Effects of a Paleolithic diet and exercise on liver fat, muscle fat and insulin sensitivity

Julia Otten
Everything should be made as simple as possible, but not simpler.

Albert Einstein (1879–1955)
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Results

Weight loss by the Paleolithic diet
All study participants reduced liver fat through the Paleolithic diet
Improvement of insulin sensitivity by the Paleolithic diet
No strong association between liver fat and hepatic insulin sensitivity
The reduction in liver fat and muscle fat by the Paleolithic diet is reversed by exercise training
Improvement in blood lipids with the Paleolithic diet
Blood pressure reduction through the Paleolithic diet
The fasting index Revised QUICKI is the most appropriate measure of insulin sensitivity in large-scale clinical studies

Discussion

Paleolithic diet improves cardiovascular risk factors
Liver fat
Blood lipids
Insulin sensitivity
Blood pressure
Is eating a Paleolithic diet possible?
Exercise does not improve cardiovascular risk factors beyond the improvement from the Paleolithic diet
Exercise reverses the effect of a Paleolithic diet on muscle and liver fat
No straightforward connection between fat content and insulin sensitivity in muscle and liver
Measuring insulin sensitivity
Limitations

Conclusions and future perspectives
Acknowledgements
References
Abstract

Finding ways to reduce risk for obesity-related disorders, including type 2 diabetes and cardiovascular disease, is important. Such approaches can include lifestyle interventions by diet and exercise. Our ancestors in the Paleolithic Era ate a diet based on vegetables, fruit, berries, lean meat, fish, seafood, nuts and eggs. Cereals, dairy products and legumes were not a significant part of the diet before the agricultural revolution, and neither were added sugar or salt. Furthermore, our ancestors were much more physically active compared to the average Western population.

Contemporary hunter-gatherers like the Kitava Islanders and the Greenlandic Inuit eat a diet similar to that of the Paleolithic Era and have a strikingly low frequency of cardiovascular events. Detailed studies of the metabolic effects of the Paleolithic diet, with and without exercise, are therefore warranted.

Impaired insulin sensitivity is a key factor in the development of type 2 diabetes and cardiovascular disease. In this thesis, insulin sensitivity was measured with the gold-standard examination – the hyperinsulinemic–euglycemic clamp – and also with fasting blood samples and the oral glucose tolerance test. We found the fasting index Revised QUICKI to be the best choice if the time-consuming gold-standard examination is not feasible. However, to distinguish insulin sensitivity of different tissues like skeletal muscle, liver and adipose tissue, the hyperinsulinemic–euglycemic clamp is preferred.

In our studies, the Paleolithic diet improved cardiovascular risk factors like overweight, insulin sensitivity, liver fat, triglycerides and blood pressure in obese, postmenopausal women. All study participants decreased liver fat when eating a Paleolithic diet. Six months of Paleolithic diet improved weight, liver fat and triglycerides significantly more than a conventional low-fat diet in obese, postmenopausal women. It was difficult for the women to remain adherent to the Paleolithic diet for 2 years, however, and most cardiovascular risk factors showed some degree of deterioration between 6 and 24 months. In individuals with type 2 diabetes, a Paleolithic diet for 12 weeks improved weight, insulin sensitivity, HbA1c, triglycerides and blood pressure. Exercise training did not improve these cardiovascular risk factors beyond the changes observed with the Paleolithic diet alone. The 12-week Paleolithic diet intervention also reduced muscle fat and liver fat, but exercise training reversed this effect.

A Paleolithic diet has strong effects on fat content in liver and muscle and on insulin sensitivity. Our present results indicate reduced metabolic flexibility in the fat content in liver and muscle tissue among patient with type 2 diabetes, which may improve through diet and exercise intervention.
Original papers


Abbreviations

AU  Arbitrary units
AUC  Area under the curve
C   Carbon
C   Concentration of unlabeled glucose
C*  Concentration of labeled glucose
CI  Confidence interval
da/dt Change of enrichment over a period of 30 min
EMCL Extramyocellular lipid
FIRI Fasting insulin resistance index
G0  Fasting glucose
G90, G120 Glucose 90 and 120 min after 75 g glucose
Gmean Mean glucose during OGTT
GIR Glucose infusion rate
H   Hydrogen
H*  Deuterium
H0  External magnetic field
HDL High-density lipoprotein
HOMA-IR Homeostasis model assessment of insulin resistance (fasting index of insulin sensitivity)
HOMA-%S HOMA of insulin sensitivity (computer-generated fasting index of insulin sensitivity)
I0  Fasting insulin
I30/I60/I90/I120 Insulin 30, 60, 90 and 120 min after 75 g glucose
Imean Mean insulin during OGTT
IMCL Intramyocellular lipid
LDL Low-density lipoprotein
Liver IR index Liver insulin resistance index (OGTT-based index of insulin sensitivity)
MRI Magnetic resonance imaging
NAFLD Non-alcoholic fatty liver disease
NEFA Non-esterified fatty acids
O   Oxygen
OGIS Oral glucose insulin sensitivity (OGTT-based index of insulin sensitivity)
OGTT Oral glucose tolerance test
QUICKI Quantitative insulin sensitivity check index (fasting index of insulin sensitivity)
ppm Parts per million
Ra  Rate of appearance of endogenous glucose
Ra* Rate of appearance of labeled glucose
Rd  Rate of disappearance of glucose
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<tr>
<td>$R_a^*$</td>
<td>Rate of disappearance of labeled glucose</td>
</tr>
<tr>
<td>$r$</td>
<td>Pearson’s correlation coefficient</td>
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<tr>
<td>$r_s$</td>
<td>Spearman's correlation coefficient</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>Stumvoll ISI</td>
<td>Stumvoll insulin sensitivity index (OGTT-based index of insulin sensitivity)</td>
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<td>Stumvoll MCR</td>
<td>Stumvoll metabolic clearance rate (OGTT-based index of insulin sensitivity)</td>
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<td>TG</td>
<td>Triglycerides</td>
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<td>V</td>
<td>Volume of distribution</td>
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<td>VLDL</td>
<td>Very low-density lipoprotein</td>
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Sammanfattning på svenska


Nutida ursprungsbefolkningar på ön Kitava och Grönland lever fortfarande som jägare-samlare och åter en diet liknande den man åt under den paleolitiska perioden. De har en påfallande låg frekvens av kardiovaskulära sjukdom vilket gör det högintressant att studera de metabola effekterna av paleolitisk kost, med och utan tillägg av fysisk aktivitet.

Nedsatt insulinkänslighet är en nyckelfaktor för utveckling av typ 2-diabetes och kardiovaskulära sjukdom. I denna avhandling mättes insulinkänslighet med referensmetoden hyperinsulinemisk euglykemisk clamp samt med fasteblodprover och oral glukosbelastning. Fasteindexmätten Revised QUICKI var det bästa alternativet om inte den tidskrävande referensmetoden var möjlig att utföra. För att studera insulinkänslighet i olika vävnader som skelettmskel, lever och fett är den hyperinsulinemiska euglykemiska clampen att föredra.

I våra studier förbättrade paleolitisk kost kardiovaskulära riskfaktorer som övervikt, insulinkänslighet, leverfett, triglycerider och blodtryck hos överviktiga, postmenopausala kvinnor. Alla studiedeltagare som åt paleolitisk kost minskade sitt leverfett. Efter sex månader med paleolitisk kost minskade kroppsvikt, leverfett och triglycerider signifikant mer än efter en kost enligt Nordiska Näringsrekommendationer hos överviktiga, postmenopausala kvinnor. Fölsamheten till den paleolitiska kosten avtog under studien och de flesta kardiovaskulära riskfaktorerna uppvisade en viss försämring mellan 6 och 24 månader.

Tolv veckor med paleolitisk kost förbättrade vikt, insulinkänslighet, HbA1c, triglycerider och blodtryck hos patienter med typ 2-diabetes. Fysisk träning hade ingen tilläggseffekt utöver de förändringar som observerades med enbart den paleolitiska kosten. Dessutom minskade den paleolitiska kosten fetthalten i muskel och lever, men fysisk träning reverserade denna effekt.

En paleolitisk kost har kraftiga effekter på fettnäring i muskel och lever samt på insulinkänslighet. Hos patienter med typ 2-diabetes har fetthalten i muskel och lever en sämre metabol flexibilitet, vilken dock kan förbättras genom kostförändringar och träning.
Background

Western lifestyle causes disease
Cardiovascular disease is the leading cause of death in Sweden and other developed countries (1). A Western lifestyle may be the most important reason for cardiovascular disease and also for overweight, type 2 diabetes, cancer, depression and Alzheimer’s disease (2-4). This association may be explained by a mismatch between a modern lifestyle and a metabolism programmed for hunter-gatherers (5). This hypothesis is strengthened by the fact that contemporary hunter-gatherers have a strikingly low frequency of these Western diseases (6-9). Both dietary habits and physical activity have changed since humans were hunter-gatherers but may not be equally important for the development of diseases of modern civilization.

Humans have lived on earth for at least 2.3 million years (10) and for more than 99% of that time as hunter-gatherers. The Old Stone Age (Paleolithic Era) began 2.3 million years ago and lasted until 10,000 years BC, when the New Stone Age (Neolithic Era) started. During the New Stone Age, our ancestors started to grow grains and keep livestock. Not more than 200 years ago, the industrial revolution took place, which changed dietary habits even further and decreased physical activity substantially. The past 12,000 years have not been long enough for the human genome to adapt to new alimentary conditions and physical inactivity (11, 12). Our metabolism is still unchanged compared to our Paleolithic ancestors (13).

How has lifestyle changed since the Paleolithic Era?

Physical activity
During the Paleolithic Era, males hunted game for 1–4 days per week, and the women gathered food every 2–3 days (12). Thus, our ancestors practiced high- and low-intensity physical activity with periods of less activity in-between. Compared to a modern Western sedentary individual, our Paleolithic ancestors engaged in four times more physical activity (14). Industrialization caused the most marked decline in physical activity: before the industrial revolution, workers expended 3000 kcal/day, but individuals of a contemporary Western population expend only 2000 kcal/day or less (15). The reduction in physical activity continues: during the past 50 years, energy expenditure due to work has decreased by at least 100 kcal/day (16).
Diet
During the Paleolithic Era, the staple foods of our ancestors were fruit, shoots, roots, bulbs, nuts, non-grass seeds, insects, larvae, eggs, shellfish, fish and meat (17).

The diet of our ancestors 2–6 million years ago was mostly plant-based (18). Starchy vegetables like roots and tubers became staple food during periods of dry and cold climate 1–2 million years ago or even before that (19, 20). Two million years ago, Homo erectus and their descendants became capable of hunting, which is more energy efficient compared to gathering plants (11, 21). About 230,000 years ago, our ancestors started to cook their food, making it easier to digest (19, 22). How much of the energy intake that was plant-based or animal-based may have varied widely (5). Wild vegetables and fruits consumed during the Paleolithic Era were different from their cultivated counterparts that we consume today. For example, uncultivated fruits contain more than three times more fibers than commercial ones (23), as illustrated in Figure 1 showing a wild banana compared to a cultured one.

![Figure 1 Wild banana (A) and cultured banana (B)](image)

Also, the game meat hunted by our ancestors is not comparable to the meat we buy at the supermarket today. Wild animals are leaner and have a higher proportion of mono- and polyunsaturated fatty acids compared to domestic animals (24). Our ancestors in the Paleolithic Era consumed large quantities of fish and shellfish, which resulted in a high intake of omega-3 polyunsaturated fatty acids (25). The mammalian brain consists mostly of long-chain polyunsaturated fatty acids. The high consumption of marine animal flesh may have played a central role in the evolution of the human brain (25).

Before the Neolithic Era, cereals (grass seeds) and dairy products were not a noticeable part of the human diet. When humans started to use agriculture, their life expectancy decreased from about 40 to 20 years. This
was caused by lower nutritional quality, which according to archeological findings also resulted in lower final height and an increase in nutritional stress and infections (26-28). Since the industrial revolution, dietary habits have changed even further, and refined carbohydrates like white flour and sugar have become staple foods. If we compare our contemporary Western diet to the food of our Paleolithic ancestors, only 25% of our modern food has the same origin, and the remaining calories come from grains, dairy products, refined fat, sugar and legumes (17). Because of its high proportion of refined fat, sugar and cereals, the Western diet has a much lower content of minerals, vitamins and trace elements compared to the Paleolithic diet with its high representation of fruit, vegetables, meat and fish (29).

**Hunter-gatherers today**

Because of the obvious difficulties in examining hunter-gatherers of the Paleolithic Era, it is compelling to investigate contemporary hunter-gatherers.

Lindeberg and colleagues evaluated the 2,300 inhabitants of Kitava, an island in the archipelago of Papua New Guinea (7). The residents live exclusively on vegetables, fruit, fish and coconut. Fruit and root vegetables like sweet potatoes are staple foods, which results in a high carbohydrate intake. Coconut is consumed in large amounts, resulting in a saturated fat intake comparable to the Swedish population (30). Strikingly, ischemic heart disease and stroke are virtually absent in the Kitava population although 6% are 60 to 95 years old. The conclusion of this observational study calls into question the role of a high intake of carbohydrates and saturated fat for the development of cardiovascular disease.

The traditional diet of Greenlandic Inuit is exclusively based on animal food. Cardiovascular disease is nearly absent in this hunting tribe (8, 9). Compared to Danes, Inuit have a higher intake of protein, monounsaturated fatty acids and polyunsaturated fatty acids (mostly omega-3). However, they eat comparably less saturated fat and carbohydrates (8, 9). The blood lipids of the Inuit showed lower triglycerides and LDL cholesterol and higher HDL cholesterol compared to the Danes. The implication is that a high consumption of animal products is not directly linked to dyslipidemia and cardiovascular disease.

The Yanomamo Indian tribe in Northern Brazil does not use added salt. The mean blood pressure in this population is 96/61, and blood pressure does not increase with age (31). In women, systolic blood pressure actually falls with age. We can therefore conclude that the rise in blood pressure we see in the Western world is not due to age but rather is related to our lifestyle.
What is the underlying cause of cardiovascular disease?
In seeking to understand the connection between lifestyle and cardiovascular disease, it is tempting to search for a link in human metabolism that connects these two.

**High cholesterol causes cardiovascular disease**
The American Heart Association proposed in 1961 that cholesterol is the link between Western lifestyle and cardiovascular disease (32). The authors therefore recommended a diet low in saturated fat and cholesterol to prevent cardiovascular disease. This suggestion has influenced national nutrition recommendations for decades and still does. However, the nutritional advice of the American Heart Association was based on two assumptions: 1) Cardiovascular disease is caused by high blood cholesterol levels. 2) Blood cholesterol levels are caused by dietary intake of saturated fat and cholesterol. Neither of these two assumptions has been proven. During the past decades, though, cholesterol-lowering drugs (mostly statins) were able to substantially decrease the risk for coronary heart disease and stroke. Whether statins decrease the risk of cardiovascular disease by decreasing cholesterol or by other mechanisms is not known. The biostatistician Richard Kronmal explained this non-causal relationship: “Saying that statins reduce heart-disease risk by lowering cholesterol is like saying that aspirin reduces heart-disease risk by reducing headaches” (33). That the reduction of saturated fat in the diet reduces cardiovascular risk is yet another issue. A Cochrane meta-analysis showed a small decrease in cardiovascular events when saturated fat was replaced by unsaturated fat (34). However, neither total nor cardiovascular mortality decreased, and women did not reduce their risk for cardiovascular events at all. The same meta-analysis could not prove a decrease in cardiovascular events by reducing fat intake.

**Insulin resistance causes cardiovascular disease**
Gerald Reaven declared in his famous Banting lecture in 1988 that insulin resistance is the underlying cause of cardiovascular disease (35). He had observed the cluster of cardiovascular risk factors of overweight, hypertension, high triglycerides levels, low HDL levels and glucose intolerance that later on was called the metabolic syndrome (36). Reaven preferred the term ‘insulin resistance syndrome’ to suggest a causal relationship (37). However, a direct causal relationship between high insulin levels and atherosclerosis has not been proven. It is possible that insulin acts on blood vessels directly (38), by e.g. inducing growth of smooth muscle cells (39). Reaven proposed to treat insulin resistance by weight loss and to reduce insulin levels through carbohydrate restriction (37).
**Insulin sensitivity**

Impaired insulin sensitivity (insulin resistance) together with insufficient insulin secretion is the underlying cause of type 2 diabetes. On a population basis, insulin sensitivity decreases with increasing weight (40), but this association has exceptions, and individuals with impaired insulin sensitivity can be normal weight and vice versa (40). Insulin sensitivity can change within a few days, for example through disease or exercise training. If insulin sensitivity deteriorates, insulin secretion needs to increase to prevent blood glucose levels from rising. If insulin secretion cannot increase any further, blood glucose rises and the individual develops type 2 diabetes. The level of insulin sensitivity at which type 2 diabetes arises depends on individual ability to increase insulin secretion. Thus, individuals without diabetes can have poor insulin sensitivity if they have a sufficient ability to secret insulin.

Insulin acts on many different tissues in the human body, but I will limit my discussion to skeletal muscle, liver and adipose tissue (Figure 2). Before discussing insulin sensitivity in different organs, it is important to note that not all tissues have the same level of insulin sensitivity. If impaired insulin sensitivity of one organ causes insulin levels to rise, other insulin-sensitive tissues may experience those insulin levels as extremely high and can react pathologically (37). For example, the kidney stays sensitive to insulin even if muscle and adipose tissue are insulin resistant (41). Thus high insulin levels increase renal sodium retention, which increases blood pressure.

**Skeletal muscle**

Insulin sensitivity in the skeletal muscle is by far the most studied. After binding to its receptor in the skeletal muscle cell membrane, insulin facilitates glucose uptake into the muscle cell by the glucose transporter GLUT4. In case of impaired insulin sensitivity, higher amounts of insulin are needed for the same amount of glucose transported into the muscle cell. If insulin secretion cannot increase any further, not all glucose can be taken up into the muscle, and blood glucose rise (Figure 2).

**Liver**

The liver keeps blood glucose levels stable in the fasting state, which is most important for tissues that can metabolize only glucose and not fat, e.g. erythrocytes and the brain. This organ and these cells need a continuous glucose supply via the bloodstream even in the fasting state. After a meal, blood glucose and insulin secretion rise simultaneously. Insulin is the signal for the liver to cease glucose output and instead to store glucose as glycogen for later use. Glucose output continues if the liver is not sensitive to insulin, which causes blood glucose to rise (Figure 2).
**Adipose tissue**

During the fasting state, adipose tissue is responsible for energy supply of most tissues in the body. Energy is stored as triglycerides in the adipocyte. With fasting, triglycerides are broken down into three non-esterified fatty acids (NEFA) and one glycerol, which are all secreted into the bloodstream. After a meal, the rising insulin secretion is supposed to suppress triglyceride breakdown, which results in decreasing NEFA in the blood. With impaired insulin sensitivity of the adipose tissue, insulin cannot suppress NEFA production. As a consequence, NEFA rises in the bloodstream (Figure 2).
One could argue that a continuous breakdown of adipose tissue would be of advantage and promote weight loss. However, a healthy body is supposed to save its energy stores if energy is supplied by food. Of importance, high NEFA levels in the bloodstream may lead to fat storage in organs other than the adipose tissue.

**Fat storage in organs other than adipose tissue**

If fat is stored in organs other than adipose tissue, that process is called ectopic lipid deposition. High amounts of lipid accumulation may affect organ function. So far, most work has been published on ectopic fat deposition in muscle and liver, but fat accumulation in pancreas and heart also has been investigated. Fat storage in liver and muscle is more common in patients with type 2 diabetes and in overweight subjects compared to lean and healthy individuals (42, 43). It is debated if fat deposition in liver and muscle impairs insulin sensitivity or if the association is not causal (44, 45).

To store ectopic fat, the organs take up fat from the plasma pool (Figure 3). Most ectopic fat probably comes from lipolysis of adipose tissue, which results in albumin-bound NEFA in the plasma pool (46). Moreover, fat in the plasma pool originates from chylomicrons and very low-density lipoprotein (VLDL) particles. After food intake, chylomicrons, which contain triglycerides from dietary fat, enter the plasma pool. In addition, the liver secretes VLDL particles containing triglycerides. Triglycerides in chylomicrons and VLDL particles are hydrolyzed in smaller capillaries, and the different organs take up the resulting NEFA.

Even if the plasma supply of fat is the most important player for ectopic fat deposition, at least two other factors affect the actual amount of fat in the specific organ:

1) *De novo lipogenesis* (synthesis of NEFA from carbohydrates)
2) NEFA oxidation (how much fat is used as energy supply for the actual organ)
Figure 3 Ectopic fat deposition: Where does the fat come from and where does it go? Triglycerides (TG) enter the plasma fat pool in the form of chylomicrons from food or as very low-density lipoprotein (VLDL) particles from the liver. Albumin-bound non-esterified fatty acids (Albumin-NEFA) enter the plasma fat pool from the adipose tissue. Liver, pancreas, heart and skeletal muscle take up Albumin-NEFA and TG from VLDL particles and chylomicrons to store as ectopic fat.
**Lipid metabolism in the liver**

The liver plays a central role in glucose and lipid metabolism and links the two. All fat taken up by the liver enters a common NEFA pool (47, 48). From there, it is directed to its different fates (Figure 4). Most of the NEFA in the liver pool come from lipolysis of adipose tissue (46). The uptake of plasma NEFA in the liver cell is independent of insulin and depends only on the concentration of plasma NEFA (49). This feature is a major difference from glucose metabolism in the liver, which is tightly regulated by insulin. After a meal, dietary fat enters the liver NEFA pool via chylomicron remnants and NEFA (Figure 4). Dietary carbohydrates may enter the liver NEFA pool after being reduced by *de novo lipogenesis*.

NEFA from the common liver pool may either be oxidized to meet the liver energy needs or excreted as triglycerides in VLDL particles (Figure 4). If the influx of NEFA to the common liver pool exceeds the capacity of removal by oxidation or excretion as VLDL particles, NEFA will be stored in the liver cells as triglycerides (50).

Triglycerides excreted as VLDL particles and those stored as liver fat contain the same amount of NEFA originating from adipose tissue, dietary fat and *de novo lipogenesis*, respectively (46). This similarity confirms the existence of a common NEFA pool in the liver. It also suggests the possibility that liver fat and VLDL-triglycerides act in a similar way during intervention.

A healthy liver can change its metabolism rapidly from the fed state, where the plasma supply of carbohydrate and fat is high, to the fasted state, where glucose output is an essential task of the liver. An example of this flexibility is the ability of the liver to change its rate of *de novo lipogenesis*, which should be high if carbohydrates are available and low otherwise. In the fasting state, *de novo lipogenesis* accounts for 5% of all triglycerides appearing in plasma in healthy individuals and for 24% in individuals with high liver fat content (46, 51). Healthy individuals increase *de novo lipogenesis* 5-fold after a carbohydrate-containing meal (51). In individuals with high liver fat, no increase in *de novo lipogenesis* is seen after a meal (46). We may therefore conclude that *de novo lipogenesis* already has reached its maximum in individuals with high liver fat content and that the flexibility in substrate metabolism is lost.
Figure 4 Lipid metabolism in the liver. Non-esterified fatty acids (NEFA) enter the common NEFA pool from adipose tissue and from food. If NEFA do not leave the common NEFA pool by excretion as very low-density lipoprotein (VLDL) particles into the blood or by oxidation, they stay in the liver as liver fat.
Non-alcoholic fatty liver disease (NAFLD)
Liver fat content over 5.6% is considered abnormal and is called non-alcoholic fatty liver disease (NAFLD) if other underlying causes – mainly alcohol consumption – are excluded (52). NAFLD is a very common condition: in the United States, it is the most common cause of elevated liver enzymes (53). Obesity and type 2 diabetes are closely associated with NAFLD (54, 55). Patients with type 2 diabetes have a NAFLD prevalence of about 70%. NAFLD may cause steatohepatitis, cirrhosis and hepatocellular carcinoma, and effective therapy is therefore of great importance (56). Moreover, NAFLD is a risk factor for cardiovascular disease and death (57).

Treatment of NAFLD

Diet
Weight loss is the most effective way to treat NAFLD (56). Calorie restriction decreases liver fat in a matter of days: a reduction of energy intake by about 1000 kcal decreases liver fat content 30% after 2 days and 45% after 6 days in individuals with NAFLD (58, 59). However, it is debated which diet composition is most effective. It is difficult to decide on a theoretical background if a diet low in carbohydrate or low in fat would be the better option. A low-carbohydrate diet would decrease de novo lipogenesis in the liver. By contrast, with a diet low in fat, fewer chylomicron remnants and NEFA from the diet would enter the liver. A higher carbohydrate proportion would at the same time increase insulin secretion, which would block lipolysis in adipose tissue. Because most liver fat comes from adipose tissue lipolysis, a diet with higher carbohydrate content is more promising on a theoretical basis. Two isocaloric studies showed a decrease in liver fat only for the low-fat diet and an increase in liver fat for the comparison low-carbohydrate diet (60, 61). However, two short hypocaloric studies showed the low-carbohydrate diet to be more effective than the low-fat diet in reducing liver fat (58, 62), although hypocaloric studies of longer duration could not confirm this different effect on liver fat (58, 63). In conclusion, the issue of whether a diet low in fat or low in carbohydrate should be recommended to decrease liver fat is not resolved.

Another option to treat NAFLD is to influence fatty acid composition. In an isocaloric 10-week trial, polyunsaturated fatty acids decreased liver fat, but saturated fatty acids increased liver fat (64). Overfeeding with saturated fatty acids for 7 weeks increased liver fat, but during overfeeding with polyunsaturated fatty acids, liver fat remained unchanged (65). Two studies showed liver fat improvement by supplementation with omega-3 polyunsaturated fatty acids (66, 67). It has been suggested that polyunsaturated fatty acids decrease liver fat because they are more easily oxidized compared to saturated fatty acids, they inhibit
de novo lipogenesis and they are more often used as phospholipids to build cell membranes (64, 68).

It has been discussed if fructose can cause NAFLD. High fructose corn syrup was introduced in the United States in 1967 and is widely used in sweetened beverages. Its consumption increased during the past decades and overweight and metabolic diseases like NAFLD were increasing simultaneously. Human studies showed that de novo lipogenesis is doubled if fructose is consumed together with glucose compared with glucose alone (69). Results from mostly animal studies have suggested increased liver fat with high fructose corn syrup consumption (70). However, a meta-analysis of human studies concludes that fructose increases liver fat only if consumed as excess energy and not as isocaloric exchange for other carbohydrates (71).

Physical activity
It is uncertain if exercise training decreases liver fat independently of weight reduction. A study with either aerobic or resistance training for 4 months showed decreased liver fat in patients with type 2 diabetes, but the intervention resulted in a small but significant weight loss (72). However, aerobic exercise combined with diet intervention has not been shown to reduce liver fat beyond the effect of the diet intervention alone (73, 74).

Treatment of type 2 diabetes with diet and physical activity
According to observational studies, 91% of type 2 diabetes and 82% of coronary heart disease are caused by Western lifestyle (75, 76), which implies that a healthy lifestyle may protect from these diseases of civilization. We may therefore recommend lifestyle changes to patients with type 2 diabetes. However, observational studies do not prove that individuals with type 2 diabetes will decrease their risk for cardiovascular disease and death if they change their lifestyle. Large randomized controlled trials over long time periods are needed. With this intention, the Look AHEAD trial was started. Standard care for patients with type 2 diabetes was compared to intensive lifestyle intervention with calorie restriction and increased physical activity. After a median follow-up of 9.6 years, the trial was terminated early because intensive lifestyle intervention did not reduce cardiovascular events (77). Aside from the Look AHEAD trial, hardly any long-term studies have investigated the effect of diet or exercise on cardiovascular events.

A Mediterranean diet is the only diet that has decreased cardiovascular risk in two long-term studies (78, 79). In one of the studies, half of the study population had type 2 diabetes (78). Hence, a Mediterranean-type diet is the only evidence-based diet to be recommended to patients with type 2 diabetes. It is therefore of interest that a Paleolithic diet proved superior to a Mediterranean diet in improving glucose tolerance.
in a 3-month trial in individuals with type 2 diabetes (80). So far, long-term prospective studies on the Paleolithic diet are lacking.

Sedentary behavior is a strong contributor to death in many individuals with coronary heart disease and diabetes (81). In prospective observational studies, increased physical activity resulted in a decreased mortality in individuals with type 2 diabetes (82). However, long-term randomized studies that prove the effect of physical activity on cardiovascular risk in individuals with type 2 diabetes are lacking. Short-term randomized trials have investigated the effect of different exercise modalities on cardiovascular risk factors. The combination of resistance and aerobic exercise has been shown to improve glycemic control compared to either resistance or aerobic exercise alone (83-85). For best effect, exercise duration should be at least 150 min per week (86).
Hypotheses

The main aim of this thesis was to investigate the effect of a Paleolithic diet on liver fat and insulin sensitivity in comparison to a conventional low-fat diet and in comparison to the combination of Paleolithic diet and exercise.

A Paleolithic diet was combined with exercise in a randomized controlled trial for the first time. Moreover, our research group is, to the best of our knowledge, the first to investigate the effects of a Paleolithic diet on fat content in liver and muscle, including the gold-standard examination for insulin sensitivity to assess the effects of a Paleolithic diet on hepatic and adipose tissue insulin sensitivity. We compared a Paleolithic diet to a conventional low-fat diet during a 2-year randomized trial. So far, the longest randomized trial with a Paleolithic diet has been 3 months.

Specific hypotheses:

Paper I: A Paleolithic diet improves liver fat and insulin sensitivity more than a conventional low-fat diet.

Paper II: A Paleolithic diet improves body composition and metabolic balance. The addition of aerobic exercise and resistance training improves these outcomes further.

Paper III: A Paleolithic diet decreases liver fat and muscle fat and increases insulin sensitivity. The addition of aerobic exercise and resistance training increases insulin sensitivity and muscle fat but decreases liver fat.

Paper IV: OGTT-based indices correlate more strongly than fasting indices to the gold-standard measurement of insulin sensitivity, the hyperinsulinemic–euglycemic clamp.
Study design

Paper I
Postmenopausal women with a BMI above 27 kg/m² were randomized to either a Paleolithic diet or a conventional low-fat diet (Figure 5). Exclusion criteria were smoking, diabetes, hypertension, heart disease, and any other severe illness, medication with statins or beta-blockers and consumption of a restricted diet. A total of 210 women were interested in participating in the study, but only 70 met the inclusion criteria. Forty-one women completed the examinations for liver fat and were included in the analyses (Figure 5).

Figure 5 Study design for paper I. Not all women could perform liver fat examinations with MRI because they were bearing implanted metal, refused examination or the liver spectroscopy failed. For each time point, the number of participants with liver fat examination is depicted in the figure.

Papers II and III
Patients with type 2 diabetes were randomized to either a Paleolithic diet with exercise training under supervision 3 hours per week (Paleolithic diet + Exercise group) or to eating the Paleolithic diet only (Paleolithic diet group, Figure 6). The study period was 12 weeks. Both intervention groups received exercise recommendations based on the existing guidelines for patients with type 2 diabetes. Study participants were included if they had a BMI between 25 and 40 kg/m² and were 30 to 70 years of age. Women were included only after menopause. Exclusion criteria were smoking, cardiovascular disease, use of beta-blockers, severe illness and higher amounts of training. A total of 261 individuals were interested in participating, but only 32 fulfilled the inclusion criteria. Twenty-nine subjects performed the final examinations at 12 weeks and were included in paper II. Twenty-six individuals completed the hyperinsulinemic–euglycemic clamp and were included in paper III (Figure 6).
Figure 6 Study design for papers II and III. After randomization, two individuals did not continue the study because of a lack of time and disappointment with allocation. One participant did not perform the final measurements at 12 weeks. Three individuals could not be analyzed: two did not perform the clamp examination due to illness, and one clamp examination did not succeed.

Paper IV
We searched Medline (1979–2012) to find all articles reporting bivariate correlations between the reference measurement of insulin sensitivity, the hyperinsulinemic–euglycemic clamp and surrogate indices. We included studies that reported surrogate measures based on the oral glucose tolerance test (OGTT) or on fasting blood samples.
Methods

The Paleolithic diet
Study participants were instructed to eat a Paleolithic diet for 2 years in paper I and for 12 weeks in papers II and III. The diet was based on fish, seafood, lean meat, vegetables, fruit, berries, nuts and eggs (Figure 7A). Dairy products, cereals, legumes and added salt and sugar were excluded from the diet. The diet was consumed without restriction of calorie intake. Study participants had group sessions led by a trained dietician during the whole study period and learned to prepare the Paleolithic diet in a cooking class.

The conventional low-fat diet
The conventional low-fat diet in paper I was based on the Nordic Nutrition Recommendations (87). The participants were advised to increase their intake of whole grain, fruit, vegetables and fish (Figure 7B). Dairy products and meat were low fat and calorie intake was not restricted. Also participants eating the low-fat diet had group sessions with a trained dietician and learned to prepare the diet in a cooking class.

Exercise training
The participants in the Paleolithic diet + Exercise group in papers II and III underwent an exercise program with 1-h sessions three times weekly of combined aerobic and resistance training. All sessions started with aerobic exercise. The first session each week consisted of exercise at 70% of the maximum heart rate on a cross-trainer. The second session of the week started after warm-up with 10 sprint intervals at 100% of the maximal workload on a cycle-ergometer. The third session each week comprised six 5-min intervals at 45 to 60% of maximal workload on a cycle-ergometer. After aerobic exercise, the sessions progressed to resistance training with both lower and upper body exercises, including lat pull-downs, seated rows, dumbbell rows, shoulder raises, flat and incline bench presses, leg curls, hip raises, leg presses, seated leg extensions, back extensions, burpees, sit-ups, step-ups, and wall ball shots. The intensity of the training increased during the 12-week study period and was adjusted to the participant’s performance.
Figure 7 A Paleolithic diet breakfast (A) and a conventional low-fat diet breakfast (B).
Measuring liver fat with proton magnetic resonance spectroscopy

To assess liver fat content, the study participants were examined in a MRI scanner. We examined a cube of 2 cm × 2 cm × 2 cm in the right liver lobe (Figure 8). To estimate liver fat percentage, we measured the proportion of fatty acids and water molecules in this area. Fatty acids and water contain hydrogen atoms (H), which can be detected by magnetic resonance spectroscopy due to the magnetic properties of the nucleus of the hydrogen atom. The nucleus of a hydrogen atom contains one single proton and no neutron and therefore the examination is called proton magnetic resonance spectroscopy.

The single proton makes the hydrogen nucleus a magnetic dipole; in other words, the nucleus behaves like a small bar magnet. It is therefore possible to detect the hydrogen nucleus with spectroscopy. In contrast, the nucleus of the most common isotope of carbon (¹²C) is made up of 6 protons and 6 neutrons and has no magnetic property.

Figure 8 MR image of the liver. A cube of 2 cm × 2 cm × 2 cm is outlined in the right liver lobe and examined with spectroscopy to determine the proportion of fatty acids and water molecules in this area.
Normally, the orientations of the magnetic dipoles of the hydrogen nuclei are random (Figure 9A). When a subject is placed in a MRI scanner, a strong magnetic field ($H_0$) is applied to this individual (Figure 9B). This external magnetic field causes the magnetic dipoles of the study subject to align in the same direction. If energy in the form of radio waves is applied to the cube in the right liver lobe, the magnetic fields of the hydrogen nuclei flip to the opposite direction (Figure 9C). When the radio waves are turned off, the magnetic fields of the hydrogen nuclei relax back to their initial state, which is in alignment with the external magnetic field (Figure 9D).

**Figure 9** The basics of proton magnetic resonance spectroscopy. Initially, the magnetic orientation of a person’s hydrogen nuclei is random (A). When an external magnetic field $H_0$ is applied, the magnetic dipoles of the hydrogen nuclei align in the same direction as $H_0$ (B). If in addition radio waves are applied, the magnetic fields of the hydrogen nuclei flip to the opposite direction (C). When the radio waves are turned off, the magnetic fields of the hydrogen nuclei relax back into alignment with the external magnetic field $H_0$, causing a resonance frequency (D).
Relaxation of the hydrogen nuclei creates electromagnetic radiation with the same frequency as the applied radio waves and is called resonance (Figure 9D). The frequency of the radio waves that are able to flip the magnetic field of the hydrogen nuclei to the opposite direction depends directly on the external magnetic field $H_0$. The stronger the $H_0$, the higher the frequency needed. This relationship also means that the resonance of the hydrogen nuclei relaxing back into alignment with the external field $H_0$ depends on the strength of the magnetic field $H_0$. Because MRI scanners have different magnetic fields $H_0$, resonance frequency is instead reported as chemical shift, which is the actual resonance frequency compared to a reference compound. Chemical shift is independent of $H_0$ and therefore equal between different MRI scanners.

Hydrogen atoms in fatty acid molecules (Figure 10A) have different resonance frequencies and concomitantly different chemical shifts compared to hydrogen atoms in water molecules (Figure 10B). With the different chemical shifts, it is obvious that hydrogen nuclei in fatty acid molecules must experience a different external magnetic field compared to hydrogen nuclei in water molecules although the applied external magnetic field $H_0$ is the same. This difference arises from small magnetic fields in the nearest surroundings of the hydrogen nuclei that either increase the shielding against the external magnetic field $H_0$ or decrease it. Important players in that context are binding electrons (Figure 10).

![Chemical structures](image)

*Figure 10* Chemical structures of the fatty acid palmitate (A) and a water molecule (B).
When an external magnetic field $H_0$ is applied to a molecule, the electrons start circulating and generate a weak magnetic field that happens to point in the opposite direction than $H_0$ (Figure 11). The magnetic fields generated by binding electrons in a fatty acid molecule (Figure 11A) decrease the magnetic field strength that the hydrogen nuclei experience from the applied external magnetic field $H_0$. However, in water molecules, the electronegative oxygen atoms pull the binding electrons towards it (Figure 11B). Because the hydrogen nuclei of the water molecule are not as much influenced by the magnetic fields of the binding electrons, they experience more of the external magnetic field $H_0$. Therefore, hydrogen nuclei in a water molecule need higher frequency radio waves to flip their magnetic field to the opposite direction and consequently generate a higher chemical shift compared to hydrogen nuclei in a fatty acid molecule.

**Figure 11** The magnetic field experienced by a hydrogen nucleus depends on the external magnetic field $H_0$ and the proximity of the nucleus to electrons. (A) Part of a fatty acid molecule farthest away from the carboxyl group where small magnetic fields generated by the binding electrons shield the hydrogen nuclei from the external magnetic field $H_0$. In a water molecule (B), the hydrogen nuclei do not experience the same shielding effect from the small magnetic fields of the electrons because the electrons are pulled towards the electronegative oxygen atom and are therefore farther away from the hydrogen nuclei.
The resonance frequency from hydrogen nuclei relaxing back into alignment with the external magnetic field \( H_0 \) is registered as chemical shift in a spectrum (Figure 12). Hydrogen nuclei of water molecules have a chemical shift of about 4.7 parts per million (ppm), which is much higher than the chemical shift of saturated fatty acids, which is mostly between 0.9 and 1.6 ppm. The chemical shift of hydrogen nuclei in a fatty acid molecule depends on the localization of the hydrogen, e.g. its proximity to the oxygen atoms bound to C, (Figure 10A). Liver fat measurements are based on the following hydrogen atoms: hydrogens from the \( \text{CH}_3 \)-group at the far end of the fatty acid molecule (C\(_{16}\) in palmitate, Figure 10A) generating a chemical shift at 0.9 ppm; hydrogens from \( \text{CH}_2 \)-groups in the middle of the fatty acid molecule (C\(_4\)-C\(_{15}\) in palmitate, Figure 10A) creating a chemical shift at 1.3 ppm, and hydrogens from the \( \text{CH}_2 \)-group at C\(_3\) that is two carbons away from the oxygen atoms bound to C, (Figure 10A) and gives a chemical shift at 1.6 ppm (88).

In the liver spectrum (Figure 12), the area under the curve (AUC) represents the amount of hydrogen nuclei measured. The liver spectrum in Figure 12 shows that the liver contains much more hydrogen nuclei associated with water molecules (4.7 ppm) than hydrogen nuclei from fatty acids (0.9 and 1.3 ppm). A computer program estimates the AUCs (89). The percentage of fat in the liver is calculated as the ratio between fatty acid hydrogen nuclei and water hydrogen nuclei plus fatty acid hydrogen nuclei.

**Measuring muscle fat with proton magnetic resonance spectroscopy**

Fat is stored in muscle either as intramyocellular lipid (IMCL) or as extramyocellular lipid (EMCL). IMCL is stored in small lipid droplets inside the muscle cell (90). EMCLs form larger areas of fat in an elongated shape between the muscle cells. Interestingly, IMCLs and EMCLs have different chemical shifts. It is speculated that EMCL, with its orientation along the muscle cells, has its own magnetic moment, which has the same orientation as the muscle fibers (90). In contrast, the lipid droplets of IMCL have random orientation in the cell. The hydrogen nuclei of EMCL therefore experience a stronger magnetic field compared to those of IMCL. For this reason, EMCL creates a chemical shift at 1.5 ppm compared to IMCL at 1.3 ppm. If the muscle fibers are not parallel to the external magnetic field, the chemical shift for IMCL and EMCL will overlap.

IMCL is evenly distributed in muscle, which is not the case for EMCL. Proton magnetic resonance spectroscopy is therefore not recommended for measuring EMCL because a small dislocation of the measured area will alter the results (91).
Figure 12 Spectrum of the liver measured with proton magnetic resonance spectroscopy (89). When hydrogen nuclei of water molecules relax back into alignment with the external magnetic field, they create a chemical shift at 4.7 ppm. Hydrogen nuclei in fatty acid molecules create several chemical shifts depending on the hydrogen’s position in the molecule. The CH$_3$-group at the far end of the fatty acid molecule (C$_{16}$ in palmitate, Figure 10A) shows a chemical shift at 0.9 ppm. CH$_2$-groups in the middle of the fatty acid molecule (C$_{4}$-C$_{15}$ in palmitate, Figure 10A) create a chemical shift at 1.3 ppm.

We examined the soleus and the tibialis anterior muscle. The soleus muscle consists mostly of type I fibers (slow-twitch fibers) and uses predominantly oxidative metabolism as the energy supply. For that reason, it has a high IMCL content (92). In contrast, the tibialis anterior muscle has more glycolytic fibers and lower IMCL content.
In contrast to the measurement of liver fat, we used creatine instead of water as the reference substance to estimate muscle fat (Figure 13). Creatine levels in muscle are stable, even during exercise (92). A computer program estimates the AUCs for the CH$_2$-groups of IMCL and the CH$_3$-group of creatine (89). IMCL content is calculated as the ratio between the CH$_2$-groups of IMCL and the CH$_3$-group of creatine in arbitrary units (AU).

**Figure 13** Measuring fat in soleus muscle with proton magnetic resonance spectroscopy (89). The CH$_2$-groups of extramyocellular lipid (EMCL) create a chemical shift at 1.5 ppm. The CH$_2$-groups of intramyocellular lipid (IMCL) show a chemical shift at 1.3 ppm. The CH$_3$-group of the reference substance creatine has its chemical shift at 3.03 ppm. The signals of the CH$_2$-groups of EMCL and IMCL are not used for calculation of fat content in muscle.
Measuring insulin sensitivity

**Hyperinsulinemic–euglycemic clamp: the gold standard**
The hyperinsulinemic–euglycemic clamp can assess skeletal muscle insulin sensitivity and insulin sensitivity of the adipose tissue. If labeled glucose is infused during the examination, hepatic insulin sensitivity also can be estimated. We labeled glucose in our study with two deuterium atoms (H*) at the sixth carbon of the glucose molecule ([6,6-$^{2}$H$_{2}$]glucose, Figure 14). The labeled molecule is metabolized the same way as unlabeled glucose. By infusing labeled glucose, it is possible to estimate glucose production of the liver.

![Figure 14](image)

*Figure 14 [6,6-$^{2}$H$_{2}$]glucose. Glucose labeled with two deuterium atoms (H*) at the sixth carbon (C$_{6}$).*
On the examination day, the study participant comes to the research facility in the fasting state. After blood sampling, an infusion with labeled glucose is started and continued for 6 h (Figure 15). After 3 h of continuous infusion, the labeled glucose is well mixed with the unlabeled glucose pool (steady state), and endogenous glucose production of the liver (rate of appearance, $R_a^*$) can be assessed. During the last 3 h of the examination, insulin is infused with 40 mIU/m²/min. Blood glucose is measured every 5 minutes, and the rate of a glucose infusion is adjusted to hold blood glucose at 8 mmol/L. The variable glucose administration to keep blood sugar stable at a certain level is the reason why this examination is called clamp. At the end of the examination (after 3 h of insulin infusion), the second steady state is reached. Glucose production of the liver during insulin infusion (rate of appearance during insulin infusion, $R_a$(clamp)) and skeletal muscle insulin sensitivity (rate of disappearance, $R_d$(clamp)) can be assessed.

Figure 15 Operating procedure for the hyperinsulinemic–euglycemic clamp. C, concentration of unlabeled glucose. $C^*$, concentration of labeled glucose. NEFA, non-esterified fatty acids.

The assessment of insulin sensitivity with the hyperinsulinemic–euglycemic clamp is based on the assumption that one single, well-mixed glucose pool exists (Figure 16) (93). We assumed the glucose pool to be 16% of the body weight, which includes not only blood plasma but also large parts of the interstitium.
The examination starts with the infusion of labeled glucose (rate of appearance of labeled glucose, $R_a^*$, Figure 16). At this time point, there is only a small amount of labeled glucose ($C^*$) in the glucose pool, and labeled glucose is leaving the glucose pool ($R_d^*$) more slowly than it is entering it ($R_a^*$). After 2 h 30 min of continuous infusion of labeled glucose ($R_a^*$), the glucose pool is in steady state, which means that labeled glucose enters and leaves the glucose pool at the same rate ($R_a^* = R_d^*$). In the fasting study, participant endogenous glucose production and rate of disappearance of glucose have been in steady state since the beginning of the examination ($R_a = R_d$). With those assumptions, we can calculate the rate of appearance of endogenous glucose ($R_{a,E}$, Equation a). Because inflow and outflow of glucose are not always in steady state, we introduce a second part into the equation for endogenous glucose production that accounts for the concentration changes of glucose and labeled glucose between 2 h 30 min and 3 h ($R_{a,F}$, Equation b).

In analogy with the calculation of endogenous glucose production during the first steady state ($R_a$(basal), between 2 h 30 min and 3 h), we can estimate endogenous glucose production during insulin infusion ($R_a$(clamp), between 5 h 30 min and 6 h). Endogenous glucose production during insulin infusion is supposed to be much lower compared to basal conditions. Hepatic insulin sensitivity is calculated as the suppression of endogenous glucose production during insulin infusion compared to baseline conditions (Equation c).

During the second part of the examination, insulin is infused in a fixed dose depending on body surface area (Figure 15). The amount of glucose needed (GIR) to keep blood glucose at 8 mmol/L is a measure of
skeletal muscle insulin sensitivity \((R_d(\text{clamp})\text{, Equation d})\). \(R_d(\text{clamp})\) is corrected for endogenous glucose production \((R_{a}(\text{clamp}))\) and for the variation in glucose concentration during the measurement time (between 5 h 30 min and 6 h).

Adipose tissue insulin sensitivity is calculated as suppression of NEFA during insulin infusion compared to baseline conditions (Figure 15, Equation e).

\[
\text{(a) } R_a = \frac{R_{a}^{*}}{a}
\]
\[
\text{(b) } R_a = \frac{R_{a}^{*}}{a} - \left[ \frac{V \times C}{a} \times \frac{da}{dt} \times 1000 \right] \div \text{body weight}
\]
\[
\text{(c) Suppression of endogenous glucose production (\%)} = \frac{R_{a}(\text{basal}) - R_{a}(\text{clamp})}{R_{a}(\text{basal})} \times 100
\]
\[
\text{(d) } R_{d}(\text{clamp}) = R_{d}(\text{clamp}) + \text{GIR} - \frac{dC}{dt}
\]
\[
\text{(e) Suppression of NEFA (\%)} = \frac{\text{NEFA at 0h} - \frac{\text{NEFA at 4h} + \text{NEFA at 5h} + \text{NEFA at 6h}}{3}}{\text{NEFA at 0h}} \times 100
\]

Equations for calculation of insulin sensitivity during the hyperinsulinemic–euglycemic clamp. Rate of appearance in steady state (a), rate of appearance in non-steady state (b), hepatic insulin sensitivity as suppression of endogenous glucose production (c), skeletal muscle insulin sensitivity as rate of disappearance in the clamped period (d) and adipose tissue insulin sensitivity as suppression of NEFA (e). Abbreviations: \(R_a\), rate of appearance of endogenous glucose. \(R_{a}^{*}\), rate of appearance of labeled glucose. \(a\), enrichment \((a=C^{*}÷[C+C^{*}]\). \(C\), concentration of glucose. \(C^{*}\), concentration of labeled glucose. \(V\), volume of distribution \((V=0.1625 \times \text{body weight})\). \(\frac{da}{dt}\), change of enrichment over a period of 30 min. \(R_{a}^{*}\), rate of disappearance of labeled glucose. \(R_a\), rate of disappearance of glucose. \(R_{a}(\text{basal})\), rate of appearance of endogenous glucose during the basal state of the clamp (measured from 2 h 30 min to 3 h). \(R_{d}(\text{clamp})\), rate of appearance of endogenous glucose during the clamped period (measured from 5 h 30 min to 6 h). \(R_{d}(\text{clamp})\), rate of disappearance during the clamped period (measured from 5 h 30 min to 6 h). \(\text{GIR}\), glucose infusion rate from 5 h 30 min to 6 h. \(dC/dt\), change of glucose concentration from 5 h 30 min to 6 h. NEFA, non-esterified fatty acids.
**OGTT-based indices**

If the hyperinsulinemic–euglycemic clamp is not feasible, surrogate measures of insulin sensitivity based on fasting blood samples or the OGTT can be used instead.

For the OGTT, 75 g glucose is ingested after an overnight fast. Glucose and insulin are analyzed at baseline and after 30, 60, 90 and 120 min. OGTT-based indices use a selection of those measurements (Table 1). Besides insulin and glucose, other variables like body weight and HDL cholesterol are included in the calculation of the various insulin sensitivity indices.

### Table 1 OGTT-based indices of insulin sensitivity

<table>
<thead>
<tr>
<th>OGTT-based index</th>
<th>Formula</th>
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</thead>
<tbody>
<tr>
<td><strong>Skeletal muscle insulin sensitivity</strong></td>
<td></td>
</tr>
<tr>
<td>Matsuda (94)</td>
<td>( \frac{10000}{G_0 \times I_0 \times G_{\text{mean}} \times I_{\text{mean}}} )</td>
</tr>
<tr>
<td>Stumvoll ISI (95)</td>
<td>( 0.226 - 0.0032 \times \text{BMI} - 0.0000645 \times I_{120} - 0.00375 \times G_{90} )</td>
</tr>
<tr>
<td>Stumvoll MCR (95)</td>
<td>( 18.8 - 0.271 \times \text{BMI} - 0.0052 \times I_{120} - 0.27 \times G_{90} )</td>
</tr>
<tr>
<td>Gutt (96)</td>
<td>( \frac{[75000 + (G_0-G_{120}) \times 0.19 \times \text{body weight}]}{[120 \times \log ([I_0 + I_{120}] / 2) \times [(G_0 + G_{120}) / 2]]} )</td>
</tr>
<tr>
<td>OGIS (97)</td>
<td>webmet.pd.cnr.it/ogis</td>
</tr>
<tr>
<td>Belfiore (98)</td>
<td>( \frac{2}{(\text{AUC insulin} + \text{AUC glucose}^a) + 1} )</td>
</tr>
<tr>
<td>Cederholm (99)</td>
<td>( \frac{[75000 + G_0-G_{120}]}{\times 1.15 \times 180 \times 0.19 \times \text{BW}]/(120 \times \log I_{\text{mean}} \times G_{\text{mean}}) )</td>
</tr>
<tr>
<td>AUC insulin^b</td>
<td>( 0.25 \times I_0 + 0.5 \times I_{30} + 0.5 \times I_{60} + 0.5 \times I_{90} + 0.25 \times I_{120} )</td>
</tr>
<tr>
<td><strong>Hepatic insulin sensitivity</strong></td>
<td></td>
</tr>
<tr>
<td>Liver IR (100)</td>
<td>( -0.091 + \log (\text{AUC insulin}) \times 0.4 + \log (\text{fat mass} %) \times 0.346 + \log (\text{HDL}) \times 0.408 + \log (\text{BMI}) \times 0.435 )</td>
</tr>
</tbody>
</table>

^aAUC glucose is calculated according to the trapezoidal rule = \( 0.25 \times G_0 + 0.5 \times G_{30} + 0.5 \times G_{60} + 0.5 \times G_{90} + 0.25 \times G_{120} \). ^bAUC insulin is calculated according to the trapezoidal rule; however, the formula can differ if other measurement times are available. \( I_0 \), fasting insulin. \( I_{30} / I_{60} / I_{90} / I_{120} \), insulin 30, 60, 90, and 120 min after the administration of 75 g glucose. \( G_0 \), fasting glucose. \( G_{90} / G_{120} \), glucose 90 and 120 min after the administration of 75 g glucose. \( I_{\text{mean}} \), mean insulin during OGTT. \( G_{\text{mean}} \), mean glucose during OGTT.
Most surrogate indices based on the OGTT are designed to measure skeletal muscle insulin sensitivity. The Liver IR index, measuring hepatic insulin sensitivity, is one of the few exceptions (Table 1).

Some authors developed their indices of insulin sensitivity by using regression analysis (Stumvoll ISI, Stumvoll MCR, Liver IR). Other researchers created their indices on a theoretical background (Matsuda, Gutt). Mari and colleagues developed the OGIS index based on principles of insulin sensitivity and optimized it with the hyperinsulinemic–euglycemic clamp. This approach resulted in a complicated formula consisting of several equations, which can be found as a spreadsheet on the authors’ homepage (97).

Matsuda and colleagues developed an index that would represent both hepatic and skeletal muscle insulin sensitivity (Table 1) (94). They proposed that fasting glucose and fasting insulin would represent hepatic insulin sensitivity. Mean glucose and mean insulin during the OGTT were suggested as a measure of skeletal muscle insulin sensitivity. The Matsuda index shows a strong correlation to the hyperinsulinemic–euglycemic clamp, indicating that this index is a good surrogate of skeletal muscle insulin sensitivity.
**Fasting indices**

For large-scale clinical studies, fasting indices of insulin sensitivity (Table 2) are an alternative because they are less time-consuming compared to OGTT-based measures and the hyperinsulinemic–euglycemic clamp. On a theoretical background, indices based on fasting insulin and glucose reflect primarily hepatic insulin sensitivity (101, 102). However, insulin sensitivity of liver and skeletal muscle is closely related in most individuals. Fasting indices of insulin sensitivity may therefore be a less costly option to measure skeletal muscle insulin sensitivity.

<table>
<thead>
<tr>
<th>Fasting index</th>
<th>Formula</th>
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<tbody>
<tr>
<td>HOMA-IR (103)</td>
<td>( \frac{G_0 \times I_0}{22.5} )</td>
</tr>
<tr>
<td>QUICKI (104)</td>
<td>( \frac{1}{\log G_0 + \log I_0} )</td>
</tr>
<tr>
<td>Revised QUICKI (105)</td>
<td>( \frac{1}{\log G_0 + \log I_0 + \log \text{NEFA}} )</td>
</tr>
<tr>
<td>HOMA-%S (103)</td>
<td><a href="http://www.dtu.ox.ac.uk/homacalculator/index.php">www.dtu.ox.ac.uk/homacalculator/index.php</a></td>
</tr>
<tr>
<td>FIRI (106)</td>
<td>( \frac{G_0 \times I_0}{25} )</td>
</tr>
<tr>
<td>McAuley (107)</td>
<td>( e^{2.63 - 0.28 \times \ln(I_0) - 0.31 \times \ln(TG)} )</td>
</tr>
</tbody>
</table>

\( G_0 \), fasting glucose. \( I_0 \), fasting insulin. \( \text{NEFA} \), fasting non-esterified fatty acids. \( TG \), triglycerides.

Table 2 Fasting indices of insulin sensitivity
Results

Weight loss by the Paleolithic diet
Two years of the Paleolithic diet without calorie restriction resulted in a mean weight loss of 8 kg in obese postmenopausal women (Paper I). After 6 months, the weight reduction was more pronounced in the women eating a Paleolithic diet compared to the women eating a conventional low-fat diet (Figure 17A). In individuals with type 2 diabetes, 12 weeks of Paleolithic diet without calorie restriction induced a mean weight reduction of 8 kg (Paper II). The addition of combined resistance and aerobic exercise did not result in any further weight loss (Figure 17B) despite an improvement in maximum oxygen uptake (P<0.01 for the difference between both groups).

Figure 17 Two years of Paleolithic diet without calorie restriction compared to a low-fat diet in obese postmenopausal women (A). 12 weeks of Paleolithic diet with and without supervised exercise in individuals with type 2 diabetes (B). Data represent mean ± SEM. ***P<0.001 for the difference between diet groups.
All study participants reduced liver fat through the Paleolithic diet

Six months of a Paleolithic diet reduced liver fat in all participating obese postmenopausal women (Figure 18B and Paper I). This liver fat reduction was more pronounced in the women eating the Paleolithic diet compared to the participants eating the conventional low-fat diet (P<0.01, Paper I). Liver fat reduction was associated with weight reduction only in the low-fat diet group (r_s=0.66, P<0.01, Paper I).

Our research group performed an earlier study in 10 obese postmenopausal women eating a Paleolithic diet without calorie restriction for 5 weeks (108). Nine participants reduced liver fat, and one woman increased liver fat by 3% compared to baseline values (Figure 18A). All individuals with type 2 diabetes eating a Paleolithic diet without calorie restriction for 12 weeks reduced liver fat (Figure 18C and Paper III).

**Figure 18** Liver fat changes in healthy, obese women eating a Paleolithic diet for 5 weeks (A, (108)) and 24 months, respectively (B, (109)). Liver fat changes in individuals with type 2 diabetes eating a Paleolithic diet for 12 weeks (C). Data are presented for each study participant as percent change from baseline. *P<0.05, **P<0.01 and ***P<0.001 for the difference compared to baseline values for the whole study group.
Improvement of insulin sensitivity by the Paleolithic diet

In individuals with type 2 diabetes, a Paleolithic diet for 12 weeks increased skeletal muscle insulin sensitivity, but supervised exercise did not give any further improvement (Figure 19A and Paper III). Some individuals improved hepatic insulin sensitivity during the 12 weeks of intervention, but there was no significant change at the group level (Figure 19B). Adipose tissue insulin sensitivity improved in both study groups (Figure 19C). Insulin sensitivity measured with the fasting measures Revised QUICKI improved significantly in both groups (Figure 19D and Paper II).

In obese postmenopausal women, a Paleolithic diet for 6 months improved hepatic insulin sensitivity measured with HOMA-IR and Liver IR index (Figure 20 and Paper I). However, hepatic insulin sensitivity deteriorated between 6 and 24 months. There were no significant changes in hepatic insulin sensitivity among the women eating a low-fat diet for 24 months (Figure 20). Skeletal muscle insulin sensitivity did not change (Paper I).

No strong association between liver fat and hepatic insulin sensitivity

In obese, postmenopausal women, we found a moderately strong association between liver fat and hepatic insulin sensitivity measured with HOMA-IR at baseline (Figure 21A and Paper I). However, if hepatic insulin sensitivity was measured with Liver IR index, there was no such association (Paper I). Changes in liver fat during the 2-year intervention did not correlate with changes in hepatic insulin sensitivity either in the Paleolithic diet group or in the low-fat diet group (Paper I).

In individuals with type 2 diabetes, liver fat and hepatic insulin sensitivity were not associated at baseline (Figure 21B and Paper III). The decrease in liver fat during the 12-week intervention was significantly correlated with the improvement in hepatic insulin sensitivity within the Paleolithic diet group ($r_s=-0.62$, $P<0.05$), but not in the group exercising under supervision in addition to eating the Paleolithic diet (Paper III).

Liver fat was associated with adipose tissue insulin sensitivity at baseline in individuals with type 2 diabetes ($r_s=-0.58$, $P<0.01$).
Figure 19 Insulin sensitivity in individuals with type 2 diabetes eating a Paleolithic diet for 12 weeks and randomized to either exercise under supervision (Paleolithic diet + Exercise) or standard care exercise recommendations (Paleolithic diet). *P<0.05, **P<0.01 and ***P<0.001 for the change over time within each group. There was no significant difference between groups.
Figure 20 Hepatic insulin sensitivity measured with HOMA-IR (A) and Liver IR index (B) in obese, postmenopausal women eating either a Paleolithic diet or a low-fat diet. *P<0.05, ***P<0.001.
Figure 21 Association between liver fat and hepatic insulin sensitivity in obese, postmenopausal women (A) and individuals with type 2 diabetes (B). Hepatic insulin resistance is measured with HOMA-IR in Figure A. Hepatic insulin sensitivity is measured as suppression of endogenous glucose production (%) in Figure B.
The reduction in liver fat and muscle fat by the Paleolithic diet is reversed by exercise training

Intramyocellular lipid in the soleus muscle decreased by 40% in individuals with type 2 diabetes eating a Paleolithic diet for 12 weeks (Figure 22A and Paper III). In contrast, lipid in this muscle remained unchanged in study participants eating a Paleolithic diet and exercising under supervision. Liver fat decreased by 74% in patients with type 2 diabetes eating a Paleolithic diet for 12 weeks but remained unchanged in the study participants who also exercised under supervision (Figure 22B and Paper III).

Fat content in the soleus muscle was not associated with peripheral insulin sensitivity, either at baseline or during the intervention.

![Figure 22 Intramyocellular lipid in soleus muscle (A) and liver fat (B) in patients with type 2 diabetes eating a Paleolithic diet with or without supervised exercise training for 12 weeks. Boxes represent the interquartile range. The line in the middle of the box is the median. The whiskers represent the most extreme values. If values are farther from the box than 1.5 of the interquartile range, they are printed as filled circles. *P<0.05, **P<0.01, ***P<0.001.]

Improvement in blood lipids with the Paleolithic diet

Six months of a Paleolithic diet without calorie restriction decreased triglycerides, total cholesterol and LDL cholesterol more than a low-fat diet in obese, postmenopausal women (Paper I). Triglycerides were still lower after 24 months in women eating the Paleolithic diet compared to women eating the low-fat diet.

In patients with type 2 diabetes, a Paleolithic diet for 12 weeks decreased triglycerides in both study groups (Paper II).
**Blood pressure reduction through the Paleolithic diet**

In obese postmenopausal women, systolic blood pressure decreased after 6 months of a Paleolithic diet or a low-fat diet (Paper I). After 24 months, there was no difference in systolic blood pressure levels compared to baseline levels. Diastolic blood pressure at 6 and 24 months decreased among women eating a Paleolithic diet but not with a conventional low-fat diet.

In individuals with type 2 diabetes, a Paleolithic diet for 12 weeks decreased systolic and diastolic blood pressure (Paper II). Adding exercise training under supervision gave no further improvement.
The fasting index Revised QUICKI is the most appropriate measure of insulin sensitivity in large-scale clinical studies

According to our meta-analysis, the following measures of insulin sensitivity have the strongest correlation with the hyperinsulinemic–euglycemic clamp results: the OGTT-based indices Stumvoll MCR, OGIS, Matsuda, Stumvoll ISI and Gutt (Table 3, paper IV). Of the indices based on fasting blood samples, only the Revised QUICKI correlates as strongly with the gold-standard clamp examination as the best OGTT-based measures (Table 3).

Table 3 Pooled Pearson’s correlation (r) between surrogate indices of insulin sensitivity and hyperinsulinemic–euglycemic clamp

<table>
<thead>
<tr>
<th>Name of surrogate index</th>
<th>Pooled correlation r (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OGTT-based indices</strong></td>
<td></td>
</tr>
<tr>
<td>Stumvoll MCR</td>
<td>0.70 (0.61 – 0.77)</td>
</tr>
<tr>
<td>OGIS</td>
<td>0.70 (0.57 – 0.80)</td>
</tr>
<tr>
<td>Matsuda</td>
<td>0.67 (0.61 – 0.73)</td>
</tr>
<tr>
<td>Stumvoll ISI</td>
<td>0.67 (0.60 – 0.72)</td>
</tr>
<tr>
<td>Gutt</td>
<td>0.65 (0.60 – 0.69)</td>
</tr>
<tr>
<td>Belfiore</td>
<td>0.64 (0.55 – 0.71)</td>
</tr>
<tr>
<td>Cederholm</td>
<td>0.60 (0.51 – 0.68)</td>
</tr>
<tr>
<td>AUC insulin</td>
<td>-0.59 (-0.64, -0.54)</td>
</tr>
<tr>
<td>Glucose (120 min)</td>
<td>-0.52 (-0.58, -0.45)</td>
</tr>
<tr>
<td>Insulin (120 min)</td>
<td>-0.40 (-0.66, -0.06)</td>
</tr>
<tr>
<td><strong>Fasting indices</strong></td>
<td></td>
</tr>
<tr>
<td>Revised QUICKI</td>
<td>0.68 (0.58 – 0.77)</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.61 (0.55 – 0.65)</td>
</tr>
<tr>
<td>FIRI</td>
<td>-0.58 (-0.80, -0.21)</td>
</tr>
<tr>
<td>HOMA-%S</td>
<td>0.57 (0.46 – 0.67)</td>
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<tr>
<td>HOMA-IR</td>
<td>-0.53 (-0.60, -0.46)</td>
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<tr>
<td>Fasting insulin</td>
<td>-0.53 (-0.56, -0.49)</td>
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<tr>
<td>McAuley</td>
<td>0.52 (0.40 – 0.63)</td>
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<tr>
<td>Fasting glucose / fasting insulin</td>
<td>0.44 (0.23 – 0.61)</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>-0.35 (-0.43, -0.27)</td>
</tr>
<tr>
<td>Fasting insulin / fasting glucose</td>
<td>-0.29 (-0.44, -0.12)</td>
</tr>
</tbody>
</table>
Discussion

Paleolithic diet improves cardiovascular risk factors

Liver fat
A Paleolithic diet caused a substantial reduction in liver fat in our studies, which was more pronounced with the Paleolithic diet compared to a conventional low-fat diet. The simplest conclusion is that the pronounced weight reduction was causing liver fat reduction, as weight loss is the most effective therapy to decrease liver fat. Indeed, among study participants eating the conventional low-fat diet, weight loss was associated with liver fat improvement. However, for the individuals eating the Paleolithic diet, liver fat reduction was not correlated with weight loss. This result implies that the composition of the Paleolithic diet may be crucial. It is therefore of interest to dissect out possible mediators of this differential effect. Polyunsaturated fatty acids are possible candidates because they have reduced liver fat in several studies (64-67). Intake of polyunsaturated fatty acids is usually high with the Paleolithic diet because of a high intake of fish and nuts. Our study group has recently found that individuals eating the Paleolithic diet have higher levels of polyunsaturated fatty acids compared to participants eating the conventional low-fat diet, reflecting the difference in diet composition (Blomquist et al., unpublished data). Concomitantly, study participants eating the Paleolithic diet reported lower carbohydrate intake, which may have improved liver fat by decreasing de novo lipogenesis.

Blood lipids
A Paleolithic diet decreases triglyceride levels. This improvement is more pronounced compared to a conventional low-fat diet not only after 6 months but also after 24 months. Because weight reduction is equal after 24 months of diet intervention, the decrease in triglyceride levels should be due to the composition of the Paleolithic diet. Most of the triglycerides we measure in blood originate from the liver as VLDL particles. As described above, liver fat and VLDL particles originate from the same liver NEFA pool. Therefore, not only the improvement of liver fat but also the decrease in triglyceride levels may be caused by the increased intake of polyunsaturated fatty acids and the decreased carbohydrate intake. This inference is in line with the results of an earlier short-term study where a Paleolithic diet improved triglycerides more than a consensus diabetes diet did (110).

The Paleolithic diet improved total cholesterol and LDL cholesterol more than the conventional low-fat diet in obese, postmenopausal women. However, total cholesterol and LDL cholesterol remained unchanged in our
study participants with type 2 diabetes. A possible explanation is that half of the participating subjects were on statin therapy.

**Insulin sensitivity**
The Paleolithic diet increased skeletal muscle and adipose tissue insulin sensitivity in individuals with type 2 diabetes and hepatic insulin sensitivity in postmenopausal women. Earlier studies have found a greater improvement in glucose tolerance and HbA1c with 3 months of a Paleolithic diet compared to a Mediterranean diet and a conventional diabetes diet (80, 110).

**Blood pressure**
We found a decrease in blood pressure with the Paleolithic diet. It is noteworthy that only the individuals eating the Paleolithic diet decreased diastolic blood pressure whereas those eating the conventional low-fat diet did not. This finding is in line with a study from Lindeberg's group, who found that a Paleolithic diet improved diastolic blood pressure more than a conventional diabetes diet did (110). The most probable explanation for blood pressure improvement is that study participants eating a Paleolithic diet are not adding salt to their food. However, an additional explanation could be that the increase in insulin sensitivity in muscle and adipose tissue causes insulin levels to decrease. Lower insulin levels may decrease sodium retention in the kidney, which would reduce blood pressure.

**Is eating a Paleolithic diet possible?**
The Paleolithic diet excludes dairy products, cereals, and added sugar and salt. Most of the meals of our Western diet contain one or several of these ingredients. The Paleolithic diet can therefore be difficult to implement because meals may have to be prepared from scratch. Consequently, it is not easy to be adherent to a Paleolithic diet over longer time periods. We could verify this lack of adherence in our 2-year study. During the first 6 months, weight, liver fat, total cholesterol and LDL cholesterol improved significantly more with the Paleolithic diet compared to the conventional low-fat diet. However, between 6 and 24 months, all of these measurements deteriorated in the Paleolithic diet group, and at 24 months there was no longer a significant difference from the conventional low-fat diet group. The lack of adherence to the Paleolithic diet was also proven by the analysis of polyunsaturated fatty acids in serum. These increased significantly after 6 months in the Paleolithic diet group compared to the conventional low-fat diet group, but after 24 months of intervention, there was no significant difference in polyunsaturated fatty acids between diet groups (Blomquist et al., unpublished data).
The Paleolithic diet is supposed to consist of the same food our ancestors ate in the Paleolithic Era. However, the food we buy in the supermarket today is different from the animals and plants of that period: e.g. fruit had a higher fiber content and meat had a different fatty acid composition. The term ‘Paleolithic-type diet’ may therefore be more appropriate. This term also embraces the likelihood that different research groups may propagate different compositions of the Paleolithic diet. However, the diet in the Paleolithic Era may also have varied widely. Today, hunter-gatherers have variable diet compositions: Kitava islanders eat mostly starchy vegetables, and Greenlandic Inuit rely on animal products. Eating a Paleolithic diet may include the freedom to choose between animal and plant products, which we implemented in our studies.

If the Paleolithic diet is based on animal products, the food will be costly and, from a population perspective, unfortunately not sustainable in the long run. However, if mostly plant-based products are chosen, the Paleolithic diet could be an affordable and sustainable alternative.

It has been argued that a Paleolithic diet cannot be recommended as balanced nutrition. Because dairy products are excluded, calcium intake may be below recommended levels to prevent osteoporosis. However, calcium uptake from the intestine is usually very low, partly because of chelate bonding with phytic acid from cereals and beans (111). Calcium uptake from vegetables may therefore increase if cereals and legumes are excluded from the diet. In the Western world, sufficient iodine intake is guaranteed by iodine enrichment of table salt (29). Individuals eating a Paleolithic diet without added salt may therefore decrease their iodine intake substantially. However, if fish and shellfish are consumed in a sufficient amount, iodine deficiency can be avoided because seafood is an exceptionally good source of iodine (29).

**Exercise does not improve cardiovascular risk factors beyond the improvement from the Paleolithic diet**

In individuals with type 2 diabetes, exercise training did not improve insulin sensitivity, blood pressure and triglyceride levels beyond the improvements observed with the Paleolithic diet alone. Previous studies including a calorie-restricted diet did not show additional effects on insulin sensitivity, glycemic control, blood pressure and triglycerides by exercise training (112, 113). However, participants in our study decreased their weight substantially even though our diet was without calorie restriction. The weight reduction may have masked any potential additive effects of exercise on insulin sensitivity, blood pressure and triglycerides. Moreover, most study participants were using metformin or statins on a daily basis. These drugs may blunt the positive effects of exercise (114-116). This interaction is of major clinical relevance because most patients with type 2 diabetes are treated with statins.
and metformin. Usually, regular exercise training increases mitochondrial content in skeletal muscle, but statins cause a decrease (116). Both exercise and metformin improve insulin sensitivity. However, if insulin sensitivity is studied directly after a single exercise bout, only individuals without metformin treatment improve insulin sensitivity (115).

**Exercise reverses the effect of a Paleolithic diet on muscle and liver fat**

A lean sedentary individual (Figure 23A) has a lower fat content in muscle and liver compared to an obese sedentary individual (Figure 23B). We found a decrease in fat content of muscle and liver with the Paleolithic diet. Previous studies have shown that weight loss reduces lipid accumulation in muscle and liver (56, 117, 118). This means that weight loss causes the obese individual with increased fat content in liver and muscle (Figure 23B) to move towards the lean individual profile with less fat content in these organs (Figure 23A).

Lean trained individuals (Figure 23C) have equally high fat content in muscle after rest as sedentary individuals with obesity and type 2 diabetes (Figure 23B). This phenomenon has been called the athlete’s paradox (45). In lean trained individuals (Figure 23C), fat content decreases during exercise because fat is used as an energy substrate (119, 120). In these individuals (Figure 23C), two components are involved in the adaptation of skeletal muscle to exercise training: the increase in muscle fat content during rest and the ability to use all stored muscle fat as energy supply during exercise. Thus, muscle fat content in a lean trained individual (Figure 23C) is highly variable and depends on supply and demand.

This highly dynamic adaptation of the muscle in lean trained individuals (Figure 23C) is not seen in obese trained individuals (Figure 23D). Obese subjects do not decrease muscle fat content during an exercise session (121). However, obese individuals can increase muscle fat content at rest after periods of training (122). In our study, the Paleolithic diet decreased muscle fat content, but if the Paleolithic diet was combined with exercise training, muscle fat content remained unchanged. We conclude that exercise training caused an increase in muscle fat content, which is a known adaptation for lean and obese trained individuals.

In our study, not only was the diet-induced reduction of muscle fat content reversed by exercise training, but the reduction in liver fat was, as well. An increase in liver fat due to regular exercise over a longer time period has not been described before. However, liver fat increases substantially after a single exercise bout in lean trained individuals (Figure 23C) (123). We conclude that comparable to the dynamic response to energy demand and supply in the muscle, this phenomenon may also exist in the liver of a lean trained individual (Figure 23C).
To explain this further, we have to consider the liver’s lipid metabolism as described above (Figure 4). If NEFA in the common NEFA pool are high, the liver of a lean trained individual will increase its fat content. During an exercise session, plasma NEFA can become extremely high to meet the energy needs of the working muscle (123). Consequently, the influx of NEFA to the common NEFA pool is high, which causes liver fat to increase substantially after an exercise bout in lean trained individuals (Figure 23C).
Obese individuals are not equally capable of increasing liver fat after exercise despite high plasma NEFA levels (124). Obviously, the liver of obese trained individuals lacks the dynamic response to high plasma NEFA supply (Figure 23D). In analogy to the muscle, the healthy liver should deplete its fat content if energy supply is low. Calorie restriction could be one such situation that decreases liver fat over the course of a few days in lean and obese individuals (58, 59). In our study, the Paleolithic diet was consumed without calorie restriction. However, all study participants lost weight, which implies that energy intake was lower than energy needs during the study period.

In our study, some participants exercising under supervision increased their liver fat content and some decreased it. We hypothesize that individuals with lower liver fat content had a low calorie intake after the last exercise bout, which decreased fat content. In contrast, those with high liver fat content had high energy intake after the last exercise bout, and liver fat that had increased during exercise remained unchanged during the following days. This interpretation entails that the livers of our study participants were capable of a highly dynamic response to energy supply and energy demand. We conclude that the intervention with Paleolithic diet and exercise may have shifted the liver fat metabolism of our obese trained participants (Figure 23D) closer to that of lean trained individuals (Figure 23C).

**No straightforward connection between fat content and insulin sensitivity in muscle and liver**

Obesity, associated with increased muscle fat content, is linked to impaired skeletal muscle insulin sensitivity (45). However, lean endurance-trained athletes have muscular fat content nearly as high as that of overweight individuals with type 2 diabetes but have an excellent insulin sensitivity (45).

Also for the liver, an association between overweight, high fat content and impaired hepatic insulin sensitivity has been reported (54, 125, 126). However, this relationship has not been verified in all study populations (127). In our studies, changes in liver fat content were associated only with changes in hepatic insulin sensitivity for patients with type 2 diabetes eating the Paleolithic diet, but not for those exercising or for postmenopausal women. Given the dynamic response of the liver to react to fat demand and supply (Figure 23C), this lack of association is not unexpected. It is e.g. unlikely that the liver changes its insulin sensitivity when liver fat increases acutely during exercise. We therefore conclude that hepatic insulin sensitivity may be influenced by many factors other than total liver fat content, including the putative effects of lipid subspecies like ceramides and diacylglycerols (128).

Most of the triglycerides stored as liver fat come from plasma NEFA originating from adipose tissue. On theoretical background, poor insulin
sensitivity of the adipose tissue increases plasma NEFA levels, which may cause higher liver fat content. In our study, adipose tissue insulin sensitivity was associated with liver fat content, which strengthens this hypothesis.

**Measuring insulin sensitivity**

Insulin sensitivity is closely linked to cardiovascular risk factors like overweight, high triglycerides, liver fat, hypertension and glucose intolerance. This relationship is often not as straightforward as we might think. We examined with the hyperinsulinemic–euglycemic clamp not only hepatic insulin sensitivity but also insulin sensitivity in skeletal muscle and adipose tissue. We found that insulin sensitivity of adipose tissue was associated with liver fat content, suggesting a link between increased plasma NEFA levels and liver fat (127). This result stresses the importance of measuring insulin sensitivity in different tissues because liver, skeletal muscle and adipose tissue may not have the same level of insulin resistance.

The hyperinsulinemic–euglycemic clamp is the gold-standard examination for insulin sensitivity of skeletal muscle, liver and adipose tissue. However, when we measure skeletal muscle insulin sensitivity with the clamp, only 70% of the glucose is taken up by skeletal muscle. The remaining glucose is taken up by other peripheral tissues like kidney, heart and adipose tissue (129). We therefore often refer to peripheral insulin sensitivity instead of skeletal muscle insulin sensitivity.

When measuring hepatic insulin sensitivity, we are mostly interested in glucose production of the liver and to what extent insulin can suppress this glucose output. However, the liver is not the only organ capable of glucose production. The kidney may account for as much as 25% of whole body glucose output (130). We therefore often refer to suppression of endogenous glucose production instead for hepatic insulin sensitivity.

When measuring adipose tissue insulin sensitivity, we are interested in the ability of insulin to suppress adipose tissue lipolysis and therefore measure NEFA in plasma. However, NEFA can originate not only from adipose tissue but also from hydrolyzed triglycerides in chylomicrons or VLDL particles, which may influence results.

Of importance, the hyperinsulinemic–euglycemic clamp does not reflect physiological conditions. In normal metabolism, glucose and insulin are continuously changing, but during the clamp examination, insulin is infused at a constant rate, which does not take into account the feedback mechanism between glucose and insulin (131). Moreover, insulin secreted by the pancreas is to a large part extracted by the liver, which is not the case for insulin infused intravenously. The gold-standard examination for insulin sensitivity, besides being costly and time consuming, thus has some physiological drawbacks. One could argue that the OGTT enables a more physiological measurement of insulin sensitivity because the glucose load
mimics a meal. However, the results of an oral glucose challenge will not only be influenced by insulin sensitivity but also by gastric emptying, glucose absorption, insulin secretion, and the release of incretin hormones.

Because of its connection to cardiovascular risk factors, insulin sensitivity measurement is an important tool, not the least to evaluate lifestyle interventions. OGTT-based indices and fasting measurements have been developed for use in clinical studies when the accepted reference method – the hyperinsulinemic–euglycemic clamp – is not practicable. Based on our meta-analysis, we recommend using the fasting index Revised QUICKI for estimating insulin sensitivity in large-scale clinical studies. This index is easy to perform because only fasting blood samples are needed, and it measures insulin sensitivity as accurately as OGTT-based indices. However, plasma NEFA analyses are needed to construct this index. If NEFA analyses are not available, any of the following OGTT-based indices could be employed as a second-choice measurement: Stumvoll MCR, OGIS, Matsuda, Stumvoll ISI or Gutt. If estimations must rely on fasting levels without NEFA analysis, the best choice would be the QUICKI, the log-transformed HOMA-IR or the HOMA-%S.

Limitations

**Paper I**

Diet studies in free-living individuals are always challenging because it is difficult to estimate true food intake. Food records most likely influence the diet on the recorded days, and food intake will be different on non-recorded days. Our hypothesis was that high protein intake could be a major contributor to the effects of a Paleolithic diet. However, the individuals in the Paleolithic diet group did not increase protein intake during the intervention compared to the conventional low-fat diet group, based on nitrogen excretion (132). We also measured fatty acid composition in serum and found an increase in polyunsaturated fatty acids in the Paleolithic diet group. However, this analysis reflects only a short time period. It would have been advantageous to analyze fatty acid composition in erythrocyte membranes, which reflects fatty acid intake over several months.

The Paleolithic diet improved hepatic insulin sensitivity but not skeletal muscle insulin sensitivity. In contrast, we saw a clear improvement of skeletal muscle insulin sensitivity by the Paleolithic diet, measured with the hyperinsulinemic–euglycemic clamp, in paper III. The lack of improvement in skeletal muscle insulin sensitivity in paper I probably reflects the measurement method. The OGTT measurement used is influenced by insulin secretion, glucose absorption and incretin hormones and is therefore less sensitive to changes in insulin sensitivity.
Paper II
We added an observational group to our study. However, we did this first after we had started the study, which means that participants in that group were not randomized. Individuals excluded from the randomized study because of cardiovascular disease or beta-blocker usage were offered the opportunity to participate in this group, but because of differences compared to the intervention groups and the lack of randomization, we could not draw any conclusions based on the observational group.

Paper III
We measured hepatic insulin sensitivity with the hyperinsulinemic–euglycemic clamp but did not find any significant changes caused by the Paleolithic diet. This outcome was unexpected because hepatic insulin sensitivity improved with the Paleolithic diet in paper I. The insulin dose of 40 mIU/m²·min was chosen to measure hepatic insulin sensitivity in type 2 diabetes patients, in line with previous studies (133-135); however, insulin sensitivity in our study participants may have been better than expected and the insulin dose therefore too high to detect changes in hepatic insulin sensitivity. Moreover, we calculated negative values for endogenous glucose production for some examinations. The explanation is that labeled glucose is difficult to measure exactly if the ratio between labeled and unlabeled glucose in the blood is too low. This problem can be reduced by adding labeled glucose to the unlabeled glucose infusion, which we did not do in this study.

Another limitation is that we had only a small number of measurements of soleus muscle fat content. We had to exclude measurements of three participants because we could not separate the signal of IMCL from the signal of EMCL. This problem is a known technical difficulty because soleus muscle fibers are not aligned in parallel to the main magnetic field.

Paper IV
A major limitation of this meta-analysis is that we were not two independent assessors deciding which studies to include. The goal of the meta-analysis was to decide which surrogate measure of insulin sensitivity can be recommended for large-scale clinical studies. These surrogate measures were compared to the gold standard examination, the hyperinsulinemic–euglycemic clamp. Even if the clamp examination is the reference method, it does not necessarily measure the correct insulin sensitivity. The insulin dose during the clamp may be inadequate or the equilibration time too short. This possible inadequacy of the reference method leads to uncertainty when deciding about the most suitable surrogate measure.
Conclusions and future perspectives

This thesis shows that the Paleolithic diet improves cardiovascular risk factors like overweight, liver fat, insulin sensitivity, triglycerides and blood pressure. Would I recommend this diet to patients with type 2 diabetes or other individuals who want to adopt a healthy lifestyle? The absence of cardiovascular disease in contemporary hunter-gatherers and the improvement of cardiovascular risk factor in randomized studies like ours point in this direction. However, no study so far has compared the Paleolithic diet to other diet alternatives with respect to cardiovascular events like myocardial infarction and stroke. To support recommending the Paleolithic diet over other diets, such long-term studies are needed.

Liver fat content is very dynamic in the healthy liver. It may take days to decrease liver fat through calorie restriction, but during exercise, liver fat increases within a couple of hours. Liver fat regulation is more dynamic in the lean compared to the obese individual. In future studies, the dynamics of liver fat regulation should be compared between healthy individuals and subjects with obesity and type 2 diabetes. Furthermore, the effect of diet and exercise interventions on liver fat regulation should be investigated further.
Acknowledgements

Thank you to all study participants! You have surprised me many times with your enthusiasm about participating in our studies and your eagerness to help increase knowledge about health and disease. Without you, this thesis would not have been possible!

Thanks to my main supervisor Tommy Olsson! I am amazed that it was more important to you that I learned scientific skills like statistics and evaluation of scientific work than that the papers were finished in time. I am impressed that you often convinced me with your arguments despite my being quite a determined person (some would call it stubborn). With your 30 years of research experience, the odds are quite high that your solution is better than mine. However, you let me choose my own path, which I am most grateful for. Altogether, I could not have had a better mentor!

Mats Ryberg, my co-supervisor, you helped me with small and big issues, regardless of whether they were about patient care, computer trouble, spelling or something else. How can you keep up this positive attitude all the time?

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References


