 kg (about 20%) after 3 months of VLCD and more than 85% of that mass was fat (Foster et al., 1992). Despite significant initial weight loss during VLCD, the weight loss is difficult to sustain. In a meta-analysis of the long-term effects of VLCDs (< 800 kcal/day) in combination with dietary counseling in obese patients, the effect of the VLCD was approximately 3.0 kg (3.2%) after 2-5 years of treatment despite a weight loss of over 20 kg (21.3%) during the VLCD (Anderson et al., 2001).
2.4.2. Exercise

Increasing physical activity has been shown to be a key element for successful long-term weight maintenance. National Weight Control Registry contains the weight loss data of 4000 adults who have lost at least -13.6 kg and kept it off at least 1 year. The results suggested that regular high-intensity exercise was the most important indicator for long-term success at weight loss (Wing and Phelan, 2005). Exercise alone decreased body weight by 4.0 kg and the change was accompanied with improvements in LDL and HDL profile. When exercise program was combined with dietary prescription, weight loss of 7.2 kg was achieved (Wood et al., 1988). A Cochrane review of 43 studies found 1.1 kg reduction in weight in the exercise and diet group compared with the diet alone group. High intensity exercise produces additional 1.5 kg weight loss than low intensity exercise. Moreover, exercise reduces diastolic blood pressure 2 mmHg more than no exercise (Shaw et al., 2006).

In addition, benefits of physical activity on glucose metabolism have been observed independently of decrease in body fat. It has been shown that exercise increases insulin-stimulated glycogen synthesis and glucose transport leading to improved plasma glucose concentrations (Perseghin et al., 1996). In addition, elevated capillary proliferation and increased muscle mass leads to improved insulin sensitivity (Goodyear and Kahn, 1998). Exercise has been shown to decrease low-grade systemic inflammation that is known to be a hallmark feature of MetS and type 2 diabetes. Petersen et al. (2005) suggested that acute release of IL-6 from muscle observed during exercise may suppress TNF-α and lead to overall suppression of systemic inflammation. Consistently, exercise has proven efficient in prevention of type 2 diabetes. More than 2.5 hours/week of walking was associated with 63% lower risk of type 2 diabetes (Laaksonen et al., 2005a). A recent meta-analysis of 10 studies showed that regular physical activity of moderate intensity decreases incidence of type 2 diabetes by 30% (Jeon et al., 2007). Thus, 30 min or more of daily activity of moderate intensity as recommended by guidelines can substantially reduce the risk of type 2 diabetes (Pate et al., 1995).

Yet data in humans is lacking, improvement in systolic blood pressure in response to physical activity is accompanied with upregulation of apelin/APJ expression in aorta and myocardium in spontaneously hypertensive rats (Zhang et al., 2006). The effect of exercise on plasma ghrelin in humans is currently controversial. Single aerobic exercise has either slightly decreased (Vestergaard et al., 2007) or had no effects on total plasma ghrelin (Burns et al., 2007; Jürimäe et al., 2007a). Also increase in total ghrelin levels during prolonged (3h) medium-intensity exercise has been described (Christ et al., 2006). However, acylated ghrelin levels have been shown to decrease during to treadmill running in healthy males. Unfortunately, the total ghrelin concentration was not
measured (Broom et al., 2007). Using bicycle exercise on an ergometer, increase in total ghrelin was observed rather in low than high-intensity training. These changes did not correlate with variations in hunger (Erdmann et al., 2007). However, prolonged regular exercise of 6 hours/day was associated with slightly higher acylated ghrelin levels in adolescent girls compared to inactive age and BMI matched controls (Jürimäe et al., 2007b).

2.4.3. Drugs

Currently available drugs for obesity are designed to reduce food intake either by acting directly on neurons in the brain or decreasing absorption of nutrients in the gut. When used as monotherapy, current drugs have reduced weight less than 10%. When the treatment is discontinued, weight returns back to the level before the treatment. In addition, current drugs have significant side effects such as nausea, diarrhea and depression leading to reduced compliance (Bray and Greenway, 2007). Therefore, considerable effort for development of novel approaches for treatment of obesity is being carried out.

Orlistat is a selective pancreatic lipase inhibitor and inhibits digestion of fat in the gut lumen. Thus, the amount of absorbed triglycerides and fatty acids is reduced. A 4-year randomized trial found that orlistat combined with hypocaloric diet produces approximately a 10 kg weight loss during the first year compared with 6 kg on a hypocaloric diet alone. After 3 years, slight weight regain occurs resulting in 2.8% weight loss compared with placebo. Weight loss was accompanied with 37% reduction in impaired glucose tolerance and diabetes (Torgerson et al., 2004). In a randomized double-blind multicentre study Richelsen et al. (Richelsen et al., 2007) found that orlistat provides additional 2.2 kg weight loss compared with placebo after 3 years of weight maintenance, slightly less than the 3 kg weight loss found in meta-analyses (Rucker et al., 2007). Orlistat has little systemic side effects, since it is not absorbed to a significant degree. However, initial loss of fecal fat and GI symptoms are common and may lead to poor compliance. Studies regarding the effect of orlistat treatment on plasma inflammatory markers have yielded variable results. Samuelsson et al. (2003) observed slightly lower TNF-α levels in the orlistat treated group than in the placebo group after 12-month caloric restriction, while difference in IL-6 was observed only in patients with type 2 diabetes and hypertension. Similarly, TNF-α levels were decreased in non-diabetic women after 6-month hypocaloric diet with orlistat treatment compared with diet alone.

Sibutramine is a serotonin-norepinephrine reuptake inhibitor. Serotonin 5-HT\(_{2C}\) receptors have been shown to modulate fat and food intake in animals. 5-HT\(_{2C}\) receptor deficient mice are obese and have increased food intake possibly through modulation of downstream melanocortin-4
receptors (Lam et al., 2008). Activity of α- and β-receptors in the brain also modulate food intake in a reciprocal manner. Stimulation of α2-receptors increases while activation of β2-receptors reduces food intake (Jackson et al., 1997). In a meta-analysis of long-term treatment, sibutramine produced 4.16 kg weight loss during 1-year follow-up (Rucker et al., 2007). When sibutramine was combined to behavioral therapy, weight loss of 6.7 kg was obtained during a 12-month study. Intensive lifestyle intervention combined to sibutramine increases weight loss to 12.1 kg (Wadden et al., 2005). Sibutramine has been shown to increase systolic and diastolic blood pressure in normotensive patients by 0.8 mmHg and 0.6 mmHg, respectively (Kim et al., 2003). In addition, sibutramine should not be used simultaneously with selective serotonin reuptake inhibitors or monoamine oxidase inhibitors. These things significantly limit the use of sibutramine in patients with history of coronary artery disease, cardiac insufficiency and arrythmias, stroke and depression.

Rimonabant is an antagonist of cannabinoid receptor 1 that is distributed in the brain areas related to regulation of feeding. Mice lacking cannabinoid receptor 1 are lean and resistant to diet-induced obesity (Di Marzo et al., 2001). Rimonabant was approved by European Medicines Evaluation Agency for treatment of obesity, but not by Food and Drug Administration (FDA) in the United States. Initial studies with obese patients showed that 20 mg of rimonabant administered daily produces additional 6.3 kg weight loss compared with placebo during a 1-year follow-up. The weight loss was accompanied with improvements in LDL, HDL and insulin sensitivity. The drop out rate was as high as 39% at end of the treatment period due to psychiatric, nervous system and GI tract symptoms (Van Gaal et al., 2005). A subsequent study in overweighted and dyslipidemic patients showed similar results. Administration of 20mg rimonabant produced 6.5 kg weight loss compared with the control group. Drop-out rate reached 40% during 1-year trial. Adverse effects included nausea, diarrhea, vomiting, dizziness fatigue and anxiety, which were generally mild or moderate. Rimonabant also increased plasma adiponectin by 58% compared with baseline (Despres et al., 2005). A recent double-blind multi-center study in 839 obese patients with the MetS showed that despite weight loss, rimonabant did not improve atheroma plaque volume. However, psychiatric adverse effects were two-fold greater in the treatment group (43% and 28%, p < 0.001) (Nissen et al., 2008). Therefore, rimonabant was withdrawn from the market also in Europe in January 2009.

2.4.4. Obesity surgery

Obesity surgery is the most efficient treatment option for morbidly obese patients with history of several failed weight loss attempts by dietary and pharmacological methods. The National Institute of Health recommends consideration of bariatric surgery for patients with BMI greater than 40 kg/m² and for those with BMI greater than 35 kg/m² who also have serious medical problems
due to obesity such as diabetes and sleep apnea (Hubbard and Hall, 1991). Several surgical
techniques have been developed (Pories. 2008), but the Roux-en-Y gastric bypass and laparoscopic
adjustable gastric banding are currently the most widely used (Buchwald and Williams, 2004).

Classical Roux-en-Y surgery involves a creation of 20 - 30 ml pouch in the cardia of the
stomach. The jejunum is dissected distal to the Treitz ligament and the distal end is anastomosed to
the pouch created to upper stomach. The proximal jejunum is anastomosed to distal jejunum.
Weight loss after Roux-en-Y has been varied 65 - 75% of excess weight corresponding
approximately 35% of initial weight (Brolin, 2002).

In gastric banding surgery, a silicone band is placed around the cardia of the stomach to create a
30 ml pouch similarly to a Roux-en-Y bypass. Laparoscopic gastric banding surgery was improved
by the invention of adjustable gastric banding. The caliber of the silicone band may be regulated by
filling or emptying the inflatable saline reservoir placed under the skin of the patient (Figure 2).

Figure 2. Adjustable gastric banding. Modified from (Crookes, 2006).

The efficacy of bariatric surgery was demonstrated in a study of over 2000 patients. During 10
years of follow-up, the weight losses in gastric bypass, vertical-banded gastroplasty and gastric
banding were 25%, 16%, and 14%, respectively. There were 101 deaths in the bariatric surgery
group and 129 deaths in the control group receiving conventional weight loss therapy (hazard ratio
0.76, p = 0.04). Among the main causes of death were cardiac infarction and cancer (Sjostrom et al.,
Another study of 1000 patients found that 67.1% of the excess weight loss is maintained 16 years after the surgery. Mortality and morbidity were significantly reduced compared with controls during 5 years of follow-up (0.68% and 6.17%). The prevalences of cancer, cardiovascular disease, endocrinological disorders and respiratory conditions are also reduced 5 years after the surgery (Christou et al., 2004). Remarkably, complete resolution or improvements in diabetes, hypertension, hyperlipidemia, and obstructive sleep apnea have been observed (Pories et al., 1987; Hickey et al., 1998; Tice et al., 2008) (Figure 3). In a meta-analysis of 10 studies including 4594 patients, adjustable gastric banding resulted in over 50% of excess weight loss accompanied by resolution of type 2 diabetes and hypertension (Cunneen, 2008).

![Figure 3](image_url)

Figure 3. The resolution of obesity associated co-morbidities after Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding. Values are reported as median values from meta-analysis of 14 comparative studies. Modified from (Tice et al., 2008).

According to a recent meta-analysis of 14 clinical trials, Roux-en-Y is considered more effective compared with adjustable gastric banding producing a 26% greater loss of excess body weight 1 year after the operation (Tice et al., 2008). The follow-up data from Kuopio University Hospital clinic showed that postoperative reduction of excess weight is 36 ± 24% 1 year and 21 ± 5% nine years after the operation, respectively. Most patients started to regain weight after 1-3 years and the final average weight decrease from baseline was 19.5 kg (Martikainen et al., 2004). However, 52% of 200 patients required re-operation, which is high compared with the 19% observed in a larger study (Chevallier et al., 2004).

Gastric banding is considered safer in short-term, yet the overall mortality rate is low (0.06% vs. 0.17%) after both gastric banding and Roux-en-Y during the follow-up period of 1-5 years, respectively (Tice et al., 2008). Common complications of the gastric banding include band slippage...
and pouch dilation, while bowel obstruction has been the most common problem after the Roux-en-Y gastric bypass. Short-term complications of different laparoscopic bariatric surgery techniques include conversion to open procedure, bleeding and anastomose leakage. Band erosion, gallbladder problems, and incisional hernias are relatively uncommon long-term complications. A study of 1000 laparoscopic gastric banding operations reported 193 complications (19.3%) during 7 years of follow-up. Twelve of these complications (1.2%) were lifethreatening. In addition, an abdominal reoperation was required in 111 (11%) cases (Chevallier et al., 2004). These complication rates need to be weighted against the individual risk for obesity-related complications when considering the bariatric surgery as a treatment option in obese patients.

Obvious mechanisms leading to weight loss after the bariatric surgery are malabsorption of the nutrients due to intestinal bypass and mechanical restriction of the stomach. In Roux-en-Y, food is directed pass the stomach, duodenum and upper parts of jejunum leading to poor absorption. Creation of small pouch limits the amount of ingested food after both operations. However, improvement or full resolution of obesity-associated diabetes and insulin resistance immediately after the surgery, before the occurrence of weight loss (Pories et al., 1987; Hickey et al., 1998). The mechanism of action is currently under intensive research. In response to gastric banding, leptin levels decreased and ghrelin significantly increased (Hanusch-Enserer et al., 2004; Coupaye et al., 2005). However, weight loss after Roux-en-Y has been associated with decreases in ghrelin levels in some (Cummings et al., 2002b; Fruhbeck et al., 2004), but not in all studies (Borg et al., 2006). The conflicting data may be due to the different surgical techniques that affect the functionality of the gastric fundus from which ghrelin is mainly secreted (Fruhbeck et al., 2004). Adiponectin levels increased in morbidly obese patients after Roux-en-Y bypass and pre-operative levels predict post-operative weight loss (Faraj et al., 2003). In contrast, no change in fasting serum peptide YY and GLP-1 values has been observed after Roux-en-Y bypass (Clements et al., 2004; Borg et al., 2006), yet peptide YY and GLP-1 responses to a 420kJ mixed test meal has been increased 6 months after the operation (Borg et al., 2006). Thus, further studies are required to understand the rapid metabolic improvements observed in patients undergoing bariatric surgery.
3. **AIMS OF THE STUDY**

The current study was designed assess the possible roles of apelin, OXA and ghrelin in obesity and MetS. More specifically, the following questions were addressed:

1. Are plasma apelin levels altered in morbidly obese patients? What is the effect of diet-induced weight loss and 6 months of weight maintenance on plasma apelin in patients with MetS? Are apelin levels associated with changes in body fat mass, inflammation and blood pressure?
2. Are plasma OXA levels altered in morbidly obese patients undergoing bariatric surgery?
3. Are circulating OXA levels altered in children with PWS?
4. Is the post-prandial ghrelin response blunted in individuals with MetS and is this response altered differently by two high-carbohydrate meals producing different insulin levels?
4. MATERIALS AND METHODS

4.1. Study protocols

4.1.1. Apelin and OXA levels in morbidly obese patients

A study group of 32 morbidly obese patients undergoing gastric banding were recruited for the study at the Department of Surgery, Kuopio University Hospital. Characteristics of the participants are given in Table 3. All patients had participated in long-term non-surgical weight reduction programs at the Division of Clinical Nutrition of Kuopio University Hospital by a multidisciplinary team, including surgeon, endocrinologist and clinical nutritionist before undergoing surgery. Criteria for the bariatric surgery were: age > 18 y, BMI > 40 kg/m$^2$ or > 35 kg/m$^2$ with significant co-morbidities, including diabetes, hypertension, hyperlipidemia, hypertension, sleep apnea and osteoarthritis. The patients were required to be obese for 15 y and possess no acute psychiatric illness or eating disorder. Information regarding the benefits, risks and side effects of surgery were discussed in detail and the patients were informed of the necessity to undergo postoperative lifelong follow-up and the importance to change dietary habits.

Table 3. Characteristics of morbidly obese patients undergoing gastric banding, subgroup of morbidly obese patients analyzed before and 1 year after the gastric banding and healthy lean controls (*morbidly obese-lean controls, $^b$subgroup-lean controls).

<table>
<thead>
<tr>
<th></th>
<th>Morbidly obese</th>
<th>Subgroup</th>
<th>Lean controls</th>
<th>p value$^a$</th>
<th>p value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>10/22</td>
<td>1/7</td>
<td>2/10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (y)</td>
<td>44 ± 2</td>
<td>37 ± 1</td>
<td>49 ± 6</td>
<td>0.31</td>
<td>0.12</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>134 ± 4</td>
<td>135 ± 7</td>
<td>61 ± 8</td>
<td>0.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>48 ± 1</td>
<td>48 ± 2</td>
<td>22 ± 2</td>
<td>0.99</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

In the morning prior to surgery, blood samples were drawn from antecubital vein after an overnight fast. In a subgroup of 8 obese patients, fasting blood samples were obtained one year after surgery. An adjustable silicone gastric band (Lab-Band, BioEnterics Corporation, California) was placed either in open surgery or using a laparoscopic technique. The surgery followed the principles described by Kuzmak (1989). The results of the gastric banding procedure on weight loss, late complications and quality of life have been reported (Martikainen et al., 2004). A control group of 12 normal weighted and age matched volunteers was recruited from the university staff (Table 3). Participants were required to have BMI < 25 kg/m$^2$ and no interfering medical history, diabetes, or use of corticosteroids.
4.1.2. The effect of diet-induced weight loss

A study group of 35 obese subjects with a BMI between 30 and 45 kg/m\(^2\) and waist circumference \(\geq 102\) cm (men) and \(\geq 92\) cm (women) (Table 4) was recruited from the Kuopio center of the Scandinavian multicenter study of obese subjects with the metabolic syndrome (SMOMS) (Laaksonen et al., 2003b; Richelsen et al., 2007). The study was initiated to investigate the effects of lipase inhibitor orlistat on weight maintenance after a VLCD. Participants were required to have plasma glucose concentrations \(\geq 7.0\) mmol/l treated by diet only, or at least one of the following criteria: impaired fasting glucose (plasma glucose \(\geq 6.1\) mmol/l), dyslipidemia (HDL \(\leq 0.9\) mmol/l [men] and \(\leq 1.2\) mmol/l [women]) and/or serum triglycerides \(\geq 2.0\) mmol/l. Exclusion criteria were poorly controlled diabetes (HbA1c \(\geq 10\%\)), hypertension exceeding \(\geq 180/120\) mmHg, ischemic heart disease, psychiatric disorders and kidney insufficiency.

Table 4. Characteristics of patients with MetS at baseline (p value between sexes).

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>19</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>52 ± 2</td>
<td>53 ± 2</td>
<td>0.84</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>106.9 ± 2.5</td>
<td>95.6 ± 2.7</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>34.0 ± 0.7</td>
<td>36.2 ± 1.0</td>
<td>0.07</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>116 ± 2</td>
<td>111 ± 2</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130 ± 2</td>
<td>128 ± 2</td>
<td>0.60</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80 ± 1</td>
<td>79 ± 1</td>
<td>0.91</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.9 ± 0.4</td>
<td>6.4 ± 0.4</td>
<td>0.37</td>
</tr>
<tr>
<td>Hb1Ac (%)</td>
<td>5.9 ± 0.1</td>
<td>6.4 ± 0.3</td>
<td>0.06</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.11 ± 0.07</td>
<td>1.23 ± 0.06</td>
<td>0.20</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>3.95 ± 0.32</td>
<td>3.81 ± 0.27</td>
<td>0.96</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.86 ± 0.47</td>
<td>2.03 ± 0.20</td>
<td>0.14</td>
</tr>
</tbody>
</table>

After the baseline measurements were performed, subjects who lost \(\geq 5\%\) of the initial weight during the VLCD were included into the study. Participants consumed a VLCD of 800 kcal day for 8 weeks (Nutrilett; Leiras Co., Finland). They supplemented the Nutrilett diet with low-calorie vegetables as desired. Subjects were randomized into two groups receiving either 120 mg of orlistat or placebo three times a day during a 6-month weight maintenace period (WM). WM consisted of a hypocaloric low-fat (< 30 E\%) diet of at least 1200 kcal/day individualized according to estimated daily energy expenditure allowing a deficit of 600 kcal/day. In addition, similar dietary and exercise counselling was given to both study groups (Laaksonen et al. 2003a; Richelsen et al. 2007). Blood samples were taken at baseline, after VLCD and after the 6-month WM. Antropometric data was
also collected at given time points and ambulatory blood pressure measurements and CT scan were performed as described in 9.5 and 9.6.

### 4.1.3. OXA levels in children with PWS

Eight children with diagnosed PWS were recruited for the study in the Poznan University of Medical Sciences (Table 5). Two of the patients were taking GH treatment. Eighteen lean and healthy young adults were recruited from the students of Poznan University of Medical Sciences and Poznan University of Life Sciences. They were all non-smokers and taking no medication. All of the healthy controls were young adults, since the use of children in the current study was considered unethical. All participants were informed written consent and the study plan was approved by the Joint Ethics Committee of Poznan University Hospital.

<table>
<thead>
<tr>
<th>Women/Men</th>
<th>PWS</th>
<th>Healthy</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>11 ± 2</td>
<td>23 ± 1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.38 ± 0.06</td>
<td>1.73 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>48.3 ± 6.1</td>
<td>63.0 ± 2.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.5 ± 1.6</td>
<td>21.1 ± 0.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>BMI (Z-score)</td>
<td>1.76 ± 0.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.0 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>42.5 ± 5.3</td>
<td>42.4 ± 3.0</td>
<td>0.99</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.4 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>0.58</td>
</tr>
</tbody>
</table>

In the morning after an overnight fast, basal blood samples were taken. The study participants consumed liquid meal consisting of two packages of Nutridrink (2 x 200 mL; N.V. Nutricia, Zoetermeer, The Netherlands) containing 600 kcal of energy. The meal consisted of 12 g protein (E 16%), 37g carbohydrates (E 49%), 11.6 % fat (E 35%) and a variety of vitamins. After the meal, blood samples were drawn at 30 and 90 min time points from the antecubital vein.

### 4.1.4. Ghrelin levels after two meals producing different insulin responses

Eight obese individuals (3 men and 5 women; BMI 33.7 ± 0.7 kg/m²) were recruited from a larger study cohort for the effect of carbohydrate modification to metabolic parameters in patients with MetS (Laaksonen et al., 2005b). Participants were required to fulfill three of the following criteria: waist circumference > 102 cm (men) and > 92 cm (women), fasting serum triglycerides concentration > 1.7 mmol/l, fasting HDL < 1.0 mmol/l (men) or < 1.2 mmol/l (women), fasting plasma glucose concentration between 6.1 and 6.9 mmol/l, blood pressure ≥ 130/85 mmHg or blood
pressure medication. Exclusion criteria were diagnosed diabetes and lipid lowering medication and corticosteroids. The control group of eight healthy volunteers (3 men and 5 women, BMI 22.5 ± 0.5 kg/m²) was recruited from another intervention study (Karhunen, 2005; Obes Rev, abstract). They had normal glucose tolerance, serum lipid profile and blood pressure levels. They were not taking any medication and had no significant weight loss 1 year prior to the experiment. Characteristics of the patients with MetS are given in Table 6.

**Table 6. Characteristics of obese subjects with MetS.**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>55.6 ± 1.8</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>33.7 ± 0.7</td>
</tr>
<tr>
<td>Waist circumference, men (cm)</td>
<td>109 ± 1</td>
</tr>
<tr>
<td>Waist circumference, women (cm)</td>
<td>114 ± 1</td>
</tr>
<tr>
<td>Blood pressure, systolic (mmHg)</td>
<td>152 ± 5</td>
</tr>
<tr>
<td>Blood pressure, diastolic (mmHg)</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Serum triglycerides (mmol/L)</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

After an overnight fast (12 h), the obese study group consumed rye bread or wheat bread meals (A or B) known to produce low and high insulin responses in separate days a week apart (Table 7). The control group consumed only wheat bread meal. Blood samples were collected via intravenous catheter in obese study group at 15, 30, 45, 60, 90 and 120 min after the meal. In the control group, blood samples were collected at 20, 40, 60 and 90 min.

**Table 7. Portion size and the calculated nutrient composition of the test bread baskets.**

<table>
<thead>
<tr>
<th></th>
<th>Rye bread meal</th>
<th>Wheat bread meal A</th>
<th>Wheat bread meal B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portion size (g)</td>
<td>113</td>
<td>125</td>
<td>114</td>
</tr>
<tr>
<td>Available carbohydrate (g)</td>
<td>50</td>
<td>50</td>
<td>52</td>
</tr>
<tr>
<td>Total dietary fiber (g)</td>
<td>10.2</td>
<td>7.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>6.9</td>
<td>13.9</td>
<td>9.2</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>2.6</td>
<td>5.1</td>
<td>5.7</td>
</tr>
<tr>
<td>Energy content (kJ)</td>
<td>1093</td>
<td>1260</td>
<td>1250</td>
</tr>
<tr>
<td>Energy content (kcal)</td>
<td>259</td>
<td>299</td>
<td>298</td>
</tr>
</tbody>
</table>
4.2. Ethical approval

Study protocols of studies I, II and IV were approved by the Joint Ethical Committee of Kuopio University Hospital. In study III, the study protocol was approved by Joint Ethics Committee of Poznan University Hospital. All participants were informed about the methodology used in the study. All participants gave informed written consent.

4.3. Blood samples

After an overnight fast, blood was drawn from antecubital vein into Vacutainer tubes (BD, Franklin Lakes, NJ, USA) and samples were immediately cooled on ice. Serum or plasma was separated with centrifugation 1700-3000 g x 12-15 min in +4 C within 10 min of sampling and samples were stored -70 C until the analysis.

4.4. Plasma peptide measurements

Plasma apelin was measured with commercially available EIA (EK-057-23; Apelin-12; Phoenix Pharmaceuticals Inc., CA, USA) that detected human apelin-12, -13 and -36 and intra- and inter-assay CV% reported by the manufacturer were < 5% and < 14%, respectively. Plasma OXA was measured using RIA or EIA (Phoenix Pharmaceuticals Inc., CA, USA). Prior to the analysis, plasma was extracted using Sep-Pak C-18 (Waters Associates, Milford, MA, USA) columns using a slightly modified method by Arihara et al. (Arihara et al. 2001). In study I, 1-2 ml of was applied to the Sep-Pak column previously activated with methanol and sterile water. The column and washed with 20 ml of water and samples were eluted slowly with 80% ACN. Resulting volume was reduced to 0.4 ml under nitrogen flow and the aliquot was evaporated into dryness using Speedvac (Savant Instruments, Holbrook, NY). The dry residue was dissolved in sterile water and used for EIA. The intra- and inter-assay CV% for OXA EIA reported by the manufacturer were < 5% and < 14%, respectively. In study III, 1 ml of plasma was acidified with 1 ml of 0.1% TFA and applied to Sep-Pak column previously equilibrated with methanol, 60% ACN in 0.1% TFA and 0.1% TFA. The column was washed with 10 ml of 0.1% TFA and peptides were eluted with 2 ml of 60 % ACN in 0.1% TFA. Samples were evaporated into dryness and dissolved in 0.1 ml of RIA buffer. RIA was conducted in the same tube without additional dilutions to avoid loss of the peptide. RIA detected full length human orexin A, but did not crossreact with orexin B. Ghrelin RIA kit detected human ghrelin, des-octanoyl-Ser3-ghrelin and o-n-octanoyl-ser3-ghrelin(1-27). Intra- and inter-assay CV% determined in our lab were 5% and 20%, respectively. TNF-α and IL-6 were measured with Quantikine HS EIA kit (R&D Systems Inc., MN, USA). Intra- and inter-assay CV% for
Quantikine HS kits were 3.1 - 8.5% and 6.5 - 10.6%, respectively. Plasma adiponectin and leptin were determined with EIA and RIA, respectively (Linco Research Inc., MO, USA). Intra- and inter-assay CV% for leptin RIA were 10.2% and 13% and for adiponectin EIA 7.4% and 8.4%, respectively. In study IV, also EIA by Cayman Chemicals Inc. (MI, USA) was used. Intra- and interassay CV% reported by the manufacturer were 9%. Plasma glucose was determined using glucose dehydrogenase method after precipitation of protein by trichloroacetic acid or glucose hexokinase method (Roche, Basel, Switzerland). Serum insulin was measured with chemiluminescent immunoassay (ACS 180 Plus, Bayer Diagnostics, Germany) or RIA (Pharmacia Diagnostics, Uppsala, Sweden; Millipore, MA, USA). Insulin sensitivity was assessed by the homeostasis assessment model (HOMA-IR \[\text{mmol/l} \times \mu \text{U/ml}\] = fasting glucose \[\text{mmol/l}\] x fasting insulin \[\mu \text{U/ml}\]/22.5). LDL and HDL fractions were separated from serum by combined ultracentrifugation and precipitation. Cholesterol and triglyceride contents were measured enzymatically. All samples were measured in duplicates except in OXA RIA (Study III) that was measured in single due to the lack of sample material.

4.5. Ambulatory blood pressure measurements

Participants underwent 24-h ambulatory measurements using a digital ambulatory blood pressure system (SpaceLabs 90207; SpaceLabs Medical Inc., Redmond, WS, USA) at baseline and after VLCD and 6-month weight maintenance period. The blood pressure was measured in 15 min intervals in the daytime and 30 min in the night. The mean heart rate and mean MAP (\[2 \times \text{diastolic blood pressure} + \text{systolic blood pressure}/3\]) during 24-h measurements were used in the calculations.

4.6. Determination of body adiposity

The amount of visceral and subcutaneous fat was measured at the L4-L5 level using computational tomography (CT; Somatom Plus S, Siemens, Erlangen, Germany) scan before and after VLCD of 15 study participants. CT derived VAT and SAT were measured as described (Sjostrom et al., 1986). Briefly, a 10 mm thick cross-sectional scan centred on the L4 vertebra was obtained at 120 kV with a scanning time of two seconds and a 512 matrix. The areas of total and visceral fat were determined by delineating them with a graph pen and then computing the areas at an attenuation ranging from -30 to -190 HU. The area of visceral fat was defined by drawing a line inside the muscle wall outlining the abdominal cavity, and the area of subcutaneous fat was calculated by subtracting the area of visceral fat from the area of total fat.
4.7. Statistics

All values are reported as mean ± SEM and p values lower than 0.05 were considered significant. Normality of the data was tested using the Kolgomorov-Smirnov or Shapiro-Wilk tests. In study I, peptide levels between the morbidly obese and healthy controls and levels before and after the surgery were compared using Student’s t-test and paired t-test when appropriate. Correlations were calculated using Pearson correlation. In study II, changes in anthropometric measures and plasma cytokine values at different time points and differences between sexes and orlistat and placebo groups were compared using repeated measures ANOVA. Post-hoc tests were based on comparing estimated marginal means. A relative decrease in VAT and SAT was compared using paired samples t-test. Pearson correlations were calculated at each timepoint. Plasma apelin, TNF-α, IL-6, ghrelin, insulin and adiponectin levels were not normally distributed and therefore Spearman correlation was used between these variables. Multivariate regression analysis was performed to assess the effect of individual parameters on changes in plasma apelin. Univariate analysis of variance was used in analysis of the effect of degree of weight loss on changes in plasma markers after the WM. The Bonferroni post test was used to determine the differences between groups. Differences from baseline after the VLCD and WM in weight loss groups were determined with repeated measures ANOVA and the Bonferroni post-hoc tests. In study III, peptide levels between PWS patients and healthy adults were compared using Student’s t-test. Peptide levels between the groups at different time points were compared using repeated measures ANOVA with Bonferroni post-hoc test. In study IV, post-prandial variations within the group were determined using repeated measures ANOVA with Bonferroni post-test. Differences between the obese groups were determined using two-way ANOVA with Bonferroni post-hoc test and lean and obese groups were compared using Student’s t-test in each overlapping time point. AUCs were compared using ANOVA with Bonferroni post-hoc test. Statistical tests were performed using Graphpad Prism 4.0 (Graphpad Software Inc., CA, USA) and SPSS 14.0 (SPSS Inc., IL, USA).
5. RESULTS

5.1. Apelin and OXA in morbid obesity

We found that plasma apelin levels were elevated in morbidly obese patients compared with healthy normal-weight controls (736 ± 50 pg/ml and 174 ± 14 pg/ml; p < 0.001). Apelin levels correlated positively with BMI in morbidly obese patients and lean controls (r = 0.77, p < 0.001). OXA levels determined with EIA were increased in morbidly obese patients undergoing gastric banding (75.3 ± 24.1 pg/ml and 0.8 ± 0.4 pg/ml; I). A weak positive correlation to BMI was observed (r = 34, p < 0.05). Gastric banding was accompanied with significant weight loss (-16 ± 6 kg, p < 0.001) and improvements in glucose levels (-0.8 mmol/l, p < 0.001). Despite pronounced weight loss, minor decrease in plasma OXA level did not reach statistical significance (56.1 ± 16.1 pg/ml, p = 0.19).

5.2. Apelin, adipokine and cytokine levels after weight loss

In response to weight loss induced by VLCD and WM, plasma apelin levels decreased in 23 (66%) individuals, yet this decrease did not reach statistical significance (1003 ± 69 pg/ml to 913 ± 67 pg/ml; p = 0.26; Table 8). Furthermore, plasma IL-6 and TNF-α did not change significantly from baseline during the VLCD and WM. Substantial reductions in body weight and BMI, body adiposity, MAP and enhancement of glucose metabolism and a modest improvement in blood pressure in response to a VLCD and WM were observed (Table 8). Participants lost on average 14.8 ± 0.8 kg of weight during the VLCD and weight loss of 15.1 ± 1.0 kg was maintained after WM. Both VAT and SAT significantly decreased after the VLCD (p < 0.001). The proportional decrease was higher in VAT than in SAT (-28.7 ± 4.0% and -17.9 ± 3.4%; p < 0.05). Systolic and diastolic blood pressures decreased during the VLCD.

Adiponectin levels acutely increased in response to the VLCD and remained increased during the WM (Table 8). Leptin decreased in response to the VLCD and increased slightly towards the baseline during the WM. Leptin levels remained higher in females throughout the study. The decrease after the VLCD was more pronounced in females than in men (-16.6 pg/ml vs. -9.5 pg/ml; p < 0.01).
Table 8. Changes in metabolic parameters of patients with metabolic syndrome after the VLCD and WM (a baseline-VLCD values; b baseline-WM values).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>VLCD</th>
<th>WM</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>101.7 ± 2.0</td>
<td>86.9 ± 1.7</td>
<td>86.6 ± 1.8</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>35.0 ± 0.6</td>
<td>30.0 ± 0.6</td>
<td>29.9 ± 0.6</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Visceral fat (cm²)</td>
<td>217 ± 48</td>
<td>161 ± 56</td>
<td>-</td>
<td>&lt; 0.001</td>
<td>-</td>
</tr>
<tr>
<td>Subcutaneous fat (cm²)</td>
<td>415 ± 128</td>
<td>319 ± 110</td>
<td>-</td>
<td>&lt; 0.001</td>
<td>-</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>129 ± 1</td>
<td>120 ± 2</td>
<td>125 ± 1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80 ± 1</td>
<td>75 ± 1</td>
<td>78 ± 1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>6.2 ± 0.3</td>
<td>5.5 ± 0.1</td>
<td>5.6 ± 0.1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>92.0 ± 6.8</td>
<td>48.6 ± 4.7</td>
<td>60.6 ± 4.2</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HOMA-IR (mmol/L x mU/L)</td>
<td>3.8 ± 0.4</td>
<td>1.8 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>20.7 ± 1.7</td>
<td>7.9 ± 0.9</td>
<td>11.1 ± 1.1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Adiponectin (ug/mL)</td>
<td>5.6 ± 0.4</td>
<td>7.6 ± 0.6</td>
<td>7.7 ± 0.5</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ghrelin (pg/mL)</td>
<td>763 ± 44</td>
<td>897 ± 66</td>
<td>823 ± 61</td>
<td>&lt; 0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Apelin EIA (pg/mL)</td>
<td>1003 ± 69</td>
<td>900 ± 65</td>
<td>913 ± 67</td>
<td>0.18</td>
<td>0.26</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.55 ± 0.28</td>
<td>1.76 ± 0.29</td>
<td>1.30 ± 0.20</td>
<td>0.38</td>
<td>0.49</td>
</tr>
<tr>
<td>TNF-alpha (pg/mL)</td>
<td>1.68 ± 0.15</td>
<td>1.71 ± 0.14</td>
<td>1.64 ± 0.14</td>
<td>0.62</td>
<td>0.89</td>
</tr>
</tbody>
</table>

To further investigate the overall effect of weight loss after VLCD and WM, participants of the study were divided into three subgroups: high weight loss (HWL, > 16.5 kg, n = 12), medium weight loss (MWL, 12 - 16.5 kg, n = 12) and low weight loss (LWL, < 12 kg, n = 11; Figure 4). Decreases in plasma apelin, MAP, TNF-α and IL-6 tended to occur only in HWL group, yet significance was reached only in MAP and TNF-α. Instead, leptin decreased in all subgroups (at least p < 0.01) and adiponectin increased in HWL and MWL subgroups (at least p < 0.05).

5.3. Correlations between variables in patients with MetS

Apelin correlated with TNF-α at baseline (r = 0.41, p < 0.05) and minor changes in plasma apelin during the VLCD and WM correlated with ΔTNF-α and ΔMAP. During the VLCD, changes in plasma apelin correlated with ΔBMI (r = 0.40, p < 0.05), but this correlation was non-significant after the WM. In a multiple linear regression model using Δapelin as the dependent variable and ΔBMI, ΔTNF-α and ΔMAP as independent variables, ΔMAP (β = -0.45; p = 0.01) and ΔTNF-α (β = 0.34; p < 0.05) were significantly associated with Δapelin (baseline-WM levels). The model significantly predicted overall changes in plasma apelin level (r² = 0.28, p < 0.01) during the VLCD and WM and ΔMAP accounted for 45% of the variation.
Figure 4. The overall effects of the degree of weight loss during the VLCD and WM on measured variables (baseline-WM). Participants were divided into subgroups losing > 16.5 kg (HWL), 12-16.5 kg (MWL) and < 12 kg (LWL) of initial weight. TNF-α, MAP, adiponectin and leptin, but not apelin changed significantly from the baseline in the indicated subgroups (**p < 0.01, *p < 0.05).

At baseline, adiponectin levels correlated positively with ghrelin (r = 0.40, p < 0.05) and negatively with VAT (r = -0.55; p < 0.05). After the WM, adiponectin correlated negatively with TNF-α (r = -0.35; p < 0.05). Significant correlations were detected between ghrelin and circulating insulin (r = -0.37, p < 0.05) and HOMA-IR (r = 0.39; p < 0.05) at baseline. IL-6 correlated significantly with VAT at baseline (r = 0.68, p < 0.01). A strong correlation between TNF-α and IL-6 was observed throughout the study (at least r = 0.584, p < 0.01).

Plasma leptin levels correlated positively with BMI (at least r = 0.40; p < 0.05) throughout the study. Significant correlations to insulin (r = 0.60, p < 0.001), VAT (r = 0.47, p < 0.01) and SAT (r = 0.72, p < 0.001) were detected after the VLCD. Overall changes in leptin after the VLCD and WM correlated with Δweight (r = 0.70, p < 0.001) and ΔBMI (r = 0.50, p < 0.01).

Significant correlations were detected between ghrelin and circulating insulin (r = -0.37, p < 0.05) and HOMA-IR (r = 0.39; p < 0.05) at baseline, but not after VLCD and WM periods.

5.4. OXA levels in PWS children

In PWS children, plasma OXA levels determined by RIA were lower than in healthy adult controls (12.6 ± 1.3 pg/ml and 15.9 ± 0.7 pg/ml, p < 0.05). Furthermore, no significant postprandial
response in plasma OXA levels or significant correlations between OXA, BMI, glucose, insulin and age were observed.

5.5. Ghrelin responses to carbohydrate meal and diet-induced weight loss

The postprandial suppression of plasma ghrelin observed in the healthy subjects (524 ± 52pg/ml to 458 ± 45pg/ml, p < 0.05) was absent in patients with the MetS, whose ghrelin concentrations remained similar to the mean preprandial values (445 ± 25 pg/ml) throughout the experiment. Plasma ghrelin levels tended to be higher in healthy subjects, but this difference did not reach statistical significance. Postprandial insulin response 30 – 60 min after the wheat bread meal was higher than after the rye bread meal (P < 0.05) in patients with the MetS. Despite different insulin levels, no difference in plasma ghrelin levels was observed.

A transient increase in plasma ghrelin level was seen after the VLCD, but the elevation was no longer significant during the WM (Table 8).
6. DISCUSSION

6.1. Apelin levels in obesity and effect of diet-induced weight loss

Apelin has been previously detected in the circulation and in this thesis we showed that apelin levels are increased in morbid obesity. However, despite pronounced weight loss, no significant decrease in plasma apelin was observed in obese patients with MetS. The apelin assay used in the current study was chosen to detect apelin-12, -13 and -36 fragments, since those peptides have been shown to contain the biological activity of apelin (Tatemoto et al., 1998; Tatemoto et al., 2001; Szokodi et al., 2002; Berry et al., 2004). Moreover, apelin-13 and -36 have been the most abundant endogenous fragments detected in colostrum and human tissues (Hosoya et al., 2000; Kawamata et al., 2001). In agreement with our findings, adipose tissue apelin mRNA and plasma apelin levels have been increased in obesity, impaired glucose tolerance and diabetes (Boucher et al., 2005; Li et al., 2006; Castan-Laurell et al., 2008).

Because it is present in the circulation, apelin could function in an endocrine manner activating cells expressing APJ. Based receptor studies using synthetic peptides, circulating apelin may indeed be sufficient to increase intracellular cAMP levels in cells expressing APJ. The EC₅₀ for APJ binding for apelin-36 in HEK-293 cells transfected with the human APJ receptor is 2.5 nmol/l (10 ng/ml), whereas cAMP accumulation was observed already at 0.1 nmol/l (0.5 ng/ml). Ca²⁺ mobilization required a concentration of 20 nmol/l (80 ng/ml) of apelin-36 (Medhurst et al., 2003). However, studies showing that apelin regulates arterial tone, heart contraction and food and water intake have used pharmacological doses of apelin. Thus, it is not certain whether physiological plasma concentrations of endogenous apelin may exert those effects. Further studies utilizing physiological doses of apelin are required to verify whether apelin may function as endocrine hormone regulating arterial tone, inflammation and food and water intake.

In contrast to our findings, plasma apelin levels were decreased in overweighted patients with newly diagnosed diabetes suggesting that factors other than adiposity may regulate apelin levels (Erdem et al., 2008). The minor changes in plasma apelin correlated with ΔBMI after the VLCD, but not after the WM. Recently, Castan-Laurell et al (Castan-Laurell et al., 2008) reported that plasma apelin and adipose tissue apelin mRNA levels decrease in response to a 3-month diet-induced weight loss in obese women (-6.7 ± 3.7 kg). Changes in apelin levels correlate significantly with TNF-α and insulin in a subgroup analysis of individuals with highest improvement (> 20%) in insulin resistance. Previously, apelin expression in adipose tissue has been shown to be up-regulated by TNF-α in mice (Daviaud et al., 2006). In addition, administration of an APJ antagonist decreases inflammatory cytokines including TNF-α in rats (Tiani et al., 2008). In humans, plasma apelin
levels are up-regulated by insulin and TNF-α (Boucher et al., 2005; Castan-Laurell et al., 2008), although a negative correlation has also been described (Tasci et al., 2008). In the current study, minor changes in circulating apelin correlate positively with TNF-α supporting the role of TNF-α as a regulator of apelin levels.

Since apelin is secreted by adipocytes in vitro (Boucher et al., 2005), we hypothesized that plasma apelin would correlate with abdominal fat deposits. However, the minor changes in apelin levels did not correlate with abdominal adipose tissue deposits measured by CT. In agreement with previous findings showing a close relation between leptin and body fat (Havel et al., 1996), leptin levels were correlated with BMI, VAT and SAT. Moreover, a negative correlation between adiponectin and VAT was observed. When the study group was divided into three groups according to the amount of weight loss, small decreases in plasma apelin, TNF-α, IL-6 tended to occur in individuals losing > 16.5 kg of weight. These findings contrast the pattern observed in leptin and adiponectin where significant changes occurred already after 6 and 12 kg weight losses, respectively. In addition, improvement of hypercholesterolemia without changes in body weight was accompanied with pronounced increase in plasma apelin, suggesting that body adiposity may not be a major regulator of apelin (Tasci et al., 2008). Thus, these results suggest that apelin is not that strongly correlated with the fat mass like the more abundant adipokines adiponectin or leptin.

The minor changes in circulating apelin in response to weight loss were linked to MAP. Systemic administration of apelin in anesthetized rats has been shown to decrease MAP via a NO-mediated mechanism (Tatemoto et al., 2001), the effects of peripherally administered apelin have been variable (Kagiyama et al., 2005; Mitra et al., 2006). In addition, APJ KO mice have normal blood pressure and heart rate and blockage of APJ does not affect blood pressure and heart rate in rats with portal hypertension (Ishida et al., 2004; Tiani et al., 2008). In healthy volunteers, local infusions of apelin-36 and [Pyr]-apelin-13 (from 420 pg to 1300 pg/kg min) into brachial artery and dorsal hand vein causes a NO-dependent arterial vasodilatation with no apparent affect on venous tone, systemic blood pressure or heart rate (Japp et al., 2008). Our results suggest that apelin may be involved in the regulation of systemic cardiovascular tone in humans via regulation of arterial rather than venous vasodilatation.

6.2. OXA levels in morbid obesity and PWS children

OXA is a neuropeptide abundantly expressed in CNS and GI tract regulating food intake, wakefullness, sleep and gastric motility and secretion. Although its physiological role in the circulation is currently unclear, we found that basal OXA levels are elevated in morbidly obese patients compared with lean and healthy age-matched controls when plasma OXA was measured
using EIA. Despite significant improvements in BMI, leptin and glucose levels, the decreases observed in OXA and lipid levels did not reach statistical significance. Elevated plasma OXA levels in morbid obesity measured with EIA contrast the RIA measured data in the literature. Adam et al. (Adam et al., 2002) found a negative correlation between plasma OXA levels and BMI in individuals with BMI ranging 19.59 kg/m². However, the overall variations in plasma OXA were minor (40-61.4 pg/ml). A negative correlation between BMI and OXA has also been described in obese women (Baranowska et al., 2005) and weight loss in obese children has been associated with increased plasma OXA (Bronsky et al., 2006). Plasma OXA levels have increased in fasting subjects and decreased upon refeeding with a negative correlation to leptin in lean men and women (Komaki et al., 2001).

Animal data mainly support the data obtained from RIA measurements. Orexin expression is down regulated in LHA in genetically obese ob/ob an db/db mice with high basal glucose levels (Yamamoto et al., 1999). In obese Zucker fatty rats, hypothalamic PPO mRNA levels are decreased and weight gain further decreases PPO expression. Surprisingly, in extremely obese and diabetic Zucker fatty rats, PPO expression is similar to lean and non-diabetic controls (Cai et al., 2000). In another study, hypothalamic OXA levels in obese fatty Zucker rats were similar to lean control rats (Taheri et al., 1999). Thus, plasma OXA levels in extreme obesity and diabetes may differ that of observed in modest uncomplicated obesity.

The discrepancy between our data and the literature in morbidly obese patients may also be caused by different methodology used. Orexin EIA detected full-length OXA and did not crossreact with OXA fragment (16-33), OXB, agouti-related protein (83-132)-amide, neuropeptide Y or leptin. The minimal detectable concentration given by the manufacturer was 0.37 ng/ml and all measured values were below that level. However, the standard curve enabled the comparison of the results between 10 and 100 pg/ml and hence, this exception was accepted. In addition, plasma was extracted prior to EIA analysis using methodology different from RIA measurements. Peptide was eluted in 80% ACN without 0.1 - 1% TFA widely used in Study III and in the literature (Adam et al., 2002; Baranowska et al., 2005). Using the same EIA after Sep-Pak extraction, Tomasik et al. (2004) reported plasma levels of 1000 pg/ml in healthy children of varying ages. These results are significantly higher than our EIA results and RIA values reported in the literature. Unfortunately, comparison of RIA and EIA was not possible, since the EIA used in the study I was discontinued by the manufacturer.

Decreased plasma OXA levels in PWS children measured with RIA are in line with previous data showing that PWS is characterized by low CSF OXA levels (Dauvilliers et al., 2003; Arii et al., 2004; Nevsimalova et al., 2005). Our findings indicate that in addition to central OXA deficiency observed in PWS, also peripheral OXA levels are suppressed. These findings suggest that decreased
OXA levels may reflect impaired orexin signaling in PWS. Sleep apnea is frequent in PWS (Holm et al., 1993; Nevsimalova et al., 2005). In addition, PWS patients have shown abnormal chemoreceptor response to hypoxia and hypercapnia (Arens et al., 1994; Gozal et al., 1994) and blunted arousal response to hypercapnia (Livingston et al., 1995). These findings suggest that ventilatory problems may not be explained by muscle weakness and obesity alone (Burman et al., 2001). OXA stimulates breathing via medullary and spinal pathways in rats (Young et al., 2005) and a lack of OXA in pre-proorexin knock-out mice leads to decreased peripheral chemosensitivity to carbon dioxide during wakefulness. The effect could be partially restored by i.c.v. supplementation of OXA (Deng et al., 2007). Thus, reduced OXA levels might contribute to the disturbed sleep pattern, wakefulness and impaired chemoreceptor response observed in PWS. In mice deficient of Magel2, one of four genes within PWS region in chromosome 15q11-q13, OXA peptide concentrations in the hypothalamus were 60% lower than for wild-type. However, no reduction in number of hypothalamic OXA neurons was observed in post mortem analysis of PWS patients (Fronczek et al., 2005).

Yet children with PWS had BMI < 25 kg/m², they were obese according to the BMI z-score, which is commonly used in the evaluation of obesity in children. Therefore, we cannot exclude the possibility that decreased OXA level is reinforced by obesity rather than PWS alone. However, PWS children had normal glucose tolerance, suggesting that complications of obesity have not developed. The control group was also significantly older and consisted of young adults, since use of children was considered unethical. We did not observe correlation between plasma OXA levels, which agrees with some, but not all earlier results. Kanbayashi et al. (2002) studied CSF levels of OXA in a large cohort of apparently healthy individuals with ages ranging from 4-months to 79-years and revealed no significant differences. Consistently, no correlation between age and OXA levels were observed in either study group in the current study. Tomaskik et al. (2004) analyzed plasma OXA in children and young adults with ages varying 0-18 years using EIA and found higher OXA levels in newborns and adolescents (10-15 y) than in young adults (16-18 y). In agreement with our data, no correlation to age was detected. Another study found that OXA levels correlated with age in 39-60 years old adults (Matsumura et al., 2002). These results suggest that lower OXA levels are not explained by different age in our study group.

The physiological significance and source of circulating OXA is currently unclear. Plasma OXA levels measured with RIA have varied between 1-100 pg/ml corresponding to 0.4-28 pmol/l in most studies. Some studies reported even higher levels of 175-847 pg/ml (50-240 pmol/l) (Dalal et al., 2001). Concentration of OXA in the circulation is comparable to other circulating peptides, including GLP-1 (16-50 pg/ml) (Carr et al., 2008) and CCK-8 (1-5 pg/ml) (Enck et al., 2009). Fasting serum gastrin concentration is usually 14-25 pg/ml and intact gastric inhibitory polypeptide
concentration varies 77-260 pg/ml in healthy volunteers (Carr et al., 2008). Plasma OXA levels are lower than most affinities of recombinant OX₁ and OX₂ in vitro. Sakurai et al. (Sakurai et al., 1998) used an iodinated [125I-Tyr^{17}]-OXA tracer and OX₁ and OX₂ transfected CHO cell line and found that IC₅₀ for OXA binding to OX₁ was 20 nmol/l. Another study utilizing [125I]-OXA tracer in OX₁ and OX₂ expressing CHO cells found EC₅₀ 20 nmol/l for OXA. (Shibahara et al., 1999). Similar concentrations have been required to trigger Ca^{2+} transient in CHO cells expressing human orexin receptors. OXA EC₅₀ for OX₁ was 30 nmol/l and 34 nmol/l for OX₂ (Sakurai et al., 1998). In locus coeruleus, administration of 10-30 nmol/l OXA was required to increase firing rate of neurons (Hagan et al., 1999). Surprisingly, Okumura et al. (Okumura et al., 2001) reported that EC₅₀ for OXA induced Ca^{2+} influx was as low as 68 pmol/l (240 pg/ml) for OX₁ and 57 pmol/l (200 pg/ml) for OX₂ using similar study setup and the same CHO cell line. The discrepancy in the results is not known, yet latter results are close to OXA levels detected in the circulation.

Comparable results have been obtained with the GI peptides GLP-1 and CCK. Studies with HEK293 cells overexpressing human GLP-1 receptors showed that EC₅₀ for GLP-1 was 2.6 nmol/l (8.6 ng/ml) and the concentration of 3.6 pmol/l (12 pg/ml) was required for activation of the receptor mediated transcription (Murage et al., 2008). For CCK-8, EC₅₀ values for receptor binding were 4.1 nmol/l (4.3 ng/ml) and Ca^{2+} mobilization 230 pmol/l (255 pg/ml) (Wu et al., 2008). Yet receptor binding affinity of OXA is slightly higher, plasma OXA levels are in the same range with other GI peptides detected in the circulation.

When administered to the circulation, half-life of OXA was 27 min which is long compared with insulin (6 min) or GLP-1 (3.3 min) (Ehrstrom et al., 2004). In CSF, OXA was more stable than OXB and its concentration after i.c.v. injection lasts for 4h, which is significantly longer than in blood stream (Yoshida et al., 2003). Iodinated OXA, but not OXB, has been shown to penetrate the blood-brain barrier by simple diffusion (Kastin and Akerstrom, 1999). Iodination has been observed to modify the properties of the peptide and thus, these results should be interpreted with caution (Bauer et al., 1996). Despite significantly reduced CSF OXA concentration in narcoleptic patients, no differences in plasma levels were observed (Dalal et al., 2001). Bingham et al. (2001) found that i.v. infusion of pharmacological doses of OXA (30 mg/kg) in rat and mouse was not reflected in the peptide levels in the brain. Therefore, it has been suggested that OXA in CNS and periphery may form two different pools (Ehrstrom et al., 2005a).

As discussed earlier in this thesis, OXA and its receptors are located in ENS. Administration of pharmacological doses of OXA has been shown to modulate glucose homeostasis, intestinal motility, CCK release, gastric acid secretion and duodenal bicarbonate secretion. Therefore, OXA detected in the blood stream may be originated from ENS reflecting the activity of orexigenic neurons in GI tract and pancreas. OXA-immunoreactivity has been located in pancreatic nerve
fibers and paravascular nerve bundles associated with blood vessels (Kirchgessner and Liu, 1999). In addition, orexin receptors have been detected in vagal afferent neurons (Burdyga et al., 2003). Orexin is co-stored with insulin in secretory granules in pancreatic β-cells, where it was released in response to hypoglycemia (Ouedraogo et al., 2003). Subcutaneous administration of OXA stimulated insulin release from β-cells in rats and both OXA and OXB stimulated insulin release in vitro perfused islets (Nowak et al., 2000; Nowak et al., 2005). In this thesis, we did not observe significant postprandial response in plasma OXA levels in PWS children and healthy individuals. In addition to GI tract and pancreas, orexins have been detected in several peripheral tissues, including adrenal gland, testis, ovary, cardiovascular system and adipose tissue (Heinonen et al., 2008). Therefore, further identification of factors regulating OXA signalling are required to determine the physiological significance of circulating OXA.

6.3. **Ghrelin responses to diet-induced weight loss and carbohydrate meal**

Ghrelin is an orexigenic peptide secreted by stomach that increases food intake and weight in animals and humans when administered in pharmacological doses (Tschop et al., 2000; Wren et al., 2001a; Wren et al., 2001b; Murakami et al., 2002). Currently, the physiological significance of ghrelin is controversial, since ghrelin-deficient and ghrelin receptor-deficient mice have normal growth rate and appetite (Sun et al., 2003; Wortley et al., 2004). In the current study, weight loss during the VLCD is accompanied with increase in plasma ghrelin, but the increase is attenuated and no longer significant after the 6 months of WM. Plasma ghrelin levels have been shown to increase in response to weight loss in obese subjects (Cummings et al., 2002b; Hansen et al., 2002) and during a 6-month diet and exercise induced weight loss in hyperlipidemic women (Santosa et al., 2007). Garcia et al. (2006) found an increase in plasma ghrelin after 6-month low caloric diet combined with orlistat treatment and exercise. However, after 1 year ghrelin levels were returned to the baseline. These findings suggest that plasma ghrelin level is attenuated in obesity and MetS. In addition, ghrelin increases in response to weight loss, but the increase may not be sustained during prolonged weight reduction.

Despite different insulin response in obese subjects with the metabolic syndrome, there was no difference in postprandial plasma ghrelin levels. The interaction between insulin and ghrelin is currently under debate. Administration of insulin has been shown to suppress plasma ghrelin levels in both healthy non-obese and obese individuals under hypo-, eu- and hyperglycemic clamp conditions (Saad et al., 2002; Flanagan et al., 2003; Leonetti et al., 2003). Significant negative correlation between plasma insulin and ghrelin levels has been observed in some studies.
(Monteleone et al., 2003; Tannous dit El Khoury et al., 2006), whereas no such correlation was observed in others (Shiiya et al., 2002; Leonetti et al., 2003). Postprandial ghrelin secretion profile resembles inversely that of insulin in response to macronutrients and isocaloric glucose sweetened beverages producing different insulin levels (Teff et al., 2004; Foster-Schubert et al., 2008). However, the postprandial decrease in plasma ghrelin levels in type 1 diabetic patients lacking insulin secretion was comparable to healthy and lean controls (Spranger et al., 2003). Insulin dependent modulation of ghrelin was less pronounced in insulin resistant and type 2 diabetic patients (Anderwald et al., 2003; McLaughlin et al., 2004). Therefore, it is possible that insulin resistance of the ghrelin producing cells may be a determinant of the postprandial ghrelin secretion (Erdmann et al., 2005; Krohn et al., 2006). However, mice lacking ghrelin and ghrelin receptor display improved insulin sensitivity, supporting involvement of ghrelin in the regulation of glucose homeostasis (Sun et al., 2006).
A widely used method for measuring peptide levels in biological samples is to utilize commercially available RIA and EIA kits. However, variations in the assay performance are likely to occur between laboratories. In the current study, two different commercially available methods were used to measure plasma OXA levels. The results from the EIA measurements in Study I contrast results obtained in obese subjects in other laboratories and the PWS children in Study III. The reliability of the RIA assay could be tested, while the EIA assay was no longer available.

The performance of the OXA RIA assay was analyzed in our lab (Heinonen et al. unpublished results). Ionization spectra of the standard peptide of the OXA RIA was compared with synthetic OXA purchased from NeoMPS (San Diego, CA, USA) using electro spray ionization tandem mass spectrometry (ESI-MS/MS) (LTQ, Thermo, San Jose, CA) after Sep-Pak C-18 (Waters Associates, Milford, MA, USA) extraction and subsequent fractionating with reverse-phase high-pressure liquid chromatography (RP-HPLC; Waters, Milford, MA, USA; Vydac C-18 column, 218TP510, Western Analytical Products Inc., Murrieta, CA, USA). Linear gradient from 0.1% TFA to 80% ACN in 0.1% TFA (40 min; 2 ml/min) was used and 1 min fractions were collected, dried overnight and used for RIA or ESI-MS/MS. OXA standard peptide added to extracted plasma eluted from the RP-HPLC in a single immunoreactive peak at 32 min (Figure 5C). Identical mass and tandem mass spectra to synthetic OXA was observed in the 32 min fraction (results not shown). To determine the linearity and recovery of RIA, 1 ml of pooled plasma was spiked with serial dilutions of OXA standard peptide (128, 64, 32, 16, 8, 4, 2, 1 pg) provided with the kit before applying to Sep-Pak. Same dilutions were added to extracted plasma prior to RIA. The recovery of added OXA above 32 pg/tube in plasma samples was poor (Figure 5A). This was not due to Sep-Pak, since OXA added to plasma after Sep-Pak displayed similar trend. The increase in plasma volume did not lead to a corresponding increase in OXA immunoreactivity, as observed earlier by Nishino et al. (2002) (Figure 5B). However, the sensitivity of the current ESI-MS/MS system was not sufficient to detect endogenous OXA from 1 or 2 ml of human plasma. These findings indicate that OXA RIA used in the measurement underestimates OXA concentrations above 32 pg/ml. However, values measured with RIA in study III were mainly below that and therefore values between PWS children and adult controls may be compared.

Total intra-assay CV% of the OXA RIA including Sep-Pak extraction was 16.7%, yet for RIA it was 4.2%. Inter-assay CV% of the RIA was 18%. The limit of detection using serial dilutions of plasma was determined between 4.6 pg/tube and 2.3 pg/tube. OXA immunoreactivity remained constant in plasma for 6 hours in room temperature. The recovery of 37 pg OXA after Sep-Pak was 77.8 ± 17.5%. Intra- and inter-assay CV% of the RIA were in line with the values reported by the
manufacturer. However, when Sep-Pak extraction was taken into account, the intra-assay CV% increased to 18.8% exceeding the generally accepted level (< 10%). Thus, the use of internal standards to compensate the variability between the assays is highly recommended.

Figure 5. The linearity OXA RIA in plasma. (A) Measured concentrations of serial dilutions standard peptide in RIA buffer and in 1ml of pooled plasma added before or after Sep-Pak (n = 4; ***p < 0.001). (B) Recovery of 37 pg OXA added either before or after the extraction in different amounts of pooled plasma (n = 4). (C) OXA detected in RP-HPLC separated fractions of 1 ml plasma with or without added OXA and 2 ml of plasma.
8. FUTURE ASPECTS

Apelin stands in the cross-roads of obesity, inflammation and cardiovascular system. Apelin was named an adipokine, since it is secreted by adipocytes and its expression is increased in obesity (Boucher et al., 2005). In contrast to well established adipokines leptin and adiponectin, which are produced mainly by adipocytes, apelin was discovered from rat stomach. A wide expression pattern in non-adipose tissues, including vascular endothelium, heart, lung, mammary gland, hypothalamus and GI tract also indicates that apelin may not be secreted by adipose tissue alone. In addition, apelin mRNA expression is higher in stromal-vascular fraction than in adipocytes in rat subcutaneous and retroperitoneal fat pads (Garcia-Diaz et al., 2007). These findings suggest that in addition to its role as an adipokine, apelin is likely to possess other functions. In the current study, plasma apelin levels were increased in obesity, yet correlations to arterial pressure and inflammation rather than body adiposity were observed. Animal studies have shown that apelin may stimulate cardiac contractility and cause arterial vasodilatation, which would be beneficial for patients with cardiac insufficiency. However, Japp et al. (2008) did not observe changes in systemic blood pressure and heart rate after local intravenous and intra-arterial administrations of apelin in humans and therefore, studies using systemic administrations of apelin in physiological concentrations are indicated.

Adipose tissue has an exceptional ability to grow and regress throughout adulthood. It is highly vascularized and an extensive capillary network surrounds each adipocyte. Angiogenesis has been suggested to be a limiting factor of adipose tissue expansion (Rupnick et al., 2002). Intriguingly, apelin has been shown to induce retinal angiogenesis and retardation of retinal vascular development was observed in apelin KO mice (Kasai et al., 2004; Kasai et al., 2008). Inhibition of apelin-APJ signaling reduces splanchic neovascularization to half and suppresses important proangiogenic factors in rats with portal hypertension (Tiani et al., 2008). Proangiogenic effect of apelin has been also detected in adipose tissue (Kuduzova et al., 2008). Hypoxia is a major driver of angiogenesis also in adipose tissue and stimulates expression of leptin and angiogenic factors (Lolmede et al., 2003). Apelin expression is stimulated by hypoxia in cultured rat cardiomyocytes (Ronkainen et al., 2007) and adipocytes (Kuduzova et al., 2008). Therefore, apelin might potentiate the increase of adipose tissue mass in obesity as well as regulate vascular endothelial functions in other tissues.

In addition, apelin has been shown to induce intestinal endothelial proliferation and CCK release in rats suggesting that apelin might stimulate endothelial growth in the gut (Wang et al., 2004). Interestingly, apelin has been expressed in mammary gland and it is abundant secreted into colostrum (Habata et al., 1999). Apelin mRNA expression is increased in fetal and post-natal
stomach than in the adult rat stomach (Wang et al., 2004). Thus, apelin could modulate the maturation of the intestinal endothelium in fetal and post-natal states.

In addition to its central role in the regulation of food intake, sleep and wakefulness, OXA has been shown to participate in several functions in the peripheral tissues. As shown in the current study, orexin A is detected in plasma and its level varies in response to the metabolic state. Thus, studies further defining the modulatory role of OXA in peripheral tissues are implicated. Recently, orexin receptor expression was shown to be strongly upregulated during proestrus in rat ovaries and similar upregulation was observed in the hypothalamus and pituitary indicating the involvement in ovulation. Since PPO has not been detected in the ovaries, these results suggest a role for circulating OXA (Silveyra et al., 2007).

Ghrelin is an orexigenic peptide whose role in the regulation of short and long-term energy balance seems likely. However, since phenotypic changes in ghrelin-deficient and ghrelin-receptor deficient transgenic animals are subtle, the relative importance of ghrelin in energy homeostasis is controversial (Cummings, 2006). Ghrelin levels are decreased in obesity and as shown also in the current study, ghrelin levels increase in response to weight loss. Attenuated plasma ghrelin in obesity and MetS therefore might reflect transient protective response against overfeeding. The efficacy of anti-ghrelin treatment is currently unproven in human obesity. Fasting ghrelin levels are increased in anorexia nervosa and cachexia and they return to normal levels after weight recovery (Hosoda et al., 2006). Thus, administration of ghrelin and ghrelin antibodies may offer potential for pharmacologic treatment of eating disorders and obesity.
9. SUMMARY

In the current study:

I Plasma apelin levels were increased in morbid obesity, but correlation to body adiposity during diet-induced weight loss was weaker than for the abundant adipokines leptin and adiponectin. The minor changes in apelin levels in response to a pronounced diet-induced weight loss were related to arterial pressure and inflammation in patients with MetS.

II Plasma OXA levels measured with EIA were increased in morbidly obese patients.

III Plasma OXA levels measured with RIA were decreased in children with PWS.

IV Postprandial suppression of plasma ghrelin was impaired in patients with MetS independently of insulin after different carbohydrate-rich meals. In addition, ghrelin increased in response to diet-induced weight loss in patients with MetS, but the increase was not sustained during prolonged weight maintenance.
10. REFERENCES


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