# Effects of diet intervention on body composition and ectopic fat accumulation in obese postmenopausal women

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***I rörelse***

*Den mätta dagen, den är aldrig störst.*
*Den bästa dagen är en dag av törst.*

*Nog finns det mål och mening i vår färd -*
*men det är vägen, som är mödan värd.*

*Det bästa målet är en nattlång rast,*
*där elden tänds och brödet bryts i hast.*

*På ställen, där man sover blott en gång,*
*blir sömnen trygg och drömmen full av sång.*

*Bryt upp, bryt upp! Den nya dagen gryr.*
*Oändligt är vårt stora äventyr.*

*Karin Boye*

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# Abstract

**Background** Obesity is increasing worldwide and is a major contributor to morbidity and mortality. Notably, abdominal (central) obesity carries a high risk of obesity-related diseases, while peripheral fat accumulation can act in a protective manner. A redistribution of fat from peripheral to central depots is seen after the menopause and is associated with an increasing prevalence of diabetes and cardiovascular disease. A key mediator may be ectopic fat accumulation in the liver. Our hypothesis was that a Palaeolithic-type diet (PD) consumed *ad libitum* improves body composition and metabolic risk markers, including liver fat and insulin sensitivity, in obese postmenopausal women.

**Methods** In study I the study subjects (n=10) used a PD during 5 weeks. In study II and III (n=70) the effect of a Palaeolithic-type diet (PD) was compared to a diet according to the Nordic Nutrition Recommendations diet (NNR) during a 2-year randomized clinical trial (RCT). Food records and nitrogen excretion in urine validated food intake. Anthropometric measurements were performed in a standardized manner. Body composition was calculated using Dual Energy X-ray Absorptiometry (DXA). Total energy expenditure was calculated by accelerometry (Actiheart®) in combination with indirect calorimetry. Liver and muscle fat content was estimated by magnet resonance spectroscopy ($^{1}$H-MRS). Insulin sensitivity was measured either with hyperinsulinemic euglycemic clamps (paper I) or oral glucose tolerance tests (OGTTs) (paper III).

**Results** In study I a significant weight loss, linked to improved lipid and blood pressure levels, was associated with a 49% decrease in liver fat. Concomitantly, hepatic insulin sensitivity improved, while peripheral insulin sensitivity (and muscle fat) was unaltered. In study II/III both groups had a significant and sustained weight loss after 2 years. The PD was more effective than the NNR diet regarding loss of weight and fat mass after 6 months, but not after 24 months. Serum triglyceride levels were significantly lower at 24 months in the PD group. Liver fat decreased throughout the study in both groups. Hepatic insulin sensitivity improved during the first 6 months of the study, while peripheral insulin sensitivity did not change. Hepatic insulin sensitivity was associated with liver fat at baseline, but not during the diet intervention. Energy expenditure did not change in any of the study groups.

**Conclusion** *Ad libitum* diets can have sustained beneficial effects on weight and body composition in obese postmenopausal women, a PD being more

effective on short-term than a diet according to the NNR. This is associated with a reduction in liver fat that may reduce the risk of future diabetes and cardiovascular disease. Further studies are needed in order to explore the association between liver fat and metabolic dysfunction, including insulin sensitivity.

# Original papers

The thesis is based on the following papers:

I. Ryberg M, Sandberg S, **Mellberg C**, Stegle O, Lindahl B, Larsson C, Hauksson J, Olsson T. A Palaeolithic-type diet causes strong tissue-specific effects on ectopic fat deposition in obese postmenopausal women. J Intern Med 2013; 274: 67-76.

II. **Mellberg C**, Sandberg S, Ryberg M, Brage S, Larsson C, Olsson T, Lindahl B. Long-term effects of a Palaeolithic-type diet in obese postmenopausal women: a 2-year randomized trial. Eur J Clin Nutr 2014; 68: 350-7.

III. **Mellberg C**, Otten J, Ryberg M, Sandberg S, Hauksson J, Lindahl B, Larsson C, Olsson T. Decreased liver fat during a two-year diet intervention was not associated with improvement in hepatic insulin sensitivity. Manuscript.

# Abbreviations

| | |
|---|---|
| BMI | body mass index |
| cAMP | cyclic adenosine monophosphate |
| CT | computer tomography |
| CVD | cardiovascular disease |
| DAG | diacylglycerol |
| DGAT | diacylglycerol acyltransferase |
| DXA | dual-energy X-ray absorptiometry |
| E% | energy % |
| FFA | free fatty acids |
| GEE | generalized estimating equations |
| GLUT-4 | glucose transporter type 4 |
| $^{1}$H-MRS | proton magnetic resonance spectroscopy |
| HDL | high-density lipoprotein |
| HOMA | homeostasis model assessment |
| hsCRP | high sensitive C-reactive protein |
| IL-6 | interleukin 6 |
| IMCL | intramyocellular lipid |
| LPL | lipoprotein lipase |
| MCP-1 | monocyte chemotactic protein-1 |
| MRI | magnet resonance imaging |
| MUFA | mono-unsaturated fatty acids |
| NAFLD | non-alcoholic fatty liver disease |
| NNR | Nordic Nutrition Recommendations |
| nPKC | novel protein kinase C |
| NU | nitrogen excretion in urine |
| OGTT | oral glucose tolerance test |
| PABA | para-aminobenzoic acid |
| PAEE | physical activity energy expenditure |
| PAI-1 | plasminogen activator inhibitor-1 |
| PD | Palaeolithic-type diet |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PUFA | polyunsaturated fatty acids |
| RMR | resting metabolic rate |
| SAT | subcutaneous adipose tissue |
| SFA | saturated fatty acids |
| T2DM | type 2 diabetes mellitus |
| TEE | total energy expenditure |
| TF | trans-unsaturated fatty acids |
| TG | triglycerides |
| TNFalpha | tumour necrosis factor alpha |
| VAT | visceral adipose tissue |
| VLCD | very low calorie diet |
| VLDL | very low-density lipoprotein |
| WHO | World Health Organisation |

# Sammanfattning på svenska

Fetma är ett tilltagande hälsoproblem i hela världen och fetma-relaterad sjuklighet står för en betydande del av samhällets sjukvårdskostnader, förutom det lidande på individnivå som sjukdomen orsakar. En nyckel till sjuklighet vid fetma kan vara ansamling av fett utanför fettväven, s.k. ektopisk fettinlagring, i olika organ. Detta kan bidra till nedsatt insulinkänslighet med åtföljande höga insulinnivåer i blodet. Följderna kan bli utveckling av en rad olika sjukdomar, inklusive diabetes och hjärt-kärlsjukdom. Att öka förståelsen för fetmarelaterad sjuklighet samt att förebygga och behandla fetma och associerade sjukdomar är därför av stor betydelse.

Dietinterventioner med lågkalorikost har på kort sikt visat sig ha god effekt på viktnedgång samt på ektopisk fettinlagring, framför allt i levern. Dock behövs studier avseende olika dieters förmåga att på kort och lång sikt ge gynnsamma metabola effekter inklusive reduktion av ektopisk fettinlagring.

En paleolitisk diet bygger på studier av urbefolkningar där förekomsten av fetma, diabetes och hjärt-kärlsjukdom är mycket låg. Kosten består i huvudsak av magert kött, bär, frukt, grönsaker och nötter. Mängden mättade fettsyror i kosten är låg. Spannmålsprodukter och mjölkprodukter ingår inte i kosten. I våra studier har vi använt en paleolitisk kost med ett relativt högt proteininnehåll och ett något lägre kolhydratinnehåll. Även mängden enkel- och fleromättade fettsyror är ökad i denna kost, vilket i andra studier visats ha goda effekter på mättnadskänsla och metabol balans. Vi har delvis gjort jämförelser med en kost enligt de nordiska näringsrekommendationerna (NNR).

I en första studie användes en paleolitisk diet under fem veckor i en grupp överviktiga kvinnor som genomgått menopaus. Dieten reducerade energiintag, kroppsvikt, bukomfång och blodtryck samtidigt som en rad metabola parametrar förbättrades, inklusive blodfetter. Leverns fettinnehåll reducerades kraftfullt (49% minskning) medan fettinnehållet i muskulaturen inte förändrades. Parallellt med detta förbättrades leverns insulinkänslighet påtagligt, avspeglat i lägre nivåer av glukos och insulin i blodet. Muskulaturens insulinkänslighet påverkades inte av dietinterventionen.

En två-årsstudie med paleolitisk diet gav en kraftfull och bestående minskning av fettmassa, vikt och bukomfång. Effekten var mer uttalad i gruppen med paleolitkost under de första sex månaderna, jämfört med gruppen med kost enligt NNR. Vid uppföljning efter två år förelåg inga signifikanta skillnader mellan grupperna, förutom att gruppen med paleolitisk diet hade en bättre balans av blodfetter (lägre nivå av triglycerider

i blodet). I båda dietgrupperna sågs en signifikant sänkning av leverfett under studieperioden. Leverns insulinkänslighet förbättrades de första sex månaderna i båda studiegrupperna, mera uttalat i gruppen som intog en paleolitisk diet. Vid studiens slut hade insulinkänsligheten återgått till ursprungsnivån i båda grupperna.

En paleolitisk kost kan ha kraftfulla effekter på energiintag och kroppssammansättning liksom på fettinlagring i levern. Ytterligare studier av kombinationen av denna diet med fysisk aktivitet är av stort intresse, särskilt vid metabola störningar inklusive typ 2-diabetes.

x

# Introduction

## Obesity

Following a steady increase since 1980, the global prevalence of overweight and obesity in 2013 was estimated to be 37% among males and 38% among females [1]. The WHO [2] classifies obesity as being a disease in itself, as well as associated with major co-morbidity. While general obesity is a major health concern, abdominal obesity is of greater clinical relevance. In particular, central (abdominal) obesity is linked to increased risks of type 2 diabetes mellitus (T2DM), cardiovascular disease, cancer, and mental illness. Obesity and its comorbidities have major health consequences for the affected individuals, as well as a huge impact on national healthcare and the economy. Therefore, the prevention and treatment of obesity are crucial tasks within current medical research.

Obesity stems from multifactorial causes. Heredity is an important factor, with 40–70% of obesity suggested to be dependent on genetic factors [3]. Notably, epigenetics—i.e. changes in gene functions throughout growth in relation to foetal life, childhood, stress, socioeconomic factors, and perhaps infections—may also contribute to hereditary effects on the development of obesity. However, since genes do not change rapidly, the major causes of obesity development in our society relate to changes in lifestyle and environment. Consistently greater energy intake than energy expenditure can lead to adipose tissue accumulation. Adipose tissue is both a storage organ for energy and an active endocrine organ. As adipose tissue expands, it produces a wide range of bioactive substances (adipokines) that can influence multiple processes in different organs.

## Central obesity and ectopic lipid deposition

Disease risk is more strongly impacted by fat tissue distribution than by body mass index (BMI). Individuals with normal weight and BMI in the normal range (<25 kg/m$^2$), but with central obesity, have a clearly higher mortality risk compared to those with a more peripheral fat mass distribution [4]. It has been suggested that the increased risk for metabolic and cardiovascular complications linked to abdominal obesity is partly due to fat accumulation outside of fat cells [5]. The presence of fat within non-adipose tissues that do not normally contain a lot of fat (e.g. the abdomen, liver, pancreas, heart muscle, and skeletal muscle) is called ectopic fat deposition. Most cells contain lipid droplets that vary in size from small droplets in skeletal

muscles to very large droplets in adipocytes. These droplets were initially considered to be storages depots, but they are now recognized as highly dynamic organelles that are involved in various cellular processes [6].

Ectopic fat in the liver may play a central role in the adverse effects of central obesity [7]. Therefore, the interplay between adipose tissue and the liver is a focus of interest for research, including investigations of the possibility to intervene against ectopic fat accumulation and its consequences. Additionally, skeletal muscle plays a major role in the development of insulin resistance and T2DM in obesity. Notably, insulin-resistant obese subjects significantly differ from insulin-sensitive obese subjects with regard to lipid accumulation in muscles and the liver, without showing significantly different subcutaneous or visceral fat mass [8, 9].

## The interplay between adipose tissue and liver

Adipose tissue plays a major role in fat storage as triglycerides and fat release as free fatty acids (FFAs). Fatty acids continuously flow to and from the adipose tissue with minute-to-minute regulation. The fat stored in adipose tissue and other organs comes from two major sources: uptake of triglycerides (TG) from the plasma and *de novo* lipogenesis. Fatty acids are not water-soluble and are dependent on special transport systems.

Fat uptake into the liver (and muscle, as described below) leads to insulin resistance, regardless of the total fat mass and fat distribution. Overexpression of lipoprotein lipase (LPL) promotes lipid accumulation and thus insulin resistance [10]. Correspondingly, removal of LPL and other proteins involved in fat transport is protective against lipid accumulation and insulin resistance [10]. Choi et al. showed that increased energy expenditure also protects against lipid accumulation in mouse models that overexpress muscle-specific uncoupling protein 3 or acetyl CoA carboxylase 2 [11, 12]. Studies in humans and rodents with lipodystrophy/lipoatrophy further demonstrate that ectopic lipid accumulation can occur without fat mass expansion. Interestingly, transplantation of fat into peripheral depots reverses liver steatosis and insulin resistance in lipotropic mice [13].

Non-alcohol fatty liver disease (NAFLD)—i.e. fat accumulation of >5% within the liver—is associated with major health risks, including liver cirrhosis and liver cancer. Indeed, obesity is now the leading cause of end-stage liver disease in the USA [14], where obesity rates are high. Furthermore, NAFLD is linked to increased risks of T2DM and cardiovascular disease [14]. It is presently unclear how NAFLD and its associated diseases develop. One possible major source is FFA derived from ectopic fat in the abdomen that is released into the portal vein. High FFA concentrations force the liver to secrete more triglycerides (TG) in very low-

density lipoprotein (VLDL) particles, which is a major factor in atherosclerosis development. FFAs may also contribute to hepatic insulin resistance (discussed below).

Chronic low-grade inflammation in VAT, followed by increased lipolysis with a major FFA release into the portal vein, may be a contributing factor to NAFLD. Notably, obesity is associated with adipose tissue containing an increased number of macrophages that secrete inflammatory cytokines (e.g. TNF-alpha, IL-6, and MCP-1). Studies in rodents have clearly shown that adipose tissue expansion is accompanied by macrophage infiltration and a switch to a more proinflammatory state [15]. The heightened FFA influx to the liver may raise circulating glucose levels through increases in the hepatic gluconeogenesis rates and the conversion of glycerol to glucose [13]. The proinflammatory response may also per se contribute to T2DM development via impaired functions of pancreatic beta and alpha cells [13]. Interestingly, it has also recently been suggested that adipocyte inflammation is important for adipose tissue expansion, as an impaired proinflammatory response in fat reportedly led to liver steatosis and metabolic dysfunction in an experimental setting [16].

Ectopic lipid accumulation in the liver is closely associated with insulin resistance, possibly due to accumulation of “toxic” lipid intermediates, particularly ceramides and diacylglycerols (DAGs) [17]. Ceramides and DAGS can activate different kinases—such as novel protein kinase C (nPKC) epsilon—which have a negative feedback on insulin signalling [18]. This can contribute to the insulin resistance observed in patients with NAFLD [19]. Skeletal muscle is a second target organ linking ectopic lipid deposition with insulin resistance.

## Muscles

Skeletal muscle is the predominant site of postprandial insulin-mediated glucose uptake [20], and is capable of switching between use of glucose and lipids depending on availability and energy requirements [21]. Glucose uptake is mediated by insulin-sensitive glucose transporters, mainly glucose transporter type 4 (GLUT4). Low insulin concentrations are associated with low glucose uptake, while higher insulin concentrations activate more transporters, thus increasing glucose uptake from the blood. Glucose is then used for glycogen synthesis or for glycolysis within muscle. In muscles, glycogen synthase can be phosphorylated by protein kinase-A (PKA), which is not present in the liver. Via cAMP and PKA, adrenaline has the ability to turn off glycogen synthase in muscles and stimulate glycogen breakdown, for example, during exercise.

Defective insulin-stimulated glucose transport results in decreased muscle glycogen synthesis, followed by fat accumulation and lipid-induced insulin resistance. Intracellular lipid accumulation triggers protein kinase activation, which leads to muscle insulin resistance [13]. nPKCs have a strong affinity for DAGs. DAGs accumulation within skeletal muscle activates nPKCtheta, which is linked to decreased insulin signalling via translocation to the cell membrane [22]. On the other hand, greater diacylglycerol acyltransferase (DGAT) activity increases the conversion of DAGs to triglycerides, thus preventing a lipid-induced increase of muscle DAG content. This may at least partly explain the lower lipid-induced increase in DAGs among physically active subjects, and could contribute to the "athletes paradox" in which athletes show increased levels of intramyocellular fat without concomitant insulin resistance [23]. Notably, a single dose of exercise can induce changes similar to those observed in mice overexpressing DGAT. [22]. One contributing factor may be the compartmentalization of DAGs to lipid droplets rather than into cell cytosol or membrane, which prevents the inhibition of insulin signalling via plasma membrane binding [13].

Furthermore, mitochondrial fat oxidation in muscle cells is crucial for lipid accumulation and insulin resistance. Mitochondrial activity is reduced with increasing age, with corresponding increases in lipid accumulation and reductions in insulin sensitivity [24]. Studies have also reported that the offspring of individuals with T2DM show a 40% reduction in mitochondrial oxidative and phosphorylation activity. This suggests that improving muscle insulin sensitivity via increased mitochondrial function may be crucial for preventing T2DM associated with ectopic lipid deposition.

Finally, increased methylation of key genes regulating mitochondrial function and biogenesis in skeletal muscle may contribute to insulin resistance associated with obesity and T2DM, as well as in subjects with a family history of T2DM [25]. Importantly, in healthy volunteers, exercise results in a rapid but transient decrease in DNA promoter methylation of genes related to insulin signalling [26]. It remains to be studied whether similar effects can be achieved with exercise in humans with obesity and T2DM.

## Postmenopausal women

Altered body fat distribution with fat centralization after menopause may partly explain the increased risk of cardiometabolic diseases in postmenopausal women [27]. Studies using dual-energy X-ray absorptiometry (DXA), computed tomography (CT), and magnetic resonance imaging (MRI) have demonstrated an association between the menopause

transition and central fat accumulation, particularly intra-abdominal fat [28]. Lowered oestrogen levels during the menopausal transition may contribute to body fat centralization as well as to changes in plasma lipoprotein levels and a higher prevalence of NAFLD [29]. In Northern Sweden since 1990, there has been a steady increase in the prevalence of abdominal obesity, most markedly among women aged 45–64 years [30]. Therefore, our studies have focused on obese postmenopausal women to further understand and promote intervention against obesity-related diseases in this population.

## Diet interventions

The first-line therapy against obesity and its comorbidities is lifestyle modification, including weight loss and increased physical activity [31]. Our studies have focused on the possible role of dietary intervention on weight loss and ectopic fat accumulation. Regardless of macronutrient composition, caloric restriction has strong beneficial effects on weight loss (up to two years), insulin resistance, and the risk of developing diabetes [32, 33]. It has also recently been suggested that substantial weight loss can be achieved by following any low-carbohydrate or low-fat diet for up to twelve months [34]. Additionally, diets including a moderate increase in protein intake are suggested to be beneficial for weight loss when used *ad libitum*, for at least up to 12 months [35]. Furthermore, a Mediterranean diet supplemented with extra-virgin olive oil or nuts has shown beneficial effects on weight balance, as well as protective effects against cardiovascular disease [36]. This suggests that both macronutrient composition and specific food items can have beneficial effects on body weight balance and metabolic functions.

## Fat

Reducing one's intake of trans-unsaturated fatty acids (TFs) to as low as possible clearly shows beneficial effects on insulin sensitivity, while increased TF intake is associated with a dose-dependent increase in diabetes risk. Furthermore, TF intake is associated with increased LDL levels, lower HDL levels, increased levels of proinflammatory cytokines, and increased risk of CVD [37, 38].

A reduction of total fat intake (such that <30% of energy comes from fat) has a modest effect on weight loss and insulin resistance, while increased/excessive fat intake reportedly decreases insulin sensitivity. Furthermore, a reduction in the total fat content of one's diet lowers LDL cholesterol and reduces CVD risk [33].

A reduced dietary intake of saturated fatty acids (SFA) seems to be beneficial for improving insulin sensitivity. However, it is unclear whether this confers a lower risk for developing T2DM. The debate concerning the role of saturated fatty acids in cardiovascular disease has lately become more intensive. Two recently published large reviews and meta-analyses could not find an association between the intake of saturated fat and cardiovascular disease [38, 39]. One reason for this could be a difficulty in evaluating the exposure, i.e. the intake of saturated fat, on an individual level. Another reason could be that the association between saturated fat and cardiovascular disease is much weaker than presented in established cardiovascular guidelines or non-existent. Notably, dairy products, that are relatively high in SFA, have in observational studies and partly supported by randomized trials, been associated with anti-obesity effects and protection against development of cardiovascular disease. This may be due to several components in these products, including probiotic effects [40].

Substitution with monounsaturated fatty acids (MUFA) reduces insulin resistance by 10% [41] and lowers LDL, TG, and blood pressure levels [42], but has not been shown to be beneficial regarding diabetes risk [43]. Polyunsaturated fatty acids (PUFA) appear to have variable effects depending on the ratio between n-3 and/or n-6 PUFA. In animal models, n-3 PUFA, but not n-6 PUFA, show beneficial effects on insulin sensitivity [42], whereas the opposite effect has been reported in human studies. Both classes of PUFA lower circulating levels of proinflammatory cytokines, adipokines, and LDL [44]. Interestingly, n-6 PUFA has also been shown to decrease liver fat content [45].

## Carbohydrates

Low-carbohydrate diets have a relatively modest impact on weight loss, but they may be more effective than low-fat diets, at least during the first 12 months [46]. It remains unknown whether low-carbohydrate diets have a positive impact on insulin resistance. Carbohydrate restriction could potentially have adverse metabolic effects under isocaloric conditions and especially in combination with high-protein diets [47, 48]. Low-carbohydrate diets result in increased HDL and lowered TG, but may lead to higher LDL compared to low-fat diets [49]. Reduced carbohydrate intake seems to beneficially impact triglyceride levels [50, 51], while excessive intake of simple sugars apparently has a negative influence on liver fat content, which is thought to be mediated through increased *de novo* lipogenesis [52].

## Protein

High-protein diets (25–30% of energy from protein), commonly applied in combination with lower carbohydrate intake, have beneficial effects on weight loss and body composition as well as on blood lipids and blood pressure at least in the short-term [49, 53]. Protein has high satiating and thermogenic effects [54], which may explain the beneficial weight loss found with the use of high-protein diets compared with lower protein intake (15% of energy from protein) [55]. It has also been shown that adhering to a high-protein diet can improve maintenance of weight loss [56]. High protein intake also carries the possibility of adverse effects on insulin sensitivity. Healthy persons exposed to high amino acid levels via infusions reportedly develop insulin resistance [48]. Correspondingly, long-term high protein intake can decrease whole-body insulin sensitivity [57] via increased stimulation of insulin and glucagon release, high glycogen turnover, and stimulation of gluconeogenesis [57].

Since weight gain occurs as a direct effect of caloric surplus, dietary interventions and caloric restrictions are used to prevent/reverse the effects of obesity. Several studies of short-term dietary interventions have shown major effects on weight loss and cardiometabolic parameters, most of which have included caloric restriction in addition to different macronutrient modifications. In general, calorie and carbohydrate restrictions lead to rapid significant reductions in liver fat, with concomitant improvement of insulin sensitivity and glucose levels [58]. However, there is a lack of long-term studies of the putative effects of *ad libitum* diets, particularly regarding weight change and ectopic fat deposition. Importantly, an average of only about 20% of participants reportedly succeed in adhering to long-term lifestyle interventions [59].

## Our intervention studies

In our studies, we hypothesized that altering macronutrient composition without caloric restriction would beneficially affect ectopic fat accumulation. We compared a diet determined according to the Nordic Nutrition Recommendations (NNR) [60] with a Palaeolithic-type diet (PD), which has become increasingly popular in Sweden over the last decade.

The NNR diet [60] emphasises high fibre ingestion and consumption of low-fat dairy products, and has been recommended by the Swedish National Food Agency for more than 30 years. On the other hand, the PD includes a moderately high protein intake combined with a moderate decrease in carbohydrates and an increased intake of MUFA and n-3 fatty acids. This diet is thought to be beneficial based on the low age-adjusted rates of

cardiovascular disease among contemporary hunter gatherers and indigenous people [61] who eat a diet based on sweet and ripe fruits and berries, shoots, flower, buds and young leaves, meat, bone marrow, organ meats, fish, shellfish, insects, larvae, eggs, roots, bulbs, nuts and non-grass seeds [62]. Short-term studies of this diet ingested *ad libitum* have shown beneficial effects on energy intake, weight, waist circumference, and metabolic balance, including insulin sensitivity and cardiovascular risk markers [63-66].

Our hypothesis was that the PD would be advantageous regarding body composition and cardiovascular risk markers compared to a conventional low-fat high-fibre diet under *ad libitum* conditions. We also aimed to specifically investigate whether the PD had significant effects on ectopic lipid deposition among obese postmenopausal women.

# Materials and Methods

All parts of this study were conducted in accordance with the Helsinki declaration and were approved by the Regional Ethical Review Board at Umeå University, Umeå, Sweden.

## Study I

This study enrolled ten non-smoking overweight or obese (BMI 27–35 kg/m$^2$) but otherwise healthy postmenopausal women. Menopause was defined as >1 year after the last menstruation. At inclusion, none of the participants showed any sign of heart or kidney disease, hyper- or hypothyroidism, osteoporosis, or diabetes. They did not use any prescribed drugs and were not on any specific diet prior to the start of the intervention. Women were excluded if they could not tolerate the key foods in the Paleolithic-type diet due to allergies or other reasons (e.g. vegetarians). Each subject acted as her own control. All variables were measured before and after five weeks of intervention.

### *Dietary intervention and assessment*

The study subjects were given prepared protein portions that were intended to provide an average of 30 energy percent (E%) of the total daily intake. They were also given 40 grams/day of nuts (walnuts and sweet almonds). Participants were instructed to complete the meal with carbohydrates (30 E%) and fat (40 E%) from a food list (Table 1), *ad libitum*. The women received menus and recipes along with advice regarding portion sizes.

***Table 1***. *List of foods allowed and restricted in the Palaeolithic type diet.*

| **Foods included in the diet** | **Foods excluded from the diet** |
|---|---|
| Lean meat | Dairy products |
| Fish, shellfish | Cereals |
| Fruit, berries | Beans |
| Vegetables | Refined fat |
| Root vegetables | Refined sugar |
| Eggs | Added salt |
| Nuts | Bakery products |
| Avocados | Soft drinks |
| Rapeseed or olive oil for cooking | |

The recommended alcohol intake was a maximum of two glasses of wine per week, preferably red wine. Throughout the intervention, daily food records were kept. The women were instructed to weigh the leftovers of the provided food, and to estimate their food amounts using household utensils and a book of photographs of food portions. The participants met with the study dietician once per week to collect their prepared meals, submit their food records, and check their weight. Energy and nutrient intakes were calculated using the personal computer nutrient software package MATS version 4.06 (Rudans Lättdata, Västerås, Sweden) and the Swedish Food Database PC version 02_1 (National Food Agency, Uppsala, Sweden).

## Studies II and III

We performed a two-year randomized clinical trial investigating the effects of two diets with different macronutrient composition—the PD and NNR (as described earlier)—on weight-loss and metabolic risk profile. The trial was conducted between 2007 and 2010 in Umeå, Sweden. Advertisements in local newspapers were used to recruit the subjects from the county of Västerbotten in Northern Sweden. A total of 210 subjects responded with interest in participating in the study (Table 1), and completed a phone interview to determine whether they fulfilled the entry criteria.

Eligible volunteers were postmenopausal, non-smoking, and had a BMI of ≥27 kg/m$^2$. Subjects were excluded if they were already consuming a restricted or vegetarian diet or if they reported an allergy to key components of the intervention diets (Table 1). Other exclusion criteria included history of heart disease or kidney disease; hyper- or hypothyroidism; osteoporosis; diabetes; abnormal fasting plasma glucose levels; blood pressure >150/90 mm Hg; or current treatment with oestrogens, statins, beta-blockers, or any medication for psychiatric disorders. Three subjects were on monotherapy with an angiotensin-converting enzyme inhibitor for mild hypertension. After reading and signing the consent form, each participant met with a physician for a clinical assessment followed by a series of baseline tests at our clinical research centre.

A total of 70 subjects fulfilled the inclusion criteria and were randomized to consume either a Palaeolithic-type diet (PD) or a diet following the Nordic Nutrition Recommendations (NNR) for 24 months. The study was conducted in three cohorts: the first starting in August 2007, the second in November 2007, and the third in March 2008. The 24-month follow-up for the final cohort was concluded in March 2010.

### *Dietary intervention and assessment*

The PD was designed to provide 30 E% protein, 40 E% fat, and 30 E% carbohydrates, and included a recommendation for increased unsaturated fat intake. The NNR diet was lower in protein and fat content and consequently higher in carbohydrates than the PD, designed to provide a daily intake of 15 E% protein, 25–30 E% fat, and 55–60 E% carbohydrates. Neither diet included a recommendation of energy restriction, i.e. the diets were consumed *ad libitum*.

Each participant attended a total of 12 group sessions held by a trained dietician. Eight of these sessions occurred during the first 6 months, with the last 4 sessions taking place at 9, 12, 18, and 24 months. The group sessions included cooking classes, information regarding behavioural changes, putative effects of diet on health, and group discussions.

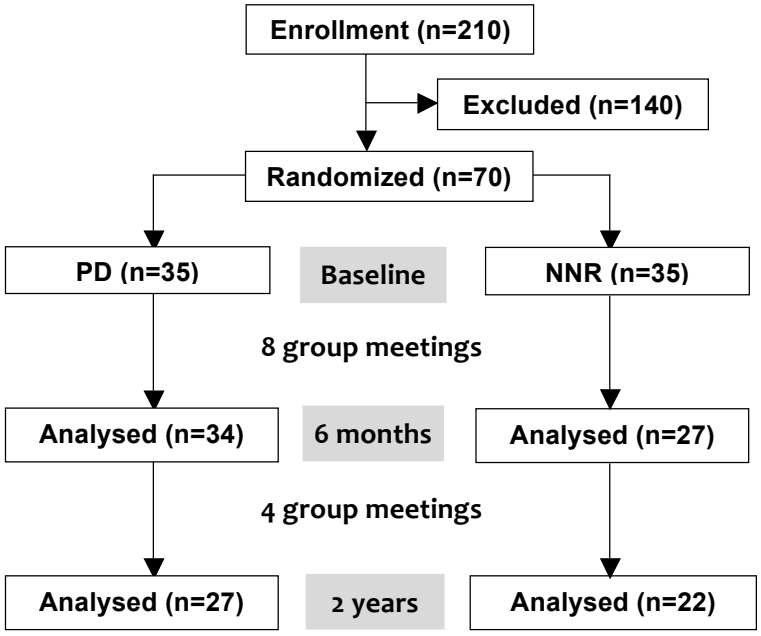


***Figure 1.*** *Flow chart showing numbers of participants in each diet group at different time-points. Participants were instructed to eat either a Palaeolithic-type diet (PD) or a diet following the Nordic Nutrition Recommendations (NNR). Group meetings refer to sessions with the study dietician.*

### *Anthropometry*

Body weight was measured to the nearest 0.1 kg with the participants wearing light indoor clothing and no shoes, using a calibrated electronic digital scale (Tanita BWB-800 MA; Umedico AB, Rosersberg, Sweden). Body height was measured to the nearest 0.5 cm with the participants not wearing shoes, using a wall-mounted stadiometer (Hultafors, Sweden). BMI was calculated as body weight in kilograms divided by body height in meters squared (kg/m$^2$). Waist circumference was measured to the nearest 0.5 cm with a tape measure, midway between the lower rib margin and the iliac crest during a gentle exhale. The sagittal diameter of the abdomen was measured as the abdominal height at the umbilical level with the participant lying down on the examination couch with her legs in an extended position.

### *Dual-Energy X-ray Absorptiometry*

Dual-energy X-ray absorptiometry (DXA) is based on the body's differential attenuation of photons at two energy levels [67]. DXA provides a safe and practical method of quantifying both whole body composition and regional changes in body composition. A body scan takes 10–20 minutes. Unlike chemical testing, hydrodensitometry, or anthropometry, DXA measures the regional distribution of bone, fat, and lean tissue mass. Here we used DXA equipment (Lunar Prodigy X-ray Tube Housing Assembly, Brand BX-1L, Model 8743; GE Medical Systems, Madison, WI, USA) for measuring body composition. The absolute values of fat mass and lean mass were measured for each subject, and the results were expressed as ratios and percent of total body mass, as well as in grams.

### *Estimation of insulin sensitivity*

#### *Hyperinsulinaemic euglycaemic clamp*

After an overnight fast, participants were administered a hyperphysiological insulin infusion of 56 mU/m$^2$ per minute for 120 minutes. In parallel, the participants received a glucose infusion that was adjusted to maintain euglycaemia. The infused glucose levels were then used to estimate insulin sensitivity [68]. The M-value was calculated by dividing the glucose infusion rate during the last 60 minutes of the clamp by the participant's body weight. The infused amount of glucose to maintain euglycaemia is a measure of insulin sensitivity [69].

*Homeostasis model assessment*

Homeostasis model assessment (HOMA) was performed using the formula $(G_o \times I_o)/22.5$ where $G_o$ is fasting plasma glucose (mmol/L) and $I_o$ is fasting plasma insulin (mIU/L) [69].

*Oral glucose tolerance test (OGTT)*

After an overnight fast, baseline blood samples were drawn for glucose and insulin measurements. The participant was then given a solution containing 75 g glucose to drink within 5 min. Blood samples for glucose and insulin monitoring were drawn every 30 min for a total of 2 hours.

*Indices for insulin sensitivity*

To determine hepatic insulin sensitivity, the Liver IR index was calculated as follows: −0.091 + (log insulin AUC 0 – 120 min × 0.4) + (log fat mass% × 0.346) – (log HDL-cholesterol × 0.408) + (log BMI × 0.435) [70]. Based on the fact that hepatic insulin sensitivity mostly influences fasting levels of insulin and glucose, we also estimated hepatic insulin sensitivity using HOMA-IR calculated as (fasting glucose × fasting insulin)/22.5. In accordance with the findings of a recent meta-analysis, we chose oral glucose insulin sensitivity (OGIS) and the Matsuda and Gutt indices, as surrogate measures for peripheral insulin sensitivity [71-74]. These three chosen indices were based on the OGTT (not only on fasting samples) and did not account for BMI, which we wanted to relate to in our study.

## *Magnetic resonance imaging and spectroscopy*

Magnetic resonance (MR) imaging and spectroscopy were performed with a 1.5-T ACS NT MR scanner (Philips, Best, The Netherlands), using the SENSE 5 element cardiac coil or an extremity coil (Philips) as the receiver coil. Participants lay in the supine position during imaging, positioned head-first in the bore of the magnet.

For spectroscopic investigations, high-resolution $T_1$-weighted MR images were used for placement of the spectroscopic volumes in the right lobe of the liver, carefully avoiding vascular structures and subcutaneous fat tissue. MR spectra were recorded using the point-resolved spectroscopy (PRESS) sequence with a repetition time (TR) of 3000 ms and an echo time (TE) of 25 ms, acquiring 64 signal averages from a volume element of 20 × 20 × 20 $mm^3$. A total of 1024 data points were acquired over a 1000-Hz spectral width, without presaturation of the water resonance. Respiratory triggering

of spectroscopic data acquisition was accomplished using a software patch written by GyrTools Ltd. Zürich, Switzerland.

Spectra were analysed using LCModel version 6.2, written by Dr. Stephen Provencher. The area of the combined lipid peak from the LCModel analysis (L16 + L09 + L13) and the area of the water peak were calculated and used to determine the hepatic liver content expressed as the ratio of lipid protons to water protons. Correction for the $T_2$ relaxation effects was performed using a $T_2$ time of 78 ms for lipids a $T_2$ time of 40 ms for water [75].

*Visceral and subcutaneous adipose tissue (VAT and SAT)*

VAT and SAT images were obtained using the body coil and a T1-weighted two-dimensional (2D) multislice spoiled gradient echo acquisition of 16 contiguous axial slices of the abdomen, denoted 1–16 from caudal to cranial. The volume was centred over L4–L5, and the acquired volumes included the subjects' arms. To simplify the automated image processing, the subjects' arms were manually "erased" from all image slices. The utilized scan parameters were as follows: TR, 129 ms; TE, 4.6 ms; Flip, 80 degrees; FOV, 430 mm; Matrix, 256 × 256 mm; and slice thickness, 10 mm. An anterior–posterior phase coding direction was used, and the scan time was 16 s.

### *Resting metabolic rate*

Resting metabolic rate (RMR) was evaluated using indirect calorimetry (Datex-Ohmeda Deltatrac II; Datex-Ohmeda Inc., Madison, WI, USA) to measure oxygen consumption through breath-by-breath sampling. Measurements were performed during 30 minutes with the subjects lying in a supine position with a fully transparent plastic canopy placed over the head. Participants were instructed not to eat during the four hours prior to the examination, and to avoid coffee or physical activity before the test.

### *Physical activity energy expenditure*

Free-living physical activity energy expenditure (PAEE) was estimated using data collected with a combined heart rate recorder and accelerometer (Actiheart®; CamNtech Ltd., Cambridge, UK), described in detail elsewhere [76]. The sensor was attached to the left side of the chest using standard ECG pads. For individual calibration of heart rate to PAEE, we used an 8-minute step test on a 20-cm-high step board [77]. Acceleration and heart frequency were recorded over a 7-day period, during which the subjects carried the sensors 24 hours/day (except when showering or swimming). Data collected

during free-living periods were downloaded to a computer, and each heart rate trace was processed using a robust Gaussian Process regression method to handle potential measurement noise [78]. Instantaneous PAEE (J/min/kg) was estimated from the combination of individually calibrated heart rate and movement data [77] using a branched equation framework [79]. Periods of non-wear were inferred from the combination of non-physiological heart rate and prolonged periods of inactivity, which were accounted for to minimize diurnal information bias when summarizing the intensity time-series into PAEE (kJ/kg/day). Values were then multiplied by body weight and expressed in megajoule (MJ)/day. Branched equation model estimates of PAEE are viewed favourably compared with the double-labelled water method [76]. Diet-induced thermogenesis—i.e. the production of heat after eating—was fixed as 10% of total energy expenditure (TEE). The value of TEE was calculated for each participant as the sum of PAEE and RMR, divided by 0.9 and expressed as MJ/day (24 h).

### *Dietary assessment*

Baseline dietary intake was assessed according to two four-day estimated self-reported food records. Subjects were instructed to record all food items and drinks consumed over four consecutive days (three weekdays and one weekend day), with the amounts described and estimated using coloured food portion photographs representing known weights, household measures, and standard weights of food items. The food records were reviewed by a dietician, and the subjects were asked to complete any missing information. The reported food intake was then converted to estimates of energy and nutrient intake using the nutritional analysis package Dietist XP (version 3.0) and the food composition database by the Swedish National Food Administration (2008-03-06). Reported nitrogen intake (NI) was obtained by dividing the reported protein intake by the protein factor for mixed food (i.e. protein intake/6.25) [80].

### *Measurement of nitrogen excretion in urine (NU)*

Participants were instructed to collect three 24-h urine samples. Prior to sampling, they were provided with written instructions, tablets containing para-aminobenzoic acid (PABA), and questionnaires regarding the urine collection. The sampling was performed during the same period (± one week) as the food records and measurements of RMR and PAEE. Subjects were asked to return the collected urine to the laboratory on the same day that the collection was completed, together with a written description of any

problems encountered during the collections. The urine samples were weighed (g) and the volume was measured (mL). From each collection, ten 15-mL aliquots of urine were frozen and stored at −20°C until analysis.

The PABA check method [81] was used to verify urine collection completeness. Each subject took three 80 mg PABA tablets immediately before beginning each 24-h urine collection. A sample from each collection was colourimetrically analysed for PABA (Department of Clinical Nutrition, University of Gothenburg, Sweden). Urine collections containing 85–110% recovery of the oral dose of PABA were considered complete, while collections containing <50% or >110% PABA were excluded. For incomplete collections with a 50-85% PAPA recovery, the nitrogen content was adjusted using a linear regression equation method (compensated 24-hour NU = N excreted + [0.088 × (93 − PABA)]) [82]. NU was determined following the Kjeldahl technique using a Kjeltec analyser (model NMKL nr 6; Eurofins Food & Agro AB, Lidköping, Sweden). For quality control, duplicates were analysed for 10% of the samples. The analytical precision was ±10%. The measured protein intake (g/d) was calculated from NU using the following equation: [protein = 6.25 × (24-hour NU/0.81)], where 6.25 is the protein factor for mixed food and 0.81 is a factor considering extra-renal loss [80, 83].

### *Metabolic parameters in blood and urine*

All participants underwent routine laboratory function analyses. Venous blood samples were drawn by cannulating the cephalic vein, and all samples were drawn under fasting conditions after 15 minutes of rest. Portions that were not analysed immediately were frozen and stored at −20°C until analysis. The methods section of each paper describes the techniques used for analyses of blood and urine including glucose, insulin, c-peptide, lipids, inflammatory markers, fibrinolytic function and adipokines.

## Statistics

### *Study I*

Wilcoxon's signed-rank test was used to test differences between baseline and after five weeks, using SPSS v. 19 (IBM SPSS, Chicago, IL, USA).

### *Studies II and III*

The primary outcome was the change in fat mass over a two-year period. The study was powered to detect a 10% decrease of fat mass in the PD group vs. a 3% decrease in the NNR group after 24 months. Thirty-five participants in each group was calculated to be sufficient to determine a significant outcome ($p$ <0.05).

*Randomisation and stratification*

We applied block randomisation with a block size of four and an allocation ratio of 1:1. To achieve balanced diet groups, the randomisation was stratified by BMI. The randomisation sequence was created using SPSS v. 19 statistical software (IBM SPSS, Chicago, IL, USA), and the randomisation code was kept intact until all measured variables were analysed and the statistical evaluation was completed.

The normality of the distribution of outcome variables was assessed both visually (by plots and histograms) and numerically (using skewness, Kolmogorov-Smirnov, and Shapiro-Wilk tests). Most variables were approximately normally distributed. Serum insulin, triglycerides, PAI-1, high sensitive CRP, and polyunsaturated fat (g) required logarithmic transformation in order to not deviate from the normality assumption. Between-group differences at baseline were tested by independent samples *t*-tests.

*Generalized estimating equation (GEE)*

We evaluated repeated measurements over time using a generalized estimating equation (GEE), assuming a correlation structure for repeated measurements within the same subject. Time-point and diet group were used as explanatory variables. The time*diet interaction was included in the model to test whether the effect over the whole time interval (0–24 months) differed between diet groups. If significant difference were found, we further performed *post-hoc* diet group comparisons of change from baseline until 6 months and until 24 months. If the time*diet variable was non-significant, a separate GEE analysis was performed using time as the only explanatory variable. Data were expressed as mean and standard error of the mean, unless otherwise stated. A two-sided $p$ value of <0.05 was considered significant. Statistical analyses were performed using SPSS for Windows (version 20.0; IBM Corporation, Armonk, NY, USA).

*Imputations*

The primary analysis in this trial (GEE) was an intention-to-treat analysis. For individuals who dropped out, their drop-out values (weight only) and/or baseline values were imputed, assuming that individuals who left the study returned to their old habits. Baseline values were imputed to the nearest time-point after the individual announced their drop out without carrying forward. When individuals were excluded due to illness, no imputation was performed.

# Results and Discussion

Detailed descriptions of the results of these three studies can be found in the respective papers.

## Study I

### A Palaeolithic-type diet has strong tissue-specific effects on ectopic fat deposition in obese postmenopausal women

Our hypothesis was that adhering to a PD that included increased protein and unsaturated fat with relatively low carbohydrates and saturated fat would reduce the ectopic fat contents of the liver and the skeletal muscle, thereby improving insulin sensitivity.

Table 2 presents the baseline characteristics of the study population (PD 5 weeks). Although food intake was not restricted, the participants decreased their daily energy intake by an average of 500 kcal and their body weight was reduced by an average of 5% from baseline. All anthropometric measurements changed significantly over the study period. We also observed beneficial changes in blood lipid profiles, except for a slight decrease in HDL. ApoA1 and ApoB levels decreased significantly. Furthermore, both systolic and diastolic blood pressure improved over the course of the five-week dietary intervention.

| | PD 5 weeks | PD 2 years | NNR 2 years |
|---|---|---|---|
| Age (years) | 56.2 ± 3.8 | 59.5 ± 5.6 | 60.3 ± 7.0 |
| Body weight (kg) | 86.4 ± 8.3 | 87.0 ± 10.6 | 86.8 ± 9.6 |
| BMI (kg/m$^2$) | 31.3 ± 2.6 | 32.7 ± 3.6 | 32.6 ± 3.3 |
| Waist circumference (cm) | 104.8 ± 6.2 | 105.4 ± 10.0 | 104.7 ± 10.4 |
| Hip circumference (cm) | 112.4 ± 4.2 | 113.5 ± 7.2 | 114.4 ± 8.1 |
| Sagittal diameter (cm) | 27.0 ± 2.3 | 21.7 ± 2.2 | 21.7 ± 2.2 |
| Total fat mass (kg) | 39.1 ± 4.5 | 39.8 ± 7.2 | 40.1 ± 7.2 |
| Total lean mass (kg) | 41.1 ± 4.4 | 42.6 ± 5.0 | 41.7 ± 4.0 |

***Table 2.*** *Baseline characteristics (mean ± standard deviation) of participants on a Palaeolithic-type diet (PD) or a diet following the Nordic Nutrition Recommendations (NNR; studies I-III).*

Liver triglyceride content drastically decreased, in association with lower fasting glucose and insulin levels (Figure 2). In contrast, we did not observe any changes in muscle fat content at the end of the intervention. We also found no changes in peripheral insulin sensitivity, as measured using euglycaemic hyperinsulinaemic clamps.

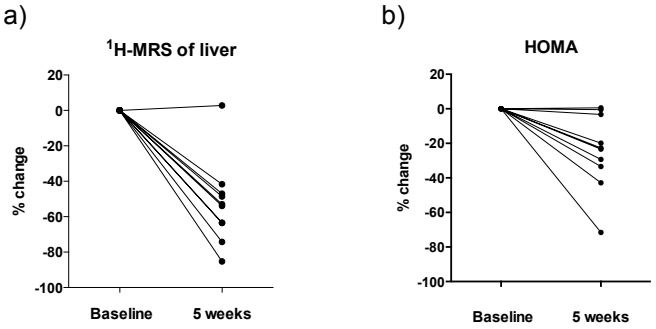


***Figure 2.*** *Changes in liver fat content (a), HOMA indices (b), between baseline and after 5 weeks on a Palaeolithic-type diet.*

After 5 weeks on PD *ad libitum*, the study participants exhibited major effects on liver fat content that were comparable with results reported after more drastic interventions, for example, gastric bypass and very-low calorie diets (VLCDs) [84, 85]. Additionally, the weight loss seen after this *ad libitum* PD intervention was comparable with the weight loss and metabolic changes seen with a very-restricted caloric intake (600–1200 kcal/24 h) [86, 87].

Despite the *ad libitum* conditions, the women in this study lost substantial amounts of weight and were thus in a catabolic state. The average weight loss observed corresponds to an energy deficit of approximately 1000 kcal/day. However, the reported energy intake of 1900 kcal was equal to the estimated total energy expenditure (TEE = RMR + PAEE; RMR, resting metabolic rate; PAEE, physical activity energy expenditure), suggesting an energy balance. It is unclear why weight loss occurred despite this energy balance. Contributing factors likely include over-reporting of energy intake, increased PAEE (when the volunteers were not wearing the Actiheart monitor), the increased thermogenic effects of protein, and uncertainties regarding the measurement of all energy components on each side of the equation.

While all of the quantifiable beneficial effects of this dietary intervention could be explained by a catabolic state, there are also other possible contributing factors, i.e. change in dietary components, including

macronutrient composition and food choices. It has previously been shown that isoenergetic diets high in PUFA and/or MUFA have beneficial effects on liver fat content [45, 88]. This supports the possibility that the effect of the PD on liver fat content may be due to factors other than energy deficiency.

The women more than doubled their PUFA intake and increased their MUFA intake by almost 40%, while decreasing their saturated fat and carbohydrate intakes. Furthermore, during the 5-week intervention, the women decreased their average sucrose (glucose + fructose) intake from 69.5 g/day to 27.5 g/day, equalling a 50% reduction of fructose. Simple sugars (monosaccharides), especially fructose, have been suggested to be obesogenic [52, 89]. Fructose uptake is not insulin-dependent, and fructose consumption leads to lower postprandial glucose and higher triglyceride levels (via *de novo* lipogenesis) compared to consumption of other types of carbohydrates. Fructose intake also has a putative effect on energy intake, as it exhibits a decreased ability to cause satiety compared to other carbohydrates [90].

Furthermore, protein intake was nearly doubled in the PD. Bortolotti et al. demonstrated that protein intake as part of a hypercaloric diet played a protective role regarding liver fat content [91]. This effect was associated with bile acid concentrations that may inhibit lipogenesis via stimulation of liver X factor and farnesoid receptor A [91]. High-protein diets can spare muscle tissue under hypocaloric conditions [92], thus allowing maintenance of RMR/BMR despite lower weight. This is crucial, especially with a long-term hypocaloric state. Compared to fat, muscle mass/fat-free mass requires more energy in resting conditions. Since the resting metabolic rate accounts for a majority of the total energy used (TEE), it is important to maintain muscle mass despite weight reduction. Furthermore, the observed decreased liver fat content was associated with lower fasting insulin and glucose levels, suggesting improved hepatic insulin sensitivity. In contrast, we observed no changes in the whole-body insulin sensitivity as measured with the hyperinsulinaemic euglycaemic clamp. This makes sense, since we also found no significant changes in IMCL content. Under conditions of euglycaemic hyperinsulinaemia, 80–90% of infused glucose is taken up by skeletal muscle. Thus, insulin sensitivity measured with the insulin clamp technique primarily reflects that of the skeletal muscle [20]. Our present findings are in agreement with earlier studies, showing significantly reduced liver fat content without concomitant improvement in peripheral insulin sensitivity [87].

## Study II

### Long-term effects of a Palaeolithic-type diet in obese postmenopausal women: a 2-year randomized trial

Table 1 shows baseline and 2-year follow-up characteristics of the study participants (also see Tables 1 and 2 in the original publication). In this long-term intervention we found that although diet intake was *ad libitum*, both groups decreased their energy intake (Figure 3a). This decrease was associated with significant weight loss after 2 years, despite unaltered physical activity levels. The PD group showed more pronounced weight loss during the first 6 months (9.2 ± 5.5% loss versus 3.6 ± 4.9% loss in the NNR group) (Figure 3b). At six months, the PD group also showed significantly greater decreases in all anthropometric variables compared to the NNR group. However, these differences were not sustained after two years. Notably, serum triglyceride levels were significantly lower in the PD group at the end of the study.

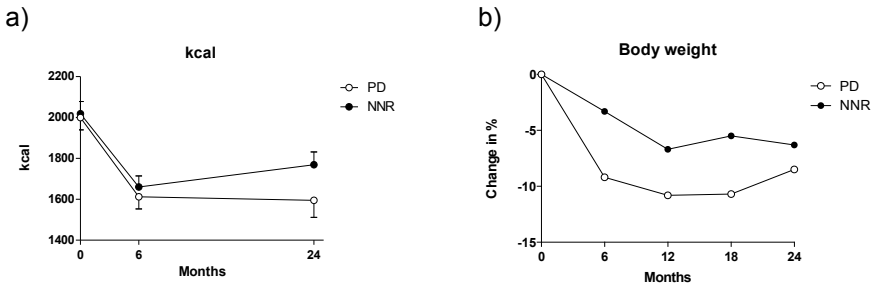


***Figure 3.*** *a) Mean reported kcal intake at baseline and at 6 and 24 months. b) Changes in body weight. Generalized estimating equations were used, and data are presented as mean. PD, Palaeolithic-type diet; NNR, Nordic Nutrition Recommendations***.**

The PD had substantial and long-lasting effects on body composition in postmenopausal women. Compared to the NNR diet, the effects of the PD were greater during the first half of the study, but the between-group differences diminished after two years. Both diet groups maintained a substantial weight loss after two years, which makes this study unique. Long-term diet interventions usually show a pattern of weight loss during the first 6- to 12-month period of the intervention, followed by a rebound period [93, 94].

Triglyceride levels showed significantly greater improvement in the PD group compared to the NNR group at both 6 and 24 months. Reducing circulating triglyceride levels can be important for controlling/reversing the effects of ectopic fat accumulation, as blood lipid concentrations and composition appear to be important factors in the pathogenesis. Our data are in agreement with a study from Denmark that compared a low-fat high-protein diet with a low-fat high-carbohydrate diet, and found that the high-protein diet group showed significantly decreased triglyceride and FFA levels, whereas no such changes were observed in the high-carbohydrate diet group or the control group [95].

Despite efforts to encourage increased protein intake among the participants in the PD group, validation of this intake by measurements of nitrogen in urine revealed that they were unable to reach the target protein intake. Importantly, the two diet groups did not significantly differ in urine nitrogen excretion, suggesting no difference in protein consumption. This suggests that the positive effect of the PD originates from other factors than protein intake. In support of this, Lindeberg has investigated Native populations in Papua New Guinea [96], which have very low age-adjusted rates of cardiovascular disease, despite a relatively low protein content and high carbohydrate intake in their diet. This suggests that food quality and choice may be central for the positive effects of this type of diet, rather than macronutrient composition.

## Study III

### Decreased liver fat during a two-year diet intervention is not associated with improvement in hepatic insulin sensitivity

Two years of diet intervention led to significant mean decreases in VAT (16% decrease) and SAT (11% decrease). Liver fat also decreased throughout the study in both the PD (63% decrease) and NNR (50% decrease) groups. Insulin sensitivity improved during the first 6 months of the study but returned to baseline levels after two years. Hepatic insulin sensitivity was associated with liver fat at baseline, but was not associated with the changes in liver fat throughout the diet intervention.

Although the participants maintained lower levels of VAT and liver fat, the initial increase in insulin sensitivity was surprisingly not sustained throughout the study period. This finding is important for interpreting the correlation between liver and metabolic dysfunction. Liver fat has clearly been shown to be associated with insulin sensitivity among patients with T2DM and severely obese women [97, 98]. Korenblat also reported a clear association between insulin action in the liver, muscle, and adipose tissue

among non-diabetic individuals [99]. However, our results contradict the general link between liver fat and insulin sensitivity.

There is an on-going debate regarding whether ectopic fat accumulation is a cause or consequence of insulin resistance. In Study I, we found a clear association between reduced liver fat content and improved hepatic insulin sensitivity (measured with HOMA) after a 5-week diet intervention. Studies of gastric bypass and VLCD have shown similar changes in hepatic insulin sensitivity after a short duration (1–2 weeks) [100, 101]. Therefore, it seems that both ectopic fat accumulation and insulin sensitivity can change rapidly with diet interventions. Conflicting data are present regarding long-term effects on liver fat after diet interventions (see general discussion below).

Exercise can also influence liver fat. In healthy subjects, aerobic exercise results in acutely increased liver fat content, along with decreases in intramyocellular and intracardiomyocellular lipid contents, without accompanying changes in insulin sensitivity [102, 103]. The effect of exercise on ectopic fat accumulation has been studied in subjects with T2DM, with the results indicating that exercise training, independent of dietary modification, may reduce ectopic fat accumulation in the liver [104].

Notably, the participants in our study showed relatively wide variation of baseline liver fat contents, ranging from 0.5–28%, with 22 out of 65 having a liver fat content of >5%, consistent with NAFLD (Figur 4). Therefore, there may have been a ceiling effect, reducing the chance of substantially improving fat content in a major part of the study population. Our study group could also be characterized as "metabolically" healthy with a lower risk for immediate metabolic/cardiovascular complications.

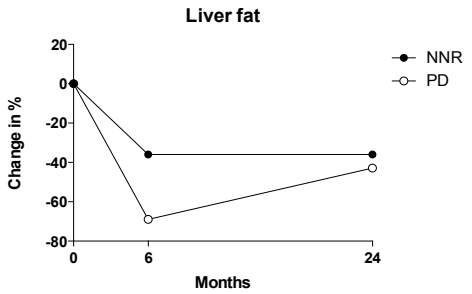


***Figure 4.*** *Mean change in liver fat content at 0, 6 and 24 months. Generalized estimating equations were used. PD, Palaeolithic-type diet; NNR, Nordic Nutrition Recommendations.*

## Missing data

In the two-year intervention, we found that the participants who dropped out differed from those who completed the study. Notably, fewer participants dropped out from the PD group than from the NNR group. Additionally, the volunteers who left the study had a higher mean weight at baseline compared to those who completed the study (Figure 5).

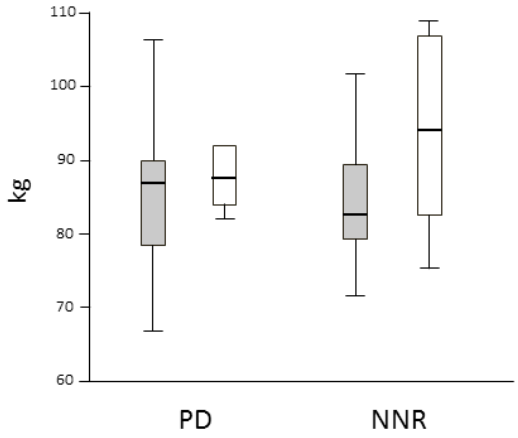


***Figure 5****. Differences in weight for drop-outs and those who completed the study between the Palaeolithic-type diet (PD) group and the Nordic Nutrition Recommendations (NNR) group. Grey boxes show participants who completed the study, white boxes show participants who dropped out.*

This trial primarily involved an intention-to-treat analysis, the General Estimating Equation (GEE). For individuals who dropped out, their drop-out values (weight only) and/or baseline values were imputed, under the assumption that individuals who left the study would resume their old habits. Baseline values were imputed to the nearest time-point, without carrying forward after announcement of drop-out. When individuals were excluded due to illness, no imputation was done.

Drop-outs pose a major methodological problem in diet interventions studies. Participants who choose to leave the study might be systematically different from those who remain, creating a potential risk of bias. In this situation, there are several different approaches to analysis, including deletion methods and imputation.

Deletion methods involve the omission of cases with missing observations, such that all remaining cases are complete. With this method, all analyses can be calculated with the same set of cases, making it possible to track individual changes over the whole study period. However, if the omission of data is not random, the resulting complete datasets may not be representative of the whole population, and the analysis may be biased. Moreover, the deletion of a large number of individuals will reduce the power of the analysis.

Imputation methods involve the replacement of missing data with new plausible values. This technique results in no data loss, making it more efficient. However, imputation can be difficult to implement and may disturb relationships. Various imputation methods are available, which differ regarding the information used to determine plausible values to impute. Two commonly used methods are to use baseline data or the last value carried forward.

In the GEE method, drop-outs with missing values are handled using an algorithm that calculates a development over time for the drop-outs, which follows the development of the remaining study participants. In this manner, the GEE creates an intention-to-treat (ITT) analysis. Weight loss studies typically observe a rapid weight decline during the first months from the study start, with many subjects regaining most of this lost weight after a year. In this situation, choosing the "last value carried forward" option of handling missing values could lead to larger effects of weight loss in an ITT analysis, while choosing to carry forward the baseline value would result in a more conservative estimation (Figure 6). In our study, we chose to impute the baseline value.

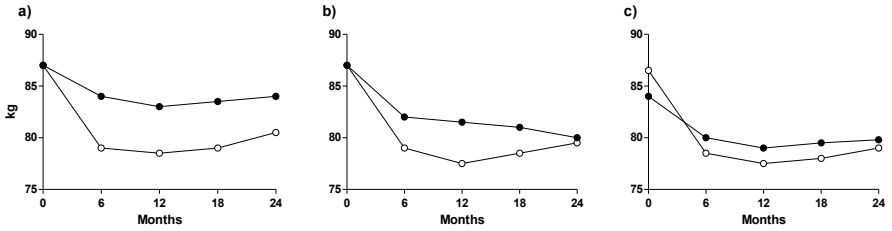


***Figure 6.*** *Participant weights over the course of the two-year study analysed using different methods to handle missing values. a) Drop-out weight included, with last value carried forward thereafter. b) GEE used to adjust data with no further action and no imputation. c) Deletion of drop-outs, inclusion of only complete cases.*

# General discussion and future challenges

The growing obesity epidemic has prompted numerous studies searching for an optimal diet to prevent and reverse obesity. Diets with different macronutrient contents have demonstrated small differences in their ability to prompt weight loss and modify metabolic risk factors [34]. The only consistent finding from several meta-analyses has been that intervention adherence is apparently the strongest predictor of weight loss and improvement in cardio-metabolic parameters [105-107]. However, this does not preclude that specific food components may influence long-term outcome, including metabolic balance, after diet interventions.

Our diet interventions have consistently included *ad libitum* consumption and, despite this or perhaps as a consequence of this, have demonstrated substantial effects on anthropometric variables. Weight loss over time in diet interventions often follows a pattern of substantial weight decline during the first 6–12 months, followed by a period of weight regain [94]. In our long-term intervention, we also observed substantial weight reduction during the first 12 months, but the participants did not show significant weight regain during the second year. This was even more impressive considering that the measures undertaken to increased adherence to the diet intervention (e.g. regular group meetings with the participants) diminished substantially during the second year of our two-year study. There are several possible reasons for the high intervention adherence observed in our study.

Our study cohorts largely comprised women who were motivated to lose weight and had tested several diets over previous years. Macronutrient composition can influence dietary adherence via different properties regarding satiation and fullness, and we expected that the increased protein intake in the PD would contribute to increased satiety. However, in our two-year diet intervention, estimations of nitrogen excretion in urine revealed that the participants in the PD group failed to reach their protein intake goal. In agreement with our findings, Shai et al. previously investigated several different diets with variable protein content [93] and their results suggest that dietary factors other than protein intake may have been beneficial in reducing body weight in the PD group. Thus, macronutrient composition is likely only one of many factors influencing adherence. Social, cultural, and religious factors, as well as food availability and costs also probably influence adherence to diet [108]. Notably, the increased PUFA intake in the PD group may have been beneficial in several ways, including increased satiety [109].

The PD is considered as a “healthy” diet, and its popularity has been increasing. This diet involves the avoidance of energy-dense food, it is relatively easy to follow, and it includes increased amounts of unsaturated

fats, which can have several beneficial effects. Replacing saturated fats (and trans-unsaturated fatty acids) with unsaturated fats improves insulin sensitivity and may reduce one's future risk of T2DM [42]. Avoiding gluten (grains) and dairy products has also attracted a lot of media attention. It remains unclear whether this type of diet may be associated with negative effects, including possible negative impact on iodine balance, and this subject must be studied further.

Iodine deficiency is among the most common nutritional disorders worldwide. It is the most common cause of goitre [110] and is also linked to development of thyroid dysfunction. In Sweden, the major dietary source of iodine is salt added to cooking. Dairy products and seafood are also important iodine sources [111]. Some have expressed concerns that reduced salt intake may result in some of the population becoming iodine insufficient, which is of particular concern for pregnant and breast-feeding women. Further studies are therefore underway to evaluate the effects of a PD on iodine balance.

Another concern relates to the costs associated with consuming foods with increased protein content, since protein-rich food is generally more expensive than high-fat or high-carbohydrate food [112]. A comparison of the costs of different foods relative to their energy and nutritive value demonstrated the highest prices per serving for meats, poultry, and fish, and the lowest prices for fats [113].

We used the NNR diet as a comparison in our two-year intervention. This was the diet recommended at study start by The Swedish National Food Agency for general use as a "healthy" diet. Recently the Nordic Nutrition Recommendations diet were updated for the fifth time (NNR5). A focus was put on healthy food patterns instead of isolated nutrients, and on quality of carbohydrates and fats instead of quantity. An allowance was also made for increasing the total amount of fat in the diet as long as the fat quality was in order, i.e. mainly monounsaturated fatty acids as in olive and rapeseed oils [114].

It is possible that the negative energy balance in both groups in our two-year study may have consequences beyond weight loss. Interestingly, an MD noticed a very low incidence of cardiovascular events in the late 1920s/early 1930s in the county of Västerbotten in Northern Sweden [115]. This was associated with very poor living conditions, in which a major portion of the population was starving. This observation suggests that a negative energy balance can reduce cardiovascular risk. It remains to be studied whether this translates to a diminished cardiovascular risk in subjects with a high risk for cardiovascular disease, including those with T2DM.

We stated that the participants in our studies were healthy, suggesting a low future risk for obesity-related complications. However, this can be debated. It seems that obesity-related disturbances vary among obese

individuals, despite similar fat mass and age. Cross-sectional studies have suggested that there is a phenotype considered metabolically healthy obese. These subjects have the ability to store fat within adipose tissue, away from insulin-sensitive tissues, such as the liver and skeletal muscle [116, 117]. Associated with this, these individuals have no hypertension, have favourable blood lipid profiles and normal hormone levels, and show no signs of low-grade systemic inflammation with relatively low hsCRP levels [117]. Notably, not all metabolically healthy obese subjects remain healthy over time. A recent meta-analysis by Kramer et al. concluded that obese individuals are at increased risk for adverse long-term outcome even without metabolic abnormalities, suggesting that there is no long-term healthy pattern of obesity [118]. However, some studies using a stricter definition of metabolic health have found no increased mortality risk among healthy obese individuals [119, 120]. All of these studies have defined individuals with only one solitary risk factor (hypertension, increased fasting glucose, or cholesterol) as metabolically healthy. Importantly, each of these factors are themselves risk markers of cardiovascular disease. Elevated blood pressure contributes to about 54% of stroke and 47% of ischaemic heart disease risk worldwide [121]. Indeed, 30% of metabolically healthy obese subjects experience cardio-metabolic complications over a 5–10 year period [122]. In this respect, two important risk factors have been suggested: ageing and additional weight gain.

Aging per se may contribute to ectopic fat accumulation. Healthy lean elderly persons have impaired insulin-stimulated glucose uptake in muscles compared to healthy lean young individuals [13]. This impairment results from reduced mitochondrial function and predisposes elderly persons to ectopic fat accumulation and insulin resistance. The development of skeletal muscle insulin resistance may be a primary starting point in the development of ectopic fat accumulation in both muscle and liver tissue [13]. This underscores the importance of exercise for long-term effects on insulin sensitivity and cardiometabolic risk reduction.

Our studies included obese postmenopausal women. After the menopause transition, women exhibit higher incidences of cardiometabolic disorders and T2DM, which has been explained by the redistribution of fat from more peripheral/subcutaneous to central depots. Oestrogen deficiency may contribute to this change. Importantly, oestrogen has been suggested to have a protective role against NAFLD development. Oestradiol reportedly affects the ability of Apo lipoprotein C3 to inhibit lipoprotein lipase activity [13]. Therefore, the decreased oestrogen levels with menopause can increase fat accumulation in non-adipose tissues.

Haufe et al. recently demonstrated that a diet intervention had effects on liver fat content and insulin sensitivity that were maintained at two years after the end of the intervention, despite weight regain [123]. This study was

performed in overweight and obese men (n = 35) and women (n = 135) with mean ages of 42–46 years in the different intervention groups [124]. The different ages of the participants and the corresponding differences in oestrogen levels, may possibly contribute to the partly contradictive results between our study and the study by Haufe et al. regarding the association between liver fat and hepatic insulin sensitivity. Future investigations are warranted to investigate the effects of lifestyle recommendations in combination with oestrogen supplementation in postmenopausal women with metabolic abnormalities, including decreased insulin sensitivity. However it is currently debated whether oestrogens have a net beneficial cardiometabolic effect, especially in postmenopausal women with atherosclerotic manifestations.

There also remains a need to further explore the importance of "lipotoxic" effects of lipid intermediate accumulation in subjects with varying degrees of ectopic lipid accumulation and insulin sensitivity. This includes measurements of diacylglycerols (DAGs), ceramides, and acylcarnitines, at both the circulation and tissue levels. These lipid intermediates could be the major culprits for the development of metabolic complications with ectopic lipid deposition, may also predict the possibility of improving insulin sensitivity after lifestyle interventions. Tissue analyses could further explore the possibility that cellular compartmentalization of DAGs within lipid droplets or other biochemical effects may explain the lack of association between changes in ectopic lipid deposition and insulin sensitivity, as has also been described in endurance athletes [13].

Alteration of physical activity during the study period is a confounding factor in analysing the effects of diet interventions. Obesity is a consequence of a positive energy balance, created by a too-large energy intake and/or a too-small energy output. Ideally, when studying diet interventions, physical activity should remain at the same level during the study as before the study start. However, knowing the beneficial effects of physical activity, the advice to the participants had to be formulated in accordance with the customary health care guidelines, recommending a daily dose of at least 30 minutes of moderately intensive physical activity. This was the advice given to the participants in our randomised study. Importantly, we calculated the daily energy expenditure in physical activity using the Actiheart monitors, such that we could demonstrate that there were no changes in physical activity over time between or within study groups.

# Summary and conclusions

To the best of our knowledge, the studies in this thesis are the first diet interventions that have evaluated short- and long-term effects of *ad libitum* diets with special emphasis on liver fat accumulation. We found that a Palaeolithic-type diet (PD) had beneficial effects on anthropometric measurements, including weight and fat mass, with concomitant reduction of liver fat content. In contrast, we found only short-term effects (up to 6 months) on hepatic insulin sensitivity. In our 5-week intervention, we also found that a PD did not influence muscle fat content, and that there was a corresponding lack of effect on peripheral insulin sensitivity. The long-term effects on anthropometric variables and liver fat did not differ between the PD and a diet according to the Nordic Nutritional Recommendations (NNR), but the PD group showed significantly lower triglyceride levels after 24 months.

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