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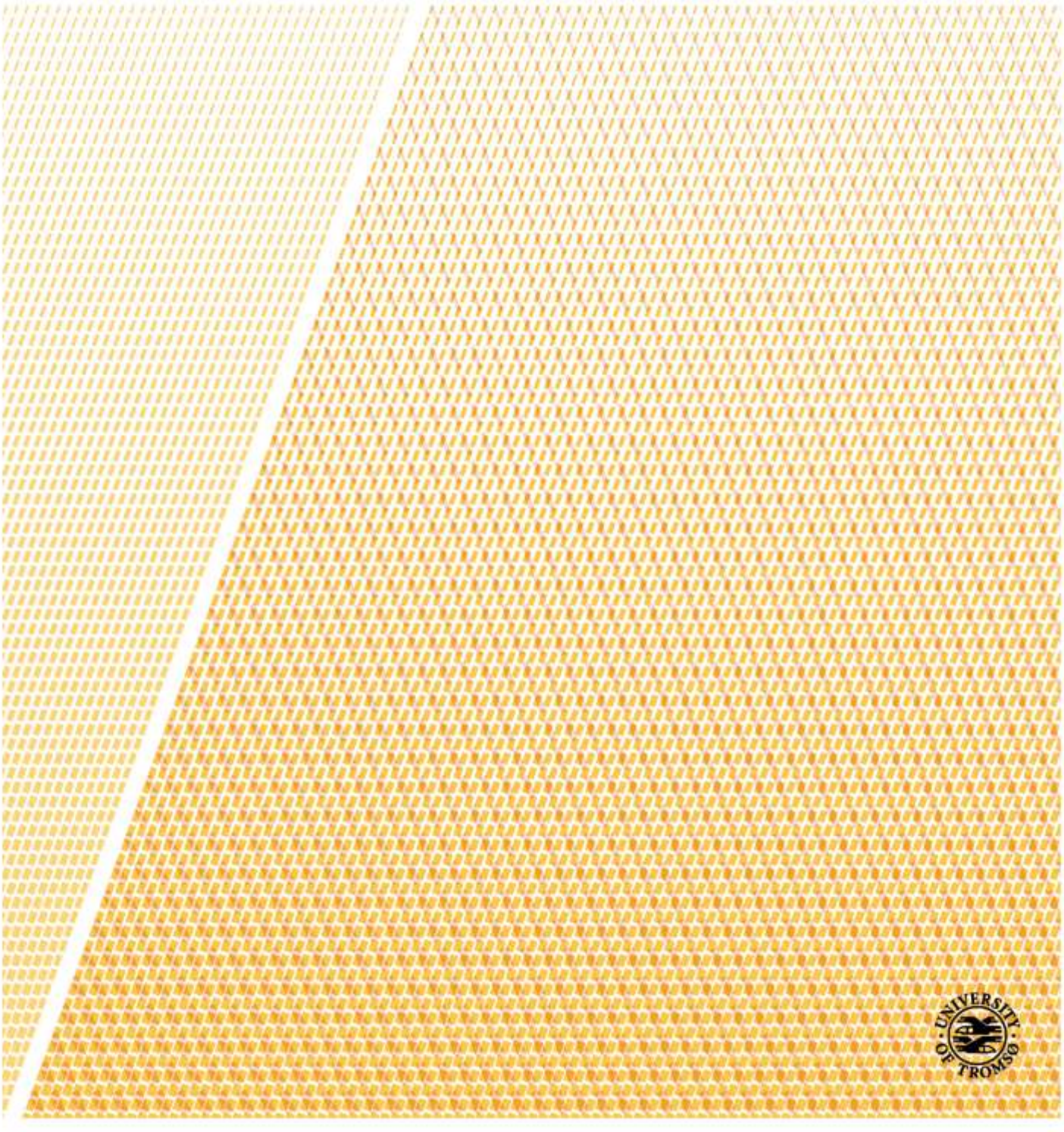
Early markers of metabolic disease in obesity

A study of postprandial triglycerides, leptin and adiponectin interactions in the view of normal and dysregulated metabolism



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I dedicate this thesis to my dear mum, Anita Elida Larsen.

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List of papers

Larsen MA, Goll R, Lekahl S, Moen OS, Florholmen J. Delayed clearance of triglyceride-rich lipoproteins in young, healthy obese subjects. Clin Obes. 2015 Dec;5(6):349-57. doi: 10.1111/cob.12118. Epub 2015 Oct 15. PubMed PMID: 26469529; PubMed Central PMCID: PMC5111784.

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Abbreviations

ApoA-I, Apolipoprotein A-I
ApoB-100, Apolipoprotein B- 100
ApoB-48, Apolipoprotein- B48
ATP-III, Adult Treatment panel III
BMI, Body mass index
CM, Chylomicrons
CM-TGR, Chylomicron triglyceride response
CRP, C-reactive protein
CVD, Cardiovascular disease
DEXA, Dual- X-ray- absorptiometry
DHA, Docosahexaenoic acid
EPA, Eicosapentaenoic acid
HDL, High density lipoproteins
HDL-C, High density lipoproteins cholesterol
HOMA-IR, Insulin resistance by the homeostasis model assessment
IR, Insulin resistance
IFG, Impaired fasting glucose
L:A ratio: Leptin to Adiponectin ratio
LDL, Low density lipoprotein
LPL, Lipoprotein lipase
LR, Leptin resistance
MDO, Metabolic dysregulated obese
MHO, Metabolically healthy obese
NaCl, Natrium Chloride
NCEP/ATPIII, National Cholesterol Education Panel/Adult Treatment panel III
NGT, Normal glucose tolerance
NSD, Norwegian Science Data Service
NPV, Negative predictive value
OFTT, Oral fat- tolerance test
OGTT, Oral glucose tolerance test
PUFA, polyunsaturated fatty acids
PPV, Positive predictive value
REE, Resting energy expenditure
RM- ANOVA, repeated measure analysis of variance
ROC, Receiver operating characteristic
SE-TG, Serum triglyceride
TG, Triglycerides
TGR, Triglyceride response
TRL, Triglyceride-rich lipoproteins
T2DM, Type 2 diabetes mellitus
USA, United States of America
VLDL, Very low-density lipoproteins
WBISI, Whole body insulin sensitivity index

1 Introduction

1.1 Obesity at a glance

Overweight and obesity are increasing global health problems with several metabolic disturbances and co-morbidities, such as type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). CVD is the leading cause of morbidity and mortality in industrialized countries, with obesity as an independent risk factor[1], and it was the main cause of death worldwide in 2012[2]. In the United States of America (USA), diabetes indirect and direct costs have been estimated to rise 26% from 2012 to 2017, now estimated to cost in total 327 billion USD yearly in 2017[2]. Furthermore, T2DM is calculated to rise from 175 million people in 2000, to 353 million people in 2030, worldwide[3]. Also, the incidence of diabetes is expected to be especially high in developing countries, the next 25 years[3]. If this was not enough, obesity, and especially abdominal obesity is also linked to the risk, and the prognosis of common cancers as colon cancer, breast cancer, endometrium cancer and prostate cancer[4].

The diagnosis of obesity is often based on body mass index (BMI), calculated as bodyweight in kilograms (kg) divided by height in meters (m) squared (kg/m^2). Individuals with a BMI from 25 to 29.9 are classified as overweight, whilst those with a BMI ≥30 are classified as obese. Abdominal obesity is defined as waist circumference ≥88 cm for women, and ≥102 cm for men[5]. A high BMI is considered the sixth most important risk factor for global death and disease burden[6]. Environmental challenges including a sedentary lifestyle and excessive intake of processed food are factors that are thought to contribute to the increased prevalence of obesity[7,8]. However, the reason for the obesity epidemic in the world is complex, and still not fully understood. What we do know is that both environmental and genetic factors are of importance.

1.2 Metabolic dysregulation and obesity

1.2.1 Insulin resistance

Insulin resistance (IR) is the precursor for T2DM, and has a high prevalence in abdominal obesity. A study[9] on the prevalence of IR in non-diabetic individuals (defined as a fasting glucose <6.7 mmol/L), found that 26% of individuals with a BMI≥30, and 60% of individuals with a BMI ≥ 35 had IR. In this study IR was measured by euglycemic clamp, the gold standard method for defining IR[10]. Because this method is both expensive, and not very suitable in a clinical setting, other methods have been developed including the homeostasis model assessment (HOMA-IR)[11], and measurement of insulin sensitivity (IS) by the Whole body insulin sensitivity index (WBISI)[12]. Fasting insulin is also used as a marker of IR, and a significant correlation between fasting insulin and glucose uptake during euglycemic clamp has been reported[13]. Furthermore, in a clinical setting the connecting peptide, also known as the c-peptide, is measured to mirror the insulin level in the body. The main difference between HOMA-IR and WBISI as methods, is that HOMA-IR is calculated by fasting values of insulin and glucose, whilst WBISI is calculated also by postprandial values. HOMA-IR might therefore be more suitable for a clinical setting. There is no standardized cut-off values for either HOMA-IR or WBISI, and as there is no standardized way to measure insulin[14], this complicates the standardization of these two methods.

1.2.2 Hypertriglyceridemia

Hypertriglyceridemia is the typical lipid disturbance in overweight and obesity [15], and contributes to atherosclerosis. A study from 2013 found that elevated TG was independently associated with the metabolic syndrome (MetS), and also a likely predictor for IR in individuals with an increased waist circumference (≥85 cm for women, ≥102 cm for men) [16]. The same study also found TG to have a negative correlation with the adipokines, adiponectin, and have a positive correlation with high sensitivity C-reactive protein (CRP) and fasting insulin levels. We know that lipid disturbances are central for the development of atherosclerosis, with hypertriglyceridemia as an independent risk factor

[17,18]. However, our knowledge about different levels of hypertriglyceridemia, and how it might affect other risk factors in obese individuals is limited, and needs more investigation.

1.2.3 Obesity and stress

Stress-related cortisol secretion is associated with abdominal obesity and its metabolic complications[19,20]. One of the first to propose this was Bjørntorps group in 1996[21], and later reviewed in 2000[22] and 2001[23] . Moreover, it is well documented that glucocorticoid treatment increases leptin levels, and at the same time eating behavior, in obese women[24]. During the last years there has been numerous publications covering this issue, recently published by a systematic review and meta-analysis in 2018 by Tenk et al[25]. The authors conclude that perceived stress correlates with visceral obesity, and lipid parameters of metabolic syndrome, but the results contradict mainly due to gender differences between the individual studies. So far, theoretical framework for a causal etiology is unclear. Most likely, gender and stress require additional genetic factors to induce abdominal obesity. The glucocorticoid metabolism inside the adipocyte has been shown to be altered in morbidly obese subjects, with a BMI ≥40, while restored following surgery induced fat-loss[26]. The potential key role of visceral obesity in the association between perceived stress and dyslipidaemia, or diastolic blood pressure are discussed together with potential moderators (e.g. sex-differences, variations in stress assessment and metabolic syndrome criteria) that may explain the inconsistent, contradictory results of the different studies.

1.2.4 The metabolic syndrome

As mentioned, obesity is closely related to metabolic disturbances, and in particular the MetS. The MetS [5] is a cluster of metabolic disturbances; There are mainly two different definitions of the MetS, but they all consist of the risks factors: IR, hypertension, dyslipidemia and abdominal obesity, which in turn are major risk factors for T2DM and CVD [27,28]. To be diagnosed with the MetS, individuals have to have 3 out of the 5 of the following criteria (NCEP/ATPIII[3]): abdominal obesity (women ≥88 cm, men ≥102 cm, Europe: women ≥80 cm, men ≥94 cm), elevated fasting TG ≥1.7 mmol/L, hypertension ≥130/≥85 mmHg, reduced high density lipoprotein cholesterol (HDL-C)

(Women: < 1.29 mmol/l, men <1.03 mmol/L) and elevated fasting glucose (≥5.6 mmol/L) [5,29]. Or they have to have abdominal obesity and 2 out of the other risk factors mentioned above (International diabetes federation, IDF[29]). Different ethnicities also have different cut-off values for waist circumference. In Norway, 70% of the obese patients referred to weight-loss treatment, had the MetS[30]. In another report 53 % of Italian, obese adults had the MetS[31]. Other studies have shown that as much as 80% of adults with diabetes have MetS[32], while 15% of adults without T2DM are estimated to have the MetS[33]. Individuals with the MetS have doubled risk to develop CVD events in a 5-10 year period, and they have five times as high a risk to develop diabetes compared to individuals without the MetS[5,32]. In a clinical setting, the aim is to calculate the patients overall risk, and treat the different diseases individually, in addition to lifestyle interventions. The different components of the MetS affect each other with complex mechanisms, directly or indirectly, warranting further investigation.

Table 1. Definition of the metabolic syndrome

Definition of the metabolic syndrome[5]. *In order to make a diagnosis of the metabolic syndrome the patient must present with three or more out of the five criteria below*	
Waist circumference	Men ≥102 cm Women: ≥88 cm Europeans: Men: ≥94 cm Women: ≥80 cm) *Other ethnicities have their own cut-off values.*
Hypertriglyceridemia (fasting) *or treatment for hypertriglyceridemia*	≥1.7 mmol/L
Reduced HDL-C *or treatment for reduced HDL-C*	Men: <1.0 mmol/l (<1.04) Woman: <1.3 mmol/l (1.29)
Hypertension *or treatment for hypertension*	Systolic blood pressure ≥135 mmHg and/or diastolic blood pressure ≥85 mmHg.
Impaired fasting glucose *or treatment for dysglycemia or T2DM*	≥5.6 mmol/L

1.2.5 The metabolic healthy obese individual

Not all obese individuals have established metabolic disease. Approximately 32 % of obese adults (age ≥ 20 years old) in the USA are considered metabolically healthy obese (MHO) individuals, having less than two metabolic disturbances (elevated blood pressure; elevated triglyceride and glucose levels; IR (HOMA-IR ≥5.13); systemic inflammation (elevated CRP); and decreased HDL-C level)[34]. When considering the NCEP/ATP-III criteria for Met S the prevalence of MHO were higher at 39%[34]. In the same study, 51% of overweight individuals were considered metabolically healthy[34].

However, studies with long follow-up periods have demonstrated that apparently MHO individuals are at an increased risk of major CVD events[35,36] and total death[35], as compared to metabolically healthy normal weight individuals. This supports the belief that MHO is not a benign condition, and that further studies are needed to know more about the prognosis of this group.

1.2.6 The pro-inflammatory state in obesity

Obesity causes increased oxidative stress[37], and also chronic subclinical inflammation. The latter is related to the pro-inflammatory actions of the adipocytokines (for review see [38]). In addition to endothelial dysfunction and hypertriglyceridemia, oxidative stress and subclinical inflammation are

contributing mechanisms to CVD[39]. In a study among obese adolescents, oxidative stress was increased, and might be associated to TG metabolism and dyslipidemia[37]. Another study showed that macrophages and fibrosis in adipose tissue were linked to both liver damage, and metabolic risk in obese children[40]. In addition, adipose tissue-resident macrophages are positively correlated to clinical measures for metabolic dysregulation such as IR (measured by HOMA-IR), serum leptin and total cholesterol: HDL-C ratio[41]. Also, both metabolically healthy and unhealthy overweight and obese individuals have higher levels of high-sensitivity CRP (≥3 mg/L) and higher levels of hepatic steatosis (by abdominal ultrasound) than normal weight individuals according to a study from 2015[42]. The main unresolved question is whether this oxidative stress and low-grade inflammation are primary or secondary events to obesity. So far, it is well documented that adipose tissue-resident macrophages play a crucial role in the pathogenesis of obesity, and a secondary-driven inflammation and metabolic complications (for review, see [43]). In addition, adipose tissue-resident T-lymphocytes increase, the higher the level of adiposity in modest overweight and obese men[41]. However, these adipose tissue-resident T-lymphocytes were not related to any typical clinical measures for metabolic dysregulation, as the adipose tissue-resident macrophages were, except for the gene expression of leptin and serum leptin. Most likely, there is a combination of obesity and genetics, but further studies are needed to explore this, and other potential explanations before a comprehensible understanding of this so far complex issue.

1.3 Postprandial lipoprotein metabolism

Individuals in industrialized countries spend a most part of their lives in the non-fasting, postprandial state. Consuming food with fatty acids elevates the TG in the blood. As mentioned hypertriglyceridemia is the typical lipid disturbance in overweight and obesity [15]. Of interest is that postprandial hyperlipidemia also has been associated to overweight [15], and especially to abdominal obesity [44-47]. The plasma lipoproteins consist of five major classes, in addition to several subclasses. They all differ in size, composition, and can be separated by ultracentrifugation. TG is mainly transported by triglyceride rich-lipoproteins (TRL), which contain liver derived apolipoprotein B100 (ApoB-100), containing Very-Low-Density-Lipoproteins (VLDL), which mainly transport endogen

(body derived) TG. On the contrary, the intestine-derived Apolipoprotein B48 (ApoB-48), containing the largest lipoprotein, chylomicron (CM), transport diet-derived TG and cholesterol from the intestine to peripheral cells and their remnants[48]. Furthermore, CM remnant particles can penetrate the endothelial wall efficiently; they are retained selectively in early atherosclerotic lesions of the vessel wall[49,50], and contribute to CVD by delayed elimination of postprandial TRL[51,52]. The postprandial TG is not measured in a clinical setting, because it is time consuming and expensive. Finding other biomarkers, that mirrors the postprandial triglycerides, and especially CM, would be of clinical importance, because of its connection to CVD.

1.3.1 Lipoprotein lipase

Lipases are water-soluble enzymes that hydrolyze ester bonds of water insoluble substrates such as TG, phospholipids and cholesteryl esters. The enzyme lipoprotein lipase (LPL) is synthesized in the parenchymal cell, primarily in adipose tissue and myocytes. LPL plays an important role in the lipoprotein metabolism by hydrolyzing TG in CM and VLDL. To initiate this LPL must transfer to endothelial cells, and translocate from the abluminal to the luminal side, where LPL associates in complex with heparan sulphate proteoglycans[53]. Formation of TRL remnants is a result of activation of LPL, and results in hydrolyzing of CM and development of TG in small CM remnants[54]. Furthermore, hydrolyzing TG in VLDL assembled in the liver, contributing fatty acids to the vascular endothelium, and finally remove them from the bloodstream [55]. CM and VLDL compete for LPL. At plasma TG levels of more than 5.6 mmol/L, LPL actions are saturated, leading to defects in clearance of both VLDL and CM[56]. However, our state of the art knowledge of LPL actions is still limited. Further investigations are needed, for a more or less complete understanding of the interaction between TG, and other risk factors for metabolic complications.

1.3.2 HDL-cholesterol and postprandial lipoprotein metabolism

For several decades, studies have consistently pointed out HDL-C as an independent risk factor for CVD[57]. However, recently there have been some studies that failed to show improvement in CVD risk, despite increased HDL-C, when subjects were treated with HDL-C raising agents[58,59]. This has raised the question of HDL-C as a potential biomarker, rather than directly participating in the process in

developing CVD. The lipids of HDL-C are mainly cholesterol esters (85%) and a small amount of TG (15%). HDL-C is usually divided into two subclasses; HDL2 (large) and HDL3 (small). Moreover, the greater the magnitude, and duration of the postprandial TG response, the arterial wall will be more exposed to postprandial TRL. Longer duration of the postprandial TRL in the bloodstream will give more time to replace cholesterol ester in LDL-C and HDL-C, favoring the transformation of LDL-C to be a smaller and more pro-atherogenic particle, and making HDL-C more dysfunctional. Moreover, the mechanism of TRLs influence on lowering the HDL-C level, is believed to be due to the enrichment of TG to the HDL-C particle, which leads to increased catabolism of Apo-A-I HDL-C (for review see[60]). HDL-C is often[61,62], but not always [63] inversely correlated with postprandial triglyceridemia, which is an in vivo measurement of LPL action. Via this indirect analysis, HDL-C levels are sometimes viewed as an index of the activity of LPL in vivo. Our knowledge of HDL-C in relation to the other risk factors is limited in obese individuals, and further investigations are needed.

1.3.3 Insulin resistance and postprandial lipemia

Under fasting conditions, the hepatic production of VLDL is induced, whilst the increase of postprandial insulin reduces VLDL production. In addition, LPL activity in the vascular endothelium is regulated by insulin; The IR typical found in overweight and obesity [64] may contribute to a delayed removal of postprandial TRL and is highly association to overweight [15] and especially to abdominal obesity [44-47]. Individuals with an impaired fasting glucose (IFG) and impaired glucose tolerance have an increased postprandial TG response, and also higher muscle TG extraction, compared to normal glucose tolerant (NGT) subjects[65]. These metabolic disturbances most likely contribute to the development of IR[65], by complex and not fully understood mechanisms. Therefore, more studies are needed to bridge the gap between insulin sensitivity, IR and postprandial TG.

1.3.4 Postprandial lipoprotein metabolism and atherosclerosis

Total cholesterol, LDL-C and HDL-C are established as independent risk factors for atherosclerosis and CVD[57]. However, the importance of TG as a clinical parameter regarding CVD risk has not yet been fully established. TG is commonly measured in the fasting state, however TG increases significantly in the postprandial state, and an important role in the pathogenesis of atherosclerosis-

related diseases has been postulated for postprandial lipids. In 1979, there was a milestone in the understanding of the atherosclerotic process, when a reduced and prolonged clearance of postprandial accumulation of TRL, was found as one of the main pathophysiological events in the atherosclerotic process[66]. This was later supported by studies showing that postprandial triglyceridemia contribute to CVD by delayed elimination of postprandial TRL[51,52], and CM remnant particles penetrate efficiently, and are retained selectively in the early atherosclerotic lesions of the vessel wall[49,50].

Several recent studies have shown TRL as independent risk factor for CVD[67,68], including an increased risk of ischemic stroke[69]. In addition, a recent Mendelian randomization study suggested that lifelong exposure to remnant TRLs is causal for CVD risk, independent of low plasma HDL-C[70]. Despite this, our knowledge of postprandial TG is limited in obese individuals, especially for postprandial TG metabolism focusing on CM.

1.3.5 Factors affecting postprandial triglycerides

There are several factors affecting postprandial TG, including dietary habits, physical activity and medical treatment. Dietary habits affect both postprandial and fasting levels of TG. One study from 2013 reported that reduced meal frequency of 3 meals vs. 6 meals per day resulted in reduced total postprandial TG concentrations[71]. A 2-week diet, where 25% of the daily energy requirements (E) consisted of high- fructose corn syrup in young adults (BMI 18-35) resulted in increased 24 h postprandial TG, increased fasting LDL-C and Apo-B, comparable with fructose and more than glucose[72,73]. The effect on postprandial TG was highest 4 to 6 h post dinner, in the evening[72]. Similarly, in a small study of overweight and obese women the 14 h postprandial TG was 141% higher, and fasting Apo-B 19% higher than baseline after a 10- week diet with 25% E consisting of fructose, however no significant changes were observed in fasting LDL-C[74]. When it comes to dietary intake of meals containing olive oils, with oleic acid (which is rich in N-9 poly unsaturated fatty acids (PUFA)), results in a higher ApoB-48 response compared to palm oil, safflower oil (rich in N-6 PUFA) and a mixture of fish- and safflower oil[75], and other dietary oils[76,77]. However, recently it has also been reported that a meal containing extra virgin olive oil decreased postprandial TG and apo-B48 in individuals, in addition to postprandial glucose in individuals with IFG[78]. Furthermore, N-3

PUFA has shown to reduce the hepatic production of VLDL particles, favor free fatty acid (FFA) oxidation, enhance both chylomicron and VLDL clearance[79], and increasing LPL activity[80]. At high levels of chronic intake (3-4 g/day), N-3- PUFA also lower postprandial TG[79,80]. However acute intake of doses ≥10 grams, long chain N-3 PUFA, in a fatty meal can decrease the postprandial TG response, partly through an increase of post-heparin LPL- activity[81]. Both Eicosapentaenoic acid (EPA), or Docosahexaenoic acid (DHA), in N-3 PUFA, has been shown to be equally effective[80]. Traditionally low-fat high carbohydrate diets were considered the best anti-atherogenic alternative[82]. This is supported by a study with a 12- week diet where postprandial TG was improved by a low- fat high complex-carbohydrate diet, supplemented with N-3 PUFA and high-monounsaturated fatty acid, in individuals with the MetS, compared to other diets[83]. Polyphenols has also shown to reduce postprandial TG, in addition to reduce oxidative stress in obese individuals with the MetS[84]. Also, physical activity and regular activity breaks compared to prolonged sitting and inactivity, lowers postprandial TG[85]. Furthermore, the medicine Ezetimibe has in some studies shown to improve postprandial hypertriglyceridemiae[86], in addition to IR[87]. Despite these studies reported above, our knowledge of postprandial TG in obese individuals is far from completely understood. Furthermore, adipocytes play a pivotal role in obesity, but how they may affect the postprandial TG is so far unsettled.

1.4 The adipocyte as an endocrine organ

White adipose tissue (WAT) is a highly metabolically active endocrine organ (for review see [88]), and more than 600 adipokines has been described[89]. Several of these adipokines are pro-inflammatory, so called adipocytokines. Adipocytokines have direct influence on cellular metabolism. Among them are tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), which in turn are believed to contribute to metabolic disturbances associated with obesity[90]. TNF-α, for example, directly decreases insulin sensitivity[91], and increases lipolysis in the adipocyte[92]. IL-6 on the other hand leads to hypertriglyceridemia in vivo by increasing lipolysis and hepatic TG secretion in rats[93]. It is the elevated amount of activated macrophages in the WAT that are found to account for the elevated production of TNF-α and IL-6, and the number of macrophages are increased when the adipocyte size

increases[90]. In addition, the adipokines leptin and adiponectin are mainly secreted from WAT[88]. The adipocyte as an endocrine organ, is also linked to breast cancer in postmenopausal women[94]. Furthermore, adipokines play a pivotal role in the inflammation process and in the development of non-alcoholic-fatty liver disease [95](review). In summary, the adipocytes as an endocrine organ play a multifunctional physiological and pathophysiological role in normal weight and obese individuals, respectively. However, our knowledge of adipokines is far from complete, and needs further investigation.

1.4.1 Leptin

Leptin, mainly secreted from WAT, is stimulated by insulin, and leptin significantly correlates with insulin[96,97]. Furthermore, leptin is most commonly known as a satiety-, fertility- and weight regulating hormone in low-leptin states. Leptin levels are pulsatile[98], and have been implicated in the regulation of satiety, fertility, the immune system, bone metabolism and resting energy expenditure (REE) [99](review). Also, leptin suppresses adipocyte lipogenesis, increases TG hydrolysis and FFA and glucose oxidation [100]. Leptin, plays a pivotal role in gating surplus of lipids from circulation to the adipose tissue, whilst leptin sensitive individuals seem to protect non-adipose tissue from lipid uptake, and hence lipotoxity [101]. Furthermore, leptin is also linked to cancer, through gastric leptin signaling and gastric cancers[102], and has a pro-carcinogenic role in breast cancer (for review see [103]). In the obese and diabetic state circulating levels of leptin are increased. Studies show that increased levels of leptin are directly or indirectly associated with atherogenicity and cardiovascular health [104] (review). Supporting this, an association between leptin and oxidized LDL cholesterol has been found in postmenopausal women[105]. Moreover, when the level of serum leptin reaches 25-30 mcg/L, the concentration of leptin in the cerebrospinal fluid and brain tissues does not further increase[106], potentially driving central leptin resistance (LR). The concept of LR in obesity is still complex, and no validated cut-off value or standard measurement for leptin and LR have been validated. However, LR can be calculated indirectly by the REE to serum leptin ratio[107]. A diet rich in N3-PUFA has shown to reduce plasma leptin, and a individuals consuming a diet rich in fish compared to a vegetarian diet had significantly reduced leptin levels[108]. When it comes to leptin in the postprandial state, the results are

diverging, with reports of no postprandial changes in leptin[109-111], as well as increased postprandial leptin levels in normal weight individuals and decreased in obese individuals[112,113]. Furthermore, a few studies have examined the fasting adipokine profiles of MHO individuals [114-116]. Two of the studies found significant difference in leptin [114,116], whilst the study by Philips et al did not[115]. Our knowledge of postprandial leptin in normal weight and obese individuals, as well as fasting leptin levels in MHO individuals is limited. Finally, the relationship of adipokines and postprandial lipidemia in MHO is unknown.

1.4.2 Adiponectin

Adiponectin is circulating in the blood in three different isoforms; trimeric, hexameric, and multimeric high–molecular weight (HMW) isoforms. All levels of circulating adiponectin isoforms are 30-80% heritable, suggesting a genetic link[117] (review). Interestingly, adiponectin is reduced in the obese and diabetic state, and has shown to have protective and anti-atherogenic actions [118] (review). Some studies have found increased fasting adiponectin levels in MHO[119,120]. In addition, there are diverging reports about adiponectin. In individuals with a low BMI and chronic illness, and in elderly individuals, recent studies show that adiponectin might be associated to increased all-cause mortality and increased cardiovascular mortality [117,121-123]. There are several hypothesis that suggest an increased adiponectin is associated with a higher mortality in some subjects, and potentially explained by adiponectin resistance[124]. Adiponectin has two different receptors, Adiponectin receptor 1 and 2, and these have been demonstrated to be altered in diabetic states, and in animal models with high-fat diets[125]. Several reports show that adiponectin resistance is connected to reduced adiponectin receptor sensitivity, decreased receptor expression, in addition to dysfunctional downstream signaling [124]. There is also evidence that a modest weight gain of 5% in healthy normal weight individuals increase the fasting levels of adiponectin, which correlated positively with changes in leptin suggesting a protective role in weight gain[126]. However, impaired leptin signaling, in relation to increased caveolin-1-expression in obesity, may prevent concordant increases in adiponectin despite high levels of leptin[126]. While leptin has a pro-carcinogenic role, adiponectin has the opposite; an anti-carcinogenic role in breast cancer (for review see [103]). As the case for leptin, our knowledge of postprandial

adiponectin and its potential actions in the postprandial state, in normal weight and obese individuals is low. Recently, much attention has been on the fibroblast growth factor-21 (FGF-21)-adiponectin axis [127], which has been proposed to protect against a various cardio-metabolic disorders via mediating multi-organ communications (for review, see Hui [128]). One study of healthy, normal weight men, found that walnuts, rich in N-3-PUFA increased postprandial adiponectin[129]. Another study showed significantly lower postprandial adiponectin, compared to baseline levels, in normal weight men with non-alcoholic-fatty-liver disease, compared to healthy normal weight controls[130]. The reports of the postprandial response of adiponectin are diverging, and reports have found both increased [130-132], and unchanged [129,132-135] postprandial adiponectin levels for both normal weight and obese individuals. As with leptin, there are varying results also on postprandial adiponectin levels in MHO. One study from 2010 finding significant higher adiponectin levels in MHO[116], whilst the other studies did not find any difference[114,115]. As the results are diverging, further studies are needed to understand the complex mechanism of adiponectin.

1.4.3 The Leptin:Adiponectin ratio

The relationship between leptin and adiponectin have made the basis for the Leptin:Adiponectin ratio (L:A ratio), which has shown to be a sensitive marker for the MetS[136], insulin sensitivity and a potential atherogenic index in both healthy individuals[137] as well as in individuals with T2DM[138,139]. There is clinical evidence of a close link between the L:A ratio, IR and atherosclerosis [136-139]. However, to this date, there is no validated cut-off value for the L:A ratio. Furthermore, in a clinical setting it is not standard procedure to measure adiponectin and leptin. More knowledge in this field is needed, both to further understand the L:A ratio in different subject groups, in relation to other risk factors, such as postprandial TG, and also with a view to get closer to a potential cut-off value. Further studies are needed to understand the L:A ratio as a potential early biomarker of metabolic disease, to potentially be suitable for a clinical setting.

1.5 Summary of introduction

A high BMI is the sixth most important risk factor for global death and disease burden[6]. However, approximately 32-39 % of obese adults are classified MHO[34]. Individuals in industrialized countries

spend most part of their lives in the postprandial, non-fasting state. Postprandial hyperlipidemia has been associated with overweight [15] and especially with abdominal obesity [44-47]. TRL is an independent risk factor for CAD[67,68], and associated with an increased risk of ischemic stroke [69]. However, our knowledge about postprandial TG is limited in obese individuals, especially in MHO, and for postprandial TG metabolism focusing on CM, in these individuals.

The adipocyte is an active endocrine organ, and the adipokines, leptin and adiponectin seem to be central for development of metabolic disease in obesity. There is clinical evidence of a close link between L:A ratio, IR and atherosclerosis [136-139]. We also know that lipid disturbances are central for the development of atherosclerosis, with hypertriglyceridemia as an independent risk factor [17,18]. There is a lack of knowledge about the relationship between, if any, the L:A ratio and the postprandial TG, both in MHO and MDO. Most of life is spent in the postprandial state, however, there is a knowledge gap of leptin and adiponectin in the postprandial state in obese and normal weight individuals, and possible regulatory functions. Most importantly, detection of early subclinical signs of metabolic disturbances, to both prevent and treat early, in obese subjects would be of strategic and important value in the management of obesity.

1.6 Hypothesis

1. Young, apparently healthy obese individuals have delayed postprandial TG and CM-TG clearance compared to normal weight individuals.
2. The adipokines leptin and adiponectin are interactive with TG metabolism, and insulin resistance, mirror metabolic disease in obese individuals, and are surrogate biomarkers with high clinical utility.
3. Adiponectin and leptin have a regulatory role in postprandial metabolism, and are dysregulated in obese individuals compared to normal weight individuals.

2 Aims of the thesis

The lack of knowledge in the field of postprandial TG, leptin and adiponectin, and early markers of metabolic disease, is the basis for this doctoral thesis. The general purpose of this thesis was therefore

to examine postprandial TG metabolism, leptin and adiponectin in obese indnividuals with, and without established disease, to find a potential surrogate biomarker of metabolic disease in obese individuals. The aims were:

1. To study the postprandial TG clearance in young, apparently healthy obese individuals.
2. To test the L:A ratio as a potential surrogate biomarker of postprandial TG clearance, IR or LR in an adult population of obese individuals with and without established metabolic disease.
3. To explore postprandial leptin and adiponectin in healthy controls and obese individuals with and without established disease, and the connection if any on TG clearance.

3 Summary of results

3.1 Approvals

Participants included in the study were informed and signed a written consent. The study was approved by The Regional Committee for Medical and Health Research Ethics of Northern Norway (2007, ID: 200704595-10/MRO/400), and the data bank approved by Norwegian Social Science Data Services (ID: 2206). Registered the 15th of April 2008.

3.2 Paper I

Delayed Clearance of Triglyceride Rich Lipoproteins in Young, Healthy Obese Subjects

Larsen MA, Goll R, Lekhal S, Moen OS, Florholmen J.

Clinical Obesity, December 2015. PMID: 26469529.

Aims. Obesity is associated with the metabolic syndrome. The primary aim was to study the postprandial TG clearance in young, healthy obese subjects. The secondary aim was to investigate if fasting TG can predict delayed postprandial triglyceride (TG) clearance.

Methods. Eighteen apparently healthy, obese subjects (BMI≥30) with no clinical signs of metabolic disturbances participated. Controls were age- and sex-matched, healthy, normal weight (BMI<25) subjects. All subjects were non-smokers. Subclinical markers of metabolic disturbances were assessed by measuring postprandial TG in serum and in CM by oral-fat-tolerance-test (OFTT). Postprandial TG clearance during 8 h was assessed indirectly as removal of the lipid from serum during the OFTT. IR was measured by the HOMA-IR.

Results. Twelve (66%) of the apparently healthy obese individuals had IR measured by HOMA-IR (≥1.83). There was a delayed clearance of serum TG (SE-TG) (P< 0.001) and CM-TG (P=0.011) at 6 h when compared to the control group, while at 8 h the differences were only detected for the CM-TG clearance (P=0.007). TG response (TGR) (P=0.013) and CM-TGR (P=0.006) was significantly greater in the obese subjects. When adjusted for fasting TG at baseline, the obese subjects still had higher postprandial SE-TG levels, compared to the normal weight controls. The obese subjects with fasting SE-TG in the upper normal range had a significantly delayed SE-TG- and CM-TG clearance (TG ≥ 1.02 mmol/L), and pathological insulin sensitivity (TG ≥1.13 mmol/L).

Strengths and limitations. The strength of this study is that it focuses on TG- rich lipoproteins, with a specific focus on CM on apparently healthy, non-dieting, obese subjects. The limitations of this study is the unbalanced sex distribution, and that we did not measure LPL-activity[55] or include adipokines. Furthermore, a quantitative and direct estimate of TG clearance is the gold standard, but we have

measured TG indirectly. However, OFTT has proven to have a strong correlation to triglyceride clearance, when compared to other methods[140].

Conclusion. In young, apparently healthy, obese subjects, early metabolic disturbances including IR and delayed postprandial TG clearance can be detected. Fasting SE-TG in upper-normal level predicted delayed postprandial TG clearance and IR. This might be a potential marker of early metabolic dysregulations, and an easier way to map out if there is a chance that a patient has delayed postprandial TG clearance.

3.3 Paper II

Leptin to Adiponectin ratio – a surrogate biomarker for early detection of metabolic disturbances in obesity

Larsen MA, Isaksen VT, Moen OS, Wilsgaard L, Remijn M, Paulssen EJ, Florholmen J, Goll R. Nutrition, metabolism and cardiovascular diseases, November 2018. PMID: 30145019.

Aims. To study if the leptin to adiponectin (L:A) ratio, can be a potential biomarker for postprandial triglyceride clearance, insulin resistance (IR) or leptin resistance (LR) in apparently healthy obese, and obese individuals with established metabolic disease.

Material and methods. Fifty adult subjects with obesity (BMI ≥30); of which 36 metabolic healthy obese (MHO), and 14 metabolic dysregulated obese (MDO), with clinical and/or biochemical signs of metabolic disease were included. Seventeen healthy, normal weight subjects represented the control group. Postprandial triglyceride (TG) levels were measured in an 8 h oral fat tolerance test (OFTT). IR by homeostasis model assessment of IR (HOMA-IR), L:A ratio and indirect LR were measured.

Results. In the MHO group, 71.4% had delayed TG clearance, 69.4% had IR and 86.1% had LR; whereas in the MDO group this was detected in 85.7%, 71.4% and 91.7%, respectively. A combination of all three metabolic risk factors was found in 39.8% of the MHO and in 42.9% of the MDO patients. Receiver operating characteristics (ROC) analysis revealed that a cut-off value for the

L:A ratio of ≥1.65 for the control group (PPV 1.0, NPV 0.91) and ≥3.65 for the obese subjects (PPV 0.86, NPV 0.48) predicted the delayed TG clearance with a good specificity and sensitivity. Detecting a combined risk with at least 2/3 metabolic risk factors, the ROC yielded the most suitable L:A ratio cut-off at ≥1.88.

Strengths and limitations. Strengths of this study: Firstly, subjects were included from the everyday practice at the obesity out-patient clinic, which underlines the clinical utility and transferability of our observations. Secondly, we have performed a thorough simultaneous characterization of the three axes of developing metabolic disturbances (delayed TG clearance, IR and LR). The most prominent weaknesses: Firstly, a lack of match between the three groups studied according to number of subjects, sex and age. Secondly, lack of statistical power, as a larger study would yield safer conclusions. Thirdly, by setting cut-off values for the target variables from the 95% CI of normal controls, we intentionally detect very early disturbances of metabolism; however, this choice may be controversial.

Conclusion. L:A ratio was able to detect early metabolic disturbances in obese individuals, and may be a potential useful clinical surrogate biomarker of metabolic disorders and dysregulation, for earlier prevention, detection and treatment of disease in obese patients.

3.4 Paper III

Postprandial leptin and adiponectin in response to sugar and fat in obese and normal weight subjects

Larsen MA, Isaksen VT, Paulssen EJ, Goll R, Florholmen J.

Endocrinology, 2019.

Purpose. Adipokines, produced by white adipose tissue are central in the development of lifestyle diseases. Individuals in industrialized countries spend a substantial part of life in the non-fasting, postprandial state, which is associated with increased oxidation and inflammation. The aim was to study postprandial adiponectin and leptin levels after an oral fat tolerance test (OFTT) and oral glucose tolerance test (OGTT) in obese (OB) and healthy, normal weight subjects (NW).

Methods. Fifty adult subjects with obesity (BMI ≥30) and 17 NW were included. Postprandial triglyceride (TG), adiponectin and leptin levels were measured every second hour during an 8 h OFTT, and every half hour during a 2 h OGTT.

Results. Compared to the basal level, postprandial levels of adiponectin following OFTT showed a slight initial peak, followed by a significant decrease at 8 h, in the NW. In the OB these changes were abolished. Postprandial levels of leptin decreased significantly from basal in the OFTT, in the NW, whereas in the OB, leptin was unchanged except for a slight increase from 2 h to 8 h (Figure 1). During the OGTT both adiponectin and leptin levels remained unchanged in the NW, but decreased significantly in the OB (Figure 2). In addition, the OB had delayed TG clearance at 6 h (Figure 1).

Strengths and limitations. The strengths of this study are; the postprandial measurements of the adipokines were done over a longer observation time than most studies, and also had an higher number of study participants than previous studies. The most prominent weaknesses are, firstly, a lack of match between the groups studied according to number of subjects, sex and age. Finally, a model of adipokine measurements directly in interstitial fat tissue is highly preferable to get a more precise postprandial response profile of adipokines.

Conclusion. A fatty meal gives postprandial changes in the secretion of adiponectin and leptin in NW, but not in OB. Our observations indicate that a potential postprandial regulatory role of adiponectin and leptin is impaired in OB, and of importance in a more comprehensive understanding of the delayed postprandial TG clearance in obese subjects. This is of importance to further understand the complex physiology behind the development and treatment of metabolic disturbances.

4 General discussion

4.1 Methodological considerations

4.1.1 Selection of study population and design

In this thesis a cross-sectional, case-control design was chosen. Volunteers were recruited from the University Hospital of North Norway (paper I-III) and the Norwegian institute for Sports medicine (paper II-III) by posters and leaflets. Participants included in the study were informed and signed a

written consent. The inclusion criteria for obese individuals were BMI ≥30 kg/m^2 and age 18-70 years. An obese individual was considered a MHO when documented normotensive, normal thyroid function tests, normal liver function tests, normal kidney function, normolipemic and normoglycemic, none of the metabolic syndrome criteria, excluding the waist circumference criteria [141]. An obese individual was considered metabolically dysregulated obese (MDO) when he or she had two or more of the metabolic syndrome criteria according to the NCEP/ATPIII guidelines [141], excluding the waist circumference criteria, which all of the obese individuals had.

Table 2. Definition of MHO and MDO

MHO	**MDO**
Normotensive, normal thyroid function tests, normal liver function tests, normal kidney function, normolipemic and normoglycemic, none of the metabolic syndrome criteria (excluding the waist circumference criteria)	Had metabolic syndrome by definition [141], or treated for hypertension, diabetes or hyperlipidemia

All study participants were euthyreot including normal laboratory tests. Exclusion criteria were pregnancy, current smoking, serious mental illness, and the use of medications to induce weight loss. The inclusion and exclusion criteria for the age and sex matched, healthy controls were the same, except for having to be of normal weight (BMI <25 kg/m^2). The normal weight individuals were recruited by age and sex (Paper I). In total sixty-seven subjects were included in this thesis: 17 normal weight individuals, 36 MHO and 14 MDO. There was an unbalance in sex in the obese individuals, approximately 80% women, and 20% men. This is in line with a study of 190 005 participants, where approximately 80% of those seeking help to lose weight through bariatric surgery were women[142].

Table 3. Inclusion and exclusion criteria for obese individuals

Inclusion criteria	**Exclusion criteria**
BMI ≥30 kg/m^2, age 18-70 years.	Pregnancy, current smoking, serious mental illness, the use of medications to induce weight loss.

The case-control design is a feasible and low-cost approach for studies where the outcome is rare, as is the case with MHO in our studies. In addition, it is a good option when there is a long time from the

exposure to the development of the disease. Because this study design is retrospect it is not possible to calculate the relative risk. However, the odds ratio can be calculated, which again approximates the relative risk. Also, the nature of the case-control design is that information about exposure is gathered after the disease has been diagnosed, and that the exposure was assumed to be of importance in development of the disease[143]. This might give systematic bias, which in addition to recall bias and selection bias is a challenge with case-control studies. The disease may influence the subjects to change lifestyle which may subsequently affect the exposure variable, postprandial TG and adipokines in our studies. Inaccurate and incomplete case selection can cause selection bias, as well as reduce precision. For example, how we define the MDO and the MHO individuals can cause selection bias. However, this is the reality and risk for bias, especially when there are no validated definitions per se. As the purpose of the studies was to detect early disease, we had a relatively strict definition of the MDO and the MHO. That said, most of the apparently MHO had early metabolic dysregulations, not detected in regular examinations done in the clinical practice. Having this in mind, one might speculate that the MHO is just a close path on the way to become MDO. Another selection bias is the unbalance in sex, with 80% of the obese subjects in our study to be women. However, this is in line with a study of 190 705 participants, where approximately 80% of those seeking help to lose weight, through bariatric surgery, were women[142]. Case-control studies are suited to generate hypothesis of causality, but they are not suitable to establish a cause-effect relationship. For the latter, randomized controlled trials (RCT) or cohort studies should be conducted. RCT is considered the gold standard for establishing cause-effect relationships, and further studies on this field should be designed as RCTs with larger study populations, in addition to cohorts.

As case-control studies are observational, they are vulnerable to confounding. We cannot definitively establish whether the observed difference in outcome (i.e. MHO or MDO) is attributed to the studied exposure (i.e. postprandial TG or adipokines levels) rather than other factors. This factor is called confounding, and it is associated with the risk of disease. Confounding factors represents a bias in estimating causal effects[144]. A regression analysis can be valuable to rule out confounding factors. However, if the study population or groups are small, as in our study, it might be difficult to get enough power to run the regression analysis.

4.1.2 Methods

Oral fat- tolerance test

In this doctoral thesis a 8 h oral fat- tolerance test (OFTT) was used to indirectly measure TG clearance, where blood tests were drawn from the antecubital vein at baseline, and every second hour. The OFTT has proven to be a good, indirect and qualitative measure of triglyceride clearance[140], There is no gold-standard for conducting OFTT, and studies perform them with various postprandial length, mixed meals and different macro nutrient intake. There are also several methods for measuring postprandial TG. In this doctoral thesis participants were instructed to have a normal food intake; no meals with very high fat content, no alcohol intake and had abstained from heavy physical activity, three days prior to the OFTT. Also, the subjects did a 12 h fast before the OFTT, in agreement with other studies[145-147]. A study in animal models, show that FGF-21 increases after a 7 day fast, but not after a 2 day fast[148]. One might speculate that this could affect the adiponectin level, and potentially also the TG level, as FGF-21 has been shown to be a potential regulator of both. If our study participants had a longer fasting period, one might hypothesize an even lower postprandial TG level and different level of adiponectin levels in both groups. However to explore real- life reflection of postprandial TG in the study participants a 12 h fast is considered enough to reach a fasting state, and a longer fasting period might be more difficult for the study participants to complete. The 12 h fast before the OFTT is also in agreement with other studies[145-147].

The strength of the OFTT performed in this thesis is that we performed a 8 h measurement, whereas several other studies report shorter postprandial observation times. We also fed the participants with weight-adjusted amounts of fat. Furthermore, we measured CM in parts of the study group (paper I), which many studies on postprandial TG do not measure. CM is the specific postprandial TG. Our test meal consisted mainly of the macronutrient fat, and minimally of protein and carbohydrate, as we wanted to focus on the fat metabolism. However as well as strength, this could also be a weakness, as most meals in everyday life are mixed meals, with a combination of all the three macronutrients. Other weaknesses of the OFTT were that we did not include a dietary record or a specific record for physical

activity. Also, we did not ask specifically about intake of food containing N-3-PUFA, supplements of this or abnormal intake of fructose. Moreover, the participants did not follow a specific diet at inclusion, as we wanted to explore the reference population in a non-dieting group. Furthermore, the participants consumed a calorie-free beverage and a fruit midway through the test, to prevent an un-physiological condition of dehydration and hunger. There are no reports that a fruit can interfere with postprandial TG. Measuring postprandial TG is expensive and time consuming, both for the patient, and healthcare workers, so getting closer to a standard method that considers this would be of importance for the future. There is a need for finding a suitable biomarker that can predict postprandial TG clearance, without having to perform the costly and time consuming OFTT.

In paper I, we analyzed CM, apoB-48, the meal specific TG. The CM was prepared and isolated[149] by Swedberg flotation (Sf) rates of >400 x-10^{-13}, and at these flotation rates CM predominates, while Sf 60-400 x 10^{-13} rates, VLDL (ApoB-100) predominates. However, similar fractions of both apoB-48 and apoB-100 have been found in Sf >400[150], meaning that at these rates, apoB-100 might also be included to some extent. Furthermore, CM was determined by an enzymatic colorimetric test (GPO PAP). Approximately 82% of the TG in the postprandial increase can be accounted to be CM, and individual variations of VLDL, apo B-100, postprandial, has shown to vary from 3-27% of the increase[151]. Because of this, we measured total TG in paper II-III, when including more patients.

Oral glucose tolerance test

We used a standard OGTT, first described in the 1960s[152], using an oral intake of 75 g glucose in solution, after a 12 h overnight-fast, and normal food intake and usual activity, the days before the test. Blood tests were drawn from the antecubital vein at baseline and every 30 minutes for 2 h. The OGTT can be performed with different amounts of glucose, from 50 g to 100 g, and with 8-16 h fast. We chose to use the standard 75 g glucose load, as recommended by the World health organization, and 12 h fast. We could have drawn blood tests for a longer time, as some studies have 3 h measurements. However, a 2 h measurement is the standard length, recommended by the WHO, and more often used in clinical settings. However, recently, a shorter 1 h OGTT has been explored. The NGT subjects with

a postprandial glucose value at 1 h ≥8.6 mmol/L, has shown to have significant reduced peripheral insulin sensitivity and beta cell function, compared to IFG subjects, and those with postprandial glucose levels at 1 h <8.6 mmol/L, but not with IGT subjects[153]. It has also been suggested that a 1 h OGTT may be a useful tool to recognize those NGT subjects at risk to develop T2DM and cardiovascular diseases[154](review). As we wanted to calculate IR through WBISI and HOMA-IR, in addition to the postprandial adipokines, we chose a test with several measurements, and standard time of 2 h.

Measurements for insulin, adiponectin and leptin

Insulin and insulin resistance. Serum insulin was measured by Elisa-kit. As insulin has a narrow reference level, there is a bigger chance for reduced precision. Furthermore, insulin is most often used in a research setting, and there is no standardized assay for measuring insulin[14]. This might give different values of HOMA-IR[11] and WBISI[12], in comparison with other studies. A strength of the measurements is that we included both fasting and postprandial values of insulin, so that we could calculate both HOMA-IR and WBISI. A weakness of this thesis is that we did not perform the euglycemic clamp measurement[10], which is the gold-standard for measurement of IR, but it is also expensive, time consuming, and not very suitable in a clinical setting. HOMA-IR and WBISI are both recognized methods for measurement of insulin resistance[11,12]. Furthermore, HOMA-IR might be more suitable for a clinical setting, as one only needs to measure fasting glucose and insulin. We will need a more standardized measurement for insulin, if it should be used in a clinical setting, in addition to a standardized cut-off value in different subject groups, age groups, genders, BMI and for ethnicity. The cut-off value of 95% CI of HOMA-IR in the normal weight individuals, was considered as the limits of normality[155-157]. Choosing this cut-off value from our control group, with 95% CI, might be considered a bit strict. However, the cut-off levels are approximately in line with other cut-off values for IR[155-157]. There are some factors related to IR, that we did not measure in these studies. Among them are hepatic lipid content, gastric motility and modified VLDL export. It would be of interest to investigate the combined relationship among these factors IR, and the other metabolic disturbances in a future study.

Leptin and leptin resistance. Leptin was measured by ELISA-kit (sandwich ref. EIA-2395, DRG Diagnostics), and LR measured indirectly with the REE:leptin ratio. REE can be calculated by different equations, without measuring it, but the equations have been criticized to over- and underestimate the REE, especially in individuals that are not healthy and normal weight[158]. In our study we used indirect calorimetry, as a more accurate way to measure it, where the Weir equation is used to calculate the REE[159]. For the measurement of leptin resistance, there is no validated method. We chose to use the experimental REE:leptin[107] ratio to measure this. However, a weakness about this, is that it is not validated in larger study populations or cohorts. However, until a validated method, and a more comprehensive understanding of LR, this is a method feasible to be used.

Adiponectin. Adiponectin was measured by ELISA-kit (human, ref. EIA-4574, DRG Diagnostics). Since the adiponectin has a narrow reference area, and most human values are between 2-10 mcg/L, there is a need of a more accurate essay. To improve the precision in these measurements we used duplicates. Moreover, the observational period for the OFTT test was 8 h. This is a strength, as we did indeed observe changes during the observation time. However, we measured total adiponectin, and not high- molecular weight (HMW) adiponectin. This might be a weakness, as the HMW is thought to be a more biologically active form[160], but it is still unclear how HMW adiponectin is regulated. Most studies before have studied total adiponectin, but it would have been of value to measure both HMW- and total adiponectin. Also, to calculate the L:A ratio, total adiponectin is used. Furthermore, adiponectin has also shown to have some day and night variations in the fed state, with one study of 8 healthy and normal weight men showing peaks at 12:00 h and at 20:00 h[161]. The diurnal variations have not yet been studied in MHO or MDO subjects, and not in women, to our knowledge, so we chose to use the morning measurement after a 12 h fast as the baseline value of adiponectin. Furthermore, adiponectin has shown higher levels in women than men[123], this is because of testosterone lowering HMW adiponectin by inhibiting secretion from the adipocytes[162]. This might also affect the results.

L:A ratio. The L:A ratio was calculated in Excel. The L:A ratio was included to find a more sensitive method to detect metabolic dysregulations. There is no validated cut-off value for the L:A ratio, and

we calculated the cut-offs ratio in this thesis to be 95% CI of the normal weight controls. It would also be interesting to include HMW adiponectin into this equation, but this was not an aim of the studies. As leptin and adiponectin varies among the genders, and different ethnicities, different cut-off values are needed. In our study we only included Caucasians, and mostly women, and this is also a strength of the studies.

Statistics

Statistics were calculated on SPSS 19-24 IBM for Windows (SPSS Inc., Chicago, Illinois, USA). Microsoft Excel was used for calculating HOMA-IR, WBISI, LR, L:A ratio, TG clearance and TGR. Normal distribution was detected by determination of skewness and histograms. Parametric statistics were performed when either raw or transformed data resembled normal distribution; otherwise non-parametric tests were used. Tests for independent or paired samples were used as appropriate. Two sided p-values <0.05 were considered statistically significant.

RM-ANOVA. A repeated measure ANOVA (RM- ANOVA) was used to analyze data from the postprandial measurements. Corrections for deviation from the assumption of sphericity were used as appropriate. Two sided p- values <0.05 were considered statistically significant.

ROC analysis. The ROC analysis was performed, and ROC curves. HOMA-IR, indirect LR, L:A ratio and fasting TG was analysed to different variables, to explore if they were suitable to predict those variables, in different groups. The cut-off values of ROC targets were determined by the appropriate upper or lower limit of the 95% CI for the normal weight control group. Optimal cut-off values were defined by highest Youden index. For each cut-off value we performed a logistic regression to estimate the odds-ratio (95% CI, p-value) for a given state based on for example a positive L:A ratio by that cut-off (corrected for sex and age).

Descriptive analysis. To compare differences between different groups, the appropriate test was used; independent sample t-test for normally distributed data or Mann-Whitney or Wilcoxon rank test for non-normally distributed data.

Correlation. Correlation was used as appropriate. Depending on if the data was normally distributed or not, the Pearsons coefficient or the Spearmans coefficient was used.

4.2 General discussion of main results

Human beings spend most parts of their lives in the non-fasting state, and postprandial TG is a well-known risk factor for atherosclerosis. Furthermore, the adipokines play a crucial role in the regulation of energy homeostasis, and this seems to be altered in obese individuals. There is lacking evidence about MHO, and the development of metabolic dysregulations, in addition to potential surrogate biomarkers to highlight these metabolic dysregulations. The most important findings of this doctoral thesis is that young, apparently healthy obese individuals have a postprandial delayed metabolism of SE-TG and in CM-TG, compared to healthy normal weight individuals, when measured indirectly by OFTT, adjusted for fasting TG (paper I). Also, 71.4% of the apparently MHO have delayed TG clearance (paper II). Furthermore, almost 40% of the apparently MHO had a combined delayed TG clearance, IR and LR, indicating metabolic dysregulations. The L:A ratio proved to be a sensitive, surrogate biomarker for delayed TG clearance, also in combination with IR and LR (paper II). There are postprandial changes in the adipokines adiponectin and leptin in normal weight individuals, after a fatty meal, but not in obese individuals. This sets the focus on a potential postprandial regulatory role of these adipokines that might be impaired in obese individuals (paper III).

4.2.1 Postprandial triglycerides in MHO and MDO

Using an 8 h OFTT, there was a delayed peak and clearance of postprandial SE- TG and CM- TG in the MHO individuals compared to the normal weight individuals. Moreover, CM-TG was the most sensitive test as significant differences were observed both after 6 h and 8 h postprandial. In this thesis we used TG response (TGR) (paper I) and TG clearance at 6 h (Paper I-III) and 8 h (paper I-II) to explore postprandial TG patterns. TGR says something about how high the TG levels are postprandial, but what is more interesting is how fast the TG is reduced after a fat-load, as this say something about how long the TG is inside the blood vessel, and can do damage. We found that the TG clearance at 6 h

was most useful, and therefore used this in the following papers (paper II-III). However, when it comes to measuring TG clearance, a quantitative and direct estimate of the TG clearance would be the gold standard, like isotopic labeling; but in this thesis, we estimated TG clearance indirectly. However OFTT has proven to have a strong correlation to TG clearance, when compared to other methods[140]. A postprandial delayed SE-TG clearance is well documented in overweight and obese individuals with fasting hypertriglyceridemia (for review, see [163]), whereas diverging results exist for CM-TG[46]. Moreover, in similar studies including MHO with normal fasting TG, few reports exist, and especially for CM-TG. In two studies obese individuals with normal fasting TG, a delayed postprandial metabolism of TG was observed, but the study groups were small[164,165]. As far as we know, no reports exist for postprandial CM-TG in MHO. The postprandial TG in serum and in CM have been reported to be a key marker, and a more sensitive risk factor for atherosclerosis than the corresponding fasting levels [166] (for review see[167]). The greater the magnitude and duration of the postprandial TG response, the arterial wall will be more exposed to postprandial TRL. The longer duration of the postprandial TRL in the bloodstream will give more time to replace cholesterol ester in LDL and HDL, favoring the transformation of LDL to be a smaller and more pro-atherogenic particle, and making HDL more dysfunctional. Moreover, the mechanism of TRLs influence on lowering the HDL- level, is believed to be due to the enrichment of TG to the HDL particle, which leads to increased catabolism of Apo-A-I HDL (for review see[60]). All of the MHO had TG levels within the reference level (<1.7 mmol/L). It is well known that fasting TG levels are strongly associated to the postprandial TG metabolism[168]. Another strong influence on the postprandial TG profile is abdominal obesity[44-47]. All of the apparently MHO had abdominal obesity according to the WHO and International diabetes foundation (waist circumference ≥88 cm (≥80 cm) for women and ≥102 cm (≥94 cm) for men). In our study (paper I) abdominal fat percent was strongly correlated to postprandial TG profile, as expected [44-47].

In the postprandial state IR is associated with increased intestinal production of CM[169]. In our study, as expected, the postprandial TG clearance, SE-TGR and CM-TGR were significantly correlated to the insulin sensitivity, reflecting the LPL activity in the endothelium[163].

4.2.2 MHO and potential biomarkers for early metabolic dysregulation in obesity

In the literature, the concept of MHO have been used for obese individuals with none and up to two clinical established metabolic disturbances [34]. The MHO in our study did not have any clinically significant metabolic disturbances, as indicated by fasting cholesterol, fasting TG, fasting glucose and blood pressure, nor did they use any medicines for such. In one study, approximately one-third of obese subjects were classified as MHO, having less than 2 metabolic disturbances [34]. Previous studies with long follow-up periods have demonstrated that these MHO individuals are at an increased risk of major CVD events [35,36], and overall mortality [35], as compared to healthy, normal weight individuals. Without a good biomarker it is difficult to predict which individuals of the MHO that is at risk. In our study, close to 90% of the individuals had LR, but no differences were seen between the MHO and the MDO. Leptin was somewhat non-significant higher in the MHO group than in the MDO group, most likely explained by non-significant differences in the body fat percent. Other studies have also found no significant difference in fasting leptin between MHO and MDO [114,116]. A study from 2014 including over 11000 subjects found fasting leptin to have moderate sensitivity and specificity, for identifying cardio-metabolic abnormalities and leptin sensitivity [170].

In our study, adiponectin was significantly lower in obese individuals compared to healthy, normal weight individuals, as expected, whereas no differences were observed between the MHO and MDO. Finally, 76% of the obese individuals (both MHO and MDO) had low adiponectin values (95% CI of normal weight: <9.6 μmol/L). A few studies have examined the adipokine profiles of MHO [114-116]. One study from 2010 reported higher adiponectin levels in MHO, compared to MDO [116]. None of the individuals in this study were elderly, nor did they have low BMI, going through weight loss, had CVD, chronic kidney disease or heart failure. In such individuals studies have shown that high circulating levels of adiponectin might be associated to increased mortality, this may be due to smaller, differentiated adipocytes, increased production from non-adipocyte tissue, decreased elimination or direct stimulation through natriuretic peptides [117,121,122], and a potential adiponectin resistance[124], may also be involved . The L:A ratio might therefore not be of clinical value for this

patient group, with already prominent chronic disease. Predicting delayed TG clearance, an increased risk of CVD, may be of great clinical value in the daily treatment of the obese patient. All of the apparently MHO included in the study had fasting TG in the normal range. In addition, the obese individuals with high-normal fasting TG (≥1.02 mmol/L) had delayed postprandial TG clearance, compared to both the obese individuals with low normal-fasting TG and the healthy, normal weight individuals. L:A ratio was in this thesis, found to be sensitive to detect delayed TG clearance in all individuals, and performed better than fasting TG, as a biomarker. Furthermore, we also found that fasting TG, in the normal range predicted IR with a sensitivity of 83% and the specificity of 86% with a cut-off value of TG ≥1.13 mmol/L. Our findings that fasting TG in the normal range in obese individuals can predict IR is of great utility in clinical practice. Therefore, the L:A ratio and fasting TG may detect obese individuals with high risk of development of the various metabolic disturbances, such as CVD and Type 2 Diabetes. Further studies are needed to validate the L:A ratio and fasting TG as biomarkers, and they should include more patients, including men, women, different ages, and ethnicities.

4.2.3 The role of leptin and adiponectin in regulation of metabolism

We have found that postprandial adiponectin and leptin regulation, following a fat load, is altered in obese individuals, compared to normal weight individuals. These adipokines might have a regulatory role in postprandial metabolism that is more or less abolished in obese individuals. Interestingly, our data is in conflict with other studies. One study from 2003 found no difference in postprandial adiponectin profile in normal weight individuals compared to fasting levels after an oral-fat-tolerance-test (OFTT)[134]. A small study found a significant increase in adiponectin after 60 min, in 11 obese individuals, but not in the normal weight individuals[132]. However, the postprandial period was limited to 200 min, there was a higher prevalence of males in the study, and they were served a mixed meal (56.5% carbohydrates, 12,1% protein and 31% fat) which promotes insulin increase. Interestingly, in a study of 25 non-obese, non-diabetic patients with non-alcoholic-steatohepatitis (NASH), a significant decrease in adiponectin profile was found, after an OFTT at 8 h and 10 h compared to baseline levels,

whereas the healthy controls showed a significant increase in adiponectin after 6 h. In addition, the NASH patients had significantly higher postprandial triglycerides and FFA[130].

In mice, exogen adiponectin has shown to enhance FFA oxidation by activating the adenosine monophosphate-activated protein kinase, and to reduce the postprandial FFA increase[171,172]. Furthermore, obese mice, fed on a high-fat diet, also showed a reduction of blood glucose levels and body weight[173,174], when treated with exogen adiponectin, through improved insulin sensitivity[173], regulating inflammatory responses and increased fat oxidation[174]. Also, low levels of adiponectin independently predicted post-heparin LPL activity in both diabetic and non-diabetic patients, accounting for variation of 25% of the LPL activity[175]. Adiponectin, but not leptin, has also been found as an important regulator of VLDL apoB catabolism, independently of other adipokines or IR[176]. To this date, there haven't been any studies on exogen adiponectin administration in humans, as it has not yet been approved[95]. Furthermore, it has also been proposed that FGF-2 regulate postprandial lipid metabolism and permits better clearance of triglyceride-rich lipoprotein fractions [177], especially in healthy individuals, and that adiponectin might mediate this response. Also, both adiponectin and leptin might facilitate response of FGF-21, which has an effect on energy expenditure, and whole-body glucose metabolism. FGF-21 is also a regulator of adiponectin secretion [127]. The regulation of adipokines, the unbalance in adipokines, and their role in postprandial metabolism is complex and we are far from understanding the full picture. Further studies are of importance to get closer to a understanding of these complex physiological mechanisms.

5 Concluding remarks- evaluation of hypothesis and implications

1. *Hypothesis:* Young, apparently healthy, obese individuals have delayed postprandial TG and CM-TG clearance compared to normal weight individuals.

   Evaluation of hypothesis: In apparently healthy, obese individuals, early metabolic disturbances with delayed metabolism of postprandial SE-TG and CM can be observed. When

adjusted for fasting TG at baseline the obese individuals still had higher postprandial serum TG levels, compared to normal weight controls. Furthermore, obese individuals with fasting TG in the upper reference level had significant delayed postprandial TG clearance.

2. *Hypothesis:* The adipokines leptin and adiponectin are interactive with TG metabolism, and IR, mirror metabolic disease in obese individuals, and is a surrogate biomarker, with high clinical utility.

   Evaluation of hypothesis: The L:A ratio is a sensitive biomarker for delayed TG clearance, also in combination with IR and LR.

3. *Hypothesis:* Adiponectin and leptin have a regulatory role in postprandial metabolism, and is dysregulated in obese individuals compared to normal weight individuals.

   Evaluation of thesis: A fatty meal gives postprandial changes in the secretion of adiponectin and leptin in normal weight individuals, but not in obese individuals. Our observations indicate that a potential postprandial regulatory role of adiponectin and leptin is impaired in obese individuals, and of importance in a more comprehensive understanding of the delayed postprandial TG clearance in obese individuals.

6 Implications

- This thesis adds novel knowledge in the field of regulation of energy metabolism in obese individuals, proving that apparently MHO have early metabolic dysregulations
- Sensitive, surrogate biomarkers can help us diagnose metabolic dysregulation at an early stage, to further lower the risk for development of metabolic diseases and potentially treat at an earlier stage.
- Fasting glucose, insulin and TG are easy tests to do, and can be measured in a busy clinical practice. However, the L:A ratio might be the most suitable biomarker, as it was sensitive to predict delayed TG clearance, leptin resistance and insulin resistance. Further studies are needed to find cut-off values for different patient groups, especially for L:A ratio.
- The postprandial regulation of adiponectin and leptin is altered in obese subjects, and this is of importance to further understand the complex regulations of energy metabolism.
- As obesity and diabetes are on the rise, , early diagnosis and treatment of metabolic diseases, and more knowledge about it, is of great importance not only to the individual patient health and quality of life, but also of great importance in a socioeconomical perspective.

7 References

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Paper I

Delayed Clearance of Triglyceride Rich Lipoproteins in Young, Healthy Obese Subjects

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Running title: metabolic changes in healthy obese subjects

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What is already known in this field?

- Up to 10% of obese subjects can be considered metabolically healthy to date.
- Postprandial triglyceridemia is well documented in overweight and obese subjects with fasting hypertriglyceridemia.
- Diverging results exist for chylomicron triglycerides.

What does this study add?

- As far as we know this is the first report that shows that metabolically apparently healthy obese subjects have a delayed postprandial clearance of chylomicron triglycerides.
- This study also points out the possible need of a new-, and lower reference level for fasting triglyceride levels in obese subjects.

Abstract

Aims

Obesity is associated to the metabolic syndrome. The aims were first to study the postprandial triglyceride clearance in young, healthy obese subjects; second to investigate if fasting triglycerides can predict delayed postprandial triglyceride clearance.

Methods

Eighteen apparently healthy, obese subjects with no clinical signs of metabolic disturbances participated. Controls were age- and sex-matched, healthy, normal weight subjects. Subclinical markers of metabolic disturbances were assessed by measuring postprandial triglycerides in serum and in chylomicrons by oral-fat-tolerance-test. Postprandial triglyceride clearance during 8 h was assessed indirectly as removal of the lipid from serum during the oral-fat-tolerance test. Insulin resistance was measured by the homeostasis-model-assessment of insulin resistance (HOMA-IR).

Results

Twelve (66%) of the apparently healthy obese individuals had insulin resistance measured by HOMA-IR. There was a delayed clearance of serum triglycerides and chylomicron triglycerides at 6 h when compared to the control group, while at 8 h the differences were only detected for the chylomicron triglyceride clearance. Triglyceride response was significantly greater in the obese subjects. Fasting triglycerides in upper-normal level predicted a delayed postprandial triglyceride clearance and insulin resistance.

Conclusion

In young, apparently healthy obese subjects early metabolic disturbances including insulin resistance and delayed postprandial triglyceride clearance can be detected. Fasting serum triglyceride in upper-normal level predicted delayed postprandial triglyceride clearance and insulin resistance.

Keywords: Chylomicrons, fasting triglycerides, abdominal obesity, triglyceride-rich lipoproteins, triglyceride response, insulin resistance, metabolic healthy obese

Abbreviations

TRL, triglyceride-rich lipoproteins; LPL, lipoprotein lipase; TG, triglycerides; SE-TG, serum triglyceride; CM, chylomicrons; VLDL, very low-density lipoproteins; IR, insulin resistance; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoproteins; DEXA, dual- X-ray- absorptiometry; OGTT, oral glucose tolerance test; OFTT, oral fat- tolerance test; HOMA-IR, Insulin resistance by the homeostasis model assessment; WBISI, whole body insulin sensitivity index; NaCl, Natrium Chloride; Apo A-I, apolipoprotein A-I; ApoB-100, apolipoprotein B- 100; TGR, Triglyceride response; CM-TGR, Chylomicron triglyceride response; RM- ANOVA, repeated measure ANOVA; ROC, receiver operating characteristic

Introduction

Overweight and obesity, the sixth most important risk factor for global death and disease burden[1], are raising global health problems with several metabolic disturbances and co-morbidities, such as type 2 diabetes and cardiovascular disease (CVD). Environmental challenges such as a sedentary lifestyle and excessive intake of processed food contribute to the increased prevalence of obesity[2,3] with a substantial part of life in the postprandial state. Hypertriglyceridemia is the typical lipid disturbance in overweight and obesity [4] and contributes to atherosclerosis. A milestone in the understanding of the atherosclerotic process was the proposal of Zilversmith in 1979[5] of a reduced and prolonged clearance of postprandial accumulation of triglyceride-rich lipoproteins (TRL) as one of the main pathophysiological events in the atherosclerotic process. This was later supported by studies showing that chylomicrons (CM) remnant particles penetrate efficiently and are retained selectively in early atherosclerotic lesions of the vessel wall[6,7] and contributes to coronary atherosclerotic disease by delayed elimination of postprandial TRL[8,9]. The enzyme lipoprotein lipase (LPL) plays a pivotal role in the lipoprotein metabolism by hydrolyzing TG in CM. Activation of LPL results in hydrolyzing of CM and development of TG in small CM remnants[10], hydrolyzing TG in very low-density lipoproteins (VLDL) assembled in the liver, contributing fatty acids to the vascular endothelium, and finally remove them from the bloodstream [11]. LPL- activity in the vascular endothelium is regulated by insulin, and the insulin resistance typical found in overweight and obesity [12] may contribute to a delayed removal of postprandial TRL and its highly association to overweight [4] and especially to abdominal obesity [13-16].

Our knowledge of lipid disturbances in young, healthy overweight and obese subjects is poor, especially for postprandial triglyceride metabolism focusing on CM. Both delayed postprandial plasma TG response, in obese men and women[17], and serum TG (SE-TG)[18] in overweight men have been detected, but as far as we know no differences have been found in the CM compartment.

Therefore, in this report postprandial TG metabolism in serum and in CM have been studied in young, apparently healthy, obese subjects without signs of metabolic disturbances, including normolipidemia.

Methods

Participants

Volunteers were recruited from the Centre of Obesity, Department of Gastroenterology and Nutrition, University hospital of North- Norway. Posters were also used to recruit obese individuals and healthy controls to participate in the study. The inclusion criteria for the obese subjects were: body mass index (BMI) >30 kg/m^2, age 18- 40 years old, normotensive, normoglycemic, normolipemic, no history of diabetes and not pregnant. Exclusion criteria were smoking, serious mental and somatic diseases, and patients on anti-obesity drugs. The inclusion and exclusion criteria for the age- and sex-matched, healthy controls were the same, except being normal weight (BMI<25).

Participants were informed and signed a written consent. Of the 40 obese subjects who were screened for participation, 13 did not answer after receiving more info, 9 was excluded mainly because of high blood pressure and high blood lipids. The remaining 18 subjects were eligible to participate, and were defined as "healthy obese". A "healthy obese" individual is in this study defined as an obese individual whom would be classified as apparently healthy by standard clinical evaluation and biochemical measurements by a general practitioner.

Height, body weight, BMI and waist circumference were measured and blood tests were drawn. These included fasting glucose, total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoproteins (HDL) cholesterol and TG.

Dual- X-ray- absorptiometry (DEXA, Lunar Prodigy Advance, GE healthcare, USA), were collected at baseline for all subjects. The DEXA measured total fat percent, abdominal fat percent, total fat mass (kg) and total muscle mass (kg).

Oral glucose tolerance test (OGTT)

OGTT was conducted using an oral intake of 75 g glucose in solution, after a 12 h night-fast. Blood tests were drawn from the antecubital vein before ingestion (0 min) and at 30 min- 60 min- 90 min-120 min after intake. Glucose and insulin were measured at all time-points.

Oral fat- tolerance test

On the test day the participants did an 8 h oral fat- tolerance test (OFTT) to indirectly measure TG clearance. OFTT has proven to be a good, indirect and qualitative measure of triglyceride

clearance[19].The three days prior to the test the subjects had a normal food intake, no alcohol intake and had abstained from heavy physical activity. The participants fasted 12 h before start of the test. They were at rest, not allowed to smoke, chew gum or drink anything other than water during the test day. The OFTT was conducted using a test meal prepared from standard sour cream porridge and double cream, together containing 70% calories of fat, of which 66% was saturated fat, 32% was monounsaturated fat and 2% was polyunsaturated fat [20]. A freshly prepared test meal was served with two teaspoons of white sugar (10 g carbohydrates), cinnamon and one glass (100 ml) of calorie-free lemonade. The participants were served a weight-adjusted meal (1 g fat per kg body weight) at 08:00 hours (baseline) and the meal was consumed within a 15-min period. The participants were offered a 500 ml calorie-free beverage and one fruit (pear or apple) at 12:00 hours (4 h). Blood samples for serum and EDTA- plasma for isolation of CM were collected before the test meal (baseline) and every second hour over the next 8 h. The TG clearances at 6 h and 8 h were calculated by the following formula: Clearance 6 h = 100 * (1-([TG (6 h) – TG (0 h)]/[TG (max)-TG (0 h)])). Triglyceride response (TGR) in CM and in SE-TG was calculated as the mean of the two highest postprandial values minus the baseline value of serum TG[21].

Isolation of chylomicrons

CM were isolated by over layering 8 ml EDTA plasma with 5 ml of Natrium Chloride (NaCl) solution (a density of 1.006 kg/l NaCl solution with 0.02% sodium azide and 0.01% EDTA) in a cellulose nitrate tube (Beckman Instruments Inc., CA, USA) and centrifuged in a Beckman SW40 Ti swinging bucket rotor at 20 000 rpm for 1 h at 20 º Celsius[22]. The CM, with Svedberg flotation (Sf) rates >400 x 10^{-13}s, were carefully removed by aspiration from the top of the tubes by[23], divided into three aliquots in cryovials, flushed with nitrogen, and frozen at -70° Celsius until further analysis.

Serum lipid and apolipoprotein measurements

Serum lipids were analyzed on a Hitachi 737 Automatic Analyzer (Boehringer Mannheim, Germany) according to manufacturer's recommendations. Total cholesterol (our labs reference value was 18 - 29 years: 2.9 – 6.1 mmol/L, 30 - 49 years: 3.3 – 6.9 mmol/L, >= 50 years: 3.9 – 7.8 mmol/L) was measured with an enzymatic colorimetric method (CHOD-PAP) and HDL-

cholesterol (our labs reference value was for women: 1.0-2.7 mmol/L, and men: 0.8-2.1 mmol/L) was assayed by the same procedure after precipitation LDL with heparin and manganese chloride as described by Burstein et al. [24]. TG concentration in serum (our labs reference value was 0.5-2.6 mmol/L) and in CM was determined with an enzymatic colorimetric test (GPO-PAP). LDL cholesterol was calculated by the formula of Friedewald et al. [25]: LDL cholesterol = Total cholesterol - HDL cholesterol - 0.47 x serum triglycerides. Apolipoproteins A-I (Apo A-I) and B- 100 (ApoB-100) were measured immunochemically by rate nephelometry, using the Array Protein System from Beckman Instruments Inc. (Brea, CA, USA).

Measurements for insulin sensitivity

Serum insulin was analyzed directly through a commercial ELISA kit (DRG Insulin Elisa kit, DRG Instruments GmbH, Germany). Insulin resistance (IR) determination by the homeostasis model assessment (HOMA-IR), and supplementary by the Whole body insulin sensitivity index (WBISI) were calculated. Insulin resistance was calculated as follow: HOMA-IR = Fasting insulin (FI) (mU/L) × Fasting glucose (FG) (mmol/L)/22.5[26], WBISI = 10000/[FI (mU/L) × FG (mg/dL) × mean insulin (mU/L) × mean glucose (mg/dL)]1/2 [27]. The cut-off value of 95% CI of HOMA-IR in the normal weight subjects, was considered as the limits of normality[28-30]

Statistics

Statistics were calculated on SPSS 19 IBM for Windows (SPSS Inc., Chicago, Illinois, USA). Microsoft Excel was used for calculating HOMA-IR, WBISI, TG clearance and TGR. Normal distribution was detected by determination of skewness and histograms. Parametric statistics were performed when either raw or transformed data resembled normal distribution; otherwise non-parametric tests were used. Tests for independent or paired samples were used as appropriate. A repeated measure ANOVA (RM- ANOVA) was used to analyze data from the OFTT. Corrections for deviation from the assumption of sphericity were used as appropriate. An RM- ANOVA was performed to modulate postprandial TG profile as predicted by weight group and fasting TG group (all subjects were ranked according to fasting TG and split in two equal groups with cut-off of 1.02mmol/L). Two sided p- values <0.05 were considered

statistically significant. The receiver operating characteristic (ROC) curves of HOMA-IR for fasting TG, total fat percent, abdominal fat percent and BMI were depicted, and the optimal cut-offs were determined by 95% CI for the normal weight controls

The study was approved by The Regional Committee of Medical Ethics of North Norway and the Norwegian Social Science Data Services.

Results

Subject Characteristics

The anthropometric-, clinical- and metabolic characteristics for the normal weight subjects and the healthy obese subjects are shown in Table 1. As expected, the anthropometric data showed several differences. The healthy obese subjects had several metabolic parameters that were significantly increased compared to controls, though still within the normal range (table 1).

Insulin sensitivity

There was a significant difference in fasting insulin and a close to significant difference in fasting plasma glucose at baseline (table 1). The cut-off value of the HOMA-IR defined by 95% CI on the normal weight subjects was defined as IR and was calculated to be > 1.83. Twelve of the 18 healthy obese subjects (66 %) had IR measured by HOMA-IR. Furthermore, insulin sensitivity was significant lower (higher HOMA-IR, lower WBISI) in the healthy obese subjects (table 1).

Postprandial triglyceride profiles

Results from the OFTT were analyzed by RM-ANOVA, and estimated marginal means are shown in figure 1.

Serum-triglyceride Grand (all time points) mean of the total SE-TG levels was 0.54 mmol/L (0.20 – 0.94) higher in healthy obese subjects when compared to the normal weight subjects (P=0.01). A significant interaction between time and subject group was detected (P=0.004; Greenhouse-Geisser). The contrast showed significantly higher SE-TG levels for the obese subjects at time points 4h (P=0.015) and 6h (P=0.009) compared to the normal weight subjects (figure 1).

Chylomicron-triglyceride The healthy obese subjects had overall ×1.8 (1.3 – 2.7) time higher CM-TG (grand mean, transformed raw data, P=0.002) (figure 1). No difference in time course between subject groups was detected.

Triglyceride clearance The results of total SE-TG and CM-TG clearance calculations are presented in table 2. At 6h a significant difference between subject groups in both SE-TG (P< 0.001)- and CM-TG (P=0.011) clearance is noted, while at 8h the differences between subject groups can only be detected in CM-TG clearance (P=0.007).

Triglyceride response (TGR) The results of the serum TGR (SE-TGR) and chylomicron TGR (CM-TGR) are shown in table 2. There was a significant difference between subject groups in both SE-TGR (P=0.013) and CM-TGR (P=0.006). Significant correlations were also found between SE-TGR and each of the variables BMI, total bodyweight (kg), total fat mass (kg) and abdominal fat percent (data not shown). The cut-off value of the grand SE-TGR and CM-TGR defined by 95% CI on the normal weight subjects is defined abnormal (pathological), and was calculated to be SE-TGR > 0.64. Nine (50%) of the healthy obese subjects had an abnormal high SE-TGR (> 0.64). The cut-off value of the grand CM-TGR and was calculated to be CM-TGR > 0.28. Seven (39%) of the healthy obese subjects had an abnormal high CM-TGR (> 0.28). Significant correlations were also found between CM-TGR and each of the variables BMI, total bodyweight (kg), total fat mass (kg) and abdominal fat percent (data not shown).

Fasting - triglyceride as predictor of postprandial triglyceride clearance

We then studied if fasting TG could reflect the postprandial TG profile. A significant interaction between fasting TG versus SE-TG at various time points was detected in each group (P=0.008; Greenhouse-Geisser). The contrasts showed significantly higher SE-TG levels for the obese subjects at time points 4 h (P=0.023) and 6 h (P=0.013) compared to the normal weight subjects, when adjusted for fasting TG level. The results of SE-TG clearance calculations are presented in table 2.

A significant interaction between time, subject group and fasting TG category (low-normal or high-normal; cut-off 1.02mmol/L) was found (P=0.007; Greenhouse-Geisser). The contrast showed significantly higher postprandial SE-TG levels for the obese subjects with a higher fasting SE-TG (>1.02 mmol/L) at time points 4h (P=0.006), 6h (P=0.006) and 8 h

(P=0.028). The subjects with high-normal fasting SE-TG also had significantly higher SE-TG- (P=0.029) - and CM-TG clearance (P=0.027) at 6 h, while the difference at 8 h only was detected in CM-TG clearance (P=0.044). Thus, the 12 obese subjects with high-normal fasting TG calculated as TG >1.02 mmol/L had a significant delay in TG clearance (figure 2). Finally, there was a significant non-parametric correlation between insulin sensitivity and postprandial SE-TG clearance at 6h (HOMA-IR; ρ= -0.636, p=0.000, WBISI; ρ=0.632, p=0.000; see figure 3) and CM-TG clearance at 6 h (HOMA-IR; ρ=-0.493, p= 0.005, WBISI: ρ=0.555, p=0.001), while at 8 h the significant correlation was only found between insulin sensitivity and CM-TG clearance (HOMA-IR; ρ=-0.527, p=0.002, WBISI; ρ=0.528, p=0.002). In addition fasting HDL cholesterol significantly correlated to the postprandial CM-TGR (ρ=-0.520, p=0.004), SE-TGR (ρ=-0.429, p=0.013), SE-TG clearance (ρ=0.757, p=0.000) and CM-TG clearance at 6 h (ρ=0.540, p=0.002), and CM- TG at 8 h (ρ=0.608, p=0.000).

Correlation between insulin sensitivity and postprandial triglyceride response

There was significant non-parametric correlations between HOMA-IR and WBISI and both SE-TGR (HOMA-IR; ρ= 0.395, p=0.021, WBISI; ρ=-0.384, p=0.025) and CM-TGR (HOMA-IR; ρ=0.393, p=0.021, WBISI ρ= -0.375, p=0.049) respectively. Furthermore, 77% of the obese subjects who had a pathological SE-TGR (> 0.64), and 86% who had a pathological CM-TGR (> 0.28), also had IR measured by HOMA-IR. Of the 12 subjects having IR measured by HOMA-IR, 58% of these subjects also had a pathological SE-TGR, and 50% of the 12 had an abnormal high CM-TGR.

Fasting triglycerides as predictor of insulin sensitivity

Fasting TG as a predictor for IR defined as HOMA-IR > 1.83 (see above) had the sensitivity of 83% and the specificity of 86% with a cut-off value of TG >1.13 mmol/L. BMI as a predictor of IR measured by HOMA-IR had the sensitivity of 92% and the specificity of 81%, with a BMI cut-off >29.95. In this study, total body fat percent as even a better documented predictor of IR[31], had the sensitivity of 92% and the specificity of 72%, with a cut-off total body fat percent >41%. Abdominal fat percent as a predictor of IR had in this study the best

sensitivity of 100% and the specificity of 82%, with a cut-off >51%. With a model combining BMI and fasting TG (BMI x fasting TG) as predictors of IR measured by HOMA-IR the sensitivity was 92 % and the specificity was found to be 86% with a cut- off value of >31.3.

Discussion

In this study we have shown that young, apparently healthy obese subjects have a postprandial delayed metabolism of SE-TG and in CM-TG when measured indirectly by OFTT. When adjusted for fasting TG at baseline, the obese subjects still had higher postprandial SE-TG levels, compared to the normal weight controls. The obese subjects with a fasting TG > 1.02 mmol/L and TG >1.13 mmol/L had a significantly delayed SE-TG- and CM-TG clearance, and pathological insulin sensitivity, respectively. Our data indicate that young, apparently healthy obese subjects have early metabolic disturbances.

Insulin sensitivity

In agreement with other studies [28-30] , 66 % of the apparently healthy obese subjects had IR defined by HOMA-IR (>1.83) based on 95% CI of the normal weight subjects. This limit is in well agreement with other studies performing comparisons of HOMA-IR to the gold standard method glucose clamp[29]. In our study with a median BMI around 35, 2/3 had IR, and this is similar to that observed in other reports with the same BMI [28-30]. In the postprandial state IR is associated with increased intestinal production of CM[32]. The RM-ANOVA model of postprandial TG clearance did not include HOMA-IR in addition to fasting TG in part due to low statistical power, and in part because the statistical model becomes unstable when entering closely correlated variables, such as HOMA-IR and fasting TG. To explore this further, we tested replacing fasting TG with HOMA-IR; however this model was inferior to the model presented here and explained less of the variance in the data set. In our study, as expected, the postprandial TG clearance, SE-TGR and CM-TGR was significantly correlated to the insulin sensitivity, reflecting the LPL activity in the endothelium[33]. There are some factors related to insulin resistance, that we did not measure in this study. Among them are hepatic lipid content, gastric motility, leptin, adiponectin and modified VLDL export. It would be of interest to investigate the combined relationship among these factors and postprandial triglyceride clearance in a future study.

Characteristics of postprandial triglyceride metabolism

Using an OFTT test there was a delayed peak and clearance of postprandial SE- TG and CM-TG in the obese subjects compared to the normal weight controls. Moreover, CM-TG was the most sensitive test as significant differences were observed both after 6 h and 8 h postprandial. A postprandial delayed SE-TG clearance is well documented in overweight and obese subjects with fasting hypertriglyceridemia (for review, see [33]), whereas diverging results exist for CM-TG[15]. Moreover, similar studies in healthy, obese subjects with normal fasting TG few reports exist, and especially for CM-TG. In two studies of obese subjects with normal fasting TG levels, a delayed postprandial metabolism of TG was observed, but the study groups were small[17,18]. As far as we know, no reports exist for postprandial CM-TG. The postprandial TG in serum and in CM have been reported to be a key marker, and a more sensitive risk factor for atherosclerosis than the corresponding fasting levels [34] (for review see[35]). The greater the magnitude and duration of the postprandial TG response, the arterial wall will be more exposed to postprandial TRL. The longer duration of the postprandial TRL in the bloodstream will give more time to replace cholesterol ester in LDL and HDL, favoring the transformation of LDL to be a smaller and more pro-atherogenic particle, and making HDL more dysfunctional. Moreover, the mechanism of TRLs influence on lowering the HDL- level, is believed to be due to the enrichment of TG to the HDL particle, which leads to increased catabolism of Apo-A-I HDL (for review see[36]).

In our study the fasting TG levels were significantly higher, and the fasting HDL cholesterol was significantly lower in the healthy obese subjects than in the normal weight controls, although within the normal range. As expected the fasting HDL cholesterol significantly correlated to the postprandial CM-TGR, SE-TGR, SE-TG clearance and CM-TG clearance at 6 h, and CM- TG at 8 h. This implies that an OFTT can be performed to unmask early changes in the TG metabolism in overweight and obese subjects which may have strategic therapeutical implications. The OFTT may be a way to identify overweight and obese subjects at high risk of developing the metabolic disturbances.

There are several lifestyle factors contributing to delayed clearance of postprandial triglycerides. Among them are: low level of physical activity, low intake of omega 3 fatty acids and high alcohol intake. Some studies have shown that low fat and high carbohydrate diets may increase the postprandial triglycerides. The study subjects did a 12 h fast before the OFTT, in agreement with other studies[20,21,37]. However if the subjects would have a longer fasting period, one might hypothesize an even lower post prandial triglyceride level in both groups. However to explore real- life reflection of postprandial triglycerides in the study subjects, 12 h fast is considered enough to reach a fasting state. This was also reflected on the TG profile during the OFTT of duration of 8 h. In addition the study subjects had a "normal" food intake, without extensive amount of fat and no alcohol 3 days prior to the OFTT, to reflect the reference population. However they did not fill out a 3 day food- or activity diary and the amount of omega- 3 fatty acids in the diet were not investigated; this may be a weakness of the study. On the other hand, the participants did not follow a specific diet at inclusion, as we wanted to explore the reference population in a non-dieting group.

Fasting triglyceride as predictor of postprandial lipid profile

All of the apparently healthy obese subjects included in the study had fasting TG in the normal range. In addition, the obese subjects with high-normal fasting TG (> 1.02 mmol/L) had delayed postprandial TG clearance compared to both the obese subjects with low normal-fasting TG and normal weight subjects. This indicates that in healthy, obese subjects fasting TG in the upper- normal range predicts a delayed postprandial TG clearance both in serum and in chylomicron. It is well known that fasting TG levels are strongly associated to the postprandial TG metabolism[38]. Another strong influence on the postprandial TG profile is abdominal obesity[13-16]. All of the apparently healthy, obese subjects had abdominal obesity according to the WHO and International diabetes foundation (waist circumference >88 cm (80) for women and >102 cm (94) for men. In our study abdominal fat percent was strongly correlated to postprandial TG profile, as expected [13-16].

Fasting triglycerides as a predictor for insulin sensitivity

IR is closely related to postprandial lipid metabolism because of insulin's influence on LPL-activity in the vascular endothelium[12]. In our study 77% of the obese subjects who had a

pathological SE-TGR, and 86% who had a pathological CM-TGR, also had insulin resistance measured by HOMA-IR. Moreover, we also found that fasting TG, in the normal range predicted IR with a sensitivity of 83% and the specificity of 86% with a cut-off value of TG > 1.13 mmol/L. Our findings that fasting TG in the normal range in obese subjects can predict IR is of great utility in clinical practice. The calibrated cut-off of fasting TG of 1.13 mmol/L should therefore be followed up with a validation study.

The advantage of this study is that it focuses on triglyceride- rich lipoproteins, with a specific focus on chylomicrons on apparently healthy, non-dieting, obese subjects.
However, in our study there are also some areas with limitations. First, the sex distribution is unbalanced among our participants. This indicates a need to confirm our results in a larger study population to explore sex differences. Second, a quantitative and direct estimate of the triglyceride clearance like isotopic labeling is the gold standard; in our study, triglyceride clearance was estimated indirectly. However OFTT has proven to have a strong correlation to triglyceride clearance, when compared to other methods[19]. Third, LPL- activity[11] was not measured which would also be of importance in a future study.

Conclusion

In apparently healthy obese subjects early metabolic disturbances with delayed metabolism of postprandial SE-TG and CM can be observed. When adjusted for fasting TG at baseline the obese subjects still had higher postprandial serum TG levels, compared to normal weight controls. A sub analysis revealed that fasting TG level in the higher normal range predicted delayed postprandial TG clearance and insulin resistance, but only in obese subjects. Further studies should focus on a new and possibly lower normal range for fasting TG in obese subjects, as this may have therapeutic implications.

Conflict of interest

The authors report no conflict of interest.

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Author contributions

M.A. Larsen designed the study, conceived and carried out the experiments, data collection, analyzed the data and wrote the manuscript. R. Goll analyzed the data, supervised the manuscript and generated figures. S. Lekhal contributed to the design of the study. OS. Moen carried out some of the tests. J. Florholmen designed the study and supervised the manuscript. All the authors had final approval of the article before submission.

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Table 1. Anthropometric, metabolic- and clinical characteristics at baseline between normal weight- and obese subjects. Values are mean (95% CI).

	Baseline	
Variables	**Normal weight subjects (n=17)**	**Obese subjects (n=18)**
Sex (M/F)	2/15	3/16
BMI (kg/m2)	22 (21, 23)	37 (34, 38) ***
Total fat percent (%)	27(24, 30)	49 (46, 51) ***
Abdominal fat percent (%)	28 (24, 31)	56 (54, 58) ***
Systolic BP (mmHg)	108 (102, 114)	124 (118, 130) ***
Diastolic BP (mmHg)	65 (62, 68)	73 (69, 76) ***
Glucose (mmol/L)	4.5 (4.3, 4.7)	4.8 (4.5, 5.0)
Insulin (mikromol/L)	5.1 (4.0, 6.2)	12.1 (9.8, 14.5) [M] ***
HOMA-IR	1 (0.8, 1.2)	2.6 (2.0, 3.2)[M] ***
WBISI	157 (130, 191)[G]	62 (52, 74)[G] ***
Total cholesterol (mmol/L)	4.5 (4.1, 4.8)	4.2 (3.9, 4.5)
LDL cholesterol (mmol/L)	2.7 (2.3, 3.0)	2.7 (2.4, 3.1)
HDL cholesterol (mmol/L)	1.6 (1.4,1.8)	1.2 (1.0-1.3) ***
HDL/ LDL ratio	0.7 (0.5, 0.8)	0.5 (0.4, 0.6) [M] *
TG (mmol/L)	0.9 (0.7,1.0)	1.3 (1.1, 1.6) **

*p<0.05, ** p<0.005, ***p<0.0001. [G] Geometric mean. [M] Mann- Whitney non-parametric test.

Table 2. Postprandial triglyceride (TG) clearance, chylomicron triglyceride (CM-TG) clearance, Triglyceride response (TGR) and chylomicron triglyceride response (CM-TGR) after oral fat- tolerance test at baseline. Values are median (range).

	Normal weight	**Obese**	**P**
6h SE-TG clearance (%)[M]	115 (163)	61 (96)	0.000
8h SE- TG clearance (%)[M]	125 (257)	103 (100)	0.123
6h CM-TG clearance (%)[M]	88 (110)	60 (104)	0.011
8h CM-TG clearance (%)[M]	98 (97)	78 (114)	0.007
TGR [M]	0.34 (1.8)	0.63 (1.5)	0.013
CM-TGR [M]	0.15 (0.36)	0.22 (0.5)	0.006

[M] Mann Whitney U test.

Legends to figures

Figure 1: Oral fat- tolerance test in apparently healthy, obese and normal weight subjects at study baseline. Upper part shows total serum triglycerides (SE-TG) and lower part shows chylomicron triglycerides (CM-TG). At 0 h the subject ingested a standard meal. Asterisk denotes significant difference (RM-ANOVA: interaction time×group; post-hoc comparison, Bonferroni correction). Dagger denotes a significant difference in grand mean (RM-ANOVA, between subjects). Double dagger denotes significant difference in triglyceride clearance at 6 h or 8 h (Wilcoxon). Triangles represent obese subjects; circles represent normal weight subjects. Values are estimated marginal means (95 %CI).

Figure 2: SE-TG in oral fat- tolerance test in normal weight and apparently healthy, obese subjects. Estimated marginal means (SEM) of the observed interaction between weight group, fasting triglyceride group, and time is plotted. Triangles represent obese subjects, circles represent normal weight subjects. Solid lines indicate fasting triglyceride level > 1.02 mmol/L; dashed lines indicate fasting triglyceride level ≤ 1.02 mmol/L. Asterisk indicate significant contrast. Values are estimated marginal means (95 %CI).

Figure 3: Indirect SE- TG clearance at 6 h vs. insulin sensitivity (WBISI; panel A), and insulin resistance (HOMA-IR; panel B). Normal weight subjects are shown as squares, obese subjects as circles. Linear regression line is based on all subjects. Dashed lines indicate 95% confidence interval for prediction.

Paper II

Leptin to Adiponectin ratio – a surrogate biomarker for early detection of metabolic disturbances in obesity

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The authors report no conflicts of interest.

Abbreviations:

CVD: Cardiovascular disease, L:A ratio: Leptin to Adiponectin ratio, HOMA-IR: homeostasis model assessment of insulin resistance, MHO: Metabolically healthy obese, MDO: Metabolic dysregulated obese, Triglycerides : TG, Body mass index : BMI, National Cholesterol Education Panel/Adult Treatment panel: NCEP/ATPIII, LDL: Low density lipoprotein, HDL: High density lipoprotein, , Dual X-ray absorptiometry: DEXA, Oral glucose tolerance test : OGTT, Oral fat tolerance test : OFTT, Resting energy expenditure: REE, Insulin resistance : IR, Leptin resistance: LR, Receiver operating characteristics: ROC, Positive predictive value: PPV, Negative predictive value: NPV.

Abstract

Aim. To study if the leptin to adiponectin (L:A) ratio, can be a potential biomarker for postprandial triglyceride clearance, insulin resistance (IR) or leptin resistance (LR) in apparently healthy obese, and obese individuals with established metabolic disease.

Material and methods. Fifty adult subjects with obesity (BMI ≥30); of which 36 metabolic healthy obese (MHO), and 14 metabolic dysregulated obese (MDO), with clinical and/or biochemical signs of metabolic disease were included. Seventeen healthy, normal weight subjects represented the control group. Postprandial triglyceride (TG) levels were measured in an 8 h oral fat tolerance test (OFTT). IR by HOMA-IR, L:A ratio and indirect LR were measured.

Results. In the MHO group, 71.4%, 69.4% and 86.1%, had delayed TG clearance, IR and LR, respectively; whereas in the MDO group this was detected in 85.7%, 71.4% and 91.7%, respectively. A combination of all three metabolic risk factors was found in 39.8% of the MHO and in 42.9% of the MDO patients. Receiver operating characteristics (ROC) analysis revealed that a cut-off value for the L:A ratio of >1.65 for the control group (PPV 1.0, NPV 0.91) and >3.65 for the obese subjects (PPV 0.86, NPV 0.48) predicted the delayed TG clearance with a good specificity and sensitivity. Detecting a combined risk with at least 2/3 metabolic risk factors, the ROC yielded the most suitable L:A ratio cut-off at >1.88.

Conclusion. L:A ratio was able to detect early metabolic disturbances in obese individuals, and may be a potential useful clinical surrogate biomarker of metabolic disorders.

Introduction

Postprandial hyperlipidaemia, a risk factor for cardiovascular disease (CVD) [1, 2] and ischemic stroke [3] mediated via atherosclerosis, has been associated to overweight [4] and especially to abdominal obesity [5] . We have previously showed a prolonged postprandial clearance of triglycerides (TG) in metabolically healthy obese (MHO) adults, indicating metabolic disturbances in these apparently healthy subjects [6]. It is of importance to detect individuals at high risk for further disease development, so that prophylactic actions can be taken. At this point, no clinical screening tools exist that are sensitive enough to detect early metabolic disturbances in MHO.

Adipokines, produced by white adipose tissue, have been shown to play a pivotal pathophysiological role in the metabolic disease in obesity and low-grade inflammation. Increased levels of leptin, which is the case in obesity and leptin resistance (LR), are directly or indirectly associated to cardiovascular health [7]. In contrast, adiponectin has shown to have protective and anti-atherogenic actions [8]. Also, there are diverging reports about adiponectin. Especially in subjects with low BMI and chronical illness, recent studies show that it might be associated to increased all-cause mortality and increased cardiovascular mortality [9-11]. However, the relationship between leptin and adiponectin has made the basis for the use of the leptin to adiponectin (L:A) ratio, first described in the literature in 2004 [12]. The L:A ratio has been shown to be a sensitive marker for established metabolic syndrome and insulin sensitivity [13], and is a potential atherogenic index in both healthy subjects [14] as well as subjects with Type 2 diabetes [12, 15].

There is clinical evidence of a close link between L:A ratio, insulin resistance (IR) and atherosclerosis [12-15]. We also know that lipid disturbances are central for the development of atherosclerosis, with hypertriglyceridemia as an independent risk factor [16, 17]. However,

to our knowledge there is no documentation of an association between the L:A ratio and postprandial hyperlipidemia. Therefore, the aim of this study was to test the L:A ratio as a potential surrogate biomarker of postprandial TG clearance, IR or LR in an adult population of obese subjects with and without established metabolic disease.

Methods

Participants

Volunteers were recruited from the Centre of Obesity, Department of Gastroenterology, at the University Hospital of North Norway. The inclusion criteria for the obese subjects were body mass index (BMI) ≥30 kg/m^2 and age 18-70 years. An obese patient was considered a MHO when documented normotensive, normal thyroid function tests, normal liver function tests, normal kidney function, normolipemic and normoglycemic, none of the metabolic syndrome criteria, excluding the waist circumference criteria [18]. An obese patient was considered metabolically dysregulated obese (MDO) when he or she had two or more of the metabolic syndrome criteria according to the NCEP/ATPIII guidelines [18], excluding the waist circumference criteria, which all subjects had. Within the MDO group, five patients had elevated fasting TG (≥1.7 mmol/L), three patients had untreated hypertension (≥ 130/≥ 85 mmHg), six patients had reduced high density lipoprotein (HDL) cholesterol (Women: < 1.29 mmol/l, men <1.03 mmol/L) and six patients had elevated fasting glucose (≥ 5.6 mmol/L). Furthermore, ten subjects had hypertension regulated within the normal range with antihypertensive medication, five subjects had diabetes mellitus type II (regulated with lifestyle, no anti-diabetes medication), eight patients used lipid lowering drugs and four patients were treated for hypothyreosis. All of the study subjects had T4 and TSH within the normal range. Exclusion criteria were pregnancy, current smoking, serious mental illness, and the use of medications to induce weight loss. The inclusion and exclusion criteria for the age and sex

matched, healthy controls were the same, except for having to be of normal weight (BMI <25 kg/m^2).

Height, body weight, and waist circumference were measured, and BMI calculated. Blood pressure was measured 3 times on the right arm, after a 15 minute rest. Appropriate cuff size was used. The mean of the two last measurements were used. All the blood samples were collected at the laboratory, and at the same day, for the analysis of fasting glucose, total cholesterol, low density lipoprotein (LDL) cholesterol, HDL cholesterol and fasting TG. They were taken from the antecubital vein, with the patient in a seated position. Serum lipids and apolipoprotein were measured according to a previous report from our group [6].

Dual X-ray absorptiometry (DEXA, Lunar Prodigy Advance, GE healthcare, USA) measurements were collected at baseline for all subjects. The DEXA measured total fat percent, abdominal fat percent, total fat mass (kg), and total muscle mass (kg).

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was conducted using an oral intake of 75 g glucose as previously described [6].

Oral fat tolerance test

The oral fat tolerance test (OFTT) has proven to be a good, indirect and qualitative measure of postprandial TG clearance [19]. OFTT was performed as previously described [6]. The participants had an 8 h oral OFTT to measure TG clearance: The subjects were instructed to have a normal food intake, no intake of alcohol, and to abstain from moderate to heavy physical activity for three days, and fasted 12 h before the start of the test. Halfway through the test they had one fruit and 500 mL of sugar-free soda. They were at rest, not allowed to smoke or chew gum, and were only allowed to drink water during the test day. Blood samples

for serum TG were collected before the test meal (baseline) and every second hour over the next 8 h. The TG clearances at 6 h and 8 h were calculated by the following formula: $Clearance\ (6h) = 100 * \left(1 - \frac{TG(6h) - TG(0h)}{TG(max) - TG(0h)}\right)$. We have previously demonstrated that the postprandial TG clearance at 6 h was the most suitable measure [6].

Insulin resistance

Serum insulin was analyzed directly by ELISA (DRG Insulin Elisa kit, DRG Instruments GmbH, Germany). IR determination was by the homeostasis model assessment for IR (HOMA-IR), and the cut-off value defined as the upper limit of the 95% CI of HOMA-IR in the normal weight control group [20-22].

Leptin resistance

Resting energy expenditure (REE) measurements were performed by a canopy test with an indirect calorimetry device from Medical Graphics CPX metabolic cart (St Paul, MN, USA). The indirect calorimetry was performed in a supine position. Before start, the O_2 and CO_2 analyzers were calibrated (a combined internal and manual adjustment system), based on the ambient temperature and barometric pressure. In addition, the breathing capacity analyzer was calibrated with a three-calibration syringe using multiple measures. Measurements were taken in a resting and fasting state for 30 min. REE was derived from the respiratory exchange ratio and the respiratory quotient. At the completion of the REE, blood samples for measurements of serum leptin and adiponectin were obtained. LR was calculated as an indirect measure; REE to serum leptin ratio [23].

Measurements for adipokines

ELISA kits (DRG Diagnostics, Marburg, Germany) were used to analyze the adipokines leptin (sandwich ref. EIA-2395) and adiponectin (human, ref. EIA-4574).

Statistics

Statistics were calculated on IBM SPSS 23 for Windows (SPSS Inc., IBM Corporation, Armonk, New York, USA). Parametric statistics were performed when the raw data or transformed data followed a normal distribution; otherwise, non-parametric tests were used. Tests for independent or paired samples were used as appropriate. Two sided p-values <0.05 were considered statistically significant. The cut-off values of receiver operating characteristics (ROC) targets were determined by the appropriate upper or lower limit of the 95% CI for the normal weight control group. Optimal cut-off values were defined by highest Youden index. For each cut-off value we performed a logistic regression to estimate the odds-ratio (95% CI, p-value) for a given state based on a positive L:A ratio by that cut-off (corrected for sex and age).

The study was approved by The Regional Committee for Medical and Health Research Ethics of Northern Norway and the data bank approved by Norwegian Social Science Data Services (2007, ID: 200704595-10/MRO/400).

Results

Subject Characteristics

Sixty-seven subjects were included: 17 normal weight subjects, 36 MHO and 14 MDO. Table 1 shows the anthropometric, and table 2 the clinical and metabolic characteristics for the study population. As expected, the anthropometric, clinical and metabolic data showed several differences between the groups; fasting glucose, serum adiponectin, serum leptin, LR, serum

triglycerides, HDL- cholesterol, L:A ratio, fasting insulin and HOMA-IR were all significantly different in all of the obese subjects compared to the normal weight subjects (table 2). Total cholesterol and LDL- cholesterol were not significantly different between the groups (table 2). Age, diastolic blood pressure and fasting glucose (table 1) were significantly higher in the MDO group compared to the MHO group. Total fat percent and abdominal fat percent were slightly higher in the MHO group than in the MDO group, although not significantly different (table 1). There were no significant differences found in LR, L:A ratio, leptin and adiponectin levels between the MHO and the MDO (table 2). There was an unbalance no significant difference in sex among the three subgroups (Chi-square).

Cut-off values for target variables

Leptin resistance. The cut-off value for LR was defined as below the 95% CI of the measurements in normal weight subjects, and was found to be <114.5. Forty-two (87.5%) of the 48 obese subjects had LR according to this cut-off value. Of these, 31 of 36 (86.1%) were MHO, and 11 of 12 (91.7%) were MDO.

Insulin resistance. The cut-off value of HOMA-IR defined by the 95% CI of the normal weight subjects, was calculated to be >1.83. Thirty-five (70%) of all obese subjects had IR according to HOMA-IR, of these, 25 of 36 (69.4%) were MHO and 10 of 14 (71.4%) were MDO.

Delayed TG clearance. A delayed TG clearance, defined by the 95% CI of the normal weight subjects, was calculated to be <88.8%. According to this, 37/49 (75.5%) had delayed TG clearance at 6 h, 25/35 (71.4%) of the MHO and 12/14 (85.7%) of the MDO.

Moreover, 38.9% of the MHO and 42.9% of the MDO had a combined delayed TG clearance, IR and LR.

L:A ratio as a predictor single metabolic disturbances

A L:A ratio ROC curve was made to detect delayed TG clearance defined as <88.8% at 6 hours (Figure 1, panel A). Using Youden index (J = sensitivity + specificity - 1) we found two optimal cut-off values; first at 1.36 (J 0.41; PPV 0.79; NPV 0.71), and second at 3.65 (J 0.42; PPV 0.87; NPV 0.50). By logistic regression, the cut-off value of 1.36 yielded an OR of 8 (95%CI: 2-31; P = 0.004) for pathologic TG clearance, classifying 79% of the cases correctly; the cut-off value of 3.65 yielded an OR of 7 (2-28; P = 0.004), classifying 76% of the cases correctly. However, looking at the subgroups, the cut-off ratio of >1.36 had good characteristics for normal weight subjects (PPV 1.0, NPV 0.91), while the cut off at >3.65 was more suitable to predict delayed TG clearance in MHO (PPV 0.86, NPV 0.48). Most of the subjects in the MDO group (85%) had a delayed TG clearance. A subgroup logistic regression was not possible due to the limited number of observations.

A similar analysis of L:A ratio vs. IR (Figure 1, panel B), showed the most suitable cut-off value for L:A ratio at >2.2 (J 0.59; PPV 0.78; NPV 0.86). By logistic regression, the L:A ratio cut-off of 2.2 yielded an OR of 31 (6 – 166; P <0.0005) for pathologic insulin resistance, classifying 81% of the cases correctly. As leptin is part of both calculations we did not conduct a ROC analysis to predict LR by L:A ratio.

L:A ratio as a predictor of combined metabolic risk

We then explored if L:A ratio could predict combined delayed TG clearance and IR. A ROC analysis detecting at least one of delayed TG clearance or IR in all groups (Figure 1, panel C) found the most suitable cut-off value to be L:A ratio >1.36 (J 0.74; PPV 0.96 NPV 0.69). When detecting subjects with both delayed TG clearance and IR (Figure 1, panel D), the most suitable cut-off value for L:A ratio was found to be >3.6 (J 0.48; PPV 0.67; NPV 0.81). By logistic regression, L:A ratio of >1.36 gave an OR of 59 (7 – 479; P < 0.0005) for having at least one of pathologic TG clearance or IR, classifying 92% of the cases correctly.

Combining delayed TG clearance, IR, and LR in a ROC analysis, we defined two levels: at least 1 of 3 and at least 2 of 3 as target variables. The first ROC curve (at least 1 of 3) (Figure 1, panel E) showed an optimal cut-off at L:A ratio >1.12 (J 0.96; PPV 1.00; NPV 0.78), however, all obese subjects had at least one metabolic disturbance. Using the higher target variable (at least 2 of 3), the ROC curve (Figure 1, panel F) yielded the most suitable L:A ratio cut-off of >1.88 (J 0.76; PPV 0.95; NPV 0.72). By logistic regression, the L:A ratio cut-off of 1.88 yielded an OR of 48 (8 – 296; $P < 0.0005$) for having at least two disturbances, classifying 88 % of the cases correctly.

Fasting TG as a predictor of delayed TG clearance

In comparison, drawing a ROC curve for fasting TG as predictor of delayed TG clearance at 6 h (Figure 1, panel G) yielded a cut-off value of >1.09 (J 0.28; PPV 0.76, NPV 0.50). This cut-off could not predict pathologic TG clearance significantly by logistic regression.

Fasting insulin as a predictor of combined metabolic risk

We additionally calculated a ROC curve for fasting insulin levels (data not shown). The most suitable cut-off value overall was fasting insulin >12 μmol/L, and it was more suitable to predict the higher target variable (at least 2 out of 3) (J 0.53 PPV 1.0; NPV 0.42), compared to when the subjects only had one metabolic risk.

Discussion

In the present study, we have explored L:A ratio as a possible surrogate biomarker for the detection of subclinical disturbances in metabolism due to obesity. L:A ratio had good test characteristics for detection of delayed TG clearance, IR or LR alone, and even better for the detection of combined early metabolic disturbances. Thus a L:A ratio above cut-off may

indicate any of delayed TG clearance, IR or LR, and may therefore represent a sensitive test for early metabolic disturbances.

We chose cut-off values for the target variables using the 95% CI of the results of the normal weight control group for delayed TG clearance, IR and LR. There is no clear consensus on the cut-off values of these parameters. Our intention was to detect subclinical disturbances of metabolism in order to identify subjects at risk of developing overt metabolic disturbances. However, using a case-control design, we can only indicate possible outcomes, and further prospective studies are necessary to investigate this hypothesis.

In the literature, the concept of MHO has been used for obese with none and up to two clinical established metabolic disturbances [24]. The MHO in our study did not have any clinically significant metabolic disturbances, as indicated by cholesterol, fasting triglycerides, fasting glucose and blood pressure. In one study, approximately one-third of obese subjects were considered MHO, having less than 2 metabolic disturbances [24]. When considering the ATP-III criteria [25] for the metabolic syndrome, the prevalence of MHO were slightly higher at 39% [24]. Previous studies with long follow-up periods have demonstrated that these MHO individuals are at an increased risk of major CVD events [26, 27] and overall mortality [27] as compared to metabolically healthy normal weight individuals. Without a good biomarker it is difficult to predict which individuals in the MHO group that is at risk.

Leptin reflects fat mass, and as expected, leptin levels were increased in all of the obese subjects. In our study, close to 90% of the obese subjects had LR, but no differences were seen between the two obese subgroups. Leptin was somewhat non-significantly higher in the MHO group than in the MDO group, most likely explained by non-significant differences in the body fat percent. Other studies have also found no significant difference in fasting leptin between MHO and MDO [28, 29]. A study from 2014 including over 11000 subjects found

fasting leptin to have moderate sensitivity and specificity for identifying cardio-metabolic abnormalities and leptin sensitivity [30].

In our study, adiponectin was significantly lower in obese subjects than in healthy normal weight subjects, as expected, whereas no differences were observed between the MHO and MDO. Finally, 76% of the obese subjects (both MHO and MDO) had low adiponectin values (95% CI of normal weight: <9.6 μmol/L). A few studies have examined the adipokine profiles of MHO [28, 29, 31]. One study from 2010 reported higher adiponectin levels in MHO, compared to MDO [29]. None of the subjects in this study were elderly, nor did they have low BMI, going through weight loss, had CVD, chronic kidney disease or heart failure. In such subjects studies have shown that high circulating levels of adiponectin might be associated to increased mortality, this may be due to smaller, differentiated adipocytes, increased production from non-adipocyte tissue, decreased elimination or direct stimulation through natriuretic peptides [9-11] . The L:A ratio might therefore not be of clinical value for this patient group, with already prominent chronic disease.

In this report we have found that 71.4% of the MHO subjects had delayed TG clearance. Furthermore 38.9% of the MHO and 42.9% of the MDO had a combined delayed TG clearance, IR and LR, indicating that almost all of the obese subjects had a dysregulated metabolism. L:A ratio has shown to be associated with a clustering of metabolic risk factors in adolescents [32]. However, as far as we know this is the first report to show that the L:A ratio is a sensitive biomarker for delayed TG clearance, also in combination with IR and LR. Fasting insulin was found to be less sensitive to detect early metabolic changes, but more suitable when the subjects had developed more than two metabolic changes. Predicting delayed TG clearance, an increased risk of CVD, may be of great clinical value in the daily treatment of the obese patient. L:A ratio was found to be sensitive to detect delayed TG clearance in all subjects, and performed better than fasting TG. Therefore, the L:A ratio may

detect obese subjects in high risk of development of the various metabolic disturbances such as CVD and Type 2 Diabetes.

Strengths of this study: First, subjects were included from the everyday practice at the obesity out-patient clinic, which underlines the clinical utility and transferability of our observations. Second, metabolic disturbances is the product of various interactive and apparently complex pathophysiological mechanisms summarized in the L:A ratio as a surrogate biomarker of clinical utility. Third, we have performed a thorough simultaneous characterization of the three axes of developing metabolic disturbances (delayed TG clearance, IR and LR).

The most prominent weaknesses: First, a lack of match between the three groups studied according to number of subjects, sex and age. There was an unbalance between sex to an extent that sex specific analysis was not possible to perform, however this unbalance was not significant. Second, lack of statistical power, as a larger study would yield safer conclusions. The number of participants was limited due to the extensive data collection and testing involved in each participant. Third, by setting cut-off values for the target variables from the 95% CI of normal controls, we intentionally detect very early disturbances of metabolism; however, this choice may be controversial. Fourth, the cross sectional design, as our suggested L:A ratio cut-off values need verification in a prospective study of metabolic disturbances in a larger study group with more balance between sex.

Conclusion

We suggest that L:A ratio may be a good surrogate biomarker of early obesity-related metabolic disturbances of either kind. This may enable early, directed intervention and prevention of developing metabolic disturbances and related diseases.

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Table 1. Anthropometric characteristics at baseline between normal weight subjects and obese subjects. Values are median (Interquartile range). † Significantly different from normal weight. ‡ Significantly different from MHO. ^{K} Kruskal- Wallis test. Abbreviations: MHO: metabolic healthy obese, MDO: metabolic dysregulated obese, BMI: body mass index, BP: blood pressure, n.s: Non-significant.

Table 2. Metabolic and clinical characteristics at baseline between normal weight and obese subjects. Values are median (Interquartile range). † Significantly different from normal weight. ‡ Significantly different from healthy obese. [K] Kruskal- Wallis test. Abbreviations: HOMA-IR; homeostasis model assessment of insulin resistance, L:A ratio; Leptin to Adiponectin ratio, REE; Resting energy expenditure, LDL; Low density lipoprotein, HDL; high density lipoprotein, TG; Triglycerides, n.s: Non-significant.

Figure 1. Receiver operating characteristics curves for the detection of delayed triglyceride clearance, insulin resistance and leptin resistance by the leptin to adiponectin ratio.

Panel A: Detection of delayed TG clearance by L:A ratio: AUC 0.74, P= 0.002. Panel B: Detection of IR by L:A ratio: AUC 0.83, P= 0.000. Panel C: Detection of at least one of delayed TG clearance or IR by L:A ratio: AUC 0.83, P= 0.000. Panel D: Detection of at both delayed TG clearance and IR by L:A ratio: AUC 0.776, P= 0.000. Panel E: Detection of at least one of delayed TG clearance, IR, or LR by L:A ratio: AUC 0.98, P= 0.000. Panel F: Detection of at least one of delayed TG clearance, IR, or LR by L:A ratio: AUC 0.94, P= 0.000. Panel G: Detection of delayed TG clearance by fasting TG: AUC 0.68, P= 0.020.Abbreviations: TG: triglyceride, IR: Insulin resistance, L:A ratio: Leptin to adiponectin ratio.

Paper III

Postprandial leptin and adiponectin in response to sugar and fat in obese and normal weight subjects

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Keywords: postprandial; oral fat tolerance test; adiponectin; leptin; triglycerides; adipokines; obesity.

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Trial registration information: The study was approved by The Regional Committee for Medical and Health Research Ethics of Northern Norway (2007, ID: 200704595-10/MRO/400), and the data bank approved by Norwegian Social Science Data Services (ID: 2206). Registered the 15th of April 2008.

Abstract

Purpose. Adipokines, produced by white adipose tissue are central in the development of lifestyle diseases. Individuals in industrialized countries spend a substantial part of life in the non-fasting, postprandial state, which is associated with increased oxidation and inflammation. The aim was to study postprandial adiponectin and leptin levels after an oral fat tolerance test (OFTT) and oral glucose tolerance test (OGTT) in obese (OB) and healthy, normal weight subjects (NW).

Methods. Fifty adult subjects with obesity (BMI ≥30) and 17 NW were included. Postprandial triglyceride (TG), adiponectin and leptin levels were measured every second hour during an 8 h OFTT, and every half hour during a 2 h OGTT.

Results. Compared to the basal level, postprandial levels of adiponectin following OFTT showed a slight initial peak, followed by a significant decrease at 8 h, in the NW. In the OB these changes were abolished. Postprandial levels of leptin decreased significantly from basal in the OFTT, in the NW, whereas in the OB, leptin was unchanged except for a slight increase from 2 h to 8 h. During the OGTT both adiponectin and leptin levels remained unchanged in the NW, but decreased significantly in the OB. In addition the OB had delayed TG clearance at 6 h.

Conclusion. A fatty meal gives postprandial changes in the secretion of adiponectin and leptin in NW, but not in OB. Our observations indicate that a potential postprandial regulatory role of adiponectin and leptin is impaired in OB, and of importance in a more comprehensive understanding of the delayed postprandial TG clearance in obese subjects.

Introduction

Overweight and obesity are raising global health problems with several metabolic disturbances and co-morbidities, such as type 2 diabetes mellitus and cardiovascular disease (CVD). CVD by itself is the leading cause of morbidity and mortality in industrialized countries, with obesity as an independent risk factor[1], and it was the main cause of death worldwide in 2012[2].

In obesity the amount of white adipose tissue (WAT) is increased. WAT is a highly metabolically active endocrine organ (for review, see Ahima [3]). More than 600 adipokines has been described thus far [4], among which are leptin and adiponectin[3]. Increased levels of leptin, which is the case in obesity and leptin resistance (LR), are directly or indirectly associated to CVD [5]. In contrast, adiponectin, which is reduced in the obese and diabetic state, has shown to have protective and anti-atherogenic actions, opposing hyperglycemia, inflammation, lipotoxic damage and insulin resistance (IR) [6,7]. Furthermore, both leptin and adiponectin may facilitate responses of fibroblast growth factor 21 (FGF-21), which has effect on energy expenditure and whole-body glucose metabolism. FGF-21 is also a potent regulator of adiponectin secretion [8]. However, there are diverging reports about the role of adiponectin. In subjects with chronic illness and low body mass index (BMI), recent studies show that adiponectin might be associated with increased all-cause mortality, as well as increased cardiovascular mortality [9-11]. Furthermore, adipokines play a pivotal role in the inflammation process and in the development of non-alcoholic-fatty liver disease (NAFLD) (for review, see Boutari [12]).

Individuals in industrialized countries spend a substantial part of life in the non-fasting, postprandial state, which is associated with increased oxidation and inflammation. Postprandial hyperlipidemia has been associated with overweight [13] and abdominal obesity

[14-17] and is an independent risk factor for atherosclerosis. Furthermore, our knowledge of postprandial changes of leptin and adiponectin in normal weight and obese subjects is limited. The results are diverging, both for postprandial leptin and adiponectin secretion, with reports of no postprandial changes in leptin[18-20], as well as increased postprandial leptin levels in normal weight controls and decreased in obese subjects[21,22]. For adiponectin, reports have found both increased [23-25] and unchanged [26-28,25,29] for both normal weight and obese subjects.

Due to the diverging results of the postprandial profile of adiponectin and leptin secretions both in normal weight and obese subjects, the aim of this study was to explore leptin and adiponectin in the postprandial state, in response to fat and a carbohydrate load, separately, in obese and healthy, normal weight subjects.

Methods

Participants

Volunteers were recruited from the Centre of Obesity, Department of Gastroenterology, at the University Hospital of North Norway (UNN). The inclusion criteria for the obese subjects were BMI ≥30 kg/m^2 and age 18-70 years. Fifty obese subjects were included. Of these five patients had elevated fasting TG (≥1.7 mmol/L), three patients had untreated hypertension (≥ 130/≥ 85 mmHg), six patients had reduced high density lipoprotein (HDL) cholesterol (Women: < 1.29 mmol/l, men <1.03 mmol/L) and six patients had elevated fasting glucose (≥ 5.6 mmol/L). Furthermore, ten subjects had hypertension regulated within the normal range with antihypertensive medication, five subjects had diabetes mellitus type II (regulated with lifestyle, no anti-diabetes medication), eight patients used lipid lowering drugs and four patients were treated for hypothyreosis. All of the study subjects had thyroxin (T4) and thyroid-stimulation hormone (TSH) levels within the normal range. Exclusion criteria were pregnancy, current smoking, serious mental illness, and the use of

medications to induce weight loss. The inclusion and exclusion criteria for the normal weight (BMI <25 kg/m^2) were the same. All of the participants in the study were Caucasian.

Height, body weight, and waist circumference was measured. Blood pressure was measured 3 times on the right arm, after a 15 minute rest. Appropriate cuff size was used. The mean of the two last measurements were used. All blood samples were collected at the laboratory, and at the same day, for the analysis of fasting glucose, total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol and fasting TG. The samples were taken from the antecubital vein, with the patient in a seated position. Serum lipids and apolipoprotein were measured according to a previous report from our group [30].

Dual X-ray absorptiometry (DEXA, Lunar Prodigy Advance, GE healthcare, USA) measurements were collected of all the study participants. The DEXA measured total fat percent, abdominal fat percent, total fat mass (kg), and total muscle mass (kg).

Oral fat tolerance test

The oral fat tolerance test (OFTT) has proven to be a good, indirect and qualitative measure of postprandial TG clearance[31]. OFTT was performed as previously described whereas most of the data from normal weight subjects have been published previously [30]. In short, blood samples for serum TG were collected at baseline before the high fat test meal (1 g fat per kg body weight) and thereafter every second hour over the next 8 h. The TG clearance at 6 h was calculated by the following formula: $Clearance\ (6h) = 100 * \left(1 - \frac{TG(6h) - TG(0h)}{TG(max) - TG(0h)}\right)$. We have previously demonstrated that the postprandial TG clearance at 6 h was the most suitable measure [30].

Oral glucose tolerance test

A standard oral glucose tolerance test (OGTT) was conducted after 12 h fasting, using an oral intake of 75 g glucose as previously described [30]. Glucose and insulin were measured every half hour for 2 h, and the subjects were at rest during the whole test. Serum insulin was analyzed directly by ELISA (DRG Insulin Elisa kit, DRG Instruments GmbH, Germany). IR determination was done by the homeostasis model assessment for IR (HOMA-IR) [32-34], and was calculated as followed: HOMA-IR = Fasting insulin (μmol /L) × Fasting glucose (mmol/L)/22.5 [35].

Indirect leptin resistance

We wanted to measure indirect LR, for comparison between the normal weight and the obese individuals. Resting energy expenditure (REE) measurements were performed by a canopy test with an indirect calorimetry device from Medical Graphics CPX metabolic cart (St Paul, MN, USA). The indirect calorimetry was performed in a supine position. Before start, the O_2 and CO_2 analyzers were calibrated (a combined internal and manual adjustment system), based on the ambient temperature and barometric pressure. In addition, the breathing capacity analyzer was calibrated with a three-calibration syringe using multiple measures. Measurements were taken in a resting and fasting state for 30 min. REE was derived from the respiratory exchange ratio and the respiratory quotient. At the completion of the REE, blood samples for measurements of serum leptin and adiponectin were obtained. Indirect LR was measured as; REE to serum leptin ratio[36].

Measurements for adipokines

ELISA kits (DRG Diagnostics, Marburg, Germany) were used to analyze the adipokines leptin (sandwich ref. EIA-2395) and adiponectin (human, ref. EIA-4574) at baseline and postprandial during OFTT and OGTT.

Statistics

Statistics were calculated on IBM SPSS 25 for Windows (SPSS Inc., IBM Corporation, Armonk, New York, USA). Parametric statistics were performed when the raw data (fasting glucose, fasting leptin and L:A ratio) or transformed data (Indirect leptin resistance, HOMA-IR and WBISI), using log transformation, followed a normal distribution using; otherwise, non-parametric tests were used. Tests for independent or paired samples were used as appropriate. Two sided p-values <0.05 were considered statistically significant. Repeated measures analysis of variance (RM-ANOVA) was used to analyze the postprandial timeline for normal weight subjects and obese subjects. Corrections of violations of sphericity were used as appropriate, according to the epsilon value.

Results

Anthropometric, metabolic- and clinical characteristics

Anthropometric, metabolic- and clinical characteristics of obese subjects and normal weight subjects are shown in table 1. As expected, there were several significant differences; especially a higher baseline adiponectin and lower baseline leptin in the normal weight subjects compared to the obese subjects. Furthermore, delayed postprandial TG clearance at 6 h, lower insulin sensitivity, and higher indirect LR, were found in obese subjects compared to normal weight subjects (Table 1).

Oral fat tolerance test

Postprandial triglyceride after oral fat tolerance test

Normal weight subjects. When comparing fasting TG to postprandial TG levels, in normal weight subjects, there was a significant increase in TG at 2 h (p=0.000), and close to

significant increase at 4 h (p=0.062) and 8 h (p=0.084) during the OFTT (Figure 1C and table 2).

Obese subjects. When comparing fasting TG to postprandial TG levels, in obese subjects, there was a significant increase in TG at 2 h (p=0.000), 4 h (p=0.000) and 6 h (p=0.000) (Figure 1C and table 2).

Postprandial adiponectin after oral fat tolerance test

Normal weight subjects. For the healthy, normal weight subjects, when compared to baseline values there was a slight, non-significant, increase of adiponectin at 2 h (p=0.052), and a subsequent significant decrease towards 8 h postprandial (p= 0.046)(Figure 1A and table 2).

Obese subjects. In the obese subjects, when compared to baseline values there were no significant differences in adiponectin levels postprandial during the OFTT (Figure 1A and table 2).

Postprandial leptin after oral fat tolerance test

Normal weight subjects. When compared to baseline values the postprandial leptin levels were significantly decreased at 2 h (p= 0.001), 4 h (p= 0.001), 6 h (p=0.001) and 8h (p=0.004). Compared to adiponectin an initial increase of leptin was not observed (Figure 1B and table 2).

Obese subjects. When compared to baseline values the postprandial leptin levels were unchanged at all time points (Figure 1B and table 2), but they had a slight, non-significant, increase at 8h (p=0.052).

Oral glucose tolerance test

Postprandial adiponectin after oral glucose tolerance test

Normal weight subjects. When compared to baseline levels there was a trend towards increased adiponectin at 30 min (p=0.064) (Figure 2A and table 3).

Obese subjects. Compared to baseline values of adiponectin there was a significant decrease of adiponectin at 90 min (p=0.009) (Figure 2A and table 3).

Postprandial leptin in oral glucose tolerance test

Normal weight subjects. When compared to the baseline values leptin decreased slightly (Figure 2B and table 3), but not significant (n.s.).

Obese subjects. When compared to baseline levels, leptin had a gradual and significant decrease at all time points 30 min, 60 min, 90 min and 120 min (all: p=0.000) during OGTT (Figure 2B and table 3).

As expected both insulin and glucose increased significantly at all time points in both groups during the OGTT (Fig 2C, 2D and table 3). The obese subjects had significant higher basal (Table 1) and postprandial levels of both insulin and glucose, as expected. No measurements of glucose and insulin were done in OFTT.

Discussion

In this study we report postprandial adiponectin, leptin and TG responses after an 8 h fat load and a 2 h carbohydrate load in normal weight and obese subjects. In addition to insulin resistance, indirect leptin resistance was observed in the obese subjects, in addition to delayed TG clearance. In general, an apparent time effect of a postprandial suppression of leptin and adiponectin was observed in normal weight subjects in response to a fat load, whereas these regulations were more or less abolished in the obese subjects. Our data indicates that

adiponectin and leptin might have a postprandial regulatory role that can be overruled in obese subjects. These interactions are tightly associated to postprandial TG clearance in an apparent complex and not well understood regulatory mechanisms in the white adipose tissue.

The postprandial adiponectin levels observed in response to a fat load in our study is in conflict with other studies. In normal weight subjects, other studies have found postprandial adiponectin to be both increased [23,24], or unchanged [26-28,25], whereas in obese subjects both increased [25] and unchanged levels [26,29] has been observed. The early, slight increase of adiponectin seen in our study, after a fat load, indicates that there might be a triggered exocytic pathway in the adipocyte. This is supported from studies in mice where a response time of 10-45 min for translocation of adiponectin to the plasma membranesome [8]. In mice exogenous adiponectin enhanced free fatty acid (FFA) oxidation by activating the adenosine monophosphate-activated protein kinase, to reduce the postprandial FFA increase [37,38]. Based on these reports, and our observations, the exact physiological role of adiponectin is still hard to understand. Most likely, adiponectin appears to play a tuning role in FFA oxidation especially in fat tissue to accommodate storage of postprandial excess of TG, to enhance FFA oxidation in skeletal muscle, to improve insulin sensitivity and to suppress glucose production in in liver (for review, see Wang[39]). This is mainly achieved by a large number of hormones released from each organ.

Recently, much attention has been on the FGF-21-adiponectin axis [8], which has been proposed to protect against a various cardio-metabolic disorders via mediating multi-organ communications (for review, see Hui [40]). It has been proposed that FGF-21 regulates postprandial lipid metabolism and permits better clearance of triglyceride-rich lipoprotein fractions [41], especially in healthy subjects, and that adiponectin might mediate this response. In our study there was a trend towards a slight, non-significant, increase of adiponectin 2 h in the OFTT, followed by a significant decrease, in normal weight subjects,

whereas no changes in the obese subjects. Therefore, it is tempting to speculate that the fat induced response of adiponectin observed in the normal weight subjects in our study, could be explained by a FGF-21 mechanism, but this FGF-21-adiponectin axis is overruled in obesity, perhaps due to FGF21 resistance. Furthermore, a report has also shown that impaired leptin signaling, in relation to increased caveolin-1-expression, in obesity, may prevent a concordant increase in adiponectin despite high levels of leptin [42]. This might indicate that leptin resistance and adiponectin resistance is connected in complex mechanisms. One might also speculate that leptin resistant subjects, has a different postprandial profile than leptin sensitive subjects. However, this awaits future studies that have the correct study design to explore this further.

The postprandial leptin response to a fat load was significantly decreased at 2-8 h in normal weight subjects; on the contrary, the obese subjects had a slightly increased leptin at 8 h, however non-significant. These results contradict for the most other reports. For normal weight subjects, one study showed that leptin decreased at 6 h [43] such as in our study, unchanged in other studies [19,18], whereas an increase was observed in other reports [22,20,21]. In obese subjects two reports showed a significant postprandial decrease in leptin [21,22], as well as no postprandial leptin changes were observed in other studies [19,18]. Finally, opposite to our observation of the secretion of adiponectin, no initial close to significant, increase of leptin was observed. This is in agreement with a report that human leptin secretion has a constitutively slow profile [44]. The discrepancies between these reports are hard to explain, but can to some extent be explained by differences in the postprandial observation time, that for most studies were less than 3 h. In addition the studies often have a small study groups. Altogether, the exact postprandial physiological role of leptin, if any, is still unsettled. However, it is tempting to speculate that leptin increases the postprandial FFA oxidation (expenditure) in healthy normal weight subjects, at least in muscle tissue[45], and

that this is abolished in obese subjects with established leptin resistance. This may explain the delayed TG clearance; however, this awaits further studies.

There is increasing knowledge of adipocyte physiology that act to nutritional changes, that by systemic effect either can be beneficial, or harmful with various metabolic disturbances, such as in obesity. This is most likely a fine tuning of interactions in the adipokine and myokine secretome. Moreover, the cell biology of the fat expansion is complex, but the increased understanding of the pathophysiological changes in the fat tissue explaining the detrimental systemic effects (for review, see Rutkowski [46]). Our observations suggest that a postprandial increase of TG trigger a fine tuning adipokine response in normal weight subjects, but is overruled in obese patients with leptin resistance, and most likely also with adiponectin resistance[47].

The strengths of this study are, first, subjects were included from the everyday practice at the obesity out-patient clinic, which underlines the clinical utility and transferability of our observations. Second, the postprandial measurements of the adipokines were done over a long observation time of 8 h for the fat-load and 2 h for the carbohydrate load, and documented the TG clearance. Third, the number of study participants was higher than previous studies that have investigated postprandial adipokines. The most prominent weaknesses are, first, a lack of match between the groups studied according to number of subjects, sex and age. Moreover, we did not measure or record any type of exercise, nor did we monitor the diet, or intake of different types of fat, as for example N-3-PUFA in the period before the postprandial studies. Finally, a model of adipokine measurements directly in interstitial fat tissue is highly preferable to get a more precise postprandial response profile of adipokines.

Conclusion

In conclusion, postprandial changes were observed in both adiponectin and leptin suggesting a physiological role after a fatty meal in normal weight subjects. In obese subjects with leptin

resistance and delayed TG clearance these regulatory mechanisms seems to be overruled, but of importance in a more comprehensive understanding of the delayed postprandial TG clearance in obese subjects.

Declarations

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Ethics approval and consent to participate

The study was approved by The Regional Committee for Medical and Health Research Ethics of Northern Norway (2007, ID: 200704595-10/MRO/400) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The data bank approved by Norwegian Social Science Data Services (ID: 2206), registered on the 15th of April 2008. Informed consent was obtained from all individual participants included in the study.

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Larsen MA is a medical doctor specializing in obesity, weight-loss and lifestyle disorders through her PhD, obesity education from Harvard University and daily clinical work. Larsens expertise in the obesity field have been of importance for developing private obesity clinics and interdisciplinary obesity teams within the secondary health care service.

List of abbreviations

CVD: Cardiovascular disease, WAT; white adipose tissue, FGF-21: fibroblast growth factor 21, L:A ratio: Leptin to Adiponectin ratio, HOMA-IR: homeostasis model assessment of insulin resistance, NAFLD; Non-alcoholic fatty liver disease, TG: Triglycerides, BMI: Body

mass index, PUFA: poly-unsaturated fatty acids, UNN: University Hospital of North Norway, kg: kilograms, T4: thyroxin, TSH: thyroid-stimulation hormone, NCEP/ATPIII: National Cholesterol Education Panel/Adult Treatment panel, LDL: Low density lipoprotein, HDL: High density lipoprotein, DEXA: Dual X-ray absorptiometry, OGTT: Oral glucose tolerance test, OFTT: Oral fat tolerance test, REE: Resting energy expenditure, IR; Insulin resistance, RM-ANOVA: Repeated measures analysis of variance, n.s.: not significant, FFA: free fatty acid.

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Table 1. Anthropometric, metabolic- and clinical characteristics between normal weight- and healthy obese subjects. Values are median (Interquartile range).

	Baseline		
Variables	**Normal weight subjects (n=17)**	**Obese subjects (n=50)**	**Sig. (*p*)**
Sex (M/F)	2/15	10/40	n.s.
Age (years)	31.0 (24.5; 37.5)	39.8 (30.8; 48.8)	<0.01
BMI (kg/m2)	21.3 (20.2; 22.4)	39.8 (36.0; 43.6)	<0.001
Total fat percent (%)	26.6 (23.7; 29.6)	50.4 (46.4; 54.5)	<0.001
Abdominal fat percent (%)	27.5 (24.3; 30.7)	57.6 (54.7; 60.6)	<0.001
Systolic BP (mmHg)	105 (98; 113)	127 (118; 136)	<0.001
Diastolic BP (mmHg)	65 (60; 70)	75 (68; 81)	<0.001
Fasting Glucose (mmol/L)	4.4 (4.0; 4.7)	5.3 (4.8; 5.7)	0.001[M]
Fasting Insulin (µmol/L)	5.53 (4.08; 6.99)	12.12 (8.14; 16.10)	0.001
HOMA-IR	1.09 (0.37; 1.11)	2.76 (1.82; 4.17)	<0.001[G]
WBISI	147.8 (97.1; 198.6)	59.6 (42.7; 83.2)	<0.001[G]
Fasting Leptin (µmol/L)	8.5 (4.8; 12.2)	39.2 (24.3; 54.1)	<0.001[M]
Fasting Adiponectin (µmol/L)	11.8 (8.2; 15.3)	8.1 (5.9; 10.3)	0.001
Leptin/Adiponectin ratio	0.77 (0.31; 1.23)	4.26 (1.55; 6.97)	<0.001[M]
Resting Energy Expenditure, REE (kcal)	1356 (1263; 1448)	1734 (1526; 1943)	<0.001
Indirect leptin resistance (REE/leptin OFTT)	142.5 (75.5; 209.5)	47.5 (31.0; 72.7)	<0.001[G]
Total cholesterol (mmol/L)	4.2 (3.8; 4.7)	4.4 (3.9; 4.9)	n.s.
LDL cholesterol (mmol/L)	2.6 (2.0; 3.3)	2.9 (2.4; 3.4)	n.s
HDL cholesterol (mmol/L)	1.6 (1.4; 1.9)	1.1 (1.0; 1.3)	<0.001
HDL/ LDL ratio	0.57 (0.33; 0.82)	0.41 (0.29; 0.54)	<0.01
Fasting Triglycerides (mmol/L)	1.0 (0.8; 1.2)	1.4 (1.1; 1.8)	<0.001
Triglyceride clearance 6 h (%)	115.4 (39; 226.5)	58.0 (0; 193)	<0.001

[G] Geometric median. [M] Mann- Whithey non-parametric test.

Table 2. Postprandial leptin, adiponectin and triglycerides following OFTT in obese and normal weight. Values are mean and standard deviation (STD).

Measure	**Group**	**Timeline postprandial OFTT**				
		0 h	2 h	4 h	6 h	8 h
Adiponectin (µmol/L)	Normal weight	13.4 (7.1)	14.5 (8.26)	12.9 (6.25)	12.2 (5.23)	11.5* (5.56)
	Obese	8.77 (3.34)	8.84 (3.43)	8.94 (3.16)	8.57 (2.94)	8.74 (3.14)
Leptin (µmol/L)	Normal weight	9.31 (5.51)	7.86* (5.08)	7.70* (4.72)	7.80* (5.24)	8.29* (5.32)
	Obese	39.6 (20.1)	38.8 (21.0)	39.1 (20.5)	40.5 (22.6)	42.1 (23.8)
Triglycerides (mmol/L)	Normal weight	0.933 (0.316)	1.50* (0.545)	1.18 (0.500)	0.890 (0.381)	0.790 (0.274)
	Obese	1.47 (0.670)	2.37* (1.05)	2.67* (1.52)	2.19* (1.42)	1.56 (1.03)

*Sig. difference from fasting values (0h) in individual groups.

Table 3. Postprandial leptin, adiponectin, glucose and insulin following OGTT in obese and normal weight individuals. Values are mean and standard deviation (STD).

Measure	**Group**	**Timeline postprandial OGTT**				
		0 min	30 min	60 min	90 min	120 min
Adiponectin (µmol/L)	Normal weight	12.4 (7.26)	13.4 (6.95)	12.7 (12.8)	12.8 (7.00)	12.1 (7.94)
	Obese	8.57 (3.40)	8.57 (2.98)	8.37 (3.22)	8.10* (2.81)	8.38 (3.03)
Leptin (µmol/L)	Normal weight	8.84 (4.24)	8.50 (4.12)	8.39 (4.37)	8.49 (4.24)	8.40 (4.31)
	Obese	40.4 (22.3)	37.7* (20.6)	36.9* (20.0)	36.05* (18.8)	36.49* (19.2)
Insulin (µmol/L)	Normal weight	5.09 (2.04)	34.8 (11.0)	38.6 (12.9)	27.2 (13.5)	23.4 (10.3)
	Obese	14.27 (10.9)	70.2 (31.5)	87.5 (49.6)	80.3 (50.2)	67.7 (51.9)
Glucose (mmol/L)	Normal weight	4.36 (0.317)	7.38* (0.700)	7.12* (0.941)	6.03* (1.16)	6.03* (1.06)
	Obese	5.37 (1.04)	8.31* (2.10)	8.97* (2.81)	8.51* (2.81)	7.49* (2.67)

*Sig. difference ($p<0.05$) from fasting values (0h) in individual groups.

Figure 1. Oral fat tolerance test Eight hour Oral fat tolerance test in normal weight (circle) and obese (square) subjects measuring adiponectin, leptin and triglycerides. Significant differences ($p<0.05$) from baseline values are marked * in the separate groups.

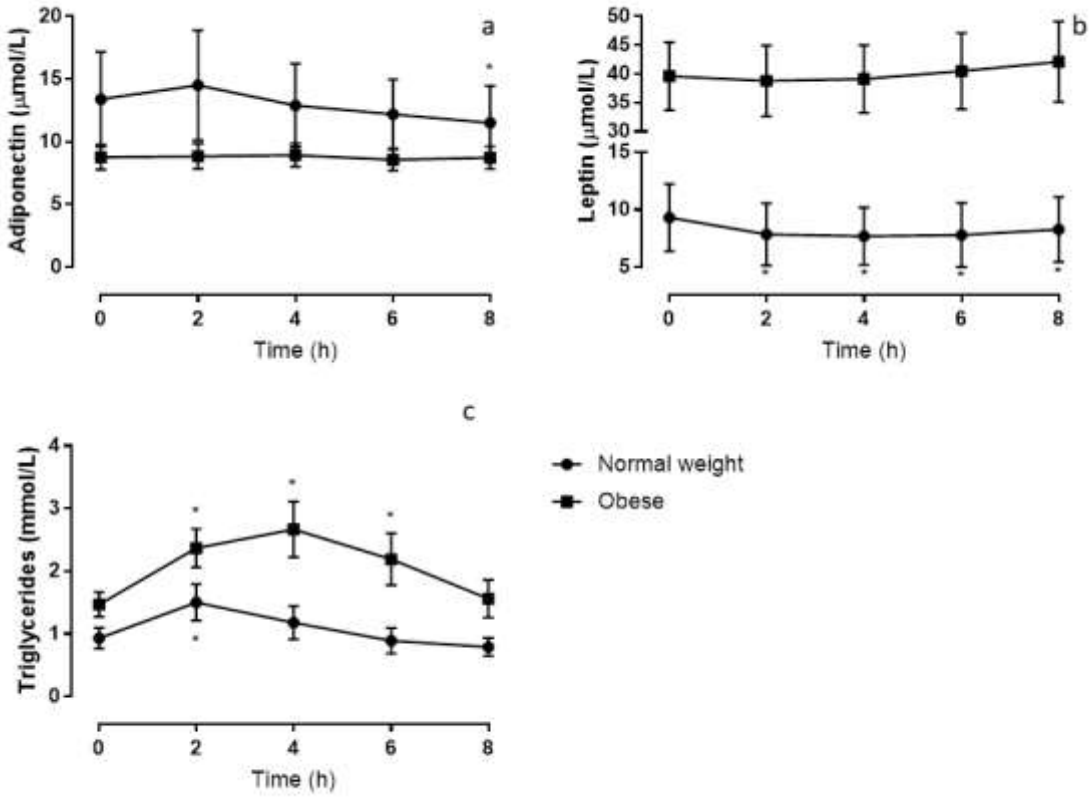

Figure 2. Oral glucose tolerance test Two hour Oral glucose tolerance test in normal weight (circle) and obese (square) subjects measuring adiponectin, leptin, insulin and glucose. Significant differences ($p<0.05$) from baseline values are marked * in the separate groups.