Paleolithic Diet in Type 2 Diabetes

Effects on glycaemic control when body weight is kept stable and on leptin

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2025

Document Version:
Publisher's PDF, also known as Version of record

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 18th of June 2025 at 09.00 in Agardhsalen, Department of Clinical Sciences, Jan Waldenströms gata 35, Malmö, Sweden.

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Organization: LUND UNIVERSITY

Department of Clinical Sciences in Malmö.

Document name: Doctoral dissertation. **Date of issue: 18th of June 2025.**

Author: Maelán Fontes Villalba. **Sponsoring organization:**

Title and subtitle: Paleolithic Diet in Type 2 Diabetes – Effects on glycaemic control when body weight is kept stable and on leptin

Abstract:

Background: Previous studies indicate comparably greater improvement in glycaemic control from Paleolithic diet, possibly due to an accompanying greater reduction in body weight, which, in turn, could be due to an accompanying greater reduction in leptin.

Aims: To assess in type 2 diabetes the effect of Paleolithic diet on glycaemic control when body weight is kept stable and diets matched for macronutrients, glycaemic load and fibre (Papers I and II); and on leptin and leptin receptor binding (Papers III and IV).

Methods: Effect on glycaemic control was assessed in a crossover trial in 14 adults with type 2 diabetes comparing 1 month each of Paleolithic and diabetes diets matched for macronutrients, glycaemic load, and fibre, and adjusted during study for body weight stability (Papers I and II). Fasting leptin and biologically active leptin (bioLep) were analysed in stored blood samples from a crossover trial in 13 adults with type 2 diabetes comparing 3 months each of Paleolithic and diabetes diets (Papers III and IV). BioLep was analysed for known recombinant leptin concentrations incubated with digested wheat gluten (Paper IV).

Results: Paleolithic diet did not result in comparably greater improvements in glycaemic control when body weight was kept stable and macronutrients and glycaemic load were matched (Papers I and II). Fasting leptin and bioLep were both comparably lower after Paleolithic diet with no difference between them (Papers III and IV). Wheat gluten digest inhibited leptin receptor binding, but not after heat treatment (Paper IV).

Conclusion: The findings indicate that the comparably greater improvement in glycaemic control from Paleolithic diet is due to the accompanying greater reduction in body weight. The findings also strengthen previous results of comparably greater reduction in leptin from Paleolithic diet and indicate that diet does not affect leptin receptor binding in the fasting state.

Keywords: Type 2 diabetes, Paleolithic diet, Glycaemic control, Leptin, Body weight loss.

Classification system and/or index terms (if any) Supplementary bibliographical information

Language English **ISSN and key title**: 1652-8220

ISBN: 978-91-8021-721-7

Recipient's notes **Number of pages**: 94

Price Security classification

Signature  Date 2025-04-23

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Cover photo by Maelán Fontes Villalba of a painting by Amada de León Moséguez depicting the author's mother and a drop of blood on one finger in a Lanzarote mountainous landscape with a volcano.

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ISBN 978-91-8021-721-7

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University

Lund 2025

This thesis is dedicated to my friend, mentor and supervisor Staffan Lindeberg. Everything burns with the right spark, and you were a true master of fire.

I hope this thesis provides food for thought.

Table of Contents

Abstract

Background: Previous studies indicate comparably greater improvement in glycaemic control from Paleolithic diet, possibly due to an accompanying greater reduction in body weight, which, in turn, could be due to an accompanying greater reduction in leptin.

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Populärvetenskaplig sammanfattning

Diabetes är en sjukdom som uppstår när kroppen har svårt att kontrollera blodsockernivåerna. Höga blodsockernivåer kan orsaka skador på blodkärl i ögon, njurar och nerver, och ökar risken för hjärt-kärlsjukdom och dödlighet. Långsiktig kontroll av blodsockernivåerna, så kallad glykemisk kontroll, är viktig för att minska dessa komplikationer. Glykemisk kontroll förbättras bland annat genom viktnedgång. Kroppsvikten kontrolleras delvis av ett hungerreglerande hormon som kallas leptin, vilket framkallar mättnad när det binder till sin receptor i hjärnan. Andelen personer som påverkas av diabetes globalt varierar från 3 % i östra subsahariska Afrika till 12 % i Oceanien, med en global genomsnittsprevalens på 10.5 %. Över 90 % av dessa fall är typ 2-diabetes. Forskare har observerat att typ 2-diabetes var ovanligt eller till och med frånvarande bland jägare-samlare. Denna observation ledde till att forskarna föreslog att en jägare-samlar-kost, även kallad en paleolitisk kost, skulle kunna vara idealisk för förebyggande och behandling av typ 2-diabetes.

En paleolitisk kost tros mest likna kosten hos våra mänskliga förfäder precis innan jordbrukets tillkomst för omkring 10,000 år sedan. En paleolitisk kost kan därför innehålla många livsmedel som fanns tillgängliga utan jordbruk, såsom frukt, rotfrukt, nötter, magert kött, larver, insekter, fisk, skaldjur, ägg, honung och grönsaker. Livsmedel som produceras efter jordbrukets uppkomst, såsom spannmål, mejeriprodukter, baljväxter, raffinerade oljor och socker, utesluts. Tidigare studier har visat på en större förbättring av glykemisk kontroll med en paleolitisk kost jämfört med en diabeteskost. Detta kan bero på en större viktnedgång. I sin tur kan viktnedgången bero på en större minskning av leptin, vilket innebär en förbättrad funktion av leptin, aptitreglering och därmed att man äter mindre mat.

Denna avhandling syftade till att bedöma effekten av en paleolitisk kost (matchad avseende glykemisk belastning och innehåll av makronutrienter och fiber), jämfört med en diabeteskost vid typ 2-diabetes på glykemisk kontroll när kroppsvikten hålls stabil (Artikel I och II) samt på leptin och leptinreceptorbinding (Artikel III och IV). För att uppnå dessa syften genomfördes en kosttinterventionsstudie på Lanzarote (Artikel I och II), och leptin mättes i sparade blodprover från en äldre kostinterventionsstudie från Lundaområdet i Sverige (Artikel III och IV).

Det fanns inga skillnader i glykemisk kontroll mellan en paleolitisk kost och en diabeteskost när kroppsvikten hölls stabil hos 14 försökspersoner med typ 2-diabetes (Artikel I och II). Resultaten tyder på att den större förbättringen av glykemisk kontroll med en paleolitisk kost jämfört med en diabeteskost sannolikt beror på en större minskning av kroppsvikten. Studierna visade också lägre leptinnivåer från en paleolitisk kost jämfört med en diabeteskost hos 13 personer med typ 2-diabetes, och att ingen av kosterna påverkade leptinreceptorbinding (Artikel III och IV). Resultaten stärker tidigare resultat om större minskning av

leptin vid typ 2-diabetes från en paleolitisk kost och indikerar att kost inte påverkar leptinreceptorbindingen.

Lay summary

Diabetes is a disease that occurs when the body struggles to control blood sugar levels. High blood sugar levels can cause damage to blood vessels of the eyes, kidneys and nerves, and increases the risk of cardiovascular disease and mortality. Long-term control of blood sugar levels, so-called glycaemic control, is important to reduce these complications. Glycaemic control is, among other things, improved by body weight loss. Body weight is controlled, in part, by a hunger-regulating hormone called leptin which induces satiety when binding to its receptor in the brain. The percentage of people affected worldwide by diabetes varies from 3% in Eastern sub-Saharan Africa to 12% in Oceania, with a global average prevalence of 10.5%. Over 90% of these cases are type 2 diabetes. Scientists observed that type 2 diabetes was rare or even absent among hunter-gatherers. The observation led scientists to propose that a hunter-gatherer diet, also called a Paleolithic diet, could be ideal for the prevention and treatment of type 2 diabetes.

A Paleolithic diet is thought to most closely resemble the diet of our human ancestors just preceding the advent of agriculture around 10,000 years ago. A Paleolithic diet can therefore include many food staples available without agriculture, such as fruits, tubers, nuts, lean meat, larvae, insects, fish, shellfish, eggs, honey, and vegetables. Food staples produced after the advent of agriculture, such as cereal grains, dairy products, legumes, refined oils and sugars, are excluded. Previous studies have indicated a greater improvement in glycaemic control from a Paleolithic diet compared with a diabetes diet. This could be due to a greater reduction in body weight. In turn, the body weight loss could be due to a greater reduction in leptin, meaning improved function of leptin, appetite regulation and consequently eating less food.

This thesis aimed to assess the effect of a Paleolithic diet (matched for macronutrient composition, glycaemic load and fibre content), compared with a diabetes diet in type 2 diabetes on glycaemic control when body weight is kept stable (Papers I and II) and on leptin and leptin receptor binding (Papers III and IV). To achieve these aims, a diet intervention study was performed in Lanzarote, Canary Islands (Papers I and II), and leptin was measured in stored blood samples from an older diet intervention study from the Lund area, Sweden (Papers III and IV).

There were no differences in glycaemic control between a Paleolithic diet and a diabetes diet when body weight was kept stable in 14 subjects with type 2 diabetes (Papers I and II). The results indicate that the greater improvement in glycaemic control from a Paleolithic diet compared with a diabetes diet is likely due to a greater reduction in body weight. The studies also found lower leptin levels from a Paleolithic diet compared with a diabetes diet in 13 subjects with type 2 diabetes, and that neither diet affected leptin receptor binding (Papers III and IV). The results

strengthen previous results of greater reduction in leptin in type 2 diabetes from a Paleolithic diet and indicate that diet does not affect leptin receptor binding.

Resumen divulgativo

La diabetes es una enfermedad que se produce cuando el cuerpo tiene dificultad para controlar los niveles de azúcar en sangre. Los niveles altos de azúcar en sangre dañan los vasos sanguíneos de los ojos, riñones y nervios, y aumentan el riesgo de enfermedades del corazón y de mortalidad. Por eso, es importante el control de azúcar en sangre a largo plazo, también llamado control glucémico, para reducir estas complicaciones. El control glucémico es mejorado, entre otras cosas, por la pérdida de peso corporal. La pérdida de peso corporal está controlada, en parte, por una hormona que regula el hambre llamada leptina. La leptina genera sensación de saciedad al unirse a su receptor en el cerebro, ayudando así a que comamos menos.

El porcentaje de personas afectadas por la diabetes en el mundo varía desde un 3% en África sub-Sahariana del Este a un 12% en Oceanía, con una media mundial de 10,5%. Más del 90% de todas las personas con diabetes tiene diabetes tipo 2. Interesantemente, algunos científicos observaron que la diabetes tipo 2 era poco común, o incluso inexistente, en cazadores-recolectores. Esto llevó a pensar que la dieta de los cazadores-recolectores, conocida como dieta Paleolítica, podría ser ideal para prevenir y tratar la diabetes tipo 2.

Se piensa que una dieta Paleolítica se asemeja mucho a la dieta de nuestros ancestros humanos justo antes de la aparición de la agricultura hace unos 10.000 años. Por tanto, una dieta Paleolítica puede incluir alimentos disponibles previamente a la introducción de la agricultura como frutas, tubérculos, frutos secos, carnes magras, larvas, insectos, pescados, mariscos, huevos, miel y una larga variedad de vegetales. Pero, se excluyen alimentos introducidos después de la agricultura como los cereales, los lácteos, las legumbres, los aceites refinados y los azúcares. Los resultados de estudios previos indican que una dieta Paleolítica mejora más el control glucémico comparado con otras dietas, y también suele reducir más el peso corporal. Esa reducción del peso corporal, por tanto, podría estar asociada a una mayor reducción de la leptina, lo que indicaría una mejor función en la regulación del apetito y, en consecuencia, una menor ingesta de alimentos.

Esto plantea una pregunta importante: ¿las mejoras en el control glucémico y en los niveles de leptina se deben a la dieta en sí o a la pérdida de peso asociada a ella? Para responder a esta pregunta, los objetivos de esta tesis fueron comparar los efectos de una dieta Paleolítica con los de una dieta para la diabetes sobre el control glucémico, manteniendo estable el peso corporal (artículos I y II), y en los niveles de leptina y su capacidad de unirse a su receptor (artículos III y IV). Para ello, se realizó un estudio de intervención dietético en Lanzarote, Islas Canarias (artículos I y II), y se analizó la leptina en muestras de sangre de otro estudio previo de intervención dietética realizado en el área de Lund, Suecia (artículos III y IV).

Los resultados no mostraron diferencias en el control glucémico entre una dieta Paleolítica y una dieta para la diabetes cuando el peso corporal se mantuvo estable

en 14 personas con diabetes tipo 2 (artículos I y II). Los resultados indican que las mejoras en el control glucémico de una dieta Paleolítica se deben probablemente a una mayor reducción del peso corporal, más que a la dieta en sí. Además, se encontró niveles más bajos de leptina con una dieta Paleolítica comparado con una dieta para la diabetes en 13 personas con diabetes tipo 2, pero que ninguna de las dos dietas afectó a la capacidad de la leptina para unirse a su receptor (artículos III y IV). Los resultados refuerzan los hallazgos de estudios previos que indican una mayor reducción de la leptina en la diabetes tipo 2 con una dieta Paleolítica y que ninguna de las dos dietas afecta a la capacidad de la leptina de unirse a su receptor.

List of papers

Paper I

Fontes-Villalba M., Jönsson T., Granfeldt Y., Frassetto L. A., Sundquist J., Sundquist K., Carrera-Bastos P., Fika-Hernando M., Picazo O., & Lindeberg S. (2014). A healthy diet with and without cereal grains and dairy products in patients with type 2 diabetes: study protocol for a random-order cross-over pilot study - Alimentation and Diabetes in Lanzarote -ADILAN. *Trials*, 15(1), 2.

Paper II

Fontes-Villalba M., Fika-Hernando M., Picazo O., Frassetto L. A., Carrera-Bastos P., Memon A. A., Lippi G., Montagnana M., Granfeldt Y., Sundquist K., Sundquist J., & Jönsson T. Randomised controlled trial of dietary interventions for glycaemic control when body weight is kept stable. Submitted. 2024.

Paper III

Fontes-Villalba M., Lindeberg S., Granfeldt Y., Knop F. K., Memon A. A., Carrera-Bastos P., Picazo O., Chanrai M., Sundquist J., Sundquist K., & Jönsson T (2016). Palaeolithic diet decreases fasting plasma leptin concentrations more than a diabetes diet in patients with type 2 diabetes: a randomised cross-over trial. *Cardiovasc Diabetol*, 15(1), 80.

Paper IV

Fontes-Villalba, M., Granfeldt, Y., Sundquist, K., Memon, A. A., Hedelius, A., Carrera-Bastos, P., & Jönsson, T. (2024). Effects of a Paleolithic diet compared to a diabetes diet on leptin binding inhibition in secondary analysis of a randomised cross-over study. *BMC Endocrine Disorders*, *24*(1), 176.

Authors' contribution to the papers

Paper I

MFV and SL conceived of the study. MFV coordinated with hospital staff and secured funding. MFV, SL, TJ and YG designed the study. MFV, SL and TJ wrote the article. PCB, AAM, YG, KS, MFH, OP, LF and JS participated in the drafting of the article, as well as revising it for important intellectual content. All authors read and approved the final manuscript.

Paper II

MFV and SL conceived of the study. MFV coordinated with hospital staff, managed secure data capture using ®REDCap and secured funding. MFV, SL, TJ and YG designed the study. MFV and TJ analysed and interpreted the data and wrote the manuscript. PCB, AAM, YG, KS, MFH, OP, LF and JS participated in the drafting of the article, as well as revising it for important intellectual content. SL passed away during the preparation of this manuscript. We acknowledge his invaluable contributions to the study design and conceptualisation. All other authors read and approved the final manuscript.

Paper III

TJ and SL conceived of the study. MFV, TJ and SL participated in the design and execution of the study. MFV and TJ analysed, interpreted the data and wrote the article. SL, YG, FKK, AAM, PCB, OP, MC, JS and KS participated in the drafting of the article, as well as revising it for important intellectual content. All authors read and approved the final manuscript.

Paper IV

TJ conceived of and participated in the design and execution of the study. MFV and TJ analysed, interpreted the data and wrote the article. KS, YG, AH and AAM participated in the design and execution of the study and the drafting of the article, as well as revising it for important intellectual content. PCB participated in the drafting of the article, as well as revising it for important intellectual content. All authors read and approved the final manuscript.

Abbreviations

AUC	Area Under the Curve
BioLep	Biologically active leptin
BMI	Body mass index
GADA	Glutamate decarboxylase autoantibodies
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein cholesterol
kDa	Kilodaltons
LDL	Low-density lipoprotein cholesterol
MARD	Middle age-related diabetes
MOD	Mild obesity-related diabetes
OGTT	Oral glucose tolerance test
RCT	Randomised controlled trial
REDCap®	Research Electronic Data Capture
SAID	Severe autoimmune diabetes
SIDD	Severe insulin-deficient diabetes
SIRD	Severe insulin-resistant diabetes

In papers I and II, the intervention diets were named as “Healthy diet with cereal grains, dairy products and legumes” (Diet A), and “Healthy diet without cereal grains, dairy products and legumes” (Diet B). For clarity and simplicity, in this thesis, “Healthy diet A with cereal grains and dairy products” is named “diabetes diet” and “Healthy diet B without cereal grains and dairy products” is named “Paleolithic diet”.

Introduction

Diabetes

Overview

The term Diabetes originates from the Greek *diabaínō*, which means "to pass through", a description first used by the Greek physician Aretaeus of Cappadocia in the 2nd century AD (Ahmed, 2002). The term was used to characterise the excessive urination seen in people with diabetes, which is one of the hallmarks of the condition.

Diabetes is a chronic disease that occurs when the body either cannot effectively use the insulin it produces (insulin resistance) or when the body does not produce enough insulin, or both (WHO, 2022). Insulin is a hormone produced and secreted by pancreatic ß-cells into the bloodstream that is necessary for glucose to enter the cells in the body. Uncontrolled diabetes leads to elevated levels of glucose in the bloodstream (hyperglycaemia), which over time can cause damage to blood vessels in the heart, eyes, kidneys and nerves (WHO, 2022). Diabetes is a major cause of disability and mortality, affecting 14% of the global adult population in 2022 (WHO, 2022).

There are three common types of diabetes: type 1, type 2 and gestational diabetes.

Type 1 diabetes (previously known as insulin-dependent, juvenile or childhood-onset diabetes) is an autoimmune condition in which the immune system attacks and destroys the insulin-producing pancreatic ß-cells (Ilonen et al., 2019; WHO, 2022). Insulin production is consequently reduced, making people reliant on exogenous insulin therapy for survival. Type 1 diabetes is the most common type of diabetes in childhood, although it can occur at any age (WHO, 2022). In 2021, there were about 8-9 million people worldwide with type 1 diabetes (Gregory et al., 2022). Neither the cause nor the means to prevent type 1 diabetes are known (Ilonen et al., 2019; WHO, 2022).

Type 2 diabetes is the most common type of diabetes affecting more than 90% of all people with diabetes. The condition arises due to a combination of insulin resistance and initial hyperinsulinemia, followed by a reduced capacity of pancreatic ß-cells to produce insulin (Ahmad et al., 2022; Chatterjee et al., 2017). At diagnosis,

40-80% of ß-cell function is lost, however, with good glycaemic control or remission of the condition substantial functional ß-cell mass can be recovered (Ahmad et al., 2022).

Gestational diabetes occurs during pregnancy and is characterised by hyperglycaemia at levels below those diagnostic of type 1 and 2 diabetes (Simmons & Sweeting, 2023; WHO, 2022). The condition arises when placental hormones reduce the production and/or use of insulin (Usman et al., 2023). Gestational diabetes is associated with an increased risk of developing type 2 diabetes (WHO, 2022).

More recently, Ahlqvist et al. (2020) have suggested five subtypes, although these are not used yet in clinical practice (Ahlqvist et al., 2020). Ahlqvist et al. used six clinical parameters, namely glutamate decarboxylase autoantibodies (GADA), age at diabetes onset, HbA1c, body mass index (BMI) and measures of insulin resistance and insulin secretion, to cluster adult-onset diabetes into five subtypes. The five subtypes comprised severe autoimmune diabetes (SAID), severe insulin-deficient diabetes (SIDD), severe insulin-resistant diabetes (SIRD), mild obesity-related diabetes (MOD) and middle age-related diabetes (MARD). SAID is defined by the presence of GADA and includes autoantibodies-positive subjects traditionally classified as type 1 diabetes, as well as subjects with latent autoimmune diabetes in adults. These subjects are characterised by relatively early disease onset, low insulin secretion, relatively low BMI and poor glycaemic control. SIDD include subjects with similar characteristics as SAID but without GADA. SIRD subjects are characterised by very high insulin resistance and compensatory insulin secretion, high BMI but low HbA1c. MOD is characterised by high BMI but not insulin resistance. MARD is the largest cluster (39%) and is characterised by late-onset of the disease but no extreme characteristics (Ahlqvist et al., 2020).

Prediabetes is a term used to describe people with impaired glucose tolerance and/or impaired fasting glucose, the former detected by an oral glucose tolerance test (OGTT), and the latter characterised by elevated fasting blood glucose levels that are higher than normal but below the threshold for diagnosing type 1 and type 2 diabetes (Echouffo-Tcheugui et al., 2023; WHO, 2022). These criteria are similar to those used to diagnose gestational diabetes. Prediabetes is an intermediate condition between normal glucose homeostasis and diabetes conditions, which is associated with an increased risk of developing type 2 diabetes (Echouffo-Tcheugui et al., 2023; WHO, 2022).

Type 2 diabetes

Type 2 diabetes is on the rise because of an increase in associated risk factors such as obesity, a sedentary lifestyle and an ageing population (Ahmad et al., 2022; *IDF Diabetes Atlas 10th Edition*, 2021; Ong et al., 2023), being the seventh leading cause

of Disability-Adjusted Life Years in 2021 (Ferrari et al., 2024). High BMI is the primary risk factor for type 2 diabetes, contributing to more than 50% of type 2 diabetes related Disability-Adjusted Life Years in 2021 (Ong et al., 2023). Type 2 diabetes affects just above half a billion people worldwide at an estimated socioeconomic cost of US$966 billion in 2021 (*IDF Diabetes Atlas 10th Edition*, 2021). Furthermore, just as many people are estimated to have impaired glucose tolerance (*IDF Diabetes Atlas 10th Edition*, 2021). People with type 2 diabetes have a two to fourfold increased risk of death from cardiovascular disease compared with people without type 2 diabetes (Canto et al., 2019). In 2021, almost 7 million adults died as a result of diabetes or its complications (*IDF Diabetes Atlas 10th Edition*, 2021).

Type 2 diabetes is diagnosed if one or more of the following criteria are met (Ahmad et al., 2022; *IDF Diabetes Atlas 10th Edition*, 2021): fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL), 2-hour plasma glucose after 75 g oral glucose load (OGTT) ≥ 11.1 mmol/L (200 mg/dL), HbA1c ≥ 48 mmol/mol (6.5%), and random plasma glucose ≥ 11.1 mmol/L (200 mg/dL) in the presence of symptoms of hyperglycaemia.

The symptoms of type 2 diabetes are related to hyperglycaemia and include excessive thirst, frequent urination, fatigue, constant hunger, sudden body weight loss, and blurred vision (Chatterjee et al., 2017). However, the condition is often essentially symptomless. If the condition progresses over a long period of time without noticeable symptoms, it is often diagnosed only after complications such as visual impairment, poorly healing lower-limb ulcers, heart disease, or stroke have developed (Chatterjee et al., 2017).

Glycaemic control refers to the overall management of blood glucose levels over a period of time (Bergman et al., 2020). Glycaemic control is commonly assessed through HbA1c – a blood measure which reflects average blood glucose levels over the preceding 2- to 3-months – providing a comprehensive view of how well blood sugar levels are being regulated on a long-term basis (Bergman et al., 2020). Other blood measures of glycaemic control are fructosamine and glycated albumin which reflect glycaemic control over the preceding two to four weeks (Bergman et al., 2020).

Glycaemic control is associated with risk of all-cause and cause-specific mortality and cardiovascular disease (Nichols et al., 2013; Palta et al., 2017). The association is U-shaped, with both high and low HbA1c levels being associated with increased risk of cardiovascular disease and all-cause mortality (Anyanwagu et al., 2019; Nichols et al., 2013). Accordingly, results from several large randomised controlled trials (RCTs) suggest that glucose-lowering interventions reduce micro- (Patel et al., 2008; UK Prospective Diabetes Study (UKPDS) Group, 1998) and macrovascular complications in subjects with type 2 diabetes (Holman et al., 2008, 2014), while results from some large RCTs indicate that intensive glucose-lowering interventions

to target normal HbA1c levels do not significantly reduce cardiovascular events, and the ACCORD study indicates an increased risk of mortality events (Duckworth et al., 2009; Gerstein et al., 2008; Patel et al., 2008). Glycaemic control is in type 2 diabetes associated with age, sex, genetics, ethnicity, waist circumference, BMI, alcohol intake, smoking and physical activity, among other factors (Bergman et al., 2020; Wisgerhof et al., 2020). Importantly, especially for this thesis, glycaemic control is in type 2 diabetes associated in a dose-dependent manner with body weight loss (Franz et al., 2015; Gummesson et al., 2017; Lean et al., 2018; Lingvay et al., 2021). In subjects with type 2 diabetes who are overweight or obese, body weight loss is deemed an effective intervention to improve glycaemic control. However, the effect of body weight loss on glycaemic control in subjects with type 2 diabetes and normal weight (BMI $\leq$25kg/m^2) is not so obvious (Taylor et al., 2023; Taylor & Holman, 2014). In the UKPDS study, one in three of the studied subjects had normal body weight (Taylor & Holman, 2014). Among these subjects, normal glycaemic control was achieved with a mean body weight loss of 13%, whereas a reduction of 21% of body weight was required in the whole sample. In the LookAHEAD study some subjects achieved normal glycaemic control with a modest body weight loss of 4.7% after 4 years (Taylor & Holman, 2014). Taken together, these results indicate that normal glycaemic control can be achieved with varying degrees of body weight loss, even in subjects with normal body weight. In consequence, the personal fat threshold hypothesis was proposed to explain why normal body weight subjects can achieve normal glycaemic control with body weight loss. This hypothesis postulates that each subject has a personal fat threshold which, if exceeded increases the risk of developing type 2 diabetes independently of their BMI (Taylor et al., 2023; Taylor & Holman, 2014). Diabetes remission is defined as HbA1c less than 6.5% (48 mmol/mol) or fasting plasma glucose less than 7.0 mmol/L (126mg/dL), or both, measured at least 3 months after cessation of glucose-lowering medication (Riddle et al., 2021). The DiRECT trial aimed at remission of type 2 diabetes through body weight management, with withdrawal of glucose-lowering medication and antihypertensive drugs, total diet replacement (825-853 kcal/day formula for 3-5 months), a stepped food reintroduction phase, and structured support for long-term body weight loss maintenance (Lean et al., 2018). The results indicate that diabetes remission was associated with degree of body weight loss. Diabetes remission was achieved in 31 (86%) of 36 participants who lost 15 kg or more, 16 (57%) of 28 participants who lost 10-15 kg, 19 (34%) of 56 participants who lost 5-10 kg, six (7%) of 89 participants who lost 0-5 kg and in none of the 76 participants who gained body weight (Lean et al., 2018). A systematic review and meta-regression analysis of RCTs indicates a robust dose-response association between body weight loss and diabetes remission, which is independent of age, diabetes duration, HbA1c, BMI and type of intervention (Kanbour et al., 2025). Overall, the results from RCTs indicate that body weight loss plays a key role in the management of type 2 diabetes and its complications.

In addition, body weight loss is in turn associated with satiety through satiety hormones such as leptin (Considine et al., 1996; Mendoza-Herrera et al., 2021).

Leptin

Leptin is a hormone primarily secreted by adipose tissue which regulates energy homeostasis and appetite (Gruzdeva et al., 2019; Zhang & Chua, 2017). Leptin exerts its effects by binding to its specific receptors, particularly in the neurons of the hypothalamus (Gruzdeva et al., 2019; Zhang & Chua, 2017). By signalling the hypothalamus, leptin reduces food intake (by reducing appetite) (Gruzdeva et al., 2019; Zhang & Chua, 2017), and promotes energy expenditure (by increasing sympathetic nervous system activity, which in turn stimulates thermogenesis in brown adipose tissue) (Pandit et al., 2017). An increase in circulating leptin concentration, therefore, reduces appetite and facilitates body weight loss (Gruzdeva et al., 2019; Zhang & Chua, 2017). In people with obesity, leptin's regulatory effects are impaired despite elevated circulating leptin levels; a condition termed leptin resistance (Git & Adan, 2015; Gruzdeva et al., 2019; Myers et al., 2012). Leptin resistance may arise from defective intracellular signalling at the leptin receptor or impaired transport of leptin across the blood-brain barrier (Gruzdeva et al., 2019). One common contributor to leptin resistance is diet-induced obesity, although animal studies indicate that leptin resistance typically precedes obesity (de Git et al., 2018; Scarpace & Zhang, 2007, 2009).

Leptin is correlated with total body fat mass and body weight fluctuations (Benini et al., 2001; Considine et al., 1996). Short-term energy restriction, even without accompanying body weight loss, reduces leptin concentrations, which return to baseline or increase upon resumption of an unrestricted diet (Mars et al., 2005, 2006; Mendoza-Herrera et al., 2021). Long-term studies also demonstrate sustained reduction in leptin levels with consistent body weight loss (Mendoza-Herrera et al., 2021; Wadden et al., 1998; Wisse et al., 1999).

Prevalence of type 2 diabetes

In 2021, age-standardised total diabetes prevalence rates varied at the super-region level, going from almost 3% in Eastern sub-Saharan Africa to just above 12% in Oceania (Ong et al., 2023), with a global average prevalence of 10.5% (*IDF Diabetes Atlas 10th Edition*, 2021). In contrast, type 2 diabetes appears to be rare or absent among modern hunter-gatherers around the world who still maintain traditional hunter-gatherer lifestyles (Joffe et al., 1971; Lindeberg et al., 1999; Lindeberg, 2009a; Merimee et al., 2003; O'Dea, 1992; Spielman et al., 1982; Zimmet, 1979). The observed low or absent prevalence of type 2 diabetes among hunter-gatherers is supported by accompanying favourable levels in risk factors for type 2 diabetes such as fasting blood glucose, glucose tolerance, insulin, obesity and

abdominal fat accumulation (Carrera-Bastos et al., 2011; O'Dea, 1992). Genetic susceptibility does not appear to account for the low prevalence of type 2 diabetes among hunter-gatherer populations since the prevalence increases upon their transition to an urbanised lifestyle (O'Dea, 1992).

Paleolithic diet

Proposition

Following the split of human and chimpanzee lineages around 5.5 million years ago, early human predecessor Ardipithecus Ramidus probably lived in a wooden habitat with access to a generalized plant-based diet (White et al., 2009). Around 4 million years ago, human predecessors started to exploit more open landscapes and incorporated more hard and abrasive foods such as seeds and underground storage organs (White et al., 2009). This transition can be demonstrated by the adaptations seen with enlarged teeth and thickened enamel (Ungar, 2011, 2019; White et al., 2009). Beginning about 3 million years ago, human predecessors began to include more animal foods, enabled by their use of stone tools (Cordain, 2007). The controlled use of fire by early humans from about 300-400 thousand years ago allowed for easier digestion of cooked food (Roebroeks & Villa, 2011; Shimelmitz et al., 2014). Another dietary shift began 10,000 years ago with the introduction of food from agriculture and animal husbandry (Cordain et al., 2005; Eaton & Konner, 1985; Lindeberg, 2009a; Lindeberg et al., 2017). In archeology, the time period from about 3 million years ago, when humans started using stone tools, up until 10,000 years ago, with the advent of agriculture, is termed the Paleolithic (from Greek: παλαιός, palaios, "old"; and λίθος, lithos, "stone", meaning "old age of the stone" or "Old Stone Age").

The observation that the prevalence of type 2 diabetes was comparably low among contemporary hunter-gatherers led to the proposition by Eaton & Konner in 1985 that their lifestyle, and especially their diet, could be ideal for the prevention and treatment of type 2 diabetes (Eaton & Konner, 1985; Konner & Eaton, 2010, 2023). The diet of hunter-gatherers was thought to most closely resemble that of our human ancestors living during the Late Paleolithic period, hence the diet was termed the Late Paleolithic diet (Eaton & Konner, 1985; Konner & Eaton, 2010, 2023). The theoretical foundation of the proposition was that modern chronic diseases arise from insufficient genetic adaptation to agricultural diets, which were introduced relatively recently from an evolutionary perspective (Carrera-Bastos et al., 2011; Cordain et al., 2005; Eaton et al., 2002; Eaton & Konner, 1985; Kuipers et al., 2010; Lindeberg, 2009b, 2012; Lindeberg et al., 2017; Muskiet & Kuipers, 2010). In

subsequent publications, the Late Paleolithic diet was shortened to just the Paleolithic diet.

Definition

As mentioned above, a Paleolithic diet represents the presumed food habits of hunter-gatherers during the Late Paleolithic period, just before the advent of agriculture and animal husbandry. Fruits, tubers, nuts, lean meat, larvae, insects, fish, shellfish, eggs, honey, and a large variety of vegetables would have been the available food staples (Cordain et al., 2005; Kuipers et al., 2012; Lindeberg, 2009a, 2012; Lindeberg et al., 2017), whose constituents and availability would have varied depending on geography, ecological niche, season and glaciations (Carrera-Bastos et al., 2011; Lindeberg, 2012). On the other hand, some foods related to the advent of agriculture, such as cereal grains, dairy products, refined oils and sugars, would consistently not have been available food staples (Cordain et al., 2005; Lindeberg, 2005; Lindeberg et al., 2017). On average, 75% of the calories consumed in many industrialised countries are derived from wheat and other cereal grains, dairy products, refined fats and sugar (Cordain et al., 2005).

Results from previous studies about type 2 diabetes

Several trials have investigated the potential benefits of a Paleolithic diet related to type 2 diabetes, both in subjects with and without type 2 diabetes.

In two 3-month RCTs – one including 13 participants (three women and 10 men, mean age 63; *SD* 6 years, and mean age 66; *SD* 6 years, for those starting with the diabetes diet first and the Paleolithic diet first, respectively) with type 2 diabetes (Jönsson et al., 2009), and the other including 29 male participants (mean age 65; *SD* 10 years, and mean age 57; *SD* 7 years, for the Paleolithic and Mediterranean-like diets, respectively) with type 2 diabetes or decreased glucose tolerance (Lindeberg et al., 2007) – a Paleolithic diet resulted in significantly greater improvements in glycaemic control (as measured by HbA1c) and glucose tolerance compared with the reference diets (diabetes and Mediterranean-like diet, respectively) (Jönsson et al., 2009; Lindeberg et al., 2007). In another 2-week RCT including 24 participants (mean age 56; *SD* 13 years, and mean age 58; *SD* 8 years, for the diabetes and Paleolithic diets, respectively) with type 2 diabetes, there was a trend towards greater improvement in glycaemic control (as measured by fructosamine) during a Paleolithic diet compared with a diabetes diet (Masharani et al., 2015). Another 12-week RCT also including 29 participants [five women and 10 men, mean age 60; range (53-64) years, and five women and nine men, mean age 61; range (58-66) years, for the Paleolithic diet and Paleolithic diet + exercise, respectively] with type 2 diabetes resulted in significantly greater improvement in glycaemic control (as measured by HbA1c) in the Paleolithic diet groups (Otten et

al., 2016). In another 12-week RCT including 14 participants [two women and four men, mean age 50; *SD* 9 years, three women and one man, mean age 52; *SD* 12 years, and two women and two men, mean age 54; *SD* 12 years, for the Paleolithic, ketogenic and control diets, respectively] with multiple sclerosis there were no between-group differences in clinical outcomes related to glycaemic control or insulin sensitivity with a modified Paleolithic diet compared with a ketogenic diet and a control diet (Lee et al., 2021). Another 3-day plus 7-day plus 10-day non-controlled study evaluated the effects of a Paleolithic diet compared with previous usual diet in nine healthy nonobese sedentary participants (three women and six men, mean age 38; *SD* 12 years) ensuring no body weight loss by daily weighing. The participants ate their usual diet for 3 days, followed by three ramp-up diets of increasing potassium and fibre for 7 days, followed by the Paleolithic diet for 10 days. After the intervention, the Paleolithic diet led to a significant decrease in plasma insulin during OGTT, regardless of no body weight loss (Frassetto et al., 2009). In another 5-week non-controlled study including 10 healthy nonsmoking postmenopausal women a Paleolithic diet resulted in a significant reduction in fasting glucose (Ryberg et al., 2013). In 10 Australian Aborigines (mean age 54; *SD* 2 years) with type 2 diabetes, a transition to their traditional hunter-gatherer lifestyle significantly improved their glucose clearance after 7 weeks (O'Dea, 1984). In a similar study, 13 Australian Aborigines without diabetes, who initially lived in a modern urban lifestyle, transitioned to their traditional lifestyle for 3 months, during which their high insulin response to glucose improved significantly, before returning to their urban lifestyle, which caused their insulin response to increase again (O'Dea et al., 1980).

In observational studies, the Paleolithic diet has been assessed for its association with incidence of type 2 diabetes in two studies (Rydhög et al., 2024; Shah et al., 2021).

In the study by Shah et al. (2021) a Paleolithic Diet Score – an aggregated score based on an individual's intake of Paleolithic food groups relative to a population – was inversely associated with incidence of type 2 diabetes in 70,991 women (aged 40–65 years at baseline) from the E3N cohort (Etude Epidémiologique auprès de femmes de la Mutuelle Générale de l'Education Nationale) with a follow-up of nearly 20 years (Shah et al., 2021). In the study by Rydhög et al. (2024) the Paleolithic Diet Fraction – a dietary measure that estimates how large a portion of the absolute dietary intake stems from food groups included in a Paleolithic diet – was similarly albeit non-significantly inversely associated with incidence of type 2 diabetes in 24,104 subjects (aged 44–74 years at baseline, 63% women) from the Malmö Diet and Cancer Study cohort with a median follow-up of 18 years (Rydhög et al., 2024).

The above-reviewed dietary studies indicate a comparably greater improvement in glycaemic control from practising a Paleolithic diet. This inference is supported by a network meta-analysis in which a Paleolithic diet was found to be an efficacious

dietary approach for glycaemic control in type 2 diabetes (Schwingshackl et al., 2018). Furthermore, a scoping review concluded that a Paleolithic diet may have favourable effects on type 2 diabetes, although the available evidence remains limited (Yan & Louie, 2024). Importantly, especially for this thesis, the improved glycaemic control from a Paleolithic diet in the above-reviewed studies were generally accompanied by a significant reduction in body weight. This finding is also supported by a network meta-analysis aiming to determine the effects of a Mediterranean diet compared with other dietary interventions, including a Paleolithic diet, on glycaemic control (Carter et al., 2014). Additionally, the accompanying reduction in body weight in the above-reviewed studies was significantly greater for a Paleolithic diet in one of the RCTs (Jönsson et al., 2009). This finding was supported by a meta-analysis in which a Paleolithic diet was accompanied by greater reductions in body weight compared with recommended diets (de Menezes et al., 2019). Reduction in body weight is associated with improved glycaemic control (Gummesson et al., 2017; Lean et al., 2018), and reduction in body weight has, in dietary intervention studies, resulted in improved glycaemic control (Emadian et al., 2015; Schwingshackl et al., 2018). This suggests that the comparably greater improvement in glycaemic control in type 2 diabetes from a Paleolithic diet could be due to an accompanying greater reduction in body weight. The suggested causality could be assessed by examining the effect of a Paleolithic diet compared with a recommended diabetes diet on glycaemic control in type 2 diabetes when body weight is kept stable (Papers I and II) (Figure 1).

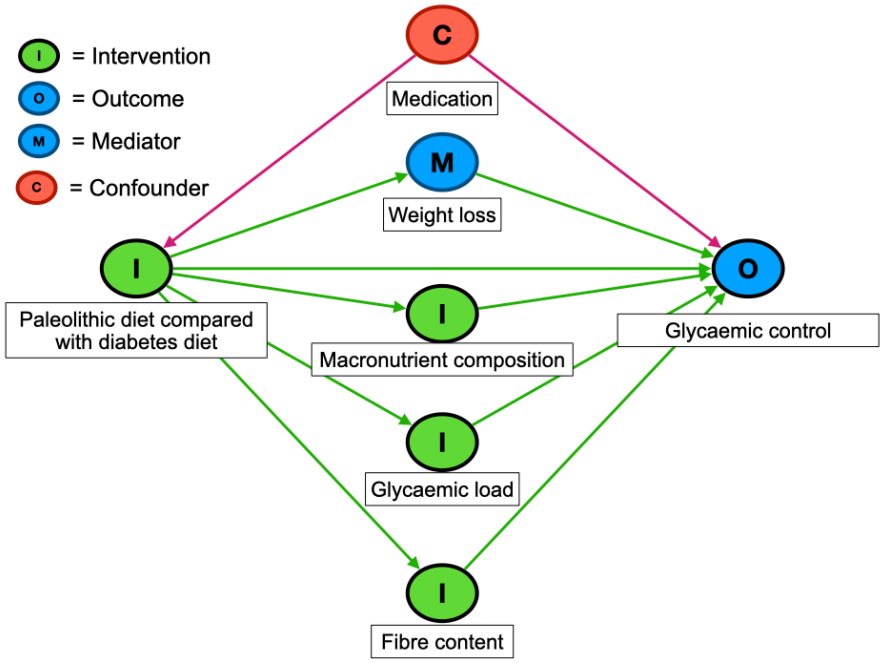

Figure 1. Graphical Representation of Suggested Causality for Papers I and II

Note. Graphical representation of the suggested causality of effects from a Paleolithic diet compared with a diabetes diet on glycaemic control. In papers I and II the effect of a Paleolithic diet compared with a diabetes diet on glycaemic control will be assessed when participants' body weight is kept stable, medication is accounted for, and diets are matched for macronutrient composition, glycaemic load and fibre content.

Regarding reduction in body weight, it is also interesting that in two of the RCTs reviewed above, including participants with type 2 diabetes, the participants ate significantly fewer calories practising a Paleolithic diet compared with the reference diets (Mediterranean-like and diabetes diet, respectively), despite being equally satiated during both diets (Jönsson et al., 2009, 2010, 2013; Lindeberg et al., 2007). Furthermore, in the aforementioned study by Lindeberg et al., which compared the

effects of a Paleolithic diet with a Mediterranean-like diet in participants with type 2 diabetes or impaired glucose tolerance, both diets resulted in a significant reduction in the satiety hormone leptin, but there was a trend toward a greater reduction in leptin levels from a Paleolithic diet, which reached statistical significance after removing a cereal grain intake outlier (Jönsson et al., 2010). Additionally, the strongest correlation between change in leptin and dietary variables was with cereal grains (excluding rice) (Jönsson et al., 2010). The reduction in leptin levels was also significantly correlated with changes in body weight from a Paleolithic diet, but not from the Mediterranean-like diet (Jönsson et al., 2010). Another study investigated the effects of a Paleolithic diet, with and without supervised exercise, in participants with type 2 diabetes (Otten et al., 2016). Both intervention groups resulted in a significant reduction in leptin levels, accompanied by a significant within-group body weight loss, with no significant differences between the two groups (Otten et al., 2016). In an acute meal study in 55 healthy male participants (mean age 28; *SD* 13 years, mean age 27; *SD* 13 years, and mean age 27; *SD* 13 years, for the Paleolithic meal 1, Paleolithic meal 2, and reference meal, respectively) a Paleolithic meal, compared with a reference meal based on WHO recommendations, resulted in a significantly greater increase in satiety scores and in the satiety-related hormones GLP-1 and GIP, but not in leptin (Bligh et al., 2015). The possibility of a direct effect from a Paleolithic diet on the satiety hormone leptin was investigated in an in vitro study, which found that digested wheat gluten inhibited leptin binding to its receptor at clinically relevant concentrations, although the inhibition was abolished by heat treatment of the digested wheat gluten (Jönsson et al., 2015). The basis for this in vitro study was the previous observation that the strongest correlation between changes in leptin levels and dietary variables was with cereal grain intake (Jönsson et al., 2010). The above-mentioned results suggest that the comparably greater reduction in body weight accompanying a Paleolithic diet in type 2 diabetes could also be due to an accompanying greater effect on satiety and reduction in the satiety hormone leptin. The suggested causality could be assessed by examining the effects of a Paleolithic diet compared with a recommended diabetes diet on leptin and leptin receptor binding (bioLep) in type 2 diabetes (Papers III and IV) (Figure 2).

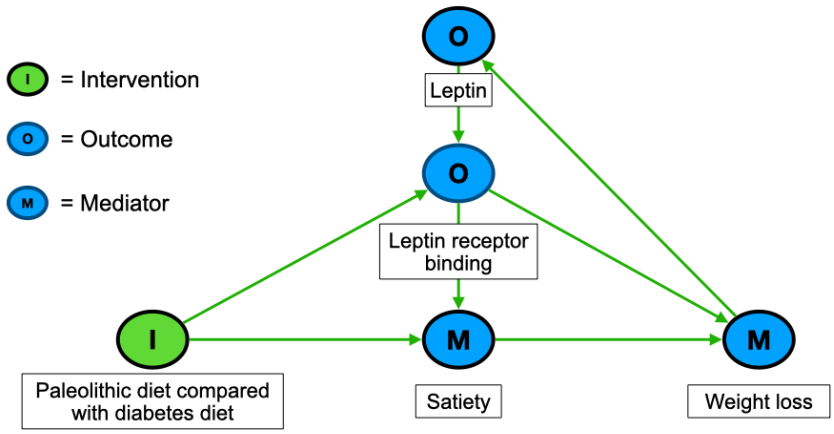

Figure 2. Graphical Representation of Suggested Causality for Papers III and IV

Note. Graphical representation of suggested causality of effects from a Paleolithic diet compared with a diabetes diet on leptin and leptin receptor binding. In papers III and IV the effect of a Paleolithic diet compared with a diabetes diet on leptin and leptin receptor binding will be assessed by comparing fasting levels of leptin and leptin receptor-binding leptin (bioLep) from a previous trial.

Results from previous studies not about type 2 diabetes

Overview of RCTs

A 2-year RCT investigated the effects of a Paleolithic diet compared with the Nordic Nutrition Recommendations diet, which included 70 postmenopausal women (mean age 59; *SD* 5 years, and mean age 60; *SD* 6 years, for the Paleolithic and Nordic Nutrition Recommendations diets, respectively) with obesity (Mellberg et al.,

2014). Over the course of the study, the Paleolithic diet produced a significant greater reduction in fat mass at 6 months and greater body weight loss at 6, 12 and 24 months. Additionally, the Paleolithic diet improved insulin sensitivity and reduced triglycerides more than the control diet. Another 2-week RCT investigated the effects of a Paleolithic diet compared with an isoenergetic healthy reference diet based on the guidelines of the Dutch Health Council, which included 34 participants (25 women and nine men in the total sample; 72% women, mean age 52; *SD* 12 years, and 75% women, mean age 55; *SD* 9 years, for the Paleolithic and Reference diets, respectively) with the metabolic syndrome (Boers et al., 2014). Individuals with other conditions such as type 2 diabetes, cardiovascular disease or smokers were not included. Although the researchers aimed to prevent body weight loss, participants in the Paleolithic diet group experienced a significantly greater reduction in body weight compared with those in the control group. The Paleolithic diet led to significant improvements in cardiovascular risk factors, including reductions in systolic blood pressure, diastolic blood pressure, total cholesterol, and triglycerides, while HDL cholesterol levels increased. Another 4-week RCT investigated the effects of a Paleolithic diet compared with the Australian Guide to Healthy Eating, which included 39 healthy women (mean age 47; *SD* 13 years) (Genoni et al., 2016). Despite the significantly greater body weight loss observed in the Paleolithic diet group, there were no differences in cardiovascular risk factors between the groups, suggesting that the Paleolithic diet's benefits in healthy populations may be more limited. This is still an important finding since it indicates that a Paleolithic diet's ability to reduce body weight is not confined to clinical populations but also include healthy people. Another 24-week RCT investigated the effects of a modified Paleolithic diet compared with a low-saturated fat diet, which included 77 subjects (32 women and seven men, mean age 46; *SD* 1 years, and 35 women and three men, mean age 47; *SD* 2 years, for the modified Paleolithic and low-saturated fat diets, respectively) with relapsing-remitting multiple sclerosis (Wahls et al., 2021). Both groups improved markers of fatigue and quality of life without between-group differences. Another 10-week RCT investigated the effects of Paleolithic-based low-carbohydrate diets compared with moderate-carbohydrate diets, with either calorie-counting or portion-control method, which included 80 Iranian adults (12 women and eight men, mean age 43; *SD* 10 years, 12 women and eight men, mean age 44; *SD* 10 years, 12 women and eight men, mean age 44; *SD* 9 years, and 12 women and eight men, mean age 41; *SD* 8 years, for the Paleolithic Low-Carbohydrate/Calorie Counting, Paleolithic Low-Carbohydrate/Portion Control, Moderate Carbohydrate/Calorie Counting and Moderate Carbohydrate/Portion Control diets, respectively) with the metabolic syndrome (Shemirani et al., 2022). Significant reduction in body weight, waist circumference, body fat, visceral fat and waist-to-hip ratio was observed in all four intervention groups, with no between-group differences. Notably, the authors also found significant reduction in leptin in all intervention groups with no between-group differences.

A short-term non-randomised controlled trial evaluated the effects of a Paleolithic diet and lifestyle intervention (daily outdoor activity of at least 30 minutes), which included 11 women [mean age 58, range (37-72) years] with breast cancer while undergoing radiotherapy for an average of 39 (range 21-43 days) (Klement et al., 2021). A control group including 11 women [mean age 58; range (35-67) years] on an unspecified standard diet was assigned by propensity score matching. The Paleolithic diet produced a significant reduction in body weight (~ 0.4 kg/week), mainly due to fat mass which decreased on average by 0.34 kg/week. In contrast, the control group led to a significant average reduction of fat free mass by 0.13 kg/week with a concomitant non-significant increase of fat mass by 0.14 kg/week.

Overview of non-controlled trials (NCT)

A 3-week NCT investigated the effects of a Paleolithic diet, which included 14 healthy participants (nine women and five men) (Österdahl et al., 2008). After 3 weeks, there was a significant decrease in body weight, BMI, waist circumference, and systolic blood pressure. Another 4-month plus 4-month NCT evaluated the effects of a Paleolithic diet compared with a previous heart-healthy diet, which included 20 individuals (10 women, mean age 52; *SD* 7 years, and 10 men, mean age 53; *SD* 7 years) with a medical diagnosis of hypercholesterolemia (Pastore et al., 2015). Compared with the previous diet, the Paleolithic diet produced a significant reduction in total and LDL cholesterol, and increased HDL cholesterol. Even though the participants lost significantly more body weight after switching to the Paleolithic diet, the authors reported that there was no statistical association between change in any blood lipid concentration value and body weight loss. Another 11-week NCT investigated the effects of the autoimmune protocol diet – which is an extension of the Paleolithic diet – on clinical response in inflammatory bowel disease, which included 15 participants (11 women and four men, mean age of total sample 44; *SD* 19 years) (Konijeti et al., 2017). The intervention resulted in a reduction in endoscopic inflammation and disease related symptoms.

Overview of observational studies

Several studies suggest that a Paleolithic diet is inversely associated with risk of all-cause mortality.

In the study mentioned above by Rydhög et al. (2024) in 24,104 subjects (aged 44–74 years at baseline, 63% women), from the Malmö Diet and Cancer Study cohort, the Paleolithic Diet Fraction was also inversely associated with both all-cause and cause-specific mortality as well as with incidence of cardiovascular disease (Rydhög et al., 2024). A similar result for inverse association with all-cause mortality was found by Whalen et al. (2017) for the Paleolithic Diet Score in the REGARDS (REasons for Geographic and Racial Differences in Stroke) study, a prospective cohort study of 21,423 black and white men and women from all

contiguous 48 US states aged ≥ 45 years (mean age 66; *SD* 9 years, 56% women), with a median follow-up of 6 years (Whalen et al., 2017). In another study, the Paleolithic Diet Score used by Whalen et al. (2014) was also inversely associated with risk of death from all causes in the Moli-sani Study, a prospective cohort study of 22,849 men and women in Italy aged ≥ 35 years (mean age 55; *SD* 12 years, 52% women), with a median follow-up of 8 years (Bonaccio et al., 2021). The same Paleolithic Diet Score, re-termed Evolutionary-Concordance Diet Pattern Score, was similarly albeit non-significantly inversely associated with risk of death from all causes in the Iowa Women's Health Study, a prospective cohort study of 41,836 women in Iowa, USA, aged 55–69 years (mean age 62; *SD* 4 years) (Cheng et al., 2018).

Similarly to in the study by Rydhög et al. (2024) incidence of cardiovascular disease was also inversely associated with the Paleolithic Diet Score in the 18,210 subjects (mean age 38; *SD* 12 years, 61% females) from the Seguimiento Universidad de Navarra (SUN) cohort study with a follow-up of 12 years (de la O et al., 2022). Congruent inverse association was found for non-alcoholic fatty liver disease and the Paleolithic Diet Score in a case-control study among 206 patients (mean age 38; *SD* 8 years, 60% women) and 306 healthy subjects (mean age 38; *SD* 9, 76% women) (Sohouli, Fatahi, et al., 2022).

Several studies highlight the potential of a Paleolithic diet in reducing risks for various cancers.

The Paleolithic Diet Score was inversely associated with incidence of colorectal cancer in 74,721 subjects (mean age 65; *SD* 6 years, 53% women) from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial cohort with a median follow-up of 9 years (Xiao et al., 2023). In a second study, the Paleolithic Diet Score was inversely associated with incidence of breast cancer in 65,574 women (mean age 52; *SD* 7 years) from the E3N (Etude Epidémiologique auprès de femmes de la Mutuelle Générale de l'Education Nationale) with a mean follow-up of 20 years (Shah et al., 2023). In a third study, the Paleolithic Diet Score was inversely associated with incidence of breast cancer in a case-control study among 253 Iranian women with breast cancer (mean age 49; *SD* 10 years) and 267 Iranian healthy women (mean age 47; *SD* 10 years) (Sohouli, Baniasadi, et al., 2022). In a fourth study, the Paleolithic Diet Score was inversely associated with incidence of brain cancer in a case-control study among 506 pairs of Chinese subjects with and without glioma (Zhang et al., 2023). Among participants aged ≤ 40 years, 44% were women in both the case and control groups, with a mean age of 31 years in both groups. Among those aged >40 years, 43% of cases and 44% of controls were women, with a mean age of 53 years in both groups. In a fifth study, the Paleolithic Diet Score was inversely associated with incidence of colorectal adenomas in a case-control study among 564 subjects with endoscopy findings of colorectal adenomas (mean age 58; *SD* 10 years, 62% men) and 1,202 endoscopy-negative controls (mean age 46; *SD* 6 years, 39% men) (Whalen et al., 2014).

Adherence to a Paleolithic diet has also been associated with reduced risk of psychological disorders such as anxiety, depression, and stress.

The Paleolithic Diet Score was inversely associated with anxiety in a cross-sectional study among 7,165 subjects (41%, 51%, 50% and 50% of women in the first to fourth quartile, respectively), with an age range of 20 to 69 years old, in the Yazd Health Study (YaHS) and Yazd Nutrition Study (TAMYZ) cohorts in Iran (Khodadadi et al., 2024). In another study, the Paleolithic Diet Score was inversely associated with depression, anxiety, and stress in a cross-sectional study among 435 women (mean age 31; *SD* 8 years) that refer to health care centers in Iran (Zamani et al., 2023).

Lastly, the Paleolithic diet has also been assessed for association with systemic inflammation and gut microbiome biodiversity.

The Paleolithic Diet Score was inversely associated with concentration of circulating inflammatory markers C-reactive protein and F_2-isoprostane in a cross-sectional study among 646 subjects (mean age of 60 years) in an elective outpatient colonoscopy population (Whalen et al., 2016). The gut microbiome response to a Paleolithic diet was investigated in a cross-sectional study among 15 healthy subjects [three women and 12 men, mean age 39; range (26-57) years], in Italy (Barone et al., 2019). A 7-day weighted food record, with the total food and beverage consumption, was filled by the participants to assess the dietary intake. The results indicated a higher gut microbiome biodiversity among subjects adhering to a Paleolithic diet compared with subjects adhering to a Mediterranean diet.

Aims of the thesis

The general aim of this thesis was to assess in type 2 diabetes the effect of a Paleolithic diet compared with a diabetes diet on glycaemic control when body weight is kept stable (Papers I and II); and on leptin and leptin receptor binding (Papers III and IV).

The specific aim for each paper was:

I. To develop the study protocol for Paper II in detail before study subject recruitment started.

II. To assess in type 2 diabetes the effect of characteristic food groups differences between a Paleolithic and a diabetes diet on glycaemic control when body weight is kept stable, and diets are matched for macronutrient composition, glycaemic load and fibre content.

III. To assess the differences in type 2 diabetes from a Palaeolithic diet compared with a diabetes diet in adipokines such as leptin.

IV. To assess in type 2 diabetes the effect of a Paleolithic diet compared with a diabetes diet on leptin and leptin receptor binding, and to replicate an in vitro study on leptin receptor binding using another method.

Methods

Papers I and II

Design

Paper I is a detailed study protocol of Paper II published before study subject recruitment started. Paper II is a randomised crossover dietary intervention trial in subjects with type 2 diabetes and increased waist circumference. The study compared two, according to official dietary guidelines, healthy diets, with characteristic food groups differences between a Paleolithic diet and a diabetes diet, while body weight was kept stable throughout the study.

Population

The study population consisted of adults with a medical diagnosis of type 2 diabetes and increased waist circumference (≥ 80 cm for women and ≥ 94 cm for men), with or without medication (including insulin treatment), with stable body weight for 3 months prior to the start of the study, who had received no change in dose of beta blocker or thyroxine for 3 months prior to the start of the study, no anticoagulant or oral steroid treatment, and had HbA1c ≥ 6.0% (≥ 42.1 mmol/mol), with no upper boundary, creatinine < 130 μmol/L and liver enzymes less than four times above the upper reference value. Subjects lived on the island of Lanzarote, in the archipelago of the Canary Islands (Spain), located off the western coast of Africa.

Eligible participants were given general oral and written information about the study. They were informed that the objective was to compare two healthy diets because it was not known which, if any, was the better one. If a person was interested, their name and telephone number were registered for further contact. After recruiting 15 potential participants, a meeting was arranged to provide further details about the study.

Interventions

Participants were randomised to start with one of two healthy diets for a 4-week period separated by a 6-week period. The intervention was based on two diets:

healthy diet A which included fruit, vegetables, fish, shellfish, lean meat, nuts, eggs, olive oil and substantial amounts of whole grains, low-fat dairy products and legumes (termed "healthy diet with cereal grains and dairy products") and healthy diet B which included fruit, vegetables, fish, shellfish, lean meat, nuts, eggs and olive oil, and excluded cereal grains, dairy products and legumes (termed "healthy diet without cereal grains and dairy products"). For clarity and simplicity, in this thesis, "healthy diet A with cereal grains and dairy products" is named "diabetes diet" and "healthy diet B without cereal grains and dairy products" is named "Paleolithic diet". Both diets were matched for macronutrient composition (19% protein, 28% fat and 53% carbohydrates), glycaemic load (116-118/2,000 kcal) and fibre content (~ 50g).

Following randomisation, all subjects received oral and written information about their respective initial dietary intervention. Since all participants in both groups started the intervention at the same time, information about the specific intervention was provided in group sessions, one for each group before the study commenced. Prior to the beginning of the second dietary period, participants were instructed about the new dietary intervention, also in group sessions. Each session lasted 1 hour and was performed in the meeting room at the Hospital Insular de Lanzarote after the laboratory tests. The degree of behavioural support was the same in both interventions to minimise differences in motivation. Written information with dietary advice, a 7-day menu plan and food recipes were similarly formulated for both diets. Depending on body weight as measured every week, each participant was reassigned to another (with higher or lower energy) menu plan if their body weight changed by ≥ 1 kg. All the different menu versions maintained the same macronutrient composition, glycaemic load and fibre content.

Advice about regular physical activity was given equally to all participants. Specifically, they were recommended not to change their current physical activity levels during the whole trial.

Outcomes

Before and after each intervention period, an OGTT was performed in the morning after obtaining venous blood samples in the fasting state, and measurements of primary and secondary outcomes were performed. Height was only measured at the start of the first intervention period. For the OGTT the participants ingested 75 g of glucose and blood samples were drawn for glucose and glucagon AUC analysis at 0, 15, 30, 60, 90 and 120 min. For safety reasons, capillary glucose was assessed using a glucometer before performing the OGTT. If the result was greater than or equal to 190 mg/dL (10.5 mmol/L) the OGTT would not be performed; notwithstanding, this threshold was never reached during the study.

Predefined primary outcomes were HbA1C and glucagon. Two weeks after randomisation, and the concurrent start of the study, fructosamine was added as a primary outcome, thinking its shorter evaluation period of glucose exposure over the last month would improve the study by being a better fit to the 1-month diet interventions than the 2- to 3-month evaluation period of HbA1C (Bergman et al., 2020). For the same reason, after the study completion, glycated albumin was also added as a secondary outcome (Bergman et al., 2020). The predefined secondary outcomes were fasting plasma glucose, total cholesterol, LDL, HDL, triglycerides, high-sensitivity C-reactive protein, blood pressure, AUC for glucose and glucagon during OGTT, anthropometric measurements (body weight, waist and hip circumference, sagittal abdominal diameter, and skinfold thickness at triceps, biceps, suprailiac and subscapular regions), quality of life as assessed with the SF-36v2 questionnaire (standard version), satiety quotient, experience with the dietary patterns and change in medication. A 4-day weighed food record, including 1 weekend day, with weighing of each food item on an electronic weighing scale (that could be set to zero), was completed by the participants before the start and end of each intervention period. In parallel with the 4-day weighed food records, the participants recorded their subjective rating of satiety by means of a 7-point Likert-type scale. Nutrient composition was assessed using the nutritional software package DIAL that was used to create the meal plans (Ortega et al., n.d.).

Statistical analysis

A pre-study power calculation showed that to detect, with 80% power and at a significance level of 5%, a 0.6 percent point (4 mmol/mol) difference between diets in a change in HbA1c, 13 participants were estimated to be required. Similarly, for a 26 μmol/L difference in change in fructosamine, 12 participants would be required. Normal Q-Q plot assessments and the Shapiro-Wilk test were performed to examine if variables were normally distributed. Normally distributed variables are presented as mean with standard deviation. When variables were not normally distributed, logarithmic transformation was applied to see if normal distribution could be achieved. Transformed variables with normal distribution are presented as median [95% CI]. Variables not normally distributed, neither before nor after transformation, are presented as median (range). Mean comparisons between participants were performed using a two-tailed unpaired *t* test or a Mann-Whitney *U* test, as appropriate. Mean comparisons within participants were performed using a two-tailed paired *t* test or a Wilcoxon signed-ranks test, as appropriate. All statistical tests were two-sided, and statistical significance was assumed at $p < .05$. The SPSS statistical computer package (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses.

Papers III and IV

Design

Papers III and IV are measurements of fasting leptin, bioLep and other adipokines in stored blood samples from a randomised crossover dietary intervention trial in participants with type 2 diabetes comparing a Paleolithic diet with a diabetes diet (Jönsson et al., 2009). BioLep is a measurement of the levels of leptin with the ability to bind to its receptor. BioLep thereby provides an assessment of how "biologically active" or "functional" leptin is in terms of its ability to bind to its receptor. Paper IV also replicates an in vitro surface plasmon resonance technology study on leptin receptor binding inhibition using bioLep measurements.

Population

The study population consisted of adults with type 2 diabetes without insulin treatment and a C-peptide value above zero, unaltered medical diabetes treatment with stable body weight for 3 months before the start of the study, who had received no change in dose of beta blocker or thyroxin for 6 months prior to the start of the study, no anticoagulant or oral/injected steroid treatment, and had HbA1c above 5.5% by Mono-S standard, creatinine < 130 μmol/L, liver enzymes below four times their respective upper reference value, and no acute coronary event. Physical or psychological illness or changes in personal circumstances, which would make further study participation impossible, were exclusion criteria. Subjects were recruited from three primary health care units in the Lund area in Sweden.

Interventions

All eligible subjects were informed of the intention to compare two healthy diets for the treatment of type 2 diabetes and that it was unknown if any of them would be superior to the other. At study start all eligible subjects were randomised to start with either a diabetes diet in accordance with current guidelines or a Paleolithic diet. Immediately after randomisation, all participants received oral and written information individually about their respective initial diet. After 3 months all participants switched diets and received new oral and written information individually about the diet for the following 3 months. Written information with dietary advice and food recipes were similarly formulated for both diets.

The information on the diabetes diet stated that it should aim at evenly distributed meals with increased intake of vegetables, root vegetables, dietary fibre, whole-grain bread and other whole-grain cereal products, fruits and berries, and decreased intake of total fat with more unsaturated fat. The majority of dietary energy should

come from carbohydrates from foods naturally rich in carbohydrates and dietary fibre. The concepts of glycaemic index and varied meals through meal planning using the Plate Model were explained. Salt intake was recommended to be kept below 6 g per day.

The information on the Paleolithic diet stated that it should be based on lean meat, fish, fruit, leafy and cruciferous vegetables, root vegetables, eggs and nuts, while excluding dairy products, cereal grains, beans, refined fats, sugar, candy, soft drinks, beer and extra addition of salt. The following items were recommended in limited amounts for the Paleolithic diet: eggs (two per day), nuts (preferentially walnuts), dried fruit, potatoes (one medium-sized per day), rapeseed or olive oil (one tablespoon per day), wine (one glass per day). The intake of other foods was not restricted, and no advice was given regarding proportions of food categories (e.g. animal versus plant foods). The evolutionary rationale for a Paleolithic diet and the potential benefits were explained.

Advice about regular physical activity was given equally to all participants.

Outcomes

For Paper III, measurements of leptin and adiponectin, adipsin, C-peptide, ghrelin, GIP, GLP-1, leptin, insulin, glucagon, insulin, resistin and visfatin were made using the Bio-Plex pro™ human diabetes panel (Bio-Rad Inc., Hercules, CA, USA).

For Paper IV, measurements of leptin and bioLep were made using LEP ELISA E07 and bioLep ELISA L07 (Mediagnost), respectively.

An OGTT was performed in the morning after obtaining fasting venous blood samples and measurements of blood pressure, body weight and waist circumference using standard methods at the start of the study, after 3 months (when switching to a new diet) and at the end of the study (after 6 months). Samples were collected in EDTA-containing tubes and centrifuged for 10 min at 4°C. Plasma was then aliquoted and stored at -80°C until analysis. A 4-day weighed food record on 4 consecutive days, including 1 weekend day, with weighing of each food item on a digital weighing scale (that could be set to zero), was completed by the participants, starting 6 weeks after initiating each diet. Nutrient compositions were calculated using data from The Swedish Food Database of the National Food Administration in Sweden.

In vitro

BioLep was measured in triplets for recombinant leptin at 10 and 50 ng/ml concentrations (recombinant human leptin, R&D Systems 398-LP, the same product purchased for use in the in vitro study using surface plasmon resonance technology) (Jönsson et al., 2015), incubated with a series of increasing gluten digest

concentrations ranging from 0 to 320 µg/ml. The recombinant leptin concentration of 10 ng/ml was chosen as a close representation of mean in vivo leptin concentrations in a previous randomised crossover trial (Fontes-Villalba et al., 2016), and the recombinant leptin concentration of 50 ng/ml was chosen as a representation of a much higher but still clinically plausible concentration, both of which are well within the detection limits of the laboratory kit. BioLep was also measured for recombinant leptin at 10 ng/ml concentration incubated with gluten digest concentrations ranging from 5 to 320 µg/ml after the gluten digest had been subjected to heat treatment at 100ºC for 30 min, heat treatment at 100ºC for 30 min followed by centrifugation at 13,000 g for 10 min, or only centrifugation at 13,000 g for 10 min without heat treatment.

The gluten digest used was the same -80°C stored gluten digest used in the previous in vitro study using surface plasmon resonance technology (Jönsson et al., 2015). Briefly, to mimic physiological conditions in the human intestine, gluten from wheat was digested with the gut enzymes pepsin and trypsin (Fontes-Villalba et al., 2024). Enzyme activity from pepsin and trypsin (molecular weight ~ 40 and 25 kDa, respectively) was subsequently removed from the gluten digest by spin-filtering through a 10 kDa filter (Fontes-Villalba et al., 2024).

The effects of heat treatment and centrifugation on gluten digest concentration were assessed by measuring the concentration of gluten digest before and after heat treatment at 100°C for 30 min, heat treatment at 100°C for 30 min followed by centrifugation at 13,000 g for 10 min, or only centrifugation at 13,000 g for 10 min without heat treatment.

Statistical analysis

Data were analysed for normality (determined by Q–Q plots and the Shapiro–Wilk test in SPSS) and logarithmically transformed when necessary. If data did not show reasonable normal distribution after logarithmic transformation, the Wilcoxon matched pairs signed rank sum test was used, otherwise a paired *t* test was used for paired samples. For nonpaired samples, the Mann-Whitney *U* test was used if data did not show reasonable normal distribution after logarithmic transformation, otherwise an unpaired *t* test was used. To analyse the difference between diets concerning their effects on outcomes, the absolute values at the end of each diet were compared. Significance was set at $p < .05$. All *t* tests were two-sided. The SPSS statistical computer package (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses.

For the study on leptin binding inhibition in Paper IV, the sample size was deemed sufficient based on the previous finding of a half-maximal inhibition of leptin binding at a gluten digest concentration of 10 ng/ml, which is well below the 41 ng/ml mean concentration reported for undegraded gliadin in about half (14 of

31) of healthy adult human sera (Chirdo et al., 1998a). Gliadin is the prolamin protein of wheat and makes up about half of all wheat gluten. Assuming that the leptin binding inhibitory effect of gluten digest concentrations is at least roughly translatable to similar gliadin concentrations, a greater than half-maximal inhibition of leptin binding could be expected in about half of the samples. The study would then require a sample size of at least seven participants to achieve a power of 80% and a level of significance of 5% (two-sided), for detecting a mean of the differences of 0.25 between pairs, assuming a pooled standard deviation of 0.18 for the differences.

Ethical considerations

Papers I and II

The study was approved by the Ethics Committee of Clinical Investigation (CEIC) of the Hospital Universitario de Gran Canaria Doctor Negrín (Code CEIC Negrín: 130030). Due to technical issues, C-peptide could not be assessed at the Hospital Insular de Lanzarote, where the study was conducted. As a C-peptide value ≥ 0.3 nmol/L was an inclusion criterion, an amendment to the protocol was required to remove C-peptide as a criterion. Instead, participants were required to certify their diagnosis of type 2 diabetes through a medical certificate. No adverse effects from the intervention were anticipated beyond those commonly associated with routine clinical practice. All participants received a written informed consent form, which explicitly detailed the study's aims, description, procedures, duration, number of participants, potential risks and benefits, confidentiality of personal data, and the principal investigator's contact information. The consent form was written in language that was clear and comprehensible to ensure participants fully understood its content. All participants provided their written informed consent form before taking part in the study. A requirement from the ethics committee was that participants be insured against medical complications arising from the interventions, with a policy exclusively contracted for the study. The study adhered to the recommendations and good clinical practice guidelines outlined in the Declaration of Helsinki. The scope of the study's funding did not compromise adherence to ethical principles.

Papers III and IV

For Papers III and IV, analyses in stored blood samples from a previous dietary study were performed, for which ethical approval had been acquired in 2004 from the Regional Ethical Board in Lund (LU 726/2004). The new analyses aimed to study leptin more closely and other adipokines involved in satiety regulation, and the effects on glucose metabolism. For the additional analyses, an amendment of the previously approved application was obtained (711/2013).

Results

Overview

An overview of the studies included in this thesis is presented in Table 1.

Table 1

Overview of Studies Included in this Thesis

	Paper	
	I and II	**III and IV**
Aims	To assess in type 2 diabetes the effect of characteristic food group differences between a Paleolithic and a diabetes diet on glycaemic control when body weight is kept stable, and diets are matched for macronutrient composition, glycaemic load and fibre content.	To assess in type 2 diabetes differences from a Paleolithic diet compared with a diabetes diet in adipokines such as leptin. To assess in type 2 diabetes the effect of a Paleolithic diet compared with a diabetes diet on leptin and leptin receptor binding, and to replicate an in vitro study on leptin receptor binding using another method.
Design	Randomised crossover trial 4 + 4 weeks.	Randomised crossover trial 3 + 3 months.
Population	Subjects with type 2 diabetes (n = 14).	Subjects with type 2 diabetes (n = 13).
Intervention	Paleolithic diet vs diabetes diet. Body weight kept stable, and diets matched for macronutrient composition, glycaemic load and fibre content.	Paleolithic diet vs diabetes diet. In vitro, recombinant leptin with increasing gluten digest concentrations with or without heat treatment and centrifugation.
Outcomes	HbA1c and fructosamine.	Fasting leptin and bioactive leptin (bioLep).
Main results	No differences in HbA1c or fructosamine between diets (Table 2.1).	Lower fasting leptin and bioLep after a Paleolithic diet compared with after a diabetes diet with no difference between them for either diet (Table 3.3). In vitro, dose-dependent leptin receptor binding inhibition from gluten digest that was abolished by heat treatment of the gluten digest (Figure 3).

Papers I and II

Recruitment started in August 2013 and finished in November 2013. In total, 23 subjects were assessed for eligibility and eight were excluded. A total of 15 participants met the inclusion criteria and were randomised to start the trial. One participant randomised to start with the diabetes diet discontinued the intervention after the first day because the participant found the protocol too difficult to follow.

All participants started the trial at the same time. The first diet intervention started on Monday 18th of November 2013 and finished on Sunday 15th of December 2013. The second diet intervention started on Monday 27th of January 2014 and finished on Sunday 23rd of February 2014. A total of 14 participants (seven women and seven men; seven randomised to start with the diabetes diet and seven randomised to start with the Paleolithic diet) completed the trial and were analysed per protocol for the primary outcomes. Baseline characteristics and relative change in outcomes during diets are reported in Table 2.1. There were no reported harms to the participants derived from the interventions at any time point of the trial.

There were no differences between the diets in relative change for the primary outcomes HbA1c or fructosamine (Table 2.1). No results are reported for glucagon for which most measurements failed due to sample haemolysis.

At the end of the diets, daily intake of characteristic Paleolithic food groups by energy and weight differed significantly between diets, being higher for the diabetes diet in cereal grains, legumes and dairy products compared with the Paleolithic diet (Table 2.2). Regarding macronutrient intake, except for fibre and protein, there were no significant differences between diets. Fibre intake was significantly higher during the Paleolithic diet, but well above the recommended intake (25 g per day according to WHO) during both diets. Protein intake was higher (by weight although not by energy percent) during the Paleolithic diet (Table 2.3). Body weight remained stable throughout both interventions and, therefore, no between-diets difference was observed (Table 2.1). However, to keep body weight stable (< 1.0 kg change), dietary energy intake had to be increased in seven participants during the diabetes diet and in six participants during the Paleolithic diet, while it had to be decreased in two participants during the diabetes diet and in none during the Paleolithic diet.

Table 2.1

Baseline Values and Relative Change During Diet

Variable	Baseline	Relative Change During Diet (%)			*p*
		Diabetes diet	Paleolithic diet	Difference	
Female/male, *n (%)*	7 (50%) / 7 (50%)				
Age, years *M (SD)*	64 (7)				
Diabetes duration, years *M (SD)*	8 (5)				
Height, cm *M (SD)*	164 (7)				
BMI, kg/m^2 *M (SD)*	32 (5)				
Fructosamine, µmol/L *M (SD) Mdn* (Range) *M* [95% CI]	317 (268 to 476)	-13 (8)[d]	-11 (9)[d]	-3 [-10, 4]	.40
HbA1c, % *M (SD) Mdn* (Range) *Mdn* [95% CI]	7.4 (1)	0.6 (-25 to 5)	-1.1 (7)	2 [-6, 5]	.80
Glycated albumin, % *M (SD) M* [95% CI][a]	34 (9)	-11 (15)[d]	-11 (13)[d]	1 [-12, 14]	.90
Glucometer, mg/dL *M (SD) Mdn* (Range) *M* [95% CI]	133 (123 to 224)	0 (29)	-14 (17)[d]	14 [-8, 36]	.20
Fasting glucose, mmol/L *M (SD) M* [95% CI]	8.0 (2)	-5 (27)	-8 (22)	3 [-16, 22]	.70
Fasting insulin, ng/ml *M (SD) M* [95% CI]	0.9 (1)	-12 [-32, 12][c]	-1 (34)	-6 [-37, 25]	.70
Total AUC glucose$_{0\text{-}120}$, mmol/L/min *M (SD) M* [95% CI]	1571 (263)	0 (22)	2 (18)	-2 [-20, 17]	.80
Total AUC insulin$_{0\text{-}120}$, ng/ml/min *M (SD) M* [95% CI][e]	186 (66)	7 (36)	2 (42)	5 [-8, 17]	.40
Adiponectin, mg/ml *M (SD) M* [95% CI]	3.2 (2)	-3 (27)	-11 (23)	7 [-8, 22]	.30
Leptin, ng/ml *M (SD) M* [95% CI]	7.5 (6)	-9 (32)	-11 (34)	2 [-18, 22]	.80
Total cholesterol, mmol/L *M (SD) Mdn* [95% CI]	4.2 (1)	-7 [-17, 4][c]	-15 (14)[d]	7 [-4, 18]	.30
LDL, mmol/L *M (SD) Mdn* (Range) *Mdn* [95% CI]	2.3 (0)	-17 (-36 to 67)	-26 (16)[d]	11 [-5, 26]	.20
HDL, mmol/L *M (SD) Mdn* [95% CI]	1.2 (0)	-13 (14)[d]	-6 (16)	-5 [-16, 3]	.30
Triglyceride, mmol/L *M (SD) M* [95% CI]	1.6 (1)	26 (39)[d]	-1 (23)	27 [-2, 56]	.10
C-reactive protein, mg/L *M (SD) Mdn* [95% CI][e]	3.2 (3)	-17 [-47, 20][c]	30 [-35,125][c]	-14 [-177, 36]	.60
Body weight, kg *M (SD) M* [95% CI]	86 (14)	-0.57 (1)	-1.07 (2)	0 [-1, 2]	.30
Waist circumference, cm *M (SD) M* [95% CI]	108 (11)	-1 (2)	-1 (2)	0 [-1, 2]	.60
Hip circumference, cm *M (SD) M* [95% CI]	108 (10)	-1 (2)	-1 (2)	0 [-3, 2]	.80
Sagittal abdominal height, cm *M (SD) M* [95% CI][a]	26 (3)	1 (3)	0 (3)	1 [-1, 3]	.30
Skinfold biceps, mm *M (SD) M* [95% CI][bf]	22 (12)	3 (12)	-18 (14)[d]	21 [6, 37]	.01
Skinfold triceps, mm *M (SD) M* [95% CI]	31 (12)	-1 (23)	-6 (16)	6 [-8, 19]	.40
Skinfold suprailiac, mm *M (SD) M* [95% CI]	34 (7)	-7 (10)[d]	-2 (12)	-4 [-15, 7]	.40
Skinfold subscapular, mm *M (SD) M* [95% CI]	38 (10)	-1 (15)	-2 (7)	1 [-11, 12]	.90
Systolic Blood Pressure, mmHg *M (SD) M* [95% CI]	133 (8)	3 [-2, 8][c]	2 (17)	2 [-10, 13]	.70
Diastolic Blood Pressure, mmHg *M (SD) M* [95% CI]	77 (5)	-1 (8)	4 (17)	-5 [-15, 6]	.40

Total body fat, kg *M (SD) M* [95% CI]	34 (9)	0 (6)	-1 (6)	1 [-3, 5]	.70
Visceral fat, kg *M (SD) Mdn* (Range) *M* [95% CI]	14 (3)	-3 (5)[d]	0 (-8 to 8)	-3 [-7, 1]	.10
Muscle mass, kg *M (SD) M* [95% CI]	53 (9)	0 (2)	-1 (4)	1 [-2, 3]	.60
Bone mass, kg *M (SD) Mdn* (Range) *Mdn* [95% CI]	3 (0)	0 (-4 to 5)	-1 (3)	1 [-2, 3]	.50
Body water, % *M (SD) M* [95% CI]	48 (7)	0 (2)	0 (3)	0 [-2, 2]	.90
Resting Energy Expenditure, MJ *M (SD) M* [95% CI]	7.0 (1)	0 (2)	-1 (3)	1 [-1, 3]	.50

Note. Baseline characteristics and outcome measures of participants, relative change during each diet and the difference between relative changes between a Paleolithic and a diabetes diet after 4 weeks. Normally distributed variables are presented as *M* (*SD*). Transformed variables with normal distribution are presented as *Mdn* [95% CI]. Variables not normally distributed, neither before nor after transformation, are presented as *Mdn* (Range). The table has been adapted from its original format in the manuscript.

[a]Period effect. [b]Carry-over effect, only data from first intervention period used. [c]Geometric mean. [d]$p < .05$ for mean change during diet. [e]$p < .05$ for mean difference between diets at baseline. [f]$p < .05$ for mean difference between diets in change during diet.

BMI = Body mass index. HbA1c = Glycated haemoglobin. AUC = area under curve at oral glucose tolerance test (OGTT). LDL = low-density lipoprotein cholesterol. HDL = high-density lipoprotein cholesterol.

Table 2.2

Daily Dietary Intake from Food Groups at End of Diet

Variable	Diabetes diet (N = 14)	Paleolithic diet (N = 14)	Difference (N = 14)	*p*
Vegetables, g *M* (*SD*) *Mdn* (Range) *Mdn* [95% CI]	391 (93)	1150 (259 to 1430)	-724 [-854, -643]	.001
Vegetables, kJ *M* (*SD*) *Mdn* (Range) *Mdn* [95% CI]	503 (120)	1798 (291 to 2252)	-1346 [-1495, -1196]	.001
Fruits, g *Mdn* (Range) *M* [95% CI]	700 (333 to 792)	1403 (768 to 1635)	-733 [-890, -576]	< .001
Fruits, kJ *Mdn* (Range) *M* [95% CI]	1509 (758 to 1696)	5489 (2374 to 6243)	-3697 [-4428, -2967]	< .001
Meat, g *M* (*SD*) *Mdn* [95% CI]	84 (24)	242 (75)	-137 [-207, -117]	.001
Meat, kJ *M* (*SD*) *Mdn* [95% CI]	379 (140)	1061 (362)	-575 [-899, -479]	.001
Fish, g *M* (*SD*) *Mdn* (Range) *Mdn* [95% CI]	172 (100 to 190)	259 (58)	-107 [-127, -91]	.001
Fish, kJ *Mdn* (Range) *M* [95% CI]	716 (444 to 795)	929 (574 to 1009)	-193 [-280, -106]	.001
Eggs, g *M* (*SD*) *Mdn* (Range) *M* [95% CI]	43 (10)	26 (0 to 34)	20 [14, 27]	< .001
Eggs, kJ *M* (*SD*) *Mdn* (Range) *M* [95% CI]	253 (57)	152 (0 to 200)	121 [82, 160]	< .001

Cereals, g *Mdn* (Range) *Mdn* [95% CI]	317	(176 to 369)	0	(0 to 379)	305	[214, 323]	.001
Cereals, kJ *M* (*SD*) *Mdn* (Range) *Mdn* [95% CI]	3683	(697)	0	(0 to 4932)	3736	[2818, 4125]	.001
Legumes, g *Mdn* (Range) *Mdn* [95% CI]	44	(22 to 69)	6	(4 to 26)	38	[28, 46]	< .001
Legumes, kJ *M* (*SD*) *Mdn* (Range) *Mdn* [95% CI]	607	(304 to 950)	70	(10)	537	[393, 636]	< .001
Dairy products, g *Mdn* (Range) *Mdn* [95% CI]	422	(22 to 451)	0	(0 to 452)	413	[222, 424]	.002
Dairy products, kJ *Mdn* (Range) *Mdn* [95% CI]	909	(302 to 1375)	0	(0 to 1068)	909	[605, 1038]	.001
Sugars and cakes, g *Mdn* (Range) *Mdn* [95% CI]	1	(0 to 2)	12	(1 to 14)	-11	[-13, -6]	.001
Sugars and cakes, kJ *Mdn* (Range) *M* [95% CI]	12	(5 to 40)	158	(18 to 282)	-118	[-163, -73]	< .001
Oils and fats, g *M* (*SD*) *M* [95% CI]	32	(11)	19	(3)	13	[8, 19]	< .001
Oils and fats, kJ *M* (*SD*) *M* [95% CI]	1213	(411)	724	(111)	489	[292, 686]	< .001
Drinks, g *Mdn* (Range) *Mdn* [95% CI]	167	(110 to 446)	19	(17 to 141)	148	[126, 258]	.001
Drinks, kJ *Mdn* (Range) *Mdn* [95% CI]	39	(16 to 497)	48	(39 to 211)	-9	[-20, 53]	.680
Pre-cooked foods, g *Mdn* (Range) *Mdn* [95% CI]	0	(0 to 0)	0	(0 to 1)	0	[0, 0]	.110
Pre-cooked foods, kJ *Mdn* (Range) *Mdn* [95% CI]	0	(0 to 2)	0	(0 to 8)	0	[-3, 0]	.110
Snacks, g *Mdn* (Range) *Mdn* [95% CI]	3	(0 to 3)	17	(0 to 17)	-14	[-16, -12]	.001
Snacks, kJ *Mdn* (Range) *Mdn* [95% CI]	16	(0 to 16)	26	(0 to 26)	-10	[-18, -9]	.006
Sauces and dressings, g *Mdn* (Range) *Mdn* [95% CI]	7	(6 to 37)	11	(0 to 11)	-4	[-4, 9]	.400
Sauces and dressings, kJ *Mdn* (Range) *Mdn* [95% CI]	69	(46 to 447)	25	(1 to 31)	45	[43, 110]	.001

Note. Average food eaten per day by weight and energy during a Paleolithic and a diabetes diet. Estimated from 4-day weighed food records. Normally distributed variables are presented as *M* (*SD*). Transformed variables with normal distribution are presented as *Mdn* [95% CI]. Variables not normally distributed, neither before nor after transformation, are presented as *Mdn* (Range). The table has been adapted from its original format in the manuscript.

Table 2.3

Daily Dietary Nutrient Intake at End of Diet

Variable	Diabetes diet (*N* = 14)	Paleolithic diet (*N* = 14)	Difference (*N* = 14)	*p*
Total weight, kg *M (SD) Mdn* (Range) *M* [95% CI]	2.5 (1.4 to 2.7)	3.1 (0.6)	-0.9 [-1.1, -0.7]	< .001
Total energy, MJ *Mdn* (Range) *Mdn* [95% CI]	10.4 (6.2 to 11.8)	10.9 (6.5 to 12.3)	-0.2 [-1.0, 0.0]	.048
Protein, g *M (SD) Mdn* (Range) *M* [95% CI]	103 (16)	120 (71 to 140)	-9 [-17, -1]	.030
Protein energy percent, E% *M (SD) Mdn* [95% CI]	18 (1)	18 (1)	0 [-1, 0]	.900
Carbohydrates, g *M (SD) Mdn* (Range) *M* [95% CI]	286 (52)	314 (188 to 358)	-14 [-31, 2]	.080

Carbohydrates energy percent, E% *Mdn* (Range) *M* [95% CI]	54	(48 to 57)	55	(51 to 56)	-1	[-3, 0]	.080
Fibre, g *M (SD) M* [95% CI]	47	(9)	62	(15)	-15	[-23, -7]	< .001
Fat, g *M (SD) M* [95% CI]	75	(19)	75	(17)	0	[-7, 6]	.900
Fat energy percent, E% *Mdn* (Range) *M* [95% CI]	28	[27, 30][a]	26	(26 to 31)	2	[0, 3]	.100
Cholesterol, mg *M (SD) Mdn* (Range) *M* [95% CI]	326	(181 to 379)	314	(71)	-11	[-36, 14]	.400
Saturated fatty acids, g *M* [95% CI] *Mdn* [95% CI]	16	[13, 19][a]	13	[11, 16][a]	3	[1, 4]	.010
Monounsaturated fatty acids, g *M (SD) M* [95% CI]	36	(10)	36	(7)	0	[-3, 3]	.800
Polyunsaturated fatty acids, g *M (SD) Mdn* (Range) *M* [95% CI]	13	(8 to 14)	16	(4)	-4	[-5, -2]	< .001
Calcium, mg *M (SD) Mdn* (Range) *Mdn* [95% CI]	1086	(404 to 1206)	703	(166)	343	[194, 402]	.002
Iron, mg *M (SD) Mdn* (Range) *M* [95% CI]	22	(4)	22	(4)	-1	[-3, 1]	.500
Sodium, mg *M (SD) Mdn* (Range) *M* [95% CI]	1747	(587)	1024	(695 to 2558)	656	[324, 987]	.001
Vitamin A, µg *M (SD) Mdn* (Range) *Mdn* [95% CI]	1302	(654 to 1403)	3350	(1074)	-2251	[-2720, -1803]	.001
Vitamin B1, mg *M (SD) Mdn* (Range) *M* [95% CI]	2.3	(0.5)	2	(1 to 2)	0.3	[0.1, 0.44]	.004
Vitamin B2, mg *M (SD) Mdn* (Range) *M* [95% CI]	2.4	(1.1 to 2.7)	1.9	(0.4)	0.4	[0.2, 0.5]	.001
Folic acid, µg *M (SD) M* [95% CI]	431	(66)	558	(104)	-127	[-176, -78]	< .001
Vitamin C, mg *M (SD) Mdn* (Range) *Mdn* [95% CI]	219	(128 to 251)	472	(100)	-277	[-325, -244]	< .001
Vitamin B12, µg *M (SD) Mdn* [95% CI]	6.3	(1.6)	10.7	(2.6)	-4.1	[-05, -03]	< .001
Water, g *M (SD) Mdn* (Range) *M* [95% CI]	1609	(917 to 1746)	1972	(351)	-514	[-644, -384]	< .001
Alcohol, g *Mdn* (Range) *Mdn* [95% CI]	1.3	(0.5 to 6.8)	1.6	(1.3 to 3.2)	-0,3	[-01, 01]	.800
Glucose, g *Mdn* (Range) *Mdn* [95% CI]	18	(9 to 20)	83	(23 to 96)	-64	[-72, -44]	< .001
Fructose, g *Mdn* (Range) *Mdn* [95% CI]	30	(14 to 34)	86	(32 to 100)	-54	[-67, -41]	< .001
Saccharose, g *Mdn* (Range) *M* [95% CI]	27	(13 to 29)	66	(40 to 79)	-40	[-46, -33]	< .001
Myristic acid, g *Mdn* (Range) *M* [95% CI] *Mdn* [95% CI]	1.2	[1 to 1.6][a]	0.5	(0.3 to 2.8)	0.7	[0.2, 1]	.020
Palmitic acid, g *M (SD) Mdn* [95% CI]	9.7	(2.7)	9.5	(3)	0.7	[-1.6, 1.3]	.800
Stearic acid, g *M (SD) M [95% CI] Mdn* [95% CI]	3.2	(1)	2.7	[2.1, 3.7][a]	0.5	[-0.4, 1]	.020
Palmitoleic acid, g *M (SD) Mdn* [95% CI]	1.1	(0.3)	1.3	(0.3)	-0.1	[-0.2, 0.02]	.020
Oleic acid, g *M (SD) M* [95% CI]	34	(10)	35	(7)	0	[-3, 3]	.800
Linoleic acid, g *M (SD) Mdn* (Range) *M* [95% CI]	10	(7 to 11)	12	(3)	-2	[-3, -1]	< .001
Alpha-linolenic acid, g *M (SD) Mdn* [95% CI]	0.9	(0.2)	1.7	(0.4)	-0.8	[-0.9, -0.6]	< .001
Arachidonic acid, g *M (SD) Mdn* (Range) *M* [95% CI]	0.1	(0.1 to 0.2)	0.3	(0.1)	-0.2	[-0.2, -0.1]	< .001
Niacin, mg *Mdn* (Range) *M* [95% CI]	52	(31 to 58)	64	(36 to 76)	-13	[-18, -7]	< .001
Folate, µg *M (SD) M* [95% CI]	431	(66)	553	(110)	-121	[-176, -67]	< .001
Retinol, µg *M (SD) M* [95% CI]	246	(70)	103	[78, 134][a]	133	[94, 171]	< .001
Beta carotene, µg *M (SD) Mdn* (Range) *Mdn* [95% CI]	5362	(2466 to 5580)	17370	(5836)	-13450	[-15857, -10728]	.001
Vitamin D, µg *M (SD) Mdn* (Range) *M* [95% CI]	5.2	(1)	5.5	(3 to 6)	0.2	[-0.3, 0.7]	.300
Vitamin E, mg *M (SD) Mdn* (Range) *M* [95% CI]	15	(10 to 17)	26	(6)	-12	[-15, -9]	< .001

Tocopherols, mg *Mdn* (Range) *M* [95% CI]	13	(8 to 15)	22	(13 to 25)	-9	[-11, -6]	< .001
Zinc, mg *M (SD) Mdn* [95% CI]	14	(3)	12	(3)	3	[0, 3]	.020
Magnesium, mg *Mdn* (Range) *M* [95% CI]	632	(413 to 703)	563	(116)	10	[-45, 65]	.700
Potassium, mg *M (SD) Mdn* (Range) *M* [95% CI]	4300	(2536 to 4681)	7487	(1671)	-3626	[-4410, -2841]	< .001
Selenium, µg *Mdn* (Range) *M* [95% CI]	182	(97 to 200)	157	(92 to 175)	17	[8, 26]	.001
Glycaemic Index, *M (SD) M* [95% CI]	50	(2)	50	(1)	0	[-1, 1]	.600
Glycaemic load, *Mdn* (Range) *M* [95% CI]	152	(91 to 178)	159	(91 to 179)	-8	[-17, 1]	.100

Note. Average nutrient intake per day during a Paleolithic and a diabetes diet. Estimated from 4-day weighed food records. Normally distributed variables are presented as *M* (*SD*). Transformed variables with normal distribution are presented as *Mdn* [95% CI]. Variables not normally distributed, neither before nor after transformation, are presented as *Mdn* (Range). The table has been adapted from its original format in the manuscript.

[a]Geometric mean.

E% = percent energy from total macronutrient energy.

Papers III and IV

The study started in January 2005 and the last participant follow-up was completed in September 2007, after which the study ended. Out of 26 subjects assessed for eligibility, nine were not eligible since they did not meet the inclusion criteria or refused to participate. Out of the remaining 17 eligible subjects, who were all randomised and started the study, four subjects were excluded. All reported analyses are per protocol analyses on the 13 participants (three women and 10 men) who completed the trial. Baseline characteristics are summarised in Table 3.1.

Fasting leptin (Papers III and IV) and bioLep (Paper IV) levels were significantly lower after a Paleolithic diet compared with a diabetes diet (Tables 3.2 and 3.3, respectively). However, no significant differences were observed between leptin and bioLep after either diet. Consequently, there was no significant difference between diets when comparing differences between leptin and bioLep or their ratio (Paper IV) (Table 3.3). No significant differences between diets for the other adipokines were observed (Paper III) (Table 3.2). No carry-over or period effect was found.

To assess the condition of frozen blood samples, in Paper III, new analyses of insulin were compared with older ones. The newly analysed insulin was on average 27% lower, with a 66% increase in standard deviation. This notwithstanding, new and old insulin measurements were highly correlated (r = .72, p < .001). In Paper IV, new and old leptin measurements were also highly correlated (rs = .97, p < .001), with new leptin measurements being, on average, 67% higher.

Mean (*SD*) daily intake in grams of rice and other cereal grains were 7 (17) g and 11 (24) g for the Paleolithic diet and 6 (10) g and 172 (96) g for the diabetes diet, respectively. Average food eaten during the Paleolithic and diabetes diet is shown in Table 3.4.

In vitro

There was no effect on gluten digest concentration (190 μg/ml) from heat treatment (192 μg/ml) and/or centrifugation (194 μg/ml and 196 μg/ml), respectively. Gluten digest with or without centrifugation reduced bioLep in a dose-dependent manner and at similar concentrations for both 10 and 50 ng/ml recombinant leptin (Figure 3). Heat-treated gluten digest with or without centrifugation did not reduce bioLep (Figure 3).

Table 3.1

Baseline Characteristics

Variable	All	Diabetes first (6/13)	Paleolithic first (7/13)
Sex male/female (n)	10/3	4/2	6/1
Age, years	64 (6)	63 (6)	66 (6)
Height, cm	171 (5)	170 (6)	172 (4)
Body weight, kg	87 (17)	92 (20)	82 (13)
BMI, kg/m^2	30 (7)	32 (8)	28 (4)
Waist circumference, cm	103 (14)	109 (17)	97 (9)
Diabetes duration, years	8 (5)	11 (6)	6 (4)
Diabetic values at OGTT yes/no (n)	12/1	6/0	6/1
Lipid lowering drug (= statin) yes/no (n)	8/5	4/2	4/3
Drugs per day	4.3 (2.3)	3.7 (1.8)	4.9 (2.7)
Antihypertensive drugs per day	1.5 (1.5)	1.2 (1.2)	1.9 (1.7)
Beta-blocker yes/no (n)	4/9	1/5	3/4
Thiazide yes/no (n)	4/9	1/5	3/4
ACE-inhibitor yes/no (n)	5/8	2/4	3/4
Angiotensin-II receptor blocker yes/no (n)	4/9	2/4	2/5
Calcium channel blocker yes/no (n)	3/10	1/5	2/5
Anti-diabetic drugs per day	1.2 (0.9)	1.5 (0.8)	0.9 (0.9)
Metformin yes/no (n)	9/4	5/1	4/3
dosage, mg/day	1031 (864)	1283 (950)	814 (790)
Sulfonylurea yes/no (n)	3/10	2/4	1/6
Thiazolidinedione yes/no (n)	3/10	2/4	1/6
Plasma adiponectin, µg/ml	4.8 (4.2)	4.8 (2.5)	4.9 (5.4)
Plasma adipsin, ng/ml	797 (157)	804 (218)	792 (178)
Plasma C-Peptide, pg/ml	487 (275)	437 (276)	531 (289)
Plasma ghrelin, pg/ml	568 (129)	613 (165)	530 (82)
Plasma GIP, pg/ml	232 (91.6)	226 (83)	237 (105)
Plasma GLP-1, pg/ml	26.8 (3.39)	26.4 (4.5)	27.1 (2.4)
Plasma glucagon, pg/ml	425 (44.34)	435 (52.4)	417 (38.4)
Insulin, pg/ml	454 (460)	208 (100)	665 (550)
Leptin, ng/ml	9.84 (12.18)	12.1 (17)	7.9 (6.8)
Resistin, ng/ml	2.21 (0.39)	2.3 (0.4)	2.1 (0.4)
Visfatin, ng/ml	2.52 (0.75)	2.7 (0.5)	2.4 (0.7)

Note. Baseline characteristics and fasting outcome measures for all participants and for participants starting with a diabetes or Paleolithic diet first. Data are presented as *M* (*SD*), unless stated. The table has been adapted from its original format in the published article.

BMI = Body mass index. OGTT = Oral glucose tolerance test. ACE = angiotensin converting enzyme. GIP = glucose-dependent insulinotropic polypeptide. GLP-1 = glucagon-like peptide-1.

Table 3.2

Outcome Measures and Body Weight After a Paleolithic Diet and Diabetes Diet

Variable	Paleolithic diet	Diabetes diet	Delta diets	p^a
Adiponectin, µg/ml	5.2 (4.4) [2.5, 7.9]	5.7 (5.4) [2.5, 9.1]	-0.5 (1.2) [-1.3, 0.2]	.15
Adipsin, ng/ml	787 (182) [677, 896]	776 (153) [684, 869]	10 (79) [-37, 58]	.65
C-peptide, pg/ml	455 (224) [319, 590]	412 (204) [289, 535]	43 (262) [-116, 201]	.64
Ghrelin, pg/ml	540 (97) [481, 598]	566 (145) [478, 654]	-26 (74) [-70, 18]	.23
GIP, pg/ml	254 (266) [93, 415]	186 (75) [141, 232]	68 (264) [-92, 227]	.60
GLP-1, pg/ml	27 (9.3) [22, 33]	27 (3.7) [25, 29]	0.4 (7.7) [-4.3, 5.12]	.23
Glucagon, pg/ml	409 (40) [385, 433]	431 (51) [400, 463]	-22 (43) [-48, 3.9]	.09
Insulin, pg/ml	248 (138) [165, 332]	336 (327) [138, 533]	-87 (240) [-232, 58]	.27
Insulin[b], pg/ml	401 (174) [296, 506]	391 (115) [322, 461]	9.8 (172) [-94, 114]	.84
Leptin, ng/ml	5.1 (4.9) [2.1, 8.0]	7.4 (8.3) [2.4, 12]	-2.3 (4.6) [-5.1, 0.4]	.02
Resistin, ng/ml	2.5 (0.9) (1.9, 3.0]	2.3 (0.6) [2.0, 2.7]	0.2 (0.6) [-0.2, 0.5]	.36
Visfatin, ng/ml	2.4 (0.7) [2.0, 2.9)	2.5 (0.6) [2.1, 2.8]	-0.1 (0.5) [-0.3, 0.3]	.91
Body weight, kg	81 (13) [74, 88]	84 (15) [76, 92]	-3.3 (3.8) [-5.7, -1.0]	.01

Note. Fasting outcome measures and body weight after a Paleolithic diet and diabetes diet, and the difference between values after the diets. Data are presented as *M* (*SD*) [95% CI]. The table has been adapted from its original format in the published article.

[a]*p* for difference between diets. [b]Old insulin values previously published.

GIP = glucose-dependent insulinotropic polypeptide. GLP-1 = glucagon-like peptide-1.

Table 3.3

Leptin and BioLep After a Paleolithic and Diabetes Diet

Variable	Paleolithic diet	Diabetes diet	Paired difference	p^a
BioLep (Paper IV), ng/ml *M (SD)*	8.5 (6.0)	11.8 (9.3)	-3.3 (5.4)	.02
Leptin (Paper III), ng/ml *M (SD)*	5.1 (2.1)	7.4 (8.3)	-2.3 (4.6)	.02
Leptin (Paper IV), ng/ml *M (SD)*	8.8 (6.7)	12.1 (9.9)	-3.2 (5.2)	.02
BioLep minus leptin (Paper IV), ng/ml *M (SD)*	-0.3 (0.8)	-0.3 (0.9)	-0.1 (0.6)	.70
p^b	.2	.3		
BioLep to leptin ratio (Paper IV), *M (SD)*	0.96 (0.18)	1.02 (0.15)	-0.06 (0.18)	.40

Note. Fasting leptin and bioLep after a Paleolithic and diabetes diet. Data are presented as *M* (*SD*). The table has been adapted from its original format in the published article.

[a]*p* for mean comparison between diets. [b]*p* for mean comparison between leptin and bioLep for each diet.

BioLep = Biologically active leptin.

Table 3.4

Average Food and Nutrients Eaten per day During a Paleolithic and Diabetes Diet

Variable	Paleolithic diet	Diabetes diet	*p*[a]
Total weight, g	1445 (367)	1456 (312)	.900
Total energy, MJ	6.6 (1.2)	7.9 (1.6)	.005
Total energy, kcal	1581 (295)	1878 (379)	.005
Energy density, kJ/g	4.7 (0.7)	5.6 (1.1)	.020
Protein, g	94 (18)	90 (14)	.500
Protein energy percent, E%	24 (3)	20 (4)	< .001
Carbohydrate, g	125 (43)	196 (61)	< .001
Carbohydrate energy percent, E%	32 (7)	42 (7)	< .001
Fat, g	68 (11)	72 (20)	.600
Fat energy percent, E%	39 (5)	34 (6)	.040
Alcohol, g	6.3 (8.9)	3.6 (5.6)	.200
Alcohol energy percent, E%	3 (4)	1 (2)	.080
Fibre, g	21 (8)	26 (8)	.020
Fibre energy percent, E%	2.5 (0.7)	2.7 (0.7)	.400
Glycaemic load, g	63 (23)	111 (41)	< .001
Glycaemic Index	50 (5)	55 (6)	.010
Monosaccharides, g	46 (21)	33 (16)	.030
Disaccharides, g	31 (14)	39 (15)	.100
Sucrose, g	29 (13)	30 (12)	.800
Saturated fatty acid, g	19 (5)	27 (9)	.002
Monounsaturated fatty acid, g	30 (6)	26 (7)	.130
Polyunsaturated fatty acid, g	14 (4)	12 (4)	.200
Fatty acid C4:0-C10:0, g	0.3 (0.4)	2.1 (1.3)	< .001
Fatty acid C12:0, g	0.3 (0.3)	1.1 (0.8)	.002
Fatty acid C14:0, g	1.3 (0.5	2.8 (1.3)	< .001
Fatty acid C16:0, g	12 (3)	14 (4)	.020
Fatty acid C16:1, g	2.0 (0.5)	1.5 (0.6)	.030
Fatty acid C18:0, g	4.5 (1.5)	5.9 (1.9)	.053
Fatty acid C18:1, oleic acid, g	26 (6)	24 (7)	.300
Fatty acid C18:2, n-6, Linoleic acid, g	9 (4)	8 (3)	.600
Fatty acid C18:3, n-3, ALA, g	1.5 (0.7)	1.6 (0.8	.600
Fatty acid C20:0, g	0.1 (0.1)	0.1 (0.1)	.200
Fatty acid C20:4, n-6, g	0.2 (0.1)	0.1 (0.1)	.010
Fatty acid C20:5, n-3, EPA, g	0.6 (0.3)	0.3 (0.3)	.052
Fatty acid C22:5, n-3, g	0.2 (0.1)	0.1 (0.1)	.300
Fatty acid C22:6, n-3. DHA, g	1.3 (0.7	0.7 (0.7)	.060
Cholesterol, mg	577 (107)	365 (88)	< .001
Vitamin A, Retinol equivalents, µg	896 (534)	1139 (450)	.200
Vitamin A, Retinol, µg	385 (333)	673 (353)	.051
Vitamin A, Carotene, µg	5038 (3414)	4811 (5633)	.900
Vitamin D, µg	9 (4)	9 (7)	.900
Vitamin E, mg	13 (4)	11 (3)	.070
Vitamin E, Alpha-tocopherol, mg	13 (4)	11 (3)	.070
Vitamin B-1, Thiamine, mg	1.5 (0.5)	1.6 (0.5)	.800
Vitamin B-2, Riboflavin, mg	1.6 (0.3)	1.6 (0.2)	.500
Vitamin B-6, mg	3.2 (0.7)	2.4 (0.6)	.003
Vitamin B-12, µg	8.6 (4.0)	6.7 (2.4)	.200
Vitamin B, Folate, µg	340 (172)	300 (79)	.400
Vitamin C, Ascorbic acid, mg	219 (1369)	(119) (60)	.030

Niacin equivalents, mg	45 (11)	39 (8)	.080
Niacin, mg	27 (8)	22 (6)	.030
Phosphorus, mg	1233 (247)	1437 (208)	.020
Iron, mg	12 (3)	12 (3)	1.000
Potassium, mg	3669 (982)	3181 (908)	.049
Calcium, mg	356 (102)	698 (220)	< .001
Magnesium, mg	307 (84)	311 (68)	.900
Sodium, mg	2530 (924)	2963 (678)	.140
Selenium, µg	81 (20)	55 (18)	.001
Zinc, mg	11 (3)	12 (2)	.300
Ash, g	17 (4)	19 (4)	.130
Water, g	1113 (306)	1049 (258)	.500
Fruits, g	451 (200)	251 (210)	.005
Vegetables, g	346 (179)	241 (176)	.049
Potatoes, g	49 (51)	106 (84)	.030
Nuts, g	29 (24)	12 (20)	.130
Meat, g	139 (67)	73 (29)	.003
Meat products, g	97 (76)	71 (43)	.200
Fish, g	104 (55)	89 (56)	.500
Eggs, g	71 (27)	27 (24)	.001
Beans, g	4 (14)	24 (33)	.030
Cereals without rice, g	11 (24)	172 (96)	< .001
Rice, g	7 (17)	6 (10)	.900
Milk/milk products, g	16 (32)	183 (123)	< .001
Oil, g	0.3 (0.7)	1.4 (3.5)	.300
Sauce, g	13 (20)	30 (36)	.200
Bakery, g	10 (18)	34 (35)	.005
Jam, g	0 (0)	12 (22)	.070
Spirits, g	1.0 (2.8)	1.4 (4.1)	.800
Wine, g	52 (83)	20 (49)	.140
Beer, g	31 (103)	55 (80)	.400
Sweet beverages, g	0 (0)	38 (64)	.051
Juice, g	12 (35)	10 (26)	.600

Note. Average food and nutrients eaten per day during a Paleolithic and diabetes diet. Estimated from 4-day weighed food records. Data are presented as *M (SD*). The table has been adapted from its original format in the published article.

[a]*p* for mean difference between diets.

E% = percent energy from total macronutrient energy.

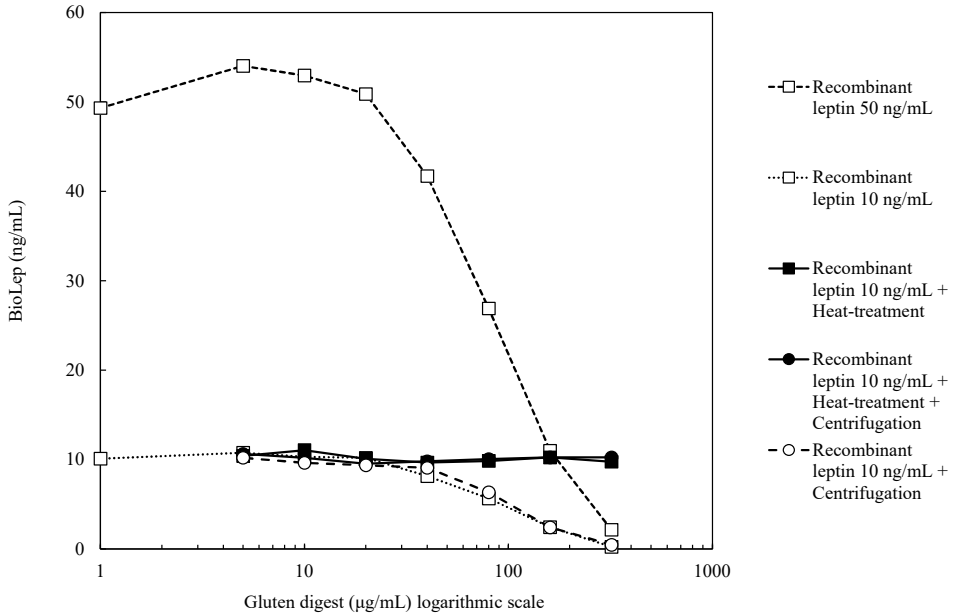

Figure 3

BioLep for Recombinant Leptin Incubated with a Series of Increasing Gluten Digest Concentrations

Note. BioLep concentrations at 0 µg/ml gluten digest concentration is here presented at 1 µg/ml gluten digest concentration since the logarithm of 0 is undefined and cannot be presented on the x-axis.

Discussion

As mentioned in the introduction, a comparably greater improvement in glycaemic control in type 2 diabetes from a Paleolithic diet could be due to an accompanying greater reduction in body weight. The suggested causality was assessed in Papers I and II by examining in type 2 diabetes the effect of a Paleolithic diet compared with a diabetes diet on glycaemic control when body weight was kept stable.

As mentioned in the introduction, a comparably greater reduction in body weight accompanying a Paleolithic diet in type 2 diabetes could also be due to an accompanying greater reduction in the satiety hormone leptin. The suggested causality was assessed in Papers III and IV by examining in type 2 diabetes the effects of a Paleolithic diet compared with a diabetes diet on leptin and leptin receptor binding.

Main findings

Papers I and II

There were no differences between the two diets in effects on glycaemic control as measured by HbA1c, fructosamine, and glycated albumin. Body weight was kept stable, and the contents of the two diets differed, as planned, regarding the food groups cereal grains, dairy products and legumes, and the diets were successfully matched for macronutrient composition and glycaemic load, but not for fibre content.

Papers III and IV

A Paleolithic diet resulted in lower fasting leptin compared with a diabetes diet with no effect from either diet on leptin receptor binding in the fasting state. Using another laboratory method, an in vitro finding of a dose-dependent leptin receptor binding inhibition from gluten digest that was abolished by heat treatment of the gluten digest was replicated.

Glycaemic control and body weight loss

The results from Paper II indicate that characteristic food group differences and accompanying fibre content differences between a Paleolithic diet and a diabetes diet do not result in greater improvements in glycaemic control when body weight is kept stable and macronutrient composition and glycaemic load are matched between diets. The result strengthens the suggestion that the comparably greater improvement in glycaemic control in type 2 diabetes from a Paleolithic diet is due to an accompanying greater reduction in body weight.

The result from Paper II is congruent with previous trials lasting 6 months or longer in body weight stable participants that reported no significant differences in glycaemic control when comparing low-carbohydrate or low-glycaemic index diets with diabetes diets (Iqbal et al., 2010; Ma et al., 2008; Milne et al., 1994; Wolever et al., 2008). In contrast, short-term RCTs (5 to 6 weeks) in body weight-stable participants with type 2 diabetes have shown that low-carbohydrate, high-protein diets improve glycaemic control (as measured by HbA1c) compared with diabetes diets (Gannon et al., 2003; Skytte et al., 2019). One RCT lasting 1 year compared a low glycaemic index versus an American Diabetes Association Diet in body weight-stable participants with type 2 diabetes (Ma et al., 2008). Both diets resulted in a significant improvement in glycaemic control (as measured by HbA1c), with no differences between the groups. However, the glycaemic index did not differ between the groups at any time point throughout the study, despite being a primary goal. In another study, a prospective analysis of a RCT in participants with newly diagnosed type 2 diabetes assessed whether glycaemic control was associated with dietary patterns independent of body weight change after 6 months (Garbutt et al., 2022). Glucose-lowering medications were unchanged, ensuring that any effect on HbA1c could be attributed solely to lifestyle. The results indicated that glycaemic control improved in those participants who adopted a dietary pattern with an increased "carbohydrate/fat balance" pattern compared with baseline after adjustment for potential confounders. Notably, this effect was independent of body weight loss (Garbutt et al., 2022). However, the effect size of the increased "carbohydrate/fat balance" was small compared with the effect of body weight loss on glycaemic control. These findings are congruent with the results from Paper II, where both diets improved glycaemic control (as measured by fructosamine and glycated albumin) without significant differences between diets. This indicates that while small changes in glycaemic control can be achieved when body weight is kept stable, body weight loss remains the main driver of improved glycaemic control.

The result from Paper II is also congruent with a systematic review of longer-term studies which found no effect from differences in macronutrient composition on glycaemic control in type 2 diabetes provided there were no differences in reductions in body weight between the groups (Emadian et al., 2015). Fibre content and glycaemic load also often differed between diets in these studies when not

matched for, which when taken together suggests that macronutrient composition, fibre content, and glycaemic load do not independently affect long-term glycaemic control beyond the effects of reductions in body weight.

Leptin

The lower leptin after a Paleolithic diet compared with after a diabetes diet in Papers III and IV concurs with previous findings, with a trend toward a greater reduction in leptin from a Paleolithic diet compared with a Mediterranean-like diet (Jönsson et al., 2010). The result strengthens the suggestion of a comparably greater accompanying leptin reduction from a Paleolithic diet.

Differences in leptin after the diets did not correlate with corresponding differences in cereal grain intake (Paper III). This contrasts with previous findings, which found a strong correlation between a change in leptin and cereal grain intake (Jönsson et al., 2010). The result does not support a direct effect from a Paleolithic diet on leptin as a causal mechanism for a greater accompanying leptin reduction from a Paleolithic diet.

Differences in leptin after the diets also did not correlate with corresponding differences in body weight loss, which is also contrary to previous findings, where changes in leptin were significantly correlated with changes in body weight (Jönsson et al., 2010). The result does not support reduction in body weight as a causal mechanism for a greater accompanying leptin reduction from a Paleolithic diet.

Neither diet affected leptin receptor binding in the fasting state since there was no significant difference between fasting leptin and bioLep after the Paleolithic or diabetes diet and consequently, also no significant difference between the diets when comparing differences between fasting leptin and bioLep or their ratio. The lack of leptin receptor binding inhibition concurs with an in vitro replicated finding that heat treatment abolishes leptin receptor binding inhibition from wheat gluten digest (Paper IV). It also concurs with a review indicating that it is still unknown if longer peptides can be easily transported into the circulation system (Miner-Williams et al., 2015; Xu et al., 2019), although a recent meal study did find immunoreactive gluten peptides in the urine of healthy volunteers during 12 hours post-ingestion (Rodríguez-Ramírez et al., 2024). The lack of leptin receptor binding inhibition does not preclude the possibility that heat-treated digests of wheat gluten, or other cereal grain proteins, can impair leptin function other than leptin receptor binding, such as disruptions in the intracellular signalling of the leptin receptor (Greco et al., 2021). Similarly, the result also does not preclude the possibility of effects from transient postprandially elevated wheat protein levels on leptin receptor binding (Chirdo et al., 1998b; Greco et al., 2021), or from wheat gluten subjected to

heat treatment before enzymatic digestion, a reversal of the in vitro study more aligned with in vivo conditions.

In summary, a Paleolithic diet lowered leptin more than a diabetes diet in type 2 diabetes and neither diet affected leptin receptor binding in the fasting state. The results strengthen previous results of a comparably greater reduction in leptin in type 2 diabetes from a Paleolithic diet and indicate that diet does not affect leptin receptor binding in the fasting state.

Strengths

Papers I and II

The aims of keeping participants' body weight stable, macronutrient composition and glycaemic load the same in both diets were all achieved, thereby negating accompanying differences in effects on glycaemic control when comparing diets. To increase compliance, a daily lunch was provided to all participants during the study. To decrease the risk of bias, great care was given to present both study diets as equally favourable. To minimise the risk of bias associated with naming a specific dietary pattern, the term "Paleolithic diet" was avoided. Instead, participants were informed that the study aimed to compare two healthy diets, as it was not yet known which, if any, was superior.

Papers III and IV

Paper IV successfully replicated the leptin measurements in Paper III with a high correlation between old and new measurements. Additionally, a previous finding using surface plasmon technology of an in vitro wheat gluten-mediated inhibition of leptin receptor binding was also replicated using the bioLep laboratory kit. Replicating previous experimental results, especially using another method as was the case with the bioLep Laboratory kit, increases both confidence and credibility of the results by reducing the chances of false positives or measurement errors.

Limitations

Papers I and II

A seemingly major limitation of this study is the unsuccessful analyses of the primary outcome variable glucagon due to a high rate of sample haemolysis. However, this limitation is rather minor since the assessment of glucagon was an exploratory aim of the study in addition to the central assessment of effects on glycaemic control, and glucagon should in hindsight more appropriately have been classified as a secondary outcome. Nevertheless, the methodology in future research should aim at better handling of samples to avoid haemolysis.

Other variable results not obtained were the subjective secondary outcomes of satiety, quality of life, and experience with the dietary patterns, which was due to participants not completing these ratings and questionnaires as planned. Satiety, in particular, would have been interesting to evaluate considering the aims and results from Papers III and IV. The failure in obtaining these variable results was likely caused by insufficient oral and written instructions, which also should be remedied in future research.

The aim of keeping fibre content the same in both diets was not achieved, thereby precluding the possibility of negating accompanying differences in effects when comparing diets. Fibre content possibly ended up differing between diets because participants did not consume or properly report their consumption of fibre added in the diabetes diet, although both diets were well above the recommended intake (25 g per day according to WHO), being almost 1.9 and 2.5 times higher during the diabetes and Paleolithic diets, respectively. This addition was intended to compensate for an expected lower fibre intake compared with the Paleolithic diet caused by greater amounts of fruit and vegetables in the latter. Future study instructions should put more emphasis on the importance of consuming and reporting the consumption of added fibre.

Papers III and IV

The small sample size precluded multivariate analyses, which would have been relevant when interpreting leptin reductions.

Leptin was analysed from stored fasting blood samples. Analyses of leptin from stored samples may have limited the ability to detect significant results due to resulting discrepancies in average values and variance. To assess the severity of the resulting limitation, analyses were conducted that showed a high degree of correlation between previous and stored sample measurements. Analyses of leptin in a fasting state precluded the assessment of potential effects from transient

postprandially elevated wheat proteins levels on leptin binding to its receptor (Chirdo et al., 1998b; Greco et al., 2021).

The in vitro studies conducted with heat treatment following enzymatic digestion of gluten represent a reversal of the order under in vivo conditions. While it is unlikely, the possibility that applying heat treatment prior to enzymatic digestion might have yielded different in vitro results cannot be ruled out.

Conclusions and future perspectives

Papers I and II

Characteristic food group differences and accompanying fibre content differences between a Paleolithic diet and a diabetes diet did not result in greater improvements in glycaemic control when body weight was kept stable and macronutrient composition and glycaemic load were matched between diets. The result strengthens the suggestion that the comparably greater improvement in glycaemic control in type 2 diabetes from a Paleolithic diet is due to an accompanying greater reduction in body weight.

Papers III and IV

A Paleolithic diet lowered leptin more than a diabetes diet in type 2 diabetes and neither diet affected leptin receptor binding in the fasting state. The results strengthen previous results of a comparably greater reduction in leptin in type 2 diabetes from a Paleolithic diet and indicate that diet does not affect leptin receptor binding in the fasting state.

General conclusion

The findings indicate that the comparably greater improvement in glycaemic control in type 2 diabetes from a Paleolithic diet is due to an accompanying greater reduction in body weight. The findings also strengthen previous results of a comparably greater reduction in leptin in type 2 diabetes from a Paleolithic diet and indicate that diet does not affect leptin receptor binding in the fasting state.

Future perspectives

Further research is needed to assess the comparably greater leptin reduction in type 2 diabetes from a Paleolithic diet. For instance, as discussed above, the findings in this thesis that diet does not affect leptin receptor binding in the fasting state do not

preclude other dietary effects from a Paleolithic diet on leptin function. Also, the findings do not preclude the possibility that the comparably greater leptin reduction in type 2 diabetes from a Paleolithic diet is due to the accompanying greater reduction in body weight. For a more direct assessment of leptin function and effects from reductions in body weight, an interesting study would be to assess the effects from exogenous leptin in a fasting and prandial state on dietary intake and metabolism in a crossover study in subjects with type 2 diabetes comparing a Paleolithic diet with a diabetes diet when body weight is kept stable and when allowed to vary (Bligh et al., 2015; Chrysafi et al., 2020; Kissileff et al., 2012). Lastly, further research is also needed to assess satiety effects from other differences between Paleolithic and non-Paleolithic diets such as palatability (Deighton et al., 2016; Guyenet, 2019; Guyenet & Schwartz, 2012; Stubbs & Whybrow, 2004) and water and fibre content (Akhlaghi, 2024; Salleh et al., 2019).

Acknowledgements

I wish to express my deepest gratitude to everyone who has walked alongside me on this journey, helping me grow into the person I am today and supporting me in accomplishing the incredible milestone of completing my doctoral thesis. I'll never walk alone!

In loving memory of my supervisor and mentor, associate professor Staffan Lindeberg – I will be eternally grateful for your inspiration, which led me into the world of science and critical thinking. Meeting you was a game changer.

My main supervisor, associate professor Tommy Jönsson, who has always believed and supported me throughout these years. You motivated me to restart this project after a difficult time and helped me fall in love with it more and more every day. I appreciate your scientific rigour in questioning every single word I wrote, which made me improve and ultimately achieve my goal. I admire your brightness and great ability to simplify complex things, all of which you deliver with a great sense of humour.

My co-supervisor, professor Kristina Sundquist, for welcoming me to the department, providing continuous advice and generously sharing your expertise throughout my thesis journey. You always provided excellent answers to complex questions. And those coffee breaks, listening to your history and political conversations, are pure gold.

My co-supervisor, professor Yvonne Granfeldt, your humility and incredible depth of knowledge never cease to amaze me. Your knowledge and expertise for designing the study in Lanzarote and for important intellectual feedback on the dietary interventions were crucial.

Professor Jan Sundquist for giving me the opportunity to enrol as a PhD student at this magnificent university, Lund University, and guidance during this thesis journey.

Carlos Hugo Abdul Jalbar, for being the nurse during the study in Lanzarote and for your tremendous efforts on the days we performed the blood extractions. I was truly amazed by your dedication, professionalism, and exceptional skills as a nurse. I will always be grateful to you!

The participants from the diabetes study in Lanzarote: thank you for your immense kindness in sharing your time and interest in contributing to making the world a better place with our research.

Associate professor Ashfaque Memon for your invaluable feedback on laboratory procedures and study design, and for your patience in explaining very complex procedures.

Anna Hedelius for your patience answering my questions and for excellent laboratory technical support.

María Luz Fika-Hernando, for your help and support on the design of the study and for your exceptional help in arranging crucial meetings in Lanzarote. Your dedication and assistance were truly indispensable.

Óscar Picazo, thank you for your friendship. I admire your unique blend of humility and deep knowledge across countless fields. Thank you for being there any time I needed your help in the design of the study in Lanzarote, as well as your technical support and guidance in informatics and emerging technologies.

Professor Lynda Frassetto, your expertise in the field and your experience in previous trials were crucial to the design of the ADILAN study, not to mention your humility and friendship. I always enjoy talking with you and meeting you!

My co-authors, Staffan Lindeberg, Tommy Jönsson, Yvonne Granfeldt, Kristina Sundquist, Jan Sundquist, María Luz Fika-Hernando, Óscar Picazo, Lynda

Frassetto, Pedro Carrera-Bastos, Ashfaque Memon, Anna Hedelius, Giuseppe Lippi, Martina Montagnana, Filip Knop and Madhvi Chanrai, your time and knowledge for important intellectual content in the papers was priceless.

Sara Larsson Lönn, for your indispensable guidance in the complex field of statistics.

Patrick O'Reilly, for scientific editing of our papers and this thesis.

Helene Rosenqvist, Kenta Okuyama, Emelie Stenman and all the colleagues already mentioned at the Center for Primary Health Care Research, who warmly welcomed me and shared insightful conversations during coffee breaks – thank you!

Marci Acuña, representing Excmo. Cabildo de Lanzarote, for your continuous support in the logistics, as well as for providing human and financial resources for the study conducted in Lanzarote.

Olivia Duque, representing Ayuntamiento de Teguise, for providing the financial resources for the study conducted in Lanzarote.

Kitchen staff at Hospital Insular de Lanzarote for kindly adapting and creating the menus, which were indispensable for conducting the study in Lanzarote.

Domingo Guzmán, director at Hospital Insular de Lanzarote, your invaluable advice on logistics and your kindness, which were crucial for the successful and smooth development of the study in Lanzarote.

Asociación de diabéticos de Lanzarote (ADILA), for providing their database and spreading the word for participant recruitment.

María Montilla, for assistance with the laboratory analysis and for arranging meetings with the kitchen staff to create the meals.

María Ángeles Díaz, for your selfless help in designing the menus for the study in Lanzarote.

On a personal level, I am grateful to:

First and foremost, my parents, Lita Villalba and Nolo Fontes, who not only brought me into this world but also guided me on the path of humility and hard work. They've always been my guiding star.

My life partner, Amada, for being an endless source of inspiration and continuous support in my life. There are so many memories related to this thesis that they could fill a book. And for painting an amazingly creative front cover. Without you, this thesis would have been impossible to achieve!

♪♪"*When the heavens burst and the stars don't shine, you'll be on my mind and I'll love you always!*"♪♪

My daughter, Arrieta, our life project who exceeded all our dreams. So strong, so bright and lovely. Without a doubt, you are the engine of my life! Your first trip, when you were just a newborn, was also my first journey to this incredible country called Sweden.

My brother Rubén, my soulmate with whom I share more interests than anyone else on this planet. You have contributed enormously to shaping the person I am today. Let's keep rocking together!

My sisters Ariadna and Rosaura and my brother Eladio, so far and so close! Thank you for your unconditional friendship and support!

Eva Lindsten, for your special friendship and for warmly welcoming my family and me into your home on countless times. We love you!

Agradecimientos

Primero que nada, a mi madre y mi padre, Lita Villalba y Nolo Fontes, que no solo me trajeron a este mundo, sino que me han guiado por el camino de la humildad y el trabajo duro. Siempre serán mi camino a seguir.

A los participantes del estudio de diabetes en Lanzarote: gracias por su amabilidad y predisposición para compartir su tiempo. Su contribución a nuestro proyecto de investigación nos ayuda a hacer de este mundo un lugar mejor.

A Carlos Hugo Abdul Jalbar, como enfermero del estudio en Lanzarote. Tu conocimiento y esfuerzo los días de extracción de muestras fueron esenciales para realizar el estudio. Me sorprendió enormemente tu dedicación, profesionalidad y exquisitas habilidades como enfermero. ¡Siempre estaré en deuda contigo!

A María Luz Fika-Hernando, por tu indispensable ayuda y apoyo en el diseño del estudio, y por la organización de reuniones cruciales en Lanzarote.

A Marci Acuña, en representación del Excmo. Cabildo de Lanzarote, por tu continuo apoyo en la logística, así como en la obtención de recursos humanos y financiación para el estudio realizado en Lanzarote.

A Olivia Duque, en representación del ayuntamiento de Teguise, por tu ayuda en la obtención de financiación para el estudio realizado en Lanzarote.

Al equipo del hospital Insular de Lanzarote por su amabilidad y disponibilidad para adaptar y crear los menús, los cuales fueron indispensables para la realización del estudio en Lanzarote.

A Domingo Guzmán, director del Hospital Insular de Lanzarote, por tu amabilidad y tus consejos en la logística del estudio en Lanzarote, los cuales fueron determinantes para el desarrollo exitoso y fluido del mismo.

A la Asociación de diabéticos de Lanzarote (ADILA), por aportar su base de datos y ayudar en el reclutamiento de participantes para el estudio en Lanzarote.

A María Montilla, por tu ayuda en los análisis de laboratorio y por la organización de las reuniones con el equipo de cocina para crear los menús.

A María Ángeles Díaz, por tu amable ayuda en el diseño de los menús para el estudio en Lanzarote.

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Paper I

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TRIALS

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A healthy diet with and without cereal grains and dairy products in patients with type 2 diabetes: study protocol for a random-order cross-over pilot study - Alimentation and Diabetes in Lanzarote -ADILAN

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Abstract

Background: Research on the role of nutrition in type 2 diabetes has largely focused on macro/micronutrient composition and dietary fiber intake, while fewer studies have tested the effects of differing food choice. Some observational studies and short-term intervention studies suggest that a food pattern mimicking the diet with which humans evolved positively influences glucose control and associated endocrine systems. Such a food pattern mainly differs from other common healthy food patterns in its absence of cereal grains and dairy products. The primary aim of this pilot study is to determine the effect of two healthy diets with or without cereal grains and dairy products on glucose control, while keeping participants' weight stable and other food parameters, such as macro/micronutrient composition, dietary fiber and glycemic load, the same in both diets.

Methods/Design: We intend to include 15 adult patients with a medical diagnosis of type 2 diabetes mellitus with or without medication and with an increased waist circumference (≥ 80 cm for women and ≥ 94 cm for men) in a random-order cross-over diet intervention study during two periods of four-weeks separated by a six-week washout period. Patients will be instructed to eat two healthy diets according to official dietary guidelines with respect to macro/micronutrient composition and fiber content, but differing in the type of food included, with one diet being without cereal grains and dairy products. Lunch will be served in a hospital kitchen for control of nutrient intake, while the rest of the meals will be eaten at home according to specific directions. The energy content of the diets will be individually adjusted to maintain a stable body weight during the two four-week intervention periods. Primary outcomes will be change in fasting plasma glucagon and fructosamine, while secondary outcomes include change in fasting glucose and glycated hemoglobin, glucose and glucagon response during oral glucose tolerance test, blood lipids, blood pressure, C-reactive protein, body composition, quality of life, subjective experience with the two diets, satiety scores and changes in medication.

Discussion: Using these results, we will assess the need to conduct larger and longer studies with similar design.
(Continued on next page)

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Trial registration: This trial was registered at clinicaltrials.gov as NCT01891955 and Spanish Agency of Medication and Sanitary Products (AEMPS) registration code: MFV-ADI-2013-01.

Keywords: Protocol, Random-order cross-over trial, Type 2 diabetes mellitus, Metabolic diseases, Dietary intervention, Grain-free diet, Dairy-free diet, Glucagon, Fructosamine

Background

The prevalence of type 2 diabetes mellitus (T2DM) has increased dramatically (more than doubled) since 1980, with a global prevalence of 9.8% and 9.25% for men and women, respectively, and affecting 347 million adults in 2008 [1]. In Europe, the prevalence of T2DM is estimated to be 8.1% of the adult population, affecting 52.8 million people in 2011. The estimated prevalence of T2DM for 2030 is 9.5% of the adult population affecting 64.2 million people. T2DM accounts for 85 to 90% of all cases of diabetes mellitus.

T2DM is one of the world's most important causes of mortality, disability and economic cost due to the associated increase in the risk of micro- [2] and macrovascular [3] complications. T2DM caused 282,400 and 317,000 deaths in 2011, for men and women, respectively [4]. Estimates indicate that at least USD 131 million were spent in Europe due to T2DM in 2011 [4]. In 2003, the prevalence of T2DM in the archipelago of Canary Islands (Spain) was estimated to be 12% of the population with an economic cost of €38.8 million, which accounts for 2.1% of all the health costs of the Canary Islands' Government [5,6]. Furthermore, micro- and macrovascular complications, including end-stage renal disease, are four to five times higher than in the rest of Spain [6,7]. In impaired glucose tolerance (IGT), lifestyle intervention reduces the incidence of T2DM [8], even when compared with insulin sensitizing medication [9]. In a study from primary health care in Stockholm, more cardiovascular risk factors were improved after an exercise intervention in people with normal glucose tolerance compared with those with IGT or T2DM [10]. However, in these studies the separate effect of exercise versus diet could not be elucidated [11]. Data from systematic reviews regarding the role of food in the prevention and treatment of T2DM indicates that there are some uncertainties with respect to the optimal dietary intervention [11,12], and results from one large interventional dietary study suggest that targeting macronutrient composition by lowering total fat intake and increasing carbohydrate intake from whole grains, may have detrimental effects in women with pre-existing T2DM [13]. A recent intensive diet and exercise program in patients with T2DM was stopped because the intervention did not reduce cardiovascular risk, despite significant weight loss at four years, compared to the control group [14]. Research on the role of nutrition in T2DM has largely focused on macro/micronutrient composition and fiber intake, while fewer studies have tested the more direct endocrine effects of food [13,15,16]. A recent systematic review and meta-analysis suggests that low-carbohydrate, low-glycemic index, Mediterranean and high-protein diets may be effective in improving cardiovascular disease risk and diabetes management compared to control diets, with no significant difference effect between the different interventions [17]. Also, the effect of weight loss over macronutrient composition precludes drawing a definitive conclusion derived from this meta-analysis.

Theoretically, dietary interventions can improve glucagon [18] and leptin physiology [19]. Thus, we speculate that the possible effects of the diets in the ADILAN study could be mediated, in part, by improving the action of those hormones.

Some observational studies [20] and two short-term intervention trials from our group [21,22] suggest that a food pattern mimicking the diet with which humans evolved, may be an optimal approach for patients with T2DM. Importantly, the short-term trials compared the experimental approach to the recommended dietary treatment in each case, namely the Mediterranean diet for patients with ischemic heart disease [21] and the American Diabetes Association diet for patients with T2DM [22], respectively. In both trials, the experimental approach resulted in better outcomes than in the control diet. A noteworthy finding in those dietary trials is the significant difference in reported macronutrient composition between interventions and controls, specifically, higher energy percentage intake for protein and fat (except in Lindeberg *et al*. [21] for fat), and lower energy percentage intake for carbohydrate in the experimental versus the control diet, taking into account that the intervention diet was not fixed to any dietary macronutrient ratio. Dietary glycemic load (GL) was also significantly lower.

Notwithstanding these findings, the beneficial effects shown in the experimental diets in both trials are not fully explained by macronutrient composition or GL after further statistical analysis [21,22]. We concluded that food choice, rather than micro-and macronutrient composition, may have been the most important factor leading to the beneficial effects observed. In a recent meta-analysis comparing Mediterranean diets to alternative dietary strategies,

the authors stated that the paleolithic diet (a healthy diet without grains and dairy) in the study by Lindeberg *et al.* [21] demonstrated the most positive effect on fasting blood glucose of all the studies included in the meta-analysis [23].

The Alimentation and Diabetes in Lanzarote (ADILAN) study

The major aim of the ADILAN study is to test the direct endocrine effect of food items beyond macro/micronutrient composition, fiber content and glycemic load of the diet. For this purpose, we will try to avoid weight loss in an attempt to isolate the effect of food, as weight loss could have been a confounding factor in the previous studies. Our objective is to run a pilot intervention for a later long-term study with a representative sample in patients with T2DM. The working hypothesis is that food choice rather than macro/micronutrient composition, fiber intake, or GL, is a major determinant for the prevention and treatment of T2DM. Hypothetically, the foods that composed the diet during most of the time of *Homo sapiens*' evolution, may be the optimal dietary approach for the prevention and treatment of T2DM [24,25].

Methods/Design

Participants

We aim to enroll 15 participants with T2DM, males and females, > 18 years old based, on the power calculations (see below) estimation of a required minimum of 13 patients, allowing for one or two drop-outs. Patients live in the island of Lanzarote, in the archipelago of Canary Islands (Spain), located off the western coast of Africa.

Recruitment

Patients will be recruited through different strategies. We are in collaboration with the *Asociación de Diabéticos de Lanzarote* (ADILA), a local organization, whose aim is helping patients to deal with the consequences of type 1 diabetes and T2DM. Data from 1996 estimated the prevalence of T2DM in Lanzarote to be 6.6% of the population aged between 15 and 75 years old, with an absolute number of 4,067 patients [26,27]. ADILA will contact the potential participants registered in their database by telephone, Email and advertisement in the association's bulletin board. In addition, we will advertise the study in the local newspapers, radio and in some institutional websites on the island of Lanzarote. Patients interested in participating in the study will receive general oral and/or written information about the study. They will be informed that our objective is to compare two healthy diets because we do not know which, if any, is the better one. If a person is interested, we will register their name and telephone number for further contact. After recruiting 15 potential participants, a meeting will be arranged to provide further details about the study.

Eligibility

We will include adults (> 18 years old), males and females, with a medical diagnosis of T2DM and increased waist circumference (≥ 80 cm for women and ≥ 94 cm for men), with or without medication (including insulin treatment), with stable weight for three months prior to the start of study, who have received no change in dose of beta blocker or thyroxine for three months prior to the start of study, and no anticoagulant or oral steroid treatment. Inclusion criteria are present glycated hemoglobin (A1c) ≥ 6.0% (≥ 42.1 mmol/mol), with no upper boundary, creatinine < 130 μmol/L and liver enzymes less than four times above the upper reference value.

Procedure

The first 15 enrolled participants will undergo baseline tests, including A1c, creatinine and liver enzymes. At this time they will be instructed, in a group session, how to perform the four-day weighed food record and fill the satiety score sheets (Figure 1). Later, participants who meet the inclusion criteria will be notified about their final inclusion in the trial and reminded how to perform the four-day weighed food record and satiety score. Participants will borrow an electronic weighing scale with tare function and will be provided with enough sheets for the four-day weighed food record and satiety score. Five to seven days later, they will return the food records. The baseline four-day food record will yield information about the pre-study food choices and nutritional composition. The four-day food record and satiety score will be carried out at the start and end of each intervention period. After receiving the first food records, participants will be appointed for the first laboratory tests and intervention allocation. Based on total energy expenditure, as estimated by a bio-electrical impedance analyzer model BC-545 (Tanita Corporation, Tokyo, Japan), we will provide each participant with a menu plan with the approximate energy intake to avoid changes in body weight.

Regarding insulin titration, the participant's physician will handle insulin titration as needed.

Randomization

The study participants will be randomly allocated to start with either diet A or diet B. The process will be performed after the first laboratory testing. Randomization will be performed by use of an Internet-based random sequence generator from the School of Computer Science and Statistics, Trinity College, Dublin [http://www.random.org/sequences/]. Due to the small sample size, and to avoid unbalanced allocation of the participants to one of the starting diets, stratified sampling by use of minimization technique will be used. Weighting variables are starting diet and duration of T2DM [28].

Fecha: __/__/____ Nombre: _____

Lugar: Hora de comienzo a comer: :

Alimentos	Preparación Crudo/cocinado	Cantidad de consumo (g, dl, cucharadas, cm)
Sobras		

Saciedad
X=al inicio de la comida
O= treinta (30) minutos tras iniciar la comida

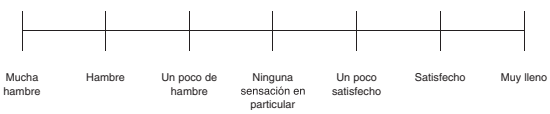

Figure 1 Four-day weighed food record and satiety score sheet.

Consent

Once 15 interested persons contact the researchers, an initial meeting with the study investigators will be arranged to give additional information about the study. Additional eligible subjects will be placed in queue for possible participation in case of drop-out(s). People who are willing to participate in the study and who meet inclusion criteria (medical diagnosis of T2DM, increased waist circumference, stable body weight for three months prior to the start of study, stable dosing of beta blockers or thyroxine for three months prior to the start of study, and absence of chronic oral steroid treatment, and anticoagulant treatment) will receive written information about the study and informed consent as approved by the ethics committee. After returning the informed consent, laboratory tests for plasma A1c, creatinine and liver enzymes will be analyzed. For each person who is excluded or declines participation, one person from the queue will be contacted and informed individually.

Ethics committee and participants insurance

The ADILAN study was approved by the Ethics Committee of Clinical Investigation (CEIC) of the Hospital Universitario de Gran Canaria, Doctor Negrín (Code CEIC Negrín: 130030). The study participants will be protected against any medical complication derived from the intervention,

with an insurance exclusively contracted for ADILAN (HDI Seguros, policy number 130/002/001897), registered at clinicaltrials.gov as NCT01891955.

Design

The ADILAN study is a randomized cross-over open label dietary intervention trial in patients with T2DM who will be instructed to eat two different dietary patterns during two four-week periods separated by a six-week washout period. The procedure for screening, eligibility and recruitment is detailed in Figure 2. Schedule of enrollment, interventions and assessments can be found in Additional file 1. Detailed information about enrollment, interventions and assessments is described in Additional file 2.

Power calculations

The predefined primary outcomes are changes in fasting fructosamine and glucagon. In order to detect, with 80% power and at a significance level of 5%, a 26 μmol/L larger reduction in fructosamine after diet B than after diet A, 12 subjects are estimated to be required. For a 37 ng/L difference in change in fasting glucagon between the diets, 13 subjects are estimated to be required.

Intervention

The intervention is based on two diets: a) healthy diet A (healthy diet with cereal grains and dairy products), and b) healthy diet B (healthy diet without cereal grains and dairy products). After randomization, there will be no blinding to dietary assignment for study participants, nor for those administering the interventions or assessing the outcomes. Following randomization, all subjects will receive oral and written information about their respective initial dietary intervention. Owing to the fact that all participants in both groups will start the intervention at the same time, information about the specific intervention will be provided in group sessions, one for each group before the study starts. Prior to the beginning of the second dietary period, participants will be instructed about the new dietary intervention, in group sessions. Each session will last one hour and will be performed in the meeting room at the Hospital Insular de Lanzarote after the laboratory tests. The degree of behavioral support will be the same in both interventions in order to minimize differences in motivation. Written information with dietary advice, a seven-day menu plan and food recipes are similarly formulated for both diets. The rationale behind the higher intake of whole grains, dairy and legumes in healthy diet A is based on the possible beneficial effect of those foods in the treatment of T2DM in some studies [29], and their prominent position in Spanish dietary guidelines for patients with T2DM [30]. The rationale behind the exclusion of grains, dairy and legumes in healthy diet B is based on the observed positive effects in some previous studies [21,22].

Written information consists of a detailed seven-day meal plan with five meals per day and corresponding recipes (Additional file 3). Participants will be requested to follow the menu plan as much as possible when eating away from home. They will be requested to report (in as much detail as possible, the ingredients) any meal taken which differs from what is stipulated in the menu plan. In that case, they will be encouraged to photograph the meal, and send it to one of the investigators, whenever possible.

Advice about regular physical activity will be given equally to all subjects. Specifically, they will be recommended not to change their current physical activity during the whole trial.

One of the objectives of the study is to avoid weight loss during the intervention. In order to accomplish this objective, participants will be weighed once a week in order to check for weight stability. If any participant loses ≥ 1 kg compared to baseline, a new meeting will be arranged for that person to adjust his/her meal plan. We have created different versions for both menu plans in order to provide different amounts of energy per day; specifically 1,500, 1,800, 2,000, 2,200, 2,500 and 2,800 kcal/day. All the different menu versions maintain the same macronutrient composition. Depending on body weight as measured every week, each participant will be reassigned to another (with higher or lower energy) menu plan if their body weight changes by ≥ 1 kg. The meals will be taken to the lunch room in two trolleys marked with menu A or B, respectively. Each trolley will contain the trays labeled with the participant's name. Finally, the lunch room will be separated in two areas for menu A and B, respectively.

Improving patients adherence to the meal plan

Despite the notion that a healthy diet can improve glucose control, adherence to dietary advice is generally poor among patients with T2DM [31]. Moreover, the particular dietary design of this trial could increase the risk of nonadherence. In order to minimize this risk, several strategies will be adopted. To facilitate the participants eating the same approximate amount of equivalent food in every meal (expressed in grams in Additional file 3), we will supply a table of equivalence. For example, 56 grams of tinned tuna equates to a small tin of tuna and 20 grams of bread corresponds to a slice of bread. Similarly, food items such as eggs or fruits will be listed as small, medium or large. In the case of more elaborated dishes, such as stewed meat, baked tubers, soups, or creams, we will provide the participants with detailed recipe directions corresponding to four servings, so as to facilitate the task in the family context. On the first day of each intervention, the investigators will review all the assigned meals, together

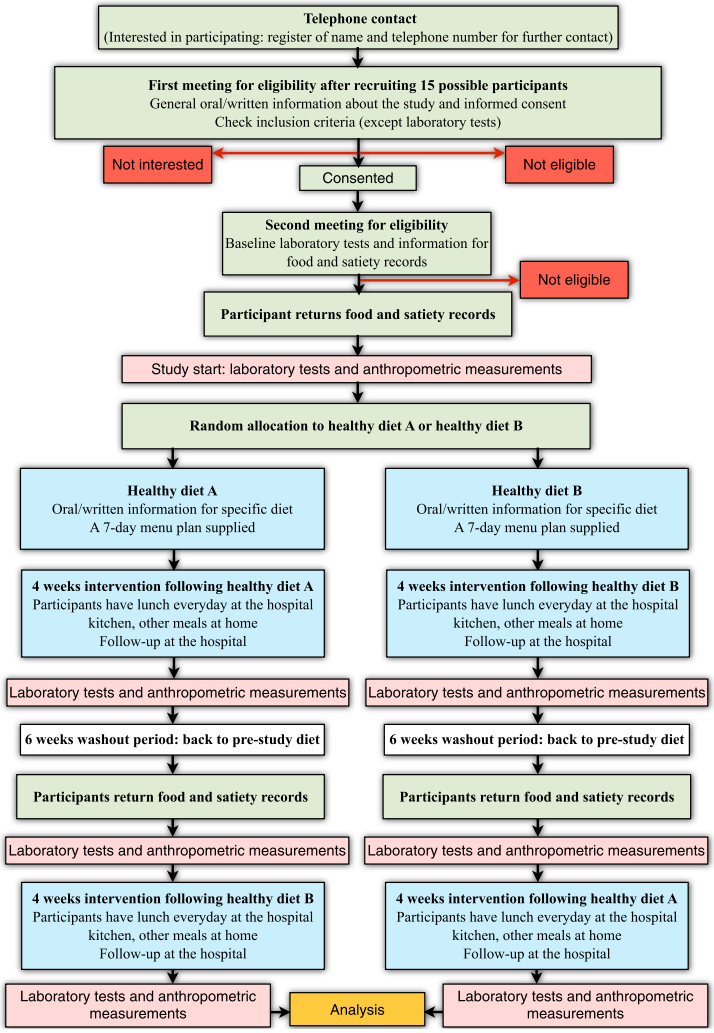

Figure 2 Diagram flow for Alimentation and Diabetes in Lanzarote (ADILAN).

with the participants and their partners, to make sure that the directions are understandable and easy to follow. Previous to starting the study, subjects not included in the trial will test the meals in order to elaborate a meal plan that is understandable and easy. We will ask the participants to call one of the investigators if they have any questions regarding the meal plan. Besides this, one of the investigators will meet the participants in the lunch room at the beginning of each week to give behavioral support. Furthermore, an assistant will be present on a daily basis in the lunch room to ensure that the meals are served as planned. Also, the assistant will be asked everyday to inquire whether the participants have any difficulties following the meal plans.

Healthy diet A

Healthy diet A will include fruit, vegetables, fish, shellfish, lean meat, nuts, eggs, olive oil and substantial amounts of whole grains, low-fat dairy products and legumes. Macronutrient composition and fiber intake will

not differ from healthy diet B. GL will also be matched to that of healthy diet B. Healthy diet A is termed 'healthy diet with cereal grains and dairy products'. This diet is classified as 'very healthy' using a Spanish validated nutritional software package [32]. It is in accordance with official Spanish dietary recommendations for people with T2DM regarding macronutrient composition, dietary fiber, minerals and vitamins (Tables 1 and 2). A detailed description of each of the meals proposed during the course of one week is included in Additional file 3.

Healthy diet B

Healthy diet B will include fruit, vegetables, fish, shellfish, lean meat, nuts, eggs and olive oil. Macronutrient composition and fiber intake will not differ from healthy diet A. GL will also be matched to that of healthy diet A. Healthy diet B will exclude cereal grains, legumes and dairy products, which will largely be replaced by root vegetables (including potatoes), vegetables, fruit and lean meat, and slightly more fish and nuts. Healthy diet B is termed 'healthy diet without cereal grains and dairy products'. Salt intake will be lower in healthy diet B. This diet is also classified as 'very healthy' using validated nutritional software package [32]. It is in accordance with official Spanish dietary recommendations for people with T2DM regarding macronutrient composition,

Table 1 Dietary composition of the two healthy diets

	Energy content, kcal (SD)		*P*-value[a]
	Healthy diet A	Healthy diet B	
Cereals	711.4 (105.2)	0	0.001
Legumes	185.4 (185.1)	0	0.07
Vegetables	102.6 (60.2)	421.4 (130.5)	< 0.0001
Fruits	259.4 (56.1)	980.1 (183.1)	0.001
Dairy products	216.6 (53.9)	0	0.001
Meats	62.9 (62.4)	173.1 (86.6)	0.02
Fish	165.7 (90.3)	186.4 (48.5)	0.6
Eggs	49.3 (41.7)	33.2 (41.4)	0.2
Sugars and cakes	0.97 (2.6)	11.8 (28.2)	0.6
Oils and fats	245.7 (45.3)	167.1 (43.7)	0.006
Drinks	8.9 (11.5)	11.5 (6.5)	0.6
Pre-cooked food	2.2 (3.0)	3.8 (5.1)	0.6
Snacks	7.1 (18.9)	7.0 (11.5)	0.5
Sauces and dressings	12.6 (26.7)	5.6 (4.4)	0.94
Other	0.5 (1.2)	0.5 (1.2)	1.0
Total energy	2,031.3 (142.3)	2,001.7 (59.5)	0.6
Total weight	1,866.8 (300.1)	2,099.8 (184.0)	0.1

Dietary composition (kcal) of the two healthy diets, as calculated from seven-day menu plans. Total weight in grams/day.
Values are means (SD).
[a]Mann-Whitney *U*-test, except for meat, fish, oils and fats, and sauces and dressings, total energy and total weight (*t*-test).

Table 2 Daily intake of macronutrients, micronutrients, fiber, glycemic load and index, cholesterol and water, as calculated from seven-day menu plans

	Diet		*P*-value[a]
	Healthy diet A	Healthy diet B	
Energy			
(kcal)	2,031 (142.3)	2,002 (59.5)	0.6
(MJ)	8.5 (0.6)	8.4 (0.2)	0.6
Protein			
g	97.2 (7.6)	93.0 (4.2)	0.2
E%	19.0 (1.8)	18.3 (1.2)	0.4
Carbohydrates			
g	245.7 (18.3)	236.7 (8.4)	0.8
E%	53.0 (3.4)	53.4 (1.5)	0.8
Fiber (g)	48.5 (10.3)	53.1 (5.7)	0.3
Glycemic load (g)	116.1 (8.0)	118.0 (11.9)	0.7
Glycemic index	47.4 (1.2)	49.8 (4.7)	0.2
Total fat (g)			
g	63.6 (12.8)	62.8 (6.9)	0.89
E%	27.6 (4.6)	27.8 (2.4)	0.95
Fatty acids			
Saturated (g)	13.3 (5.2)	10.9 (2.0)	0.3
Monounsaturated (g)	30.8 (5.7)	32.1 (4.2)	0.6
Polyunsaturated (g)	10.9 (3.0)	12.5 (2.7)	0.3
Cholesterol (mg)	257.4 (106.1)	271.4 (80.1)	0.8
Water (g)	1,395.3 (296.2)	1,708.9 (240.7)	0.05
Calcium (mg)	978.3 (123.9)	591.4 (88.6)	< 0.0001
Iron (mg)	19.9 (2.8)	18.5 (2.3)	0.3
Iodine (μg)	101.7 (27.8)	143.9 (68.1)	0.15
Magnesium (mg)	570.6 (70.7)	468.6 (49.3)	0.009
Zinc (mg)	13.6 (1.8)	9.9 (2.3)	0.006
Selenium (μg)	155.7 (23.4)	126.3 (28.8)	0.057
Sodium (mg)	1,811.9 (733.9)	1,079.3 (440.7)	0.042
Potassium (mg)	3,556.0 (305.5)	6,392.9 (575.4)	0.001
Vitamin B1 (mg)	2.2 (0.3)	1.8 (0.2)	0.004
Vitamin B2 (mg)	2.2 (0.3)	1.6 (0.2)	0.001
Vitamin B3 (mg)	45.0 (5.7)	49.0 (6.9)	0.27
Vitamin B6 (mg)	2.6 (0.6)	4.2 (0.8)	0.001
Folic acid (μg)	427.6 (133.5)	506.3 (179.3)	0.37
Vitamin B12 (μg)	6.0 (1.3)	8.6 (6.3)	0.38
Vitamin C (mg)	170.1 (46.9)	434.4 (163.5)	< 0.0001
Beta-carotene (μg)	4,233.7 (1494.5)	17,194.1 (6085.9)	< 0.0001
Vitamin A (μg)	986.3 (582.8)	3,021.1 (1027.1)	0.001
Vitamin D (μg)	5.2 (3.6)	4.8 (3.2)	0.96
Vitamin E (mg)	13.0 (5.9)	23.7 (7.4)	0.01

Values are mean (SD).
[a]*t*-test, except for carbohydrate (grams), potassium and vitamin B12 (Mann–Whitney *U*-test).

dietary fiber, minerals and vitamins (Tables 1 and 2). A detailed description of each of the meals proposed during the course of one week is included in Additional file 3.

Dietary design

The main goal of the study is to test the hypothesis that the type of food has an effect on glucose control, independently of macro/micronutrient composition, fiber content, GL and weight loss. Therefore, we generated two different seven-day meal patterns where macro/micronutrient composition and fiber intake are similar. GL is also similar between both seven-day meal patterns. Furthermore, we aim at creating two healthy food patterns, according to official nutritional guidelines in Canary Islands, utilizing a validated nutritional software package (DIAL) [32]. The nutritional composition of the menu plan was analyzed using another validated nutritional software (Dietist XP 3.1; Kost och Näringsdata AB, Bromma, Sweden) to check for possible errors. Minimal differences were observed in the results from both programs. The distribution of macronutrient energy of diets is 19% protein, 28% fat and 53% carbohydrate, and 18% protein, 28% fat, and 53% carbohydrate for healthy diet A and healthy diet B, respectively. The amount of fiber per 2,000 kcal is approximately 48.5 g and 53.1 g in healthy diet A and healthy diet B, respectively. The GL per 2,000 kcal is 116 and 118 in healthy diet A and healthy diet B, respectively. Regarding micronutrient intake, significant differences between the two diets can be seen in Table 2. Importantly, and because one goal of the ADILAN trial is to avoid weight loss, dried fruits were regularly included instead of fresh fruits, in healthy diet B, to match the total weight of food between the two food patterns. Two recent studies have shown that substituting cereal grains for fruits and vegetables increases satiety [19,33], and therefore it may be challenging for the participants to avoid weight loss while adhering to healthy diet B.

Evaluation

Before and after each intervention period, an oral glucose tolerance test (OGTT) will be performed in the morning after obtaining venous blood samples in the fasting state, and measurements of blood pressure, height (only at the start of intervention 1), weight, waist and hip circumference, sagittal abdominal diameter, triceps, biceps, suprailiac and subscapular skinfolds, at the start and end of both intervention periods will also be taken. The participants will ingest 75 grams of glucose and blood samples will be drawn for glucose and glucagon at 0, 15, 30, 60, 90 and 120 minutes. Areas under the curve (AUC) for plasma glucose (AUC glucose) and glucagon (AUC glucagon) will be calculated. Of great consequence, and for safety reasons, we will assess capillary glucose, using a glucometer, before performing the OGTT. If the result is greater than or equal to 190 mg/dL (10.5 mmol/L) the OGTT will not be performed. Information about participants' quality of life will be assessed by means of the short form quality of life questionnaire SF-36v2 (standard version), using the validated Spanish version, which will be filled by the participant during each of the OGTT procedures. During the OGTT, at the end of each intervention period, patients will also be asked to give written answers to three open-ended questions about their experience with the dietary pattern that they have been following during the previous four weeks, specifically: 'What are your thoughts about this diet?', 'Describe your positive and negative experiences with this diet' and 'How do you think this diet has affected your health?'

During both dietary interventions, free lunch will be provided every day in the kitchen of the Hospital Insular de Lanzarote. Here, our objective is two-fold: a) to control nutrient intake as much as possible and b) to give the participants behavioral support during visits to the hospital.

Outcome assessment

Primary outcomes

The primary outcomes of this study are fasting fructosamine and glucagon. These outcomes will be determined after obtaining venous blood in the fasting state at start and end of each intervention period.

Secondary outcomes

Fasting plasma glucose, A1c, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, high-sensitivity C-reactive protein, blood pressure, and AUCs for glucose and glucagon during OGTT comprise secondary outcomes. Quality of life, assessed with the SF-36v2 questionnaire (standard version), satiety quotient (see below), experience with the dietary patterns and change in medication are also included as secondary outcomes.

Anthropometric measurement and laboratory tests

Trained nurses and investigators will perform the lab tests and anthropometric measurements in the Hospital Insular de Lanzarote. All measurements and laboratory tests will follow standard certified methods [34].

Standing height without shoes will be measured with a height stadiometer. Height will be reported to the nearest 1 mm.

Weight will be measured using an electronic weighing scale placed on a hard surface with the patient in the fasting state. Patients will be asked to take off their shoes, jackets, sweaters, and any other heavy clothing, and to remove all heavy items from their pockets. Then the patients will place both feet in the center of the

platform and distribute their weight evenly between both feet. Weight is reported to the nearest 0.1 kg.

Waist circumference will be measured using a measuring tape immediately above the iliac crest. Measurements will be reported to the nearest 0.1 cm.

The hip circumference will be measured using a measuring tape at the level of the trochanter major and reported to the nearest 0.1 cm.

Sagittal abdominal diameter will be measured using a sagittometer (that is, a sliding beam caliper with a ruler) in the sagittal plane during a normal expiration at the level of iliac crest (L4 to L5) with the subject in supine position on a firm bench with the knees bent. Measurements will be reported to the nearest 0.1 cm.

Blood pressure will be measured in the upper arm (preferably right arm) at the level of the heart after five minutes rest in the sitting position (MONICA criteria) [34]. Ideally, the same person should perform all measurements in the same patient, using the same sphygmomanometer and procedure. The mean of two measurements will be calculated as the final result.

Skinfold measurements will be taken by use of a validated caliper with a precision of 0.2 mm. The following points will be measured as follows:

- tricipital skinfold: in the posterior part of the arm in the middle point between the lower border of the acromion and the vertex of the olecranon.
- bicipital skinfold: in the anterior part of the arm in the middle point between the most external and superior border of the acromion and the most external and superior of the radial bone head.
- suprailiac skinfold: approximately 2.5 cm above iliac crest in mid-axillary line.
- subscapular skinfold: in the inferior vertex of the scapular bone.

Four-day weighed food records and satiety scores

A four-day weighed food record, including one weekend day, with weighing of each food item on an electronic weighing scale (that could be set to zero), will be completed by the participants before the start and end of each intervention period (Figure 1). By completing each four-day weighed food record, participants will be asked to rate, on a scale from 0 to 100, how well the record represents their food habits in the preceding four weeks. Nutrient compositions will be assessed with the nutritional software package DIAL [32] used to create the meal plans. In parallel with the four-day weighed food records, the participants will record their subjective rating of satiety by means of a seven-point Likert-type scale (Figure 1). Satiety Quotients will be calculated, as the intra-meal quotient of change in satiety during meal and consumed energy or weight of food and drink for that specific meal.

Data management

Study data will be collected and managed using REDCap™ (Research Electronic Data Capture) electronic data capture tools hosted at Lund University [35]. REDCap™ is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

REDCap™ will be configured according to the study protocol in a way that the researchers can register all data at baseline, 4, 10 and 14 weeks after study start. Only the researchers and the hospital nurses will have access to REDCap™. This web application allows for management of the trial without violation of privacy or participant confidentiality and integrity of allocation concealment. The procedure also facilitates supervision from abroad by some of the investigators.

Data analysis

Differences in baseline characteristics between the participants will be measured using two-tailed unpaired *t*-tests. A two-tailed paired *t*-test will be used to analyze treatment effects within subjects and a two-tailed unpaired *t*-test will be used to compare mean values of outcome variables between the two diets. Normal Q-Q plots and Shapiro-Wilk test will be performed to check for normal distribution of data. Data transformation will be applied in case the data does not present normal distribution. To test for a possible period effect a two-tailed unpaired *t*-test comparing the mean difference between first and second period for outcome variables and food intake will be performed. To test a possible carry-over effect a two-tailed unpaired *t*-test comparing the average of observations for outcome variables and food intake between the two periods will be performed. $P < 0.05$ will be considered to indicate statistical significance.

Analysis will be conducted by use of SPSS for Mac Version 20 (IBM SPSS Statistics for Mac, Version 20.0. Armonk, NY: IBM Corp.)

Discussion

As discussed previously, there is uncertainty as to what is the optimal dietary approach for the prevention and treatment of T2DM [11,12,17], and previous trials from our group raise the question of whether focusing on the type of food, rather than macro/micronutrient composition, dietary fiber and GL is a better approach [21,22]. The ADILAN study aims at comparing the effects of

two healthy diets on the control of blood glucose, with no significant differences in macro/micronutrient composition, dietary fiber and GL, only different food choice. Importantly, we will try to eliminate weight loss as a confounding factor.

Of paramount importance, the nutritional software package (DIAL) used to create the seven-day menu plan generates a 'Healthy Alimentation Index' that classifies both diets as 'very healthy'. Due to its specific design, constructed on evolutionary principles, the grain/dairy-free diet scored zero in two items, 'Cereals and legumes' and 'Dairy', but in spite of this, it was classified as very healthy owing to the fact that it scored the maximum (except for 'Variety of foods with a score of 7/10) in the rest of items', namely 'Vegetables', 'Fruits', 'Meat, Fish and Eggs', 'Energy derived from fat', 'Energy derived from saturated fats', 'Cholesterol', 'Dietary sodium' and 'Variety of foods' [32]. It can be concluded that the ADILAN study will compare two healthy diets which are similar in macro/micronutrient composition, fiber intake and GL, for the treatment of T2DM but differing in the intake of cereal grains, legumes and dairy products.

The ADILAN study could serve as a pilot study to run long-term dietary intervention trials in the future, focusing on food choice, which could shed light on a better dietary approach for prevention and treatment of T2DM.

Trial status

The ADILAN study is currently under recruitment process at 22 October 2013.

Additional files

Additional file 1: Schedule of enrollment, interventions, and assessments.

Additional file 2: Detailed study diagram flow. Detailed study diagram flow of the different events with corresponding laboratory tests and measurements at each time point.

Additional file 3: Seven-day menu plan. Detailed daily description of each of the proposed meals with specific weights for both diets in a period of one week.

Abbreviations

A1c: Glycated hemoglobin; ADILA: Asociación de Diabéticos de Lanzarote; ADILAN: Alimentation and Diabetes in Lanzarote; AUC: Area under curve; GL: Glycemic load; IGT: Impaired glucose tolerance; MONICA: World Health Organization's Multinational Monitoring of Trends and Determinants in Cardiovascular Disease protocol; OGTT: Oral glucose tolerance test; REDCap®: Research Electronic Data Capture; T2DM: Type 2 diabetes mellitus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MFV designed the study, created the meal plans, arranged and participated in meetings with the hospital staff, worked in the web application for data capture, was responsible for obtaining funding for the study, performed the statistical analysis and wrote the manuscript. TJ participated in the study design, helped to create the meal plans, worked in the web application for data capture, helped in the statistical analysis and revised the manuscript for important intellectual content. YG participated in the study design, helped to create the meal plans, helped in the statistical analysis and revised the manuscript for important intellectual content. LAF helped to create the meal plans, participated in the study design and revised the manuscript for important intellectual content. JS revised the manuscript for important intellectual content. KS revised the manuscript for important intellectual content. OP participated in the study design, helped to create the meal plans, helped in the statistical analysis and revised the manuscript for important intellectual content. PCB revised the manuscript for important intellectual content. MFH participated in the study design, participated in meetings with the hospital staff and revised the manuscript for important intellectual content. SL participated in the study design, helped to create the meal plans, participated in meetings with the hospital staff, helped in the statistical analysis and participated in the design of the manuscript as well as revising it for important intellectual content. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful to Marci Acuña for continuous assistance in the logistics of the study and providing human resources, to María Montilla for helping in the laboratory analysis and arranging the meetings with the kitchen staff to create the meals, to the kitchen staff for preparing the meals and administering them to the participants, and to the Asociación de Diabéticos de Lanzarote (ADILA) for support in participant recruitment. This study received funding from the Excmo. Cabildo Insular de Lanzarote; Excmo. Ayuntamiento de Teguise and Excmo. Ayuntamiento de Arrecife. The Excmo. Cabildo Insular de Lanzarote will fund laboratory costs. The authors are very grateful to Felipa Villalba Díaz for helping in the design and testing the menu plan. The authors are also grateful to Ione Aguiar for his invaluable help in the design of the study protocol prior to be reviewed by the ethics committee, and for helping with the insurance contract.

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Received: 22 October 2013 Accepted: 16 December 2013
Published: 2 January 2014

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doi:10.1186/1745-6215-15-2
Cite this article as: Fontes-Villalba *et al.*: A healthy diet with and without cereal grains and dairy products in patients with type 2 diabetes: study protocol for a random-order cross-over pilot study - Alimentation and Diabetes in Lanzarote -ADILAN. *Trials* 2014 **15**:2.

Paper III

Fontes-Villalba *et al. Cardiovasc Diabetol (2016) 15:80*
DOI 10.1186/s12933-016-0398-1

Cardiovascular Diabetology

ORIGINAL INVESTIGATION

Open Access

Palaeolithic diet decreases fasting plasma leptin concentrations more than a diabetes diet in patients with type 2 diabetes: a randomised cross-over trial

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Abstract

Background: We have previously shown that a Palaeolithic diet consisting of the typical food groups that our ancestors ate during the Palaeolithic era, improves cardiovascular disease risk factors and glucose control compared to the currently recommended diabetes diet in patients with type 2 diabetes. To elucidate the mechanisms behind these effects, we evaluated fasting plasma concentrations of glucagon, insulin, incretins, ghrelin, C-peptide and adipokines from the same study.

Methods: In a randomised, open-label, cross-over study, 13 patients with type 2 diabetes were randomly assigned to eat a Palaeolithic diet based on lean meat, fish, fruits, vegetables, root vegetables, eggs and nuts, or a diabetes diet designed in accordance with current diabetes dietary guidelines during two consecutive 3-month periods. The patients were recruited from primary health-care units and included three women and 10 men [age (mean ± SD) 64 ± 6 years; BMI 30 ± 7 kg/m^2; diabetes duration 8 ± 5 years; glycated haemoglobin 6.6 ± 0.6 % (57.3 ± 6 mmol/mol)] with unaltered diabetes treatment and stable body weight for 3 months prior to the start of the study. Outcome variables included fasting plasma concentrations of leptin, adiponectin, adipsin, visfatin, resistin, glucagon, insulin, C-peptide, glucose-dependent insulinotropic polypeptide, glucagon-like peptide-1 and ghrelin. Dietary intake was evaluated by use of 4-day weighed food records.

Results: Seven participants started with the Palaeolithic diet and six with the diabetes diet. The Palaeolithic diet resulted in a large effect size (Cohen's $d = -1.26$) at lowering fasting plasma leptin levels compared to the diabetes diet [mean difference (95 % CI), −2.3 (−5.1 to 0.4) ng/ml, $p = 0.023$]. No statistically significant differences between the diets for the other variables, analysed in this study, were observed.

Conclusions: Over a 3-month study period, a Palaeolithic diet resulted in reduced fasting plasma leptin levels, but did not change fasting levels of insulin, C-peptide, glucagon, incretins, ghrelin and adipokines compared to the currently recommended diabetes diet.

Trial registration: ClinicalTrials.gov NCT00435240

Keywords: Palaeolithic diet, Type 2 diabetes, Glucagon, Leptin, Lipotoxicity, Adiposopathy, Evolution

Background

The metabolic syndrome represents a cluster of symptoms including abdominal obesity, insulin resistance, dyslipidemia, and high fasting glucose and blood pressure [1]. The condition is associated with a fivefold increased risk of type 2 diabetes, which is characterized by insulin resistance [2] and β-cell failure [3]. Lifestyle plays a prominent role in the pathophysiology of the metabolic syndrome and type 2 diabetes [4, 5]. Specifically, an unhealthy diet with chronic caloric surplus induces

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hyperinsulinemia leading to ectopic lipid deposition (lipotoxicity) in the heart, liver, pancreas and muscle [6–8], increasing the risk of the metabolic syndrome, fatty liver, fatty heart and type 2 diabetes [7].

Interestingly, insulin resistance has been suggested to be a consequence and a protective mechanism against lipotoxicity [6–10]. Leptin resistance is a possible player in the roadmap to the metabolic syndrome and type 2 diabetes [7], and it has been suggested that leptin can protect against lipotoxicity [7]. Lipotoxicity can generate α-cell insulin resistance resulting in hyperglucagonaemia and increased hepatic glucose production [10]. It is increasingly recognized that the recent (in an evolutionary perspective) introduction of staple food groups such as cereal grains, dairy products and refined sugars in the human diet has occurred too recently for the human genome to have completely adapted [11].

In a previous publication from this trial, we reported significant improvements in glycated haemoglobin (HbA1c), blood lipids, blood pressure, weight and waist circumference [12] along with increased satiety [13] in patients with type 2 diabetes consuming a Palaeolithic diet, as compared to the officially recommended diet for patients with type 2 diabetes (diabetes diet) [14].

The abovementioned pathophysiological changes produce adaptations in other hormones and endocrine axes. Therefore, our aim was to investigate if the beneficial effects from a Palaeolithic diet could be tentatively explained by associated changes in adipokines, glucagon, incretins and ghrelin, and here we present new data on the fasting levels of these hormones from our previous study [12] (Additional file 1).

Methods

Approval of the study was obtained from the regional Medical Ethics Committee and the trial was registered at ClinicalTrials.gov (Identifier: NCT00435240).

Participants

Patients with type 2 diabetes without insulin treatment were recruited from primary health-care units in the Lund area of Sweden. Details about inclusion and exclusion criteria and patient characteristics (Table 1) have previously been published [12]. All recruited subjects were given oral and written study information prior to signing a consent form to participate in the study and were then further assessed for eligibility.

Design

The study design and generation of random allocation sequence have been reported in detail previously [12]. In short, the study was a randomised, cross-over, dietary intervention study in which all eligible subjects were informed of the intention to compare two healthy diets and that it was not known whether one diet might be superior to the other. After randomisation, there was no blinding of dietary assignment to study participants, nor to those administering the interventions or assessing the outcomes. At study start, all subjects were randomised, by use of opaque envelopes, to start with either a diabetes diet designed in accordance with official recommendations [14] or a Palaeolithic diet [12]. Immediately after randomisation, all subjects received oral and written information about their respective initial diet individually from diabetes nurses. After 3 months, all subjects switched diets and received new oral and written information about the new diet. Advice about regular physical activity was given to all subjects.

Diets

The information on the diabetes diet stated that it aimed to provide evenly distributed meals with an increased intake of vegetables, root vegetables, dietary fibre, wholegrain bread and other whole-grain cereal products, fruits and berries, and a decreased intake of total fat with more unsaturated fat. It was recommended that salt intake should be kept below 6 g per day. The information on the Palaeolithic diet stated that it should be based on lean meat, fish, fruit, leafy and cruciferous vegetables, root vegetables, eggs and nuts, while avoiding—as far as possible—dairy products, cereal grains, beans, refined fats, sugar, candy, soft drinks, beer and added salt. The following items were recommended in limited amounts for the Palaeolithic diet: eggs (≤2 per day), nuts (preferentially walnuts), dried fruit, potatoes (≤1 medium-sized per day), rapeseed or olive oil (≤1 tablespoon per day) and wine (≤1 glass per day). The recommended intake of the other foods was not restricted and no advice was given with regard to the proportions of food categories (e.g. animal vs. plant foods). The evolutionary rationale for a Palaeolithic diet and its potential benefits have been outlined previously [15] and detailed nutritional compositions of the diets can be found in our previous report [12].

Procedures

An oral glucose tolerance test (OGTT) was performed in the morning after obtaining venous blood samples and measurements of blood pressure, weight and waist circumference using standard methods [12] at the start of the study, after 3 months (when switching to a new diet) and at the end of the study (after 6 months). Samples were collected in EDTA-containing tubes and centrifuged for 10 min at 4 °C. Plasma was then aliquoted and

Table 1 Baseline characteristics

	All	Diabetes first (6/13)	Palaeolithic first (7/13)
Sex male/female (n)	10/3	4/2	6/1
Age (years)	64 (6)	63 (6)	66 (6)
Height (cm)	171 (5)	170 (6)	172 (4)
Weight (kg)	87 (17)	92 (20)	82 (13)
BMI (kg/m^2)	30 (7)	32 (8)	28 (4)
Waist circumference (cm)	103 (14)	109 (17)	97 (9)
Diabetes duration (years)	8 (5)	11 (6)	6 (4)
Diabetic values at OGTT yes/no (n)	12/1	6/0	6/1
Lipid lowering drug (=statin) yes/no (n)	8/5	4/2	4/3
Drugs per day	4.3 (2.3)	3.7 (1.8)	4.9 (2.7)
Antihypertensive drugs per day	1.5 (1.5)	1.2 (1.2)	1.9 (1.7)
Beta-blocker yes/no (n)	4/9	1/5	3/4
Thiazide yes/no (n)	4/9	1/5	3/4
ACE inhibitor yes/no (n)	5/8	2/4	3/4
Angiotensin-II receptor blocker yes/no (n)	4/9	2/4	2/5
Calcium channel blocker yes/no (n)	3/10	1/5	2/5
Anti-diabetic drugs per day	1.2 (0.9)	1.5 (0.8)	0.9 (0.9)
Metformin yes/no (n)	9/4	5/1	4/3
Dosage, mg/day	1031 (864)	1283 (950)	814 (790)
Sulphonylurea yes/no (n)	3/10	2/4	1/6
Thiazolidinedione yes/no (n)	3/10	2/4	1/6
Plasma adiponectin (µg/ml)	4.8 (4.16)	4.8 (2.5)	4.9 (5.4)
Plasma adipsin (ng/ml)	797 (157)	804 (218)	792 (178)
Plasma C-peptide (pg/ml)	487 (275)	437 (276)	531 (289)
Plasma ghrelin (pg/ml)	568 (129)	613 (165)	530 (82)
Plasma GIP (pg/ml)	232 (91.6)	226 (83)	237 (105)
Plasma GLP-1 (pg/ml)	26.8 (3.39)	26.4 (4.5)	27.1 (2.4)
Plasma glucagon (pg/ml)	425 (44.34)	435 (52.4)	417 (38.4)
Plasma leptin (ng/ml)	9.84 (12.18)	12.1 (17)	7.9 (6.8)
Plasma resistin (ng/ml)	2.21 (0.39)	2.3 (0.4)	2.1 (0.4)
Plasma visfatin (ng/ml)	2.52 (0.75)	2.7 (0.5)	2.4 (0.7)

Data is presented as mean values with SD in brackets, unless stated

ACE angiotensin converting enzyme, *GIP* glucose-dependent insulinotropic polypeptide, *GLP-1* glucagon-like peptide-1

stored at −80 °C until analysis. Outcome variables in the present study included fasting plasma concentrations of leptin, adiponectin, adipsin, visfatin, resistin, glucagon, insulin, C-peptide, GIP, GLP-1 and ghrelin.

Assessment of conditions of frozen blood samples

To assess the condition of the frozen blood samples we compared new analyses of insulin to older ones. The newly analyzed insulin values were on average 27 % lower than older analyses and the standard deviation had increased by 66 %. However, the Pearson correlation of 0.72 (adjusted $R^2 = 0.51$) between new and old insulin values for the same individual and time were highly correlated ($p < 0.0001$).

Analyses

The Bio-Plex pro™ human diabetes panel (Bio-Rad Inc., Hercules, CA, USA) a Luminex-based magnetic bead assay, was used to quantify insulin, C-peptide, ghrelin, GIP, GLP-1, glucagon, leptin, resistin and visfatin and a separate Bio-Plex assay was used to quantify adiponectin and adipsin (due to different dilution factor) in plasma according to the manufacturer's instructions. Each run included controls of known concentration for each cytokine and a blank.

Statistics

The statistical power calculations were based on initial primary outcomes of this intervention and previously published [12]. Data were analysed for normality (determined by Q–Q plots and the Shapiro–Wilk test in SPSS) and logarithmically transformed when necessary. If data did not show reasonable normal distribution after logarithmic transformation, the Wilcoxon matched pairs signed rank sum test was used, otherwise a paired t test was used. To analyse the difference between diets in their effects on outcomes we compared the absolute values at the end of each diet. In order to check for carry-over effects, t tests were used to compare mean values of outcome variables for the group starting with the Palaeolithic diet with those for the group starting with the diabetes diet. In order to check for period effects, t tests were used to compare the effects of the first and second diets. We performed post hoc analysis using bivariate correlations between the outcome variables presented in Table 2 and outcome variables related to glucose homeostasis and satiation. Bivariate correlations were also performed between the outcome variables presented in Table 2 and dietary variables. Outcome variables with significant correlations were entered in Simple Linear Regression. Significance was set at $p < 0.05$. All t tests were two-sided. Due to multiple outcome measures problem in this post hoc analysis a multiple outcome measures correction was made using QuickCalcs online provided by GraphPad Software (http://www.graphpad.com/quickcalcs/interpretPValue1/). Statistical analysis was performed with SPSS for Mac Version 20 (IBM SPSS Statistics for Mac, Version 20.0, IBM Corp., Armonk, NY, USA).

Results

Participant flow

All reported analyses are per protocol analyses on the 13 participants (3 women, 10 men) who completed the trial (Fig. 1). Four subjects were excluded for the following reasons: one starting with Paleolithic diet was wrongly included with ongoing warfarin treatment, one starting with Paleolithic diet was unwilling to continue due to abdominal pains and bloating, one starting with diabetes diet was excluded after developing leukemia, and one starting with diabetes diet was excluded after developing heart failure. Dates defining the periods of recruitment and follow-up, and side effects have been previously published [12].

Baseline data

Individual characteristics regarding anthropometric measurements, medication and outcome variables have been reported in detail previously [12] and are summarised in Table 1. The participants starting with the Palaeolithic diet compared to those starting with the diabetes diet did not differ at baseline for any of the outcome variables (Table 1). No carry-over or period effect was observed.

Table 2 Hormone levels, and weight, after the Palaeolithic diet and diabetes diet

Outcome	Palaeolithic diet	Diabetes diet	Delta diets	p^a
Adiponectin (µg/ml)	5.2 ± 4.4 (2.5 to 7.9)	5.7 ± 5.4 (2.5 to 9.1)	−0.5 ± 1.2 (−1.3 to 0.2)	0.153
Adipsin (ng/ml)	787 ± 182 (677 to 896)	776 ± 153 (684 to 869)	10 ± 79 (−37 to 58)	0.650
C-peptide (pg/ml)	455 ± 224 (319 to 590)	412 ± 204 (289 to 535)	43 ± 262 (−116 to 201)	0.644
Ghrelin (pg/ml)	540 ± 97 (481 to 598)	566 ± 145 (478 to 654)	−26 ± 74 (−70 to 18)	0.226
GIP (pg/ml)	254 ± 266 (93 to 415)	186 ± 75 (141 to 232)	68 ± 264 (−92 to 227)	0.600[b]
GLP-1 (pg/ml)	27 ± 9.3 (22 to 33)	27 ± 3.7 (25 to 29)	0.4 ± 7.7 (−4.3 to 5.12)	0.235[b]
Glucagon (pg/ml)	409 ± 40 (385 to 433)	431 ± 51 (400 to 463)	−22 ± 43 (−48 to 3.9)	0.089
Insulin (pg/ml)	248 ± 138 (165 to 332)	336 ± 327 (138 to 533)	−87 ± 240 (−232 to 58)	0.266
Insulin[c] (pg/ml)	401 ± 174 (296 to 506)	391 ± 115 (322 to 461)	9.8 ± 172 (−94 to 114)	0.840
Leptin (ng/ml)	5.1 ± 4.9 (2.1 to 8.0)	7.4 ± 8.3 (2.4 to 12)	−2.3 ± 4.6 (−5.1 to 0.4)	*0.023*[b]
Resistin (ng/ml)	2.5 ± 0.9 (1.9 to 3.0)	2.3 ± 0.6 (2.0 to 2.7)	0.2 ± 0.6 (−0.2 to 0.5)	0.356
Visfatin (ng/ml)	2.4 ± 0.7 (2.0 to 2.9)	2.5 ± 0.6 (2.1 to 2.8)	−0.1 ± 0.5 (−0.3 to 0.3)	0.906
Weight (kg)	81 ± 13 (74 to 88)	84 ± 15 (76 to 92)	−3.3 ± 3.8 (−5.7 to −1.0)	*0.008*

Data are mean ± standard deviation (95 % CI)

Significance tests are paired t test for normally distributed data and Wilcoxon matched pairs signed rank sum test for non-normally distributed data

Significant *p* values are indicated by italics font

GIP glucose-dependent insulinotropic polypeptide, *GLP-1* glucagon-like peptide-1

[a] *p* for difference between diets

[b] Data non-normally distributed (Wilcoxon matched pairs signed rank sum test)

[c] Old insulin values previously published

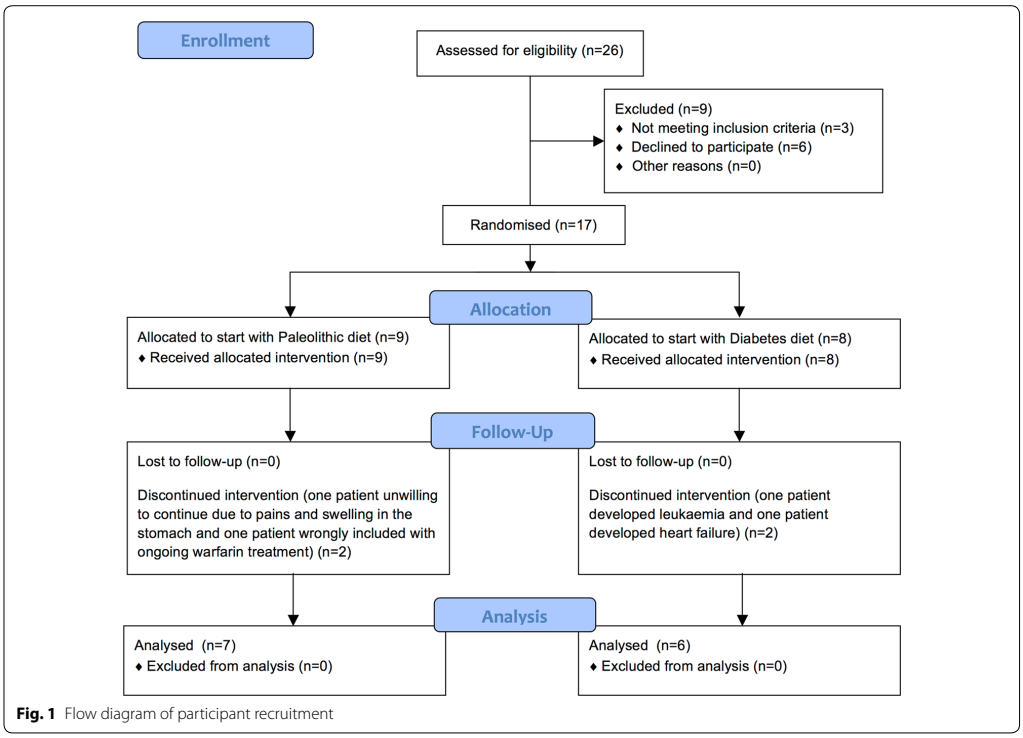

Fig. 1 Flow diagram of participant recruitment

Outcomes

The absolute level of plasma leptin after the Palaeolithic diet was lower than after the diabetes diet (large effect size, Cohen's $d = -1.26$; $p = 0.023$) (Table 2; Fig. 2) [16, 17]. When one outlier (more than 3 SDs) was excluded, the mean difference of leptin after the diets was normally distributed and the difference remained significant ($p = 0.031$). However, due to multiple outcome measures problem the probabilities of having a p value less than 0.023 just by chance in our dataset is 20.8 %.

The absolute level of glucagon at the end of the Palaeolithic diet was lower than at the end of the diabetes diet (moderate effect size, Cohen's $d = -0.51$), but this difference did not reach statistical significance ($p = 0.089$) (Table 2; Fig. 2).

As previously reported, weight loss was significantly greater (−3.3 kg) after the Palaeolithic diet than the diabetes diet ($p = 0.008$).

No statistically significant differences between the diets for the other variables were observed (Table 2).

Correlations and linear regression

In post hoc analysis of within-subject differences (value after the Palaeolithic diet minus value after the diabetes diet) we found that leptin correlated with fasting plasma insulin (Spearman's correlation 0.55, $p = 0.049$), grams of dietary fat (Spearman's correlation −0.66, $p = 0.013$), percentage of dietary fat (Spearman's correlation −0.55, $p = 0.049$), grams of dietary saturated fat (Spearman's correlation −0.59, $p = 0.033$), grams of dietary fatty acid C16:0 (Spearman's correlation −0.57, $p = 0.041$), and grams of dietary fatty acid C18:0 (Spearman's correlation −0.55, $p = 0.049$); glucagon correlated with area under the curve (AUC) for $\text{insulin}_{0-120\ \text{min}}$ (Pearson's correlation 0.94, $p = 0.015$), stimulated AUC $\text{insulin}_{0-120\ \text{min}}$ (Pearson's correlation 0.55, $p = 0.047$), fasting plasma insulin (Pearson's correlation 0.63, $p = 0.019$), satiety quotient for dietary glycaemic index per meal (Pearson's correlation −0.56, $p = 0.045$), dietary glycaemic load (Pearson's correlation 0.63, $p = 0.021$), dietary glycaemic index (Pearson's correlation 0.73, $p = 0.005$), dietary fatty acid C20:5 (EPA) (Pearson's correlation 0.58, $p = 0.037$),

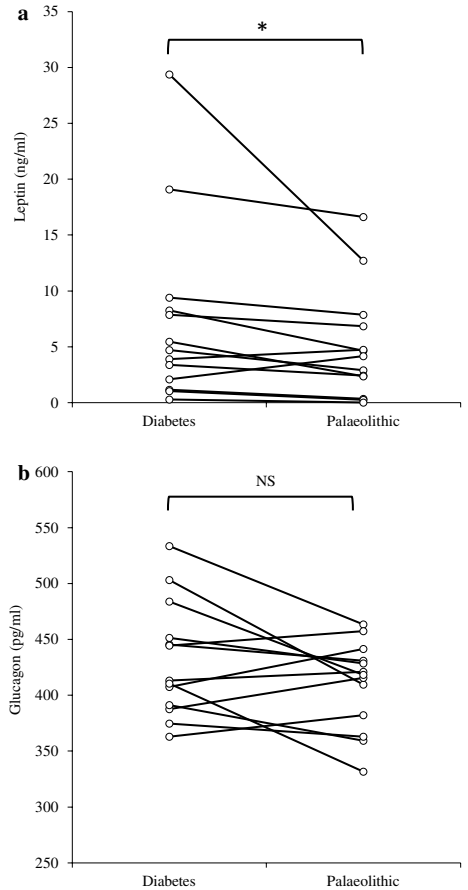

Fig. 2 Fasting hormone levels after the Palaeolithic diet and diabetes diet for leptin and glucagon. Data show individual differences in **a** leptin and **b** glucagon after 3 months in response to the Palaeolithic and diabetes diets. Significance of the difference is indicated by *asterisks* ($p < 0.05$). *NS* non-significant

dietary fatty acid C22:6 (DHA) (Pearson's correlation 0.57, $p = 0.04$) and dietary vitamin B12 (Pearson's correlation 0.57, $p = 0.041$) (Table 3).

Discussion

This small trial showed that a Palaeolithic diet decreased fasting plasma leptin, but did not affect fasting levels of insulin, C-peptide, glucagon, incretins, ghrelin and adipokines significantly compared to the currently recommended diabetes diet.

Weight loss interventions have been shown to decrease leptin concentrations [18], and in our trial leptin decreased only with the intervention that induced weight loss, i.e. the Palaeolithic diet. However, post hoc analysis revealed no correlation between difference in weight loss and leptin after the diets (Spearman's correlation 0.11, $p = 0.721$).

Interestingly, genetic and in vitro studies indicate insufficient adaptation of the human leptin system to a diet based on cereal grains [19, 20]. Therefore cereal grains could hypothetically lead to leptin resistance and higher leptin values. Our finding of lower leptin following a Palaeolithic diet virtually devoid of cereal grains compared to a diabetes diet with cereal grains supports this notion, and could represent the mechanism behind our previous findings of improved glucose control and blood lipids [12] and greater satiety per calorie from the Palaeolithic diet [13].

In our study there was a non-significant lower fasting glucagon levels after the Palaeolithic diet compared to the diabetes diet, which could be a result of the amelioration of leptin sensitivity in the pancreatic islets. However, this hypothesis should be tested in trials with adequate statistical power.

Due to the small sample size, we were not able to conduct a multivariate analysis adjusting for weight loss to explore the independent effect of the Palaeolithic diet on leptin and glucagon. Therefore, our results might be explained by the weight loss produced only during the Palaeolithic diet, as already mentioned.

Insulin plays a central role in type 2 diabetes, but despite this we found no difference in fasting insulin between the diets. Compared to baseline, there was a significant decrease in insulin ($p = 0.004$ and 0.023, for old and new insulin analysis, respectively) after the Palaeolithic diet, which may be explained by weight loss.

Adiponectin appears to play an important role in type 2 diabetes due to its anti-inflammation, antiatherogenic, and insulin-sensitizing properties [21], yet we found no difference between the diets. However, there is some controversy regarding the beneficial effects of adiponectin in type 2 diabetes [22–25].

In the exploratory analysis there was a positive correlation between change in fasting leptin and insulin, which could be explained by the mechanisms discussed above and recently reviewed by Nolan et al. [8]. This finding is consistent with other studies where a positive correlation between fasting leptin and insulin has also been shown [26, 27]. It has been shown that treatment with recombinant human leptin does not improve insulin sensitivity in obese patients with type 2 diabetes [28], contrary to what happens in patients with severe leptin deficiency [29]. This might support the notion that patients with

Table 3 Exploratory analysis

	Pearson's correlation r[a]	Spearman's correlation r[b]	Adjusted R^2	p^c
Leptin (ng/ml) versus				
Fasting insulin (ng/ml)		0.555		0.049
Fat (g)		−0.665		0.013
Fat (%)		−0.555		0.049
SAF (g)		−0.593		0.033
Fatty acid C16:0 (g)		−0.571		0.041
Fatty acid C18:0 (g)		−0.555		0.049
Glucagon (pg/ml) versus				
AUC $insulin_{0\text{-}120}$ (nmol/l min)	0.946		0.383	0.015
Stimulated AUC $insulin_{0\text{-}120}$ (nmol/l min)	0.558		0.249	0.047
Fasting plasma insulin (pmol/l)	0.637		0.352	0.019
Satiety quotient for glycaemic index per meal (RS)	−0.562		0.254	0.045
Glycaemic load (g)	0.63		0.342	0.021
Glycaemic index	0.731		0.491	0.005
Fatty acid C20:5, n-3, EPA (g)	0.581		0.277	0.037
Fatty acid C22:6, n-3, DHA (g)	0.575		0.27	0.04
Vitamin B-12 (μg)	0.571		0.265	0.041
Adipsin (ng/ml) versus				
Satiety quotient for GL per meal (RS/kg)	0.581		0.277	0.037
GIP (pg/ml) versus				
Fasting plasma insulin (pmol/l)		0.555		0.049
GLP-1 (pg/ml) versus				
Stimulated AUC insulin (nmol/l min)		0.654		0.015
AUC insulin (nmol/l min)		0.67		0.012
Resistin (ng/ml) versus				
Fasting plasma insulin (pmol/l)	−0.728		0.451	0.041
Satiety quotient for GL per meal (RS/kg)	0.810		0.598	0.015
Visfatin (ng/ml) versus				
Fasting plasma glucose (mmol/l)	−0.557		0.248	0.048

Exploratory analysis was conducted to check for significant correlations between the outcome variables presented in Table 2 and outcome variables related to glucose homeostasis and satiation. Bivariate correlations were also performed between the outcome variables presented in Table 2 and dietary variables. This analysis consisted in bivariate Pearson or Spearman's (for non normally distributed variables) correlation between within-subject differences in outcome and dietary variables. Normally distributed outcomes that were significant in Pearson's correlation were entered into simple linear regression

[a] Pearson's correlation for normally distributed variables

[b] Spearman's correlation for non normally distributed variables

[c] *p* value for bivariate correlation and simple linear regression

type 2 diabetes suffer from leptin resistance. Interestingly, within-subject differences in fasting leptin correlated negatively with the intake of total fat (in grams and percent) and C16:0 and C18:0 fatty acids. These results are consistent with a randomised controlled trial where a low-fat diet lowered leptin levels more than a high-fat diet [30]. On the other hand, in a well controlled study leptin levels were higher with a low-fat diet than a low-glycaemic index or very low–carbohydrate diet [31]. Other trials found no effect of fat restriction on leptin levels [32, 33]. This inconsistency in results may be due to differences between individuals in gene variants related to leptin physiology [34].

Comparison with findings from other studies

In a previous trial from our group, leptin decreased significantly during Palaeolithic and Mediterranean diets, respectively, with no differences between diets, but after exclusion of one outlier with a high grain intake in the Palaeolithic diet group there was a significantly greater decrease in leptin in this group [35]. Interestingly, in the same study there was a strong correlation (Pearson's correlation 0.50, $p = 0.008$) between change in leptin and intake of cereals [35]. Nevertheless, contrary to our previous finding there was no correlation in the data (Spearman's correlation 0.22, $p = 0471$). Additionally, data from the present trial indicated that the Palaeolithic diet is

more satiating than the diabetes diet [13], consistent with another trial from our group [35]. Other randomised clinical trials have shown beneficial effects of a Palaeolithic diet compared with other healthy diets on cardiovascular risk factors [36] and body fat [37]. A recent systematic review and meta-analysis, where these studies were included [12, 36–38], showed that a Palaeolithic diet improves some components of the metabolic syndrome more than the healthy control diets [39].

The Mediterranean diet has been the focus of several publications regarding its role in the metabolic syndrome and type 2 diabetes [40–43]. A systematic review and meta-analysis showed that the Mediterranean diet was superior to control diets for all components of the metabolic syndrome [44]. Another systematic review and meta-analysis concluded that the Mediterranean diet decreased HbA1c, but not fasting glucose, more than control diets but not more than the Palaeolithic diet [42]. An important consideration with respect to the characteristics of Mediterranean and Palaeolithic diets concerns their resemblance. Both emphasize a high intake of whole unprocessed foods, specifically: fruits, vegetables, fish, nuts, and olive oil, while the limitation in the intake of wholegrain cereals and legumes in the Paleolithic diet is the main difference. In light of the role that inflammation and oxidative stress might play in glycaemic control in type 2 diabetes [45], potential mechanisms behind the beneficial effects of the Mediterranean and Palaeolithic diets could be attributed to their antioxidative and anti-inflammatory capacity [41, 46]. Thus, both the Mediterranean diet and the Palaeolithic diet share common features that render them as healthy options in patients with type 2 diabetes, and represent a step forward for an optimal human diet.

Vegetarian diets are regarded as a healthy option for western diseases as well. A recent systematic review and meta-analysis investigated the effects of a vegetarian diet on glycemic control in type 2 diabetes [47], resulting in better HbA1c, but not fasting glucose, than control diets. None of the the included trials tested a vegetarian diet against a Palaeolithic or Mediterranean diet.

Importantly, a systematic review and meta-analysis assessed the effect of various diets on glycemic control in type 2 diabetes [48]. The results indicate that all the diets, namely low-carbohydrate, low-glycaemic index, Mediterranean and high-protein diets, improved HbA1c compared with their respective control diets. Consequently, the best dietary approach for the management of type 2 diabetes continues to be a matter of debate.

Limitations of the present study

A limitation of this study, as with most other dietary trials, is the lack of blinding after randomisation. To minimise this problem, all study participants were informed of the intention to compare the effect of two healthy diets for the treatment of type 2 diabetes and that it was not known which one would be superior. Also, written information with dietary advice, food recipes and behavioural support were similarly formulated for both diets. The difference in weight loss, macronutrient composition and glycaemic load between the diets precludes a definite conclusion about the specific role of different food choices on the endocrine system.

The results of this study should be interpreted with caution for other reasons as well. First, we have the limitation of multiple outcome measures problem and the probability of type I error for leptin in our study is 20.8 %. Secondly, the outcomes generated from this post hoc analysis represent exploratory investigations; the primary outcomes have been previously published. Lastly, this study has a small sample size which precludes us from performing adjusted multivariate analysis. This is specially relevant for weight loss because it decreased only during the diabetes diet and the difference after the diets was 3.3 kg ($p = 0.008$). As a result, since weight loss is a principal driver of improved leptin sensitivity we are not certain about the independent effect of the diets on the results.

Conclusion

We show that a Palaeolithic diet results in significantly lower fasting plasma leptin, non-significantly lower fasting plasma glucagon concentrations as well as weight loss, compared to a standard diabetes diet. Human beings are well adapted to food groups similar to those found in the Palaeolithic era during our evolution, and, hypothetically, the lower leptin and glucagon levels could indicate that deviations from this template is not optimal and could explain our previously reported findings on glucose control, blood lipids, blood pressure and satiety. But the small sample size of the present study makes it impossible to perform adjusted multivariate analysis and the observed weight loss after the Palaeolithic diet may also contribute to explain our results. Long-term and adequately powered trials investigating the effects of Palaeolithic diet are warranted.

Additional file

Additional file 1. Supporting data set. Data set supporting our results on hormones (adipokines, glucagon and incretins), post hoc analysis and previously published outcomes (worksheet 'Data set'). Each row numbered 1-13 in first column 'Person-ID' corresponds to data from one participant. Each column heading contains a variable name stating what individual mean has been measured, in what unit and whether on the Paleolithic diet, diabetes diet or if it is the difference between the two diets (DeltaPd, value during Paleolithic diet minus value during diabetes diet). The variable names and their description are listed below.

Abbreviations
AUC: area under the curve; GIP: glucose-dependent insulinotropic polypeptide; GLP-1: glucagon-like peptide-1; HbA1c: glycated haemoglobin; OGTT: oral glucose tolerance test.

Authors' contributions
All the authors fulfil the following conditions: (a) substantial contributions to conception and design, acquisition of data or analysis and interpretation of data; (b) drafting the article or revising it critically for important intellectual content; and (c) final approval of the version to be published. All authors read and approved the final manuscript.

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Acknowledgements
The authors are grateful to Professor Birgitta Hovelius and Dr. Kristina Haara for their participation in designing the study, to Lilian Bengtsson and Lena Kvist for technical assistance, and Anna Hedelius for excellent technical support. We are also very grateful to Amreeta Buxani for helping in the review of the manuscript.

Availability of data and materials
The dataset supporting the conclusions of this article is included within the article (and its additional file). Additional file 1.

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

Ethics approval and consent to participate
Approval of the study was obtained from the regional Medical Ethics Committee and the trial was registered at ClinicalTrials.gov (Identifier: NCT00435240). All recruited subjects were given oral and written study information prior to signing a consent form to participate in the study and were then further assessed for eligibility.

Funding
The study was funded by Crafoordska stiftelsen, Region Skåne and Lund University.

Received: 15 March 2016 Accepted: 13 May 2016
Published online: 23 May 2016

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Paper IV

BMC Endocrine Disorders

RESEARCH

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Effects of a Paleolithic diet compared to a diabetes diet on leptin binding inhibition in secondary analysis of a randomised cross-over study

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Abstract

Background Beneficial effects from practising a Paleolithic diet as compared to a diabetes diet on weight, waist circumference, satiety, leptin, HbA1c and glucose control in randomised controlled trial participants with type 2 diabetes could be due to lower leptin resistance. Support for this hypothesis comes from an in vitro experiment that showed that digested wheat gluten, which is excluded from a Paleolithic diet, inhibits leptin from binding to its receptor, thus indicating a possible dietary cause of leptin resistance. However, the clinical relevance of the latter finding is unclear since removal of enzyme activity from the gluten digest by heat treatment also abolished leptin binding inhibition. Assessment of leptin binding inhibition in vivo is possible by comparison of total leptin levels with those of 'biologically active' leptin bound to its receptor (bioLep).

Objectives To assess the effects of a Paleolithic diet compared to a diabetes diet on leptin binding inhibition and to replicate our in vitro study.

Methods BioLep and total leptin levels were measured in secondary analysis of fasting plasma samples from our open label random order three plus three-month long cross-over trial performed in 2005–2007, that compared a Paleolithic diet with a diabetes diet in participants with type 2 diabetes without insulin treatment (per protocol). BioLep was also measured in vitro for known recombinant leptin concentrations incubated with a series of concentrations of 10 kDa spin-filtered digested wheat gluten, with or without prior heat treatment, at 100ºC for 30 min and centrifugation.

Results There was no difference between diets when comparing differences between bioLep and total leptin levels and their ratio in the 13 participants, three women and 10 men, aged 52–74 years with a mean BMI of 30 kg/m^2 and a mean diabetes duration of eight years. We found no carry-over or period effect for bioLep and total leptin. In vitro, wheat gluten digest inhibited leptin binding in a dose-dependent manner but not after heat treatment.

Conclusions We found no leptin binding inhibition after the Paleolithic or diabetes diet, possibly due to its abolishment from cooking-related heat treatment of wheat gluten.

Trial registration Registered on 14/02/2007 at ClinicalTrials.gov Identifier: NCT00435240.

Keywords Paleolithic diet, Type 2 diabetes, Leptin, Leptin resistance, Wheat gluten, BioLep

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Background

Modern chronic diseases such as coronary heart disease, hypertension and diabetes appear to be absent among recent hunter-gatherer populations [1]. Based on this epidemiological observation, it has been proposed that the diet of recent hunter-gatherer populations may be ideal for helping prevent and treat modern chronic diseases such as coronary heart disease, hypertension, and diabetes [1, 2]. The suggested theoretical underpinning of the proposition is that modern chronic diseases arise due to insufficient genetic adaptation to a recently (in evolutionary terms) introduced agricultural diet [1, 3]. The diets of recent hunter-gatherer populations are thought to resemble most closely those of preagricultural human populations during the late Paleolithic period [1]. Therefore, the general dietary pattern of recent hunter-gatherer populations is now usually referred to as a "Paleolithic diet" and includes fruits and vegetables, roots and tubers, lean meats, fish, seafood, eggs and nuts, and excludes cereal grains, dairy products, legumes, refined fats and sugar [1–3]. The Paleolithic diet has been tested in randomised controlled intervention studies with encouraging results on risk factors for modern chronic diseases [4–7]. Our random order three plus three-month long cross-over diet intervention study in people affected by type 2 diabetes resulted in lower body weight, leptin and HbA1c, and higher satiety per calorie from practising the Paleolithic diet as compared to the diabetes diet [8–10]. A potential explanation for the observed beneficial effects could be a greater decrease in leptin resistance due to practising the Paleolithic diet [10].

Leptin is a hormone secreted by adipose tissue that promotes satiety and has a central role in energy balance and weight management. Leptin resistance is an unclearly defined and not fully understood state of impaired leptin function linked to food intake and is commonly seen in cases of obesity [11]. In light of the above, it is interesting that we in vitro have shown, using surface plasmon resonance technology, that wheat gluten (which is absent in the Paleolithic diet) digested with gut enzymes to mimic physiological conditions in the human intestine inhibits leptin from binding to the leptin receptor at clinically relevant concentrations, thus indicating a possible dietary cause of leptin resistance [12]. This finding, translated to the clinical setting of our cross-over study, should result in a difference in leptin binding inhibition between the wheat gluten-free Paleolithic diet and the wheat gluten-containing diabetes diet. However, the clinical relevance is unclear since the removal of enzyme activity from the gluten digest by heat treatment also abolished leptin binding inhibition in vitro [12]. Since wheat proteins are almost exclusively eaten after cooking, dietary leptin binding inhibition could in vivo be abolished or reduced. Thus, it is unclear if the in vitro finding translates to a dietary leptin binding inhibition in vivo. This uncertainty would be addressed by measurements of leptin binding inhibition in stored plasma samples from our cross-over study.

After the measurement of leptin in our clinical intervention study and our in vitro study on gluten digest and leptin binding inhibition, commercially available laboratory kits with the ability to measure biologically active leptin (bioLep) have become available [13]. The 'biologically active' aspect refers to the ability of the new kits to measure how much leptin binds to the leptin binding site of the leptin receptor [13]. Such measurements in combination with traditional measures of total leptin enable us to detect the relative amount of receptor-binding leptin and thereby also assess leptin binding inhibition. Our aim in this study was to assess the effect of a Paleolithic diet as compared to a diabetes diet on leptin binding inhibition by measurements of bioLep and total leptin in secondary analysis of stored plasma samples from our randomised cross-over trial. Another aim of this study was to replicate our in vitro surface plasmon resonance technology study using the bioLep laboratory kit.

Methods

BioLep and total leptin were measured at the Center for Primary Health Care Research lab in Malmö using bioLEP ELISA L07 and LEP ELISA E07, respectively, from Mediagnost according to the manufacturer's instructions. Each run included two controls of known concentration and a blank. The bioLEP ELISA L07 is a ligand-binding immunoassay for quantitative determination of bioLep. Briefly, recombinant extracellular domain of the human leptin receptor is immobilized on a microtiter plate. Added bioLep in gluten digest or plasma bioLep is bound to the immobilized receptor and subsequently detected by a highly specific polyclonal, biotin-conjugated anti-leptin antibody and a streptavidin-peroxidase conjugate. Non-receptor-binding leptin does not give any signal. The assay is performed according to the WHO International Standard for human leptin, NIBSC 97/594.

In vivo study design

For this part of the study, we measured bioLep and total leptin per protocol after each diet in a secondary analysis of -80 °C stored fasting plasma samples from our random order three plus three month long cross-over diet intervention study, which was performed in 2005–2007 [8]. This study compared a Paleolithic diet, based on the general dietary pattern of recent hunter-gatherer populations [14] with a diabetes diet, based on the evidence-based nutritional approaches to the treatment and prevention of diabetes mellitus by the Diabetes and Nutrition Study

Group (DNSG) of the European Association for the Study of Diabetes (EASD) [15], in individuals affected by type 2 diabetes [8]. For full details about the methods and previously reported results, please see [8–10, 16]. Approval of the study was obtained from the Regional Ethical Board in Lund (LU 726/2004 and 711/2013). All participants gave written informed consent.

Trial registered on 14/02/2007 at Clinicaltrials.gov (Identifier: NCT00435240).

Participants

Individuals affected by type 2 diabetes without insulin treatment were recruited from primary healthcare units in the Lund area of southern Sweden. Inclusion criteria were adult individuals affected by type 2 diabetes without insulin treatment and a C-peptide value above zero, unaltered medical diabetes treatment and stable weight since three months before the start of the study, HbA1c above 5.5% by Mono-S standard, creatinine below 130 μmol/L, liver enzymes below four times their respective upper reference value, no chronic oral or injection steroid treatment and no acute coronary event or change in medication of beta blockers or thyroxin since six months before the start of the study. Exclusion criteria were insulin treatment or chronic steroid treatment (not inhalation), warfarin treatment, creatinine above 130 μmol/L or liver enzymes above four times their respective upper reference value, acute coronary event, change in beta blocker or thyroxin medication, and physical or psychological illness or changes in personal circumstances that would make further study participation impossible.

Diets

The Paleolithic diet was based on lean meat, fish, fruit, vegetables, root vegetables, eggs and nuts, while avoiding dairy products, cereal grains, legumes, refined fats, sugar, candy, soft drinks, beer and added salt [14]. The diabetes diet was based on an increased intake of vegetables, root vegetables, dietary fibre, wholegrain bread and other wholegrain cereal products, fruits and berries, and decreased intake of total fat with more unsaturated fat [15]. The difference in cereal grain intake between the diets should lead to a corresponding difference in wheat gluten content.

Procedures

All eligible individuals were informed of the intention to compare two healthy diets and that it was not known whether one diet might be superior to the other. After randomisation was complete, there was no blinding of dietary assignment to study participants, nor to those administering the interventions or assessing the outcomes. Upon commencement of the study, all participants were randomised, by use of opaque envelopes, to start with either a diabetes diet designed in accordance with official recommendations or a Palaeolithic diet. Immediately after randomisation, all participants received individual oral and written information about their respective initial diet from diabetes nurses. After three months, all participants switched diets and received new oral and written information about the new diet. Advice about regular physical activity was given equally to all participants. Fasting venous blood samples were obtained in the morning, followed by an oral glucose tolerance test and measurements of blood pressure, weight and waist circumference using standard methods at the start of the study, after three months (when switching to a new diet) and at the end of the study (after six months). Samples were collected in EDTA-containing tubes and centrifuged at 1,700 g for 10 min at 4 °C. Plasma was then aliquoted and stored at − 80 °C until analysis. Food intake during each diet study was assessed from four-day weighed food records that started approximately six weeks after initiating each diet.

In vitro study design

For this part of the study, we used -80° C stored 10 kDa spin-filtered gluten digest from our surface plasmon resonance technology study [12]. For details on gluten digest manufacture, please see [12]. Briefly, to mimic physiological conditions in the human intestine, gluten from wheat was digested with the gut enzymes pepsin and trypsin. Enzyme activity from pepsin and trypsin (molecular weight ~ 40 and 25 kDa, respectively) was subsequently removed from the gluten digest by either spin-filtering through a 10 kDa filter or heat treatment at 100°C for 30 min followed by centrifugation at 13,000 g for 10 min.

Procedures

The gluten digest protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). The effects of heat treatment and centrifugation on gluten digest concentration were assessed by measuring the concentration of gluten digest before and after heat treatment at 100 °C for 30 min, heat treatment at 100 °C for 30 min followed by centrifugation at 13,000 g for 10 min, and only centrifugation at 13,000 g for 10 min without heat treatment.

BioLep was measured in triplets for recombinant leptin at 10 and 50 ng/mL concentrations (recombinant human leptin, R&D Systems 398-LP, the same as used in our in vitro study using surface plasmon resonance technology) [12], incubated with a series of increasing gluten digest concentrations ranging from 0 to 320 μg/mL. The recombinant leptin concentration of 10 ng/mL was chosen as a close representation of mean in vivo leptin

concentrations in our randomised cross-over trial and the recombinant leptin concentration of 50 ng/mL was chosen as a representation of a much higher but still clinically plausible concentration, both of which are well within the detection limits of the laboratory kit. BioLep was also measured for recombinant leptin at 10 ng/mL concentration incubated with gluten digest concentrations ranging from 5 to 320 µg/mL after the gluten digest had been subjected to heat treatment at 100ºC for 30 min, heat treatment at 100ºC for 30 min followed by centrifugation at 13,000 g for 10 min or only centrifugation at 13,000 g for 10 min.

Statistics

This is a secondary analysis of a study in which the small sample size reflects power calculations for previously reported cardiometabolic outcomes. The sample size was deemed sufficient for this study on leptin binding inhibition based on our previous finding of a half-maximal inhibition of leptin binding at a gluten digest concentration of 10 ng/mL, which is well below the 41 ng/mL mean concentration reported for undegraded gliadin in about half (14 of 31) of healthy adult human sera [17]. Gliadin is the prolamin protein of wheat and makes up about half of all wheat gluten. Assuming that the leptin binding inhibitory effect of gluten digest concentrations is at least roughly translatable to similar gliadin concentrations, a greater than half-maximal inhibition of leptin binding could be expected in about half of the samples. The study would then require a sample size of at least seven participants to achieve a power of 80% and a level of significance of 5% (two-sided), for detecting a mean of the differences of 0.25 between pairs, assuming a pooled standard deviation of 0.18 for the differences.

A two-sided paired-samples t-test or Wilcoxon matched-pairs signed rank sum test was used for group mean comparisons, as appropriate, and Spearman's rank test was used for correlation. Statistical significance was set at $p < 0.05$. Carry-over effect was tested by comparing variable means of first and second diet for the group starting with the Palaeolithic diet with those for the group starting with the diabetes diet. Period effect was tested by comparing variable means between the first and second diets. Analysis was conducted by use of IBM SPSS Statistics for Macintosh, Version 28.

Results

Previously reported

A total of 17 individuals out of 26 assessed for eligibility were randomised and started the study [8]. Four participants were excluded for the following reasons: one starting with the Paleolithic diet was wrongly included due to ongoing warfarin treatment; one starting with the Paleolithic diet was unwilling to continue due to abdominal pains and bloating; one starting with the diabetes diet was excluded after developing leukaemia; and one starting with the diabetes diet was excluded after developing heart failure [8]. The 13 participants who completed the study and were analysed consisted of three women and 10 men aged 52–74 years with a mean BMI of 30 kg/m^2 and a mean diabetes duration of eight years (baseline characteristics summarised in Table 1) [8]. Mean (SD) daily intake in grams of rice and other cereal grains were 7 (17) and 11 (24) grams for the Paleolithic diet and 6 (10) and 172 (96) grams for the diabetes diet, respectively [8]. The Paleolithic diet resulted in higher satiety, a more beneficial lipid profile, lower body weight, waist circumference, HbA1c and total leptin levels compared to the diabetes diet [8–10].

New measurements

Intra assay variance (coefficient of variability) of the bioLEP ELISA L07 and LEP ELISA E07 measurements were just below 5% with no inter assay variance since each run contained all measurements in one plate.

In vivo

There was no difference between bioLep and total leptin after the Paleolithic or diabetes diet and consequently also no difference between diets when comparing differences between bioLep and total leptin or their ratio (Table 2). Both bioLep and total leptin were lower after the Paleolithic diet compared to after the diabetes diet (Table 2). As stated in the background section, total leptin has been measured previously in these samples

Table 1 Baseline Characteristics

Variable	Mean (SD)	
Sex male/female, n	10/3	
Age, year	64	(6)
Diabetes duration, year	8	(5)
HbA1C, %, Mono-S	6.6	(0.6)
fP-glucose, mmol/L	7.8	(1.2)
Cholesterol, mmol/L	4.4	(1.1)
Low-density lipoprotein cholesterol, mmol/L	2.9	(0.9)
High-density lipoprotein cholesterol, mmol/L	1.3	(0.2)
C-reactive protein, mg/L	2.4	(1.8)
Systolic blood pressure, mmHg	150	(21)
Diastolic blood pressure, mmHg	83	(6)
Height, cm	171	(5)
Weight, kg	87	(17)
Body mass index, kg/m^2	30	(7)
Waist circumference, cm	103	(14)

Table 2 BioLep and Total Leptin After the Paleolithic and Diabetes Diet

Variable	Paleolithic diet	Diabetes diet	p^a
BioLep, ng/mL *M (SD)*	8.5 (6.0)	11.8 (9.3)	.02
Total leptin, ng/mL *M (SD)*	8.8 (6.7)	12.1 (9.9)	.02
p^b	.2	.3	
BioLep minus total leptin, ng/mL *M (SD)*	-0.3 (0.8)	-0.3 (0.9)	.7
BioLep to total leptin ratio, *M (SD)*	0.96 (0.18)	1.02 (0.15)	.4

[a] *p* for mean comparison between diets. [b] *p* for mean comparison between bioLep and total leptin for each diet

[10]. New and old measurements were highly correlated (rs = 0.97, $p < 0.001$) with new measurements being on average 67% higher compared to old measurements, most likely due to liquid evaporation during storage. We found no carry-over or period effect for bioLep and total leptin.

In vitro

There was no apparent effect on gluten digest concentration (190 µg/mL) from heat treatment (192 µg/mL) and/or centrifugation (194 µg/mL and 196 µg/mL, respectively). Heat-treated gluten digest with or without centrifugation did not reduce bioLep (Fig. 1). Gluten digest with or without centrifugation reduced bioLep in a dose-dependent manner and at similar concentrations for both 10 and 50 ng/mL recombinant leptin (Fig. 1).

Discussion

We found no difference between bioLep and total leptin after the Paleolithic or diabetes diet in a secondary analysis of stored plasma samples from our randomised cross-over trial, and consequently also no difference between the diets when comparing differences between bioLep and total leptin or their ratio. We thus found no leptin binding inhibition in vivo. Our results concur with the possibility that cooking-related heat treatment abolishes leptin binding inhibition caused by gluten digest. Other possibilities could be that gluten digest does not reach sufficient systemic concentration [18] or that leptin in vivo differs from its recombinant counterpart [19]. BioLep levels and bioLep to total leptin ratios in our study are in-line with previous results found in a clinical cohort of 409 lean and obese children and adults [13]. There were no dietary data for this cohort, but daily wheat

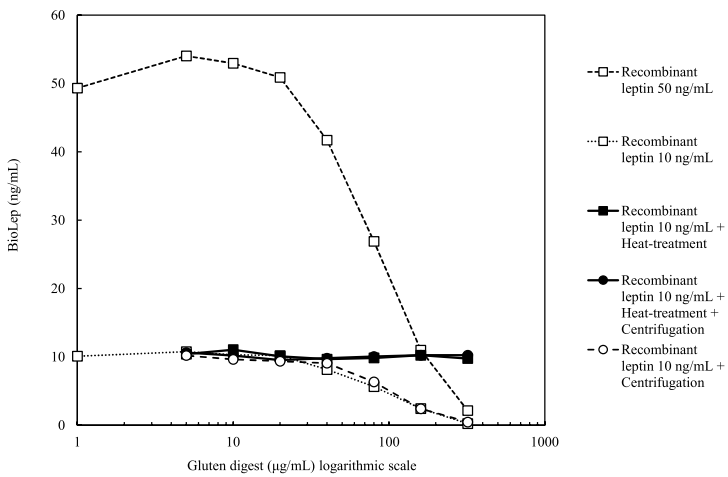

Fig. 1 BioLep for Recombinant Leptin Incubated with a Series of Increasing Gluten Digest Concentrations. *Note.* * BioLep concentrations at 0 µg/mL gluten digest concentration is here presented at 1 µg/mL gluten digest concentration since the logarithm of 0 is undefined and cannot be presented on the x-axis

gluten consumption was estimated in the Western diet thought to range from 5 to 20 g [20]. Based on estimated relative food supply quantities in Sweden in 2020 and assuming an average gluten content of wheat of around 8%, the cereal grain intake in this study translates into a mean daily wheat gluten intake of 1 g for the Paleolithic diet and 12 g for the diabetes diet [20, 21]. The daily wheat gluten intake of 12 g for the diabetes diet is well within the Western diet range but more than ten-fold higher compared to the wheat gluten intake of 1 g for the Paleolithic diet.

Furthermore, we found that gluten digest, with or without centrifugation, reduced bioLep in a dose-dependent manner similarly for both 10 and 50 ng/mL recombinant leptin. This indicates a dose-dependent leptin binding inhibition by gluten digest, which replicates our previous in vitro study findings [12]. The similar leptin binding inhibition at similar concentrations of gluten digest for both 10 ng/mL and 50 ng/mL recombinant leptin suggests that gluten digest inhibits the leptin receptor in a non-competitive way from binding to leptin. We also found that heat treatment of gluten digest abolished the leptin binding inhibition, which is in line with both our in vivo findings from this study and our previous in vitro study findings [12].

Limitations of this study

Firstly, our results do not allow us to preclude the possibility that heat-treated digests of gluten, or other cereal grain proteins, can disturb leptin function other than leptin binding. Such a disturbance may affect intracellular signalling of the leptin receptor and that would still constitute a dietary cause of leptin resistance [22].

Secondly, since our in vivo results are from blood samples taken from participants in a fasting state, they do not allow us to preclude the possibility of transient meal effects on leptin binding. Kinetics for dietary antigens in healthy adults have been assessed for ovalbumin with levels rising from undetectable to peak levels of 1.7 to 10.5 ng/ml 2–3 h after a milk and egg test meal, and the maximal total amount of dietary antigen found in the circulation corresponded to 10^{-5} fraction of the amount consumed [23]. The 41 ng/mL mean concentration reported for undegraded gliadin in only about half (14 of 31) of healthy adult human sera indicates a possibly similar meal variability [17].

Thirdly, our in vitro studies with heat treatment after enzyme digestion of gluten is an order reversal of in vivo conditions. It is possible, albeit unlikely, that heat treatment before enzyme digestion of gluten would have produced different in vitro results.

Conclusions

We found no leptin binding inhibition in vivo after the Paleolithic and the diabetes diet, which concurs with the possibility that cooking-related heat treatment abolishes leptin binding inhibition from gluten digest. Using another laboratory method, we also replicated our previous in vitro finding of a dose-dependent leptin binding inhibition from gluten digest that was abolished by heat treatment of the gluten digest.

Abbreviations

BMI	Body mass index
HbA1c	Glycated haemoglobin
kDA	Kilodaltons

Acknowledgements

The authors wish to thank the County Council in Region Skåne for providing financial and administrative support for this study. Also, we gratefully acknowledge the financial support provided by The Swedish Heart Lung Foundation and The Swedish Research Council.

Authors' contributions

TJ conceived of and participated in the design and execution of the study and wrote the article. MFV participated in the design of the study and wrote the article. KS, YG, AM and AH participated in the design and execution of the study and the drafting of the article, as well as revising it for important intellectual content. AH and AM performed the laboratory methods. PCB participated in the design of the study and the drafting of the article, as well as revising it for important intellectual content. All authors read and approved the final manuscript.

Funding

Open access funding provided by Lund University. No funding to declare.

Availability of data and materials

The dataset analysed during the current study is available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Approval of the study was obtained from the Regional Ethical Board in Lund (LU 726/2004 and 711/2013). All participants gave written informed consent. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 5 April 2024 Accepted: 30 August 2024
Published online: 04 September 2024

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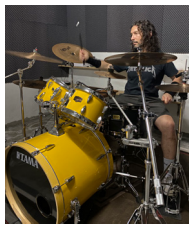

Maelán Fontes Villalba was born in Lanzarote, the Canary Islands, in 1977. His passion for healthcare, biology and sports led him to pursue a degree in physiotherapy and afterwards a master's degree in human nutrition. Meeting Staffan Lindeberg opened the door for him to enrol in a PhD program in human nutrition and type 2 diabetes. Staffan Lindeberg's research on food and modern chronic diseases was the key inspiration behind his decision to investigate the causal effects of Paleolithic diets in type 2 diabetes.

The general aim of his thesis was to assess in type 2 diabetes the effects of a Paleolithic diet compared with a diabetes diet on glycaemic control when body weight is kept stable, and on the satiety hormone leptin. The results indicate that the comparably greater improvement in glycaemic control in type 2 diabetes from a Paleolithic diet is due to an accompanying greater reduction in body weight, and the diet did not influence the satiety hormone leptin. The findings of this thesis could lay the foundation for new studies exploring which factors of a Paleolithic diet facilitate body weight loss, as a decrease in body fat appears to be the main driver for improved glycaemic control.

About the front cover: The cover image represents the key themes explored in this thesis: ancestral food, food gathering, the Paleolithic era, and type 2 diabetes. It depicts the author's mother with hands hardened by labour, with a drop of blood on one finger—a common marker in diabetes testing. The scene portrays her and other gatherers harvesting potatoes, a food often mistakenly deemed harmful for individuals with diabetes, against the ancient landscape of Lanzarote, an island millions of years old.

Acerca de la portada: La imagen de la portada representa los principales temas explorados en esta tesis: alimentación ancestral, recolección de alimentos, la era Paleolítica, y la diabetes tipo 2. Retrata a la madre del autor con sus manos curtidas por el trabajo, y una gota de sangre en un dedo; un marcador común en las pruebas para la diabetes. La escena la ilustra a ella, y a otros/as campesinos/as, cogiendo papas, un alimento que erróneamente se considera perjudicial en la diabetes, en un paisaje de Lanzarote, una isla de millones de años de antigüedad.

Printed by Media-Tryck, Lund 2025 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences in Malmö

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2025:68
ISBN 978-91-8021-721-7
ISSN 1652-8220