UiT The Arctic University of Norway

Faculty of Health Sciences

Early Markers of Metabolic Dysregulation in Obese Individuals

Identification at Baseline and Effect of Modest Weight Loss

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1 Acknowledgments

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Victoria Therese Isaksen

Ph.d. Candidate

2 Summary in English

Obesity is a condition with an elevated risk of cardiovascular disease, type 2 diabetes and cancer, among other conditions. Evidence suggests that the pathophysiological link between obesity and chronic lifestyle disease is the presence of chronic, low-grade inflammation. Leptin and adiponectin play principal roles in inducing inflammation and promoting insulin resistance, resulting in metabolic syndrome.

To this date, a cost-effective and reliable way of inducing long-term weight loss has yet to be found. Our study aimed to search for simple and clinically feasible biologic markers to identify metabolically dysregulated obese individuals in need of treatment and to study these markers' improvement with modest weight loss.

Methods: We included participants with either BMI ≥30 kg/m^2 or moderately elevated liver enzymes for ultrasonographic assessment of hepatic steatosis and calculated their hepatorenal index (HRI) based on optical density measures. Furthermore, we measured fasting and postprandial triglycerides, insulin, glucose, leptin, adiponectin, and resting energy expenditure (REE) at baseline and after conservative weight loss treatment in participants with obesity. We performed ROC analyses to assess how HRI and leptin to adiponectin (L:A) ratio can predict insulin resistance and pathologic triglyceride clearance at 6 hours, leptin- and insulin resistance, respectively. We then performed repeated measure analyses to assess improvements in the variables after a weight loss of ≥5%.

Results: HRI ≥1.17 could predict insulin resistance, defined as homeostasis model assessment of insulin resistance (HOMA-IR) ≥2.3 with 94% sensitivity and 70%

specificity in participants with BMI ≥30 kg/m^2. HRI ≥1.42 demonstrated low sensitivity and high specificity in all BMI groups.

L:A ratio ≥3.65 predicted delayed triglyceride clearance with a positive predictive value (PPV) 0.86 and negative predictive value (NPV) 0.48 in obese participants. L:A ratio ≥1.88 was most suitable for detecting two of the three metabolic risk factors insulin resistance, leptin resistance and delayed triglyceride clearance.

The HOMA-IR (-23.1%), REE:leptin ratio (+80.1%) and L:A ratio (-45.7%) all significantly improved in participants with weight loss of ≥5%, yet the triglyceride response and clearance at 6 hours improved neither in the weight loss group nor in the non-weight loss group.

Conclusion: HRI and L:A ratio are feasible markers for detecting underlying metabolic disturbances in obese individuals. The L:A and REE:leptin ratios are also feasible for monitoring the improvement of metabolic health during weight loss, whereas postprandial triglyceride measurements are not.

3 Norsk sammendrag

Overvekt og fedme er blant verdens ledende dødsårsaker gjennom sin tilknytning til kroniske følgesykdommer som hjerte-/karsykdom, kreft, autoimmune sykdommer og type 2 diabetes. Funn gjennom de senere år tilsier at disse tilstandene kan være knyttet sammen gjennom kronisk, lavgradig betennelse, der adipokinene leptin og adiponektin hos den overvektige spiller sentrale roller i å fremme betennelsen, som igjen bidrar til å øke insulinresistens og dermed metabolsk syndrom.

Vi har fortsatt ikke funnet virksom og kostnadseffektiv behandling for fedme. I et helsevesen preget av omstruktureringer ønsket vi derfor å finne enkle biologiske markører som kan skille mellom metabolsk syke og friske overvektige, og dermed hvem som trenger behandlingen mest. Videre ønsket vi å se hvordan disse endret seg med vektreduksjon.

Metoder: Vi rekrutterte pasienter med BMI ≥30 kg/m^2 og et utvalg personer med lett forhøyede leverprøver til undersøkelsen. Vi utførte ultralyd av lever og nyrer og kalkulerte hepatorenal indeks (HRI) som et mål på fettlever. Vi brukte dette til å predikere insulinresistens, vurdert ved HOMA-IR. Dernest målte vi insulin, glukose, triglyserider, leptin og adiponektin fastende og etter måltid hos pasienter med BMI ≥30 kg/m^2, både ved start og etter konservativ behandling for vektreduksjon. Vi undersøkte hvordan leptin til adiponektin (L:A) ratio kunne predikere insulin- og leptinresistens og forsinket triglyseridfall etter måltid. Videre så vi hvordan disse fire markørene bedret seg med et vekttap på mer enn 5%.

Resultater: Vi så at HRI ≥1,17 kunne finne insulinresistens med 94% sensitivitet og 70% spesifisitet hos personer med BMI ≥30. Ved å sette HRI-grensa til 1,42 hadde testen lav sensitivitet, men høy spesifisitet for å finne insulinresistens.

Deltakere med fedme hadde generelt tendens til insulin- og leptinresistens og forsinket triglyseridfall etter måltid, men metabolsk syke hadde tendens til mer forstyrrelser enn metabolsk friske overvektige. L:A ratio- verdier ≥3,65 kunne brukes til å finne forsinket triglyseridfall, og L:A ratio ≥1,88 kunne brukes til å finne forstyrrelser i to av de tre ovennevnte markørene.

HOMA-IR (-23,1%), REE:leptin ratio (+80,1%) og L:A ratio (-45,7%) bedret seg med vektreduksjon over 5%. Forsinket triglyseridfall etter måltid bedret seg ikke med vektreduksjon.

Konklusjon: HRI og L:A ratio kan begge brukes for å oppdage underliggende metabolske forstyrrelser ved fedme. L:A ratio, men ikke triglyserider etter måltid, kan også brukes til å følge metabolsk bedring ved vektreduksjon.

4 List of Papers

Paper I: Isaksen VT, Larsen MA, Goll R, Florholmen JR, Paulssen EJ *Hepatic Steatosis, detected by hepatorenal index in ultrasonography as a predictor of insulin resistance in obese subjects.* BMC obesity. 2016. DOI: 10.1186/s40608-016-0118-0

Paper II: Larsen MA, Isaksen VT, Moen OS, Wilsgaard L, Remjin M, Paulssen EJ, Florholmen J, Goll R *Leptin to adiponectin ratio – a surrogate biomarker for early detection of metabolic disturbances in obesity.* Nutrition, Metabolism and Cardiovascular Diseases. 2018. DOI: 10.1016/j.numecd.2018.06.020

Paper III: Isaksen VT, Larsen MA, Goll R, Paulssen EJ, Florholmen JR *Correlations between modest weight loss and leptin to adiponectin ratio, insulin and leptin resensitization in a small cohort of Norwegian individuals with obesity*. Endocrine and Metabolic Science. 2023. DOI: 10.1016/j.endmts.2023.100134

5 Abbreviations

A:G	Android to gynoid (ratio)
AgRP	Agouti-related peptide
B-mode	Brightness mode
BMI	Body mass index
CRP	C-reactive protein
CT	Computer tomography
CVD	Cardiovascular disease
DEXA	Dual-energy X-ray absorptiometry
EJP	Eyvind Jakob Paulssen
GLUT4	Glucose transporter 4
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
HPA	Hypothalamic-pituitary-adrenal (axis)
HRI	Hepatorenal index
IFNγ	Interferon gamma
IL	Interleukin
IR	Insulin resistance
JAK2	Janus kinase 2
L:A	Leptin to adiponectin (ratio)
LDL	Low-density lipoprotein
LFTs	Liver function tests
MDO	Metabolically dysregulated obese
MHO	Metabolically healthy obese
MAFLD	Metabolic associated fatty liver disease
MAL	Maria Arlén Larsen

MetS	Metabolic syndrome
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCEP ATP III	The national cholesterol education program adult treatment panel III
NPV	Negative predictive value
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
OFTT	Oral fat tolerance test
PDFF	Proton density fat fraction
POMC	Pro-opiomelanocortin
PPV	Positive predictive value
REE	Resting energy expenditure
ROC	Receiver operating characteristic
SNS	Sympathetic nervous system
STAT3	Signal transducer and activator of transcription 3
TNF-α	Tumor necrosis factor alpha
ULN	Upper limit of normal
VLDL	Very low-density lipoprotein
VTI	Victoria Therese Isaksen
WBISI	Whole-body insulin sensitivity index

6 Introduction

In 2016, the global prevalence of overweight and obesity in adult men and women was 39% and 40%, respectively. Of these, about 13% of the global adult population (11% men and 15% women) were obese. This equates to over 650 million obese out of 1.9 billion overweight adults (2). The prevalence of obesity is still increasing, although its rate of increase has been slowing down over the last decade, especially in developed countries (2).

In Norway, approximately 20% of middle-aged women and 25% of middle-aged men were obese in 2015. The prevalence of obesity is the highest in Finnmark County and lowest in Oslo (3). Among individuals of the younger age groups, no significant difference is observed between the sexes, whereas in the elderly population, a higher prevalence of obesity is observed among women (4).

Being overweight and obese poses a significant risk of premature death, primarily because conditions associated with these disorders, such as hypertension, insulin resistance, dyslipidemia (hypertriglyceridemia and low high-density lipoprotein (HDL) cholesterol), and visceral fat accumulation, are featured in the metabolic syndrome (MetS) (5, 6), and ultimately increase the risk of complicating diseases (7). Among the complications, cardiovascular disease (CVD) and type 2 diabetes mellitus are the most dominant (7), but complications also include several types of cancers and non-alcoholic fatty liver disease (NAFLD) (8, 9).

Obesity – an inflammatory disease

There are several paradoxes concerning obesity and the associated disease burden. It is particularly worth noting that in individuals with chronic diseases and old age, overweight and obesity have a better prognosis compared to normal or underweight

individuals (6, 10). These paradoxes have led to the coining of a new term regarding obesity and morbidity known as adiposopathy or “sick fat”, which is related to disturbances in adipokines, inflammatory pathways, insulin resistance, and pathological fat distribution (6).

Human physiology favors energy storage in the form of fat, as historically, individuals with the ability to store energy were more likely to survive during periods of starvation (11, 12).

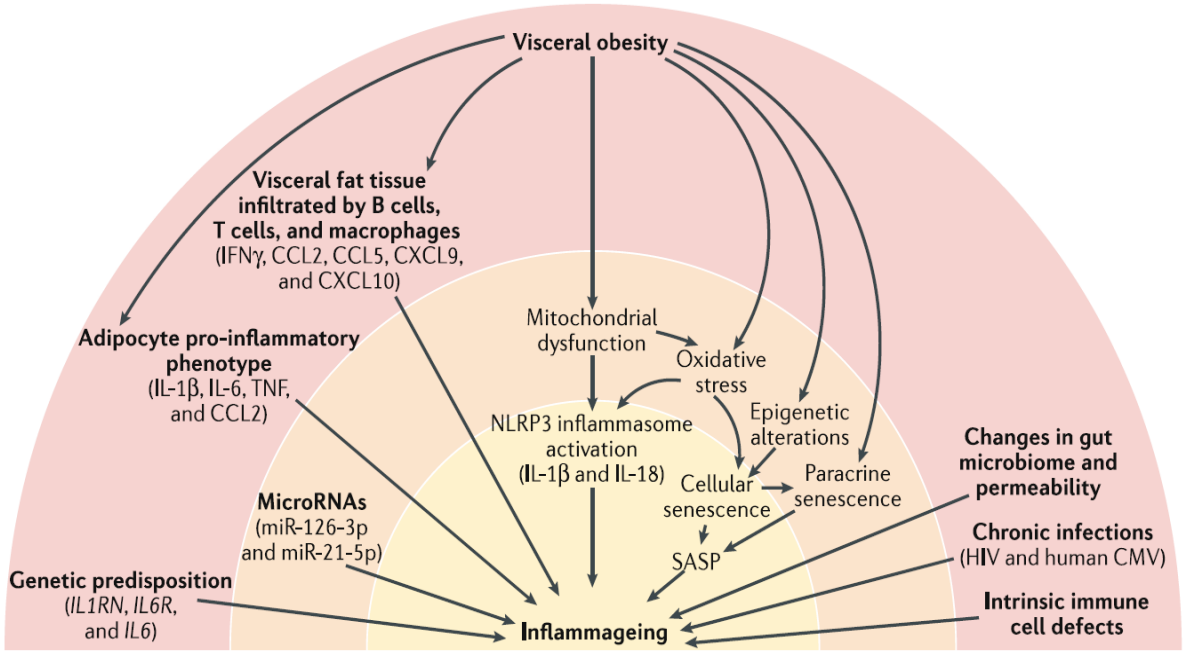

Figure 1: In central obesity, B cells, T cells, macrophages, and monocytes infiltrate the visceral adipose tissue. T cells secrete IFNγ, stimulating the production of several chemokines by adipocytes that further amplify infiltration of T cells. Cytokines released by the B cells influence the phenotypic change of adipocytes in the visceral cavity, causing the release of adipokines, other pro-inflammatory factors, and cell debris. Cell senescence can be induced by several stressors including oxidative stress and epigenetic alterations. Senescent cells, by acquisition of the senescence-associated secretory phenotype (SASP), secrete large quantities of cytokines, chemokines, and other molecules that trigger more cell senescence locally (paracrine senescence) and contribute to inflammaging (1).

Abbreviations: CC - Chemokine (C-C motif) ligand, CXCL Chemokine (C-X-C motif) ligand, CMV - Cytomegalovirus, HIV - Human immunodeficiency virus, IFNγ - Interferon gamma, IL - Interleukin, IL6R - Interleukin 6 receptor, IL1RN - Interleukin 1 receptor antagonist (gene), miR - Micro RNA, NLRP3 - nucleotide-binding oligomerization domain-like receptor protein 3, SASP - Senescence-associated secretory phenotype, SREBP1 - Sterol regulatory element binding protein, TNF - Tumor necrosis factor

Simultaneously, the capacity for inducing inflammation has been selected through evolution as it is a defense mechanism against infection (1), and cell senescence and its related secretory phenotype is favorable for tissue growth and repair in the young (13). On the other hand, the adverse health effects of chronic inflammation in individuals past the reproductive age have not exerted any selection pressure (1).

Emerging evidence in the 1990s suggested a link between obesity and adipose tissue inflammation, with studies reporting cytokine and acute phase protein production in the adipocytes (14, 15). Over the years, evidence of a clear connection has emerged between adipokine imbalance and chronic inflammation, particularly from leptin overproduction and low levels of adiponectin (16, 17).

Stress originating from inflammation, trauma, or psychosocial factors activates the hypothalamic-pituitary-adrenal (HPA) axis (18). Similar to the connection between obesity and cytokine production, activation of the HPA axis was known in the early 1990s. Chronic activation of the HPA axis, accompanied by chronically elevated cortisol levels, has multiple metabolic effects and includes all elements of the MetS (18). Long-term triggering of the HPA axis may lead to hypoactivation of the stress system with a consequent increase in inflammation as one of the many consequences of low cortisol levels (18).

Recent findings also suggest that inflammation and activation of chronic stress play a crucial role in the development of lifestyle-related chronic diseases such as CVD, type 2 diabetes, cancer, dementia, and arthritis/arthrosis, all of which are related to accelerated aging and, ultimately, frailty (1, 19, 20). The collective term used for this phenomenon is inflammaging.

Evidence suggests that the stress from inflammatory processes leads to cell senescence, in which cell cycle arrest, telomere shortening and cellular malfunctions occur (1). These processes are believed to play a crucial role in aging (21). Senescent cells also release pro-inflammatory cytokines that demonstrate paracrine and systemic effects, further promoting inflammaging (13).

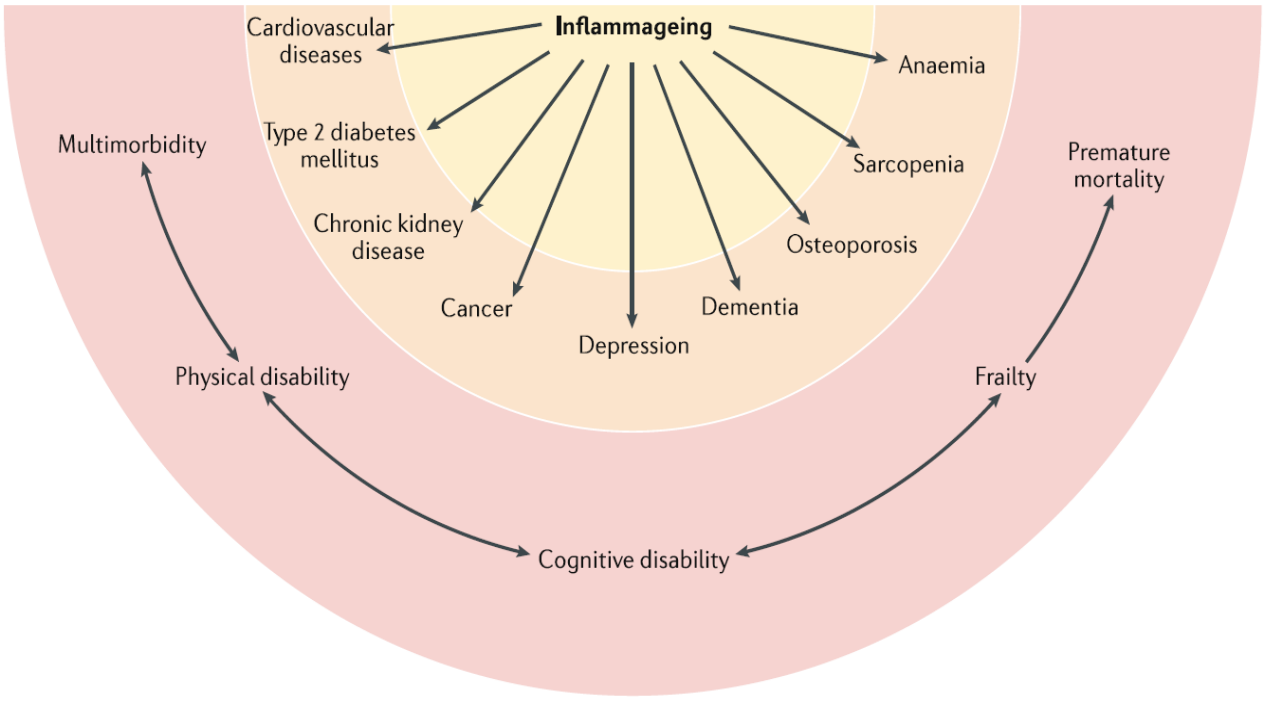

Figure 2: Inflammaging is a strong risk factor associated with numerous, highly prevalent, pathophysiologically uncorrelated diseases that are common causes of disability in old age. There is extensive evidence indicating that, in elderly individuals, inflammation contributes to the development of the diseases listed in this figure (1) but the list is far from exhaustive. Accordingly, elevated levels of pro-inflammatory markers are potent risk factors that may lead to multimorbidity. Geriatric conditions such as physical and cognitive disability, frailty and premature death are also related to inflammaging. This relationship is primarily mediated by multimorbidity but also occurs due to poor tissue maintenance and repair, leading to the accumulation of damage that contributes to frailty (1).

Obesity and insulin resistance

Insulin resistance is one of the primary conditions underlying the pathophysiology of obesity and is a crucial factor in the development of other metabolic conditions (22).

Insulin is a hormone released from pancreatic beta (β-) cells upon ingestion of carbohydrates. By binding to the insulin receptor, it translocates glucose transporter 4 (GLUT4) to the cell surface in muscle, hepatocytes and adipocytes to promote an anabolic response to available blood glucose (23, 24, 25). Insulin increases glucose transport and glycogen synthesis in skeletal muscle (23). In hepatocytes, insulin increases glycogen synthesis and glycolysis, decreases gluconeogenesis, decreases glucagon and glucocorticoid gene induction, and increases *de novo* lipogenesis. In adipocytes, insulin decreases lipolysis and increases lipogenesis, protein synthesis, and glucose transport (23, 24). Brain cells mainly express insulin-independent glucose transporters for glucose uptake. However, insulin does play a role in feedback mechanisms to suppress appetite in the pituitary gland and hypothalamus, as these areas are located outside the blood-brain barrier and express GLUT 4 receptors (23, 26).

Insulin resistance is characterized by the loss of insulin function in target cells, making it incapable of lowering blood glucose by the aforementioned anabolic responses in peripheral tissue (23, 27, 28). The factors involved in the development of insulin resistance are complex, and the primary event involved in the process is much debated.

Some evidence suggests that hyperinsulinemia, due to elevated levels of circulating free fatty acids (FFA), may occur before the development of insulin resistance because a rise in the circulating levels of insulin is detectable before changes in the levels of

blood glucose during intake of a high-fat diet in both rodents and humans (28, 29). However, this is challenged by studies on dietary macronutrient composition. Samkani and coworkers showed that postprandial glycemia and insulinemia in non-diabetic individuals were significantly reduced on a carbohydrate-reduced, high-protein diet compared to a high-carbohydrate diet (30). Furthermore, Lundsgaard and coworkers showed that insulin sensitivity is maintained on an eucaloric high-fat diet (31). This aligns with the theory that lipid dysregulation is a response to insulin resistance and not a primary event (23).

One possible mechanism leading to insulin resistance could be a high influx of fructose into the hepatocytes that occurs by bypassing the regulatory mechanisms of glycolysis, which in turn leads to *de novo* lipogenesis, elevated levels of intrahepatic triglycerides, and production of very low-density lipoproteins (VLDL) (32).

Evidence also suggests that inflammatory processes contribute to the development of insulin resistance (21). Oxidative stress, dysregulation of lipid homeostasis, adipose tissue hypoxia with subsequent cellular necrosis, endo-reticular stress, and inhibition of metabolic pathways in insulin-sensitive cells have all been proposed as conditions that may result in insulin resistance (21). Visceral adiposity, including adipokine dysregulation and ectopic adipose tissue in the liver, heart and muscles, is heavily associated with pro-inflammatory signaling (1, 33).

Once insulin resistance is established, it affects several metabolic pathways. Hyperinsulinemia and insulin resistance make up a vicious cycle that eventually leads to β-cell failure, hyperglycemia and type 2 diabetes (23, 28). There is also a loss of function of the lipoprotein lipase receptor and a decrease in the rate of triglyceride

uptake from blood to the tissues. With the failure to downregulate *de novo* lipogenesis, there is a consequent increase in serum triglyceride levels (28).

Insulin sensitivity assessment

There are two ways to assess insulin resistance; either directly, using the euglycemic hyperglycemic insulin clamp, or indirectly, using serum glucose and insulin concentrations. Among the indirect methods, the whole-body insulin sensitivity index (WBISI), obtained from the oral glucose tolerance test (OGTT), and the homeostasis model assessment of insulin resistance (HOMA-IR), obtained from fasting glucose and insulin, are the two most commonly used (34).

HOMA-IR (H) is calculated as follows:

$$H = \frac{[\text{fasting glucose}]x\ [\text{fasting insulin}]}{22.5}$$

WBISI (W) is calculated as follows:

$$W = \frac{10000}{\sqrt{[\text{fasting glucose}]\text{x}[\text{fasting insulin}]\text{x}[\text{mean OGTT glucose}]\text{x}[\text{mean OGTT insulin}]}}$$

The hyperglycemic clamp is performed by a continuous glucose infusion, acutely raising the glucose concentration to a predetermined hyperglycemic level, for example 125 mg/dL (6.9 mmol/L) above basal levels. The variable glucose infusion maintains the hyperglycemic plateau according to the body's insulin secretion rate and glucose metabolism. Since the plasma glucose concentration is kept constant, the glucose infusion rate indicates the body’s insulin secretion and glucose metabolism (35).

The euglycemic-hyperinsulinemic clamp is performed by a continuous insulin infusion, acutely raising and maintaining plasma insulin concentration to a predetermined

supraphysiological level, for example 100 µU/mL (694.5 pmol/L). Simultaneously, plasma glucose is held constant at basal levels by a variable glucose infusion. The glucose infusion rate equals the whole-body glucose uptake when steady-state is achieved and is thus a measure of tissue insulin sensitivity (35).

WBISI and HOMA-IR are deeply rooted in the physiology of glucose metabolism, as insulin sensitivity is a product of homeostatic glucose and insulin concentrations. Hence, these concentrations may be used to approximate whole-body insulin sensitivity (34). As the insulin clamp method is time-consuming and technically demanding, indirect methods of assessing insulin resistance are often preferred for clinical research.

Obesity and dyslipidemia

The definition of hypercholesterolemia is generally unclear and somewhat arbitrary, as the total and HDL/LDL cholesterol levels largely overlap between different metabolic groups (36). However, The National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) has defined elevated LDL cholesterol and non-HDL cholesterol as >3.4 and >4.1 mmol/L, respectively, as they have been found to be predictive of CVD risk (36).

The typical form of dyslipidemia found in metabolically dysregulated obese (MDO) individuals is hypertriglyceridemia (i.e., triglycerides >1.7 mmol/L (37, 38)) and a decrease in the levels of HDL cholesterol, with normal or slightly elevated levels of low-density lipoprotein (LDL) (<4.8 mmol/L in adults <50 years (38, 39)), though with a predominance of small, dense LDL particles (38, 40).

Metabolically healthy obese (MHO) individuals typically have normal cholesterol levels, similar to metabolically healthy, normal-weight individuals (40).

Evidence suggests that hypertriglyceridemia is the primary event, whereas low HDL cholesterol levels are a mere marker of the former and not an independent risk factor (37, 41). Evidence also suggests that triglyceride-rich lipoprotein remnants are more atherogenic than LDL cholesterol, as they do not require oxidation to form foam cells and cell cytotoxicity (37, 42, 43). Furthermore, recent findings suggest that a high fraction of small dense LDL particles contribute to an increased risk of CVD (43).

The mechanisms for lipid accumulation in the arterial wall have yet to be fully understood, even though much is known. Both LDL, small VLDL and their remnant particles can pass through the endothelial layer. Yet, VLDL and remnant particles are retained in the arterial wall to a much higher degree than the LDL particles due to their size, thus making them more atherogenic (44, 45).

Obese individuals manifest a dysfunctional triglyceride metabolism during the postprandial period even if they appear clinically healthy (46). This dysfunction occurs as a delayed clearance of postprandial triglycerides, especially in individuals with a fasting triglyceride level in the upper half of the normal range (46). A decreased activity of the lipoprotein lipase in adipose tissues might be the cause of this hypertriglyceridemia (47, 48). In addition to this, the levels of VLDL particles are increased, along with chylomicrons, due to *de novo* lipogenesis in hepatocytes (49).

Chylomicron levels tend to drop more rapidly than VLDL and have fewer remnant particles due to the higher affinity of the lipoprotein lipase to chylomicrons than VLDL (42). Thus, elevated levels of VLDL result in an elevated level of remnant triglycerides for several hours after a meal. The portion of the time obese individuals live under

conditions contributing to atherosclerosis is therefore increased compared to individuals with normal triglyceride metabolism (42).

Leptin

Leptin was the first adipokine discovered and is an essential factor in satiety signaling through the tissue-brain axis (50). Leptin exerts its effect in the brain by influencing the leptin receptor in pro-opiomelanocortin (POMC) and Agouti-related peptide (AgRP)/ neuropeptide Y (NPY) neurons in the arcuate nucleus of the hypothalamus, activating Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3) pathways, thus signaling satiety and inhibiting the intake of food (51). The leptin satiety signaling is delayed compared to the gut-brain axis and takes effect about 20 minutes postprandially (52).

Furthermore, leptin signaling in the hypothalamus is involved in thermogenesis regulation in brown adipose tissue, sympathetic nervous system (SNS) tone (affecting heart rate and blood pressure), thyroid hormone secretion, and fertility, among others (53). Hence, resting energy expenditure (REE) is highly affected by leptin levels, making leptin hyposecretion act as a starvation signal. The following significant REE reduction is demonstrated in both rodents and humans (53).

Animal and human models have both shown that leptin levels are inversely correlated with metabolic effects (Fig. 3) (54). Conditions related to both hypo- and hyperleptinemia are, among others, obesity, insulin resistance, hyperglycemia, hepatic steatosis and dyslipidemia. Leptin substitution in cases of leptin hyposecretion effectively reverses the conditions mentioned above (Fig. 3). However, in humans, the metabolic effects of leptin seem to be saturated at leptin levels around 30-50 ng/mL

(54). When further increasing leptin levels from a preexisting state of hyperleptinemia, no such reversing effect is seen in the above conditions (Fig 3.) (54).

Obesity is closely related to leptin resistance, characterized by the inability of high levels of circulating leptin to induce satiety signals and energy expenditure that normally would have led to weight loss (33, 55). Of the two, appetite suppression is the one most affected by leptin resistance (53). Although we now have extensive knowledge of leptin transportation across the blood-brain barrier and leptin action on the cellular level in the hypothalamus (56, 57), the anorectic effect of leptin has proved difficult to quantify in obese individuals due to the complexity of leptin actions (58, 59). In a state of elevated leptin levels, a compensatory increase in SNS tone has been demonstrated, even though the REE increase is only partial compared to the rise in

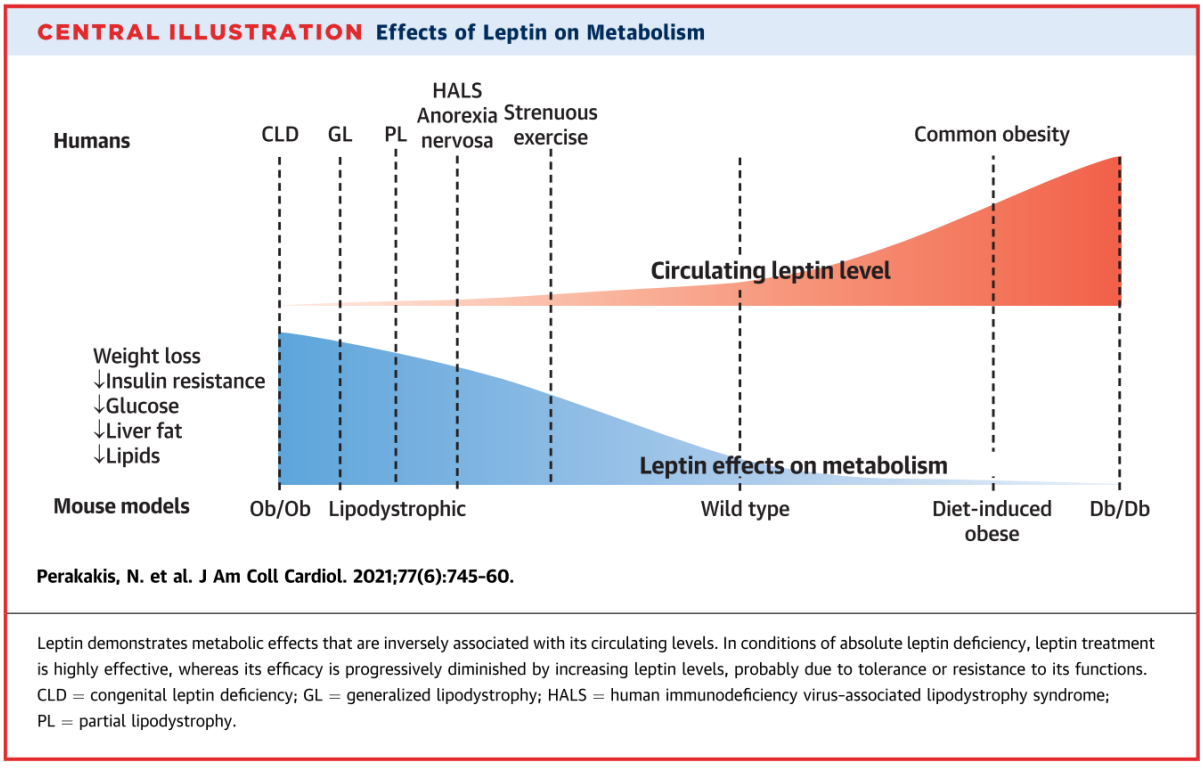

Figure 3: Effects of leptin on metabolism in different concentrations in human and mouse research models.

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circulating leptin (53). In other words, with increasing weight, higher levels of leptin are required to maintain a normal REE (60). This alteration of the ratio between REE and leptin is possible to quantify. It can thus be used as an indirect measure of leptin sensitivity in peripheral tissues (52, 61), offering a potential biological marker of metabolic function in obesity.

The role of leptin in regulating the immune system is mainly as a pro-inflammatory cytokine that increases the secretion of tumor necrosis factor-alpha (TNF-α) and several interleukins, thus acting as a component of the innate immune system. It also acts as an immune cell activator in the adaptive immune system, predominantly due to its role in activating T lymphocytes and as a promoter of dendritic cell maturation. Therefore, leptin may function as a licensor, enhancer and modulator of immune responses (19, 21), whereas adiponectin has more anti-inflammatory effects (62). Leptin is biologically active in the vascular wall: high levels of leptin impair vasodilatation, increase oxidative stress and endothelin and play a part in the expression of the type-1 angiotensin II receptor in smooth muscles, all of which contribute to hypertension and endothelial dysfunction (20). Furthermore, endogenous hyperleptinemia contributes to atherosclerosis and adverse outcomes of CVD and cardiac hypertrophy (63).

Adiponectin

Since 2001, adiponectin has been known to have anti-inflammatory, insulin-sensitizing and cardioprotective properties. It inhibits gluconeogenesis, stimulates glucose uptake, suppresses interleukin 8 (IL-8) in endothelial cells, and suppresses TNF-α in several types of cells (62, 64). Furthermore, it enhances the expression of the anti-inflammatory cytokine IL-10 in human macrophages, mitigates oxidative stress in

db/db mouse aortic endothelial cells and suppresses the formation of foam cells by inhibiting the scavenger receptor A in humans (64). Evidence suggests that adiponectin secretion is determined by adipose tissue quality rather than its quantity (63), and obese mice with adiponectin overexpression have been shown to have a metabolically healthy phenotype without insulin resistance (65).

When studying metabolic and cardiovascular effects in humans, circulating adiponectin levels were positively correlated with insulin sensitivity of skeletal muscle and liver and negatively with inflammatory markers in a Pima Indian population (66, 67, 68).

Despite this, much is debated about a positive correlation between circulating adiponectin levels and all-cause morbidity and mortality (64). We do not know how low adiponectin levels could be related to hyperinsulinemia and insulin resistance while simultaneously demonstrating this paradoxical effect on mortality (64). However, determining adiponectin levels could still be feasible for its use as a biological marker in detecting an underlying metabolic disease much before its clinical manifestation.

Leptin to adiponectin (L:A) ratio

There is clear evidence for a correlation between the L:A ratio and adipose tissue dysfunction and insulin resistance that is bigger than the correlation to leptin and adiponectin separately (69, 70, 71). L:A ratio also correlates with markers of inflammation, such as C-reactive protein (CRP) and other pro-inflammatory cytokines, hypertension and Non-alcoholic fatty liver disease (NAFLD) (69, 72). This relationship is caused by the two adipokines being regulated oppositely (63). Thus, the L:A ratio has emerged as a promising marker for cardiometabolic health in obese individuals (63, 69, 71).

The ratio can either be calculated as leptin to adiponectin, in which case the ratio is positively correlated to insulin resistance, inflammation and overweight, or it could be calculated as adiponectin to leptin, in which case the inverse correlation applies (69). Frühbeck and coworkers proposed the adiponectin to leptin ratio risk categories as normal ≥1, moderate 0.5–1, and high risk <0.5 (69). The adiponectin to leptin categories translate to our L:A ratio risk categories of normal ≤1, moderate 1–2, and high risk >2.

Non-alcoholic fatty liver disease

NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and liver failure. Furthermore, it carries a high risk of hepatocellular carcinoma (73, 74), particularly in individuals with NASH (75). NAFLD is the most common hepatic condition worldwide, with an estimated global prevalence of 25% (74), of which approximately 25-30% have steatohepatitis (75). Thus, NAFLD is the most prevalent cause of elevated liver enzymes in the general population (70) and is rapidly becoming the number one cause of liver transplantation in light of new therapeutic strategies that reduce the heavy burden of hepatitis C (74).

NAFLD often co-exists with insulin resistance and type 2 diabetes. Up to 75% of adults with type 2 diabetes also have NAFLD (76). Insulin plays a crucial role in regulating hepatic glucose metabolism. The presence of insulin resistance dramatically increases the development of NAFLD (76). Due to the close correlation between NAFLD and MetS, together with an unclear definition of excess alcoholic intake, it has been proposed to rename the condition ‘metabolic associated fatty liver disease’ (MAFLD) (77).

Day and coworkers first described the pathogenesis of NAFLD using a ‘two-hit theory.’ They determined that the first hit was an accumulation of lipids in the hepatocytes, and the second hit was an additional inflammation caused by an imbalance of pro- and anti-inflammatory factors (78).

Hepatic triglyceride metabolism is balanced between free fatty acids (FFA) formation and utilization (77). Studies using isotope labeling techniques have shown that approximately 59% of hepatic fat accumulation originates from adipose tissue, 26.1% from *de novo* lipogenesis, and 14.9% from dietary sources (74). Insulin resistance in adipose tissue leads to increased release of FFAs from adipose tissue, contributing to increased FFA uptake in the hepatocytes (77). Furthermore, hepatic insulin resistance reduces gluconeogenesis inhibition and increases *de novo* lipogenesis (77).

De novo lipogenesis is further increased with a high intake of fructose, which plays a crucial role in the accumulation of lipids in the hepatocytes, as its phosphorylation is not rate-limited to the same extent as glucose metabolism. Fructose also depletes hepatic adenosine triphosphate levels and inhibits mitochondrial beta-oxidation (79, 80). High fructose loads thereby increase insulin resistance and *de novo* lipogenesis (Fig. 3) and promote inflammation (32, 74, 81, 82).

The effect of a high dietary fat intake is much debated (74, 76, 82). Studies comparing diets high in trans-fats, high-fructose corn syrup and fast-food diets with a combination of the two revealed that the combination of fat and carbohydrates leads to a greater degree of NAFLD development than each of them does separately (74).

The second hit in NAFLD development occurs with inflammation, leading to NASH; FFA overload in hepatocytes increases mitochondrial fatty acid oxidation, leading to increased hepatic oxidative stress, dysbiosis, and increased gut permeability in obesity

(77). Another theory is increased sterile inflammation associated with endoplasmic reticulum stress, increased levels of free mitochondrial DNA, and upregulation of toll-like receptors due to lipotoxicity (83, 84).

Elevated adipocytokine levels in obesity are closely linked to the inflammatory processes in NAFLD, with reports of hyperleptinemia and hypoadiponectinemia in non-alcoholic steatosis and steatohepatitis (17, 84, 85). In lean, normoleptinemic individuals, leptin is shown to have anti-steatotic properties by inhibiting hepatic lipogenesis and glucose production (84). Together with its anorectic effect, by limiting triglyceride storage and thereby preventing lipotoxicity, leptin is a vital part of normal hepatic lipid metabolism (84).

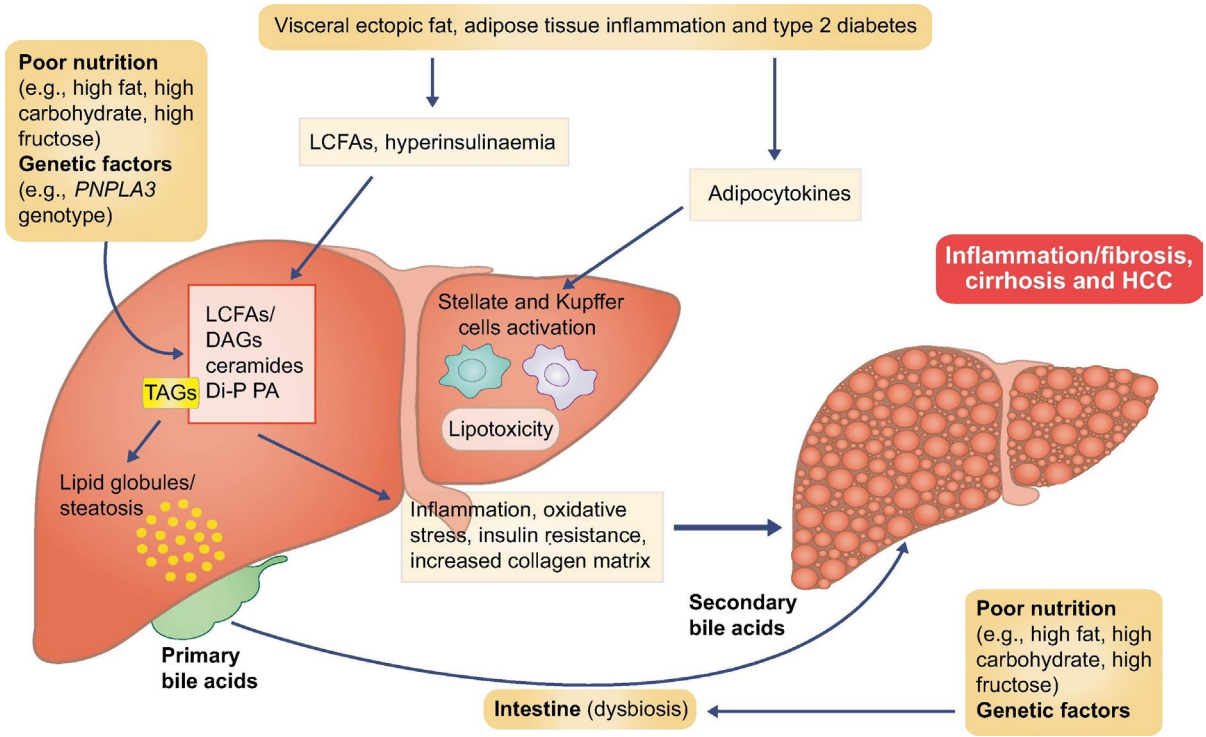

Figure 4: Development of progressive liver disease in NAFLD. Abbreviations: DAG diacyl glycerol, Di-P PA di-palmitoyl phosphatidic acid, HCC hepatocellular carcinoma, LCFA long chain fatty acids, PNPLA3 Patatin-like phospholipase domain-containing protein 3, TAG triacyl glycerol.

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In leptin resistance, these anti-steatotic effects fail. Also, leptin resistance has been shown to increase insulin resistance, contributing to NAFLD/NASH development (84). The general inflammatory response with increased cytokine levels in adipose and hepatic tissue with increasing obesity also contributes to the development of NASH (1, 86).

Quantification of hepatic fat accumulation

Despite being somewhat limited by sampling variability, liver biopsy is considered the gold standard for diagnosing hepatic steatosis. Furthermore, biopsy is the only method for diagnosing steatohepatitis (87). The histologic quantification is graded as S0, i.e., steatosis in <5% of hepatocytes; S1, 5-33% (mild); S2, 34-66% (moderate) and S3, >66% (severe) (88).

However, due to the risk of complications with an invasive procedure like biopsy, and NAFLD being a dynamic condition, radiological methods are the most widely used in clinical practice (89, 90). There is also a certain intra- and inter-observer variability between pathologists assessing biopsies (91). In recent years a new reference standard for steatosis quantification has emerged in magnetic resonance imaging (MRI) techniques: magnetic resonance spectroscopy (MRS) and proton density fat fraction (PDFF), a technique derived from magnetic resonance chemical shift imaging (90).

In hepatic tissue, most protons are found in water and fat. Both MRS and PDFF imaging of the liver utilizes the visualization of proton oscillations in water and fat based on the display of different frequencies during the procedure (90, 92). PDFF is obtained by assessing the difference in frequencies, whereas MRS measures the signals directly in a small voxel of 3x3 cm, manually selected in a liver segment minimally

affected by respiratory movement (90). Both techniques have excellent test properties compared to biopsy, correlate well with each other, and outperform computer tomography (CT) scans in accuracy (90, 91).

A limitation of the MRS is that only a small fraction of the liver parenchyma is used, which could be a problem with uneven fat distribution in the liver (90). This is not a problem with the PDFF, which assesses steatosis in the entire liver. The availability of both techniques in clinical practice might be limited in many regions due to the extensive use of MRI for other purposes. This is probably the main limitation of the procedure.

In clinical practice, ultrasonography is the most commonly used radiological modality for detecting hepatic steatosis, as it is quick, comfortable, easily accessible, and without radiation exposure (93). During a conventional brightness (B)-mode ultrasound, the operator assesses liver echogenicity and contrast, intrahepatic vessels, liver parenchyma and the diaphragm (90). The procedure is dependent on operator expertise, and the estimate of hepatic fat infiltration is subjective, making the sensitivity for detecting mild steatosis (5-33%) range between 60.9% to 65.0%, with significant intra- and inter-observer variability (90, 93). Moderate to severe steatosis could be diagnosed with 85% sensitivity and 93% specificity (94). This means that detecting hepatic steatosis on conventional ultrasound implies the presence of at least 20% fat accumulation (90).

The hepatorenal index (HRI) was developed in 1996 to improve the diagnostic properties of ultrasound. The method is based on a comparison of histogram analysis of liver and renal cortex echo, and HRI is defined as a ratio between the two measurements (95). The HRI has an accuracy of >90% and correlates well to histologic

assessments (r = .82, p < .001) and MRS (R^2 = .92, p < .001) in individuals without advanced fibrosis. In addition, it has considerably lower intra- and inter-observer variability compared to standard B-mode ultrasonography (96, 97, 98). Webb and coworkers showed the intra-observer variability to be low; the mean HRI difference between two examinations was .02 (p = .63), and the results were highly correlated (r = .77, p < .001) (96). Stahlschmidt and coworkers also found a good correlation between HRI and MRS (r = .03, p < .001) with no significant intra- or inter-observer variability (p = .283 and .135, respectively) (99).

Metabolically healthy obese (MHO) individuals

Previous studies have shown that the group of individuals with obesity is heterogeneous (6). In the last couple of decades, studies have shown that not all obese individuals show signs of the MetS and are, thus, characterized as metabolically healthy. The hallmark of MHO individuals is that insulin levels and sensitivity, CRP and IL-6, among other markers of chronic inflammation, are similar to those in a healthy, normal-weight population (100). The terms and phenotype of the group are not clearly defined, and depending on the classification, this group represents about 6 to 40% of obese individuals (100, 101).

Despite the lack of clinically manifest complications of obesity, MHO individuals have an increased risk of developing MetS over time compared to normal-weight individuals (100). Even though they have a significantly lower risk of CVD than MDO individuals (102), they have an increased risk of subclinical atherosclerosis and CVD compared to healthy, normal-weight individuals (101).

Previous work from our research group has demonstrated that young MHO individuals have a delayed clearance of postprandial triglycerides, which may contribute to the higher morbidity in this group compared to normal-weight individuals (46).

Summary of introduction

There is emerging knowledge about the pathophysiological relationship between obesity and its related diseases caused by insulin resistance, dyslipidemia and adipokine dysregulation. Inflammation appears to be the common factor linking these conditions together. Furthermore, inflammation plays a crucial role in the development of chronic lifestyle diseases, frailty and premature mortality.

The resources needed to treat each obese individual far exceed the resources available in our healthcare system. Thus, we need a way of identifying which patients urgently need treatment to reduce the risk of further morbidity and mortality and to monitor the improvement in their metabolic health and weight loss.

So far, the knowledge of a pathophysiological correlation between obesity and chronic diseases has not been transformed into the development of practical tools that are feasible for monitoring metabolic risk in obese individuals. Among others, we still need to define values that predict more advanced features of metabolic dysregulation. Our study focuses on the development of such tools.

7 Hypotheses and Aims of the Study

Paper I aimed to test if hepatic steatosis, measured by the HRI, can predict insulin resistance in individuals with obesity or previously established elevated levels of liver enzymes.

- Hypothesis I: Hepatic steatosis, quantified by ultrasonography, can reliably predict insulin resistance in obese individuals and is potentially feasible as a tool for screening in clinical practice.

Paper II aimed to test if the leptin to adiponectin (L:A) ratio can predict pathologic values of triglyceride clearance, HOMA-IR and leptin sensitivity in MHO and MDO individuals.

- Hypothesis II: The L:A ratio and leptin sensitivity are feasible markers to predict subclinical metabolic disturbances in obese individuals.

Paper III aimed to study the improvement in the postprandial triglyceride response, leptin and insulin resistance and L:A ratio after modest weight loss in obese individuals.

- Hypothesis III: Pathologic postprandial triglyceride clearance improves with a modest weight loss of 5% in obese individuals and is feasible for monitoring metabolic improvement during weight loss.
- Hypothesis IV: L:A ratio and leptin sensitivity improve with a modest weight loss of 5% and are thus useful markers for the monitoring of improvement in metabolic health during modest weight loss of 5%.

8 Material and Methods

Study population

Obese individuals were recruited either by advertisement through posters at the University of Tromsø and The University Hospital of North Norway (UNN), after admission to the Centre for Obesity (Department of Gastroenterology and Nutrition, UNN), or upon referral to an obesity rehabilitation program at Stamina Health sports center, Tromsø. The nurse at the obesity outpatient clinic and researchers VTI/MAL recruited the candidates for the study. Patients who returned a signed consent form were cross-checked per the exclusion criteria before enrolment.

We also recruited a healthy, normal-weighted control group through posters. The obese and normal weight control candidates received oral and written information and a consent form. The credibility of all candidates to participate in the study was crosschecked based on the inclusion/exclusion criteria before enrolment.

Inclusion criteria required that the obese individuals had a BMI >30 kg/m^2 and were above 18 years old. Exclusion criteria included conditions such as severe mental illness, previous heart disease, medically treated diabetes mellitus, kidney failure, pregnancy, and the use of anti-obesity drugs. The inclusion criteria for the normal-weighted individuals recruited for the control group were as follows: they had BMI <25 kg/m^2, were aged 18-40 years, and were normotensive, normoglycemic and normolipemic. The exclusion criteria for the control group were the same as that of the group with obesity. In addition, they should also have no history of diabetes mellitus, medically or diet controlled.

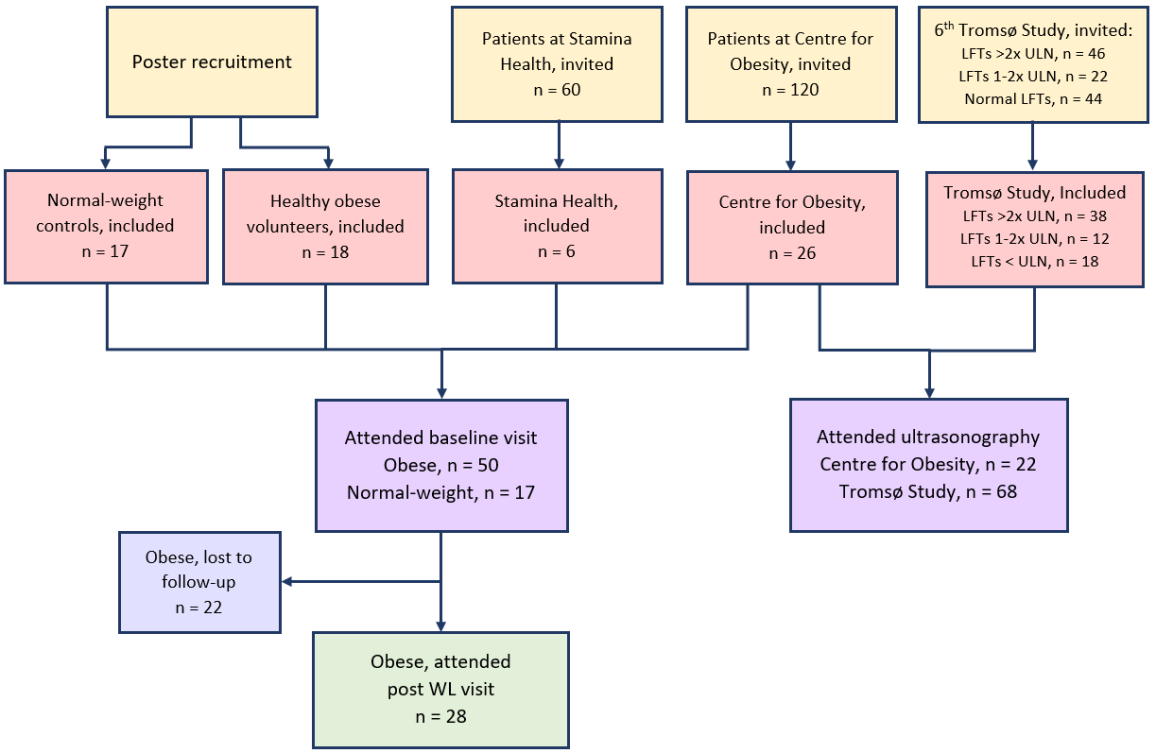

Figure 5: Flow chart of the full study. Participants who attended ultrasonography were included in sub-study I (Paper I). Participants who were part of the baseline visit were included in sub-study II (Paper II) and those who were part of the visit post weight loss were included in sub-study III (Paper III).

Abbreviations: LFTs - Liver function tests, ULN - Upper limit of normal values, WL - Weight loss.

The Sixth Tromsø Study

The Tromsø Study has been described previously by Eggen and coworkers (103). From the sixth Tromsø Study, we recruited participants with unexplained moderately elevated levels in liver function tests to supplement the participants recruited through the Centre for Obesity.

The Tromsø Study employees selected participants for our follow-up study based on the values of their aspartate aminotransferase (AST), alanine aminotransferase (ALT) or gamma-glutamyl transferase (γ-GT), which were expected to be above the Upper Limit of Normal (ULN) at the time of the primary examination in 2008. We received the

selected participants' contact information and variables chosen from the sixth Tromsø Study dataset. We then invited the participants to participate in a follow-up study.

We also invited a control group comprising individuals with normal levels of liver enzymes (n = 44) drawn randomly from the primary study population of the Tromsø Study based on the sex and age configuration of the study group with levels of liver enzymes >2x ULN. We included everyone who accepted the invitation and returned a signed consent form.

Measurements

We measured the height of participants in meters to the second decimal point. The participants stood straight, without shoes, and with their feet together. We measured their weights using a Seca scale (Seca GmbH & Co. Kg, 22089 Hamburg, Germany). The participants were wearing light clothing and were without shoes during the process of weight measurement. Weight was measured in kilograms to the first decimal. We used dual-energy X-ray absorptiometry (DEXA; Lunar Prodigy Advance, GE Healthcare, USA) for body composition measurements at baseline and follow-up. The DEXA measured the total, android and gynoid fat percentage as well as the total mass of muscle and fat (kg).

Using a measuring tape, we measured the waist and hip circumference with light clothing. Circumference of the waist was measured along the midline between the costal margin and the iliac crest, whereas that of the hip was measured at the trochanter major level (widest part of the hips). We recorded the heart rate, systolic and diastolic blood pressure in the sitting position. We calculated the mean value from the last two measurements out of three using an Omron automatic blood pressure monitor (Omron Healthcare, Inc., Vernon Hills, Illinois 60061, USA).

We measured the REE by canopy calorimetry using a SensorMedics VmaX29n apparatus (SensorMedics Corporation, 22705 Savi Ranch Parkway, Yorba Linda, California 92887, USA). The participants were instructed to fast for minimum 12 hours and refrain from engaging in vigorous physical activity three days before the exam. Before the exam, the participants were made to sit down and rest for 10 minutes. Their calorimetry lasted between 15 to 30 minutes. We selected a period of 5 minutes or longer with a continuous metabolic steady state for the REE measurement.

Missing values

Some blood samples of glucose, insulin and triglyceride were not freshly analyzed due to hemolysis or procedural mistakes. To retrieve data for these samples, we re-analyzed 20 frozen serum samples collected at the given sample time for the participant.

Postprandial triglyceride clearance

To indirectly measure the postprandial triglyceride response and clearance, we conducted an oral fat tolerance test (OFTT), a useful qualitative measure of postprandial triglyceride levels (46).

Three days before the OFTT, the participants were instructed to eat their regular diet and abstain from heavy physical activity and alcohol consumption. The participants maintained a 12-hour fasting period before the start of the test. On the test day, they were at rest and not allowed to smoke, chew gum or consume any other food or beverage outside the test protocol except water.

We prepared the test meal using commercially available sour cream porridge and full-fat cream in a 1:1 ratio, the nutritional content of which was as follows: 70% fat

containing 66% saturated, 32% monounsaturated and 2% polyunsaturated fat, 5.4% carbohydrates of which 3.1% were sugars and 2.9% protein. We served a freshly prepared test meal by adjusting its weight (1 g fat per kg body weight) with two teaspoons of white sugar (10 g carbohydrates) and cinnamon and one glass (100 mL) of calorie-free lemonade at 08:00 hours (baseline).

The participants consumed the meal within a period of 20 minutes. At noon (4 hours postprandially), the participants were offered a 500 mL calorie-free soft drink and one fruit (apple or pear, approximately 150 g). We collected blood samples to test the serum before the test meal (baseline) and every second hour postprandially over the first 8 hours.

We calculated the triglyceride (TG) clearances at 6 and 8 hours by the following formula:

$$\text{Clearance (X h)} = 100 \times \left(1 - \frac{[\text{TG (X h)}] - [\text{TG (0 h)}]}{[\text{TG (max)}] - [\text{TG (0 h)}]}\right)$$

The triglyceride response was calculated as the mean of the two highest postprandial values obtained after subtracting the fasting serum triglycerides.

Insulin sensitivity

To measure insulin sensitivity, we performed an OGTT from which the general HOMA-IR (104) and WBISI were calculated (105).

We performed the OGTT at least three days before or after the OFTT. The participants received the same instructions for the OGTT as for the OFTT. The participants were instructed to abstain from vigorous physical activity three days before the test and adhere to a 12-hour fasting period before its initiation.

At 08:00, the participants consumed 75 g of glucose dissolved in water. Blood samples were drawn every 30 minutes postprandially for 2 hours, from which we measured serum glucose and insulin (DRG insulin enzyme-linked immunosorbent assay (ELISA) kit, DRG Instruments GmbH, Germany). After glucose intake, the participants were allowed to drink water only.

Adipokines

We measured serum leptin and adiponectin in the fasting state and every 2 hours postprandially after meal intake at OFTT. We also collected fasting and postprandial samples of leptin and adiponectin every 30 minutes after glucose intake at OGTT. The serum samples were frozen at -25 °C until analyzed using ELISA kits (DRG Diagnostics, Marburg, Germany) to determine the concentrations of leptin (sandwich ref. EIA-2395) and adiponectin (human, ref. EIA-4574).

Hepatic steatosis

We performed transabdominal ultrasonography using a Hitachi EUB-6500 HW apparatus with a 5 MHz convex EUP-C524 transducer (Hitachi Medical Corporation, Tokyo, Japan). We positioned the transducer along the mid-axillary line to visualize the liver and kidney in the same picture. We recorded hepatic and renal parenchymal echogenic density with the built-in histogram function on a grayscale (values 0-255, Fig 5).

The region of interest of the liver parenchyma was selected as closely as possible to the kidney cortex. Furthermore, we avoided artifacts from rib shadows, intrahepatic vessels and kidney calyces and aimed to use homogenous areas as visually similar to the general impression of the liver as possible.

We then used an average of hepatic and renal parenchymal measurements performed in triplicates to calculate the HRI using the following formula:

$$HRI = \frac{mean\ liver\ echogenicity}{mean\ kidney\ echogenicity}$$

To reduce intra-observer variability, we performed three measurements and computed a mean from the two most similar measurements, excluding the outlier.

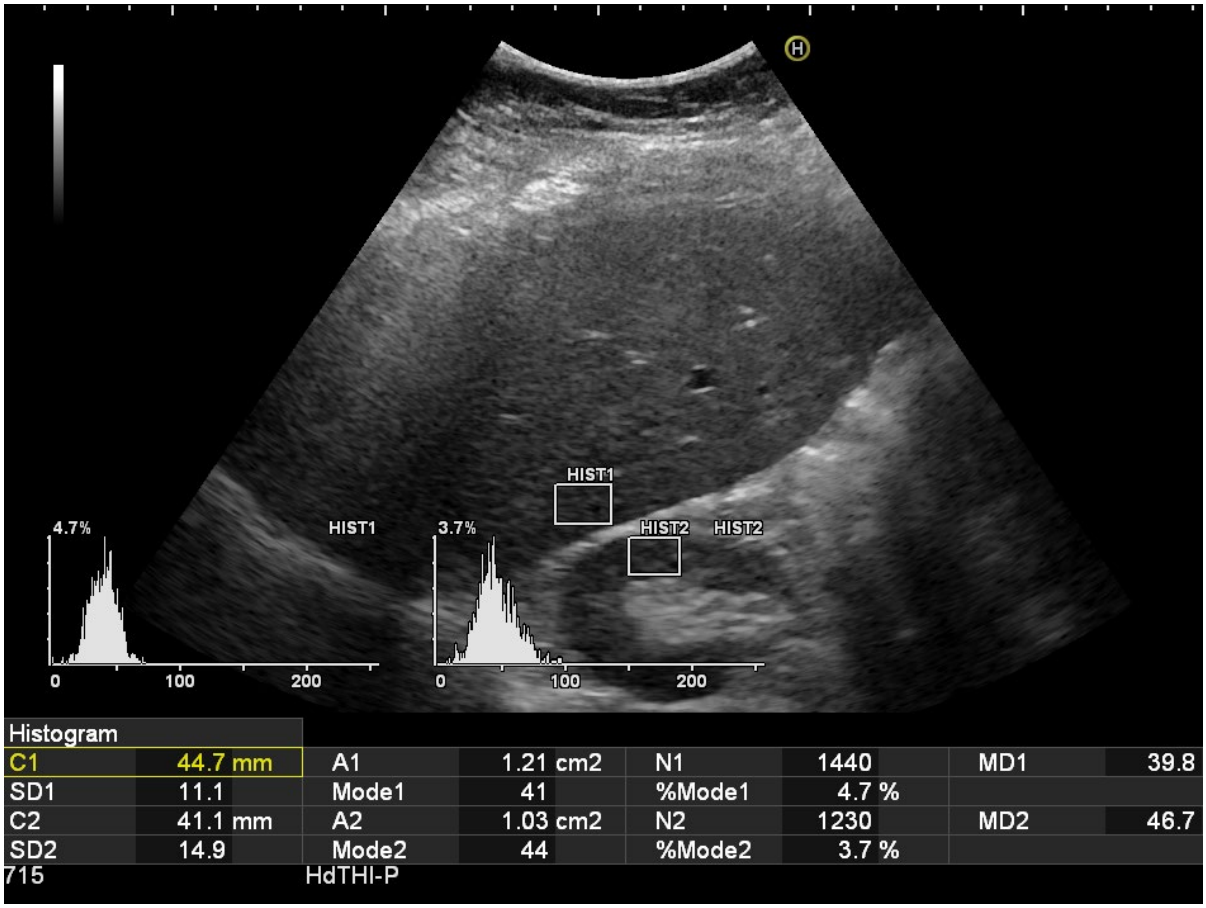

Figure 6: Ultrasonography scan for liver steatosis quantification. We selected a representative segment of each organ to produce a grayscale histogram, from which we used the mean values (MD1 and MD2) to calculate the HRI.

Ethical considerations

All participants received written and oral information before obtaining a signed written consent. The participants also approved of the use of their data in follow-up studies. The Regional Committee of Medical Ethics of Northern Norway approved the study (Reference no. 2011/1677). The approval also included the establishment of a biobank.

The Tromsø Study organization provided access to data and participants for our follow-up study from the Sixth Tromsø Study material. The initial ethical approval for the sixth Tromsø Study, given by the Regional Committee of Medical Ethics of North Norway, also covered our follow-up study.

Statistics

We performed all statistical analyses using SPSS Statistics for Windows, versions 21-25 (SPSS Inc., IBM Corporation, Armonk, New York, USA). The graphs presented in Paper III were developed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

We used the parametric tests for all the variables resembling normal distribution by visual assessment and as a measure of skewness and kurtosis. For all others, the non-parametric tests were used.

We used the ROC analyses to obtain sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) for the cut-off values examined in Papers I and II. Pearson's Chi-Square test was used to assess the improvement of markers over the cut-off values identified in Paper II.

9 Main Results

9.1 **Paper I**

Isaksen VT, Larsen MA, Goll R, Florholmen JR, Paulssen EJ: Hepatic Steatosis, detected by Hepatorenal Index in Ultrasonography, as a Predictor of Insulin Resistance in Obese Subjects. BMC obesity. 2016. DOI: 10.1186/s40608-016-0118-0

Insulin resistance is closely linked to hepatic steatosis and NAFLD. However, ultrasonographical quantification of hepatic steatosis has yet to be assessed as a tool for screening insulin resistance. Therefore, we aimed to examine the test properties of the HRI as a tool for screening insulin resistance.

Results: The HRI at level 1.17 had a sensitivity of 0.90 and a specificity of 0.70 for predicting insulin resistance (HOMA-IR >2.3) in all participants. For the subgroup with BMI ≥30, HRI at level 1.17 had a sensitivity of 0.94 and a specificity of 0.70. For the subgroup with BMI ≥35, the HRI at level 1.17 had a sensitivity of 0.93 and a specificity of 0.75 for predicting the HOMA-IR >2.3. When increasing the limit of HRI to 1.42, we found low sensitivities and high specificities in all BMI groups.

Conclusion: The detection and quantification of hepatic steatosis by ultrasonography and the HRI may be feasible as a screening tool for differentiating between individuals with low risk of insulin resistance (HRI <1.17), at risk of manifesting insulin resistance (HRI 1.17-1.41) and with likely insulin resistance (HRI ≥1.42), particularly in obese individuals (106).

9.2 **Paper II**

Larsen MA, Isaksen VT, Moen OS, Wilsgaard L, Remjin M, Paulssen EJ, Florholmen J, Goll R: Leptin to Adiponectin ratio – a surrogate biomarker for early detection of metabolic disturbances in obesity. Nutrition, Metabolism and Cardiovascular Diseases. 2018. DOI: 10.1016/j.numecd.2018.06.020

We aimed to test the L:A ratio as a potential surrogate biomarker of postprandial triglyceride clearance and resistance of insulin and leptin in obese individuals, with particular emphasis on the detection of early metabolic disturbances in MHO and MDO individuals.

Results: Among the MHO participants, 71.4%, 69.4% and 86.1% had delayed triglyceride clearance, insulin- and leptin resistance, respectively, whereas, in the MDO group, these risk factors were found in 85.7%, 71.4% and 91.7% of the participants, respectively. We found a combination of all three risk factors in 39.8% of the MHO and 42.9% of the MDO participants.

ROC analyses revealed that a cut-off value of >1.65 of the L:A ratio for the normal-weight controls (PPV 1.0, NPV 0.91) and >3.65 for the obese participants (PPV 0.86, NPV 0.48) predicted the delayed triglyceride clearance with a decent specificity and sensitivity. The ROC yielded the most suitable L:A ratio cut-off at >1.88 for detecting a combined risk with at least 2/3 risk factors.

Conclusion: The L:A ratio can detect early metabolic disturbances in apparently healthy obese individuals and is a potentially useful clinical surrogate biomarker of metabolic disorders.

9.3 **Paper III**

Isaksen VT, Larsen MA, Goll R, Paulssen EJ, Florholmen J: Correlations between modest weight loss and leptin to adiponectin ratio, insulin and leptin resensitization in a small cohort of Norwegian individuals with obesity. Endocrine and Metabolic Science. 2023. DOI: 10.1016/j.endmts.2023.100134

This study aimed to investigate if the L:A ratio, triglyceride clearance, and leptin- and insulin sensitivity improve with modest weight loss in adult individuals with obesity.

Results: 28 participants completed the study, of which 13 lost ≥ 5% body weight. The L:A ratio (-45.7%), leptin sensitivity (+80.1%) and HOMA-IR (-23.1%) significantly improved in participants who lost ≥ 5% weight, while triglyceride clearance at 6 hours and triglyceride response did not improve. No significant changes were observed in the non-weight loss group.

Conclusion: Metabolic parameters such as L:A ratio, insulin- and leptin sensitivity, but not postprandial triglycerides, improve with a modest weight loss in individuals with obesity.

10 General Discussion

10.1 Methodological Considerations

Population

We chose a study population as close to the population encountered in daily clinical practice as possible, with minimal exclusion criteria. However, we excluded participants with medically treated diabetes to avoid skewed results due to the confounding of insulin and glucose levels. Similarly, we excluded participants with severe heart and kidney failure to prevent confounding our observations from the standpoint of clinical manifestation of the MetS.

For the first sub-study, we included selected participants from the Tromsø Study with moderately elevated levels of liver enzymes, the reason for which could not be explained during the assessments in 2008. Of these participants, 24 were obese. We included these participants because many cases of hepatic steatosis are diagnosed based on pathologic levels of liver enzymes. However, this choice led to a more heterogeneous study group, which included non-obese participants.

The obese participants in this study were selected partly from the general population and partly from a population of patients intending to undergo conservative treatment for obesity. This choice was made to study the overall effect of weight loss rather than that of a specific diet. Additionally, we wanted our study population to comprise morbidly obese individuals with a fair chance of achieving weight loss through lifestyle changes by participating in a structured treatment program.

The advantage of applying only a few exclusion criteria was that our results could readily apply to the general obese population. We could also compare MHO to MDO individuals at baseline, which provided additional information regarding the L:A ratio, leptin sensitivity, and postprandial triglycerides in the absence of MetS. Conversely, the disadvantage was that, due to few exclusion criteria, our study population tended to be more heterogeneous, which might have introduced confounding factors.

Study design

The first two sub-studies (Papers I and II) were designed as analytical cross-sectional studies, whereas the last sub-study (Paper III) was a prospective case series with a repeated measures design. Both were observational studies.

A cross-sectional design is characterized by measuring all variables at a single time point, creating a snapshot of the health condition in question (107). The primary

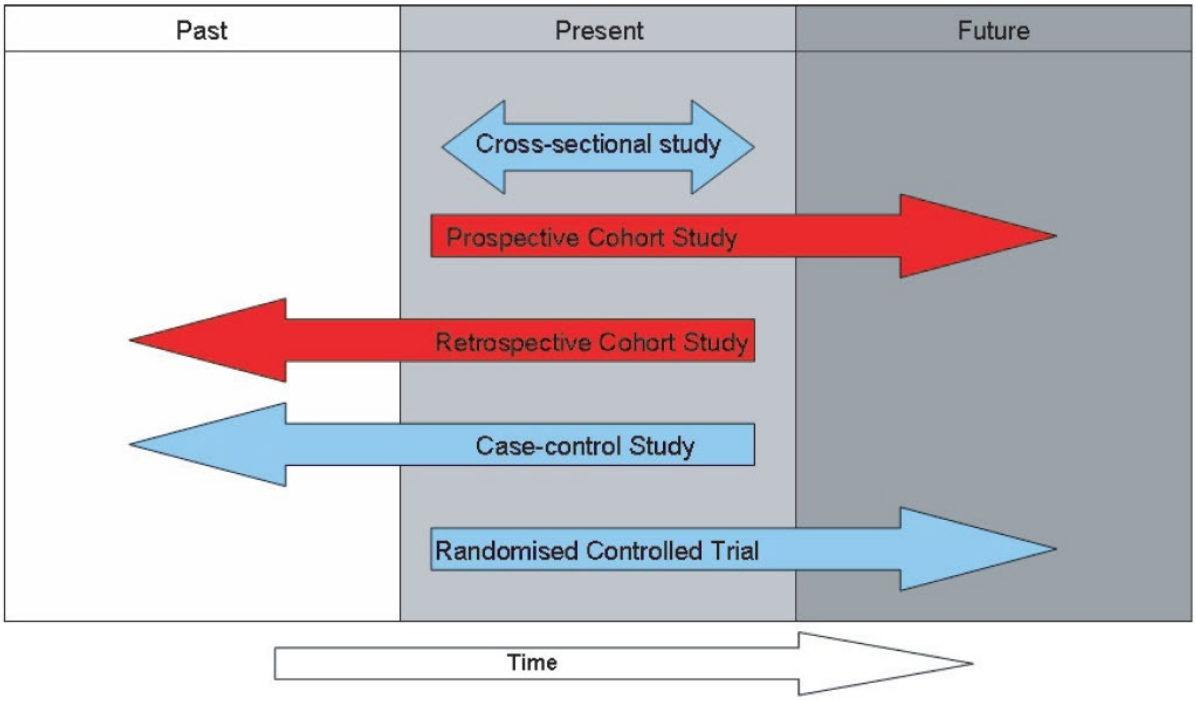

Figure 7: Schematic representation of different study designs.

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strength of this study is that this design is quick, cost-effective, and easy to conduct (107).

Furthermore, all data are measured in real-time, thus limiting recall bias. This design is perfect for creating hypotheses that may be studied using time-consuming and expensive designs (107).

The cross-sectional study is not suited for studying causality since all data are collected at the same time point. Hence, this design is suitable for assessing correlations only. Causality should be studied using prospective studies or randomized controlled trials (107). We used ROC analyses to estimate test properties based on the correlation between different features of metabolic dysregulation. We cannot determine which of the components act as causes for the others, but for a clinical marker, this causality is irrelevant.

On the other hand, the prospective design is well-suited for causality analyses since one can measure both incidence and prevalence (108). In our study, we observed the effect of weight loss intervention for an entire group, using the control group of the second sub-study.

The researchers must determine which exposure factors should be recorded and which should not, both at baseline and during follow-up visits. These choices rely heavily on existing knowledge. Failure to include an essential variable at baseline will limit the analysis and scope of the results after the outcome has occurred (108). In cases of unexpected results, this poses a real issue where existing knowledge is insufficient for predicting possible underlying factors and confounders.

A prospective study is expensive and time-consuming, especially if the study measures hard end-points, implying that the study needs to be run over several years (108). Moreover, participation in the study might alter the behavior of the participants. During inclusion and follow-up, we realized that individuals who agreed to participate often decided to participate in the study as an additional source of motivation for losing weight.

Loss to follow-up

When the study was planned, the follow-up period was estimated to one year. However, due to challenges for our participants to lose weight, the total follow-up period was extended to two years (Paper III). As the primary investigator, the candidate did regular phone follow-ups to track the participants' progression. To keep participants who managed to lose weight faster than the estimated period from dropping out, these participants were offered to perform the second round of tests earlier than estimated.

Among the weaknesses of the prospective design, loss to follow-up was prominent and proved to be a significant issue in our third sub-study (108). The main reason for withdrawal was the inability of the participants to lose weight. We needed a good balance between participants who had achieved weight loss and those who did not to study the outcome. Despite our efforts to help struggling participants to achieve the weight loss goal and remain in the study, the non-weight loss group was substantially larger than the weight loss group. For this reason, failure to lose weight was considered a valid reason for the participant to withdraw, even though all participants were offered to do the second round of tests.

On the other hand, there might be underlying differences in the metabolic statuses between the participants who completed the study and those who did not. The

comparison of biological features in participants who dropped out versus participants who completed the study was beyond the scope of the study. However, the clear impression from the follow-up phone calls and discussions with the nurse who did the primary follow-up of all patients at the obesity outpatient clinic was that several of the participants in our study were individuals with low motivation for weight loss who agreed to participate in the study as a means to improve motivation for treatment. However, their study participation did not seem to increase the success rate of our participants in achieving weight loss.

Sample size

In the first two sub-studies (Papers I and II), we included a moderate sample size of 90 and 50 participants, respectively. In contrast, loss to follow-up reduced the sample size in the third sub-study to 28 participants (Paper III).

The main challenge with small sample sizes is the increased likelihood of obtaining extreme results by pure chance. This increases the chance of both type I and type II errors (109, 110). Researchers generally underestimate the sample size needed when preparing studies, which is why sample size should always be calculated (110). In other words, an underpowered study generally has a higher variability and may be inaccurate.

Generally, underpowered studies (power <80%) are considered scientifically unsound, and many regard them as unethical, as the project may be considered a waste of resources and expose participants to undue risk (111). This view is challenged, as underpowered studies still provide information and can be used in meta-analyses to strengthen the overall knowledge of the field (111, 112).

Since small studies cannot detect small variable differences reliably, the advantage of a small study is that an actual difference detected by the study bears more clinical significance, such as the results obtained in the sub-study described in Paper III. As previously determined, we found an improvement in leptin and insulin sensitivity and L:A ratio with a 5% weight loss (113, 114). However, we could not detect changes in the fasting or postprandial triglyceride levels. Changes in triglyceride levels might require a more substantial weight loss. We performed a power analysis that pointed to a necessary sample size of 900-1600 participants to detect significant improvements in triglyceride markers with an 80% probability, suggesting that the variations in postprandial triglycerides are too large for it to be used as a clinically meaningful test in individuals with modest weight loss.

Calculating sample size when planning a study is, however, a challenge. It depends on good estimations of standard deviations of the variables studied, as a two-fold increase in standard deviation leads to a four-fold increase in sample size needed (111). The standard deviation may also be challenging to obtain unless sufficient data is available, in which case you could argue that the study is unnecessary (111).

The small sample size in Paper III limited the number of available statistical analyses, as complex regression analyses with covariates require a larger sample size. We also could not use parametric tests for most analyses since having few participants in each group made it difficult to obtain normal distributions. However, the design of the third sub-study (Paper III) enabled us to use repeated measures tests for analyzing changes with weight loss. Repeated measures tests are less restrained by a smaller sample size than independent samples tests since using the participants as their own controls significantly reduces the variability. Thus, the design seemed to be the only one feasible with the present sample size.

Furthermore, the sample size presented a challenge in ROC analyses, particularly for analyzing the BMI subgroups in the first sub-study (Paper I), as ROC curves were crude. This problem was less prominent in the second sub-study (Paper II), as the population was only subdivided into healthy and unhealthy obese individuals.

Selection bias

Most of our study population was selected from individuals intending to lose weight who had already been referred to the hospital by a general practitioner for systematic lifestyle intervention. This poses a likely selection bias since it might imply poorer participant health, judging by their need for specialist treatment.

In the groups recruited via the display of posters, young and metabolically healthy individuals were selected (46). The criteria for the selection of this group also imply bias. When comparing the MHO and MDO groups, there was a significant age difference (Paper II), which could be a confounding factor, as previous literature has shown that glucose metabolism is impaired (115) and fat mass is increased (116) in middle-aged compared to young adults. The difference in age between our study groups may also be a reflection of age as a confounder. Further investigations with a more age-matched study group may be necessary to confirm our findings.

As a whole, our study group was skewed towards women for all three sub-studies. At our baseline and second visits, 80% and 75% of the participants were women, respectively. This seemed to be mainly due to the skewed population of patients seeking help for obesity. Existing literature has shown that men are more susceptible to insulin resistance and ectopic fat deposits, while women have more subcutaneous and total fat mass (117). However, there were no significant differences between compared groups in any of the sub-studies, which minimizes the confounding impact

on our significant findings. However, it may account for an increased variability within the study groups, which may have led to more non-significant results than if we performed a study exclusively with women.

Non-responder bias

As previously mentioned, our study was skewed towards women. There is a general tendency among females to seek help for health issues more than males (118), which reflects on the behavior and results of the clinical population of obese individuals receiving treatment for their condition. This phenomenon is called non-responder bias, as the individuals who do sign up are systematically different from the ones who don't.

This factor probably accounts for most of the imbalance in the results between sexes obtained from our study population. Researchers can decide to include equal numbers of males and females to correct this effect. In the present study, we chose to include all eligible candidates despite the imbalance between sexes to increase our sample size as much as possible, as the alternative, i.e., waiting until we had enough male participants to be able to exclude females, would lead to an unacceptable long inclusion period.

Confounders

There are some possible confounders related to our study. First, the participants may not have followed the preparation instructions for the OFTT and OGTT, which could affect the test results.

Second, the study was not designed with a single specific weight-loss regime, apart from the advice offered by the clinical staff based on national guidelines (119). We did not register details about the regime of each participant, suggesting that there could

be significant underlying differences in the diet and physical activity between those who lost weight and those who did not. Hence, there might be underlying confounding factors concerning differences in the amount of exercise and micro-/macronutrient composition in the participants' diets that are not accounted for.

Third, the participants might have different gut microbiomes and habitual diets that could impact their fasting and postprandial glycemic and lipemic profiles, particularly their rate of fat uptake from the gut (120). The study of these factors was outside the scope of our study. Recent studies of gut microbiota in obese individuals and during weight loss suggest this factor warrants further investigations in relation to our findings (121).

Fourth, evidence suggests that there likely is underlying chronic stress and past traumatic experiences in the obese population (122). Adverse childhood experiences have been proven to have a dose-response relationship with chronic illnesses, including obesity (122, 123). This might be a confounding factor that could be skewed between weight loss groups and thus contributed to our participants' ability to lose weight and adhere to the project (123). Furthermore, as adverse childhood experiences and chronic stress are linked to inflammation, these factors could also affect our findings of leptin, adiponectin, insulin and triglyceride levels and hepatic steatosis (124, 125, 126). This subject was outside the scope of this study. Therefore, further studies investigating our findings in light of this knowledge would be warranted.

Fifth, unreported excess intake of alcohol, hepatitis and occult liver disease other than NAFLD might explain the test results in participants with hepatic steatosis and elevated liver enzymes. Moreover, the liver enzymes of the participants from the Tromsø Study

were measured in 2008, implying that there could be underlying confounding factors between 2008 and the follow-up in 2013.

Ultrasonographic quantification of hepatic fat accumulation

As previously described, calculating HRI as a method of hepatic fat quantification displays significantly less inter- and intra-observer variability than standard B-mode ultrasonography (93). The technique has also been proven to correlate well with MRI-based diagnostic tools (127). To ensure efficacy during participant examinations, we needed two observers. Thus, the examinations were performed by EJP and VTI, respectively, who discussed and compared results on a case-by-case basis to ensure that techniques and results aligned. A formal analysis would have been helpful to demonstrate a low inter-observer variability, but this was, unfortunately, not obtained.

The regions of interest in the liver and kidney were selected according to previous work by Cha and coworkers (93). The mean difference between the highest and lowest HRI measured for each participant was 0.19. The mean difference between the two closest measurements for each participant was 0.05. We considered outlying measurements to be artifacts and thus excluded them from the analysis. The lack of formal tests of inter-observer variability would be viewed as a weakness of the study. However, the overall variability between the measurements included in the analysis is small, which suggests that the precision in this study was sufficient.

Oral Fat Tolerance Test (OFTT)

The OFTT has been demonstrated as a robust method for measuring postprandial triglyceridemia with a correlation coefficient of 0.84 to both intravenous and duodenal fat tolerance tests (128). Previous studies have shown contradictory results regarding

the postprandial response after intake of different types of fat (129, 130). We selected sour cream porridge as our standardized test meal because it has a high fat content, yet it is reasonably easy to ingest for most people. Consuming a lipid emulsion with no carbohydrates or proteins was considered less tolerable, even though a lipid emulsion might have given the most accurate results (131).

Three participants had a delayed triglyceride response after ingestion of the meal with a triglyceride peak after 6-8 hours at one or both visits. Based on our results, it is difficult to tell whether this was due to the participants ingesting their private food during the day despite our instructions, having an underlying medical condition that was not accounted for, or having prolonged endogenous triglyceride production. The variation in time taken to reach the peak triglyceride value might also be one of the reasons for the lack of improvement in triglyceride clearance after weight loss.

Choice of insulin sensitivity assessment methods

In Paper I, we stated: "*A common and efficient way of assessing insulin resistance is by the Homeostasis Model Assessment of insulin resistance (HOMA1-IR) (…). It has been proven to be equally good as the gold standard for measuring IR, i.e., the euglycemic clamp, in addition to being a more convenient test to perform*".

This statement is inaccurate and an oversimplification of the work done by Wallace and coworkers (132) and Lorenzo and coworkers (133).

Matsuda and coworkers demonstrated in 1999 that the insulin clamp method, which is considered to be the gold standard of insulin resistance assessment, correlates well with the WBISI (r = .73, p < .0001) and reasonably well with the HOMA-IR (r = .69, p < .0001) (134). Wallace and coworkers found an even stronger correlation (r =0.73, p >

0.0001) between HOMA-IR and the euglycemic clamp (132), although the more detailed HOMA2-IR correlates better than the original HOMA1-IR estimate (132). Lorenzo and coworkers found a very similar receiver operating characteristic (ROC) curve for WBISI and HOMA-IR (0.897 and 0.875, p = .080). However, WBISI did reclassify individuals identified by HOMA-IR per the clamp assessment. The effect was most substantial (around 40%) in individuals with moderate risk of insulin resistance.

The question of whether or not the HOMA-IR is equally good as the clamp is discussed by Wallace and coworkers (132), pointing out that the HOMA-IR is considering the physiologic and homeostatic state, whereas the clamp is a complex, dynamic stress test, surpassing physiologic conditions. The two yield different aspects of insulin sensitivity (132). The WBISI considers the postprandial state and will therefore be more closely correlated with the clamp.

However, in our study material, we found a Spearman correlation of −0.922 (p <0.001, two-tailed analysis) between WBISI and HOMA1-IR (Paper I). Based on this, we decided to use HOMA-IR as our primary variable.

Choice of threshold values for disease diagnosis

In this project, we have operated with two different threshold values for HOMA-IR as follows: one was used for detecting MetS by quantification of hepatic steatosis (Paper I), and the second for assessing the L:A ratio and leptin sensitivity as markers of MetS (Paper II).

We used the first threshold value based on the work of Geloneze and coworkers in the Brazilian Metabolic Syndrome Study (BRAMS) (135), in which it was found that HOMA-IR of 2.3 was the optimal value for detecting insulin resistance in an obese population

with MetS. This value has also been confirmed in a similar study with the Korean population (136). Both studies and several others note that in a population without any other features of the MetS, insulin resistance is best detected with a HOMA-IR threshold value of 2.7. As a smaller-than-expected fraction of our 90 participants had a substantial degree of insulin resistance, we did not have enough participants with HOMA-IR values over this limit to conduct analyses against HRI, and therefore, made a compromise by establishing the threshold value of 2.5 for a stricter diagnosis of insulin resistance than 2.3. The lack of power is a limitation of this study, and we could only conclude that identifying hepatic steatosis by HRI could be used as a screening tool to warrant further diagnostic testing.

Recent work from De Cassia and coworkers suggests that a HOMA-IR threshold of 2.9 is optimal in post-pubertal non-obese adolescents (137). In contrast, both Geloneze and coworkers (138) and Gayoso and coworkers (139) suggest the threshold would be between 2.03 and 2.3 when taking obesity and elements of the MetS into account. Our identified threshold values of 2.3 and 2.5 should therefore only be applied to individuals with obesity or an otherwise higher pre-test probability of having insulin resistance.

In Paper II, we used a HOMA-IR threshold value of 1.83, the upper limit of our healthy control population's 95% confidence interval (46). This value is significantly lower than the previous study, limiting the false negatives for the diagnosis of insulin resistance. On the other hand, the false positives might account for a more substantial portion of the sample.

According to the literature, the threshold values for HOMA-IR found in different populations vary significantly, particularly with sex, age and presence of other

metabolic disorders, and a normal range has not been determined (139). Therefore, the choice of a value for diagnosing insulin resistance must be determined based on the population in question. This makes comparisons between studies challenging.

To our knowledge, threshold values for detecting pathologic triglyceride clearance and response, leptin sensitivity, and the L:A ratio have yet to be described. Therefore, we chose the upper or lower 95% confidence interval limit in our control group as threshold values for these variables.

Choice of cut-off values in ROC analyses

As noted in the description of the ROC analysis, no diagnostic test is ideal. There will always be some false positive and false negative cases. Because of this, the researcher needs to decide the strictness of the cut-off value of the diagnostic test by evaluating and comparing the importance of sensitivity versus specificity (140).

A valuable tool in this choice is the Youden index, which provides the researcher with the cut-off value that is an objectively optimal combination of sensitivity and specificity for the test, which is the point on the ROC curve farthest from the diagonal line (141, 142).

However, using this analysis in clinical practice, the desired test properties might deviate from this objective value, as the clinician might value sensitivity over specificity (140). In a screening test requiring high sensitivity, the specificity will be considered less important, whereas a precise diagnostic test will need a high specificity.

In Paper I, we emphasized sensitivity over specificity as HRI was considered a screening tool for detecting insulin resistance, in which we need to limit the number of

false negatives. The diagnosis of insulin resistance is not considered a heavy burden for the patient, and it is not difficult to rule out false positives.

The L:A ratio is also considered a screening test (69), but the diagnostic methods for ruling out leptin resistance and delayed triglyceride clearance are significantly more time-consuming and expensive. Since a greater emphasis on specificity was made in this case, the Youden index was applied (142).

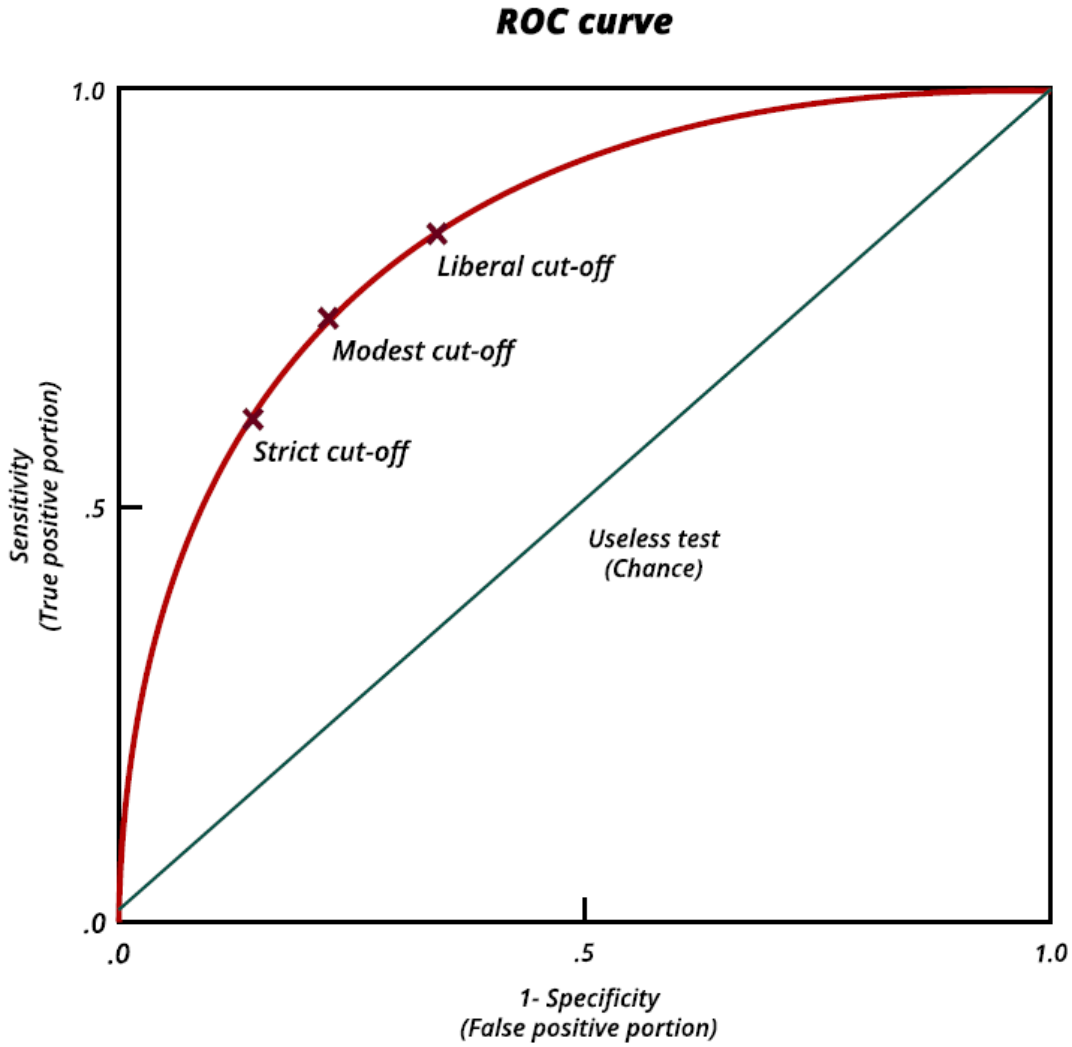

Figure 8: General ROC curve. Improved test properties tend to further the curve from the diagonal line. The diagnostic cut-off value may be chosen from any point on the curve. Each value has a corresponding sensitivity and specificity. The choice depends on whether it is more important to avoid the false positive or false negative results.

Figure inspired by Seminars in Nuclear Medicine, Vol 8/edition 4, Metz, C: Basic Principles of ROC Analysis, Pages 283-298, ©1978, with permission from Elsevier

Comparison of surrogate markers

This study was not designed to analyze markers of the MetS against endpoints such as myocardial infarction, stroke, and cancer, as this would require a large population and a follow-up period over several years. On the contrary, we compared surrogate markers with each other to determine whether simple diagnostic tools such as ultrasonography and fasting blood samples can reliably differentiate between healthy and unhealthy obese individuals.

This presents an immediate problem in which all tests involved in the analyses have individually separate test properties and none among them is considered a gold standard, introducing the issue of the test's validity. Therefore, the question arises: do the L:A ratio and HRI measure the risk of morbidity and mortality as they are supposed to?

To answer this question, all the markers need to be tested in larger prospective population studies, such as the Tromsø Study, to assess whether individuals with values now defined as pathologic have an increased risk of morbidity and mortality after adjusting the other risk factors.

10.2 Discussion of Main Results

Hepatic steatosis as a clinical marker for metabolic dysfunction

In Paper I, we demonstrated that the relationship between insulin resistance and triglyceride accumulation in hepatocytes might be used as a simple clinical marker for the occurrence of metabolic dysregulation, in particular, insulin resistance. Hepatic steatosis is a known precursor for steatohepatitis (75, 143), which aligns with other aspects of inflammation in the metabolic dysregulated state, such as insulin resistance, adipokine dysregulation and dyslipidemia (77, 144). Knowing the complications of such dysregulation, a simple and accessible diagnostic tool is of great importance.

Using ultrasonography instead of HOMA-IR as a screening tool is reasonable, as serum insulin is not a sample evaluated routinely in our local laboratory, and the most detailed methods for assessing insulin sensitivity are costly and time-consuming (133). The same could be said about MRS diagnostics of hepatic steatosis, with the added challenge of obese individuals often not being able to fit into the machine (98). Ultrasonography, on the other hand, is an excellent modality for repeated assessments as it is widely accessible and non-invasive (92).

When using ultrasonography and HRI, one should bear in mind that fibrosis is a likely confounding factor, leading to false low ratios (99). In this regard, methods like the MRS and, of course, the liver biopsy will be more accurate. It may be selected if patient history or other clinical findings suggest that other liver diseases and fibrosis might be present.

The detection of hepatic steatosis, and thus increased likelihood of insulin resistance, will indicate that the patient has metabolic dysregulation and low-grade inflammation.

This warrants further diagnostic steps (77, 99) and increases the likelihood that this patient needs intensified treatment to reduce metabolic dysregulation and complicating diseases (77).

The test properties for predicting insulin resistance based on elevated HRI were moderate. An HRI of ≥1.17 yielded a sensitivity of 0.94 and a specificity of 0.70, which are far from perfect in a diagnostic setting but can be argued to be reasonable for a screening tool. As the skill for measuring HRI is relatively easy to acquire, the findings of our study might be used to implement liver ultrasound in primary care as part of general health assessments for overweight and obese patients. The detection of hepatic steatosis might indicate that the physician should do further diagnostics towards MetS, and the patient should be prioritized for specialized weight loss intervention if they struggle to lose weight in a primary care setting.

Improvement of adipokines with modest weight loss

In paper II, we demonstrated that an elevated L:A ratio, insulin resistance, and delayed triglyceride clearance are related to and are components of the MetS (145). Leptin and adiponectin dysregulation is associated with adipose tissue inflammation, which is suggested to lead to insulin resistance (21, 63).

In Paper III, we reported a decreased leptin level with modest weight loss. This effect seems more significant than the improvement of insulin sensitivity, although the improvements of the two hormones can be correlated. The findings may be explained by decreased fat mass and inflammation, occurring as the primary event following calorie restriction, leading to improved insulin sensitivity (21). However, the reverse causality might also be possible. Thus, based on the present study, we cannot make a definite conclusion regarding the causality.

However, the normalization of adipokine levels, including the L:A ratio, is of great importance, as the reduction of adipose tissue inflammation is believed to be crucial in slowing down the development of chronic diseases and, ultimately, frailty (1, 146). With a generally increasing life expectancy, the resources for health care are more strained than ever. A frail elderly population will thus add to the social burden of our society.

The rapid response of improvement in adipokine levels with as little as 5% weight loss is promising, as conservative weight loss is a demanding process for the obese individual and is even harder to maintain over time (147). A small long-term weight loss might be achievable by a more substantial proportion of individuals, even though this, too, requires long-term dedication from the individuals.

As leptin changes the most in both L:A ratio and REE:leptin ratio (leptin sensitivity), one could ask whether the two variables describe the same phenomenon. In 2015 Matusik and coworkers demonstrated that leptin and oxidative stress were significantly higher, while adiponectin and basal metabolic rate were lower in obese, sedentary children compared to lean, sports-trained children (148). Fatouros and coworkers also demonstrated that REE and adiponectin were temporarily elevated 24-72 hours after moderate to intense resistance training (149). These studies imply a certain covariance between the L:A ratio and REE:leptin ratio, as is also demonstrated in the present work, although our study group was too small to show a change in adiponectin levels alone, as previously demonstrated by Kelly and coworkers, among others (150).

However, the clear covariance between s-leptin, L:A ratio and oxidative stress (148), together with the known proinflammatory features of hyperleptinemia (33, 151) and hypoadiponectinemia (67), suggest that the improvement of the L:A ratio is an expression of reduced inflammation in the adipose tissue (69) and a more primary

event than changes in body composition (152). According to available literature, this 5% weight loss significantly reduces the risk of developing complications from obesity (113, 153).

Leptin sensitivity and body composition

Previous literature has indicated that leptin levels not only regulate appetite but also REE in animal and human models, both in leptin deficiency and overfeeding states (53, 154, 155). The improvement of leptin sensitivity, expressed by an improved REE:leptin ratio, on the other hand, seems to be an expression of enhanced leptin function, as lower levels of circulating leptin are needed to maintain energy expenditure (61).

Moon and coworkers found that leptin signaling pathways in the brain are satiated around 30-50 mg/mL s-leptin, and exceeding leptin levels does not affect satiety or energy expenditure (156, 157). Our findings align with this literature, as our participants in the weight loss group exhibited a substantial drop in s-leptin from a median value of 32.5 ng/mL to 20 ng/mL with no significant decrease in REE. The maintenance of REE when leptin levels drop below 30 ng/mL indicates an increase in leptin sensitivity (155, 157).

However, these results must be interpreted cautiously, as important confounders to the REE:leptin ratio are fat mass (54) and, more importantly, lean body mass (61, 158). Post hoc analyses did show a positive correlation between REE and lean body mass (r = .810, p <.001 at baseline, r = .869, p <.001 post weight loss), but not between lean body mass and REE:leptin ratio (r = -.124, p = .4 at baseline, r = .274, p = .176 post weight loss). Since median s-leptin, and not REE, changed with weight loss, the improvement in leptin sensitivity may be explained by a change in body composition, specifically the maintenance of lean body mass (159).

However, the level of physical activity in weight loss and its effect on REE is debatable, as previous studies have shown a negative correlation between exercise and REE (160). Furthermore, Johansen and coworkers found a substantial decline in REE despite vigorous physical activity with a modest loss of lean body mass (161), findings further supported by comparison with weight loss in individuals who underwent bariatric surgery and had less metabolic adaptation despite a more substantial loss of fat-free mass (162), which are findings in direct opposition to the positive correlation between muscle mass and REE found in our material.

The change in REE:leptin ratio might seem more a consequence of changes in body composition than the L:A ratio. However, further studies are needed to determine to what degree leptin resensitization is explained by changes in body composition and how much can be explained by improved leptin functioning in and of itself (160).

A weakness of the study is the lack of formal recordings of what lifestyle changes and degree of physical activity each participant undertook during the weight-loss period. Further intervention studies and validating studies, therefore, need to be performed to determine to what degree resistance training affects REE:leptin ratio and whether a modified version of the REE:leptin ratio better describes leptin sensitivity, and to what degree improved leptin sensitivity could determine long term success in maintaining the obtained weight loss.

Insulin resistance

An important finding in Paper III was the improvement of insulin sensitivity, expressed through the improvement in HOMA-IR, correlating with the improvement in L:A ratio. As previously described, insulin resistance is a crucial component of metabolic dysregulation in obesity. Insulin resistance seems to be associated with chronic

inflammation, although the condition is complex, and the primary event remains elusive.

As we did not formally record macronutrient composition or amount of exercise in our participants, our ability to infer causality to the improvement of insulin sensitivity is limited. However, existing literature suggests that a critical factor in improving both insulin resistance and, as a consequence, lipid dysregulation and inflammation is lower glycemic load by dietary carbohydrates (30, 31). This can be achieved by a specific low-carbohydrate diet or by healthy low-fat diets, where refined sugars usually are discouraged, and energy deficit usually is obtained (31, 119, 163).

The lowered glycemic load seen in lifestyle intervention thus reduces *de novo* lipogenesis and lipid accumulation both peripherally and viscerally, as insulin facilitates energy storage as glycogen and triglycerides once cell metabolism is satiated (164, 165). The lowered levels of circulating insulin from reduced glycemic load in lifestyle intervention allow for increased lipolysis, thus promoting weight loss (165), as was observed by the improvement of HOMA-IR with modest weight loss in our participants. Based on previous literature (31), one can assume that the improvement of insulin sensitivity is a primary event in weight loss. However, this theory cannot be investigated further based on our current data, and further studies are warranted.

Postprandial triglycerides and weight loss

Previous literature describes a clear correlation between hypertriglyceridemia and atherosclerosis, as chylomicron and VLDL remnants have a stronger tendency to penetrate and be retained in the atherosclerotic plaque compared to LDL particles (37, 41).

Previous work in our research group has shown that young MHO individuals have elevated triglyceride response and delayed triglyceride clearance compared to normal-weight individuals, where individuals with higher (but within normal range) levels of fasting triglycerides had a stronger correlation with postprandial lipemia compared to individuals with lower levels of fasting triglycerides (46). Together, the triglyceride response and clearance accurately describe the postprandial lipemic state compared to single measurements of non-fasting triglycerides and highlight the differences between MHO and normal-weight individuals.

Furthermore, an increased triglyceride response and a delayed triglyceride clearance were both correlated to the presence of insulin resistance (HOMA-IR >1.88) (46). These findings imply that MHO individuals have a prolonged time during the day with elevated triglyceride levels and, thus, an increased risk of atherosclerosis compared to normal-weight individuals.

The results in Paper II show a significantly impaired triglyceride clearance at 6 hours in both MHO and MDO individuals. The results in Paper III suggest that postprandial triglyceride clearance does not improve, like adipokines and insulin resistance, with modest weight loss. Our study design does not allow the examination of its underlying causes, thus allowing us to speculate merely.

These results were unexpected, as known literature suggests a close link between insulin resistance, inflammation and lipid dysregulation (77). Thus, the observation of reduced insulin resistance and L:A ratio would suggest a corresponding reduction in postprandial triglyceride levels.

One explanation could be that postprandial lipids are situated further downstream of the pathophysiologic mechanism compared to adipokines and insulin resistance (166)

and also that insulin resistance to a greater degree leads to increased endogenous triglyceride production and VLDL secretion and to FFA release to the bloodstream (166). Thus, consistent improvements in triglyceride metabolism might only be detected after more significant weight loss than 5%.

Previous studies in the field support this explanation (167, 168). We could not examine whether the participants who lost ≥10% of their weight demonstrated a more consistent improvement in the levels of postprandial triglycerides, as this subgroup consisted of only five participants.

It is also possible that the levels of postprandial triglycerides are too heterogeneous to observe changes during a modest weight loss of 5-10%, as post hoc sample size calculations indicated that we would have needed several hundred participants to be able to detect significant changes in triglyceride clearance at 6 hours. Based on the current analyses, it is impossible to determine whether the levels of isolated chylomicrons and endogenous triglycerides change homogenously in the postprandial period or not. Further studies are needed to explore this question.

Development and validation of clinical markers

Papers I and II focus on the development of biological tests intended for clinical use. This process consists of multiple steps. The first step, identification of the test, is presented in Papers I and II. After the identification, validating the test in different settings and study populations is paramount.

Paper III demonstrates an improvement of most markers identified in Paper II, with several participants going from high-risk to low/moderate-risk categories with modest weight loss (fig. 2, Paper III). However, further research is needed, where the same

markers are tested in a different and larger study population, in order to confirm their test properties and validity for clinical use.

Metabolically healthy and dysregulated obese individuals – different stages of inflammaging?

Literature regarding postprandial lipemia suggests that having high levels of circulating lipoproteins for extended periods increases CVD risk (169). Pathologic postprandial triglyceride clearance might account for the observed difference in morbidity and mortality between normal-weighted healthy individuals and clinically healthy obese individuals. Evidence suggests that years spent with an unfavorable metabolic profile increases inflammation and speeds up cellular aging (7, 21, 170).

In Paper II, we compared MHO and MDO individuals. A large proportion of both groups showed early metabolic disturbances such as delayed triglyceride clearance and insulin- and leptin resistance. No significant differences were observed between the two groups undergoing weight loss. These findings are in line with existing literature (69, 71) and may explain the increased risk of morbidity and mortality in presumably healthy individuals. On the other hand, an assessment of these factors, along with the characteristic features of the MetS, might help therapists identify a smaller subgroup of MHO people who genuinely have a lower risk of developing lifestyle diseases later in life. Applying the L:A ratio as a screening tool for insulin resistance and pathologic triglyceride clearance is thus favorable (69, 71).

One of the significant differences between the metabolic groups was age, where the mean age of the MHO and the MDO was 35 years and 51 years, respectively (Paper II). Given our findings and previous knowledge (1, 171), this might reflect time under risk, i.e., increased levels of proinflammatory adipokines, insulin resistance and

adipose tissue inflammation (1), which later in life results in the clinical manifestation of MetS, which in turn results in chronic lifestyle diseases, frailty, and premature death.

11 Conclusion and Implications

11.1 Evaluation of Hypotheses

Hepatic steatosis, quantified by HRI, and L:A ratio are feasible and straightforward screening tools usable in clinical practice for detecting subclinical metabolic dysregulation in obese individuals, along with insulin resistance and pathologic triglyceride clearance.

The L:A ratio and leptin- and insulin sensitivity improve with modest weight loss and are, thus, feasible markers of improved metabolic health during weight loss. Triglyceride clearance does not improve accordingly, which makes it ill-suited for monitoring metabolic improvements with weight loss.

- Hypothesis I: Hepatic steatosis, quantified by ultrasonography, can reliably predict insulin resistance in obese individuals and can be considered a feasible tool for screening in clinical practice. *Confirmed*.
- Hypothesis II: L:A ratio and leptin sensitivity are markers that can feasibly predict subclinical metabolic disturbances in obese individuals. *Confirmed.*
- Hypothesis III: Pathologic postprandial triglyceride clearance improves with a modest weight loss of 5% in obese individuals and is feasible for monitoring metabolic improvement during weight loss. *Not confirmed*.
- Hypothesis IV: The L:A ratio and leptin sensitivity improve with a modest weight loss and are thus useful markers for monitoring improved metabolic health during modest weight loss of 5%. *Confirmed.*

11.2 Implications

Our findings may contribute towards improving patient selection for treatment in a public health care system with limited resources for a large group of patients in order to identify individuals experiencing a higher immediate risk of developing chronic disease.

The present markers' strengths are accessibility and ease of implementation. However, these markers are crude, with several confounding factors, and none of them should be used in isolation to determine which individuals need intensive lifestyle intervention. They should instead be used as screening tools, opening the gateway to more precise diagnostic tools.

Such instances could be where an individual does not fulfill standard BMI categories of obesity required to offer specialized lifestyle intervention. Still, their metabolic profile suggests their risk of complicating disease is elevated. The reverse might also be true.

Early and successful treatment of individuals with a high risk of chronic lifestyle diseases, such as CVD, cancer, type 2 diabetes, and autoimmune disease might help save public resources, increase quality of life, and prevent premature death.

The relationship between age, chronic inflammation, and metabolic dysfunction might mean that successful treatment of obesity in adolescents and young adults, long before they show signs of the clinical manifestation of metabolic dysregulation, might lead to reduced time under risk and thus less burden of chronic disease and frailty towards the end of their lives, reducing the societal cost of lifestyle disease as much as possible.

On the other hand, as the obesity epidemic is already upon us, we might be forced to prioritize those who already show signs of metabolic dysfunction and inflammation, as they are in more immediate danger of developing complications from their obesity.

11.3 Further research

The metabolic markers presented in this study need further validation in different study populations before they can be widely applied in clinical practice. Studies should both examine individuals with stable weights and monitor these individuals during weight loss.

These markers should be tested in longitudinal studies against hard endpoints such as myocardial infarction, stroke, type 2 diabetes, cancer, organ failure, and mortality. This might be done in a population-based study like the Tromsø Study, either prospectively or retrospectively, analyzing frozen plasma for leptin and adiponectin.

Further studies would also be needed to more precisely determine the degree of risk reduction for lifestyle diseases achieved with successful weight loss intervention earlier vs. later in life, as both MHO and MDO individuals display insulin and adipokine dysregulation characteristics.

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13 Papers I-III

Paper I

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RESEARCH ARTICLE Open Access

Hepatic steatosis, detected by hepatorenal index in ultrasonography, as a predictor of insulin resistance in obese subjects

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Abstract

Background: The metabolic syndrome is a worldwide health issue, with non-alcoholic fatty liver disease (liver steatosis) being one of its features, particularly closely related to insulin resistance. This study aims to investigate whether quantification of hepatic steatosis by abdominal ultrasonography, using hepatorenal index, is a feasible tool for the prediction of insulin resistance, and thus the metabolic syndrome.

Methods: Patients were recruited from the Centre of Obesity at the University Hospital of North Norway, and among participants from the Sixth Tromsø Study. Homeostasis Model Assessment of Insulin Resistance (HOMA1-IR) was measured, body mass index (BMI, kg/m^2) calculated, and hepatorenal index (HRI), i.e. the ratio of liver to kidney optical densities, was measured by transabdominal ultrasonography. Receiver operating characteristic (ROC) analyses were performed, detecting insulin resistance at HOMA1-IR cut-off values >2.3 and >2.5.

Results: Ninety participants were included in the study, of which 46 (51 %) had BMI ≥30 and 27 (30 %) had BMI ≥35. Overall, HRI at level 1.17 had sensitivity 0.90 and specificity 0.70 for predicting insulin resistance (HOMA1-IR >2.3) in all participants. For participants with BMI ≥30, HRI at level 1.17 had sensitivity 0.94 and specificity 0.70, and for BMI ≥35, HRI at level 1.17 had sensitivity 0.93 and specificity 0.75 for predicting HOMA1-IR >2.3. Setting the HRI limit at 1.42 gave low sensitivities and high specificities in all BMI groups. In the analysis predicting HOMA1-IR >2.5, sensitivity values were almost the same as in the analysis predicting HOMA1-IR >2.3, but specificity values were lower in this analysis.

Conclusion: Detection and quantification of hepatic steatosis by ultrasound and the hepatorenal index is a feasible screening tool for identifying patients with low risk of having insulin resistance (IR, HRI <1.17), patients at risk of having IR (HRI 1.17-1.41) and patients with likely IR (HRI ≥1.42), especially in obese individuals.

Keywords: Body mass index, Hepatorenal index, HOMA1-IR, Insulin resistance, Liver steatosis, Metabolic syndrome, Morbid obesity, Non-alcoholic fatty liver disease, Quantification, Ultrasonography

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Background

Obesity is a major worldwide health issue due to its association with the metabolic syndrome (MetS). In 2013, 36.9 % of men and 38.0 % of women in developed countries were overweight or obese [1]. Components of MetS are increased waist circumference, hypertension, insulin resistance (IR), type 2 diabetes and dyslipidemia, i.e. hypertriglyceridemia and low levels of high density lipoproteins (HDL) [2, 3].

Non-alcoholic fatty liver disease (NAFLD) is closely associated with both IR and MetS, as patients with IR run a high risk of also having NAFLD, and NAFLD is a predictor of the metabolic disturbances associated with MetS [4–7].

Accordingly, components of the metabolic syndrome are also strongly associated to NAFLD in normal-weight subjects without diabetes, and in obese subjects diagnosed with NAFLD the prevalence have been found to be 67–71 % [8, 9]. Similarly, in subjects with diabetes mellitus type 2 or impaired glucose tolerance, the prevalence of NAFLD is found to be 30–70 % [10–12].

The association with high body weight makes NAFLD the most common type of liver disease in the developed world today, with a prevalence of approximately 30 % [13, 14], a number that is expected to increase [15].

The hepatic lipid metabolism is vulnerable to metabolic dysfunction, resulting in the accumulation of lipid droplets in the hepatocyte. The 'two-hit theory' model by Day [16] describes the pathogenesis of NAFLD. The 'first hit' is a hepatocellular lipid accumulation due to an imbalance of lipid uptake and combustion. The progression to the 'second hit' is defined as a hepatocellular steatohepatitis (Non- alcoholic steatohepatitis (NASH)), due to the imbalance between pro- and anti-inflammatory factors [16]. Among these, adipocytokines play a central role in this pathogenesis [17].

Insulin resistance is considered to be the most important pathophysiological mechanism of MetS [18, 19]. IR is characterized by impaired lowering of blood glucose through reduced glucose uptake in muscles, and lack of insulin effect on endogenous glucose production in liver. IR is also characterized by impaired insulin effect on lipid and protein metabolism, as well as impaired effect on a number of other organs [18, 20].

A common and efficient way of assessing insulin resistance is by the Homeostasis Model Assessment of insulin resistance (HOMA1-IR) [21, 22]. This model is based on measurements of fasting blood glucose and fasting insulin only. It has been proven to be equally good as the gold standard for measuring IR, i.e. the euglycemic clamp, in addition to being a more convenient test to perform. [23] A cut-off value of HOMA1-IR >2.3 has previously been shown to have a sensitivity of 76.8 % and a specificity of 66.7 % for identifying metabolic syndrome, whereas a cut-off value of 2.7 had the same test statistics for detecting insulin resistance [24].

Measurements of increased levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (γ-GT) may be used as an indicator of NAFLD when biopsy is contraindicated or not available, in combination with radiological methods, most often transabdominal ultrasonography (US) [25].

Subjective assessments of steatosis by US display a relatively large inter- and intra-observer variability. A way of reducing this variability is by measuring the liver and kidneys' echogenicity on a histogram grayscale, and using the mean values for computing the hepatorenal index (HRI) [26]. HRI is a tool of quantifying the steatosis that is more reliable than subjective assessment alone. In a normal liver, HRI is in the range from 1.00 to 1.04. Hepatic steatosis is classified as mild (HRI 1.05–1.24), moderate (1.25–1.64) or severe (≥1.65) [26].

The availability and cost of abdominal US examinations and biochemical analyses such as that of insulin by ELISA will differ both between and within countries. In our department, ultrasonography examinations are easily accessible, whereas insulin measurements are not done on a routine basis, making use of US in the assessment of obese patients a practical approach.

As far as we know, the use of liver steatosis, assessed by HRI at ultrasonography as a predictor and screening method for IR, has not been examined in previous studies. Therefore, we aimed to investigate the test properties of this method.

Aim of study

To examine whether hepatic steatosis, quantified by HRI, is a feasible test for detecting IR.

Methods

Participants eligible for the study were adults with either obesity or elevated liver enzymes. Only individuals meeting the inclusion criteria were invited to participate in the study, and all those who accepted the invitation were included.

Obese patients were recruited from the Centre of Obesity, Department of Gastroenterology at the University Hospital of North Norway ($n = 80$). Subjects with elevated liver enzymes were recruited from the Sixth Tromsø Study population (Fig. 1).

The Sixth Tromsø Study in 2008 is previously described [27]. Participants with values of AST, ALT or γ-GT above the Upper Limit of Normal (ULN) when examined in The Tromsø Study (n = 68), were invited to participate in our study. All participants who accepted the invitation were included in the study, and divided into two groups: liver enzymes 1–2× ULN and liver enzymes >2× ULN.

We also invited a control group with normal liver enzymes ($n = 44$), drawn from the main study population of the sixth Tromsø Study. This control group was sized and adjusted for sex and age to match the group of participants with liver enzymes >2× ULN. Participants who accepted the invitation were included in the study.

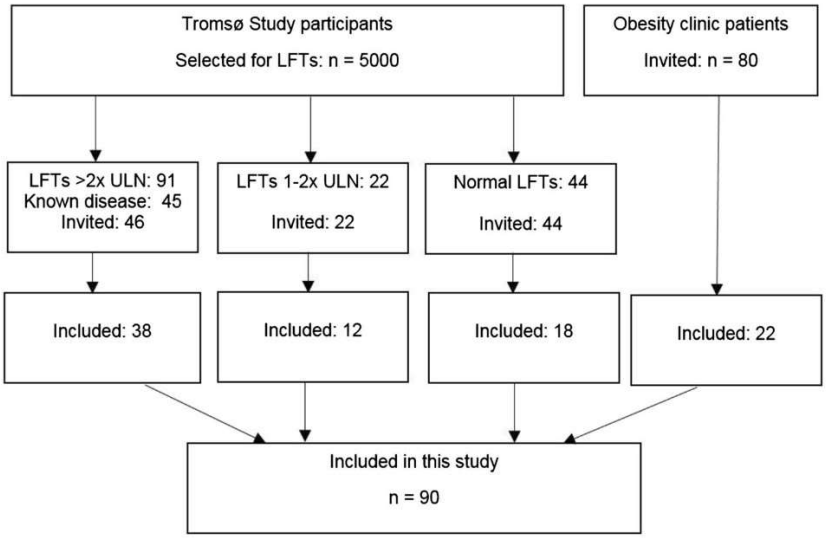

Fig. 1 Flowchart of included participants from the Sixth Tromsø Study and the University Hospital Obesity Clinic. LFT: Liver function tests (i.e. liver enzymes), ULN: Upper limit of normal

All participants signed a written consent, which included permission to use their data for follow-up studies. The Regional Committee of Medical Ethics of North Norway approved the study performed at the Centre of Obesity, including the approval of a biobank.

The Tromsø Study organisation approved access to data and participants for the follow-up study for the Sixth Tromsø Study. The main ethical approval given for the Sixth Tromsø Study by the Regional Committee of Medical Ethics of North Norway also covered the follow-up study.

Inclusion criteria for the subjects recruited from the Centre of Obesity were BMI >30 kg/m^2 and age >18 years. Exclusion criteria were medically treated diabetes mellitus, severe heart disease or severe kidney failure. Patients that met the inclusion criteria at the Centre of Obesity were invited to participate in the study on their first consultation, or at their first group seminar during their treatment period. All participants recruited from the Centre of Obesity underwent an Oral Glucose Tolerance Test (OGTT), where the participants ingested 75 g of glucose dissolved in water. Fasting and postprandial blood glucose (mmol/L) and serum insulin (μmol/L) levels were measured at 30-min intervals for up to 180 min, and HOMA1-IR and WBISI were calculated as follows:

$$HOMA1\text{–}IR = \frac{[fasting\ glucose]\ \times [fasting\ insulin]}{\frac{22.5}{10000}}$$

$$WBISI = \frac{}{\sqrt{[fasting\ glucose] \times [fasting\ insulin]\ \times [mean\ OGTT\ glucose] \times [mean\ OGTT\ insulin\ ]}}$$

Height, body weight and blood pressure were measured [27], blood samples for measurement of liver enzymes (AST, ALT, γ-GT) were collected, and transabdominal ultrasonography was performed as described below.

The participants included from the Tromsø Study follow-up were recruited from three groups: participants with either AST, ALTor γ-glutamyl transferase >2× Upper Limit of Normal, participants with values between ULN and 2× ULN, and a selection of participants with normal values, matched for sex and age of the first group (enzyme values >2× ULN).

The group with liver enzyme levels >2× ULN was followed up during the first few months after the Tromsø Study visits in 2008. The two other groups were followed up during 2013/2014. The same variables were recorded for all three groups: height, body weight, liver enzyme levels, and fasting blood glucose, serum insulin, and fasting triglyceride levels. Transabdominal ultrasonography was performed with measurement of HRI. Blood pressure was also measured from the Tromsø study visits.

Transabdominal ultrasonography was performed using a Hitachi EUB-6500 HW apparatus with a 5 MHz convex EUP-C524 transducer (Hitachi Medical Corporation,

Tokyo, Japan). Hepatic and renal parenchymal echogenic density on a grayscale (values 0–255) was recorded with the built-in histogram function. An average of three repeated measurements was used to calculate HRI by the formula:

$$HRI = \frac{mean\ liver\ echogenicity}{mean\ kidney\ echogenicity}$$

Values below 1.0 were corrected to 1.0. Steatosis was classified as mild (HRI 1.05–1.24), moderate (HRI 1.25–1.64) or severe (HRI ≥1.65) [26].

All statistical analyses were carried out using IBM SPSS Statistics, version 21. Receiver Operating Characteristic analyses (ROC) were performed, detecting IR at HOMA1-IR values >2.3 and >2.5 in both inclusion groups combined.

The cut-off value HOMA1-IR >2.3 has previously been described [24]. Other studies have previously used a value of HOMA1-IR >2.7. In this study, the cut-off value is set to 2.5 due to our relatively small study sample, with few observations of HOMA1-IR >2.7.

Subgroups of the participants with BMI ≥30 ($n = 46$) and BMI ≥35 ($n = 27$) were analysed separately.

Results

In total, 90 participants were included (20 men and 70 women), of which 22 participants were included from the Centre of Obesity and 68 participants from the Sixth Tromsø Study population as shown in Fig. 1. Baseline characteristics are shown in Table 1.

For the participants included from the Centre of Obesity, we calculated both HOMA1-IR and WBISI, in order to verify the reliability of HOMA1-IR in our data set. The correlation between HOMA1-IR and WBISI is shown in Fig. 2.

There were five missing values for either HOMA1-IR or HRI in the dataset. Hence, we performed the ROC analysis with 85 participants (all cases). Sensitivity and specificity values for different HRI levels for detecting IR measured by HOMA1-IR are shown in Table 2. We made the choice of cut-off values for HRI based on a trade-off between sensitivity and specificity of the test. The test properties and likelihood ratios derived from the ROC analysis by 2 × 2 tables are shown in Table 3.

Overall, 45 % ($n = 38$) of all subjects had HRI ≥1.17, while 13 % (n = 11) had HRI ≥1.42.

The test has a high sensitivity and a relatively low specificity for HRI values corresponding to mild hepatic steatosis (HRI = 1.17), and a low sensitivity with a high specificity for HRI values corresponding to moderate hepatic steatosis (HRI = 1.42). The corresponding ROC curves are shown in Fig. 3a–d.

Discussion

Our results show that the detection and quantification of hepatic steatosis by the measurement of abnormal hepatorenal index values is feasible as a screening tool for the detection of insulin resistance. However, HRI values corresponding to mild steatosis showed a high sensitivity but a relatively low specificity. On the other hand, HRI values corresponding to moderate steatosis had a low sensitivity but a high specificity. Therefore, one must weigh the importance of finding patients with IR against the inconvenience of having many false positive results from the test.

Table 1 Baseline characteristics of the 90 study participants

	Inclusion groups					
	Centre of Obesity			Sixth Tromsø Study		
	N	Median (SD)	Range	N	Median (SD)	Range
Age, years	22	43.0 (12.76)	21–69	68	66.0 (10.84)	32–82
Height, cm	22	167.5 (6.87)	156–179	68	166.0 (9.08)	141–189
Weight, kg	22	113.0 (16.13)	83.5–148.0	68	81.3 (15.51)	50.6–123.5
Systolic BP, mmHg	22	126.0 (11.36)	112–159	68	137.0 (22.07)	96–213
Diastolic BP, mmHg	22	74.0 (8.16)	62–94	68	78.0 (8.42)	50–102
BMI, kg/m^2	22	41.8 (5.66)	31.8–52.7	68	28.0 (5.35)	19.3–45.6
HOMA1-IR	22	2.5 (1.75)	0.8–6.5	65	0.8 (3.18)	0.2–25.5
WBISI	22	0.3 (0.77)	0.03–2.9	0	-	-
AST, U/L	22	18.0 (9.32)	12.0–53.0	66	29.5 (1.23)	14.0–71.0
ALT, U/L	4	38.5 (6.70)	29.0–45.0	66	39.0 (20.36)	14.0–102.0
γ-GT, U/L	21	29.0 (45.72)	13.0–198.0	65	82.0 (82.74)	14.0–398.0
ALP, U/L	4	88.0 (9.22)	80.0–99.0	66	81.0 (38.96)	36.0–322.0

SD Standard Deviation, *BP* Blood Pressure, *BMI* Body Mass Index, *HOMA1-IR* Homeostasis Model Assessment of Insulin Resistance 1, *WBISI* Whole Body Insulin Sensitivity Index, *AST* Aspartate Aminotransferase, *ALT* Alanine Aminotransferase, *γ-GT* Gamma-Glutamyl Transferase, *ALP* Alkaline Phosphatase

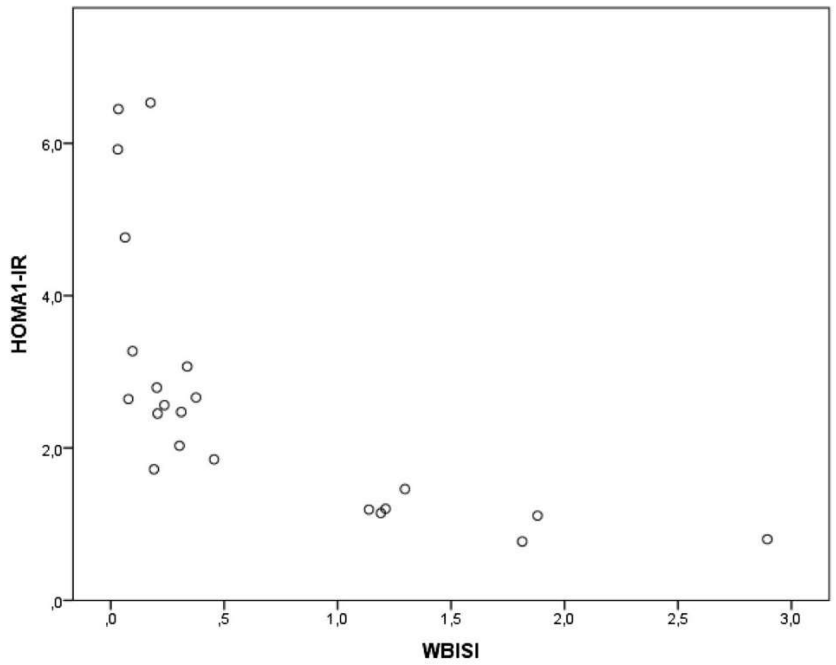

Fig. 2 Correlation between WBISI and HOMA-IR for 22 overweight or obese participants. Spearman's corr. 0.862 (p <0,001). Spearman Correlation: −0.922 (p <0.001) (two-tailed analysis)

As far as we know, the relationship between an abnormal HRI and abnormal HOMA1-IR values has not been investigated before, despite the invariable association between NAFLD, obesity and MetS [5]. In light of this association, having good screening methods for detecting early signs of MetS, especially in obese patients, is of great importance. Also in normal-weight subjects at risk, a good screening tool is useful. In normal-weight and non-diabetic individuals the correlation between the components of MetS, visceral fat accumulation and IR

Table 2 Test properties for different levels of hepatorenal index for the prediction of insulin resistance (HOMA1-IR)

	HOMA-IR >2.3					
	All participants (n = 85) (n = 21 with HOMA-IR >2.3)		BMI ≥30 (n = 45) (n = 18 with HOMA-IR >2.3)		BMI ≥35 (n = 27) (n = 15 with HOMA-IR >2.3)	
HRI cut-off	1.17	1.42	1.17	1.42	1.17	1.42
Sensitivity	0.90 (.71; .97)	0.33 (.17; .55)	0.94 (.74; .99)	0.33 (.16; .56)	0.93 (.70; .99)	0.27 (.11; .52)
Specificity	0.70 (.58; .80)	0.94 (.85; .98)	0.70 (.52; .84)	0.96 (.82; .99)	0.75 (.47; .91)	0.92 (.65; 99)
LR +	3.05 (2.04; 4.56)	5.33 (1.73; 16.4)	3.19 (1.76; 5.76)	9.0 (1.18; 68.6)	3.73 (1.39; 10.0)	3.20 (.41; 25.0)
LR -	0.14 (.04; .51)	0.71 (.52; .97)	0.08 (.01; .54)	0.69 (.50; .97)	0.09 (.01; .61)	0.80 (.56; 1.14)
	HOMA-IR >2.5					
	All participants (n = 85) (n = 17 with HOMA-IR >2.5)		BMI ≥30 (n = 45) (n = 14 with HOMA-IR >2.5)		BMI ≥35 (n = 27) (n = 12 with HOMA-IR >2.5)	
HRI cut-off	1.17	1.42	1.17	1.42	1.17	1.42
Sensitivity	0.88 (.66; .97)	0.29 (.13; .53)	0.93 (.69; .99)	0.29 (.12; .55)	0.92 (.56; .99)	0.18 (.05; .48)
Specificity	0.66 (.54; .76)	0.94 (.82; .94)	0.61 (.44; 76)	0.90 (.75; 97)	0.60 (.36; .80)	0.81 (.57; .93)
LR +	2.61 (1.79; 3.80)	4.85 (1.15; 9.63)	2.40 (1.51; 3.82)	2.95 (.76; 11.4)	2.29 (1.21; 4.36)	0.97 (.19; 4.88)
LR -	0.18 (.05; .66)	0.75 (.57; 1.06)	0.12 (.02; 79)	0.79 (.56; 1.12)	0.14 (.02; 95)	1.01 (.70; 1.45)

Test properties and likelihood ratios (LR ±) for different cut-off values of hepatorenal index (HRI) for predicting different levels of insulin resistance, defined by Homeostasis Model Assessment of Insulin Resistance (HOMA1-IR) >2.3 and 2.5, respectively. BMI subgroups are analysed specifically

Table 3 2 × 2 tables for detecting insulin resistance (HOMA1-IR >2.3) by mild hepatic steatosis (HRI ≥1.17)

		All cases		
		HOMA1-IR		
		>2.3	≤2.3	Total
HRI	≥1.17	19	19	38
	<1.17	2	45	47
	Total	21	64	85
		BMI ≥ 30		
		HOMA1-IR		
		>2.3	≤2.3	Total
HRI	≥1.17	17	8	25
	<1.17	1	19	20
	Total	18	27	45
		BMI ≥ 35		
		HOMA1-IR		
		>2.3	≤2.3	Total
HRI	≥1.17	14	3	17
	<1.17	1	9	10
	Total	15	12	27

has shown to correlate with the severity of NAFLD and all the different components of MetS are strongly associated with NAFLD [8, 28]. Transabdominal ultrasonography (US) is already in use for bedside diagnostics of NAFLD, but subjective evaluation of steatosis characteristics in US is susceptible to inter and intra observer variability, especially for mild and moderate steatosis [25]. Our results indicate that the use of HRI will improve the test properties of US in the diagnostics of MetS.

IR in obese individuals is crucial for the risk of further development of MetS, and the presence of hepatic steatosis and IR is closely linked. Although the HOMA1-IR is relatively simple to calculate both in general practice and in specialist care, it tends not to be in mind of the clinician, unless there are other factors suggesting an underlying IR.

A previous study by Geloneze et al. has shown that the optimal cut-off value of HOMA1-IR is 2.3 for detecting insulin resistance and MetS (sensitivity 77 % and specificity 67 %) [24]. The updated HOMA2-IR is more accurate, correcting for feedback relationships between insulin resistances in different organs [24]. The optimal cut-off value for HOMA2-IR is 1.4, (sensitivity 79 % and specificity 62 %). However, HOMA2-IR has a limited range of reliable values, and calculating the index is much more complicated [24].

A second method for assessing IR is the Whole Body Insulin Sensitivity Index (WBISI), which adds predictive value to HOMA-IR for assessing risk of IR, but is more inconvenient to use in clinical practice because of the need of an Oral Glucose Tolerance Test (OGTT), measuring postprandial blood glucose and serum insulin values in addition to fasting levels [23, 29]. We have demonstrated a good correlation between WBISI and HOMA1-IR, and thus the usefulness of HOMA1-IR in this study.

Our cut-off value of HOMA1-IR >2.3 was set in accordance to the study by Geloneze et al. [24]. Our results show that using HRI as a screening test for detecting IR is possible, but it should only be used in groups where the prevalence of IR is high, that is, in people with BMI >30. In this group, mild steatosis (HRI ≥1.17) diagnosed by ultrasonography, detected 94 % of patients with a HOMA1-IR of more than 2.3. However, the specificity of the test is low (70 %). Therefore, this test will identify patients with high risk of having IR.

We also calculated test properties for HOMA-IR level 2.5, because the clinical significance of the HOMA1-IR limit may vary, depending on whether one is interested in a high sensitivity or a high specificity of HOMA1-IR in diagnosing patients with true IR [24]. A limit of 2.5 gives a more clinically applicable HOMA-IR value, but with a higher number of false negative results.

The gold standard for diagnosing hepatic steatosis is by liver biopsy [30], which also is the only way of diagnosing the presence of steatohepatitis. Liver biopsy, however, remains an invasive procedure with a risk of complications, and the need for biopsy in the diagnosis of NAFLD is much debated [31]. Ultrasonography, being a risk-free non-invasive procedure, that is simple to perform, would be a good choice for clinical screening.

In a previous study of the test properties of HRI performed during abdominal ultrasonography, it was shown that a HRI cut-off value of 1.49 has a sensitivity of 100 % and a specificity of 91 % for detecting a 5 % steatosis, diagnosed by liver biopsy [26]. Our results show that a HRI cut-off at 1.42 will have a specificity of 96 % in the obese group with BMI ≥30 for having HOMA1-IR >2.3. A HRI value ≥1.49 will therefore be diagnostic, for both having an actual hepatic steatosis, and also actually having a HOMA1- IR of more than 2.3, and thus having insulin resistance.

Although a lower HRI cut-off level of ≥1.17 will give many false positive results, this is acceptable in a screening test, since the verification of IR is relatively simple through calculating HOMA1-IR.

One of the strengths of this study is that the study population is similar to the patient group that will be relevant for this screening, both in regards to overweight and obesity, as well as pathological liver enzyme levels. An extrapolation to the general population is, however, not possible.

We did not perform liver biopsy to confirm the results of the ultrasound examination. Therefore, one cannot

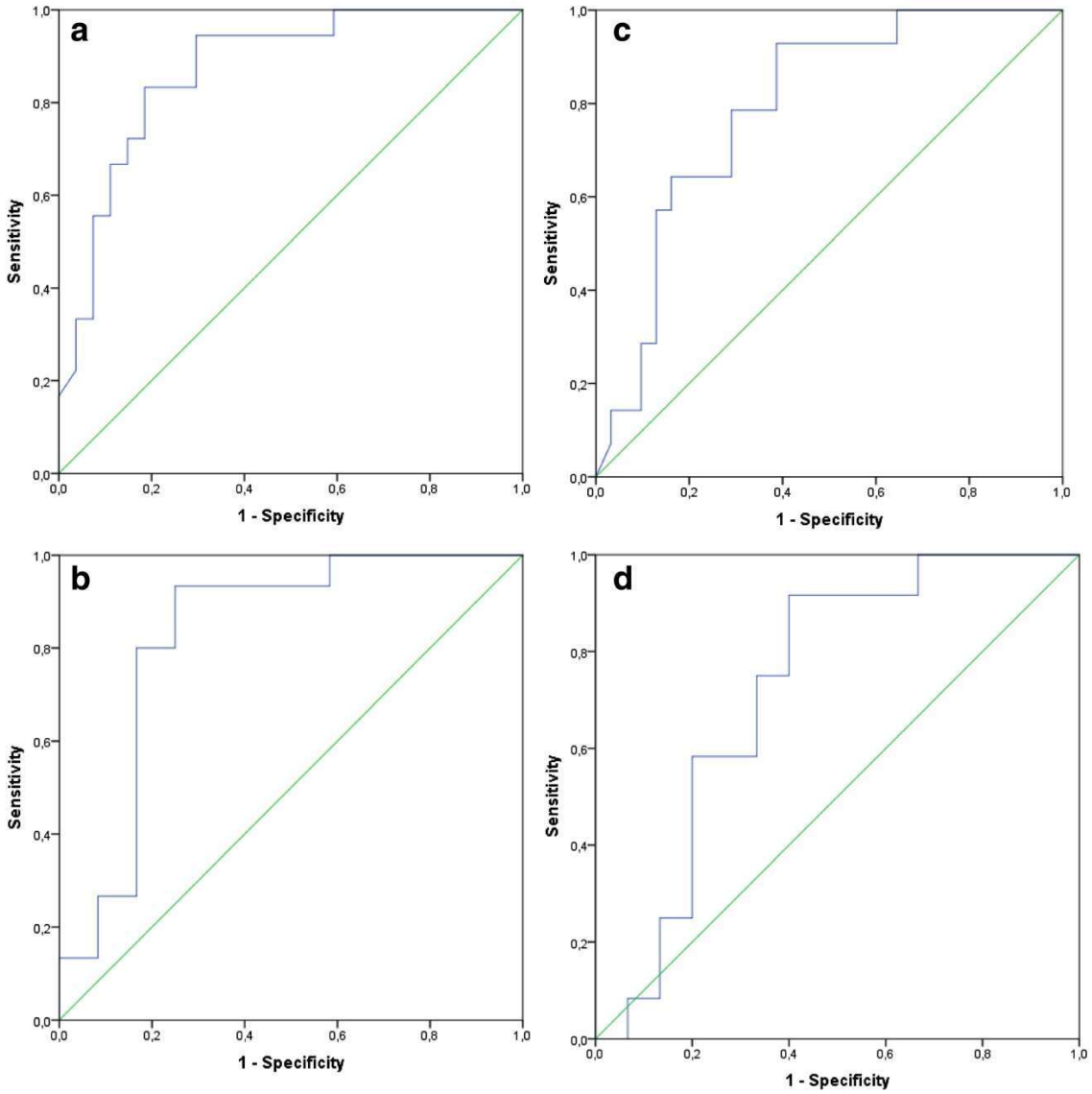

Fig. 3 **a** ROC curve of HOMA-IR >2.3 by HRI in participants with BMI ≥30. **b** ROC curve of HOMA-IR >2.3 by HRI in participants with BMI ≥35. **c** ROC curve of HOMA-IR >2.5 by HRI in participants with BMI ≥30. **d** ROC curve of HOMA-IR >2.5 by HRI in participants with BMI ≥ 35

say that participants with HRI ≥1.49 have hepatic steatosis. The correlation between the actual steatosis and IR is beside the scope of this study, since this correlation is generally accepted [13].

One of the weaknesses of using HRI as a means of assessing steatosis is its variability. There is a certain degree of both inter- and intra-observer variability, but the results are also dependent on the type of ultrasound equipment used. An example of this is one of the features available in later ultrasonography equipment models, i.e. the option to highlight the liver tissue above other tissues. Thus, the actual HRI values in different studies may not be directly comparable as a result, and one needs to be aware of this as a source of bias when choosing this method.

The participants were not examined for other chronic liver diseases, apart from a medical history. This is a possible source of bias, since the presence of liver cirrhosis could influence the HRI measurements.

The small sample size is a weakness of this study, particularly in the subgroup of morbid obese participants (BMI ≥ 35), where only 12 participants had HOMA1-IR >2.3.

Because of this, the results of our study need confirmation by further studies, with more participants included.

Conclusion

The detection of hepatic steatosis by transabdominal ultrasound, and the quantification of steatosis by measurement of the hepatorenal index, is a feasible screening tool for stratifying patients with regards to risk of having insulin resistance: patients with low risk of IR (HRI <1.17), patients at risk of having IR (HRI 1.17–1.41), and patients with likely IR (HRI ≥1.42). The test should primarily be used in obese subjects.

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Availability of data and materials
The raw data file is available through UiT Open Research Data, the institutional repository at UiT The Arctic University of Norway. Doi: 10.18710/RFTJKL.

Authors' contributions
All authors contributed to study design and manuscript preparation, including discussion of results. VTI was responsible for patient contact and collection of clinical and laboratory data. VTI and EJP performed transabdominal ultrasonography. VTI, RG, EJP did statistical analyses. JRF was principle investigator. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable

Ethics approval and consent to participate
All participants signed a written consent, which included permission to use their data for follow-up studies. The Regional Committee of Medical Ethics of North Norway approved the study.

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Paper II

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Leptin to adiponectin ratio – A surrogate biomarker for early detection of metabolic disturbances in obesity

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Postprandial triglycerides;
Leptin resistance;
Diabetes type II;
Metabolic healthy obese;
Insulinresistance;
Leptin;
Adiponectin

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.
Methods and results: 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.

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.

Abbreviations: CVD, Cardiovascular disease; L:A ratio, Leptin to Adiponectin ratio; HOMA-IR, homeostasis model assessment of insulin resistance; MHO, Metabolic healthy obese; MDO, Metabolic dysregulated obese; TG, Triglycerides; BMI, Body mass index; NCEP/ATPIII, National Cholesterol Education Panel/Adult Treatment panel III; 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; LR, Leptin resistance; ROC, Receiver operating characteristics; PPV, Positive predictive value; NPV, Negative predictive value.

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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) $\geq$30 kg/m^2 and age 18–70 years. An obese patient was considered a MHO when documented normotensive, normal thyroid function tests, normal liver function tests, normal kidney function, normolipemic and normoglycemic, none of the metabolic syndrome criteria, excluding the waist circumference criteria [18]. An obese patient was considered metabolically dysregulated obese (MDO) when he or she had two or more of the metabolic syndrome criteria according to the NCEP/ATPIII guidelines [18], excluding the waist circumference criteria, which all subjects had. Within the MDO group, five patients had elevated fasting TG ($\geq$1.7 mmol/L), three patients had untreated hypertension ($\geq$130/$\geq$ 85 mmHg), six patients had reduced high density lipoprotein (HDL) cholesterol (Women: $<$ 1.29 mmol/l, men $<$1.03 mmol/L) and six patients had elevated fasting glucose ($\geq$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 antidiabetes 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 upper arm, after a 15 min rest. Appropriate cuff size was used. The mean of the two last measurements were used. All the blood samples were collected at the laboratory, 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]. The OGTT and the OFTT were performed on different days.

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, and OGTT was performed. The measuring of the REE was done by two different trained test personnel. 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

Table 1 Anthropometric characteristics at baseline between normal weight subjects and obese subjects.

Variables	Baseline			
	Normal weight subjects (n = 17)	MHO (n = 36)	MDO (n = 14)	Sig. (*p*)[c]
Sex (M/F)	2/15	5/31	5/9	n.a.
Age (years)	31.0 (24.5; 37.5)	38.0 (29.5; 46.5)	51.6 (42.1; 61.1)[a,b]	<0.001
BMI (kg/m^2)	21.3 (20.2; 22.4)	40.0 (36.5; 43.2)[a]	39.8 (34.8; 44.8)[a]	<0.001
Total fat percent (%)	26.6 (23.7; 29.6)	50.7 (47.1; 54.1)[a]	46.3 (41.1; 51.5)[a]	<0.001
Abdominal fat percent (%)	27.5 (24.3; 30.7)	57.9 (52.7; 58.1)[a]	55.4 (51.2; 59.6)[a]	<0.001
Systolic BP (mmHg)	105 (98; 113)	124 (113; 135)[a]	130 (122; 138)[a]	<0.001
Diastolic BP (mmHg)	65 (60; 70)	74 (67; 79)[a]	80 (74; 87)[a,b]	<0.001

Values are median (Interquartile range).
Abbreviations: MHO; metabolic healthy obese, MDO; metabolic dysregulated obese, BMI; body mass index, BP; blood pressure, n.a; not applicable, M; male, F; female, Sig; significant.
[a] Significantly different from normal weight.
[b] Significantly different from MHO.
[c] Kruskal–Wallis test.

Table 2 Metabolic and clinical characteristics at baseline between normal weight and obese subjects.

Variables	Baseline			
	Normal weight subjects (n = 17)	MHO (n = 36)	MDO (n = 14)	Sig. (*p*)[c]
Fasting Glucose (mmol/L)	4.4 (4.1; 4.8)	5.1 (4.7; 5.6)[a]	5.6 (5.2; 6.1)[a,b]	0.001
Fasting Insulin (μmol/L)	5.53 (4.08; 6.99)	11.80 (7.96; 15.64)[a]	13.15 (8.08; 18.23)[a]	<0.001
HOMA-IR	1.09 (0.37; 1.11)	2.71 (1.87; 3.55)[a]	3.56 (2.26; 4.86)[a]	<0.001
Fasting Leptin (μmol/L)	8.5 (4.8; 12.2)	41.7 (25.7; 57.7)[a]	29.4 (17.3; 41.5)[a]	<0.001
Fasting Adiponectin (μmol/L)	11.8 (8.3; 15.4)	7.6 (5.9; 9.3)[a]	9.1 (6.8; 11.5)	0.01
L:A ratio	0.77 (0.33; 1.25)	5.61 (2.61; 8.62)[a]	3.60 (2.81; 8.43)[a]	<0.001
REE (kcal/day)	1356 (1264; 1449)	1729 (1544; 1915)[a]	1752 (1491; 2014)[a]	<0.001
Leptin resistance (REE/fasting leptin)	142.5 (75.5; 209.5)	43.4 (23.3; 63.5)[a]	53.8 (29.7; 77.9)[a]	<0.001
Total cholesterol (mmol/L)	4.2 (3.8; 4.7)	4.3 (3.8; 4.8)	4.7 (4.0; 5.5)	n.s.
LDL cholesterol (mmol/L)	2.6 (2.0; 3.3)	2.9 (2.4; 3.4)	2.9 (2.3; 3.5)	n.s
HDL cholesterol (mmol/L)	1.6 (1.4; 1.9)	1.1 (0.9; 1.3)[a]	1.2 (1.1; 1.3)[a]	<0.001
HDL/LDL ratio	0.57 (0.33; 0.82)	0.38 (0.25; 0.51)[a]	0.42 (0.30; 0.55)[a]	<0.02
Fasting TG (mmol/L)	1.0 (0.8; 1.2)	1.3 (1.0; 1.6)[a]	1.5 (0.9; 2.1)[a]	0.002
TG clearance 6 h (%)	115.4 (39; 226.5)	63.6[a]	36.6[a]	0.000

Values are median (Interquartile range).
Abbreviations: HOMA-IR; homeostasis model assessment for insulin resistance, L:A ratio; Leptin to Adiponectin ratio, REE; Resting energy expenditure, LDL; Low density lipoprotein, HDL; high density lipoprotein, TG; Triglycerides, MHO; metabolic healthy obese, MDO; metabolic dysregulated obese, Sig; significant, n.s; non-significant.
[a] Significantly different from normal weight.
[b] Significantly different from healthy obese.
[c] Kruskal–Wallis test.

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 imbalance in sex between the three subgroups, however not significant (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 of single metabolic disturbances

A L:A ratio ROC curve was made to detect delayed TG clearance defined as <88.8% at 6 h (Fig. 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 (Fig. 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

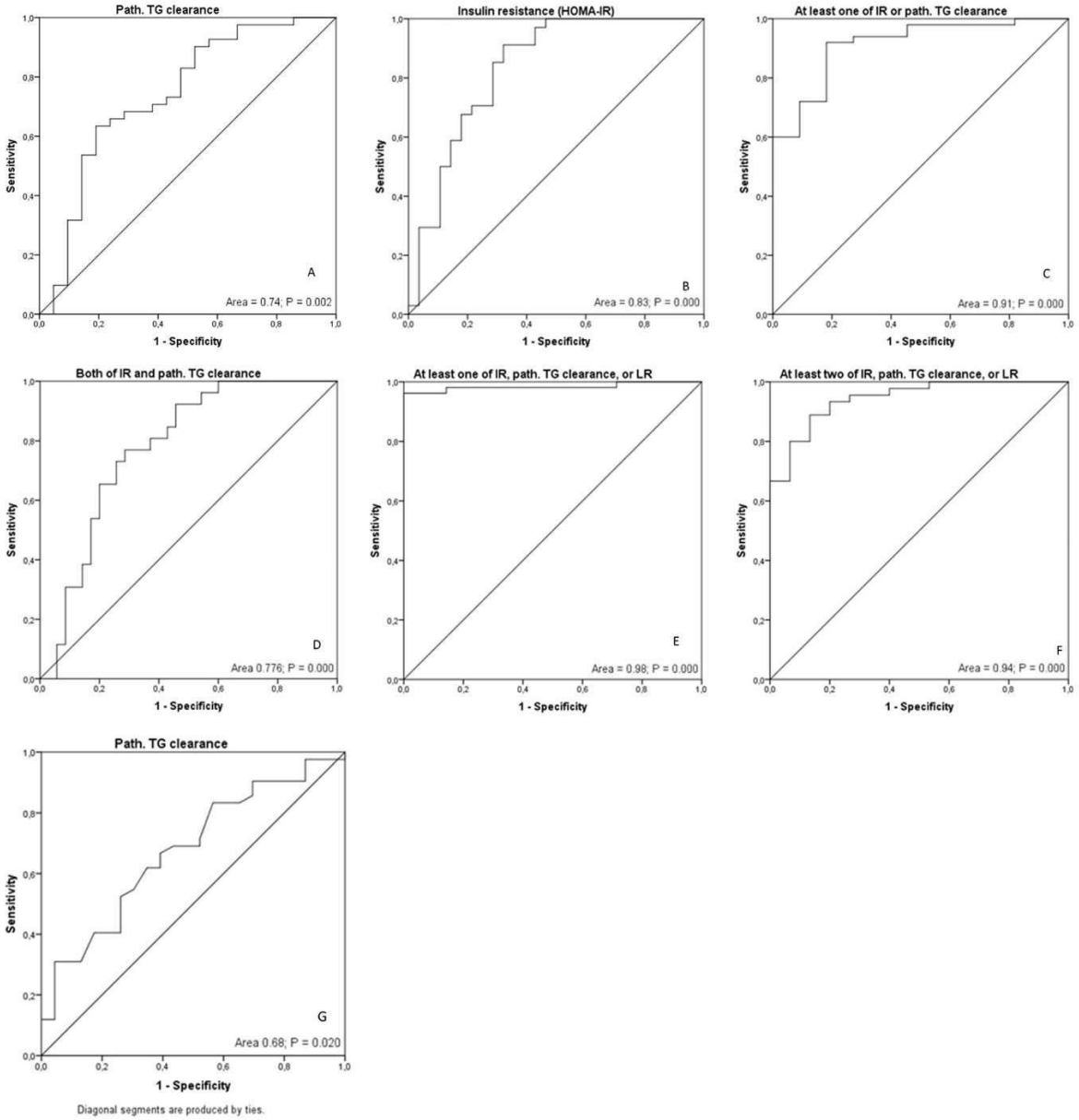

Figure 1 Receiver operating characteristics curves for the detection of delayed triglyceride clearance, insulin resistance and leptin resistance by the leptin to adiponectin ratio. Panel A: Detection of delayed TG clearance by L:A ratio: AUC 0.74, P = 0.002. Panel B: Detection of IR by L:A ratio: AUC 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: triglyc eride, IR: Insulin resistance, L:A ratio: Leptin to adiponectin ratio.

least one of delayed TG clearance or IR in all groups (Fig. 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 (Fig. 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) (Fig. 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 (Fig. 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 metabolic 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 (Fig. 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 11,000 subjects found fasting leptin to have moderate sensitivity and specificity for identifying cardiometabolic 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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Conflicts of interest

The authors report no conflicts of interest.

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Paper III

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Correlations between modest weight loss and leptin to adiponectin ratio, insulin and leptin resensitization in a small cohort of Norwegian individuals with obesity

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ABSTRACT

Background: Weight loss is important to reduce the risk of metabolic complications in obese individuals, in whom dysregulated adipokines play a central role. This study aims to investigate whether dysregulated adipokines and postprandial triglycerides (TG) improve with a modest weight loss.
Methods: Individuals with obesity (BMI ≥ 30 kg/m^2) were recruited among patients at the University Hospital of North Norway and the Stamina Health weight loss rehabilitation program. We measured resting energy expenditure (REE), and calculated the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), leptin to adiponectin (L:A) ratio, indirect leptin sensitivity (REE:leptin ratio), postprandial TG clearance at 6 h, and TG response before and after weight loss. The goal of the weight loss intervention was a loss of ≥5 % of initial total body weight.
Results: 28 participants completed the study, of which 13 lost ≥ 5 % body weight and 18 lost <5 % body weight. HOMA-IR (−23.1 %), REE:leptin ratio (+80.1 %) and L:A ratio (−45.7 %) significantly improved with weight loss, whereas there was no improvement of postprandial TG response or clearance. No significant changes were observed in the non-weight loss group.
Conclusion: The data are consistent with the general concept that modest weight loss in obese patients may restore metabolic regulation by improving L:A ratio and insulin and leptin sensitivity.

1. Background

Overweight and obesity are important risk factors for morbidity and mortality (O'Neill and O'Driscoll, 2015; WHO, 2020), mainly because of their association with metabolic dysfunction and increased risk of cardiovascular disease (CVD), cancer, and type 2 diabetes mellitus (T2DM) (O'Neill and O'Driscoll, 2015; Rhee, 2018).

Dyslipidemia is an essential part of the metabolic syndrome, in addition to abdominal obesity and insulin resistance (Vekic et al., 2019). We have previously demonstrated that the postprandial triglyceride response (TGR) is altered in healthy individuals with obesity, with a delayed postprandial triglyceride (TG) clearance compared to healthy, normal weight individuals (Larsen et al., 2015). Because humans spend most of their waking hours in the postprandial state, this increased period of triglyceride exposure in obese individuals contributes to an increased risk of both CVD and T2DM, by increasing atherosclerosis and induce insulin resistance (Tenenbaum et al., 2014; Huet et al., 2019).

The adipokines leptin and adiponectin are, together with free fatty acids (FFA), critical mediators in adipocytes to maintain metabolic homeostasis (Stern Jennifer et al., 2016; Harris, 2014; Wang and Scherer, 2016). Moreover, adipokines play a central role in modulating inflammation (Naylor and Petri, 2016; Unamuno et al., 2018), another contributing mechanism to CVD. In individuals with overweight and obesity, alterations of adipokine levels and their function are essential factors of the pathophysiologic mechanisms causing the metabolic syndrome, CVD, T2DM and other complications to obesity (Unamuno et al., 2018; Souza et al., 2017).

Abbreviations: ALT, Alanine aminotransferase; ANOVA, Analysis of variance; Apo, Apoliopoprotein (A1, B and E); AST, Aspartate aminotransferase; BMI, Body mass index; BP, Blood pressure; CI, Confidence interval; CVD, Cardiovascular disease; DEXA, Dual-energy X-ray absorptiometry; ELISA, Enzyme-linked immunosorbent assay; FFA, Free fatty acid; γ-GT, Gamma glutamyl transferase; HDL, High-density lipoprotein; HOMA-IR, Homeostasis model assessment of insulin resistance; L:A, Leptin to adiponectin (ratio); LDL, Low-density lipoprotein; OGTT, Oral glucose tolerance test; OFTT, Oral fat tolerance test; REE, Resting energy expenditure; REK, Regional etisk komité (Regional ethics committee); TG, Triglyceride; TGR, Triglyceride response; UiT, University of Tromsø; UNN, University hospital of North Norway; WBISI, Whole body insulin sensitivity index.

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Obesity is related to leptin resistance, a well-known concept first described in 2000 (Aizawa-Abe et al., 2000). Leptin levels are correlated to fat mass and thus signals energy balance (Elias and Purohit, 2013). Obese individuals are almost always hyperleptinemic while having a normal resting energy expenditure (REE), whereas normal weight individuals maintain a normal REE on lower leptin levels (Lustig et al., 2004). Reduced leptin levels signal energy deficiency, which in turn induces counter-regulatory responses, of which reduced REE is one (Elias and Purohit, 2013). Thus, the effect of leptin on REE is shown to be feasible as an indirect marker of leptin sensitivity, by using the REE to leptin (REE:leptin) ratio (Lustig et al., 2004).

Leptin and adiponectin changes are seen after a fatty meal in normal weight individuals, but not in obese individuals (Larsen et al., 2018). Furthermore, the leptin to adiponectin (L:A) ratio has been proven to correlate well with other aspects of metabolic dysregulation and risk of chronic metabolic disease (Li et al., 2017; Kang et al., 2017; Frithioff-Bøjsøe et al., 2020). Individuals with obesity and elevated L:A ratio tend to have a delayed TG clearance, as well as both insulin and leptin resistance, making the L:A ratio a useful surrogate marker for the metabolic syndrome (Larsen et al., 2018).

A modest weight loss of ≥5 % improves metabolic disturbances and clinical features of the metabolic syndrome and complications of T2DM, with the improvement of insulin sensitivity being a crucial element (Magkos et al., 2016; Clamp et al., 2017). Furthermore, because of compensatory metabolic mechanisms during weight loss, a modest weight loss might be easier to maintain in the long run than a > 10 % weight loss (Nymo et al., 2018). Improvement of central leptin satiety signalling, as expressed in feeding behaviour, with weight loss and reduced leptin levels have previously been demonstrated (Andreoli et al., 2019). However, to our knowledge, documentation of leptin resensitization has only been described in experimental animal weight loss models, not in humans (Andreoli et al., 2019).

Thus, this study aimed to investigate if a modest weight loss of ≥5 % is sufficient for substantial improvement and possibly normalization of the L:A ratio, as identified in our normal weight controls (Larsen et al., 2018), and if a corresponding improvement of other biomarkers of subclinical metabolic dysregulation occurs (Larsen et al., 2018; Ryan and Yockey, 2017).

2. Materials and methods

We recruited participants from the Centre for Obesity, Department of Gastroenterology and Nutrition, from the obesity rehabilitation program at Stamina Health Tromsø (later renamed Avonova) and by posters placed at the Department of Clinical Nutrition and Department of Endocrinology at the University Hospital of North Norway (UNN). Eligible patients were provided with oral and written information and signed a written consent to participate.

Inclusion of participants is shown in Fig. 1.

Inclusion criteria for the study population were a baseline body mass index (BMI) ≥30 kg/m^2 and age ≥ 18 years. Exclusion criteria were smoking, pregnancy, severe mental illness, previous heart disease, medically treated diabetes mellitus and kidney failure. We excluded patients who, for any reason, dropped out of the weight loss program. Height, body weight, blood pressure and pulse were measured. Total, abdominal and gynoid fat percentage, total fat mass (kg) and total muscle mass (kg) were obtained from body composition measured at baseline and follow-up after weight loss treatment, using Dual-Energy X-ray Absorptiometry (DEXA; Lunar Prodigy Advance, GE Health Care, USA).

2.1. Normal weight control group

For our control group we recruited 17 healthy, normal weight participants from the general population. Inclusion criteria were BMI in normal range, between 18 and 40 years of age, normotensive, normoglycemic, normolipemic and no history of diabetes. Exclusion criteria were otherwise the same as for our obese participants. The control participants underwent the same measurements and tests as our obese participants. Their results were used to create 95 % confidence intervals (CI) for normal values in our metabolic parameters.

2.2. Weight loss intervention

The weight loss goal for the participants with obesity was a minimum of ≥5 % of the baseline body weight, as this amount of weight loss is accepted as clinically meaningful (Ryan and Yockey, 2017). The post-weight loss tests were performed within two years after the weight loss intervention. A shortened follow-up period was selected for participants who lost weight quickly, in order to avoid loss to follow-up due to weight regain.

Weight loss intervention was performed either individually or in treatment groups in the programs from where participants were recruited. The intervention was based on Norwegian national guidelines for diet and exercise (Nordic Council of Ministers, 2014; Norwegian Directorate of Health, 2015). Since the study aimed to investigate the effects of weight loss *per se*, the instructions for weight loss methods were kept liberal and not formally recorded. Participants were free to adjust their diet and exercise according to their own preferences, within national guidelines. To avoid bias on outcome variables from differences in diet and exercise we included a three-day period with no strenuous physical exercise prior to visits and also asked participants to abstain from heavy meals and alcohol the day before visits.

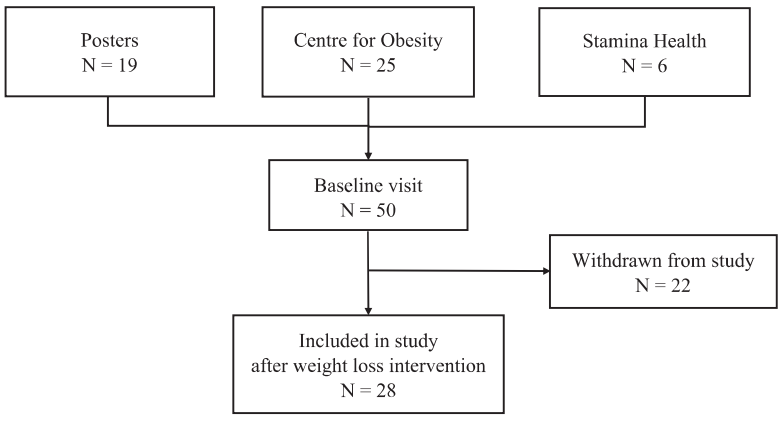

Fig. 1. Flowchart of included participants from posters at UNN, obesity out-patient clinic at UNN and Stamina obesity rehabilitation program, respectively.

All 50 participants who underwent baseline tests were offered to undergo the second round of tests regardless of the amount of weight loss during follow-up. Twenty two out of 37 participants in the non-weight loss group declined the offer and was hence lost to follow-up, while 15 accepted. All 13 participants in the weight loss group accepted the offer.

2.3. Insulin sensitivity

We performed a 2 hour (h) Oral Glucose Tolerance Test (OGTT) (Larsen et al., 2018). Participants had their regular diet and abstained from vigorous exercise three days before the test and showed up at 08:00 am after 12 h of overnight fasting. The test was conducted by oral intake of 75 g glucose dissolved in water (Matsuda and DeFronzo, 1999). We collected blood samples in both the fasting state and 30, 60, 90 and 120 min after glucose intake, in which serum (s-) glucose was measured by UV analysis (Cobas Integra Systems, Roche Diagnostics, Indianapolis, IN, USA) and s-insulin was measured using ELISA kits (DRG Insulin Elisa kit, DRG Instruments GmbH, Germany) (Larsen et al., 2015). We determined insulin sensitivity by calculation of the HOMA-IR (Matthews et al., 1985), as it has previously proven to correspond well to the whole body insulin sensitivity index (WBISI) (Matsuda and DeFronzo, 1999; Isaksen et al., 2016).

2.4. S-leptin and adiponectin measurements

Both fasting s-leptin and fasting free s-adiponectin were analysed from frozen serum drawn at all sample times, both during Oral Fat Tolerance Test (OFTT) and OGTT, using ELISA kits (DRG Diagnostics, Marburg, Germany) for leptin (sandwich ref. EIA-2395) and adiponectin (human, ref. EIA-4574), respectively. From these measurements, the L:A ratio was calculated as follows:

$$\text{L : A ratio} = \frac{\text{serum leptin}}{\text{serum adiponectin}}$$

As the intra individual variation was minimal between OFTT and OGTT measurements, OFTT values were selected for the statistical analyses.

2.5. Leptin sensitivity

Leptin sensitivity was calculated as the ratio of Resting Energy Expenditure (REE) to fasting s-leptin (Lustig et al., 2004; Bi et al., 2018). We performed REE measurements by a canopy test with an indirect calorimetry device from Medical Graphics CPX metabolic cart (St Paul, MN, USA).

The calorimetry was performed for 30 min in a supine position and in a resting and fasting state. REE was derived from the respiratory exchange ratio and the respiratory quotient (Larsen et al., 2018). After the completion of REE measurement, the OGTT was performed (Lustig et al., 2004).

2.6. Postprandial triglyceride clearance

To measure postprandial TGR and TG clearance, we performed an OFTT on a separate day from the OGTT (Cohen, 1989; Lekhal et al., 2008). Preparations for the OFTT were the same as for the OGTT. Fasting blood samples were drawn, and a meal of sour cream porridge (Fjordland sour cream porridge and full-fat cream in 1:1 ratio) was served. The meal contained 70 % calories of fat (66 % saturated fat, 32 % monounsaturated, 2 % polyunsaturated fat). The portions were adjusted to contain 1 g fat/kg body weight (Lekhal et al., 2008). The participants ingested the meal within 30 min, and blood samples were drawn from the antecubital vein in a seated position at baseline and 2, 4, 6 and 8 h postprandially.

The Department for Clinical Biochemistry at UNN analysed fasting serum lipids using a Hitachi 737 automatic analyser (Boehringer Mannheim GmbH, Mannheim, Germany) (Larsen et al., 2015).

The TGR was defined as the average of the two highest postprandial TG concentrations, minus the baseline concentration (Larsen et al., 2015; Lekhal et al., 2008).

The formula for calculating TG clearances (Lekhal et al., 2008) at time X was as follows:

$$\text{Clearance Xh} = 100 \times \left(1 - \frac{TG_X - TG_{0h}}{TG_{max} - TG_{0h}}\right)$$

2.7. Cut-off values for metabolic parameters

Normalization of metabolic parameters were defined as reaching the 95 % CI of our healthy, normal weight control group. The cut-off values were as follows: TG clearance at 6 h $\geq$ 88 %, TGR $\leq$ 0.67, HOMA-IR $\leq$ 1.83, WBISI $\geq$ 131.4, L:A ratio $\leq$ 1.19 and REE:leptin ratio $\geq$ 114.5 (Larsen et al., 2018).

2.8. Statistics

For statistical calculations, we used IBM SPSS Statistics 25 for Windows (SPSS Inc., Chicago, IL, USA). For calculating TG clearance and postprandial TGR, we used Microsoft Excel (Microsoft corp., Redmond, WA, USA). Plots were generated in GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

We used parametric tests on raw or transformed variables that resembled a normal distribution visually or by skewness/kurtosis for baseline analyses. Otherwise, Mann-Whitney non-parametric tests were performed for baseline analyses. To compare pre and post weight loss data we performed Related Samples Wilcoxon Rank Test. Due to small sample size complex analyses, such as ANOVA, were not possible to perform. Statistics for normal weight control group are previously described (Larsen et al., 2018).

3. Results

3.1. Baseline characteristics

We included 28 Caucasian participants in this study with a weight loss of 0–30 %. Seven participants (25 %) were male and 21 (75 %) were female. Among these, 15 participants (54 %) lost < 5 % body weight whereas 13 participants (46 %) lost $\geq$ 5 % body weight, including 5 participants (18 %) who lost $\geq$ 10 %. Median weight loss in the weight loss group was 10.0 kg.

Clinical, anthropometric, and metabolic characteristics are shown in Table 1. There were no significant differences in baseline characteristics between the weight loss and non-weight loss group, with the exception of REE ($p = 0.006$), TG ($p = 0.011$) and HDL-cholesterol ($p = 0.004$) levels. For leptin and adiponectin measurements, only data from OFTT samples are shown.

Four participants (three in the weight loss group, one in the non-weight loss group) used antihypertensive medication at baseline, two used thyroid replacement drugs (one in each group) and three used lipid lowering drugs (three in the weight loss and one in the non-weight loss group). Two participants in the weight loss group had hypertension at baseline. All female participants except one had LDL-cholesterol levels < 4.3 mmol/L, the upper limit of normal for females < 50 years. All male participants except one had LDL-cholesterol levels < 4.7 mmol/L, the upper limit of normal for males < 50 years.

Our normal weight controls had a median fasting s-leptin of 8.5 ng/mL and fasting free s-adiponectin of 11.8 μg/mL.

3.2. L:A ratio

There were significant improvements between pre- and post-intervention visits for fasting s-leptin and L:A ratio for the weight loss group, but not for the non- weight loss group (Table 2). Participants with weight loss had a 45.7 % ($p = 0.002$) improvement in L:A ratio (Table 2).

Fig. 2 shows the case-by-case change in central variables before and after weight loss intervention. Most notably is the significant improvement

Table 1
Baseline characteristics of the 28 overweight participants and 17 normal weight controls.

	Normal weight controls, $n = 17$	Weight loss ≥ 5 %, $n = 13$	Weight loss < 5 %, $n = 18$	Sig. (p)
Sex (M/F)	2/15	2/11	5/10	0.282
Age	31.0 (13)	39.8 (13)	36.0 (19)	0.254[M]
BMI (kg/m^2)	21.3 (2.2)	33.6 (11.5)	39.8 (7.5)	0.217[M]
Total body fat (%)	26.6 (5.9)	50.5 (9.5)	49.1 (9.1)	0.142[M]
Abdominal fat (%)	27.5 (6.4)	58.2 (7.9)	56.7 (3.9)	0.895[G]
Gynoid fat (%)	36.3 (5.8)	54.5 (9.1)	53.6 (9.8)	0.118[M]
REE (kcal/day)	1356 (185)	1604 (441)	1989 (465)	0.006[M]
Systolic blood pressure (mmHg)	105 (15)	124 (22)	128 (15)	0.525[M]
Diastolic blood pressure (mmHg)	65 (10)	80 (17)	75 (9)	0.208[G]
Fasting glucose (mmol/L)	4.4 (0.7)	5.4 (1.3)	5.1 (1.0)	0.892[M]
Fasting insulin (μmol/L)	5.5 (2.9)	10.8 (6.2)	13.2 (6.1)	0.142[M]
HOMA-IR	1.1 (0.7)	2.7 (1.8)	3.1 (1.9)	0.170[M]
WBISI	147.8 (101.5)	55.1 (45.6)	55.2 (31.2)	0.467[M]
Fasting TG (mmol/L)	1.0 (0.4)	1.2 (0.7)	1.5 (0.6)	0.011
TGR (mmol/L)	0.3 (0.3)	0.6 (0.4)	1.0 (0.9)	0.586[M]
TG clearance 6 h (%)	115.4 (62.3)	65.0 (96)	61.1 (46)	0.413[M]
Fasting leptin (ng/mL)	8.5 (7.4)	32.5 (37.0)	41.7 (21.8)	0.928[M]
Fasting adiponectin (μg/mL)	11.8 (7.1)	9.0 (3.7)	6.8 (3.7)	0.525[M]
L:A ratio	0.6 (0,9)	3.8 (2.5)	6.1 (35.2)	0.683[M]
REE:Leptin ratio	142.5 (134.0)	54.1 (51.6)	47.8 (51.6)	0.683[M]
Total cholesterol (mmol/L)	4.2 (0.9)	4.3 (1.2)	4.7 (1.1)	0.153[G]
LDL cholesterol (mmol/L)	2.6 (1.3)	2.7 (1.4)	2.9 (0.9)	0.107
HDL cholesterol (mmol/L)	1.6 (0.5)	1.3 (0.2)	1.1 (0.2)	0.004
HDL:LDL ratio	0.57 (0.49)	0.52 (0.47)	0.35 (0.17)	0.009[M]

Baseline anthropometric and metabolic characteristics for all participants. Significance tested between weight loss groups by *t*-test or Mann-Whitney non-parametric test ([M]). Parameters without normal variation distribution were transformed to geometric mean ([G]) if possible before the t-test was performed. Values shown as median (interquartile range). Abbreviations: BMI Body Mass Index. HDL High Density Lipoprotein. HOMA-IR Homeostasis Model Assessment of Insulin Resistance. L:A ratio Leptin:Adiponectin ratio. LDL Low Density Lipoprotein. REE Resting Energy Expenditure. TG Triglyceride. TGR Triglyceride Response. WBISI Whole Body Insulin Sensitivity Index.

in L:A ratio in participants in the weight loss group. The improvement was even greater in the subgroup of ≥10 % weight loss, but not reaching the level of normality (cut-off value ≥ 1.88, $p = 0.030$, Fig. 2). In the non-weight loss group no significant changes were observed ($p = 0.020$).

3.3. Leptin sensitivity

The REE:leptin ratio improved with 80.1 % ($p = 0.005$) in the weight loss group (Table 2) but not in the non-weight loss group. Furthermore, there was a tendency of greater improvements in leptin sensitivity in the subgroup of ≥10 % weight loss, compared to 5–10 % weight loss. Four of the 13 (31 %) participants in the subgroup achieved normalized leptin sensitivity after weight loss (REE:leptin ratio ≥ 114.5, Fig. 2). No significant changes were observed in the non-weight loss group ($p = 0.013$).

3.4. Insulin sensitivity

Insulin sensitivity measured by HOMA-IR improved with 23.1 % ($p = 0.011$) in the weight loss group, where nine out of the 13 (69 %)

Table 2
Post-intervention characteristics for the 28 participants.

	Weight loss ≥ 5 %			Weight loss < 5 %		
	Value	Per cent change	Sig. (p)[W]	Value	Per cent change	Sig. (p)[W]
Weight (kg)	81.1 (24.1)	−10.0 (7.1)	0.001	119.9 (18.8)	−1.1 (4.7)	0.691
BMI (kg/m^2)	30.9 (8.9)	−8.2 (6.2)	0.001	39.5 (7.1)	−1.9 (4.3)	0.379
Abdominal fat (%)	51.1 (7.6)	−7.4 (7.6)	0.001	55.6 (6.5)	0.0 (9.5)	0.917
Gynoid fat (%)	52.0 (11.3)	−5.4 (8.4)	0.002	51.2 (13.5)	−0.6 (6.5)	0.778
HOMA-IR	1.4 (1.8)	−23.1 (51.6)	0.011	2.6 (1.3)	−6.8 (52.6)	0.807
WBISI	92.1 (163.2)	49.9 (133.8)	0.008	67.2 (30.0)	13.1 (50.4)	0.477
L:A ratio	2.7 (2.2)	−45.7 (29.9)	0.002	5.0 (4.7)	−6.6 (26.8)	0.196
REE:Leptin ratio	78.5 (72.9)	80.1 (92.6)	0.005	71.3 (68.0)	0.9 (45.8)	0.480
REE (kcal/day)	1459 (427)	−8.6 (15.6)	0.196	2095 (360)	0.0 (10.4)	0.600
Fasting TG (mmol/L)	1.1 (0.6)	−9.2 (66.0)	0.649	1.3 (1.2)	−13.3 (49.7)	0.507
TGR (mmol/L)	0.51 (0.59)	−33.6 (29.6)	0.327	0.67 (1.03)	−24.4 (148.8)	0.754
TG clearance 6 h (%)	75 (62.1)	6.6 (116.6)	0.807	66.0 (42.1)	−7.1 (453.3)	0.778
Systolic blood pressure (mmHg)	121 (18)	0.0 (8.4)	0.074	120 (15)	−6.3 (15.9)	0.020
Diastolic blood pressure (mmHg)	76 (20)	0.6 (12.9)	0.248	69 (16)	1.5 (26.4)	0.409
Fasting leptin (ng/mL)	20.5 (14.3)	−50.0 (36.3)	0.004	29.8 (27.8)	−3.4 (15.2)	0.814
Fasting adiponectin (μg/mL)	8.2 (4.6)	−4.1 (42.5)	0.576	7.1 (2.4)	11.0 (19.5)	0.433
Fasting glucose (mmol/L)	5.0 (1.1)	−5.3 (11.8)	0.090	5.1 (1.6)	−2.1 (12.3)	0.339
Fasting insulin (μmol/L)	6.3 (6.6)	−16.6 (45.2)	0.006	8.3 (8.4)	−4.6 (56.6)	0.470
LDL cholesterol (mmol/L)	2.8 (1.0)	−11.1 (34.9)	0.438	4.8 (1.0)	−2.9 (32.3)	0.972
HDL cholesterol (mmol/L)	1.4 (0.3)	−0.2 (19.3)	0.872	3.0 (0.8)	10.0 (21.2)	0.715

Post-intervention characteristics and per cent change from baseline characteristics for weight loss (≥5 %) and non-weight loss (<5 %) groups, respectively. Significance tested between baseline and post-treatment values by Related Samples Wilcoxon Rank Test ([W]). Values shown as median (interquartile range). Abbreviations: BMI Body Mass Index. HDL High Density Lipoprotein. HOMA-IR Homeostasis Model Assessment of Insulin Resistance. L:A ratio Leptin:Adiponectin ratio. LDL Low Density Lipoprotein. REE Resting Energy Expenditure. TG Triglyceride. TGR Triglyceride Response. WBISI Whole Body Insulin Sensitivity Index.

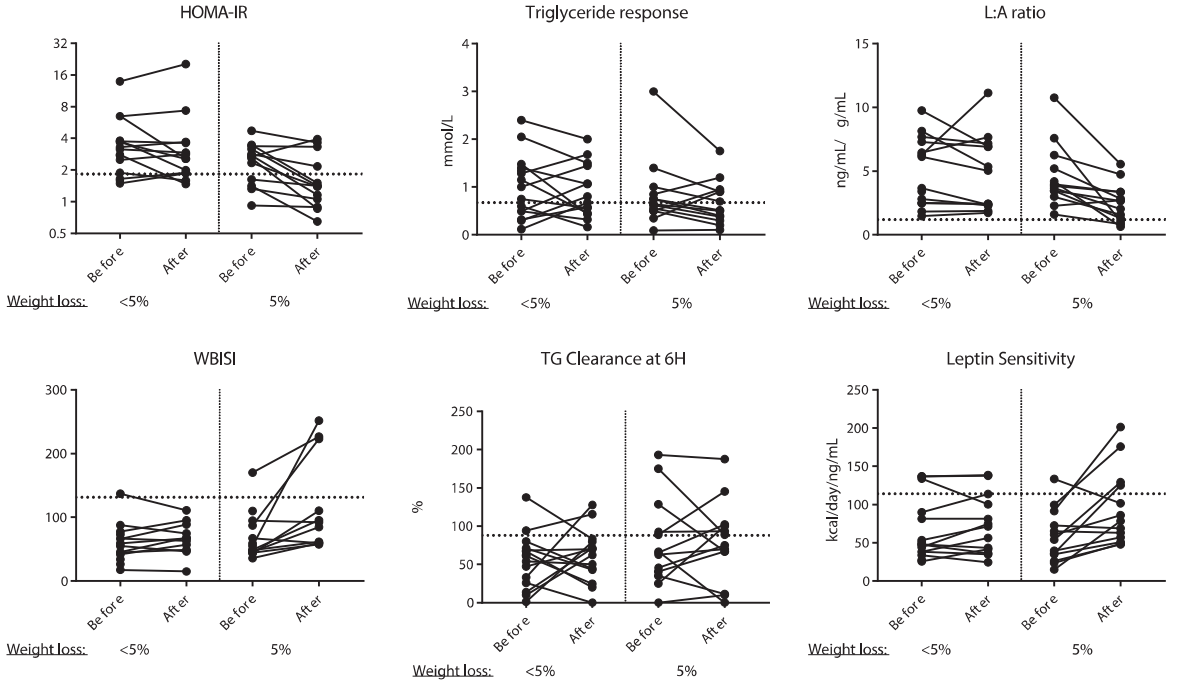

Fig. 2. Difference between baseline and post intervention values of A) L:A ratio, B) Indirect leptin sensitivity (REE:Leptin ratio) C) HOMA-IR, D) TG clearance at 6 h and E) TGR before and after <5 % and ≥ 5 % weight loss in individual participants. Horizontal lines represent the upper or lower limit of 95 % CI in healthy, normal weight controls.

participants had normal HOMA-IR values after weight loss (HOMA-IR ≤ 1.83) Of these, five participants improved from insulin resistance at baseline.

There also was a significant improvement of 49.9 % in WBISI in the weight loss group ($p = 0.008$). Two participants in this group normalized their WBISI, while one participant maintained a normal baseline WBISI. There also was a significant difference in WBISI delta values between weight loss and non-weight loss group (p = 0.013, Mann-Whitney *U* test).

No significant changes were observed in the non-weight loss group for neither HOMA-IR nor WBISI (Table 2, Fig. 2).

3.5. Improvement of postprandial triglycerides

No significant differences in postprandial TG clearance at 6 h were seen in any of the groups (6.6 %, $p = 0.807$). (Table 2, Fig. 2).

4. Discussion

In this study we have examined the L:A ratio, indirect leptin sensitivity, insulin sensitivity, and postprandial TG metabolism in obese participants before and after a modest weight loss of ≥5 %. We found significant improvements in L:A ratio (−45.7 %), leptin sensitivity (−80.1 %) and insulin sensitivity (−23.1 %) in participants who achieved weight loss, compared to participants with no weight loss. Furthermore, our study shows that as little as ≥5 % weight loss improves adipokines, but not TG metabolism.

To our knowledge, few studies have been performed to assess L:A ratio, insulin sensitivity and REE:leptin ratio in a population of otherwise largely healthy adult individuals undergoing long term lifestyle intervention to achieve weight loss. However, Ho et al. found improved insulin sensitivity and L:A ratio after a 12 month lifestyle intervention for >10 % weight loss in obese healthy adults (Ho et al., 2015) and Miller et al. found significant improved leptin and adiponectin after a one-year lifestyle intervention for >5 % weight loss in obese healthy individuals (Miller et al., 2014). Both studies also found improvements in inflammation markers. Furthermore, our results are in line with studies reporting similar improvements in leptin and adiponectin levels with weight loss in more selective groups of children/adolescents (Alaby Martins Ferreira et al., 2020), type 1 diabetes patients (Musil et al., 2015), cancer patients (Befort et al., 2020), elderly (Ilich et al., 2022; Miller et al., 2012) and patients undergoing bariatric surgery (Farias et al., 2020), weight loss >20 % (Hausmann et al., 2019), or short-term, intensive weight loss programs (Moro et al., 2016; Kelly et al., 2014). Our findings are also in line with the findings of improved adiponectin levels and cardiometabolic risk in the Look AHEAD study on T2DM patients (Belalcazar et al., 2015; Group LAR and Gregg, 2016).

We have previously reported that the L:A ratio is a feasible surrogate biomarker for early detection of metabolic disturbances in obesity (Kang et al., 2017). Moreover, we have previously reported a potential postprandial regulatory role of adiponectin and leptin which is impaired in obesity (Larsen et al., 2018). The L:A ratio has been demonstrated to improve after a weight loss of 5–10 % by Ferreira et al. and Talaei et al. among others (Alaby Martins Ferreira et al., 2020; Talaei et al., 2017).

Our present findings demonstrate an improvement in L:A ratio of −45.7 % in a small group of 13 participants with weight loss. Such a large improvement in this small dataset has major clinical significance, and supports a realistic weight loss goal that is potentially easier to maintain long term (Nymo et al., 2018). The improvement of the L:A ratio is important as it is known to correlate to low-grade inflammation and thus risk of CVD (Alaby Martins Ferreira et al., 2020; Frühbeck et al., 2018; Frühbeck et al., 2019).

Frühbeck et al. proposed adiponectin:leptin ratio cardiovascular risk category limits as follows: normal risk ≥ 1, moderate risk 0.5–1 and high risk < 0.5 (Frühbeck et al., 2019). Inversely, this translates to our L:A ratio category limits of ≤1, 1–2 and >2, respectively. According to these limits of risk, three participants with weight loss crossed from high to moderate cardiovascular risk after weight loss while one participant crossed

from moderate to low risk and one from high to low risk. In total 38 % of the participants reduced their risk category.

A study by Bi et al. suggests that leptin contributes significantly more to the variance in REE than what is explained by fat mass (Bi et al., 2018). Furthermore, Rosenbaum et al. demonstrated that leptin administration reversed the decrease in energy expenditure after weight loss (Rosenbaum et al., 2002). We found that REE:leptin ratio improved with a median of 80.1 %. In addition, approximately one third (31 %) of the participants in the weight loss group had their REE:leptin ratio normalized after weight loss (Larsen et al., 2018). Although resensitization of leptin on satiety signalling is not possible to measure clinically in humans, one can speculate whether this demonstration of indirect resensitization of leptin suggests a normalization of central leptin sensitivity as well.

Insulin sensitivity, as measured by the HOMA-IR or WBISI, improved by 23.1 % and 49.9 %, respectively, after weight loss. Moreover, five out of the 13 (39 %) participants obtained normalized insulin sensitivity as defined by HOMA-IR, while two out of 13 (13 %) normalized it as defined by WBISI (Larsen et al., 2018; Bi et al., 2018). This is in agreement with most other reports (Clamp et al., 2017). The improved insulin sensitivity observed together with an improved L:A ratio is also in agreement with previous reports, describing a correlation between the two variables (Frithioff-Bøjsøe et al., 2020). Our findings of a substantial percentage of normalized insulin sensitivity further support the conclusion that a 5 % weight loss is clinically meaningful for reducing obesity complications.

There was no significant improvement of postprandial TG clearance after ≥5 % weight loss. This could be explained by the small number of participants that lost ≥ 10 % body weight. Magkos et al. reported that improvements of TG and FFA were observed first after 11 % and 16 % weight loss, respectively (Magkos et al., 2016). Therefore, normalization of postprandial TG clearance will most likely be observed only after a substantial weight loss in obese individuals.

The strengths of the present study are the use of several different methods, particularly adipokine levels, reflecting the pathophysiological mechanisms behind metabolic disorders in obesity to make comprehensive explanations of the results obtained by a modest weight loss. In addition to this, the generalizability of the weight loss method used in the study is high, as we did not assign one specific diet or exercise plan to our participants. We also included participants from different sources with broad inclusion criteria. The study was performed in a clinical setting, which also makes the generalizability high.

There are several weaknesses of this study. The main weakness of our study design is the high dropout rate, combined with a small sample size that renders us unable to perform parametric tests and to perform more complex analyses (ANOVA, *etc.*). The final sample size was way below our estimated sample size of 50 participants, and was explained by a large dropout rate. Due to the small sample size, we mainly used repeated measures analyses, thus reducing the variability in our samples, making our results more reliable under the given circumstances.

There is also a significant imbalance between men and women included in our study. This could affect our results, as body composition and hence leptin concentrations differ between the sexes (Ruhl et al., 2007). However, the gender difference in our study is in accordance with other studies of obesity, and improvements in adipokines with weight loss seem similar in studies on males and predominantly female participants (Miller et al., 2014; Borel et al., 2017). Furthermore, our study is in line with literature on differences in the population of patients seeking help for health problems in general (Galdas et al., 2005).

There could be possible confounding factors related to the geographical location of our study, above the Arctic Circle. These would include low levels of vitamin D, which is shown to be related to obesity, hyperleptinemia and insulin resistance (Madhu et al., 2022) and circadian rhythm disruptions due to differences in daylight exposure throughout the year (Noh, 2018). One could also mention low outside temperatures, although the evidence for its' relation to obesity is contradictory, and so will not be discussed further (Speakman and Heidari-Bakavoli, 2016).

We did not formally record these factors in our study, as they are beyond the scope of this paper. There is however a general advice to the Norwegian population to take vitamin D supplements during the winter months (Health NDo, 2014). Individuals who comply with this advice usually do so every year. Our participants were not prescribed any vitamin D supplements as part of their treatment at the obesity out-patient clinic beyond this. Furthermore, people who struggle to sleep during the summer months with midnight sun usually make sure to have dark blinders on their windows to reduce light exposure while they sleep.

As our participants served as their own controls in this study, we will argue that both vitamin D levels and nocturnal light exposure are sufficiently controlled for and would not significantly hinder our results' generalizability.

4.1. Conclusion

A modest weight loss of ≥5 % improves sensitive metabolic surrogate markers like L:A ratio, leptin sensitivity (REE:leptin ratio) and insulin sensitivity. However, markers of postprandial TG clearance did not improve at the present level of weight loss. Our results support that a realistic and achievable weight loss goal of ≥5 % for obese individuals does reduce the risk of metabolic complications. The findings in this study need to be confirmed and further evaluated in a larger study population.

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Ethics

All participants signed a written consent form. The Regional Ethics Committee of North Norway (2011/1677/REK Nord) approved the study. Consent for publication of results was not applicable, as no individual participant is identifiable in this paper.

Credit authorship contribution statement

Authors VTI and MAL conducted participant inclusion and data collection. MAL performed weight loss follow-up for part of the study group. VTI and RG performed statistical analyses. JF was the principal investigator.

All authors participated in the planning of the study, the interpretation of results and the process of writing and editing the manuscript.

Data availability

The dataset supporting the conclusions of this article is available in the UiT Open Research Data repository at https://doi.org/10.18710/KRYLXN

Declaration of competing interest

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