Effects of Butter and Phytanic acid intake on metabolic parameters and T-cell polarization

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PhD Thesis

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Preface

This thesis is based on two feeding studies in mice conducted September through December 2009, and August through November 2010, at the Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark, and an immune cell study. An immune cell study was conducted with samples from a human intervention study conducted at Department of human nutrition, University of Copenhagen, Frederiksberg, Denmark, and samples from volunteers at the Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark. Immune cell studies were conducted January through June 2010, and January through April 2011, respectively. Furthermore, results, from two human interventions conducted at Department of human nutrition, University of Copenhagen, Frederiksberg, Denmark, is included, conducted September through October 2009, and January through June 2010, respectively.

During my PhD my time has primarily been spend at department of systems biology, were I have been provided with hands on experience in conducting experiments. Being in charge of conducting larger studies was new to me, and had great educational value to me as well as I have gained experience within analysis methods that were new to me.

The project was a sub-project under a cooperative project called “Green feed”, in which the effects by intake of dairy fat with altered FA-composition due to feeding various amounts of green plant material to cattle, on metabolic health was studied. “Green Feed” was part of a scientific network called “Tailored milk”. The project was funded by The Danish Council for strategic Research, Danish Cattle, and The Danish Dairy Research Board. The butter used for experiments was experimentally produced butter from Søren K. Jensen, Foulum AU, butter from ARLA Foods, Denmark, and TINE sa, Norway. High oleic sunflower oil was kindly provided from AarhusKarlshamn AB.

The studies

Five different studies are covered in this thesis; manuscripts/articles are enclosed as appendixes:

A. "*A hyper caloric diet based on monounsaturated fatty acids has more detrimental effects on metabolic parameters than diets based on butter or linoleic acid*"

   Objective: To compare effects on metabolic parameters, from high fat diets based on butter from either high- or low yield production forms, to high fat diets based on oleic acid or linoleic acid, in obese mice.

B. *"Effects of the natural PPARα and RXR agonist phytanic acid on glucose homeostasis and hepatic lipid status in obese C57bl/6j mice"*

   Objective: To investigate the effects of PA in high fat diets based on in part butter and in part grape seed oil rich in linoleic acid, on parameters related to glucose metabolism and hepatic lipid metabolism.

C. "*Implications of dairy-fat and the dairy fat component phytanic acid on T-cell cytokine shifting in activated CD3+ T-cells from a double blinded 12 week-randomized butter intervention*"

   Objective: To investigate whether butter intake and/or PA are able to alter the CD3+ T-cell polarization towards a Th2 response.

D. *"Effect of dairy fat on plasma phytanic acid in healthy volunteers - A randomized controlled study"*

   Objective: To investigate if cow feeding regimes affects concentration of plasma phytanic acid and risk markers of the metabolic syndrome in human

E. *"Effects of milk derived from mountain-pasture grazing cows on risk markers of the metabolic syndrome compared to conventional Danish milk*"

   Objective: To investigate the effect of milk delivered from mountain-pasture grazing cows (green diet) on risk markers of the metabolic syndrome and type-2 diabetes with the effect of control diet, including conventional Danish milk. The study should reveal the importance of phytanic acid content for these effects.

The overall plan by conducting these five studies were to do a broad investigation of the effects from intake of dairy fat, and the influence of feeding and production form of the dairy fat. Furthermore we wanted to examine whether dairy intake is able to alter the phytanic acid concentrations found in humans and mice, and if this would affect metabolic parameters.

By this set up we were also able to test hypothesized effects of phytanic acid (PA) on T-cell polarization. As we used mice as a model organism, we were among others also able to do studies on isolated effects from PA intake, as this was not possible by human interventions. An advantage from this larger set up of studies, is that we could use the same butters in both mice and human studies, as the grazing butter used in study A and E is the same, and the butter from conventional fed cattle is the same used in study A and D, as well as for the PA dose response in study B. Furthermore the subjects from the butter intervention of study C, is a subpopulation from study E.

Summary

The still growing obesity epidemic is a major risk for our society, as it is associated with the development of the so called metabolic syndrome, which is a clinical diagnosis correlated to development of metabolic disorders. Lack of physical activity, excess energy intake, and nutritional factors e.g. fatty acid composition of the diet, are important factors with regard to development of metabolic syndrome.

There is a controversy between the fact that several studies has shown that intake of saturated fatty acids are strongly correlated to the development of metabolic related diseases, such as cardiovascular diseases and type 2 diabetes, and against the fact that other studies has shown that intake of dairy fat, which has high saturated fatty acid content, correlates negatively with risk factors. Hence, it has even been suggested that dairy fat might have beneficial impacts in relation to metabolic disorders. Dairy fat is the most complex type of fat occurring in the nature, with more than 400 identified fatty acids. Several of these fatty acids that occur in low amounts have been suggested to have beneficial properties with regard to metabolic disorders. The concentrations of certain of these minor fatty acids are raised in dairy fat along with the amount of green plant material intake of the cattle. Phytanic acid is one of these minor fatty acids, due to agonist activities for nuclear receptors with central roles in among others the lipid and glucose metabolism.

To determine the effects of both dairy fat in general and phytanic acid on metabolic parameters, we performed several studies. First, we investigated effects on hepatic lipid metabolism, glucose homeostasis, and circulating metabolic markers, of high fat diets based on butter from high- or low-yield production, a diet based on high oleic acid sunflower oil, and a diet based on grape-seed oil with high amount of linoleic acid, in diet induced obese mice. Second, we investigated phytanic acid effects on similar parameters in obese mice, both as dose response in butter based diets, and in grape-seed oil based diets with and without addition of phytanic acid. Third, we investigated butter and phytanic acid effects on human T-cell polarization, both by *in vitro* incubation with phytanic acid, and by a 12 weeks intervention with intake of butter. Finally, we performed two human interventions, first one with intake of butter and cheese, and the second with intake of butter. In these studies we investigated whether it is possible to alter the human plasma concentration of phytanic acid due to dairy fat intake, and if butter from different feeding regimes, and production forms has different effects on metabolic parameters upon intake.

Fat type intervention in mice

Obesity was induced in mice, by addition of sucrose to the drinking water, and giving high fat diets, based on butter from either grazing or conventional fed cattle, high oleic acid (monounsaturated fatty acid) sunflower oil, or finally from grape-seed oil with high content of the n-6 poly unsaturated fatty acid linoleic acid, along with having a lean reference group. Oral glucose tolerance test was performed after 10 weeks intervention, and animals sacrificed two days later. Parameters relevant to glucose metabolism, and hepatic lipid metabolism e.g. lipid deposition, were measured, just as RT-qPCR were used to measure expression of genes relevant for lipid metabolism in the liver. Plasma lipids, adipokines, and a marker of inflammation were also measured. We found that the hyper caloric diet based on oleic acid had the most detrimental effects on metabolic parameters, of the tested fats, as it led to increased hepatic lipid deposition, and reduced glucose tolerance. The butter based diets had more unfavorable effects on concentration of blood lipids, observed as raised triacylglycerol and total cholesterol. Compared to the literature the results with regard to oleic acid are controversial, as the common advice is to substitute SFA by MUFA in the diet.

Phytanic acid effects in mice

Production of phytanic acid by organic synthesis, allowed us to investigate isolated effects of phytanic acid intake. Obesity were induced in similar manner as in the fat type intervention described above, with different amounts of phytanic acid ethyl-esters added to either butter or grape-seed oil based diets, to investigate the effects from phytanic acid intake, on parameters similar to those in the fat type intervention. We saw that PA intake have aggravating effect on glucose homeostasis in dosages of 1.0 % of total fat. We did se limited up regulation of PPARα and ACOX1 due to 1.0 % phytanic acid in butter. As we are the first to perform interventions with physiological realistic amounts of phytanic acid, which have been proposed to have protective effects due to its agonist activities for central nuclear receptors, our results most definitely, add to the knowledge of the field.

Butter and phytanic acid effects in humans, and on T-cell polarization

Two human dairy fat interventions was conducted, with healthy subjects divided into groups and given dairy fat (as butter and cheese) from cattle under different feeding regimes, resulting in among others difference in phytanic acid content. From the first intervention, we found that it is possible to alter the human plasma phytanic acid concentration due to four weeks dairy fat intervention. From the second intervention we found that butter from grazing cattle, which among others have increased phytanic acid content, increase plasma LDL cholesterol and insulin, compared to conventional butter. From a subpopulation of the second intervention, T-cells were isolated from blood before and after the intervention, to analyze the effect on T-cell polarization. Furthermore we performed an *in vitro* incubation of T-cells, from eight donors, with phytanic- and palmitic acid, to investigate if phytanic acid affects T-cell polarization as hypothesized. Phytanic acid was not found to change the T-cell polarization, neither in the incubation study nor due to the difference in concentrations in the butter intervention. We saw up regulation in mRNA expression of both IL-4 and IFN-γ due to the butter intervention, when the groups were regarded as one. This was more pronounced for IL-4 than IFN-γ, and we observed increase in the ratio IL-4: IFN-γ due to the intervention. This is pointing towards a general effect towards Th2 polarization of human T-cells due to increased intake of butter. These results add to the understanding of potential phytanic acid and butter effects, on the immune system as similar studies have not been performed on T-cells before.

Resume (dansk)

Den stadigt voksende fedme epidemi er en betydningsfuld risiko for vores samfund, da fedme er associeret til udvikling af såkaldt metabolisk syndrom, der er en klinisk diagnose korreleret til udvikling af metaboliske lidelser. Mangel på fysisk aktivitet, overskydende energi indtag, og ernæringsmæssige faktorer som eksempelvis kostens fedtsyre sammensætning, er vigtige faktorer med hensyn til udvikling af metabolisk syndrom.

Der er et kontrovers mellem kendsgerningen at adskillige studier har vist at indtag af mættede fedtsyrer er stærkt korreleret til udviklingen af metabolisk relaterede sygdomme, såsom hjertekarsygdomme og type 2 diabetes, og den kendsgerning at andre studier har vist, at indtag af mælkefedt, som har et højt indhold af mættet fedt, korrelerer negativt med risiko faktorer. Således har det endda været foreslået, at mælkefedt måske har gavnlig indflydelse i relation til metaboliske lidelser. Mælkefedt er den mest komplekse naturligt forekommende fedttype, med mere end 400 identificerede fedtsyrer. Flere af disse fedtsyrer der forekommer i små mængder har været foreslået at have gavnlige egenskaber med hensyn til metaboliske forstyrrelser. Koncentrationerne af visse af disse mindre forekommende fedtsyrer er forhøjet i takt med mængden af grønt plantemateriale som kvæget indtager. Phytansyre er en af disse fedtsyrer, eftersom den har agonist aktivitet for kernereceptorer der har centrale roller i blandt andet lipid og glukose metabolismen.

For at fastlægge effekterne af både mælkefedt generelt og phytansyre på metaboliske parametre, har vi udført en række studier. Først, har vi undersøgt effekter på leverens lipid metabolisme, glucose homeostase og cirkulerende metaboliske markører, af diæter med højt fedtindhold, givet til diæt induceret fede mus. Vi har sammenlignet diæter baseret på smør fra henholdsvis højt- og lavt-ydende produktionsform, en diæt baseret på solsikkeolie med højt indhold af oliesyre, samt en diæt baseret på vindruekerneolie med højt indhold af linolsyre. Dernæst, undersøgte vi phytansyre effekter på tilsvarende parametre i fede mus, både som et dosisrespons tilsat en smør baseret diæt, og i en vindruekerneolie baseret diæt med og uden tilsat phytansyre. Derudover, undersøgte vi smør og phytansyre effekt på polarisering af humane T-celler, både som *in vitro* inkubering med phytansyre, og ved en 12-ugers smør intervention. Endeligt, har vi udført to humane interventioner. Den første med indtag af smør og ost, og den anden kun med indtag af smør. I disse studier undersøgte vi hvorvidt det er muligt at påvirke plasmakoncentrationen af phytansyre i mennesker gennem indtag af mælkefedt, samt om smør fra forskellige fodringsstrategier og produktionsform har forskellig indflydelse på metaboliske parametre ved indtag.

Fedttype intervention i mus
Fedme blev induceret i mus, ved tilsætning af sukrose til drikkevand, og ved at give diæter med højt fedtindhold, baseret på smør fra enten græssende eller konventionelt fodret kvæg, solsikkeolie med højt oliesyre (monoumættet fedtsyre) indhold, eller vindruekerneolie med højt indhold af den n-6 fler-umættede linolsyre. Sideløbende havde vi en mager reference gruppe. Oral glukose tolerance test blev udført efter 10 ugers intervention, og dyrene blev aflivet to dage senere. Relevante parametre for glucose metabolisme og leverens fedt metabolisme, eksempelvis lipid ophobning, blev målt, ligesom der blev målt ekspressionsniveauer, for gener der er relevante for leverens fedtsyre metabolisme, ved RT-qPCR. Desuden blev plasma lipider, adipokiner, og en inflammatorisk markør målt. Vi fandt at høj energi diæten baseret på oliesyre havde de mest skadelige virkninger på metaboliske parametre, af de fedttyper, idet den førte til

forøget lipid ophobning i leveren samt reduceret glukose tolerance. De smørbaserede diæter havde mere ufavorable effekter på koncentration af blodlipider, observeret som forhøjet triglycerid og total-kolesterol. Sammenlignet med litteraturen, er resultaterne omhandlende oliesyre kontroversielle, da den gængse anbefaling er at erstatte mættet fedt med mono-umættet.

Phytansyre effekter i mus

Produktion af phytansyre ved organisk syntese, tillod os at undersøge isolerede effekter af phytansyre indtag. Fedme blev induceret på samme måde som i fedttype interventionen beskrevet ovenfor, med forskellige mængder af phytansyre-ethylester tilsat til enten smør eller vindruekerne olie baserede diæter, for at udforske effekterne af phytansyreindtag, på lignende parametre som i fedttype interventionen. Vi fandt, at phytansyreindtag har forværrende effekt på glukose homeostase mængde af 1,0 % af total fedt. Vi så desuden begrænset opregulering af PPARα og ACOX1, som følge af 1,0 % phytansyre i smør. Da vi er de første til, at udføre interventioner med fysiologisk realistiske mængder af phytansyre, som har været foreslået at have beskyttende effekter som følge af dens agonist aktivitet for centrale kerne receptorer, bidrager resultaterne bestemt til viden på området.

Smør og phytansyre effekt i mennesker og på T-celle polarisering

To humaninterventioner blev udført med raske deltagere, inddelt i grupper, blev givet mælkefedt (i form af smør og ost) fra kvæg underlagt forskellige fodringsstrategier, resulterende i blandt andet forskel i phytansyre indhold. Ved den første intervention, fandt vi ud af at det er muligt at ændre den humane plasma koncentration af phytansyre gennem fire ugers mælkefedt intervention. I det andet interventionsstudie fandt vi at smør fra græssende kvæg, som blandt andet har højere phytansyre indhold, forøger plasma kolesterol og har negativ effekt på insulinfølsomhed, sammenlignet med konventionelt smør. Fra en subpopulation i den anden intervention, isolerede vi T-celler før og efter interventionen, for at undersøge effekter på T-celle polarisering ved smørindtaget. Ydermere udførte vi en *in vitro* inkubering af T-celler, fra otte donorer, med phytansyre og palmitinsyre, for at undersøge om phytansyre påvirker T-celle polarisering som foreslået. Vi fandt ikke at phytansyre ændrer T-celle polarisering, hverken i inkuberingsstudiet eller gennem koncentrationsforskelle i smørinterventionen. Vi så upregulering i mRNA udtryk af både IL-4 og IFN-γ som følge af smør indtag, når resultaterne for de to grupper blev lagt sammen. Dette var mere udtalt for IL-4 end for IFN-γ, og vi observerede stigning i forholdet IL-4: IFN-γ som følge af interventionen. Dette peger i retning af en generel effekt i retning af Th2 polarisering af humane T-celler som følge af øget smørindtag. Disse resultater bidrager til forståelsen af potentielle phytansyre og smør effekter på immunsystemet, eftersom lignende studier ikke tidligere er udført på T-celler.

Abbreviations

ACOX	Peroxisomal acyl-coenzyme A oxidase
AKT	Protein kinase B (also abbreviated PKB)
ALA	α-linolenic acid
AMCAR	α-methylacyl-CoA racemase
AP	Activator protein
BAT	Brown adipose tissue
BCL	B-cell lymphoma protein
BMI	Body mass index
CAT	Catalase
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CETP	Cholesteryl ester transfer protein
ChREBP	Carbohydrate responsive element binding protein
CLA	Conjugated linoleic acid
CPT1b	Carnitine palmitoyltranferase-1b
CVD	Cardiovascular disease
DAG	Diacylglyceride
DBD	DNA binding domain
DHA	Docosa-hexanoic acid
DNA	Deoxyribonucleic acid
DNL	De-novo lipid synthesis
EPA	Eicosapentanoic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases (also known as MAPK)
FA	Fatty acid
FAS	Fatty acid synthase
Fasn	Gene encoding FAS
FFA	Free fatty acid, also known as non-esterified fatty acid (NEFA)
G6Pase	Glucose-6-phosphatase
GATA	Erythroid transcription factor (also known as GATA-binding factor)
GCP	Glycerol-phosphocholine
GI	Gastro intestinal
GLUT4	Glucose transporter
GSO	Grape seed oil
HDL	High density lipoprotein
HFD	High fat diet
HOMA-IR	Homeostatic model assessment–insulin resistance
ICAM	Intercellular adhesion molecule
IFN	Interferon
IKK	Inhibitor κB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRS	Insulin receptor substrate
IV	Intravenous
JNK	c-Jun N-terminal kinase
LA	linoleic acid

LBD	Ligand binding domain
LDL	Low density lipoprotein
LXR	Liver X receptor
m%	Mass %
M1	Macrophage subtype 1 (classical activated macrophage)
M2	Macrophage subtype 2 (alternatively activated macrophage)
MAG	Monoacylglyceride
MAPK	Mitogen activated Protein kinase (also known as ERK)
MCP	Monocyte specific chemokine
MI	Myocardial infarction
MIP	Macrophage inflammatory protein
MS	Metabolic syndrome (also known as syndrome X)
MUFA	Mono unsaturated fatty acid
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid, also known as free fatty acid (FFA)
NF	Nuclear factor
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
Nlrp3	NOD-like receptor family, pryin domain containing 3 (gene coding for NALP3)
OGTT	Oral glucose tolerance test
PA	Phytanic acid
PDK	Phosphoinositide dependent kinase
PhyH	Phytanoyl-CoA hydroxylase
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol-3-phosphat
PK	Protein kinase
PL	Phospholipids
PP2A	Protein-phosphatase-2A
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response element
PUFA	Poly unsaturated fatty acid
RAS	RAt Sarcoma (RAS subfamily of GTPases)
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
RXR	Retinoid X receptor
SFA	Saturated fatty acid
SM	Sphingomyelin
SOD	Superoxide dismutase
SREBP	Sterol regulatory element binding protein
TAG	Triacylglyceride
tFA	*Trans* fatty acid
Th	T -helper (cell)
TNF	Tumor necrosis factor
T_{reg}	Regulatory T-cell
tVA	*Trans*-vaccenic acid
UCP	Uncoupling protein
UPR	Unfolded protein response
VCAM	Vascular cell adhesion protein
VEGF	Vascular endothelial growth factor

VLDL	Very low density lipoprotein
WAT	White adipose tissue

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Motivation

The ongoing obesity epidemic and dramatic increase in obesity related diseases has created growing interest in prevention of obesity development. Along with lack of physical activity, changes in nutrition are a very important parameter in the development of obesity, as dietary components have been shown to affect parameters used to diagnose metabolic syndrome (MS). MS is a cluster of metabolic disorders correlated to the development of type 2 diabetes and cardiovascular disease (CVD). The belief is therefore that identification of nutrients that comprise preventive effect due to the development of MS could have a positive effect with regard to reduction of disease incidences.

Lipids and thereby fatty acids (FA) is an extremely important factor in this context. The common nutritional recommendations has, during the last decades, focused on reduction of saturated fatty acids (SFA) and trans-fatty acids (tFA), and thereby reduction of dairy fat intake, as these has been shown correlate with risk of CVD. However, a number of studies have now indicated that intake of dairy fat might not be as harmful as earlier thought, or even that intake of dairy fat might have beneficial impacts in with regard to prevention of development of metabolic disorders. Dairy fat is extremely complex, and contains many different FAs in minor concentrations. Some of these minor fatty acids might have very interesting potential with regard to prevention of metabolic disorders. One of these is phytanic acid (PA) which has agonist activity for nuclear receptors, with important functions in among others the lipid metabolism, and is therefore important in the context of metabolic disorders.

In this study we investigate the effects of energy dense diets, with high content of butter which we compare to diet high in either oleic acid, the most common mono-unsaturated fatty acid (MUFA) in the diet, and a diet high in linoleic acid, an n-6 poly-unsaturated fatty acid (PUFA), from grape seed oil, in obese mice. The focus has been on hepatic lipid metabolism and glucose metabolism. Furthermore we compare effects on metabolic disorders, from butter from either low- or high yield production forms, as the fatty acid composition in dairy fat varies due to among others feeding regimen. This is investigated both in studies with obese mice and in human interventional studies. We also investigate whether PA in physiological realistic concentrations has impact on such parameters, and whether intake of PA is able affect polarization of T-cells.

Introduction

Obesity

The world has during the last decades experienced an ongoing increase in the incidents of obesity in such a dramatic manner that it is defined as an obesity epidemic. This obesity epidemic is the most likely cause of the rising in incidences of insulin resistance, and obesity seems to be the strongest determinant of MS (Ogden *et al.*, 2006; Cornier *et al.*, 2008).

White adipose tissue is believed to have limited storage capacity of fat. When this capacity has been filled up further lipid load will lead to ectopic fat deposition in liver, muscles etc and lead to lipotoxicity in the tissues (Virtue & Vidal-Puig, 2008). Through secretion of adipokines, adipocytes communicate with other tissues and influences important processes such as energy balance, insulin sensitivity and glucose metabolism, inflammation, lipid metabolism, blood pressure, haemastasis and angiogenesis (Wood *et al.*, 2009). Adipocytes secretes cytokines and the large overfilled adipocytes secretes more pro-inflammatory cytokines than anti-inflammatory cytokines compared to small adipocytes, and furthermore also secrete more chemoatractants associated with macrophage infiltration (Skurk *et al.*, 2007; Virtue & Vidal-Puig, 2008). It has been shown that the macrophages in lean mice express many genes characteristic for alternatively activated macrophages, also called M2, and macrophages in DIO mice decreased expression of those genes while increasing expression of proinflammatory genes such as TNFα and iNOS, which are characteristic for classical acitivated macrophages, M1. In the same study it is suggested that the M2-polarized macrophages might protect against TNFα induced insulin resistance (Lumeng *et al.*, 2007). Th1 cytokines has been shown to promote M1 macrophages, while Th2 promote M2 polarization of T-cells (Bouhlel *et al.*, 2009). The combination of inflammation and lipotoxicity is believed to disturb the proper metabolism of all major organs and tissues. This might be the reason why MS is associated not only with diabetes type II and CVD, but also liver disease (such as NAFLD), kidney disease, and reproductive disorders among others (Cornier *et al.*, 2008). The type of WAT is also a very important factor, as different adipose tissue depots have distinct gene expression profiles (Zhang *et al.*, 2007). Visceral adipose tissue in humans is strongly associated with increased metabolic risk, whereas subcutaneous adipose tissue is not associated with metabolic disorders and might even have protective effects (Snijder *et al.*, 2003).

Metabolic syndrome

Metabolic syndrome (MS), also called syndrome X, is a cluster of metabolic disorders that increase the risk of developing cardiovascular disease (CVD) and diabetes mellitus. These risk factors occurs together more often than alone (Alberti *et al.*, 2009). The risk factors that make up the MS are raised blood pressure, dyslipedimia (raised TAG and lowered HDL cholesterol), raised fasting glucose, and central obesity.

Table 1 Criteria for clinical diagnosis of the metabolic syndrome (Alberti *et al.*, 2009).

Measure	Categorial cut points
Elevated waist circumference	Population- and country-specific definitions[1]
Elevated triglycerides	≥150 mg/dL (1.7 mM)
Reduced HDL cholesterol	Males: < 40 mg/dL (1.0mM) Females: < 50 mg/dL (1.3 mM)
Elevated blood pressure	Systolic ≥130 and/or diastolic ≥ 85 mm Hg
Elevated fasting glucose	≥100 mg/dL

1. There are no common categorical cut point values for waist circumference, as there are large differences in the related cardiovascular risk between ethnic groups and sexes (Danish cut-off values 94 cm for men and 80cm for women (www.hjertedoktor.dk, 2011)).

In Denmark the prevalence of MS is around 20 %, which corresponds to the world wide prevalence. The prevalence is a little higher for men than for women in Denmark, which though not are a general tendency worldwide. The prevalence between sexes differs a lot between countries, which can be due to differences in several factors such as work-related activities and cultural views on body fat (Cornier *et al.*, 2008).

Cardiovascular diseases (CVD)/atherosclerosis

Atherosclerosis is one of the most common causes of death in industrialized western societies, and CVD caused 9.9 % of all deaths in Denmark in 2009, only exceeded by the sum of all cancer types (www.danmarksstatistik.dk, 2011) The treatment against atherosclerosis has evolved as our understanding of the disease and the underlying causes evolved, and that is the primary reason why it is no longer the number one cause of death in e.g Denmark. CVD is now regarded as an inflammatory disease, involving both the immune system and the lipid metabolism (Lowenstein & Matsushita, 2004). CVD risk factors is connected to development of type 2 diabetes (D'Agostino *et al.*, 2004), as well as lipid metabolism is associated to development of CVD (Neve *et al.*, 2000).

Lipid metabolism and Dyslipidemia

The lipid metabolism is very central in the development of metabolic disorders, and especially when it is dysregulated, leading to dyslipedimia. Many lipids and lipid metabolites from different lipid classes, function as signal transducers by inhibiting or inducing metabolic processes, as in e.g. the insulin cascade, which is described below, and are thereby involved in development of metabolic disorders.

The liver plays a central role in fatty acid balance of the body, by chylomicron clearance, remnants, uptake of endogenous NEFA from circulation, and lipid neo synthesis from glucose (Adiels *et al.*, 2008; Musso *et al.*, 2009), and lipid export in VLDL particles. Increased lipid supply, as observed within obesity, leads to increased TAG-rich VLDL-particle production. By use of stable isotope methodology, Donnelly *et al.* has shown that in patients suffering from non-alcoholic-fatty-liver disease (NAFLD), 59 % of TAG in the liver comes from circulating NEFA, 26 % from de novo lipogenesis (DNL) and 15 % from the diet. They also showed that in these patients DNL was the same postpranidial as in fasting state (Donnelly *et al.*, 2005). By the actions of cholesteryl ester transfer protein (CETP) and hepatic lipase, TAG from VLDL-particles is transferred to HDL- and LDL-particles. TAG-rich lipoproteins are preferred substrates of hepatic lipase, and therefore TAG is removed from HDL and LDL. This results in formation of small dense LDL and small dense HDL. Small dense HDL is catabolized and removed from circulation, which results in reduced HDL concentrations, and small dense LDL is more atherogenic than larger subpopulations of LDL (Adiels *et al.*, 2008; Musso *et al.*, 2009).

Non-alcoholic fatty liver disease (NAFLD) has been suggested to be a main factor driving the development of MS, and is defined as: Liver fat content >5-10 % by weight in the absence of excess alcohol consumption or any other liver disease. (Vanni *et al.*, 2010). NAFLD covers variable degrees from simple and benign steatosis, to nonalcoholic steatohepatitis (NASH), and Cirrhosis. NASH is defined by the presence of hepatocyte injury, inflammation and/or fibrosis which can lead to cirrhosis, liver failure and hepatocyt carcinoma. Therefore inflammatory markers, such as TNFα, are also important factors to take into account with regard to excess hepatic lipid accumulation. When the liver gets fatty due to NAFLD, the ability of insulin to inhibit hepatic glucose production is impaired. This leads to a slight increase in concentration of plasma glucose and stimulation of insulin secretion. Therefore Vanni *et al.* suggests that hyperinsulinemia is likely to be the consequence rather than the cause of NAFLD, since exogenous insulin therapy for seven months significantly decreases liver fat content (Juurinen *et al.*, 2007; Vanni *et al.*, 2010). Dyslipidemia is an independent risk factor for type 2 diabetes, and Rhee *et al.* has identified a TAG signature of insulin resistance, which they propose is able to improve diabetes prediction in humans (Rhee *et al.*, 2011).

Insulin resistance

The pleitropic molecule insulin has many different effects and are involved in many processes, e.g.: amino acid uptake, protein synthesis, adipose tissue TAG lipolysis, lipoprotein lipase activity, very low density lipoprotein (VLDL) TAG secretion, muscle and liver glycogen synthesis, endogenous glucose production, and most commonly known muscle and adipose tissue glucose uptake(Cornier *et al.*, 2008). The many actions of insulin is mediated through a single receptor tyrosine kinase, called insulin receptor (Kido *et al.*, 2001). Insulin resistance, and the opposite insulin sensitive, is generally defined due to the response to oral or intravenous (IV)glucose or insulin stimulus (Pacini, 2006). Insulin resistance is closely coupled to obesity,

and as mentioned above also to dyslipidemia (Rhee *et al.*, 2011). Though, increased intracellular lipid concentrations in muscle cells, can together with external inflammatory stimuli interfere with the insulin signaling and glucose uptake. Insulin sensitivity is inversely correlated with intramyocellular TAG concentration; it is probably not TAG itself that is directly responsible, as it is more likely that TAG is just a marker for FFA and other lipid metabolites such as the sphingolipid ceramide and diacylglyceride (DAG). Both ceramide and DAG has been shown to activate protein kinases or –phosphatases that impair the signal from insulin receptor, with following increased insulin resistance (Chavez *et al.*, 2003; Corcoran *et al.*, 2007). Furthermore ceramide has been suggested to be involved in the activation of the Nlrp3 inflammasome during induced inflammatory development, which also seems to play a role in early stages of high fat diet (HFD)-induced insulin resistance (Vandanmagsar *et al.*, 2011).

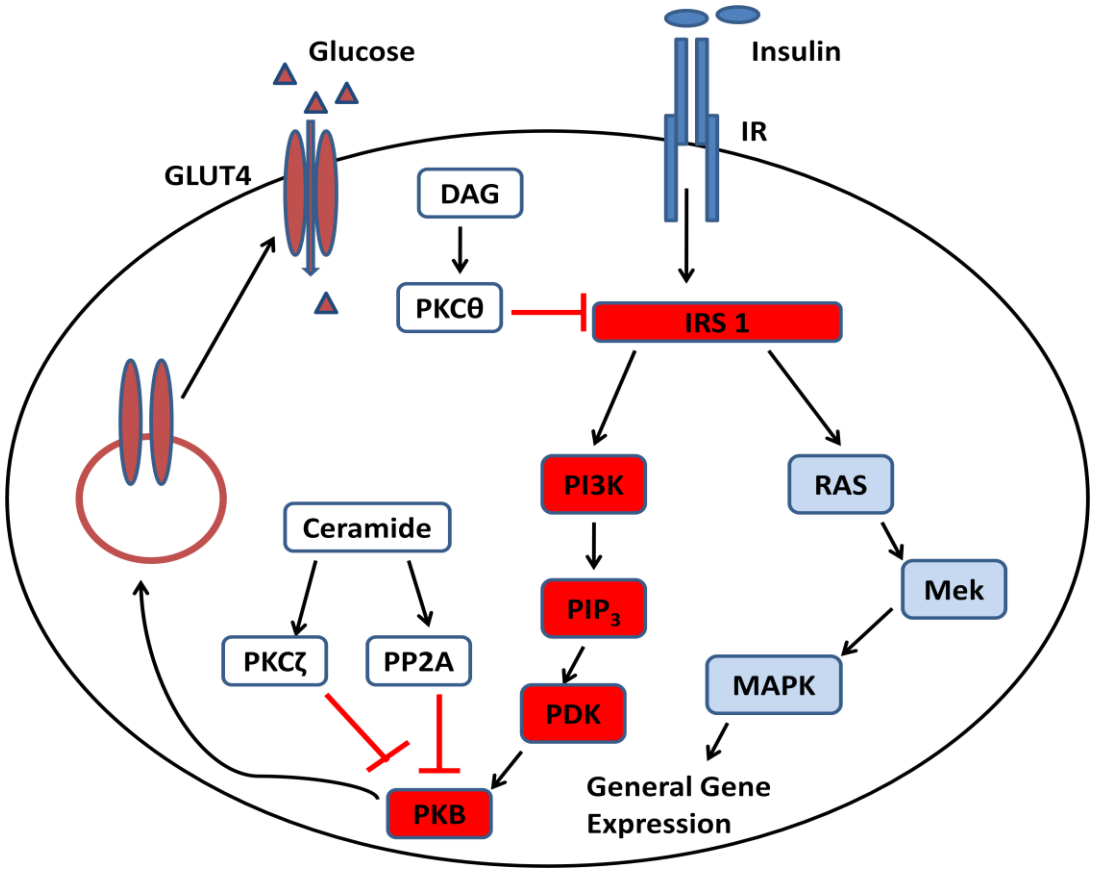

Figure 1 Insulin signalling cascade, and inhibition by Diacylglycerol (DAG) and Ceramide. IR: Insulin receptor, GLUT4: Glucose transporter 4, IRS1: Insulin receptor substrate 1, PI3K: Phosphatidylinositol-3-kinase, PIP3: Phosphatidylinositol-3-phosphat, PDK: Phosphoinositide dependent kinase, PK: Protein Kinase, PP2A: Protein-phosphatase-2a, RAS: , Mek: , MAPK: Mitogen-activated protein kinase.

Insulin resistance can also be induced by endoplasmic reticulum (ER) stress, as it leads to activation of IKK by NFκB activators (Yuan *et al.*, 2001; Fleischman *et al.*, 2008) and c-Jun N-terminal kinase (JNK), and thereby alternative phosphorylation of members of the insulin signaling cascade (Gregor *et al.*, 2009). ER stress is also called unfolded protein response (UPR) and occurs when the amount of un- or malfolded protein exceeds the capacity of ER to ensure proper folding, leading to the UPR, including activation of JNK. As treatment with chemical chaperones prevents insulin resistance in obese mice, this pathway is thought to be an important contributor to development of insulin resistance (Harding & Ron, 2002; Schenk *et al.*, 2008). ER stress is believed to induce insulin resistance in liver, adipose tissue and skeletal muscle (Gregor *et al.*, 2009).

Adipokines is another important factor in the development of insulin resistance (Rabe *et al.*, 2008). Especially adiponectin seems to be an important factor with regard to insulin resistance. The level of adiponectin is normally lowered with obesity and insulin resistance, which is unlike most other adipokines(Hotta *et al.*, 2001), and decreased expression of adiponectin correlates with insulin resistance (Yamauchi *et al.*, 2001). Administration of adiponectin leads to decreased TAG levels in muscle and liver in obese mice, due to expression of e.g. CD36, ACOX, and UCP2 (Yamauchi *et al.*, 2001). Other adipokines involved in insulin resistance are e.g. leptin, resistin, TNFα, IL-6, and IL-10 etc (Rabe *et al.*, 2008; Ropelle *et al.*, 2010). Several of these adipokines are involved in the regulation of each other, e.g. adiponectin suppres TNFα and IL-6, while they suppress adiponectin and stimulate other adipokines such as leptin and resistin (Rabe *et al.*, 2008).

Nuclear receptors

The term nuclear receptor superfamily covers a diverse array of transcription factors, from which three specific receptors will be further described, as they mediate important functions within the scope of current thesis. Both Retinoid-X-Receptor (RXR), liver-x-receptor (LXR), and the three different isoforms of peroxisome proliferator-activated receptors (PPAR) (α, γ, and δ) are transcription factors and members of the so called nuclear receptor super family, including nuclear hormone receptors and orphan nuclear receptors (Olefsky, 2001).

Common for all the nuclear receptors are that they function through so-called response elements, which are recognition/binding sites for the given at receptor dimer at the DNA strand, e.g. PPARs conduct their actions as transcription factors upon agonist activation, by binding to a PPAR response element (PPRE) on the DNA strand. All nuclear receptors therefore also have common structural features, including a DNA binding domain (DBD) which are responsible for the recognition and binding to the response elements, and a ligand binding domain (LBD) (Desvergne & Wahli, 1999; Olefsky, 2001; Desvergne, 2007).

Retinoid-X-Receptor

Retinoid-X-receptor (RXR) was originally discovered as a receptor involved in the retinoid signaling pathway, and there are three different isoforms, α, β, and γ. RXR is most known for its role as partner for a number of other nuclear receptors (e.g. PPARs and LXR), which conducts their functions as heterodimers with RXR (Shulman & Mangelsdorf, 2005). Though, RXR also act as a dimeric orphan receptor forming homodimers (Nagao & Yanagita, 2008). RXRα is abundantly expressed in liver, kidney, spleen, placenta, epidermis and a variety of visceral tissues, RXRβ is expressed ubiquitously, and RXRγ is mainly expressed in muscle and brain(Mangelsdorf *et al.*, 1992; Szanto *et al.*, 2004). RXR specific ligands has been indicated to have glucose-lowering and insulin sensitizing effects, and a RXRα agonist has been reported to have anti atherosclerotic effect in apoE mice (Nagao & Yanagita, 2008). Some of the most important effects from RXR, whit in the scope of this thesis, is conducted together with PPARs, and is described below. RXR also plays interesting roles in the immune system, as RXRα has been shown to be essential for an appropriate Th2-response, and RXRα knockout mice show a Th1-bias *in vivo* (Du *et al.*, 2005; Stephensen *et al.*, 2007). IFN-γ is *in vitro* inhibited by RXR-specific agonists as well as by 9-cis retinoic acid (Dawson *et al.*, 2008), though it is not clear whether this is mediated through RXR or retinoic acid receptor (RAR).

Peroxisome Proliferator-Activated receptors

As mentioned there is three different isoforms of PPARs, namely α, δ, and γ. PPARs are extremely important factors in e.g. the fatty acid metabolism in the liver, where PPARα operates in the catabolism of fatty acids, and PPARγ influences the storage of fatty acids in the adipose tissue, but has also controlling roles in inflammation (Kersten *et al.*, 2000; Varga *et al.*, 2011). PPARs are related to the modulation of environmental and dietary stimuli, and all three types are involved in the regulation of lipid metabolism, but they have distinctive roles, as well as they isoforms vary in tissue distributions and functions. Their cellular and systemic roles extend far beyond the control of hepatic peroxisome proliferation in rodents after which they were initially named, which among others has been reviewed in 2010 by Yessoufou and

Wahli (Yessoufou & Wahli, 2010). In the early 1990s PPARα was the first genetic sensor for fats that was identified (Evans *et al.*, 2004), and the PPARs are the main receptors acted upon by lipids (Guri *et al.*, 2006). Following ligand binding, PPARS heterodimize with RXR, inducing the detachment of co-repressors, recruitment of co-activators, some of which with acetylation activity, and subsequent binding to PPAR response element (PPRE) on DNA that initiates the transcription of responsive genes (Guri *et al.*, 2006; Nagao & Yanagita, 2008).

Table 2 Metabolic functions of PPARs. Compilation of the known main metabolic functions of PPARα, PPARδ, and PPARγ in the liver, muscle, white adipose tissue, brown adipose tissue, and pancreas. Redrawn from (Yessoufou & Wahli, 2010), information on brown adipose tissue from (Kang *et al.*, 2005; Komatsu *et al.*, 2010).

Tissue	PPARα	PPARδ	PPARγ
Liver	Increases fatty acid uptake Increases fatty acid oxidation Increases HDL apolipoproteins Decreases VLDL production Decreases acute-phase reactants Decreases inflammation	Decreases glucose production via increase of pentose phosphate shunt	Increases lipogenesis Increases insulin sensitivity
Muscle	PPARα overexpression shifts substrate utilization from glucose to fatty acids Increased fatty acid uptake Increased fatty acid oxidation Increased triglyceride lipolysis Increased glucoseintolerance/ insulin resistance Decreased glucose utilization	PPARδ (major isotype) Increased expression during starvation Increased fatty acid oxidation Increased fatty acid transport Increased thermogenesis Increases endurance capacity	Increases insulin sensitivity
White adipose tissue	Increases lipolysis during starvation	Increased fatty acid oxidation Increased fatty acid transport Increased thermogenesis	Increases adipocyte differentiation Increases adipocyte survival Increases lipogenesis Increases insulin sensitivity Increases adipokine secretion
Brown adipose tissue	Increases fatty acid oxidation during cold exposure		Involved in the differentiation of preadipocytes into brown adipocytes
Pancreas	Decreases β-cell lipotoxity Increases glucose-stimulated insulin secretion Increses fatty acid oxidation	Decreases insulin secretion in rodents	
	PPARα agonists he systemic effects in humans: Decreased triglycerides, increased HDL cholesterol, decreased hyperlipidimia and LDL, steady glycemia. PPARγ agonists are used as insulin sensitizing drugs. PPARγ ligand resistance syndrome (PLRS) – phenotypic characteristics of PLRS subjects are: Partial lipodystrothy, dyslipidemia, type 2 diabetes, insulin resistance, hypertension, hepatic steatosis, polycystic ovary syndrome. No PPARδ agosnists are on the market.		

PPARα

PPARα are highly expressed in the liver, brown adipose tissue (BAT), heart, skeletal muscle, and kidney, which are organs with high rates of FA catabolism. In other organs PPARα are expressed at lower levels. In the liver PPARα plays an extremely important role in the promotion of fatty acid oxidation and is especially active during fasting (Kersten *et al.*, 2000; Yessoufou & Wahli, 2010), but it is also involved in FA uptake through membranes, FA activation, intracellular FA trafficking, and TAG storage and lipolysis (Rakhshandehroo *et al.*, 2010). A very central enzyme in hepatic lipid oxidation, which is under transcriptional control from PPARα, is acylCoenzymeA oxidase 1 (ACOX1), as it is the first enzyme in the β oxidation of straight chain FAs.

PPARα in the liver are activated by FAs from peripheral tissue and the diet, as well as FA derivatives from diet and glycerol-phosphocholine (GCP) resulting from high sucrose intake. PPARα deficiency is associated with hepatic steatosis, decreased levels of plasma glucose and ketone bodies and elevated plasma NEFA and hypothermia, which are due to decreased expression of PPARα target genes involved in hepatic lipid metabolism (Leone *et al.*, 1999; Rakhshandehroo *et al.*, 2010).

PPARα has a broad range of both artificial and natural ligands. The artificial ligands of PPARα include hypolipidemic drugs, such as Wy-14643, clofibrate, plasticizers, and insecticides. A broad array of unsaturated fatty acids, long chain fatty acids, and branched chain fatty acids (e.g. phytanic- and pristanic-acid), are natural ligands for PPARα (Gloerich *et al.*, 2005; Rakhshandehroo *et al.*, 2010). Various artificial PPARα agonists commonly known as fibrates, has gained great success as oral agents against hypertriglyceridemia (Kota *et al.*, 2005; Berger *et al.*, 2005).

Beside the effects in the lipid metabolism, specific agonists to both PPAR-α and PPAR-γ modulate the Th1/Th2-balance in the Th2 direction, since both types attenuates the IFN-γ secretion from $CD4^+$ T-cells after CD3 crosslinking, while PPAR-α agonists also increases the production of IL-4 from murine and human lymphocytes (Marx *et al.*, 2002; Lovett-Racke *et al.*, 2004). In one study that shows increase in IL-4 due to treatment with specific PPARα agonists, one specific potent PPARα ligand (GW7,647) does not seem to augment IL-4 (Cunard *et al.*, 2002). Furthermore, PPAR-$\alpha^{-/-}$ - mice, show an impaired induction of regulatory T-cells (T_{reg}) (Dubrac *et al.*, 2011) , indicating that PPAR-α activation also are involved in differentiation of regulatory T-cells. The PPARα agonist Fenofibrate has also been shown to repress IL-17 and IFN-γ via PPARα in IL-10 deficient mice, which is normally characterized by Th1 polarized inflammation (Lee *et al.*, 2007). PPARα, as well as PPARγ, are also expressed in B cells, and PPARα activators decrease the amount of NFκB in both transformed T-cell and B-cell lines (Cunard *et al.*, 2002; Jones *et al.*, 2002). Treatment of macrophages with PPARα agonists has been showm to suppress IL-6 gene transcription by interfering with NFκB-driven promoter activation (Delerive *et al.*, 1999).

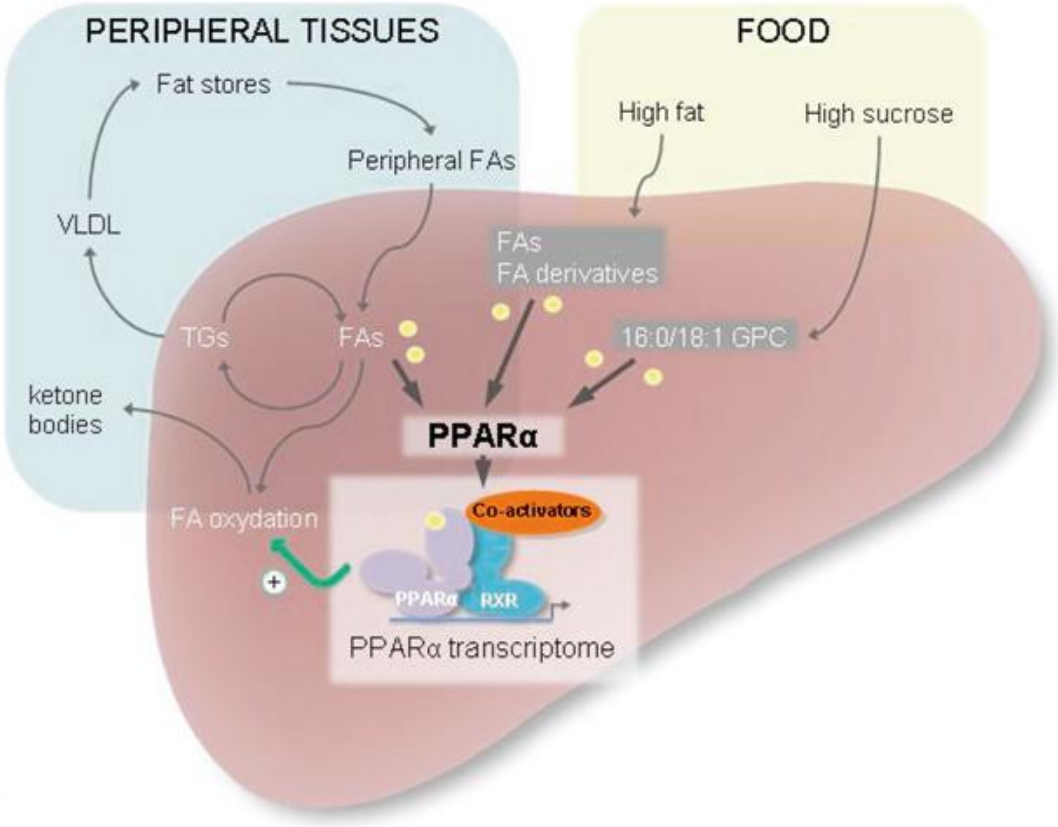

Figure 2 Simplified scheme illustrating the production of ligands activating PPARα. Dietary fatty acids and fatty acid derivatives activate PPARα. On a high sucrose (no fat diet), the production of 16:0/18:1 GPC activates PPARα. Activation of PPARα results in the transcriptional regulation of numerous genes, the so-called PPARα transcriptome, which contributes to maintaining the energy balance in part through the promotion of mitochondrial and peroxisomal beta-oxidation of fatty acids and many other metabolic pathways. FA: fatty acids; 16:0/18:1 GPC: 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; TG: triglycerides; VLDL: very low density lipoprotein. (Redrawn from (Yessoufou & Wahli, 2010) with permission from EMH Swiss Medical Publishers Ltd).

PPARγ

PPARγ exists in two isoforms, PPARγ1 and PPARγ2, which is the result of differential promoter usage and splicing. PPARγ2 is expressed primarily in adipocytes, wheras PPARγ1 is also relatively abundant in macrophages, colon epithelia, and endothelium (Lehrke & Lazar, 2005). A third PPARγ (PPARγ3) which encodes the same protein as PPARγ1 also exists (Desvergne & Wahli, 1999). PPARγ is involved in many different processes such as insulin sensitivity, body mass, atherosclerosis and macrophage biology, inflammation, and cancer (Lehrke & Lazar, 2005), but the most pronounced effects is the promotion of lipid storage in adipose tissue, together with the pivotal role in adipocyte differentiation (Madsen *et al.*, 2008; Yessoufou & Wahli, 2010). The key role in adipocyte development is controlled by PPARγ, though all three forms has been detected in adipose tissue (Madsen *et al.*, 2008). PPARγ can be activated by many different FAs, but it seems that PUFAs are better activators than MUFAs and SFAs, and highest binding affinity is achieved with FAs with 16-20 C-atoms (Madsen *et al.*, 2008).The group of PPARγ agonists called

thiazolidinediones, has been widely used as drugs for treatment of type 2 diabetes, which is often leads to increased weight gain due to the promotion of lipid storage in adipose tissue (Bloomgarden, 2011). Despite relatively low expression of PPARγ in liver, it is critical for the development of hepatic steatosis (Yu *et al.*, 2003; Patsouris *et al.*, 2006), and has recently been shown to promote osteoclast differentiation and bone resorption when activated by the agonist rosiglitazone (Wahli, 2008).

As mentioned in the section about PPARα, both PPARα and PPARγ specific agonists, has been shown to modulate the Th1/Th2-balance in the Th2 direction, since both types attenuates the IFN-γ secretion from CD-4^{+} T-cells after CD-3 crosslinking (Marx *et al.*, 2002). Furthermore PPARγ has been shown to promote human monocyte differentiation toward alternative macrophages (M2), which are normally promoted by Th2 cytokines, while Th1 cytokines promote the pro-inflammatory M1 macrophages. M2 macrophages are present in atherosclerotic lesions, where the expression of M2 markers correlates positively with expression of PPARγ (Bouhlel *et al.*, 2009), which is in accordance with the promotion of differentiation of monocytes towards M2 by PPARγ. PPARα and PPARδ does not promote differentiation of monocytes towards M2 macrophages (Bouhlel *et al.*, 2009).

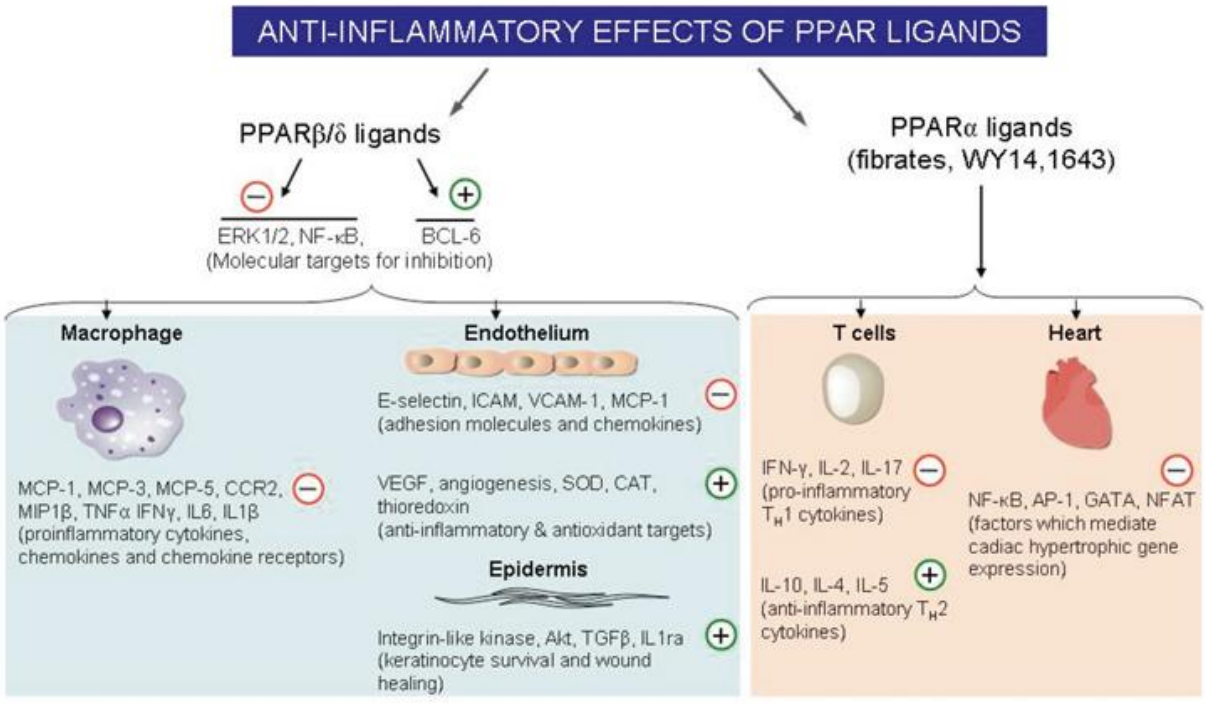

Figure 3 Anti-inflammatory effects of reroxisome proliferator-activated receptor (PPAR) ligands. ERK: Extracellular signal-regulated kinases (also known as MAPK), BCL: B cell lymphoma protein, MCP: Monocyte specific chemokine , CCR: Chemokine (C-C motif) receptor , MIP: Macrophage inflammatory protein, TNF: Tumor necrosis factor, IFN: Interferon , IL: Interleukin , E-selectin: , ICAM: Intercellular adhesion molecule, VCAM: Vascular cell adhesion protein, VEGF: Vascular endothelial growth factor, SOD: Superoxide dismutase , CAT: Catalase, Akt: Protein kinase B (PKB) , TGF: transforming growth factor, NF: Nuclear factor, AP: activator protein, GATA: Erythroid transcription factor (GATA-binding factor), NFAT: Nuclear factor of activated T-cells . Redrawn from (Yessoufou & Wahli, 2010) by permission from EMH Swiss Medical Publishers Ltd.

PPARδ

PPARδ, also known as PPARβ, did for a long time PPARδ receive much less attention than the two other isotypes of PPAR, because of its ubiquitous expression and unavailability of selective ligands. However, genetic studies and development of synthetic PPARδ agonists have helped reveal its role as a powerful regulator of fatty acid catabolism and energy homeostasis, beside implication in processes such as keratinocyte proliferation and differentiation during wound healing (Evans *et al.*, 2004; Yessoufou & Wahli, 2010). It has also been shown to be is expressed in preadipocytes and may be induced during the initial stages of adipocyte differentiation (Amri *et al.*, 1995; Madsen *et al.*, 2008). The PPAR agonist GW501516 has been shown to lower plasma triglyceride levels in obese monkeys while raising HDL levels (Oliver *et al.*, 2001), and PPARδ upregulates fatty acid oxidation and energy expenditure in skeletal muscles, to a far greater extent than does the lesser-expressed PPARα (Evans *et al.*, 2004). It appears that FAs that are unable to undergo oxidation are effective activators of PPARδ, but other FAs are also able to activate PPARδ (Madsen *et al.*, 2008).

LXR

Liver X receptors (LXR) are receptors that bind oxysterols, and there is two isoforms, α and β. LXRs form heterodimers with RXR. LXRα is expressed primarily in liver, adipose tissue, intestine, macrophages, and kidney, whereas LXRβ is ubiquitously expressed. LXRs are involved in transport of cholesterol from peripheral back to the liver and the hepatic cholesterol metabolism. Interestingly, with regard to the scope of this thesis LXR, and especially LXRα, increase the synthesis of FAs and TAGs due to up regulation of sterol regulatory binding protein 1C (SREBP-1c), which is regarded as the main regulator of FA synthesis (Repa *et al.*, 2000; Shulman & Mangelsdorf, 2005; Desvergne, 2007). An interesting feature of PUFAs with regard to LXR, is that Srebp-1c has been shown to be suppressed by PUFAs, and in particular eicosapentanoic acid (EPA), due to inhibition of LXR binding to the LXR response elements (Yoshikawa *et al.*, 2002).

Lipid intake and the risk of metabolic disease

It is commonly known that lipid intake has great influence on the metabolic status and development of various metabolic diseases, but there are many different classes and aspects of lipids, and the effects from intake, and general functions, of lipids differs a lot. Most lipids in the diet are fatty acids in the form of TAG. A Very important factor with regard to fatty acids and lipid intake is the differences in degree of saturation and chain lengths of the individual fatty acids. Within unsaturated fatty acids there are many factors with importance with regard to health effects e.g. chain length, number of double bonds, cis/trans isomerism of double bond , chain branches, placement of the double bonds e.g. n-3, n-6, and n-9 unsaturated FAs. One of the most studied types of fatty acids is the long chained polyunsaturated fatty acids (LCPUFA) mainly found in fish. This is due to great potential for health promoting effects from intake, with regard to many different metabolic parameters (Leon *et al.*, 2008; Eslick *et al.*, 2009).

Several studies has pointed out that intake of saturated fatty acids (SFA) has negative effects with regard to development of metabolic related diseases, such as cardiovascular disease (Hu *et al.*, 1997; Steyn *et al.*, 2004) and type 2 diabetes (Steyn *et al.*, 2004). Trans-unsaturated fatty (tFA) acid is another type of fatty acids that have been shown to elicit disadvantages effects with regard to health (Brouwer *et al.*, 2010). As a consequence from the knowledge provided from these studies, the general recommendations have been to decrease intake of SFA and tFA (Vartiainen *et al.*, 2010). The common nutritional recommendations for the Nordic countries from 2004, advice that the sum of SFA and tFA should be not higher than 10 E% of the total energy intake, and total fat intake not higher than 25-35 E%. Cis-MUFA should comprise 10-15 E%, and PUFAs 5-10 E% including 1 E% n-3 PUFAs (Nordic Council of Ministers, 2005). As animal fat generally contain high levels of SFA, and fat from ruminant animals also tFA, it has also been generally recommended to reduce intake of animal fat, including dairy fat.

However, a number of studies have now indicated that intake of dairy fat might not be as harmful as earlier believed (Gibson *et al.*, 2009), or even that intake of dairy fat might have beneficial impacts in relation to cardiovascular and metabolic disorders (German *et al.*, 2009), and the effects of reduction in SFA intake has been questioned (Astrup *et al.*, 2011). The three latter references all question whether the common recommendations to avoid SFA have the intentional effect on among others CVD, and German *et al*. points on positive effects from dairy intake. The viewpoint from German *et al.* is supported by several studies showing negative correlations between validated biomarkers for milk-fat intake and cardiovascular and metabolic risk markers as well as incidence of the first myocardial infarction in case-control studies (Smedman *et al.*, 1999; Warensjo *et al.*, 2004; Biong *et al.*, 2006; Hodge *et al.*, 2007; Warensjo *et al.*, 2009; Warensjo *et al.*, 2010). Smedman *et al*. found inverse associations of milk product intake and metabolic risk markers e.g. BMI and fasting plasma glucose by 7 days dietary records for 72 men, and the studies by Warensjo *et al*. indicates positive effects on risk of developing CVD by dairy fat intake. Biong *et al*. suggest that intake of dairy fat or some component within dairy products, protect persons at risk against a first myocardial infarction (MI), and Hodge *et al*. shows that dietary saturated fat intake is inversely associated with diabetes risk. Thus, the metabolic effects of dairy fat intake seem to be positively modulated by other components than the major saturated fatty acids. In 2007 (revised in 2009) The Danish Dairy Board published a report by Lars Ovesen, in which studies about intake of milk and dairy products, and risk of disease gone through. The report came up with conclusion on positive effect by intake of dairy products on among others type 2 diabetes (Ovesen, 2009), on the background of four prospective studies regarding

dairy intake and risk of type 2 diabetes (Choi *et al.*, 2005; Liu *et al.*, 2006; van Dam *et al.*, 2006; Elwood *et al.*, 2007).
As in other nutritional fats, TAG is the far most dominating lipid class in milk fat, and constitutes more than 95 % of the total lipid fraction. Diacylglycerides (DAG) constitute around 2%, Phospholipids (PL) 1% (including sphingomyelin), and the rest of the lipid fraction is covered by cholesterol, cholesteryl esters, free fatty acids (FA), monoacylglycerides (MAG) and trace amounts of hydrocarbons (Jensen, 1973; Jensen, 2002). Milk fat is the most complex type of fat occurring in nature, with at present more than 400 different fatty acids (FA) identified, which gives an enormously amount of theoretical possible different TAGs, of which probably several thousands are present (Jensen, 2002). Several factors are associated with variations in the FA-composition of bovine milk. These factors could be divided into factors correlated to the animals, and factors correlated to the feed (Palmquist *et al.*, 1993; Jensen, 2002), why there is often large seasonal variations in the FA composition. Normally only 12 different FAs each constitute more than 1% of the total fatty acid mass in milk, and the three most dominating FAs are the SFAs myristic-, palmitic-, and stearic acid. MUFAs are also accounting for a relatively large share of the FAs in milk fat, but PUFA are found in small amounts (Jensen, 2002), that are often regarded as inconsiderable.

Several FAs are almost specifically found in dairy fat and some of them can therefore be used as markers for dairy fat intake, as done in several of the studies referred above. Pentadecanoic acid (15:0) and margaric acid (17:0), which cannot be synthesized in humans, have been regarded as valid biomarkers of dairy intake, though both of them are also found in lamb, beef, venison, and fatty fish (Baylin *et al.*, 2002; Hodson *et al.*, 2008; Iggman & Riserus, 2011). Tetradecanoic acid (14:0) level in adipose tissue has been considered a valid biomarker of long-term dairy intake, although it can also be synthesized endogenously (Warensjo *et al.*, 2004; Iggman & Riserus, 2011). *Trans*-fatty acids (tFA) are another type of FAs that are quite exclusive for dairy fat, and deduce around 5 mass % of total fat. The amount varies over the season, and is found to higher in summer-butter than winter-butter (Jakobsen *et al.*, 2006).

Among the huge pool of minor fatty acids present in milk fat, there are several that might be able to abolish some of the unhealthy effects of SFA and tFA. Minor FAs that might have protective effects, either alone or synergistically, could be short-chain fatty acids (SCFA) (Legrand *et al.*, 2010), conjugated linoleic acid (CLA) (Roche *et al.*, 2001), Trans vaccenic acid, and phytanic acid (Hellgren, 2010). CLA has gained a lot of attention in the recent years, as *in-vivo* models in animals has shown that CLA has an ability to increase muscle mass and decrease adipose tissue mass, as well as repressing tumor growth, atherogenisis and diabetes (Tricon *et al.*, 2005). CLA content in milk varies with feed, and intake of linoleic- and linolenic-acid, especially from grass, leads to increased amounts of CLA in milk (Dhiman *et al.*, 2005). In Denmark the content has been shown to increase from 0.37 m% at winter to 0.57 m% (mean values) of total milk fat in summer (Jakobsen *et al.*, 2006).
A study by Legrand *et al.* indicates that the saturated short chain fatty acids (SCFA) in dairy fat, has the ability to decrease circulating cholesterol and increase the content of tissue n-3 PUFA in the rat (Legrand *et al.*, 2010). Trans-palmitoleic acid has been proposed to have metabolic benefits and have protective function against diabetes (Mozaffarian *et al.*, 2010).

Phytanic acid
The branched-chain fatty acid phytanic acid (PA, 3, 7, 11, 15-tetramethyl hexadecanoic acid) is an interesting component of dairy fat, since it, as well as its primary metabolite pristanic acid (2,6,10,14-

tetramethylpentadecanoic acid), has strong agonistic activities on the nuclear receptors RXRα and PPARα (Kitareewan *et al.*, 1996; Zomer *et al.*, 2000; Heim *et al.*, 2002). PA originates from chlorophyll, from which it is cleaved by ruminant microbiota, and therefore found in dairy- and meat products of ruminant origin. PA has been suggested to have preventive effects on the development of metabolic disorders, due to the agonistic activity on RXR (Lemotte *et al.*, 1996; Kitareewan *et al.*, 1996), and PPARα, maybe through its primary metabolite pristanic acid (Gloerich *et al.*, 2005; Fluehmann *et al.*, 2007). PA and its potential as a bioactive FA were reviewed in 2010 (Hellgren, 2010). Most of these suggestions on preventive effects of PA, builds on studies performed with its precursor phytol, see Figure 4 (Gloerich *et al.*, 2005; Gloerich *et al.*, 2007), probably as is not nearly as expensive as PA. PA has been shown to activate transcription of uncoupling protein-1 (UCP1) and suggested to be a nutrient with potential as regulator of brown adipocyte differentiation and thermogenesis (Schluter *et al.*, 2002a), and pristanic acid has been excluded as the mediator of these positive effects on brown adipocyte differentiation (Schluter *et al.*, 2002b).

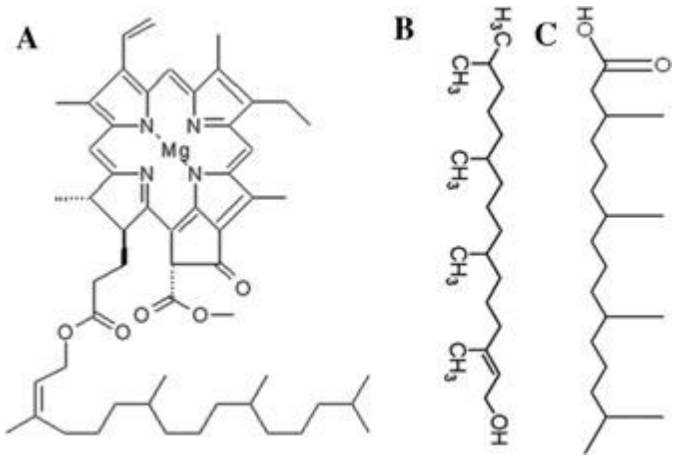

Figure 4 Structures of chlorophyll (A), phytol (B), and Phytanic acid (C)

Though PA, as well as phytol, are also associated with negative health effects. An increased level of phytanic acid in the body is toxic, so the fatty acid needs to be broken down (Gloerich *et al.*, 2007). Most important is its implication in Refsums disease, which is a disease with over-accumulation of PA, leading to among others neurologic damage and cerebellar degeneration. The reason for the increased PA accumulation in Refsums disease lies in the metabolism of PA. Before PA can enter β-oxidation, it has to undergo one round of α-oxidation, to form pristanic acid. An important enzyme in this process is phytanoyl-CoA hydroxylase (PhyH), and in general Refsums disease is caused by mutation in this enzyme (Verhoeven *et al.*, 1998). Only stereoisomers with the 2-methyl group in S-configuration can be degraded by β-oxidation. That makes α-methyl-CoA racemase (AMCAR), a very important enzyme in the oxidation process of PA, as it catalyses conversion of (2R)-methyl-branched-chain acyl-CoAs to the corresponding S-stereoisomers (Verhoeven & Jakobs, 2001). For further information on PA breakdown and metabolism of PA the articles (Verhoeven & Jakobs, 2001), as well as (van den Brink & Wanders, 2006), can be recommended.

Due to the agonistic activity towards RXR and PPARα, PA are also hypothesized to have effects in systemic inflammation, as other ligands for these nuclear receptors have been shown to impact the polarization of T-cells. Both RXR and PPARα specific ligands have been shown to repress IFN-γ (fundamental Th1 marker)and IL-17 (essential marker of Th17 cells), to increase expression of IL-4 in T-cells, reflecting a Th2-polarization of the T-cell response (Cunard *et al.*, 2002; Straus & Glass, 2007; Lee *et al.*, 2007; Hwang *et al.*, 2009). Additionally, PPARα activation seems to be involved in differentiation of T_{regs} (Dubrac *et al.*, 2011). Th1 cells are generally believed to induce pro-inflammatory immune responses, as they improve activation of cytotoxic $CD8^+$ T-cells by dendritic cells in lymph nodes, and moreover, activate macrophages in peripheral tissues in an antigen-dependent manner. $CD8^+$ T-cells mediate cytolytic effects towards infected cells within all body sites. Th2 cells, on the other hand, are involved in the humoral immune system via its essential role in propagation of B-cells to become plasma cells (producing antibodies)(Munder *et al.*, 1999; Spilianakis *et al.*, 2005; Hwang *et al.*, 2009). Th17 cells were initially described as a cellular T-cell subset implicated in autoimmunity, but are now thought to have their own distinct effector and regulatory functions, mainly mediating activation of neutrophils, controlling extracellular microbes through phagocytosis, hence being involved in balancing the number of microbes at mucosal tissue sites. However, Th17 cells can be highly proinflammatory and induce severe autoimmunity (Harrington *et al.*, 2005; Bettelli *et al.*, 2007). T_{reg}´s are crucial for maintenance of immunological tolerance. Their main role is to shut down T-cell mediated immunity towards the end of an immune reaction(Mills, 2004; Du *et al.*, 2005; Belkaid & Rouse, 2005). For functions of the cytokines relevant for the current studies, see Table 3.

Table 3 Biological functions and roles of T-cell polarizing cytokines of relevance for the studies within this thesis.

Cytokine	T-cell producers	Functions	References
IFN-γ	Th1, $CD8^+$, NKT, γδT-cells	Activates macrophages and natural killer (NK) cells. Role in delayed type hypersensitivity. Represses Th2 and Th17 differentiation. Promotes Th1 differentiation. Central for cell-mediated immunity	(Kwok *et al.*, 1993; Billiau, 1996; Smyth & Godfrey, 2000; Ansel *et al.*, 2003; Auphan-Anezin *et al.*, 2003; Korn *et al.*, 2009)
IL-4	Th2, NKT, γδT-cells	Promotes B cell proliferation, differentiation, and immunoglobulin isotype switching to IgE. Negative regulator of Th1. Promotes Th2 differentiation	(Brown & Hural, 1997; Smyth & Godfrey, 2000; Ansel *et al.*, 2003)
IL-10	T_{regs} Th1, Th2, Th17	Anti-inflammatory. Regulates growth and/or differentiation of B cells, NK cells, $CD8^+$ T-cells and Th-cells. Negative regulator of Th1 by inhibition of IFN-γ.	(Billiau, 1996; Smyth & Godfrey, 2000; Moore *et al.*, 2001; Xu *et al.*, 2009)
IL-17	Th17, NKT, γδT-cells	Antimicrobial and antifungal. Activates neutrophils Induces tissue inflammation. IL-17 does not act as a growth or differentiation factor for Th17. A reciprocal relationship between T_{regs} and Th17 exists.	(Korn *et al.*, 2009; Onishi & Gaffen, 2010)

Nutritional sources of phytanic acid

Humans and most other mammals are not able to convert chlorophyll into phytol, but the diverse microbiota in the rumen of ruminal animals is able to carry out this reaction. Hence, the human intake of phytol and PA originates form ruminal animal sources, such as dairy and meat products. There have not been many studies on phytol and PA contents in dairy products and even less in meat products. Though, due to the implication in Refsums disease some has been carried out. Most thoroughly is the study by Brown *et al.* from 1993, as they list the content of PA and phytol in many different food sources (Brown *et al.*, 1993). More recently the group of Vetter has performed studies on day to day variations in PA and pristanic acid content, and showed that the content is higher in organic than conventional dairy products on the German market (Vetter & Schroeder, 2010; Schroder *et al.*, 2011). Their finding that organic dairy products, which may be assumed to originate from cattle fed high amounts of green plant material, contain more PA than conventional dairy products, correlate with studies on PA content in milk from 72 Nordic herds, performed by our self (unpublished data), showing that organic and grazing cattle generally has higher PA content in their milk than conventional fed cattle. A frequency distribution of the PA content in milk from these herds (plus data from seven Danish butters), can be seen from Figure 5.

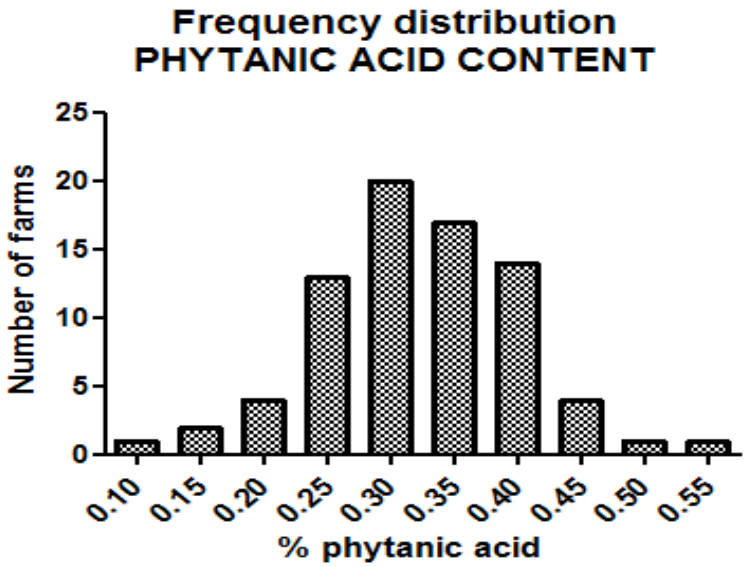

Figure 5 Frequency distribution of milk phytanic acid content. Frequency distribution of phytanic acid in milk fat from Swedish, Norwegian, and Danish farms, shown as mass% of total fat fraction, n=79.

A study from 2005 found that PA in milk from alpine cattle is raised to around 0.5 m% of fat fraction, in the grazing period of the year (Leiber *et al.*, 2005), which is just about the same amounts we measured in milk from grazing Nordic cattle. In addition a study from 1977, shows that feeding steers grass ensilage leads to a raise in their plasma PA content (Lough, 1977).

Even though not much data are avaible on PA content in various ruminant meat products, it may be assumed that both intake of ruminant dairy and meat products, have significant impact on the total PA intake of humans.

Results and Discussion

Dairy fat intake and metabolic consequences compared to intake of other fat types

Due to recent indications that dairy fat is more benign in relation to metabolic diseases (German *et al.*, 2009), than earlier thought, our hypothesis was that one or more of the minor fatty acids found in dairy fat, counteract the impairment of metabolic status caused by SFA and tFA, maybe due to synergistic effects. As the FA composition in dairy fat is dependent on among others, feeding regime we wanted to compare, effects from intake of dairy fat from cattle under different feeding regimes. As we know that the amount of green plant material in feed, e.g. grass, is an important factor with regard to the FA composition (Leiber *et al.*, 2005), this was one of our focus points. Furthermore it is important to compare intake of dairy fat against intake of other major dietary fat types. Therefore we, compared butter intake, in obese mice, with intake of oleic acid (MUFA), and linoleic acid (PUFA), as they represent the two major FA types recommended to be used as substitutes for SFA in the diet.

What we saw from the 10 weeks study on fat type in DIO mice, were that oleic acid, compared to butter and linoleic acid, had the most detrimental effects on glucose homeostasis and hepatic lipid metabolism. We observed increased hepatic ceramide deposition, a tendency to increased hepatic TAG, impaired glucose uptake, and up regulation of lipogenic genes, for mice fed a diet with high oleic acid content. Both the oleic acid and the linoleic based diets had statistical significant higher hepatic gene expression of FAS, than the two butter diets. But, as mentioned it only led to increased hepatic lipid deposition for the oleic acid fed group. Butter-based diets had the most detrimental effects on the blood-lipid parameters, observed as raised TAG, and total cholesterol, compared to high fat diets based on oleic and linoleic acid, and lean reference.

The results with regard to detrimental effects by intake of oleic acid are controversial, as the general assumption is that MUFA is a healthier alternative to SFA-rich products, and the recommendation is to replace SFA with MUFA or PUFA. Oleic acid is by far the most abundant MUFA in the human diet. It has been proposed that MUFAs protect against metabolic syndrome and CVD, as MUFA should promote healthy blood lipid profiles, mediate blood pressure, improve insulin sensitivity and regulate glucose levels (Gillingham *et al.*, 2011). This is not equivalent with the results we observed in DIO mice. An expert panel has reviewed the literature with regard to prevention of CVD by replacement of SFA with other nutrients. They concluded that evidence is strong that risk of coronary heart disease (CHD) is reduced when SFAs are replaced with PUFAs. They conclude that replacement of 1 E% SFA from SFA to PUFA, in populations who consume a Western diet, lowers LDL cholesterol and is likely to reduce CDH incidence of ≥2-3 %. Furthermore, they conclude that there is insufficient evidence to judge whether SFA replacement with MUFA, has an effect on CHD risk, and that there are no clear benefit of substituting carbohydrates for SFA. They also conclude that no clear association between SFA intake relative to carbohydrates and the risk of insulin resistance and diabetes has been shown (Astrup *et al.*, 2011).

With regard to dairy intake and development of obesity there are generally no indications that these are correlated, though factors such as calcium content (Parikh & Yanovski, 2003), and as within the scope of current thesis, the content of specific fatty acids such as CLA (Roche *et al.*, 2001), has been proposed to have positive effects on the body composition (Ovesen, 2009).

Four prospective studies concerning dairy intake and risk of type 2 diabetes has been published. The three largest ones (Choi *et al.*, 2005; Liu *et al.*, 2006; van Dam *et al.*, 2006), all distinguish between high and low fat dairy intake, and conclude that there is negative correlation between intake of low fat dairy products and the risk of type 2 diabetes. The last of the four studies conclude that intake of milk and dairy products is associated with a markedly reduced prevalence of MS, but that milk intake showed no significant trend with regard to diabetes incident (Elwood *et al.*, 2007). The positive effect on risk of type 2 diabetes, seen from low fat dairy product intake in the three studies first mentioned, is not necessarily due to effects from dairy fat, but might as well be due to other nutrients in the dairy products. A Swedish study from 2008, has shown that increased C15:0 and C17:0 FAs, which is common markers of dairy fat intake, in erythrocyte membranes, is accompanied by increased risk of type 2 diabetes (Krachler *et al.*, 2008). Though, our data from DIO mice does not indicate that dairy fat is more harmful with regard to glucose homeostasis than other fat types.

From our studies in mice, we do not see clear advantages from intake of the PUFA linoleic acid, on the measured parameters, compared to intake of butter. Though, it is very important to remember that there are many different PUFAs, and the effects from intake of these might differ a lot, and that other studies has shown very clear effects from intake of other PUFAs e.g. fish oils. Important factors are among others n-3/n-6 distribution, chain lengths, and number of double bonds. Our results that shows raised plasma lipids, TAG and total cholesterol for the butter groups compared to the other obese groups, is in line with what have been shown in other studies, and has been proposed to be mediated through PGC-β and SREBPs (Lin *et al.*, 2005). Another observation from the fat type intervention in DIO mice were substantially higher amount of EPA in hepatic phospholipids fraction of the butter groups compared to the other groups. Furthermore, the grazing butter group had statistical significant higher amount of EPA than the conventional butter group. This is explained by the linoleic acid (LA): α-linolenic acid (ALA) ratio, as seen from Figure 6.

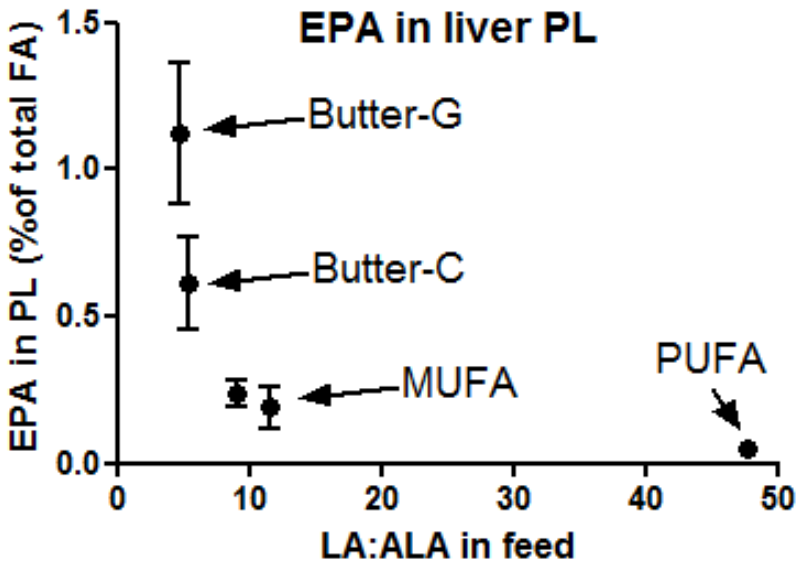

Figure 6 Hepatic EPA as function depend on LA: ALA ration. EPA content in hepatic posholipids (PL) vs. linoleic acid (LA): α-linolenic acid (ALA) ratio in the feed, G: Grazing, C: conventional, the unmarked group is lean references.

A very important factor to take into account when comparing intake of different fat types, is how it is combined with intake of other nutrients, as there might be synergistic effects from intake of different macronutrients. It has been shown that sucrose counteracts some of the beneficial effects from fish oil (Ma *et al.*, 2011), which indicates that sucrose intake combined with fat intake has aggravating effects no matter the type of fat. It is also important not to forget that lack of physical activity and excess energy intake in general, is very important factors with regard to development of obesity, and the diseases that follow. As, lipids is energy dense compared to other nutritional components, limiting intake of lipids in general seems as an apparent way to restrict excess energy intake, hence, it is both lipid quantity and quality in the diet that are relevant.
From our study on T-cell polarization it seems that increased butter intake, has the ability to change the expression pattern of IL-4 and IFN-γ, into a higher ratio of IL-4, indicating increased polarization towards Th2 as an effect of increased butter intake. It did not seem to be a PA mediated effect as we first hypothesized. Hence, we currently do not know the mechanism behind.
From the combined results, of the butter intervention in mice, and the human interventions, it does not seem that the production form or amount of green feed given to cows has clear significant effects on metabolic parameters upon intake of dairy fat. Though, we found that plasma insulin and LDL cholesterol was raised due to intake of butter from mountain-pasture grazing cattle in the second human intervention, which were not the case due to intake of conventional produced butter.

Phytanic acid intake and metabolic effects

From the compiled data on phytanic acid effects, from the studies within this thesis, it does not seem that PA deduce the hypothesized effects with regard to metabolic risk factors and T-cell polarization, which could have been expected due to its agonist activity towards the nuclear receptors PPARα and RXR. Though, we did see effects on glucose homeostasis, as well as interesting effect on hepatic TAG deposition due to addition of 0.25% PA to GSO based diet in obese mice, and minor effects on expression of genes involved in hepatic lipid metabolism.

From the study on PA intake in obese mice, the most conspicuous effect was observed in the study with addition of 0.25% pa to GSO based high energy diet, as PA addition aggravated the negative effect on hepatic TAG deposition from the high fat diet, even though total body and WAT were not affected by addition of PA. Both amount of hepatic TAG and total liver mass, were statistical significant higher for the group given GSO based diet without PA, compared to lean references, which were not the case when 0.25 % PA was added. This effect of PA were not observed in the butter based diets, though we do not know if it would have been the case, if we had been able to include a butter based diet without any PA.

So far, we have only gene expression data on hepatic lipid metabolism, from the PA dose response in butter based diet. From this we saw statistical significant up regulation of PPARα, and the PPARα dependent ACOX1 gene, in the group given 1.0 % PA, compared to the group given 0.2 % PA ($P<0.01$). This is not surprising, due to the agonistic activity of PA towards PPARα, as we also clearly showed that PA is up taken

and incorporated into the hepatic PL and TAG fractions. Other PPARα target genes (CD36 and CPT1b) were strongly affected by the DIO-diet, but not significantly up regulated by the PA-intake. This shows us that the PPARα activation by PA was not as strong as the effects from the high fat diet on these two genes. The genes coding for proteins controlling fatty acid synthesis (SREBP1 and FASN) was statistical significant up regulated in all the obese groups compared to lean references, without any effect of PA. The transcription of the glucose-6-phosphatase (G6Pase) gene was determined as an example of an insulin-regulated gene. On average did all three DIO-treatments cause increased expression, although only the increase in the group that received 0.6% PA reached statistical significant difference. The relative hepatic gene expression of α-methylacyl-CoA racemase (AMCAR) was statistically significant reduced for the 0.2 % and the 0.6 % PA groups compared to the lean references ($P<0.05$ and $P<0.01$, respectively), while this was not the case for the 1.0 % PA group. This indicates that high fat diet might impair the oxidation of PA, but that PA in a certain amount can counteract the down regulation of AMCAR gene expression, that seems impaired by the high fat diet. Noteworthy, the average expression of the macrophage marker EMR1 (F4/80), was, on average, increased in the three DIO-groups, but this did only reach significance in the 1 % PA group. This might be interpreted as a tendency to increased hepatic macrophage infiltration due to PA, and thereby a negative feature of PA, though it rather seems to be a general effect from high fat diet, that are only slightly increased by PA.

In the first human intervention, dairy fat from two different feeding regimes of cattle from same herd was compared upon human intake. The herd was divided into two groups, and fed different amounts of green plant material, resulting in among others increased content of PA in dairy fat from cattle given highest amount of green plant material. We did not see any effects on markers of the metabolic syndrome, due to the intake of cheese and butter from either of the types of dairy fat. Neither, did we see any differences in plasma PA concentrations among the two groups. What we did see, and which is important with regard to our other studies with PA, was that regardless the feeding regime and the minor differences in PA content, there was an increase in plasma PA concentrations after four weeks intervention. This is the main contribution from study D, with regard to the understanding of PAs potential as nutritional regulator of nuclear receptors.

An interesting finding from the baseline data for the subjects from the second human intervention, was that plasma phytanic acid correlated strongly with both total cholesterol ($P<0.0001$), and LDL cholesterol ($P=0.0001$). In this intervention, we compared metabolic effects due to intake of butter from grazing cattle and conventional fed cattle. The grazing cattle are a low yield production form compared to the conventional production. The butter from grazing cattle had among others increased PA content. We observed increased insulin levels for the group of subjects given butter from grazing cattle, than for the group given conventional butter. This could be interpreted as an indication that PA in physiological concentrations might have a negative effect on insulin sensitivity in humans, though it could also be due to other factors e.g. other FAs that differ between the two groups. This indication does not seem to be confirmed by the results from our mice studies. Though, we found that intake of artificially added high amounts in a butter based diet, decreases insulin sensitivity in mice significantly. This might be due to same mechanisms, even though we cannot explain how it is conducted. If it is the case that increased intake of PA leads to decreased insulin sensitivity in humans, it is of course a negative property, and indicate that increased PA concentration is a negative side effects from green feeding of cattle, at least for patients suffering from Refsums disease. Though, it is important to remember that the negative effect we observed

in obese mice, were with PA concentration almost double than what is observed in dairy fat from grazing cattle. However, it does not change the fact, that several studies have shown beneficial correlations of dairy fat markers and risk of various metabolic disorders (Gibson *et al.*, 2009; German *et al.*, 2009). Our studies does not indicate that PA based nutraceuticals has a bright future as an effective treatment against e.g. impaired glucose tolerance, as it has been claimed by patents (Eggersdorfer *et al.*, 2004; Fluehmann *et al.*, 2007), though we did see the interesting effects on hepatic TAG deposition from PA addition to PUFA high fat GSO diet.
As no other no other studies has been published, regarding *in vivo* effects of PA in concentrations similar to concentrations found in food products, we cannot compare our findings directly to other studies. Though, studies with the PA precursor phytol have been published, however, using phytol concentrations higher than what is accessible through food products. In our own lab, addition of 0.2% and 0.5% phytol of total fat mass to normal chow diet for four weeks, substantially decreased hepatic TAG in mice (Hellgren, 2010). Other groups has shown that addition of 0.5% phytol upregulates gene expression and enzyme activities of both peroxisomal and mitochondrial FA oxidation (Gloerich *et al.*, 2005; Hashimoto *et al.*, 2006). A study using 0.5 and 1.0% phytol for twelve to eighteen days, showed that phytol induce hepatotoxicity beside causing typical PPAR-α mediated effects such as decreased body mass and increased liver mass in mice (Mackie *et al.*, 2009). As the amounts of phytol used in these studies is way higher than what is physiological possible to get through diet, those results does not seem relevant with regard to a normal human diet.

As mentioned in the discussion of butter effects, we did not see the hypothesized effects on cytokine expression in human CD3+ T-cells. Hence, it does not seem that PA has the ability to affect the T-cell polarization in direction of Th_2, which we hypothesized due to PAs agonist activity towards PPARα and RXR, as other ligands of these receptors has such effects(Marx *et al.*, 2002; Lovett-Racke *et al.*, 2004; Du *et al.*, 2005; Stephensen *et al.*, 2007).

Future perspectives

The results found with regard to intake of high fat oleic acid rich diet, compared to butter end linoleic acid based diets, is quite controversial and against the common recommendations. Therefore it seems necessary to do further studies in which oleic acid is compared to other fat types, either to confirm or decline our findings.

As we have only compared dairy fat intake towards other fat types in obese mice, we do not know whether the effects from dairy fat, oleic acid, and linoleic acid, respectively, compared to each other, would also be found in similar human interventions. As we have only compared different types of butter against each other, it might be interesting to test whether the differences in between different fat types observed in mice, could also be found due to human interventions.

Due to the effects observed on hepatic TAG deposition and liver weight in the study on PA in GSO based diet, it is of course a natural step to measure the gene expression of selected genes from the hepatic lipid metabolism. This planned to be done, as we have hepatic tissue stored in appropriate manner. Furthermore it would be interesting to test whether the addition of PA to the feed affects the thermogenesis, and UCP-1 expression in BAT *in vivo*, due to the studies by Scluter *et al.* (Schluter *et al.*, 2002a; Schluter *et al.*, 2002b).

With regard to the change we observed in CD3+ T-cell cytokine expression, we observed due to the butter intervention, we do at this point not know the mechanisms behind, as PA did not seem to have the hypothesized effect compared to palmitic acid. First step could be so check whether the observed changes correlates to any other data on the subjects, and depending on these results further steps might be taken to find out the mechanism behind.

Conclusion

In the mice feeding study, in which butter intake were compared to diets high in oleic acid and linoleic acid, we saw that oleic acid, compared to butter and linoleic acid rich diets, have more detrimental effects with regard to glucose homeostasis and hepatic lipid metabolism, as we observed increased hepatic TAG and -ceramide deposition, impaired glucose uptake and up regulation of lipogenic genes, for the mice fed high amounts of oleic acid. Butter-based diets had the most detrimental effects on the blood-lipid parameters, observed as raised triacylglycerol (TAG), and total cholesterol (chol). The only significant difference we observed between the two butter fed groups, where that, mice given butter from grazing cattle had significantly higher hepatic phospholipid EPA levels, than mice given butter from cattle fed lower amounts of green plant material.

The PA acid study in mice revealed that PA is incorporated to the hepatic lipid pool. For the phospholipid fraction it was in a dose-pendent response, which was not the case with regard to TAG and NEFA. Furthermore we saw that animals given grape seed oil based diet, did not have any PA in the hepatic lipid fractions.

We observed a change in the polarization of activated human CD3+ T-cells due to 12 weeks intervention with intake of 39 g butter/day, measured as raised IL-4: IFN-γ gene expression ratio. No difference in this effect was observed between butter form grazing or conventional fed cattle. Neither did we observe any effect on expression of fundamental cytokine markers due to phytanic acid incubation.

From two human dairy fat interventions the most interesting findings were, increased plasma phytanic acid concentrations due to four weeks intervention, and tendencies to increased plasma insulin and cholesterol in subjects eating butter from grazing cattle for 12 weeks, compared to subjects eating butter from conventional fed cattle. Baseline values of plasma phytanic acid correlated strongly to total- and LDL-cholesterol in the second intervention.

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Other publications by the thesis author

Muscle ceramide content is similar after 3 weeks consumption of fat or carbohydrate diet in a crossover design in patients with type 2 diabetes
J. W. Helge, L.Tobin, **T. Drachmann,** L. I. Hellgren, F. Dela and H. Galbo
European Journal of Applied Physiology-in print

Improved glucose tolerance after intensive life style intervention occurs without changes in muscle ceramide or triacylglycerol in morbidly obese subjects
Helge JW, Stallknecht B, **Drachmann T**, Hellgren LI, Jimenez-Jimenez R, Andersen JL, Richelsen B, Bruun JM
Acta Physiol, 2010

Ceramides and barrier function in healthy skin
Jungerstedt J, Hellgren LI, **Drachmann T**, Hoegh JG, Jemec GBE, Agner T
Acta Derm Venereol, 90: 350-353, 2010

Validation of Cyanoacrylate Method for Collection of Stratum Corneum in Human Skin for Lipid Analysis
Jungersted JM, Hellgren LI, **Drachmann T**, Jemec GBE, Agner T
Skin Pharmacology and Physiology, 23:62-67, 2010

The source of dietary fatty acids alter the activity of secretory sphingomyelinase in the rat
Drachmann T, Mathiassen JH, Pedersen MH, Hellgren LI
European Journal of Lipid Science and Technology, 109:1003-9, 2007

References

Reference List

Adiels M, Olofsson SO, Taskinen MR & Boren J (2008) Overproduction of very low-density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. *Arteriosclerosis Thrombosis and Vascular Biology* **28**, 1225-1236.

Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WPT, Loria CM & Smith SC (2009) Harmonizing the Metabolic Syndrome A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* **120**, 1640-1645.

Amri EZ, Bonino F, Ailhaud G, Abumrad NA & Grimaldi PA (1995) Cloning of A Protein That Mediates Transcriptional Effects of Fatty-Acids in Preadipocytes - Homology to Peroxisome Proliferator-Activated Receptors. *Journal of Biological Chemistry* **270**, 2367-2371.

Ansel KM, Lee DU & Rao A (2003) An epigenetic view of helper T cell differentiation. *Nature Immunology* **4**, 616-623.

Astrup A, Dyerberg J, Elwood P*, et al.* (2011) The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: where does the evidence stand in 2010? *American Journal of Clinical Nutrition* **93**, 684-688.

Auphan-Anezin N, Verdeil G & Schmitt-Verhulst AM (2003) Distinct thresholds for CD8 T cell activation lead to functional heterogeneity: CD8 T cell priming can occur. independently of cell division. *Journal of Immunology* **170**, 2442-2448.

Baylin A, Kabagambe EK, Siles X & Campos H (2002) Adipose tissue biomarkers of fatty acid intake. *American Journal of Clinical Nutrition* **76**, 750-757.

Belkaid Y & Rouse BT (2005) Natural regulatory T cells in infectious disease. *Nature Immunology* **6**, 353-360.

Berger JP, Akiyama TE & Meinke PT (2005) PPARs: therapeutic targets for metabolic disease. *Trends in Pharmacological Sciences* **26**, 244-251.

Bettelli E, Oukka M & Kuchroo VK (2007) T-H-17 cells in the circle of immunity and autoimmunity. *Nature Immunology* **8**, 345-350.

Billiau A (1996) Interferon-gamma in autoimmunity. *Cytokine and Growth Factor Reviews* **7**, 25-34.

Biong AS, Veierod MB, Ringstad J, Thelle DS & Pedersen JI (2006) Intake of milk fat, reflected in adipose tissue fatty acids and risk of myocardial infarction: a case-control study. *European Journal of Clinical Nutrition* **60**, 236-244.

Bloomgarden ZT (2011) Type 2 diabetes: uses of thiazolidinediones and insulin. *Diabetes Care* **34**.

Bouhlel MA, Brozek J, Derudas B, Zawadzki C, Jude B, Staels B & Chinetti-Gbaguidi G (2009) Unlike PPAR gamma, PPAR alpha or PPAR beta/delta activation does not promote human monocyte differentiation toward alternative macrophages. *Biochemical and Biophysical Research Communications* **386**, 459-462.

Brouwer IA, Wanders AJ & Katan MB (2010) Effect of Animal and Industrial Trans Fatty Acids on HDL and LDL Cholesterol Levels in Humans - A Quantitative Review. *Plos One* **5**.

Brown MA & Hural J (1997) Functions of IL-4 and control of its expression. *Critical Reviews in Immunology* **17**, 1-32.

Brown PJ, Mei G, Gibberd FB, Burston D, Mayne PD, Mcclinchy JE & Sidey M (1993) Diet and Refsums Disease - the Determination of Phytanic Acid and Phytol in Certain Foods and the Application of This Knowledge to the Choice of Suitable Convenience Foods for Patients with Refsums Disease. *Journal of Human Nutrition and Dietetics* **6**, 295-305.

Chavez JA, Knotts TA, Wang LP, Li GB, Dobrowsky RT, Florant GL & Summers SA (2003) A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *Journal of Biological Chemistry* **278**, 10297-10303.

Choi HK, Willett WC, Stampfer MJ, Rimm E & Hu FB (2005) Dairy consumption and risk of type 2 diabetes mellitus in men - A prospective study. *Archives of Internal Medicine* **165**, 997-1003.

Corcoran MP, Lamon-Fava S & Fielding RA (2007) Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise. *American Journal of Clinical Nutrition* **85**, 662-677.

Cornier MA, Dabelea D, Hernandez TL, Lindstrom RC, Steig AJ, Stob NR, Van Pelt RE, Wang H & Eckel RH (2008) The Metabolic Syndrome. *Endocrine Reviews* **29**, 777-822.

Cunard R, Ricote M, DiCampli D, Archer DC, Kahn DA, Glass CK & Kelly CJ (2002) Regulation of cytokine expression by ligands of peroxisome proliferator activated receptors. *Journal of Immunology* **168**, 2795-2802.

D'Agostino RB, Hamman RF, Karter AJ, Mykkanen L, Wagenknecht LE & Haffner SM (2004) Cardiovascular disease risk factors predict the development of type 2 diabetes - The Insulin Resistance Atherosclerosis Study. *Diabetes Care* **27**, 2234-2240.

Dawson HD, Collins G, Pyle R, Key M & Taub DD (2008) The Retinoic Acid Receptor-alpha mediates human T-cell activation and Th2 cytokine and chemokine production. *Bmc Immunology* **9**.

Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, Fruchart JC, Tedgui A, Haegeman G & Staels B (1999) Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappa B and AP-1. *Journal of Biological Chemistry* **274**, 32048-32054.

Desvergne B (2007) *RXR: From partnership to leadership in metabolic regulations*. SAN DIEGO: ELSEVIER ACADEMIC PRESS INC.

Desvergne B & Wahli W (1999) Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocrine Reviews* **20**, 649-688.

Dhiman TR, Nam SH & Ure AL (2005) Factors affecting conjugated linoleic acid content in milk and meat. *Critical Reviews in Food Science and Nutrition* **45**, 463-482.

Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD & Parks EJ (2005) Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *Journal of Clinical Investigation* **115**, 1343-1351.

Du X, Tabeta K, Mann N, Crozat K, Mudd S & Beutler B (2005) An essential role for Rxr alpha in the development of Th2 responses. *European Journal of Immunology* **35**, 3414-3423.

Dubrac S, Elentner A, Schoonjans K, Auwerx J & Schmuth M (2011) Lack of IL-2 in PPAR-alpha-deficient mice triggers allergic contact dermatitis by affecting regulatory T cells. *European Journal of Immunology* **41**, 1980-1991.

Eggersdorfer ML, Raederstorff D, Teixeira SR, Weber P, Ludwig EM, Daniel R, Renata TS & Peter W (2004) Nutraceutical compositions, useful to treat/prevent diabetes and other conditions associated with impaired glucose tolerance, comprise biotin and pantethine, epigallocatechin gallate, phytanic acid, lipoic acid and/or policosanol. [BV DSM IP ASSETS, ML Eggersdorfer, D Raederstorff, SR Teixeira, and P Weber, editors].

Elwood PC, Pickering JE & Fehily AM (2007) Milk and dairy consumption, diabetes and the metabolic syndrome: the Caerphilly prospective study. *Journal of Epidemiology and Community Health* **61**, 695-698.

Eslick GD, Howe PRC, Smith C, Priest R & Bensoussan A (2009) Benefits of fish oil supplementation in hyperlipidemia: a systematic review and meta-analysis. *International Journal of Cardiology* **136**, 4-16.

Evans RM, Barish GD & Wang YX (2004) PPARs and the complex journey to obesity. *Nature Medicine* **10**, 355-361.

Fleischman A, Shoelson SE, Bernier R & Goldfine AB (2008) Salsalate Improves Glycemia and Inflammatory Parameters in Obese Young Adults. *Diabetes Care* **31**, 289-294.

Fluehmann B, Heim M, Hunziker W*, et al.* (2007) New composition comprising phytanic acid or its derivatives, useful for treating or preventing non-insulin dependent diabetes mellitus, impaired glucose tolerance and related obesity. [AG ROCHE VITAMINS, HOFFMANN LA ROCHE & CO AG, ROCHE VITAMINS INC, FOUN BUR RES, DSM NUTRITIONAL PROD INC, and BV DSM IP ASSETS, editors].

German JB, Gibson RA, Krauss RM, Nestel P, Lamarche B, van Staveren WA, Steijns JM, de Groot LCPG, Lock AL & Destaillats F (2009) A reappraisal of the impact of dairy foods and milk fat on cardiovascular disease risk. *European Journal of Nutrition* **48**, 191-203.

Gibson RA, Makrides M, Smithers LG, Voevodin M & Sinclair AJ (2009) The effect of dairy foods on CHD: a systematic review of prospective cohort studies. *British Journal of Nutrition* **102**, 1267-1275.

Gillingham LG, Harris-Janz S & Jones PJH (2011) Dietary Monounsaturated Fatty Acids Are Protective Against Metabolic Syndrome and Cardiovascular Disease Risk Factors. *Lipids* **46**, 209-228.

Gloerich J, van den Brink DM, Ruiter JPN, van Vlies N, Vaz FM, Wanders RJA & Ferdinandusse S (2007) Metabolism of phytol to phytanic acid in the mouse, and the role of PPAR alpha in its regulation. *Journal of Lipid Research* **48**, 77-85.

Gloerich J, van Vlies N, Jansen GA, Denis S, Ruiter JPN, van Werkhoven MA, Duran M, Vaz FM, Wanders RJA & Ferdinandusse S (2005) A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPAR alpha-dependent and -independent pathways. *Journal of Lipid Research* **46**, 716-726.

Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS & Klein S (2009) Endoplasmic Reticulum Stress Is Reduced in Tissues of Obese Subjects After Weight Loss. *Diabetes* **58**, 693-700.

Guri AJ, Hontecillas R & Bassaganya-Riera J (2006) Peroxisome proliferator-activated receptors: Bridging metabolic syndrome with molecular nutrition. *Clinical Nutrition* **25**, 871-885.

Harding HP & Ron D (2002) Endoplasmic reticulum stress and the development of diabetes - A review. *Diabetes* **51**, S455-S461.

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM & Weaver CT (2005) Interleukin 17-producing CD4(+) effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology* **6**, 1123-1132.

Hashimoto T, Shimizu N, Kimura T, Takahashi Y & Ide T (2006) Polyunsaturated fats attenuate the dietary phytol-induced increase in hepatic fatty acid oxidation in mice. *Journal of Nutrition* **136**, 882-886.

Heim M, Johnson J, Boess F, Bendik I, Weber P, Hunziker W & Fluhmann B (2002) Phytanic acid, a natural peroxisome proliferator-activated receptor agonist, regulates glucose metabolism in rat primary hepatocytes. *Faseb Journal* **16**, 718-+.

Hellgren LI (2010) *Phytanic acid-an overlooked bioactive fatty acid in dairy fat?* OXFORD: BLACKWELL PUBLISHING.

Hodge AM, English DR, O'Dea K, Sinclair AJ, Makrides M, Gibson RA & Giles GG (2007) Plasma phospholipid and dietary fatty acids as predictors of type 2 diabetes: interpreting the role of linoleic acid. *American Journal of Clinical Nutrition* **86**, 189-197.

Hodson L, Skeaff CM & Fielding BA (2008) Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Progress in Lipid Research* **47**, 348-380.

Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC & Matsuzawa Y (2001) Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes* **50**, 1126-1133.

Hu FB, Stampfer MJ, Manson JE, Rimm E, Colditz GA, Rosner BA, Hennekens CH & Willett WC (1997) Dietary fat intake and the risk of coronary heart disease in women. *New England Journal of Medicine* **337**, 1491-1499.

Hwang SS, Kim YU, Lee W & Lee GR (2009) Differential Expression of Nuclear Receptors in T Helper Cells. *Journal of Microbiology and Biotechnology* **19**, 208-214.

Iggman D & Riserus U (2011) Role of different dietary saturated fatty acids for cardiometabolic risk. *Clinical Lipidology* **6**, 209-223.

Jakobsen MU, Bysted A, Andersen NL, Heitmann BL, Hartkopp HB, Leth T, Overvad K & Dyerberg J (2006) Intake of ruminant trans fatty acids in the Danish population aged 1-80 years. *European Journal of Clinical Nutrition* **60**, 312-318.

Jensen RG (1973) Composition of Bovine Milk Lipids. *Journal of the American Oil Chemists Society* **50**, 186-192.

Jensen RG (2002) The composition of bovine milk lipids: January 1995 to December 2000. *Journal of Dairy Science* **85**, 295-350.

Jones DC, Ding XH & Daynes RA (2002) Nuclear receptor peroxisome proliferator-activated receptor a (PPAR alpha) is expressed in resting murine lymphocytes - The PPAR alpha in T and B lymphocytes is both transactivation and transrepression competent. *Journal of Biological Chemistry* **277**, 6838-6845.

Juurinen L, Tiikkainen M, Hakkinen AM, Hakkarainen A & Yki-Jarvinen H (2007) Effects of insulin therapy on liver fat content and hepatic insulin sensitivity in patients with type 2 diabetes. *American Journal of Physiology-Endocrinology and Metabolism* **292**, E829-E835.

Kang S, Bajnok L, Longo KA, Petersen RK, Hansen JB, Kristiansen K & MacDougald OA (2005) Effects of wnt signaling on brown adipocyte differentiation and metabolism mediated by PGC-1 alpha. *Molecular and Cellular Biology* **25**, 1272-1282.

Kersten S, Desvergne B & Wahli W (2000) Roles of PPARs in health and disease. *Nature* **405**, 421-424.

Kido Y, Nakae J & Accili D (2001) Clinical review 125 - The insulin receptor and its cellular targets. *Journal of Clinical Endocrinology & Metabolism* **86**, 972-979.

Kitareewan S, Burka LT, Tomer KB*, et al.* (1996) Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. *Molecular Biology of the Cell* **7**, 1153-1166.

Komatsu M, Tong YH, Li YF*, et al.* (2010) Multiple roles of PPAR alpha in brown adipose tissue under constitutive and cold conditions. *Genes to Cells* **15**, 91-100.

Korn T, Bettelli E, Oukka M & Kuchroo VK (2009) *IL-17 and Th17 Cells*. PALO ALTO: ANNUAL REVIEWS.

Kota BP, Huang THW & Roufogalis BD (2005) An overview on biological mechanisms of PPARs. *Pharmacological Research* **51**, 85-94.

Krachler B, Norberg M, Eriksson JW, Hallmans G, Johansson I, Vessby B, Weinehall L & Lindahl B (2008) Fatty acid profile of the erythrocyte membrane preceding development of Type 2 diabetes mellitus. *Nutrition Metabolism and Cardiovascular Diseases* **18**, 503-510.

Kwok AYC, Zu X, Yang C, Alfa MJ & Jay FT (1993) Human Interferon-Gamma Has 3 Domains Associated with Its Antiviral Function - A Neutralizing Epitope Typing Scheme for Human Interferon-Gamma. *Immunology* **79**, 131-137.

Lee JW, Bajwa PJ, Carson MJ, Jeske DR, Cong Y, Elson CO, Lytle C & Straus DS (2007) Fenofibrate represses interleukin-17 and interferon-gamma expression and improves colitis in interleukin-10-deficient mice. *Gastroenterology* **133**, 108-123.

Legrand P, Beauchamp E, Catheline D, Pedrono F & Rioux V (2010) Short Chain Saturated Fatty Acids Decrease Circulating Cholesterol and Increase Tissue PUFA Content in the Rat. *Lipids* **45**, 975-986.

Lehrke M & Lazar MA (2005) The many faces of PPAR gamma. *Cell* **123**, 993-999.

Leiber F, Kreuzer M, Nigg D, Wettstein HR & Scheeder MRL (2005) A study on the causes for the elevated n-3 fatty acids in cows' milk of alpine origin. *Lipids* **40**, 191-202.

Lemotte PK, Keidel S & Apfel CM (1996) Phytanic acid is a retinoid X receptor ligand. *European Journal of Biochemistry* **236**, 328-333.

Leon H, Shibata MC, Sivakumaran S, Dorgan M, Chatterley T & Tsuyuki RT (2008) Effect of fish oil on arrhythmias and mortality: systematic review. *British Medical Journal* **337**.

Leone TC, Weinheimer CJ & Kelly DP (1999) A critical role for the peroxisome proliferator-activated receptor alpha (PPAR alpha) in the cellular fasting response: The PPAR alpha-null mouse as a model of fatty acid oxidation disorders. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 7473-7478.

Lin JD, Yang RJ, Tarr PT*, et al.* (2005) Hyperlipidemic effects of dietary saturated fats mediated through PGC-1 beta cactivation of SREBP. *Cell* **120**, 261-273.

Liu SM, Choi HK, Ford E, Song YQ, Klevak A, Buring JE & Manson JE (2006) A prospective study of dairy intake and the risk of type 2 diabetes in women. *Diabetes Care* **29**, 1579-1584.

Lough AK (1977) Phytanic Acid Content of Lipids of Bovine Tissues and Milk. *Lipids* **12**, 115-119.

Lovett-Racke AE, Hussain RZ, Northrop S, Choy J, Rocchini A, Matthes L, Chavis JA, Diab A, Drew PD & Racke MK (2004) Peroxisome proliferator-activated receptor alpha agonists as therapy for autoimmune disease. *Journal of Immunology* **172**, 5790-5798.

Lowenstein C & Matsushita K (2004) The acute phase response and atherosclerosis. *Drug Discovery Today: Disease Mechanisms* **1**, 17-22.

Lumeng CN, Bodzin JL & Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *Journal of Clinical Investigation* **117**, 175-184.

Ma T, Liaset B, Hao Q*, et al.* (2011) Sucrose Counteracts the Anti-Inflammatory Effect of Fish Oil in Adipose Tissue and Increases Obesity Development in Mice. *Plos One* **6**.

Mackie JT, Atshaves BP, Payne HR, McIntosh AL, Schroeder F & Kier AB (2009) Phytol-induced Hepatotoxicity in Mice. *Toxicologic Pathology* **37**, 201-208.

Madsen L, Liaset B & Kristiansen K (2008) Macronutrients and obesity: views, news and reviews. *Future Lipidology* **3**, 43-74.

Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A & Evans RM (1992) Characterization of 3 Rxr Genes That Mediate the Action of 9-Cis Retinoic Acid. *Genes & Development* **6**, 329-344.

Marx N, Kehrle B, Kohlhammer K, Grub M, Koenig W, Hombach V, Libby P & Plutzky J (2002) PPAR activators as antiinflammatory mediators in human T lymphocytes - Implications for atherosclerosis and transplantation-associated arteriosclerosis. *Circulation Research* **90**, 703-710.

Mills KHG (2004) Regulatory T cells: Friend or foe in immunity to infection? *Nature Reviews Immunology* **4**, 841-855.

Moore KW, Malefyt RD, Coffman RL & O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology* **19**, 683-765.

Mozaffarian D, Cao HM, King IB, Lemaitre RN, Song XL, Siscovick DS & Hotamisligil GS (2010) Trans-Palmitoleic Acid, Metabolic Risk Factors, and New-Onset Diabetes in U.S. Adults A Cohort Study. *Annals of Internal Medicine* **153**, 790-+.

Munder M, Eichmann K, Moran JM, Centeno F, Soler G & Modolell M (1999) Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *Journal of Immunology* **163**, 3771-3777.

Musso G, Gambino R & Cassader M (2009) Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Progress in Lipid Research* **48**, 1-26.

Nagao K & Yanagita T (2008) Bioactive lipids in metabolic syndrome. *Progress in Lipid Research* **47**, 127-146.

Neve BP, Fruchart JC & Staels B (2000) Role of the peroxisome proliferator-activated receptors (PPAR) in atherosclerosis. *Biochemical Pharmacology* **60**, 1245-1250.

Nordic Council of Ministers (2005) *Nordic Nutrition Recommendations 2004.*, Nord 2004:13. ed.: Nordic Council of Ministers, Copenhagen
2004.

Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ & Flegal KM (2006) Prevalence of overweight and obesity in the United States, 1999-2004. *Jama-Journal of the American Medical Association* **295**, 1549-1555.

Olefsky JM (2001) Nuclear receptor minireview series. *Journal of Biological Chemistry* **276**, 36863-36864.

Oliver WR, Shenk JL, Snaith MR*, et al.* (2001) A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5306-5311.

Onishi RM & Gaffen SL (2010) Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology* **129**, 311-321.

Ovesen L (2009) *Indtag af mælk og mejeriprodukter og sygdomsrisiko - en systematisk gennemgang*. http://www.mejeri.dk/Aktuelt/2009/Ny_udredning_sundhedsegenskaber.aspx: Danish Dairy Board.

Pacini G (2006) The hyperbolic equilibrium between insulin sensitivity and secretion. *Nutrition Metabolism and Cardiovascular Diseases* **16**, S22-S27.

Palmquist DL, Beaulieu AD & Barbano DM (1993) Feed and Animal Factors Influencing Milk-Fat Composition. *Journal of Dairy Science* **76**, 1753-1771.

Parikh SJ & Yanovski JA (2003) Calcium intake and adiposity. *American Journal of Clinical Nutrition* **77**, 281-287.

Patsouris D, Reddy JK, Muller M & Kersten S (2006) Peroxisome proliferator-activated receptor alpha mediates the effects of high-fat diet on hepatic gene expression. *Endocrinology* **147**, 1508-1516.

Rabe K, Lehrke M, Parhofer KG & Broedl UC (2008) Adipokines and Insulin Resistance. *Molecular Medicine* **14**, 741-751.

Rakhshandehroo M, Knoch B, Muller M & Kersten S (2010) Peroxisome proliferator-activated receptor alpha target genes. *PPAR research* **2010**.

Repa JJ, Liang GS, Ou JF, Bashmakov Y, Lobaccaro JMA, Shimomura I, Shan B, Brown MS, Goldstein JL & Mangelsdorf DJ (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXR alpha and LXR beta. *Genes & Development* **14**, 2819-2830.

Rhee EP, Cheng S, Larson MG*, et al.* (2011) Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. *Journal of Clinical Investigation* **121**, 1402-1411.

Roche HM, Noone E, Nugent A & Gibney MJ (2001) Conjugated linoleic acid: a novel therapeutic nutrient? *Nutrition Research Reviews* **14**, 173-187.

Ropelle ER, Flores MB, Cintra DE*, et al.* (2010) IL-6 and IL-10 Anti-Inflammatory Activity Links Exercise to Hypothalamic Insulin and Leptin Sensitivity through IKK beta and ER Stress Inhibition. *Plos Biology* **8**.

Schenk S, Saberi M & Olefsky JM (2008) Insulin sensitivity: modulation by nutrients and inflammation. *Journal of Clinical Investigation* **118**, 2992-3002.

Schluter A, Barbera MJ, Iglesias R, Giralt M & Villarroya F (2002a) Phytanic acid, a novel activator of uncoupling protein-1 gene transcription and brown adipocyte differentiation. *Biochemical Journal* **362**, 61-69.

Schluter A, Giralt M, Iglesias R & Villarroya F (2002b) Phytanic acid, but not pristanic acid, mediates the positive effects of phytol derivatives on brown adipocyte differentiation. *Febs Letters* **517**, 83-86.

Schroder M, Yousefi F & Vetter W (2011) Investigating the day-to-day variations of potential marker fatty acids for organic milk in milk from conventionally and organically raised cows. *European Food Research and Technology* **232**, 167-174.

Shulman AI & Mangelsdorf DJ (2005) Mechanisms of disease: Retinoid X receptor heterodimers in the metabolic syndrome. *New England Journal of Medicine* **353**, 604-615.

Skurk T, berti-Huber C, Herder C & Hauner H (2007) Relationship between adipocyte size and adipokine expression and secretion. *Journal of Clinical Endocrinology & Metabolism* **92**, 1023-1033.

Smedman AEM, Gustafsson IB, Berglund LGT & Vessby BOH (1999) Pentadecanoic acid in serum as a marker for intake of milk fat: relations between intake of milk fat and metabolic risk factors. *American Journal of Clinical Nutrition* **69**, 22-29.

Smyth MJ & Godfrey DI (2000) NKT cells and tumor immunity - a double-edged sword. *Nature Immunology* **1**, 459-460.

Snijder MB, Dekker JM, Visser M, Bouter LM, Stehouwer CDA, Kostense PJ, Yudkin JS, Heine RJ, Nijpels G & Seidell JC (2003) Associations of hip and thigh circumferences independent of waist circumference with the incidence of type 2 diabetes: the Hoorn Study. *American Journal of Clinical Nutrition* **77**, 1192-1197.

Spilianakis CG, Lee GR & Flavell RA (2005) Twisting the Th1/Th2 immune response via the retinoid X receptor: Lessons from a genetic approach. *European Journal of Immunology* **35**, 3400-3404.

Stephensen CB, Borowsky AD & Lloyd KCK (2007) Disruption of Rxra gene in thymocytes and T lymphocytes modestly alters lymphocyte frequencies, proliferation, survival and T helper type 1/type 2 balance. *Immunology* **121**, 484-498.

Steyn NP, Mann J, Bennett PH, Temple N, Zimmet P, Tuomilehto J, Lindstrom J & Louheranta A (2004) Diet, nutrition and the prevention of type 2 diabetes. *Public Health Nutrition* **7**, 147-165.

Straus DS & Glass CK (2007) Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. *Trends in Immunology* **28**, 551-558.

Szanto A, Narkar V, Shen Q, Uray IP, Davies PJA & Nagy L (2004) Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death and Differentiation* **11**, S126-S143.

Tricon S, Burdge GC, Williams CM, Calder PC & Yaqoob P (2005) The effects of conjugated linoleic acid on human health-related outcomes. *Proceedings of the Nutrition Society* **64**, 171-182.

van Dam RM, Hu FB, Rosenberg L, Krishnan S & Palmer JR (2006) Dietary calcium and magnesium, major food sources, and risk of type 2 diabetes in US black women. *Diabetes Care* **29**, 2238-2243.

van den Brink DM & Wanders RJA (2006) Phytanic acid: production from phytol, its breakdown and role in human disease. *Cellular and Molecular Life Sciences* **63**, 1752-1765.

Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM & Dixit VD (2011) The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nature Medicine* **17**, 179-U214.

Vanni E, Bugianesi E, Kotronen A, De Minicis S, Yki-Jarvinen H & Svegliati-Baroni G (2010) From the metabolic syndrome to NAFLD or vice versa? *Digestive and Liver Disease* **42**, 320-330.

Varga T, Czimmerer Z & Nagy L (2011) PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochimica et Biophysica Acta-Molecular Basis of Disease* **1812**, 1007-1022.

Vartiainen E, Laatikainen T, Peltonen M, Juolevi A, Mannisto S, Sundvall J, Jousilahti P, Salomaa V, Valsta L & Puska P (2010) Thirty-five-year trends in cardiovascular risk factors in Finland. *International Journal of Epidemiology* **39**, 504-518.

Verhoeven NM & Jakobs C (2001) Human metabolism of phytanic acid and pristanic acid. *Progress in Lipid Research* **40**, 453-466.

Verhoeven NM, Wanders RJA, Poll-The B, Saudubray JM & Jakobs C (1998) The metabolism of phytanic acid and pristanic acid in man: A review. *Journal of Inherited Metabolic Disease* **21**, 697-728.

Vetter W & Schroeder M (2010) Concentrations of phytanic acid and pristanic acid are higher in organic than in conventional dairy products from the German market. *Food Chemistry* **119**, 746-752.

Virtue S & Vidal-Puig A (2008) It's Not How Fat You Are, It's What You Do with It That Counts. *Plos Biology* **6**, 1819-1823.

Wahli W (2008) PPAR gamma: Ally and foe in bone metabolism. *Cell Metabolism* **7**, 188-190.

Warensjo E, Jansson JH, Berglund L, Boman K, Ahren B, Weinehall L, Lindahl B, Hallmans G & Vessby B (2004) Estimated intake of milk fat is negatively associated with cardiovascular risk factors and does not increase the risk of a first acute myocardial infarction. A prospective case-control study. *British Journal of Nutrition* **91**, 635-642.

Warensjo E, Jansson JH, Cederholm T, Boman K, Eliasson M, Hallmans G, Johansson I & Sjogren P (2010) Biomarkers of milk fat and the risk of myocardial infarction in men and women: a prospective, matched case-control study. *American Journal of Clinical Nutrition* **92**, 194-202.

Warensjo E, Smedman A, Stegmayr B, Hallmans G, Weinehall L, Vessby B & Johansson I (2009) Stroke and plasma markers of milk fat intake - a prospective nested case-control study. *Nutrition Journal* **8**.

Wood IS, de Heredia FP, Wang BH & Trayhurn P (2009) Cellular hypoxia and adipose tissue dysfunction in obesity. *Proceedings of the Nutrition Society* **68**, 370-377.

www.danmarksstatistik.dk (2011) Death causes in Denmark 2009.

www.hjertedoktor.dk (2011) Danish cut of values for abdominal obesity in MS.

Xu JN, Yang Y, Qiu GX, Lal G, Wu ZH, Levy DE, Ochando JC, Bromberg JS & Ding YZ (2009) c-Maf Regulates IL-10 Expression during Th17 Polarization. *Journal of Immunology* **182**, 6226-6236.

Yamauchi T, Kamon J, Waki H*, et al.* (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine* **7**, 941-946.

Yessoufou AY & Wahli W (2010) Multifaceted roles of peroxisome proliferator-activated receptors (PPARs) at the cellular and whole organism levels. *Swiss Medical Weekly* **140**, 4-+.

Yoshikawa T, Shimano H, Yahagi N*, et al.* (2002) Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *Journal of Biological Chemistry* **277**, 1705-1711.

Yu ST, Matsusue K, Kashireddy P, Cao WQ, Yeldandi V, Yeldandi AV, Rao MS, Gonzalez FJ & Reddy JK (2003) Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma 1 (PPAR gamma 1) overexpression. *Journal of Biological Chemistry* **278**, 498-505.

Yuan MS, Konstantopoulos N, Lee JS, Hansen L, Li ZW, Karin M & Shoelson SE (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of IKK beta. *Science* **293**, 1673-1677.

Zhang YH, Bosse Y, Marceau P, Biron S, Lebel S, Richard D, Vohl MC & Tchernof A (2007) Gene expression variability in subcutaneous and omental adipose tissue of obese men. *Gene Expression* **14**, 35-46.

Zomer AWM, van der Burg B, Jansen GA, Wanders RJA, Poll-The B & van der Saag PT (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferater-activated receptor alpha. *Journal of Lipid Research* **41**, 1801-1807.

Appendix A

Manuscript for study A:

"*A hyper caloric diet based on monounsaturated fatty acids has more detrimental effects on metabolic parameters than diets based on butter or linoleic acid*"

To be submitted for: British Journal of Nutrition

A hyper caloric diet based on monounsaturated fatty acids has more detrimental effects on metabolic parameters than diets based on butter or linoleic acid.

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Abstract

Studies have given evidence that intake of saturated fatty acids (SFA) and trans-fatty acids (tFA) increase the risk of metabolic disorders. As dairy fat contains considerable amounts of both SFA and tFA, intake of dairy has been concerned as unhealthy, though studies now has shown markers of dairy fat intake correlates negatively with markers of metabolic disorders. In present study, c57bl/6J mice has been fed high fat diets and sucrose in the drinking water, to compare butter diets to diets high in either oleic acid or linoleic acid. Furthermore we have compared butter from either grazing or conventional fed cattle. This study indicates that intake of dairy fat, with its content of SFA and tFA, may not necessarily be detrimental compared to other types of fatty acids such as the MUFA oleic acid. We show that oleic acid, compared to butter and linoleic acid rich diets, have more detrimental effects with regard to glucose homeostasis and hepatic lipid metabolism, as we observed increased hepatic TAG and -ceramide deposition, impaired glucose uptake and up regulation of lipogenic genes, for the mice fed high amounts of oleic acid. Butter-based diets had the most detrimental effects on the blood-lipid parameters, observed as raised triacylglycerol (TAG), and total cholesterol (chol).

Introduction

During the recent decades a still ongoing increase in the incidence of obesity and metabolic diseases, has been seen throughout the world (Ogden *et al.*, 2006). Dietary habits are considered to be an important factor in modulating the risk of developing these pathologies and dietary fat quality has raised particular interest in this context. This is based on the fact that saturated and *trans*-unsaturated fatty acids have been shown to reduce, while mono- and polyunsaturated fatty acids of the n-6 series improve insulin-sensitivity (Riserus *et al.*, 2009). Due to the considerable amounts of saturated (SFA) and *trans* fatty acids in dairy fat, it has been regarded unfavorable for metabolic health and public advice have been to reduce dairy fat intake (Hu *et al.*, 1999; Hu *et al.*, 2001; Riserus *et al.*, 2009). However, a number of studies have now indicated that intake of dairy fat might not be as harmful as earlier thought (Gibson *et al.*, 2009), or even that intake of dairy fat might have beneficial impacts in relation to cardiovascular and metabolic disorders (German *et al.*, 2009). This latter viewpoint is supported by several studies showing a negative correlations between validated biomarkers for milk-fat intake and cardiovascular and metabolic risk markers as well as incidence of the first myocardial infarction in case-control studies (Smedman *et al.*, 1999; Warensjo *et al.*, 2004; Biong *et al.*, 2006; Hodge *et al.*, 2007; Warensjo *et al.*, 2009; Warensjo *et al.*, 2010). Thus, the metabolic effects of dairy fat intake seem to be positively modulated by other components than the major saturated fatty acids.

Although milk-fat is dominated by the saturated FA, it is characterized by a large compositional complexity. Thus, it contain more than 400 different fatty acids, although only 12 different FA constitute more than 1% of the total fatty acid mass (Jensen, 2002). Some of these minor fatty acids, such as short-chained fatty acids, conjugated linoleic acid (CLA) and phytanic acid, might have protective effects that can abolish the negative effects of SFA, either alone or synergistically (Roche *et al.*, 2001; Field *et al.*, 2009; Hellgren, 2010; Sleeth *et al.*, 2010). Hence, in the light of the recent indications that dairy fat is more benign in relation to metabolic diseases, than earlier thought, it is important that the metabolic effects of milk-fat is directly compared with the other major dietary fat types, that current public advice recommend milk-fat to be exchanged with, i. e. oleic acid (MUFA) and linoleic acid (n-6 PUFA).

However, the concentrations of several of the potentially bioactive minor fatty acids are highly dependent on feeding regimen. Thus, when the cattle are fed green plant material, rather than non-green feed, such as soy or corn, the concentration of many of the bioactive fatty acids (e. g. CLA, phytanic acid, α-linolenic acid) are increased. During the last 30-40 year, dairy-cattle farming have

gone through a dramatic change, from the use of grass, hay and other feed with origin from green plants as the dominating feed-source, to high yield production were corn and soy based feeds dominate. Therefore, a significant change in the fatty acid composition of the milk fat has occurred in this period, in particular a reduction in the content of potential bioactive fatty acids. Despite that this impact on the composition of the dairy fat-fraction is well known, the physiological effects of these changes on the consumer, have not been studied thoroughly so far. To clarify these issues, we have performed a set of experiments in which the effects on metabolic parameters by intake of either butter from high yield conventional production form or butter from low yield grazing cattle was compared, as well as we compared the effects of the butter-based diets, with diets were oleic acid or linoleic acid was the primary fat source. Our studies revealed that a diet high in oleic acid had most detrimental effects on parameters related to glucose homeostasis and hepatic lipid metabolism, while the butter based diets had negative effects on blood lipids.

Methods and materials

Animals care, feed and tissue sampling: Male C57bl/6J mice were purchased from Charles River Laboratories International, Inc (Sulzfeld, Germany). They were kept at 21 °C and 50% humidity, with a 12-h light cycle (07.00-19.00). The animals were attended daily and weighted weekly. All handling and use of animals in this study was approved by The Danish Animal Experiments Inspectorate and were carried out according to the guidelines of "The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purposes".

From 10 weeks of age, 32 mice were fed an obesity-inducing diet containing 60 E% fat and 15% (w/v) sucrose in the drinking-water for 10 weeks, to mimic western cafeteria-type diets high in both fat and sucrose. The diets were prepared by adding the experimental fats to "RD12492 px"-powder from "Research Diets Inc. (New Brunswick, NJ, USA)". Eight mice were used as a lean reference group and were given the appropriate control diet recommended by "Research Diets", diet D12450B. The tested fat types was butterfat derived from cows fed under different feeding regimes (described below), 75 % grape seed oil: 25 % lard and 75% high oleic sunflower oil : 25% lard (High oleic sunflower oil was a generous gift from AAK, Karlshamn, Sweden; lard and grape seed oil was from local supermarket). The composition of the diet is given in Table 1, and the FA-compositions in the different diets can be seen from Table 2. The mice had access to feed and water

ad libitum. After 10 weeks on the experimental diets, overnight fasted animals were anaesthetized with 0.11 mL pr 25 g animal bodyweight of 44.44 mg/mL ketamin and 2.22 mg/mL Narcoxyl(xylazin), before they were sacrificed through cardiac puncture. Blood was collected in heparin-coated tubes and stored on ice, until plasma was isolated. Tissue samples to be used for RNA extraction was transferred to RNALater (Applied Biosystems, Carlsbad, California, USA), the rest of the tissue was snap-freezed in liquid N_2 and stored at -80ºC until it was analyzed.

Table 1 Nutritional composition of diets. Values are given as wt-%.

Components	Lean	Butter diets	MUFA diet	PUFA diet
Protein	19,2	23,9	25,7	25,7
Maltodextrin 10	3,3	14,7	15,8	15,8
Sucrose	33,2	8,1	8,7	8,7
Corn startch	29,9	-	-	-
Soybean oil	2,4	2,9	3,2	3,2
Butter[1]	-	37,8	-	-
High oleic acid sunflower oil	-	-	24,8	-
Grapeseed oil	-	-	-	24,8
Lard	1,9	-	8,3	8,3
Vitamin mix[2]	0,9	1,2	1,3	1,3
Mineral mix[3]	0,9	1,2	1,3	1,3
Cellulose, BW200	4,7	5,9	6,3	6,3
Others[4]	3,5	4,4	4,7	4,4

1. Amount of butter in diet is adjusted due to watercontent to have the same total fat amount in all high fat diets
2. Vitamin mix s10026, for further information consult www.researchdiets.com
3. Mineral mix V10001, for further information consult www.researchdiets.com
4. Sum of dicalcium phosphate, calcium carbonate, potasium citrate, choline bitartrate, and dye.

Table 2 Diet fatty acid compositions. Fatty acid composition in the feed for the four different treatments and the lean references, shown as mass% of total fatty acids (nd= non-detectable).

Fatty acid	Lean	MUFA	PUFA	Grazing	Conventional
C4:0	nd	nd	nd	4,50	4,48
C6:0	nd	nd	nd	2,38	2,47
C8:0	nd	nd	nd	1,15	1,19
C10:0	0,08	0,03	0,03	2,01	2,08
C12:0	0,12	0,04	0,04	2,38	2,35
C14:0	0,79	0,41	0,41	8,51	8,58
C14:1	nd	nd	nd	0,64	0,57
C15:0	nd	0,03	0,03	0,78	0,76
C16:0	15,68	9,39	11,78	22,18	20,24
C16:1	0,85	0,64	0,65	1,00	0,99
Phytanic acid	nd	nd	nd	0,26	0,18
C18:0	7,81	5,66	6,39	13,20	13,52
11tr-Vaccennic acid	nd	nd	nd	2,09	2,46
C18:1 n-9 cis	28,39	67,42	21,97	23,66	24,15
C18:2 n-6 cis	36,94	11,58	54,42	5,81	5,89
C18:3 n-6	0,11	0,26	0,15	0,29	0,35
C18:3 n-3	4,11	1,01	1,14	1,26	1,11
9Cis, 11trans-CLA	nd	0,04	0,03	0,65	0,66
Others	5,12	3,49	2,95	7,28	7,95
ΣSFA	25,1	16,6	19,1	58,0	56,5
ΣMUFA	31,7	68,7	23,2	28,9	30,7
ΣPUFA	41,6	13,1	56,0	8,3	8,3

Production of test milk/butter

Milk for the conventional butter was produced by the experimental organic herd at Aarhus University by 23 Danish Holstein cows with an average daily milk production of 39 kg with 4.46 % fat. The cows were fed a concentrate consisting of 1:1 mixture of oat grain and rapeseed cake with 11 % fat. This concentrate constituted 40 % of the diet. The remaining 60% of the diet consisted of a silage. The silage consisted of a mixture of corn silage, pea-barley whole crop silage with a small proportion of white clover grass silage. Feeding of the cows was initiated in November 2008 and milk was collected over one week in February. The milk was transported to Thise Dairy (Roslev,

Denmark), where it was processed into butter. Total content of green plant material was 19 % of total feed.

Milk for the grazing butter was delivered from cows grazing in Norwegian mountains. Beside the grass the cows were given concentrate at the cowshed at night and when milking. While all cattle were grazing at daytime, most cows were at fertilized meadows at the farms during night time. Cattle were not given hormones, drugs or other that could be perceived as additives in excess of the normal or natural. The milk was transported to Tine Dairies (Oslo, Norway), where it was processed into butter.

Lipid analysis: Lipids were extracted using the Folch method (Folch *et al.*, 1957), with internal standards added, C19:0 NEFA, TAG, PL, long-chain base C17:0 ceramide, for the tissue samples. Fat content in feed was analyzed gravimetric. Fatty acids were analyzed as fatty acid methyls esters (FAME), preparation of FAME was performed by a method based on the method of Hamilton & Hamilton (Hamilton & Hamilton, 1992), by adding 1.0 mL 0.5 M NaOH in MeOH to the extracted lipids, and refluxed for 5 minutes at 80 °C. Mixture was cooled to room temperature, 1 mL 20% BF_3 in MeOH and 0.5 mL 0.1% hydroquinone i MeOH added, and refluxed for 2 minutes. When room temperature was achieved 2mL 0.73 % NaCL (aq) was added. After whirling of mixture 0.5 mL heptan was added, and the upper phase was transferred to another tube. Heptan extraction was repeated on the lower phase, and the new upper phase transferred to the first upper phase. FAMEs dissolved in heptan were washed with 1.0 mL of satiated alkaline NaCL (~40 g NaCL and 150 mg K_2CO_3 pr. 100 mL water). For quantification of phytanic acid, C4:0, C6:0 and C8:0, response-factors were calculated for their methyl-esteres based on the response of palmitic acid methyl ester (16:0). The FAME were separated on a 60-m Supelco SP2380 column (Sigma-Aldrich AS, Brøndby, Denmark) in a HP 6890 gas chromatograph (GC), in split mode using He as carrier gas.

Settings for the GC-program were: Injector temperature 260 °C, split ratio 20 : 1, carrier flow 1.2 mL/min, detector temperature 300 °C, air flow in detector 300,0 mL/min, hydrogen flow 35 mL/min. FAME were separated using a temperature program starting at 50 °C and rising to 160 °C at 15 °C /min; this temperature was kept for 0 min, hereafter the temperature was raised to 182 °C

at 1 °C /min, and directly the temperature was raised to 200 °C at 10 °C /min, and the oven was kept at 200 °C for 15 min before the temperature was raised to 225 °C. The final temperature was kept for 12 min (total runtime 61.97 min).
Ceramide was isolated from total lipid extract and quantified as earlier described in (Drachmann *et al.*, 2007), with the exception that ceramide with C17 long-chained base was used as internal standard.

Analysis of plasma TAG, NEFA, Cholesterol and glucose: Plasma levels of triacylglycerol (TAG), non-esterified fatty acids (NEFA), total cholesterol, and glucose, were measured using analytical kits from HORIBA abx; (Montpellier, France) (TAG, glucose, cholesterol), NEFA was analyzed using the appropriate kit from Wako Chemicals Gmbh (Neuss, Germany), on a "Cobas Mira Plus" (Roche Diagnostics Systems, Basel, Switzerland).

Oral Glucose Tolerance Test (OGTT): OGTT was performed after ten weeks on the given diets. The animals were fasted for 3 hours prior to gavage feeding the mice with a glucose-solution (1.5 g glucose per kg bodyweight). Blood-glucose levels were measured at the time points zero, one, and two hours after the glucose load and at the time points zero and two hours after infusion eye-blood was taken with 50 µL EDTA-coated capillary tubes to produce plasma for insulin measurements. Blood glucose measurements was performed with "On call® Plus Blood Glucose Meter", by adding one drop of eye-blood to the chip.

Insulin: Plasma insulin was analyzed using "Ultra sensitive mouse insulin ELISA" (Mercodia AB, Uppsala, Sweden), following the manufactures guidelines.

Leptin & TNF-α: Plasma levels of leptin and TNF-α was analyzed using the appropriate kits from Meso Scale Diagnostics (Gaithersburg, USA) on a Meso Scale Sector Imager 6000 (leptin) and a Meso Scale Sector Imager 2400 (TNF-α) following the protocol from the manufacturer, except that plates for TNF-α analyses was incubated over night with detection buffer, to increase sensitivity.

Gene expression analysis/qRT-PCR: RNA from liver-tissue was extracted on Abi Prism 6100 Nucleic acid Prepstation from Applied Biosystems (Carlsbad, California, USA), according to the manufacturer's instruction.
cDNA-synthesis was performed on Unocycler (VWR (Leuven, Belgium), using "High capacity cDNA transcription kit" from Applied Biosystems. The quantification was performed on a 7900HT Fast Real Time PCRsystem, equipped with 96 wells Fast block, using "Taqman® Environmental master mix 2.0" (Applied Biosystems), and with primers and TaqMan-probes were selected from Applied Biosystems and Integrated DNA Technologies (Coralville, IA, USA) online catalogue. SDS 2.3 and RQ-manager 1.2 software was used for data analyses.

Statistical analysis: Statistical analysis was performed using the software package GraphPad Prism (v.5.00). One way ANOVA with "Turkey" post test performed when nothing else noted. In cases of inhomogeneous variances between groups in datasets (tested by "Bartlett´s test") statistical analysis is performed on LogY transformed data. In cases where transformation of data did not homogenize the variance, data was analyzed using Welch corrected ANOVA using the R – software package (www.r-project.org). Results are given as average ± Standard deviation (SD), (n=8 for all groups, except MUFA-control, n=6, and grazing-butter group, n=5, due to loss of animals during the feeding period). Statistical outliers were detected using Grubbs test (www.graphpad.com).

Results

Body and organ masses

All groups given hypercaloric diets were found to have statistically significant higher bodyweight and WAT mass, than the lean references (P<0.001). Furthermore, WAT mass of the PUFA group was statistically significant higher than for the MUFA group (P<0.05), see

Table 3. The MUFA and the conventional butter group had statistical significant higher hepatic tissue mass, than lean references (P<0.01 for both), see

Table 3; otherwise was there no other differences in tissue weights between the groups.

Table 3 Body mass, tissue masses, and plasma lipid concentrations. Given as mean±s.d. Different letters illustrates statistical significant differences between groups. Statistical analysis performed for all parameters are one way ANOVA with Turkey post test.

	Lean	MUFA	PUFA	Grazing	Conventional
Bodyweight (g)	27.25 ± 1.28^{a}	42.67 ± 2.58^{b}	$44.75\pm3,19^{b}$	43.40 ± 2.61^{b}	44.00 ± 2.98^{b}
Hepatic tissue weight (g)	1.05 ± 0.11^{a}	1.97 ± 0.56^{b}	$1.58\pm0.22^{a,b}$	$1.73\pm0.43^{a,b}$	$1.91\pm.76^{b}$
WAT (g)[1]	0.40 ± 0.20^{a}	5.27 ± 0.48^{b}	6.30 ± 0.80^{c}	$5.64\pm0.31^{b,c}$	$5.69\pm0.39^{b,c}$
Plasma TAG (mM)	$0.64\pm0.14^{a,b}$	$0.65\pm0.15^{a,b}$	$0,62\pm0.11^{b}$	$0.88\pm0.21^{a,c}$	0.98 ± 0.14^{c}
Plasma NEFA (mM)	0.85 ± 0.11^{a}	0.96 ± 0.14^{a}	0.98 ± 0.13^{a}	0.94 ± 0.26^{a}	1.05 ± 0.18^{a}
Plasma Chol (mM)	2.41 ± 0.24^{a}	3.58 ± 0.50^{b}	$3.90\pm0.28^{b,c}$	$4.02\pm0.55^{b,c}$	$4,60\pm0.84^{c}$

1. WAT is the sum of epididymal-, kidney-, and visceral-white adipose tissue

Glucose homeostasis

Glucose tolerance was measured by an oral glucose tolerance test (OGTT). The rate of glucose clearance was measured as the difference in blood glucose values between zero to two hours after glucose dosage. This, was found to be significantly higher for both the MUFA and the PUFA groups compared to the lean references (P<0.01)(Figure 1A), while this was not the case for the two butter groups. Furthermore, the MUFA group had significantly slower rate of glucose clearance, than the conventional butter group (P<0.05).

When Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated from the fasting glucose and insulin levels, all obese groups had significantly higher values than the lean references (P<0.001), see Figure 1B.

Semi-fasting insulin and glucose levels can be seen from Figure 2B&C. Fasting insulin of all obese groups, was higher than for the lean reference group (P<0.001). Fasting glucose of the MUFA, PUFA and grazing butter group was significantly higher than for the lean references (P<0.05, P<0.001, and P<0.01, respectively), and also statistically significant higher for PUFA than for MUFA (P<0.01).

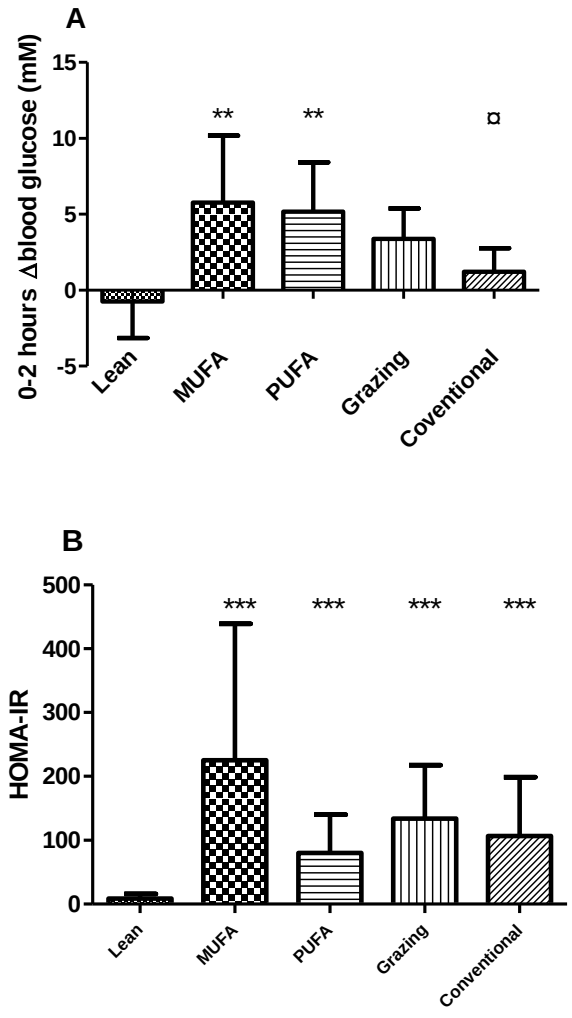

Figure 1 Glucose homeostasis. **A**: Delta blood glucose from zero to two hours from oral dosage with glucose-solution. **B**: Homeostatic Model Assessment Insulin Resistance (HOMA-IR) for the five different feeding groups given as mean±SD. * vs lean, and ¤ vs. MUFA group.

Plasma lipids

The butter-based diets did generally increase plasma triacylglycerol (TAG) concentration while all high-caloric diets caused increases in plasma cholesterol, compared to the lean reference group. However, only for the conventional butter group did the level of plasma TAG reach statistical significance to all non-butter fed groups, while the effect of the grazing-butter was slightly less pronounced, and did only reach statistically significant against the PUFA-fed and lean reference animals ($P<0.05$ against both).

We found that plasma cholesterol was significant higher for all treated groups compared to lean references ($P<0.001$for all except against MUFA group: $P <0.01$), and animals fed the conventional butter had on average the highest total cholesterol levels, and it reached statistically significance compared to the MUFA group ($P<0.01$).

No statistical significant differences on fasting plasma non-esterified fatty acids (NEFA) were observed between any groups, although the animals fed the conventional butter also had highest average NEFA-levels.

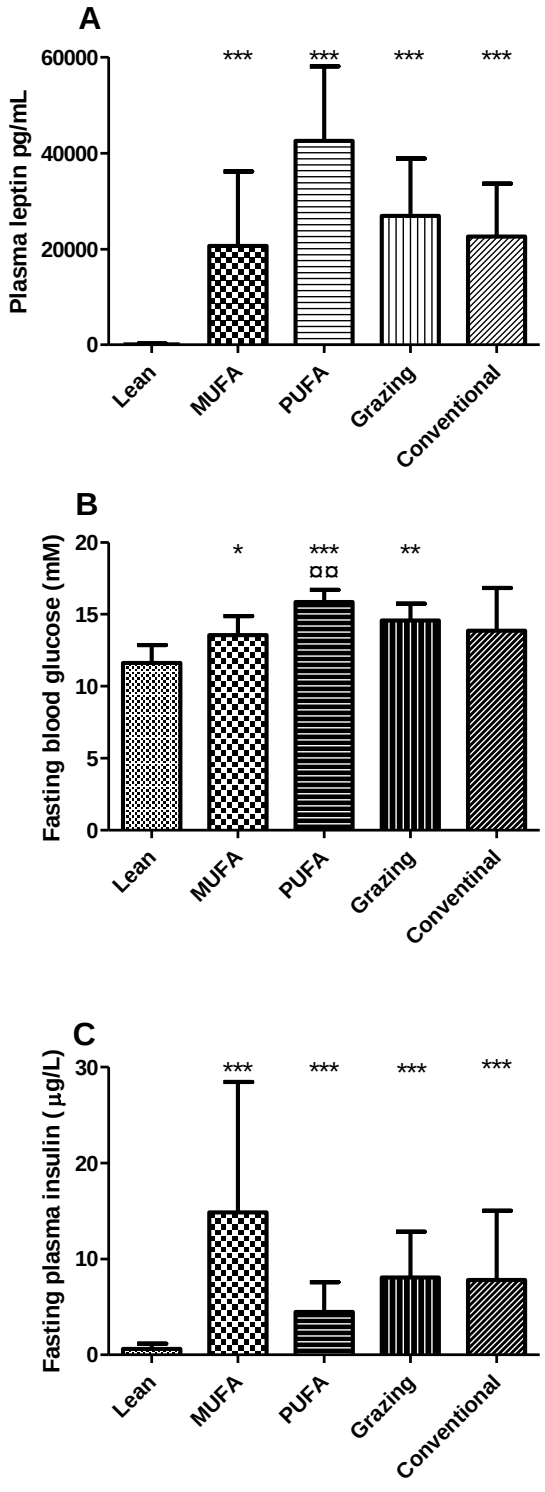

Figure 2 Fasting levels of plasma markers. A: Leptin, **B**: Glucose, and **C**: Insulin, given as mean±SD. * indicates statistical significant difference vs. lean, and ¤ vs. MUFA group.

Adipokines

Plasma leptin was statistically significant higher for all obese groups compared to the lean references ($P<0.001$ for all), see Figure 2A, but the different fat sources did not cause any differences in leptin level. The inflammatory marker TNF-α, was on the contrary substantially affected by the dietary fat source. Thus, in only one out of eighth animals in the PUFA- and conventional butter-fed groups did the TNF-α levels reach the detection limits, while it did so in all animals in the MUFA-group and 50-60% of the animals in the grazing-based butter and the lean reference-group (Figure 3).

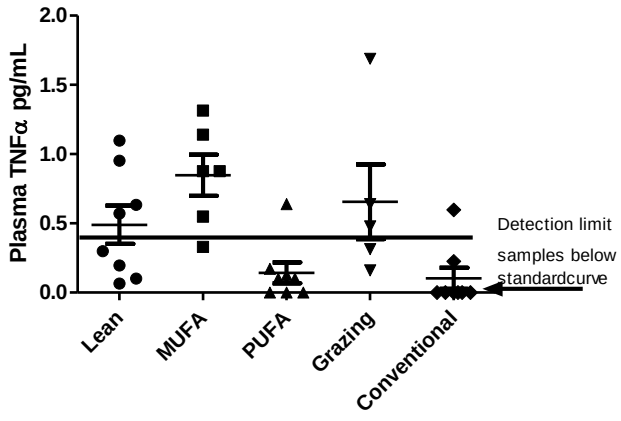

Figure 3 Fasting TNFα concentrations. Values below the limit of the standard curve set to zero, and the detection limit is marked by a horizontal line.

Hepatic lipid profile

As seen in Figure 4A, TAG pr liver mass was shown to be statistically significant for all test groups compared to lean reference group ($P<0.001$). Furthermore there is a tendency that TAG pr. liver mass is higher for the MUFA group than other groups given high fat diet ($P=0.054$ against PUFA, $P=0.076$ against grazing butter, and $P=0.18$ against conventional butter). Furthermore, the MUFA based diet caused significant increase in the hepatic ceramide levels, compared to the lean reference, as well as compared to the other obese groups ($P<0.01$ against lean references and PUFA-group, and $P<0.001$ against the two butter-groups, Figure 4B). Thus, in the liver from the

MUFA fed animals, there were 70% more ceramide, than in the butter-fed groups. There were no significant differences in the concentration of hepatic free fatty acid between the groups.

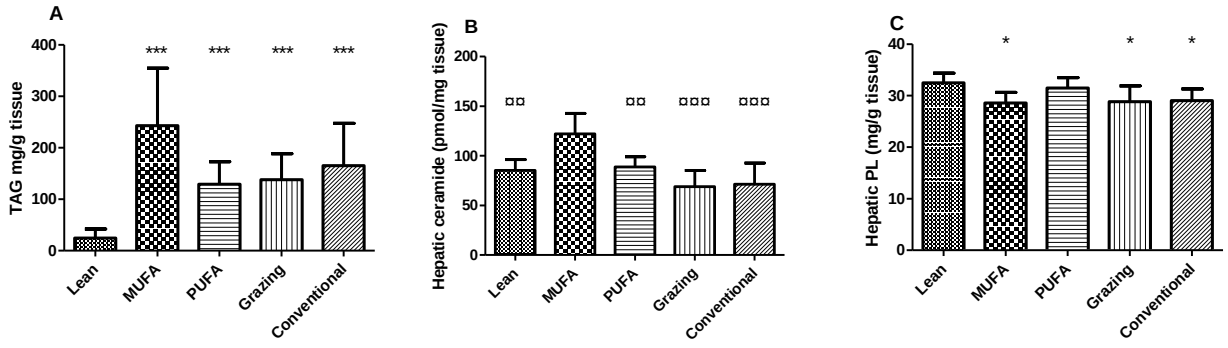

Figure 4 Hepatic lipid deposition A: TAG content showed in mg TAG pr. g hepatic tissue, **B**: Hepatic ceramide content given as as pmol pr. mg liver tissue, **C**: Hepatic phospholipid content given as mg pr. g tissue. All values given as mean+SD. * indicate statistical significant difference against lean reference and ¤ against MUFA-group.

When we compared the effect of the diets on the phospholipid fatty acid composition in the liver, the most pronounced effects was the higher level of the n-3 PUFA´s EPA and DPA in the butter fed groups compared to all other groups ($P<0.001$), and that butter from the grazing cows caused significant higher incorporation of eicosapentaenoic acid (EPA, compared to the conventional butter group (Figure 5)). EPA constituted 1.06±0.29 mass% of the total FAs in grazing butter group and 0.61±0.16 mass% in the conventional butter group ($P<0.01$), as well as significant higher for MUFA and lean than PUFA ($P<0.001$). DPA were statistically significant higher for the lean references compared to MUFA and PUFA-group ($P<0.001$). As expected, the high linoleic acid intake in the PUFA group, reduced the DHA level significantly in this group ($P<0.01$ against MUFA and grazing butter; $P<0.001$ against conventional butter) and raised the concentration of arachidonic acid, compared to the other groups. DHA was also found to be significant higher in the conventional butter group than for lean references ($P<0.01$).

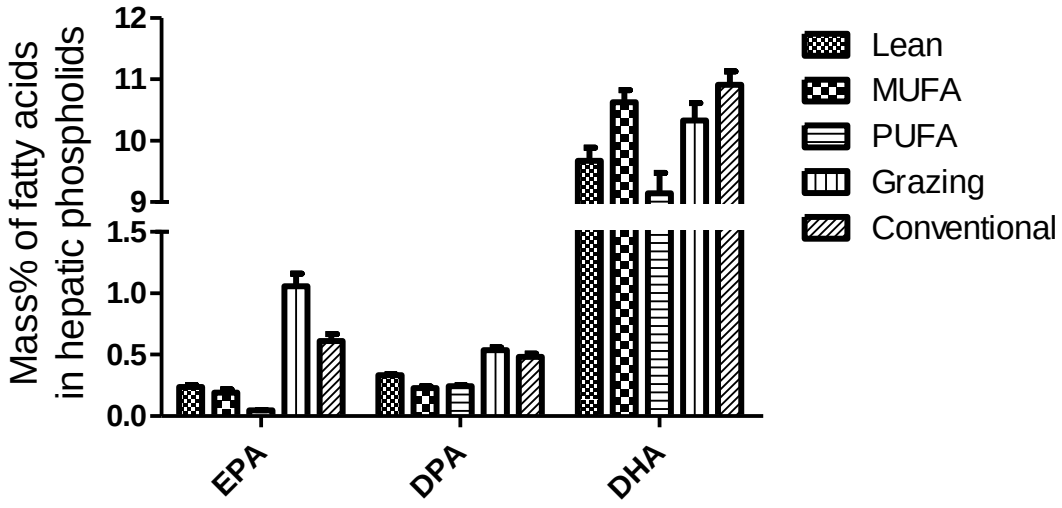

Figure 5 Hepatic phospholipid content of the three long chain poly unsaturated fatty acids; eicosapentanoic acid (EPA), docosapentanoic acid (DPA), and docosahexanoic acid (DHA), in the five groups given as mean+SD.

Hepatic gene expression

Since the main effect on hepatic lipids was seen in the MUFA group, we analyzed hepatic gene expression of genes related to fatty acid metabolism in this group compared to the two-butter groups using the lean-group as reference (Figure 6). All three high caloric diets had statistically significant higher expression of Fasn than the lean references ($P<0.001$ for all three) but it is noteworthy, that the MUFA-group has statistical significant higher expression of Fasn in the liver than the butter groups ($P<0.05$). No differences were observed between the four groups with regard to Acox1. Cpt1a was statistically significant down regulated in all the obese groups compared to the lean references ($P<0.001$). The relative expression of FA transporter CD36 was highly up regulated in all three obese groups compared to lean references ($P<0.05$ against MUFA, $P<0.01$ against Grazing butter, and $P<0.001$ against conventional butter).

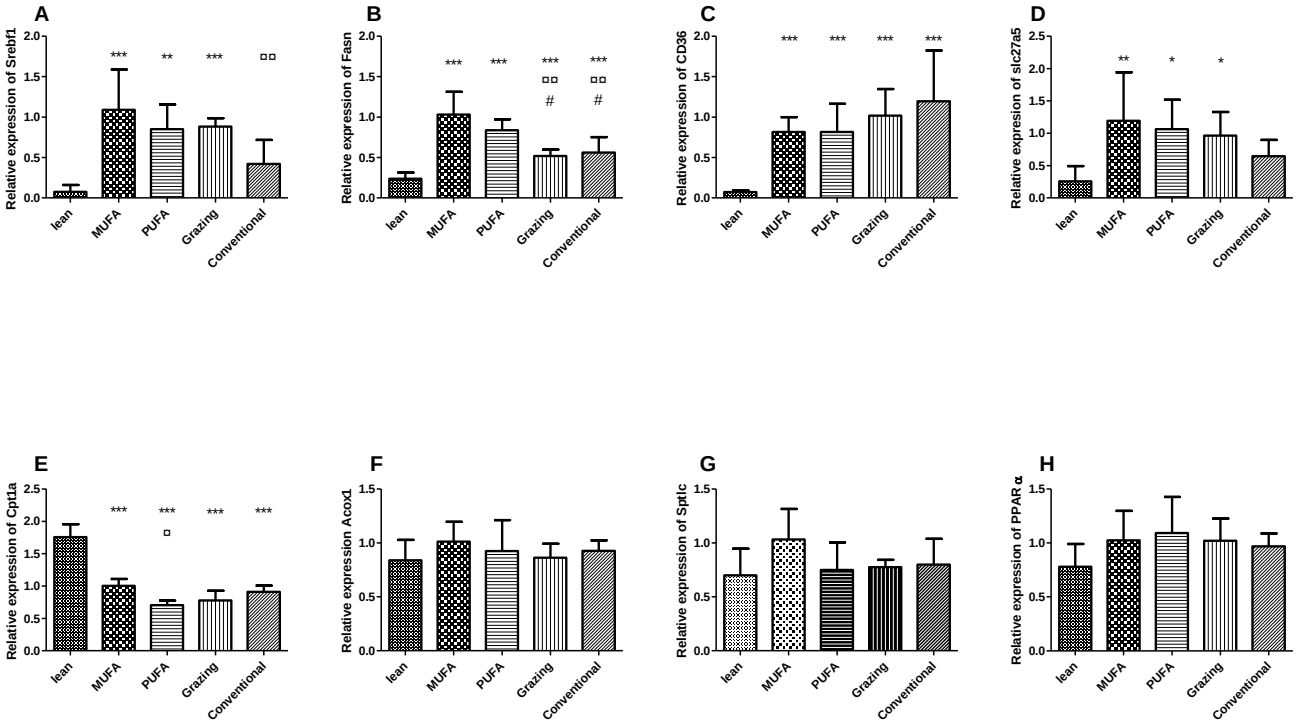

Figure 6 Gene expression of hepatic fatty acid metabolism. A: Sterol regulatory binding protein (SREBP1c), **B**: Fatty acid synthase (FASN), **C**: CD36, **D**: Fatty acid transport protein 5 (slc27a5/FATP5), **E**: Carnitine acyltransferase 1a (Cpt1a), **F**: AcylCoenzyme A oxidase1 (Acox1), **G**: Serine palmitoyltransferase, long chain subunit 1 (Sptlc), **H**: Peroxisome proliferator associated receptor-α (PPARα), given as mean+SD. * indicates statistical significant difference against lean reference, and ¤ against MUFA-group.

Discussion

Despite the fact that the only difference between the animals in body-composition was a significantly higher WAT mass in the PUFA group compared to the MUFA-group, the compiled results of this study point in the direction that the detrimental metabolic effects of high fat diets, is higher for diets high in the MUFA oleic acid, compared to butter and linoleic acid rich diets, in regard to systemic parameters describing glucose homeostasis and hepatic lipid metabolism, while butter-based diets had most detrimental effects on the analyzed blood-lipid parameters. Thus, the results from the OGTT show that the rate of glucose-clearance (measured as the difference between blood glucose levels two hours after oral glucose dosage to the semi-fasted level just before the dosage) is substantially aggravated in both the MUFA and n-6 PUFA based diet, compared to the

butter-based diets. Although the glucose clearing abilities of the PUFA group is as reduced as for the MUFA group, the PUFA-fed animals did not show the significant increase in HOMA-IR as seen in the MUFA group. This is due to the fact that the fasting insulin values of the PUFA group are substantially lower. Thus, while both linoleic and oleic acid based diets cause more detrimental effects on the animal's ability to clear glucose in the fed-state, compared with a butter-based diet, oleic acid based diets caused most severe disturbances of the glucose homeostasi in the fasted state. This could indicate that the MUFA – diet had more detrimental effects on the insulin-based regulation of gluconeogenesis than the other two types of fat. However, when we analyzed the expression of the insulin-regulated gene for glucose-6-posphatase, there was no difference between the groups (data not shown), suggesting that this was not the case. In this context, it is also worth noticing that the MUFA-fed group had highest level of systemic sub-clinical inflammation, measured as plasma TNF-α level.

The higher WAT mass of PUFA group compared to MUFA group are worth noticing. This is in accordance with previous findings that n-6 PUFA intake increases WAT, in particular when given in combination with high-sucrose diets (Ailhaud *et al.*, 2006; Madsen *et al.*, 2008a). The important finding in present study is however, that this increase in WAT-mass did not lead to more detrimental metabolic effect than what is seen in the leaner MUFA-fed group, neither in regard to hepatic lipid accumulation or glucose-tolerance.

The results for hepatic gene expression, points in the direction that the observed tendency to increase in hepatic TAG deposition for the high oleic acid group, is due to increased synthesis, and not due to increased uptake or decreased oxidation, as both the fatty acid transporters CD36 and FATP5 are up regulated in all obese groups, and ACOX expression seems unchanged. The observed expression pattern indicates that lipogenesis might play a role in the increased hepatic lipid deposition of the oleic acid group, as SREBP-1c has been coupled to *de* novo lipogenesis through ER stress (Ferre & Foufelle, 2010). The finding that a hyper-caloric diet with a high intake of oleic acid, causes a substantial increase in hepatic ceramide content , compared to both LA – and butter based diets, is an important finding. Ceramide attenuates signaling from the insulin receptor (Chavez *et al.*, 2003), are involved in the activation of the Nlrp3 inflammasome during induced inflammatory development and have been implicated to play a role in the development of non-alcoholic hepatic steatosis (Vandanmagsar *et al.*, 2011; Promrat *et al.*, 2011). Therefore, accumulation of excess tissue-ceramide is considered to be major factor, driving the development of

metabolic diseases (Lipina & Hundal, 2011). Thus, the reduced glucose tolerance in the MUFA-fed animals might be explained by the increased ceramide levels in these animals. Although there was a tendency towards higher hepatic TAG levels in the MUFA-group (P=0.054 against PUFA), the increased ceramide cannot solely be explained by a general increased fatty acid load in these animals, since the relative difference in TAG deposition was substantially smaller than the effect on ceramide concentration. However, in the MUFA group the gene for fatty acid synthase (FAS) was significantly upregulated, compared to the other obese groups, and there was a tendency toward increased expression of the rate-limiting enzyme in the ceramide synthesis pathway, serine-palmitoyl transferase (SPT) (Figure 6). The product of the FASN enzyme, palmitoyl-CoA, is one of the substrates for SPT, and the flux through this pathway is partly determined by the enzyme´s access to palmitoyl-CoA (Merrill *et al.*, 1988). Thus, enhanced *de novo* synthesis of ceramide, due to an increased *de novo* synthesis of palmitoyl-CoA can, at least partly, explain the observed increased in ceramide levels. However, the increased concentration of ceramide may also originate from hydrolysis of sphingomyelin by the neutral sphingomyelinase (N-SMase), since it recently was shown that high fat diets cause increased activity of the N-SMase but not acid sphingomyelinase in rat liver (Chocian *et al.*, 2010).

The up regulation of the gene for the key lipogenic enzyme fatty acid synthase (FASN) in MUFA group compared to the other DIO groups, are noteworthy. It has earlier been shown that the combination of high sucrose and high fat, cause activation of the FASN-gene (Madsen *et al.*, 2008b), but that excessive intake of oleic acid further enhance this activation, compared to other fatty acids, have not earlier been showed. Since it is generally considered that intake of oleic acid is nutritionally advantageous, compared to the saturated fatty acids found in butter (Riserus *et al.*, 2009), the mechanism behind this effect warrant further studies. For all the obese groups, we see a combined effect of the high fat and high sucrose intake which might be due to activation of ChREBP (no data) combined with insulin induced activation of sterol regulatory element-binding protein-1c (SREBP1c) partly via LXR activation. LXR is repressed by PUFA and EPA in particular (Yoshikawa *et al.*, 2002), thus the AA – level in the PUFA-group and the high EPA-level in the butter group, might repress this effect. Neither of these two factors is present in the MUFA group, why the glucose and insulin driven activation of SREBP-1c is unrepressed.

The proportion of linoleic acid (LA) to (ALA) α-linolenic acid in the diet seems to be decisive for the EPA content in the hepatic lipid stores, due to desaturation and elongation competition, and

recent data indicate that the FA-composition in dairy fat promote the biotransformation of ALA to EPA and DPA (Legrand *et al.*, 2010).

Butter had the most detrimental effects on plasma lipids. TAG and cholesterol tended to be higher for the butter groups, most pronounced with regard to TAG, and must be regarded as parameters supporting the view that butter-intake increase risk of CVD. It is however noteworthy, that, on average, the conventional butter increased all blood lipid parameters more than the butter produced from the grazing cows. Although the difference between the butter types did not reach statistical significance, this could indicate advantageous effects of the feeding regime based on green-plants. It has been suggested that intake of SFAs and trans-FAs raise circulating TAG and cholesterol through induction PGC-β and thereby SREBP (Lin *et al.*, 2005), which correlates with what we observed for the butter fed animals, but as discussed the raise in plasma lipid levels did not lead to raise in hepatic lipid deposition.

Conclusion

Obese mice fed either one of the butters did not have statistically significantly decreased glucose-clearance rate, compared to lean, while PUFA and MUFA-fed did have. MUFA fed obese mice had significantly higher ceramide concentration in the liver and a tendency to more TAG. The increased hepatic ceramide and trend towards higher TAG could be partly explained by a higher expression of FASN, and the increased ceramide deposition might be the explanation for the impaired glucose homeostasis observed for the group fed high amount of oleic acid. The two butters gave substantially higher EPA levels in the liver than the MUFA-diet, despite similar ALA-levels in the feed; this is explained by the low LA: ALA ratio. Intake of butter from the grazing cows gave significantly higher hepatic EPA levels, than butter from cows fed low levels of green plants. From the compiled results of this study, it seems that the detrimental metabolic effects of high fat diets, is higher for diets high in the MUFA oleic acid, compared to butter and linoleic acid rich diets, in regard to systemic parameters describing glucose homeostasis and hepatic lipid metabolism, while butter-based diets had most detrimental effects on the analyzed blood-lipid parameters. We observed increased hepatic TAG and -ceramide deposition, as well as impaired glucose uptake and up regulation of lipogenic genes, for the mice fed high amounts of oleic acid, compared for the mice fed either high butter or high linoleic acid diet. From this study it seems, that the content of SFA

and tFA in butter not necessarily makes butter fat more detrimental to metabolic health than other types of fat, although it has disadvantageous effects on blood lipids.

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Contributions of each author: Tue Drachmann; Participated in designing of the study, conducted research, statistical analysis and interpretation of results, wrote paper; Søren Krogh Jensen; Provided butter from conventional fed cattle; Jacob Holm Nielsen; Lars I. Hellgren; Designed the study, interpreted results, and participated in writing the paper.

Reference List

Ailhaud G, Massiera F, Weill P, Legrand P, Alessandri JM & Guesnet P (2006) Temporal changes in dietary fats: Role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. *Progress in Lipid Research* **45**, 203-236.

Biong AS, Veierod MB, Ringstad J, Thelle DS & Pedersen JI (2006) Intake of milk fat, reflected in adipose tissue fatty acids and risk of myocardial infarction: a case-control study. *European Journal of Clinical Nutrition* **60**, 236-244.

Chavez JA, Knotts TA, Wang LP, Li GB, Dobrowsky RT, Florant GL & Summers SA (2003) A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *Journal of Biological Chemistry* **278**, 10297-10303.

Chocian G, Chabowski A, Zendzian-Piotrowska M, Harasim E, Lukaszuk B & Gorski J (2010) High fat diet induces ceramide and sphingomyelin formation in rat's liver nuclei. *Molecular and Cellular Biochemistry* **340**, 125-131.

Drachmann T, Mathiessen JH, Pedersen MH & Hellgren LI (2007) The source of dietary fatty acids alters the activity of secretory sphingomyelinase in the rat. *European Journal of Lipid Science and Technology* **109**, 1003-1009.

Ferre P & Foufelle F (2010) Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. *Diabetes Obesity & Metabolism* **12**, 83-92.

Field CJ, Blewett HH, Proctor S & Vine D (2009) Human health benefits of vaccenic acid. *Applied Physiology Nutrition and Metabolism-Physiologie Appliquee Nutrition et Metabolisme* **34**, 979-991.

Folch J, Lees M & Stanley GHS (1957) A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. *Journal of Biological Chemistry* **226**, 497-509.

German JB, Gibson RA, Krauss RM, Nestel P, Lamarche B, van Staveren WA, Steijns JM, de Groot LCPG, Lock AL & Destaillats F (2009) A reappraisal of the impact of dairy foods and milk fat on cardiovascular disease risk. *European Journal of Nutrition* **48**, 191-203.

Gibson RA, Makrides M, Smithers LG, Voevodin M & Sinclair AJ (2009) The effect of dairy foods on CHD: a systematic review of prospective cohort studies. *British Journal of Nutrition* **102**, 1267-1275.

Hamilton S & Hamilton RJ (1992) Extraction of lipids and derivative formation. In *Lipid analysis - a practical approach*: Oxford University Press, Oxford.

Hellgren LI (2010) *Phytanic acid-an overlooked bioactive fatty acid in dairy fat?* OXFORD: BLACKWELL PUBLISHING.

Hodge AM, English DR, O'Dea K, Sinclair AJ, Makrides M, Gibson RA & Giles GG (2007) Plasma phospholipid and dietary fatty acids as predictors of type 2 diabetes: interpreting the role of linoleic acid. *American Journal of Clinical Nutrition* **86**, 189-197.

Hu FB, Stampfer MJ, Manson JE, Ascherio A, Colditz GA, Speizer FE, Hennekens CH & Willett WC (1999) Dietary saturated fats and their food sources in relation to the risk of coronary heart disease in women. *American Journal of Clinical Nutrition* **70**, 1001-1008.

Hu FB, Stampfer MJ, Solomon CG, Liu SM, Willett WC, Speizer FE, Nathan DM & Manson JE (2001) The impact of diabetes mellitus on mortality from all causes and coronary heart disease in women - 20 years of follow-up. *Archives of Internal Medicine* **161**, 1717-1723.

Jensen RG (2002) The composition of bovine milk lipids: January 1995 to December 2000. *Journal of Dairy Science* **85**, 295-350.

Legrand P, Beauchamp E, Catheline D, Pedrono F & Rioux V (2010) Short Chain Saturated Fatty Acids Decrease Circulating Cholesterol and Increase Tissue PUFA Content in the Rat. *Lipids* **45**, 975-986.

Lin JD, Yang RJ, Tarr PT*, et al.* (2005) Hyperlipidemic effects of dietary saturated fats mediated through PGC-1 beta cactivation of SREBP. *Cell* **120**, 261-273.

Lipina C & Hundal HS (2011) Sphingolipids: agents provocateurs in the pathogenesis of insulin resistance. *Diabetologia* **54**, 1596-1607.

Madsen L, Liaset B & Kristiansen K (2008a) Macronutrients and obesity: views, news and reviews. *Future Lipidology* **3**, 43-74.

Madsen L, Pedersen LM, Liaset B*, et al.* (2008b) cAMP-dependent signaling regulates the adipogenic effect of n-6 polyunsaturated fatty acids. *Journal of Biological Chemistry* **283**, 7196-7205.

Merrill AH, Wang E & Mullins RE (1988) Kinetics of Long-Chain (Sphingoid) Base Biosynthesis in Intact Lm Cells - Effects of Varying the Extracellular Concentrations of Serine and Fatty-Acid Precursors of This Pathway. *Biochemistry* **27**, 340-345.

Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ & Flegal KM (2006) Prevalence of overweight and obesity in the United States, 1999-2004. *Jama-Journal of the American Medical Association* **295**, 1549-1555.

Promrat K, Longato L, Wands JR & de la Monte SM (2011) Weight loss amelioration of non-alcoholic steatohepatitis linked to shifts in hepatic ceramide expression and serum ceramide levels. *Hepatology Research* **41**, 754-762.

Riserus U, Willett WC & Hu FB (2009) Dietary fats and prevention of type 2 diabetes. *Progress in Lipid Research* **48**, 44-51.

Roche HM, Noone E, Nugent A & Gibney MJ (2001) Conjugated linoleic acid: a novel therapeutic nutrient? *Nutrition Research Reviews* **14**, 173-187.

Sleeth ML, Thompson EL, Ford HE, Zac-Varghese SEK & Frost G (2010) Free fatty acid receptor 2 and nutrient sensing: a proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. *Nutrition Research Reviews* **23**, 135-145.

Smedman AEM, Gustafsson IB, Berglund LGT & Vessby BOH (1999) Pentadecanoic acid in serum as a marker for intake of milk fat: relations between intake of milk fat and metabolic risk factors. *American Journal of Clinical Nutrition* **69**, 22-29.

Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM & Dixit VD (2011) The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nature Medicine* **17**, 179-U214.

Warensjo E, Jansson JH, Berglund L, Boman K, Ahren B, Weinehall L, Lindahl B, Hallmans G & Vessby B (2004) Estimated intake of milk fat is negatively associated with cardiovascular risk factors and does not increase the risk of a first acute myocardial infarction. A prospective case-control study. *British Journal of Nutrition* **91**, 635-642.

Warensjo E, Jansson JH, Cederholm T, Boman K, Eliasson M, Hallmans G, Johansson I & Sjogren P (2010) Biomarkers of milk fat and the risk of myocardial infarction in men and women: a prospective, matched case-control study. *American Journal of Clinical Nutrition* **92**, 194-202.

Warensjo E, Smedman A, Stegmayr B, Hallmans G, Weinehall L, Vessby B & Johansson I (2009) Stroke and plasma markers of milk fat intake - a prospective nested case-control study. *Nutrition Journal* **8**.

Yoshikawa T, Shimano H, Yahagi N*, et al.* (2002) Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *Journal of Biological Chemistry* **277**, 1705-1711.

Appendix B

Manuscript for study B:

"Effects of the natural PPARα and RXR agonist phytanic acid on glucose homeostasis and hepatic lipid status in obese C57bl/6j mice"

To be submitted for: PPAR Research

Effects of the natural PPAR-α and RXR agonist phytanic acid on glucose homeostasis and hepatic lipid status in obese C57bl/6j mice

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Abstract

Phytanic acid (PA) and its primary metabolite, pristanic acid have been identified as potent PPAR-α agonists, as well as being agonists for retinoid-X-receptor (RXR) *in vitro.* Based on these activities, PA and its metabolites are suggested to have preventive effects on metabolic dysfunctions, and several patents claim that PA-based nutraceuticals can be applied to improve metabolic health. As there have not so far been published studies in which these hypothesized effects of PA has been tested *in vivo*, we have tested effects on metabolic parameters, due to addition of PA in realistic amounts in both butter and grapeseed oil (GSO) given to obese mice. We did not find dramatic effects due to PA intake, though addition of 0.25% PA to high fat GSO diet attenuated increased hepatic TAG deposition completely, and 1.0% PA in high fat butter diet aggravated glucose homeostasis.

Introduction

Considering the global raise in prevalence of obesity and obesity-associated metabolic diseases, identifying dietary components that have specific positive effects on metabolic control is highly prioritized in the development of health-promoting foodstuff. One mechanism, through which food components directly can affect the metabolic state of the organism, is through agonist activity toward the nuclear receptors in the PPAR -family. It is well established that many food products contains molecules, e. g. fatty acids and certain terpenoids, that have PPAR-α and PPAR-γ agonist activity *in vitro* (Goto *et al.*, 2010; Varga *et al.*, 2011), much less is known on whether dietary compounds that *in vitro* have been identified as PPAR-α agonists, actually are able to modulate lipid and glucose metabolism *in vivo*, when eaten as parts of a normal diet. The branched chain fatty acid, phytanic acid (PA) (3, 7, 11, 15-tetramethyl hexadecanoic acid) and its primary metabolite, pristanic acid have been identified as potent PPAR-α agonists (Zomer *et al.*, 2000; Gloerich *et al.*, 2005; Gloerich *et al.*, 2007), as well as being agonists for retinoid-X-receptor (RXR) *in vitro* (Lemotte *et al.*, 1996). Phytanic acid has also been found to induce uncoupler protein 1 (UCP1) expression (Schluter *et al.*, 2002), and thus suggested to induce differentiation of brown adipocytes. Based on these activities, PA and its metabolites are suggested to have preventive effects on metabolic dysfunctions (Lemotte *et al.*, 1996; McCarty, 2001; Schluter *et al.*, 2002), and several patents claim that PA-based nutraceuticals can be applied to improve metabolic health (Eggersdorfer *et al.*, 2004; Fluehmann *et al.*, 2007).

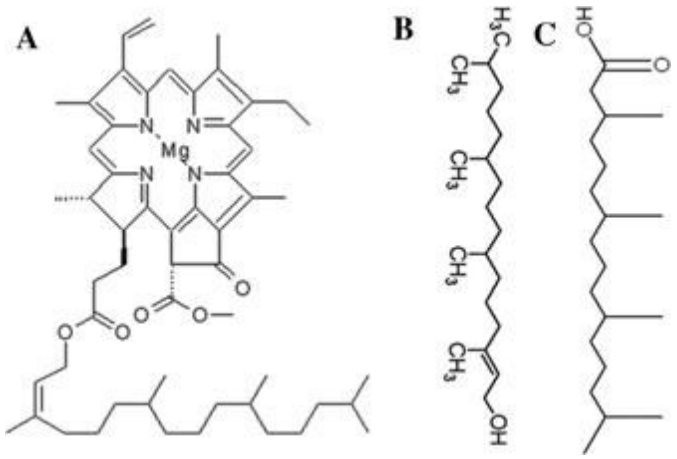

Figure 1 Structure of Chlorophyll (A), Phytol (B), and Phytanic acid (C).

Phytanic acid is a branched-chain C20 fatty acid, consisting of a C16-long chain with four methyl branches, see Figure 1. The human intake of PA primarily originate from the fat fractions of ruminant products (Hansen, 1966; Gloerich *et al.*, 2007). Phytanic acid is derived from phytol (3, 7, 11, 15-tetramethylhexadec-

2-en-1-ol), an aliphatic alcohol that are part of the chlorophyll molecule, also shown in Figure 1. The phytol moiety cannot be released from the chlorophyll-molecule in the human gastro-intestinal (GI) tract, while this occurs in the rumen of ruminant animals. In the animals the released phytol is oxidized to phytanic acid and incorporated in the ruminant meat and milk-fat (Hansen, 1966; Gloerich *et al.*, 2007). Since the precursor for its formation is chlorophyll, the concentration found in dairy products is mainly dependent on the amount of chlorophyll-containing plant material in the feed of the cattle. The concentration found in dairy products varies between 0.15 to around 0.50 percent of the fat fraction, depending on the feeding regime of the cows (Leiber *et al.*, 2005; Vetter & Schroeder, 2010).

Despite the indications from *in vitro* studies, that PA intake can improve metabolic function, through its PPAR-α and RXR agonist activity, this have not been tested *in vivo,* when PA is consumed in concentrations similar to the concentrations found in foodstuff. We have recently shown, that the concentrations of circulating PA in humans, are directly modulated by intake of dairy fat (Werner *et al.*, 2011), but to what extent dietary PA – consumption also affects fatty acid and glucose homeostasis, is not known. Until now, effects of PA *in* vivo, has been interpolated from studies using phytol in substantially higher dosages than what is found in human diets. Even though phytol is processed into PA in the metabolism, the effects of phytol-intake might differ from PA, since it has been reported that phytol also function as PPAR-α agonist (Goto *et al.*, 2005). Furthermore, to evaluate the nutritional/physiological relevance of dietary PPAR-agonists, it is essential that this is tested using concentration of the active molecule similar to what is found in foodstuffs. Therefore, we have determined the effect of PA intake on glucose-homeostasis, lipid accumulation and gene expression of genes related to glucose and lipid metabolism in the liver of diet-induced obese mice, using concentrations of PA that are similar to the concentrations in dairy fat. In one experiment, butter have been used as background diet for studying the dose-response to increased concentrations of PA, in another experiment, we also determined the effect of PA on glucose homeostasis and liver lipid levels, when a linoleic acid rich grapeseed oil (GSO) was used as background fat.

Methods and materials

Animal care, feed and tissue sampling: Male C57bl/6J mice were purchased from Charles River Laboratories International, Inc (Sulzfeld, Germany) and kept at 21 °C and 50% humidity, with a 12-h light cycle (07.00-19.00). The animals were attended daily and weighted weekly. All handling and use of animals in this study was approved by The Danish Animal Experiments Inspectorate and were carried out according to the guidelines of "The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purposes".

Table 1 Composition of the diets. Values are given as wt-%.

Components	Lean	Butter diets	PUFA diets
Protein	19,2	23,9	25,7
Maltodextrin 10	3,3	14,7	15,8
Sucrose	33,2	8,1	8,7
Corn starch	29,9	-	-
Soybean oil	2,4	2,9	3,2
Butter[1]	-	37,8	-
High oleic acid sunflower oil	-	-	-
Grapeseed oil	-	-	24,8
Lard	1,9	-	8,3
Vitamin mix[2]	0,9	1,2	1,3
Mineral mix[3]	0,9	1,2	1,3
Cellulose, BW200	4,7	5,9	6,3
Others[4]	3,5	4,4	4,4

1. Amount of butter inclusive water content
2. Vitamin mix s10026, for further information consult www.researchdiets.com
3. Mineral mix V10001, for further information consult www.researchdiets.com
4. Sum of dicalcium phosphate, calcium carbonate, potasium citrate, choline bitartrate, and dye.

From 10 weeks of age, the animals in cages randomized to DIO-diets, was given an obesity-inducing diet containing 60 E% fat in the feed and 15% (w/v) sucrose in the drinking-water for 10 weeks. The composition was chosen to mimic western cafeteria-type diets, high in both fat and sucrose. The diets were prepared by adding the experimental fats to "RD12492 px"-powder from "Research Diets Inc. (New Brunswick, NJ, USA)". Eight mice, were used as a lean reference group for each of the two experiments, and were given the appropriate control diets, as recommended by "Research Diets"; diet D12450B (Research Diets, New Brunswick, NJ, USA). In one experiment, animals were given increasing concentrations of PA, on a butter background. Thus, eight animals were given butter without added PA ethyl-ester (PA concentration = 0.20% (w/w fat)), eight

animals given the same butter, but with the addition of PA-ethyl esters to a final concentration of 0.6 % PA and finally was eight animals given the same butter with PA ethyl-esters added to a final concentration of 1.0 % PA. Production of the test butter (low PA-butter) have been described elsewhere (Werner *et al.*, 2011). For the setup with PA in a diet with high amount of linoleic acid, we used 75 % grape seed oil: 25 % lard mix (lard and grape-seed oil both from local supermarket) (GSO-diet). PA-ethyl ester was added to the concentration of 0.25 % of the fat mass to the feed for one group and another group was given identical feed without PA addition. The composition of the diets is given in Table 1, and the FA-compositions in the different diets can be seen from Table 2.

Table 2 Fatty acid composition of the feeds for the two experiments. Shown as mass% of total fatty acids. Nd= non-detectable, ta= trace amounts.

	Phytanic acid in butter				Phytanic acid in PUFA		
Fatty acid	Lean	0.2 % PA Butter	0.6 % PA Butter	1.0 % PA Butter	Lean	0.25 % PA PUFA	0 % PA PUFA
C4:0	nd	4.5	4.4	4.6	nd	nd	nd
C6:0	nd	2.5	2.4	2.5	nd	nd	nd
C8:0	nd	1.2	1.2	1.2	nd	nd	nd
C10:0	0.1	2.1	2.1	2.1	0.1	ta	ta
C12:0	0.1	2.4	2.3	2.3	0.1	ta	ta
C14:0	0.8	8.6	8.5	8.5	0.8	ta	ta
C14:1	nd	0.6	0.6	0.6	nd	nd	nd
C15:0	nd	0.8	0.8	0.8	nd	ta	ta
C16:0	15.7	20.2	20.3	20.2	15.7	12.3	12.2
C16:1	0.9	1.0	1.0	1.0	0.9	0.6	0.6
Phytanic acid	nd	0.2	0.6	1.0	nd	0.25	nd
C18:0	7.8	13.5	13.5	13.4	7.8	7.0	7.0
11tr-Vaccennic acid	nd	2.5	2.4	2.4	nd	nd	Nd
C18:1 n-9 cis	28.4	24.2	24.0	23.9	28.4	22.6	22.8
C18:2 n-6 cis	36.9	5.9	6.0	5.8	36.9	52.6	52.8
C18:3 n-6	0.1	0.4	0.3	0.3	0.1	0.2	0.2
C18:3 n-3	4.1	1.1	1.1	1.1	4.1	1.0	1.1
9Cis. 11trans-CLA	nd	0.7	0.7	0.7	nd	ta	ta
Others	5.1	8.0	7.9	7.8	5.1	2.9	2.9
ΣSFA	25.1	56.5	56.2	56.3	25.1	20.3	20.1
ΣMUFA	31.7	30.7	30.5	30.3	31.7	24.9	25.0
ΣPUFA	41.6	8.3	8.4	8.2	41.6	54.1	54.2

The mice had access to feed and water ad libitum. After 10 weeks on the experimental diets, overnight fasted animals were anaesthetized with 0.11 mL pr 25 g animal bodyweight of 44.44 mg/mL ketamin and 2.22 mg/mL Narcoxyl(xylazin), before they were sacrificed through cardiac puncture. Blood was collected in heparin-coated tubes and stored on ice, until plasma was isolated. Tissue samples to be used for RNA extraction was transferred to RNALater (Applied Biosystems, Carlsbad, California, USA), the rest of the tissue was snap-freezed in liquid N_2 and stored at -80ºC until it was analyzed.

Synthesis of phytanic acid ethyl esters

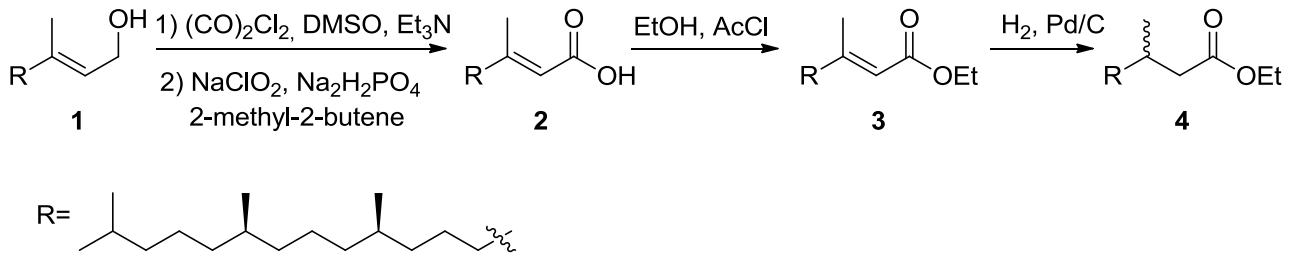

Figure 2 Organic synthesis of phytanic acid from phytol.

Phytanic acid (target molecule 4 in Figure 2) was easily prepared on large scale from commercially available phytol. The preliminary strategy was based on reduction of the double bond of phytol followed by oxidation and esterification. However, hydrogenation over Pd/C resulted in extensive deoxygenation of the β-hydroxy group. Instead phytol was first oxidized to the aldehyde via a Swern oxidation followed by a Pinnick oxidation to give the carboxylic acid **2** in excellent yield. Compound **2** was then converted into the ethyl ester **3** and finally hydrogenated to give the target molecule **4** in 81% overall yield. This strategy is advantageous compared to the former syntheses of phytanic acid due to their use of expensive reagents for either hydrogenation or oxidation. Detailed description of synthesis and purification is given in Supplemental Materials.

Lipid analysis: Lipids were extracted from tissue samples and feed using the Folch method (Folch *et al.*, 1957), with internal standards (nonadecanoic acid, C19:0 triacylglyceride (TAG), C19:0 phosphatidylcholine and ceramide with C17:1 long-chained base) added. Liver fat content and fatty acid composition in TAG, PL and FFA was determined using GC-FID after separation of the lipid

classes using preparative TLC and preparation of fatty acid methyl-esters, as earlier described (Artmann *et al.*, 2008).

The FAME were separated on a 60-m Supelco SP2380 column (Sigma-Aldrich AS, Brøndby, Denmark) in a HP 6890 gas chromatograph (GC), in split mode using He as carrier gas. Settings for the GC-program were: Injector temperature 260 °C, split ratio 20 : 1, carrier flow 1.2 mL/min, detector temperature 300 °C, air flow in detector 300,0 mL/min, hydrogen flow 35 mL/min. FAME were separated using a temperature program starting at 50 °C and rising to 160 °C at 15 °C /min; this temperature was kept for 0 min, hereafter the temperature was raised to 182 °C at 1 °C /min, and directly the temperature was raised to 200 °C at 10 °C /min, and the oven was kept at 200 °C for 15 min before the temperature was raised to 225 °C. The final temperature was kept for 12 min (total runtime 61.97 min). Response factors was calculated for methylesters of phytanic acid, C4:0, C6:0 and C8:0, based on the response of palmitic acid methyl ester (16:0). Ceramide was isolated from total lipid extract and quantified as earlier described in (Drachmann *et al.*, 2007), with the exception that ceramide with C17 long-chained base was used as internal standard.
Fat content in feed was determined by gravitational measurement after Folch extraction.
Analysis of plasma TAG, NEFA, Cholesterol and glucose: Plasma levels of TAG, non-esterified fatty acids (NEFA), total-cholesterol, and glucose, were measured with a "Cobas Mira Plus" (Roche Diagnostics Systems, Basel, Switzerland). TAG, total-cholesterol, and glucose kits were from HORIBA abx; (Montpellier, France) and NEFA was analyzed using the appropriate kit from Wako Chemicals Gmbh (Neuss, Germany). All analyses were performed according to the instruction from the manufacturer.

Oral Glucose Tolerance Test (OGTT): OGTT was performed after ten weeks on the given diets. The animals were fasted for 3 hours prior to oral glucose infusion (1.5 g glucose per kg bodyweight). Blood-glucose levels were measured to the time points zero, one, and two hours after glucose infusion and at the time points zero and two hours after infusion eye-blood was taken with 50 µL EDTA-coated capillary tubes to produce plasma for insulin measurements.

Blood glucose measurements was performed with "On call® Plus Blood Glucose Meter" (Acon Laboratories, inc., San Diego, USA), by adding one drop of eye-blood to the chip according to the manufacturers instruction.

Insulin & Leptin: Plasma insulin was analyzed using "Ultra sensitive mouse insulin ELISA" (Mercodia AB, Uppsala, Sweden), following the manufactures guidelines. Plasma levels of leptin was analyzed using the appropriate kit from from Meso Scale Diagnostics (Gaithersburg, USA) on a Meso Scale Sector Imager 6000 (leptin), according to the manufacturers recommendations.

Gene expression analysis/qRT-PCR: RNA from liver-tissue was extracted on Abi Prism 6100 Nucleic acid Prepstation from Applied Biosystems (Carlsbad, California, USA), according to the manufacturer's instruction.
cDNA-synthesis was performed on Unocycler (VWR (Leuven, Belgium), using "High capacity cDNA transcription kit" from Applied Biosystems. The quantification was performed on a 7900HT Fast Real Time PCRsystem, equipped with 96 wells Fast block, using "Taqman® Environmental master mix 2.0" (Applied Biosystems), and with primers and TaqMan-probes selected from Applied Biosystems and Integrated DNA Technologies (Coralville, IA, USA) online catalogues. SDS 2.3 and RQ-manager 1.2 software was used for data analyses. Data was analyzed using the ΔΔCt method, using glyceraldehydes-6-phosphate expression as reference-gene.

Statistical analysis: Statistical analysis was performed using the software package GraphPad Prism (v.5.00). One way ANOVA with "Turkey" post test performed when nothing else noted. In cases of unequal variances in between groups in datasets (tested by "Bartlett´s test") statistical analysis is performed on LogY transformed data. Statistical analysis of hepatic tissue mass for the PA dose response in butter, is Welch´s ANOVA performed in R. Results are given as average ± Standard deviation (SD), (n=8 for all groups). Statistical outliers were detected using Grubbs test (www.graphpad.com), data-points identified as outliers ($p<0.05$) was excluded from statistical analyses.

Results

Phytanic acid incorporation into hepatic lipid fractions

Increasing phytanic acid intake from 0.2 to 1.0 % resulted in increased incorporation in both the TAG- and PL-lipid pools of the liver (Figure 3), as expected was there no detectable PA in the reference group that only was given chow (not shown). From Figure 3A and B it is evident that PA is incorporated into PL in a strict dose-dependent response, while this was not the case for TAG, were only the highest concentration resulted in significantly higher PA-incorporation. The

concentration of PA in FFA was very similar to what was seen in TAG, with significantly higher levels of PA in the 1.0 % PA –group, compared to the 0.20%-group. As expected, the animals given the GSO diet had no PA in the hepatic TAG and PL, but that the addition of 0.25% PA to the fat fraction of the diet resulted in significant incorporation in both lipid fractions.

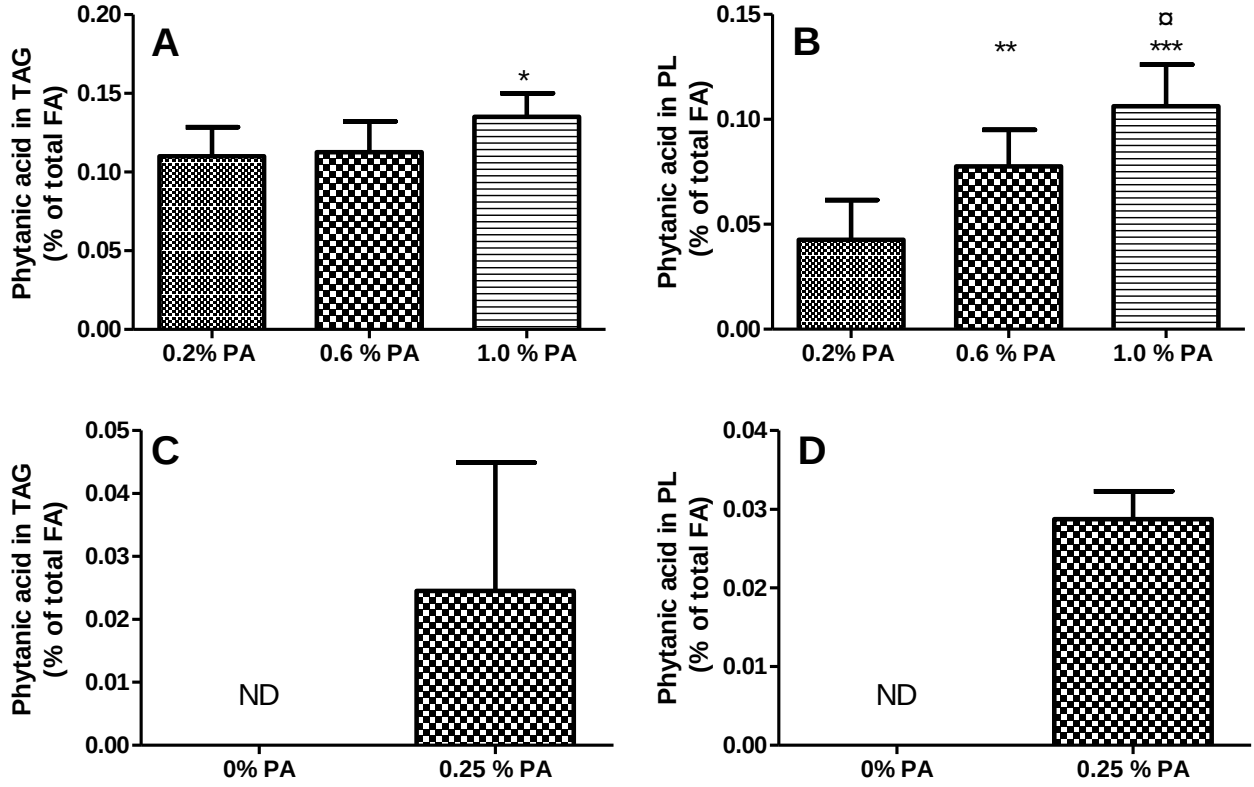

Figure 3 Phytanic acid incorporation into hepatic lipid pools. **A**: Phytanic acid incorporation into hepatic triacylglycerides after feeding with butter containing varied concentrations of phytanic acid for 10 weeks. **B**: Phytanic acid incorporation in to hepatic phospholipids, otherwise as in A. **C**: Phytanic acid incorporation in to hepatic triacylglycerides after feeding with grapeseed oil containing 0 or 0.25% PA ethyl-esters. **D**: Phytanic acid incorporation in to hepatic phospholipids, otherwise as in C. Data are given as average ± st. dev. n= 7-8, * indicates statistical significant difference against 0.2% PA group, and ¤ indicates statistical significant difference against 0.6% PA group. ND = Non detectable.

Body and organ masses

From table 3, it is evident that the DIO-groups have significantly higher body-mass and total WAT mass (sum of epididymal-, kidney-, and visceral-WAT), compared to the lean references ($P<0.001$) in both experiments, and that PA addition did not have any effect on these parameters.

Interestingly, while hepatic tissue mass was statistical significant higher for all three DIO groups in the PA-dose response experiment in butter ($P<0.05$ for the 0.2 %, and $P<0.01$ against the two other groups) compared to the lean references, 0.25% PA protected against dietary induced hepatic weight-gain, when it was given in GSO.

Table 3 Different metabolic parameters for A, phytanic acid dose response in butter high fat feed; and B, Phytanic acid in PUFA high fat feed, given as mean ± s.d. * indicates statistical significant difference against lean reference. No statistical significant differences observed between high fat groups on these parameters within the two setups.

	A: Phytanic acid dose response in butter				**B**: Phytanic acid in PUFA feed		
	Lean	0.2 % PA Butter	0.6 % PA Butter	1.0 % PA Butter	Lean	0 % PA PUFA	0.25% PA PUFA
Bodyweight (g)	27.3±1.3	44.0±3.0***	44.8±4.3***	43.6±4.8***	27.1±1.1	42.1±3.2***	41.3±3.2** *
Hepatic tissue weight (g)	1.05±0.11	1.91±0.76*	1.94±0.58**	1.98±0.58**	1.09±0.19	1.44±0.34*	1.26±0.21
WAT mass (g)	0.40±0.20	5.69±0.39***	5.72±0.74***	5.49±1.21***	0.47±0.11	5.52±0.54* **	5.52±0.68* **
Plasma TAG (mM)	0.64±0.14	0.98±0.14**	0.82±0.18	0.87±0.17*	0.90±0.24	0.68±0.13	0.77±0.17
Plasma NEFA	0.85±0.11	1.05±0.18	1.04±0.11	1.08±0.16*	1.12±0.30	1.03±0.15	1.19±0.16
Plasma Chol	2.41±0.24	4.60±0.84***	4.22±1.05***	4.50±1.06***	2.85±0.95	4.54±0.57* *	4.60±0.85* *
Fasting glucose (mM)	11.6±1.3	13.9±3.0	13.4±3.1	12.8±1.9	7.7±1.8	14.20±2.6* *	13.9±1.2**
Fasting insulin (µg/L)	0.63±0.52	7.82±7.21***	13.40±7.22** *	13.8±6.67***	0.52±0.47	3.47±0.75* *	2.71±1.22*
Leptin (ng/mL)	0.14±0.16	22.56±11.08 ***	29.26±8.60** *	30.15±5.23** *	0.21±0.26	20.72±9.67 ***	20.45±9.3 6***

Plasma lipids, insulin, leptin and glucose homeostasis

Although the average level of plasma TAG and NEFA was similar, and raised compared to the lean reference, in all three DIO-groups in the dose-response experiment, the values for TAG did only reach statistical significance at 0.2 and 1.0 % PA ($P<0.01$ and $P<0.05$, respectively) and for plasma NEFA was it only significant compared to the lean reference at 1.0 % PA ($P<0.05$). Plasma cholesterol was significantly higher in all DIO-groups, without any effect of the PA addition (Table 3).

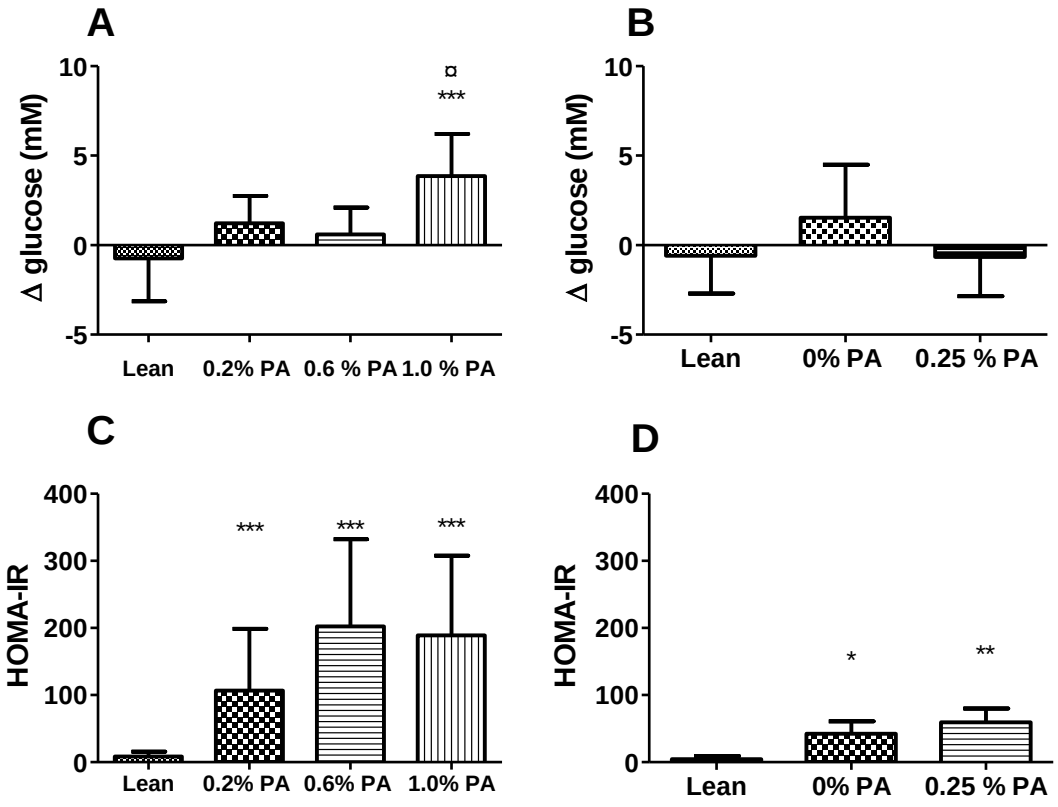

Figure 4 Glucose homeostasis measurements. **A**: Difference in blood glucose between the semi-fasted value (3.5 h fasting) and the value 2 hours after glucose gavage feeding ($\Delta_{glucose}$) at a oral glucose tolerance test (OGTT), after feeding with butter containing varied concentrations of phytanic acid for 10 weeks. **B**: Difference in blood glucose between the semi-fasted value (3.5 h fasting) and the value 2 hours after glucose gavage feeding ($\Delta_{glucose}$) at a oral glucose tolerance test (OGTT), after feeding with grapeseed oil containing 0 or 0.25% PA ethyl-esters for 10 weeks. **C**: Homeostatic model assessment of insulin resistance (HOMA-IR) otherwise as in A. **D**: Homeostatic model assessment of insulin resistance (HOMA-IR) otherwise as in B. n= 7-8, * indicates statistical significant difference against lean reference, and ¤ indicates statistical significant difference against 0.2% PA group.

In the dosis-response experiment, was there neither any effect of increasing PA concentrations in the diet on either hepatic TAG or –ceramide deposition (Figure 5A and C), while 0.25 % PA protected against increased TAG accumulation in the liver, when given in GSO (Figure 5B). The level of FFA in the liver was not affected by any dietary treatment (not shown).

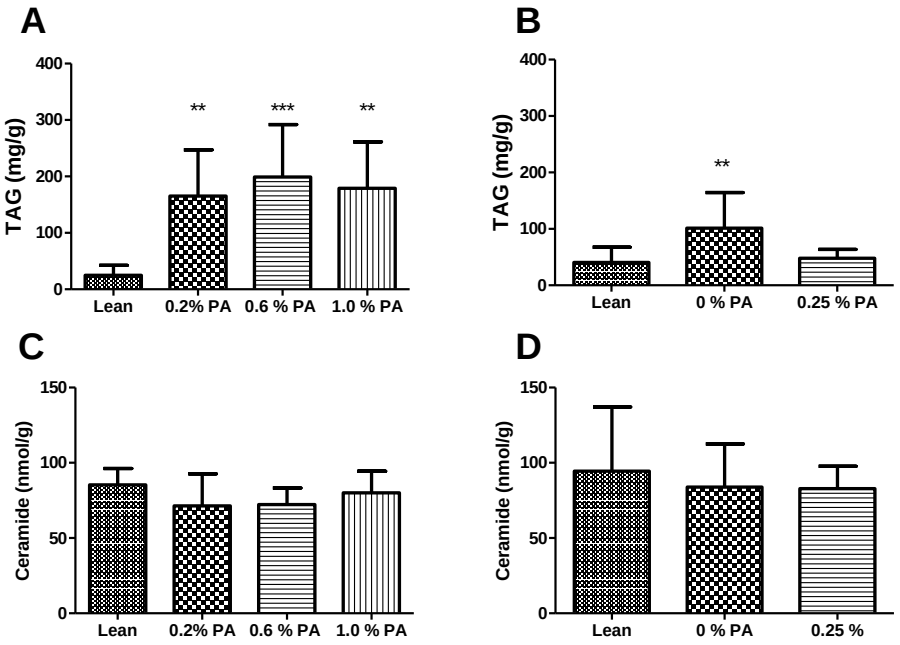

Figure 5 Effects of dietary phytanic acid on liver lipid accumulation. A: Hepatic TAG in animals given varying concentrations of PA-ethyl esters (given as % of total fat) in butter. **B**: Hepatic ceramide concentrations otherwise as in A. **C**: Hepatic TAG in animals given 0 or 0.25 % (mass % of total fat in diet) PA ethyl-esters. **D**: Hepatic ceramide, otherwise as in C.

There were no significant difference in fasting glucose between the DIO-groups and the lean references in the dose-response experiment, where butter was used as fat source, while it was significantly higher for both groups given GSO compared to the corresponding lean reference ($P<0.01$) (not shown). The results from a oral-glucose tolerance test (OGTT) in the dose-response experiment, show that the only statistical significant effect on the difference in blood glucose concentration between the fasting value (time 0) and 2h after glucose gavage ($\Delta_{glucose}$), was seen in the 1.0 % PA group, which had higher $\Delta_{glucose}$ than the lean references and the corresponding 0.6 % PA group ($P<0.001$, and $P<0.05$) (Figure 4A). There were no statistical significant difference in this value between the three groups in the experiment when PA was added to GSO (Figure 4B), although the corresponding value from one to zero hours after oral dosage of glucose, was statistically significant higher for the GSO group without PA than for the lean reference ($P<0.01$, data not shown). Fasting insulin was statistically significant higher in the animals from all DIO-

groups, compared to lean references (P<0.001 for 0.2 and 1.0 % PA in butter, P<0.01 for 0.6% PA in butter and GSO without PA, and P<0.05 for 0.25% PA in GSO diet). Likewise, leptin was statistically significant higher (P<0.001) for DIO groups compared against the lean references of the respective studies. The results of fasting insulin resulted in statistical significant higher values for all DIO groups compared for the lean reference of the respective experiments, when homeostatic model assessment for insulin resistance (HOMA-IR) was calculated (Figure 4 C and D (P<0.001 for all butter groups, P<0.01 for PUFA without PA, and P<0.05 for the PUFA diet with 0.25 % PA).

Hepatic gene expression

Hepatic gene expression was performed for the PA dose-response in butter. The expression of the genes coding for PPARα and the PPARα-regulated ACOX1, was statistically significant (P<0.01), although limited, up regulated in the 1.0% PA in butter group compared to the lean references, (seen in

Figure 6), while other PPAR-α target genes (CD36 and CPT1a) was strongly affected by the DIO-diet, but not significantly up-regulated of the PA-intake. The genes coding for proteins controlling fatty acid synthesis (SREBP1 and FASN) was statistical significant up regulated in all the obese groups compared to lean references, without effect of PA. The transcription of the glucose-6-phosphatase gene was determined as an example of an insulin-regulated gene. On average did all three DIO-treatments cause increased expression, although only the increase in the group that had received 0.6% PA reached significance. The average expression of the macrophagal marker EMR1 (F4/80), was, on average, also increased in the three DIO-groups, but this did only reach significance in the 1 % PA group. The relative hepatic gene expression of α-methylacyl-CoA racemase (AMACR) was analyzed as a measure of the induction of the phytanic acid catabolic pathway. The amount of transcript was significantly reduced in the 0.2 % and the 0.6 % PA groups compared to the lean references (P<0.05 and P<0.01, respectively), while this was not the case for the 1.0% PA group.

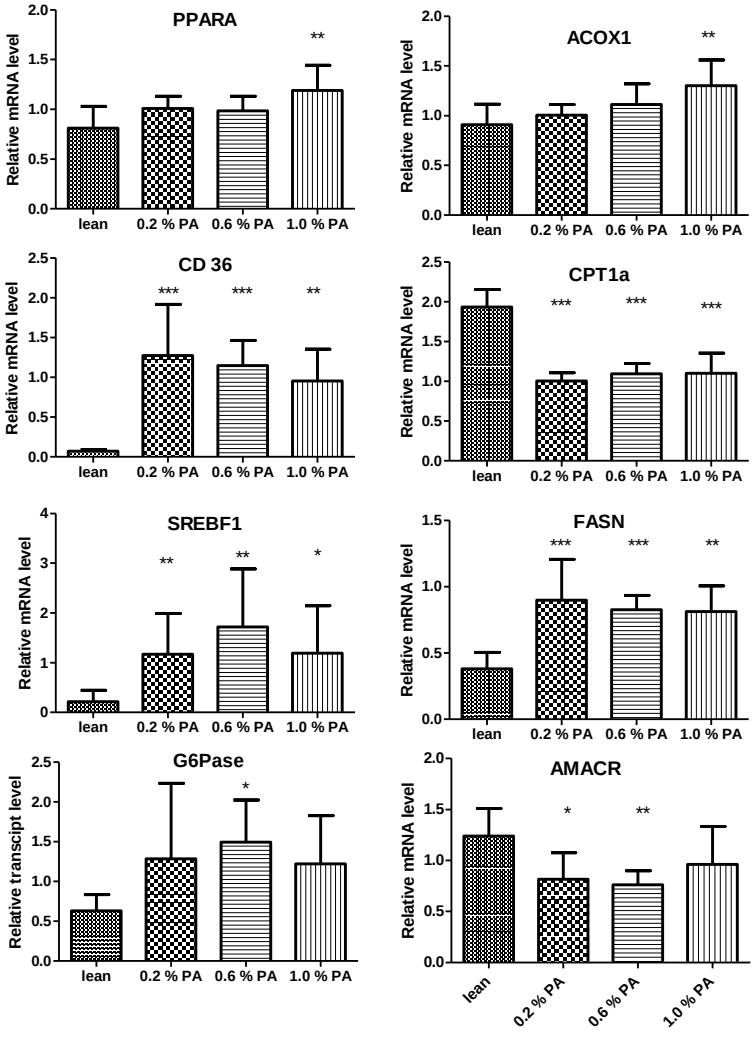

Figure 6 Relative hepatic gene expression normalized to the average value for the group given 0.2% PA. Values are based on the ΔΔCt-values using GAPDH as reference-gene. Data are given as average ± st. dev. n= 7-8, * indicates statistical significant difference against 0.2% PA group, and ¤ indicates statistical significant difference against 0.6% PA group.

Discussion

From the compiled data of this study, which is the first *in-vivo* study in which an intervention with the natural PPAR-α agonist phytanic acid that have been performed, we did not see any dramatic dose-dependent effects from physiological realistic PA dosages, when given in the butter diet, although the supra-dietary concentrations (1% PA of total fat) caused an impaired glucose tolerance. This indicates that PA intake from dairy-products do not have any physiological relevant effects on the control of lipid and glucose metabolism, but that intake of products with higher PA-concentrations than around 0.6% of total fat, might be detrimental. The lack of effect at lower concentrations than 1.0%, cannot be explained by a lack of incorporation into the relevant tissue, as evidenced by the retrieval of PA in the hepatic-lipid pools, hereunder the dose-dependent incorporation of phytanic acid into the phospholipid pool (Figure 3). However, as evidenced by the expression of the PPAR-α controlled genes PPAR-α and ACOX, it is also only after intake of the 1.0% PA-diets, that dietary PA actually cause a transcriptional activation of these genes, and, furthermore, this increase is very small (about 20 – 30 %). Thus, we can conclude that PA at concentrations found in dairy fat, do not induce PPAR-α activated gene-expression, but that highly slightly higher levels cause a minor increased transcription in some genes. It is also noteworthy that not all assayed PPAR-α controlled genes were affected by the increased PA-level, even after intake of the 1.0 %-diets. For CD 36 and Cpt1a, there was no dose-response to the addition of phytanic acid; expression of both these genes was however strongly affected by the DIO-diet. It is likely that the strong effect of the DIO-diet, have overruled the very small effect that is seen from PA-activation in PPAR-α and ACOX. From the presented data, it cannot be excluded that the observed 16-fold induction of CD36 and 50 % reduction in Cpt1a is an effect of the phytanic acid levels endogenous in the butter diets. However, we and others have shown that upregulation of CD36 and downregulation of Cpt1a are general response to the high fat/high sucrose diets in C57bl/6J (Drachmann et al. in preparation)(Koonen *et al.*, 2007).

The observed effect, that 0.25% PA totally abolishes the increased liver mass and TAG deposition, caused by high fat diet, when given in a linoleic acid rich-diet but not in a butter-based diet, is important. It shows that the effect of a dietary PPARα-agonist is highly dependent on the fatty acid composition of the diet. The effect might be explained by a PA-induced decrease in FA oxidation, increase in FA-synthesis, increased FA uptake or a combination of these. It can be hypothesized that this effect is due to synergistic effects between PA and the higher levels of PUFAs on PPAR-α on FA oxidation, in the GSO-fed groups. In these groups, total PUFA concentration in hepatic PL and TAG was around 52 and 47 %, respectively, while corresponding values was only 40 and 9 %, in the butter fed animals (not shown) . Thus, there are substantially higher levels of other natural PPARα agonists available in the liver of the GSO-fed animals.

However, others have shown that dietary phytol effects on hepatic FA oxidation is attenuated by both n-3 and n-6 PUFAs (Hashimoto *et al.*, 2006), which is contra dictionary to this hypothesis.

The most important finding of this study is the detrimental effect of PA on the glucose clearance rate measured as $\Delta_{glucose}$ values, when PA was given at 1.0 % of total fat in the dose-response study. Although the effect did not reach statistical significance, we observed tendencies toward a beneficial effect of PA in low amounts (0.6 vs 0.2 % in Figure 4A and 0.25% vs 0% in Figure 4) with regard to glucose clearance after an oral glucose-load, which could indicate that the effect of phytanic acid on glucose-tolerance is not linear, but that it might improve glucose-tolerance at lower concentrations, and reduce it at higher levels (> 0.6% of the dietary fat). When we consider the PA content in dairy products, the highest amounts of PA observed is around 0.55% PA (Leiber *et al.*, 2005), and it therefore seems that PA in dairy products would not have harmful effects with regard to glucose tolerance. With regard to insulin resistance, measured as HOMA-IR, our data does not show any effects of the addition of PA into high fat diets given to obesity prone C57bl/6j mice.

The observed reduction in expression of AMACR in the obese animals is also noteworthy. Phytanic acid is oxidized through a combination of peroxisomal α- and β-oxidation and AMACR-activity is essential for this to occur (Lloyd *et al.*, 2008). Therefore, reductions in its expression might reduce the efficiency by which phytanic acid is oxidized and enhance the tissue accumulation. Since increased tissue accumulation of PA have been associated with several serious pathologies, such as hepatotoxicity and prostate-cancer (Mackie *et al.*, 2009), and that the risk of prostate cancer also have been associated with genetic variations in the AMACR-gene (FitzGerald *et al.*, 2008), obesity induced reductions in this activity warrants further studies.

As this is the first time *in vivo* effects of PA have been tested in concentrations similar to concentrations found in food products, we cannot compare our findings directly to other studies. Though, studies with the PA precursor phytol have been performed, however, in concentrations higher than what is accessible through food products. In our own lab, addition of 0.2% and 0.5% phytol to normal chow diet for four weeks, substantially decreased hepatic TAG in balb/c mice (Hellgren, 2010). Other groups has shown that 0.5% phytol upregulates gene expression and enzyme activities of both peroxisomal and mitochondrial FA oxidation (Gloerich *et al.*, 2005; Hashimoto *et al.*, 2006). As the amounts of phytol used in the mentioned studies is substantially higher than what it is possible to intake through the diet, those results does not seem relevant with regard to a normal human diet. As we have previously shown that it is possible to affect the human plasma PA concentration through dairy product intake, it would have been relevant to human

nutrition if we had found significant effects from PA intake in the butter based diets. Though, we in this study show that relatively low levels of PA protects against hepatic TAG deposition in high fat GSO diet, it can be argued that this does not seem as interesting as PA in the diet primarily comes from dairy and ruminant meat products, which are not high in PUFA. However, it indicates that low levels of PA could be used as a nutraceutical in PUFA rich products.

Conclusion

Intake of PA in concentrations found in dairy-fat, leads to uptake and incorporation into both hepatic TAG and PL fractions. When given in a high sugar/high fat-diet, PA have no effect on glucose-clearance nor on fasting glucose and insulin, when given in concentrations up to 0.6% of total fat, while at 1.0 % PA in total fat, glucose-clearance rate was reduced. In a PUFA-rich diet, low levels of PA (0.25%) protects against obesity induced hepatic lipid-accumulation.

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Reference List

Artmann A, Petersen G, Hellgren LI, Boberg J, Skonberg C, Nellemann C, Hansen SH & Hansen HS (2008) Influence of dietary fatty acids on endocannabinoid and N-acylethanolamine levels in rat brain, liver and small intestine. *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids* **1781**, 200-212.

Drachmann T, Mathiessen JH, Pedersen MH & Hellgren LI (2007) The source of dietary fatty acids alters the activity of secretory sphingomyelinase in the rat. *European Journal of Lipid Science and Technology* **109**, 1003-1009.

Eggersdorfer ML, Raederstorff D, Teixeira SR, Weber P, Ludwig EM, Daniel R, Renata TS & Peter W (2004) Nutraceutical compositions, useful to treat/prevent diabetes and other conditions associated with impaired glucose tolerance, comprise biotin and pantethine, epigallocatechin gallate, phytanic acid, lipoic acid and/or policosanol. [BV DSM IP ASSETS, ML Eggersdorfer, D Raederstorff, SR Teixeira, and P Weber, editors].

FitzGerald LM, Thomson R, Polanowski A, Patterson B, Mckay JD, Stankovich J & Dickinson JL (2008) Sequence variants of alpha-methylacyl-CoA racemase are associated with prostate cancer risk: A replication study in an ethnically homogeneous population. *Prostate* **68**, 1373-1379.

Fluehmann B, Heim M, Hunziker W*, et al.* (2007) New composition comprising phytanic acid or its derivatives, useful for treating or preventing non-insulin dependent diabetes mellitus, impaired glucose tolerance and related obesity. [AG ROCHE VITAMINS, HOFFMANN LA ROCHE & CO AG, ROCHE VITAMINS INC, FOUN BUR RES, DSM NUTRITIONAL PROD INC, and BV DSM IP ASSETS, editors].

Folch J, Lees M & Stanley GHS (1957) A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. *Journal of Biological Chemistry* **226**, 497-509.

Gloerich J, van den Brink DM, Ruiter JPN, van Vlies N, Vaz FM, Wanders RJA & Ferdinandusse S (2007) Metabolism of phytol to phytanic acid in the mouse, and the role of PPAR alpha in its regulation. *Journal of Lipid Research* **48**, 77-85.

Gloerich J, van Vlies N, Jansen GA, Denis S, Ruiter JPN, van Werkhoven MA, Duran M, Vaz FM, Wanders RJA & Ferdinandusse S (2005) A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPAR alpha-dependent and -independent pathways. *Journal of Lipid Research* **46**, 716-726.

Goto T, Takahashi N, Hirai S & Kawada T (2010) Various Terpenoids Derived from Herbal and Dietary Plants Function as PPAR Modulators and Regulate Carbohydrate and Lipid Metabolism. *PPAR research*.

Goto T, Takahashi N, Kato S, Egawa K, Ebisu S, Moriyama T, Fushiki T & Kawada T (2005) Phytol directly activates peroxisome proliferator-activated receptor alpha (PPAR alpha) and regulates gene expression involved in lipid metabolism in PPAR alpha-expressing HepG2 hepatocytes. *Biochemical and Biophysical Research Communications* **337**, 440-445.

Hansen RP (1966) Occurrence of Phytanic Acid in Rumen Bacteria. *Nature* **210**, 841-&.

Hashimoto T, Shimizu N, Kimura T, Takahashi Y & Ide T (2006) Polyunsaturated fats attenuate the dietary phytol-induced increase in hepatic fatty acid oxidation in mice. *Journal of Nutrition* **136**, 882-886.

Hellgren LI (2010) *Phytanic acid-an overlooked bioactive fatty acid in dairy fat?* OXFORD: BLACKWELL PUBLISHING.

Koonen DPY, Jacobs RL, Febbraio M, Young ME, Soltys CLM, Ong H, Vance DE & Dyck JRB (2007) Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity. *Diabetes* **56**, 2863-2871.

Leiber F, Kreuzer M, Nigg D, Wettstein HR & Scheeder MRL (2005) A study on the causes for the elevated n-3 fatty acids in cows' milk of alpine origin. *Lipids* **40**, 191-202.

Lemotte PK, Keidel S & Apfel CM (1996) Phytanic acid is a retinoid X receptor ligand. *European Journal of Biochemistry* **236**, 328-333.

Lloyd MD, Darley DJ, Wierzbicki AS & Threadgill MD (2008) alpha-Methylacyl-CoA racemase - an 'obscure' metabolic enzyme takes centre stage. *Febs Journal* **275**, 1089-1102.

Mackie JT, Atshaves BP, Payne HR, McIntosh AL, Schroeder F & Kier AB (2009) Phytol-induced Hepatotoxicity in Mice. *Toxicologic Pathology* **37**, 201-208.

McCarty MF (2001) The chlorophyll metabolite phytanic acid is a natural rexinoid - potential for treatment and prevention of diabetes. *Medical Hypotheses* **56**, 217-219.

Schluter A, Barbera MJ, Iglesias R, Giralt M & Villarroya F (2002) Phytanic acid, a novel activator of uncoupling protein-1 gene transcription and brown adipocyte differentiation. *Biochemical Journal* **362**, 61-69.

Varga T, Czimmerer Z & Nagy L (2011) PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochimica et Biophysica Acta-Molecular Basis of Disease* **1812**, 1007-1022.

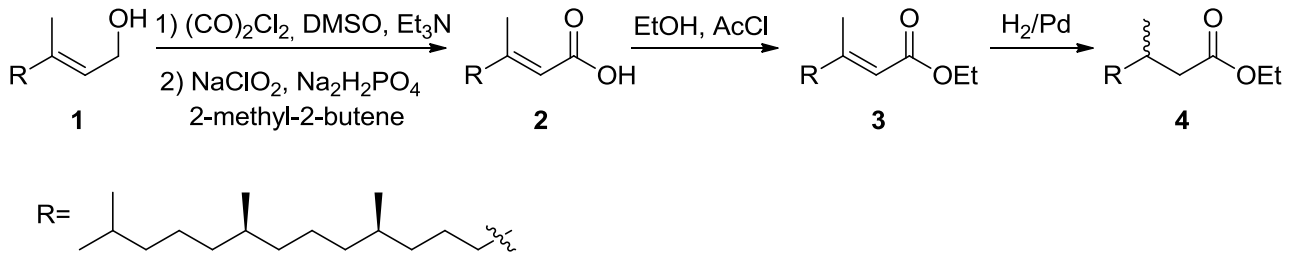
Vetter W & Schroeder M (2010) Concentrations of phytanic acid and pristanic acid are higher in organic than in conventional dairy products from the German market. *Food Chemistry* **119**, 746-752.

Werner LB, Hellgren LI, Raff M, Jensen SK, Petersen RA, Drachmann T & Tholstrup T (2011) Effect of dairy fat on plasma phytanic acid in healthy volunteers - a randomized controlled study. *Lipids in Health and Disease* **10**.

Xu JF, Thornburg T, Turner AR*, et al.* (2005) Serum levels of phytanic acid are associated with prostate cancer risk. *Prostate* **63**, 209-214.

Zomer AWM, van der Burg B, Jansen GA, Wanders RJA, Poll-The B & van der Saag PT (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferater-activated receptor alpha. *Journal of Lipid Research* **41**, 1801-1807.

Supporting information.
Synthesis of (7R, 11R)-ethyl 3, 7, 11, 15-tetramethylhexadecanoate



(7R, 11R, E)-3, 7, 11, 15-tetramethylhexadec-2-enoic acid

A mixture of CH_2Cl_2 (400 mL) and DMSO (9,6 mL; 135 mmol) was cooled to -70°C. To the mixture a solution of oxalyl chloride (5.9 mL; 67 mmol) in CH_2Cl_2 (20 mL) was added drop wise while maintaining a temperature below -60°C throughout the addition period. After 15 min a solution of phytol (10 g; 34 mmol) in CH_2Cl_2 (20 mL) was added drop wise, again maintaining a temperature below -60°C. After another 15 min Et_3N (23.5 mL; 168 mmol) was added and the reaction mixture was allowed to reach room temperature.
The reaction mixture was washed with sat. aq. NH_4Cl (300 mL) and H_2O (3x150 mL), dried over $MgSO_4$ and concentrated to give the aldehyde as a yellow oil.
The crude product was used in the following step without further purification

The aldehyde (10 g; 34 mmol) was dissolved in ^tBuOH (680 mL) and THF (340 mL). 2-methyl-2-butene (180 mL; 1.7 mol) was added followed by a solution of $NaClO_2$ (30.6 g; 0.34 mol) and $Na_2H_2PO_4$ (30.6 g; 0.26 mol) in H_2O (340 mL). The reaction mixture was stirred at room temperature until full conversion was observed (1 h).
The reaction mixture was washed with sat. aq. $Na_2H_2PO_4$ (500 mL) and the aqueous phase was extracted with EtOAc (2x500 mL). The combined organic phases were dried over $MgSO_4$ and concentrated.
The product was purified by flash chromatography to give **2** as a colorless oil.

(7R, 11R, E)-ethyl 3, 7, 11, 15-tetramethylhexadec-2-enoate

2 (10 g; 32.2 mmol) was dissolved in EtOH (65 mL). Acetyl chloride (0.34 mL; 5 mmol) was added and the reaction mixture was stirred at refluxing temperature for 48 hours.
The reaction mixture was neutralized with Et_3N and concentrated. The crude oil was dissolved in Et_2O (400mL) and washed with sat. aq. NH_4Cl (3x300 mL) and H_2O (3x300 mL). The organic phase was dried over $MgSO_4$ and concentrated.
The product was purified by flash chromatography to give **2** as a colorless oil.

(7R, 11R)-ethyl 3, 7, 11, 15-tetramethylhexadecanoate

4 (10 g; 339 mmol) was dissolved in EtOH (75 mL) and THF (25 mL). 10 wt% Pd/C (0.31 g; 0.30 mmol) was added and and an atmosphere of H_2 (1 atm.) was installed. The reaction was stirred until TLC indicated full conversion (48 h), filtered through celite, and concentrated to give a colorless oil.

Appendix C

Manuscript for study C:

"*Implications of dairy-fat and the dairy fat component phytanic acid on T-cell cytokine shifting in activated CD3+ T-cells from a double blinded 12 week-randomized butter intervention*"

To be submitted for: Molecular Nutrition and Food Research

Implications of dairy-fat and the dairy-fat component phytanic acid on T-cell cytokine shifting in activated human CD3+ T-cells from a double-blinded 12 week-randomized butter intervention.

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Abstract

Scope: To investigate whether dairy fat intake have the ability to alter the type of immune response from T-cells. We hypothesized that phytanic acid (PA), which is found in varying concentrations in dairy fat, might promote T-cell polarization towards a Th2-type response, due to its agonistic activity for the nuclear receptors PPARα and RXR.

Methods and results: In a double-blinded, randomized, 12-wk, parallel intervention study, 16 healthy subjects replaced part of their habitual dietary fat intake with 39 g fat from two test butters, that contained different amounts of PA. The butters were produced from milk delivered from mountain-pastures grazing cows or from cows fed conventional winter feed. Before and after the butter intervention, $CD3^+$ T-cells were isolated and activated for 12 hours, followed by measurement of IFN-γ, IL-4, and IL-10 gene expression levels. The direct effect of PA on T-cell cytokine patterns was further evaluated in *in vitro* incubation experiments.

No differences in-between the two butter groups were observed with regard to expression of the Th-cell signature genes IFN-γ,IL-4 and IL-10, but a general statistically significant increase in IL-4 (P=0.0009), IFN-γ (P=0.0106), IL-4/ IFN-γ (P=0.01), and IL-4/IL-10 (P=0.01) upon 12 weeks of butter intake was identified.T-cells *in vitro* incubated with PA versus palmitic acid as a control, showed that PA is not likely to contribute to the butter effect on the cytokine levels.

Conclusion: The 12 weeks butter intervention resulted in greater increases in levels of IL-4, than of IFN- γ and IL-10. The Th2 specific butter effect seems not to be caused by increased plasma or cellular PA levels, as was hypothesized.

1 Introduction

Studies have indicated that specific dietary components might have an impact on the immune response. This might occur through modulation of T-cell polarization, since the balance especially between T-helper cell type 1 (Th1), T-helper cell type 2 (Th2) or regulatory T-cell (T_{reg}), determine to what extent a certain antigen will induce a cellular or humoral immune response, or whether it may be more tolerance promoting towards the antigen. The branched-chain fatty acid phytanic acid (PA, 3, 7, 11, 15-tetramethyl hexadecanoic acid, fig. 1) is an interesting component in this regard, since it, as well as its primary metabolite pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), has strong agonistic activities on the nuclear receptors RXRα and PPARα (Kitareewan *et al.*, 1996; Zomer *et al.*, 2000) suggested to specifically affect T-cell function. RXRα is highly expressed in T-cells of the Th2 lineage, and overexpression in Jurkat T-cells caused increased expression of the Th2-derived cytokine IL-4 but not of IFN-γ, a cardinal cytokine produced by Th1 cells. It was therefore previously suggested that RXRα activity play a selective role in polarizing Th cells towards a Th2 phenotype (Hwang *et al.*, 2009). RXR-specific agonists as well as 9-cis retinoic acid (Dawson *et al.*, 2008) also selectively inhibits IFN-γ production in vitro culture systems, though it is not clear whether this is mediated through RXR or retinoic acid receptor (RAR). Furthermore, RXR-specific agonists have been shown to mediate differentiation to Th2 cells from naïve $CD4^+$ T cells (Stephensen *et al.*, 2002).

PPARα is also expressed in naive and activated T cells, and several studies have demonstrated repression of IFN-γ in activated T cells by PPARα ligands, such as fenofibrate and Wy14643 (Cunard *et al.*, 2002; Jones *et al.*, 2002; Lee *et al.*, 2007). The repression of IFN-γ seems to be linked to PPARα activity, since it is not observed in splenocytes from PPARα KO mice. Moreover, PPARα $^{-/-}$ -

mice show an impaired induction of regulatory T cells (T_{reg}) (Dubrac *et al.*, 2011), indicating that PPAR-α activation also might be involved in differentiation of T_{reg}.

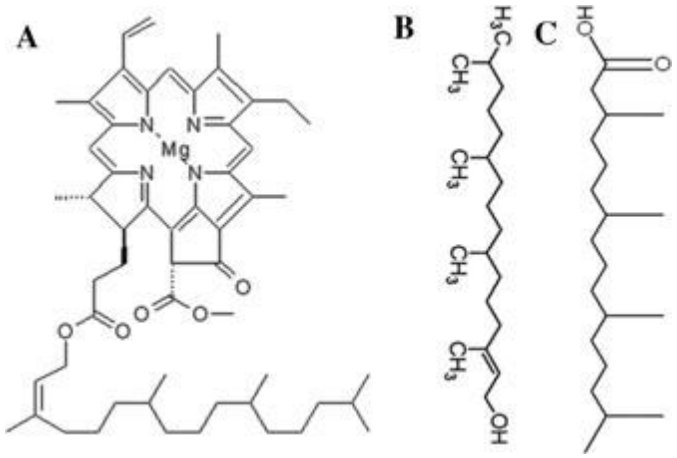

Figure 1 Structures of chlorophyll (A), phytol (B), and Phytanic acid(C).

PA is found in dairy and meat products from ruminant animals. The precursor of PA, phytol (3, 7, 11, 15-tetramethylhexadec-2-en-1-ol, also seen in fig. 1), is released from chlorophyll through microbial conversion in the rumen of ruminant animals (Hansen, 1966; Gloerich *et al.*, 2007). Since phytol cannot be released from the chlorophyll molecule in the human gastrointestinal (GI) tract, the human intake of PA primarily originate from the fat fractions of ruminant meat and dairy products. The content of PA in ruminant food products is mainly dependent on the amount of green plant material in the feed, although the composition of the ruminal microbiota also might be of importance (Lough, 1977; Vlaeminck *et al.*, 2006; Vetter & Schroeder, 2010).

Based on PA's possible role as a RXRα and PPARα agonist, we hypothesized that increased intake of PA-rich dairy products would promote Th2 type propagation. A physiological effect of PA seems plausible as we have observed that the serum level of PA is directly correlated to the intake of dairy products (Werner *et al*, 2011), and that the PA concentration in plasma in adults are within

the range of the published EC_{50} value for the binding of PA to RXR (Lemotte *et al.*, 1996; Kitareewan *et al.*, 1996; Werner *et al.*, 2011). If PA has the suggested impact on the T-cell response it is of importance, as it would indicate that intake of ruminant meat and dairy products could promote a Th2 type response.

To examine the T-cell polarizing effect during natural intake of PA in humans, we performed a 12-weeks butter fat interventional trial in 16 healthy subjects. The participants received butter derived from either grazing or conventionally fed cows. The difference in cattle feeding regimens results in varying PA concentrations within the two butter products. The CD3+ T-cell response was evaluated before and after the intervention by measurement of the expression of fundamental markers of Th1 and Th2 cells upon ex vivo activation. We found no statistically significant changes in gene expression of IFNγ, IL-4, or IL-10 that was related to the differences in the cattle feeding regimens. However, we observed a general statistically significant enhancement of IL-4 upon increased intake butter from both products during the intervention. As average plasma PA levels were raised for both butter intervention subgroups, we further tested in an in vitro incubation study if PA is able to shift the cytokine profile of activated T-cells. However, despite the overall in vivo butter effect, we did not observe any influence of PA on T-cell polarization in vitro, hence suggesting that the butter effect on Th2 cell shifting may not be mediated by PA.

2 Methods and materials

Participant information

The 16 participants in the intervention was a subpopulation from a larger human intervention with intake of butter produced from either milk from grazing cattle or from more conventional fed cattle. The selection of the sub-population among all participants was blinded and randomized. Subjects were mixed male and female, and aged 50 to 70 years. Basic physiological characteristics of the 16 subjects who completed the study are given in Table 1**.**

Table 1 Basic physiological parameters for the 16 healthy participants, and changes in plasma phytanic acid concentrations due to 12 weeks butter intervention[1].

	Treatment			
	Conventional butter		Grazing butter	
	Before intervention	After intervention	Before intervention	After intervention
Subjects, (*n)*	7		9	
Women, (*n,%)*	28.6 %		66.7 %	
Age, (*y)*	62.9 ± 4.7 (56-69)		62.0 ± 5.7 (53-68)	
Height, (*cm)*	178.8 ± 6.23 (170.2-188)		169.1± 6.33 (158-180.5)**	
Phytanic acid, (*µM)*	2.96 ± 0.69 (2.32-4.10)	3.39±0.75 (2.54-4.08)	2.78 ±0.49 (1.98-3.63)	3.36±0.61 (2.12-4.03)¤¤

[1] Values are mean ± SD; range in parentheses. BMI, Body mass index. ** indicates statistically significant difference (P<0.01) as compared to the conventional group (unpaired t-test). ¤¤ indicates statistically significant difference (P<0.01) between the baseline and the after value (paired t-test).

Exclusion criteria were: BMI > 30 kg/m^2 (though one person in the conventional butter group had BMI>30kg/m^2), current or previous chronic disease, regular use of medications, drugs and/or

alcohol. All participants were apparently healthy as indicated by a medical and lifestyle questionnaire. They all agreed to refrain from taking any dietary supplements, from donating blood 2 months before and during the study, and from taking any medication that might interfere with study measurements. All subjects were instructed to maintain the same level of physical activity throughout the study. The subjects completed a 3-day weighed-food record before the intervention and after six weeks of intervention to assess potential differences in dietary intake between the groups before and during the intervention. All records were coded before being evaluated by a clinical dietician who also calculated energy intake and dietary composition using a national database (Dankost; National Food Agency, Søborg, Denmark). The mean habitual energy intakes before the intervention were 8.3 MJ/d (range: 5.7-15.8 MJ/d); 31.1 % (24.2-45.9 %) of the energy was from fat, 16.3% (11.6-22.8%) was from protein, and 47.3 % (39.1-58.3%) was from carbohydrates. The protocol and aims of the study were fully explained (orally and in writing) to the participants, who gave written informed consent. The research protocol was approved by The Scientific Ethics Committee of the Copenhagen and Frederiksberg (H-B-2009-052).

Production of test milk

Milk fat for the human study was delivered from grazing cows from Norway or conventional Danish cows. Two different types of milk were produced with different FA composition (Table 1). The markedly higher intake of chlorophyll-containing green feed from the grazing cows resulted in a 1.7 fold higher PA content compared to the conventional Danish milk. The conventional Danish milk was delivered from Arla Food, originated from 47 farms, and was collected in October 2009. Cattle feeding routines were traditional and the farmers selected their own ingredients and made their own mixtures of feed. A conventional Danish cow feed regime consists of a mixture of corn silage (35%), wheat-barley silage (25%), clover silage (20%), soybean meal or rapeseed cake (10%)

and a small proportion molasses or beet pellets, straw, vitamins and minerals. The two types of milk were transported to Tine Dairies (Oslo, Norway), where it was processed into butter.

Table 2 Fatty acid compositions for the two butter types used in the intervention trial

	Conventional butter diet (wt%)	Grazing butter diet (wt%)
4:0	3.8±0.41	4.4±0.2
6:0	2.6±0.06	2.5±0.07
8:0	1.3±0.01	1.2±0.02
10:0	2.7±0.04	2.2±0.05
12:0	3.3±0.06	2.6±0.05
14:0	10.5±0.2	9.4±0.2
14:1	0.93±0.02	0.70±0.02
15:0	1.0±0.02	0.86±0.02
16:0	29.6±0.5	23.7±0.5
16:1 n-7	1.5±0.03	1.1±0.03
Phytanic acid	0.18±0.01	0.30±0.01
17:0	0.48±0.01	0.55±0.01
17:1	0.20±0.01	ND
18:0	10.6±0.2	14.4±0.3
18:1 *t*-11	1.6±0.03	2.1±0.05
18:1 n-9	20.5±0.2	24.5±0.6
18:1 n-7	0.90±0.02	0.74±0.02
c-9 *t*-11 CLA	0.58±0.02	$0.76\pm4*10^{-3}$
18:2 n-6	1.7±0.07	1.5±0.07
18:3 n-3	0.59±0.01	1.01±0.1

Blood sampling, T cell isolation and stimulation

Blood was collected in EDTA-coated tubes and kept at room temperature until all blood samples were collected per day (within maximum one hour). CD3+ T cells were isolated from whole blood

using the FlowComp™ Human CD3 isolation kit (Life Technologies, Carlsbad, California, USA) according to manufacturer's recommendations. T cell purity were routinely confirmed to be >98% using flow cytometry. T cells were suspended in complete cell culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 % heat-inactivated fetal bovine serum (FBS, all from Lonza)) and activated by adding $3*10^5$/well anti-CD3/CD28-coated expander beads (Life Tech). Stimulation controls were added medium without CD3/CD28-coated expander beads. After 12 hours of incubation at 37 °C and 5 % CO_2, the T-cell activation was terminated by removing 100 µL medium, followed by addition of 100 µL nucleic acid purification lysis solution from Applied Biosystems (Carlsbad, California, USA) and immediate freezing of the T cell lysate at -80 °C. T cells derived from the butter intervention participants were activated just after isolation, whereas T cells used for in vitro incubation with fatty acids (FA) were incubated over night at 37 °C and 5 % $CO_2$with the respective FAs before activation.

In vitro incubation of T cells with BSA-bound fatty acids

Prior to addition of PA and palmitic acid to T cells, they were bound to FA-free BSA (Sigma-Aldrich, Steinheim, Germany) dissolved in complete cell culture medium to a final concentration of 10 µM. The ratio of FA to BSA was ~ 1: 0.5 molar-ratios, and the mixture was vortexed for 5 minutes to ensure binding of the FA to BSA. T-cells were isolated as described, and dissolved in complete cell culture medium to a concentration of $3*10^6$ cells/mL. $3*10^5$ cells were transferred to each well, and 100 µL of the medium with FA was added to a final concentration of FA in the incubation medium of 5 µM. All samples were made in triplicates. Cells were incubated for 20 hours at 37 °C and 5 % CO_2 and the following day stimulated for 12 hours, as described above. Five µM of FA was selected, since this is close to the concentration we have determined in the subjects after the butter intervention (table 2).

Fatty acid analysis in lipids and test fats

Total lipids were extracted and analyzed from blood plasma samples as described by Tholstrup *et al* (Tholstrup *et al.*, 2004) but using TAG C19:0 as internal standard. The butter lipids were extracted according to the method of Folch (Folch J. *et al.*, 1957). Preparation of FAME was performed based on the method of Hamilton & Hamilton (Hamilton & Hamilton, 1992); to the extracted lipids 0.5 M NaOH in MeOH was added, and refluxed for 5 minutes at 80 ^{o}C. The mixture was cooled to room temperature. Twenty % BF_3 and 0.1 % hydroquinone in MeOH was added to a final concentration of 8 and 0.02%, respectively, which was further refluxed for 2 minutes at 80 ^{o}C. After cooling to room-temperature 0.8 volumes of 0.73 % NaCL (aq) was added and fatty-acid methyl esters were extracted twice with 0.2 volumes heptanes. Response factors were calculated for methyl esters of PA and short-chained fatty acids (C4-C12), based on the response of palmitic acid methyl ester (C16:0). The butter FAME were separated on a 60-m Supelco SP-2380 column (Sigma-Aldrich AS, Brøndby, Denmark) in a HP 6890 gas Chromatograph (GC), in split mode using He as carrier gas. injector temperature was 260 ^{o}C, split ratio 20 : 1, carrier flow 1.2 mL/min, detector temperature 300 ^{o}C, air flow in detector 300,0 mL/min and hydrogen flow 35 mL/min. FAME from the plasma samples and for identification of c9 t11 CLA in butter the temperature program started at 50 ^{o}C and raised to 160 ^{o}C at 15 ^{o}C /min; hereafter the temperature was raised to 182 ^{o}C at 1 ^{o}C /min, and directly to 200 ^{o}C at 10 ^{o}C /min, the oven was kept at 200 ^{o}C for 15 min before the temperature was raised to 225 ^{o}C. The temperature program used for butter samples was starting at 50 ^{o}C and rising to 160 ^{o}C at 10 ^{o}C /min; hereafter the temperature was raised to 168 ^{o}C at 0.5 ^{o}C /min and finally the temperature was raised to 225 ^{o}C at 30 ^{o}C /min and kept at this temperature for 10 min. FAME was identified with authentic standards, and PA-concentration was determined based on the peak area, compared with the internal standard.

qRT-PCR

RNA was extracted from T cell lysates on an Abi Prism 6100 Nucleic acid Prepstation from Applied Biosystems (Carlsbad, California, USA), according to the manufacturer's instruction. cDNA-synthesis was performed on Unocycler (VWR (Leuven, Belgium), using the High capacity cDNA transcription kit from Applied Biosystems. The quantification was performed on a 7900HT Fast Real Time PCRsystem (and each sample measured in duplicate), equipped with 96 wells Fast block, using the Taqman® Environmental master mix 2.0 (Applied Biosystems), with primers and TaqMan-probes from Applied Biosystems. SDS 2.3 and RQ-manager 1.2 software was used for data analyses. WBP4 (Hs00200419_m1) was used as reference gene. Other primers used were IFN-γ (Hs0098929_m1), IL-4 (Hs00174122_m1), IL-10 (Hs00174086_m1), and IL-17a (Hs00174383_m1).

Statistical analysis and methods used for quantification of gene expression

The levels of gene expression in T-cells from the in vitro incubation with FAs were determined as $2^{-\Delta Ct}$, where ΔCt is measured as the Ct-value for the respective gene minus the corresponding Ct-value for WBP4 for each donor individually. Fold changes were measured as $2^{-\Delta\Delta Ct}$, where ΔΔCt in the incubation study is the ΔCt value for PA minus the ΔCt value for palmitic acid (again each donor individually), and in the intervention study ΔΔCt is measured as ΔCt value after intervention minus the ΔCt value before intervention. Statistical analysis was performed using the software package GraphPad Prism (v.5.00). Difference in expression of the individual cytokines, and the ratios IL-4/ IFN-γ, IL-4/ IL-10, and IFN-γ/ IL-4, between the groups in the intervention, was tested by unpaired t-test and MANOVA (MANOVA was performed using R software package). Statistical analysis of cytokine expression before and after intervention was performed as paired t-test. Paired t-test was also used to compare cytokine expression after PA and palmitic acid incubation. Outlier detection performed with Grubbs test (www.graphpad.com).

3 Results

Changes in PA plasma concentrations upon 12 weeks of butter intake

A double-blinded, randomized, 12-wk, parallel intervention study in 16 healthy subjects (aged 53 to 69) was performed in which we replaced part of their habitual dietary fat intake with 39 g fat from two test butters (Table 1). The two test butters differed by 70% in their PA concentrations (Table 1). Despite the relatively great differences in PA concentration in the two butters, 12 weeks of intake of butter fat resulted in a very similar average plasma PA levels in the two butter groups (Table 2). A raise by 22 % was seen in the group receiving butter from grazing cattle (P=0.0034, Table 2) whereas an 18% increase from the baseline level was observed for the group given conventional butter (P=0.22, Table 2).

Butter intervention and cytokine profiles in activated T-cells

T-cells from the 12-weeks butter intervention participants were evaluated for their cytokine producing abilities before and after the 12 weeks to examine if intake of the two different butters may affect the cytokine profile in activated T-cells, and whether this change was correlated to changes in plasma PA-level. We activated CD3+ T-cells with anti-CD3/anti-CD28 antibody-containing beads and compared the gene expression levels of IFNG, IL-4 and IL-10 by qPCR. In preliminary experiments, the gene expression levels of IFNG, IL-4, or IL-10 were examined in T-cells activated for 3, 6 and 12 hours, and based on these pre-studies we settled on 12 hours of activation as the optimal time point for detection of all three mRNA-cytokine products in activated CD3+ T-cells. Comparisons of the effect of the two types of butter on the gene expression levels in activated T-cells (before and after intervention) showed no statistically significant differences in IFN-γ, IL-4, or IL-10 levels (Figure 2A).

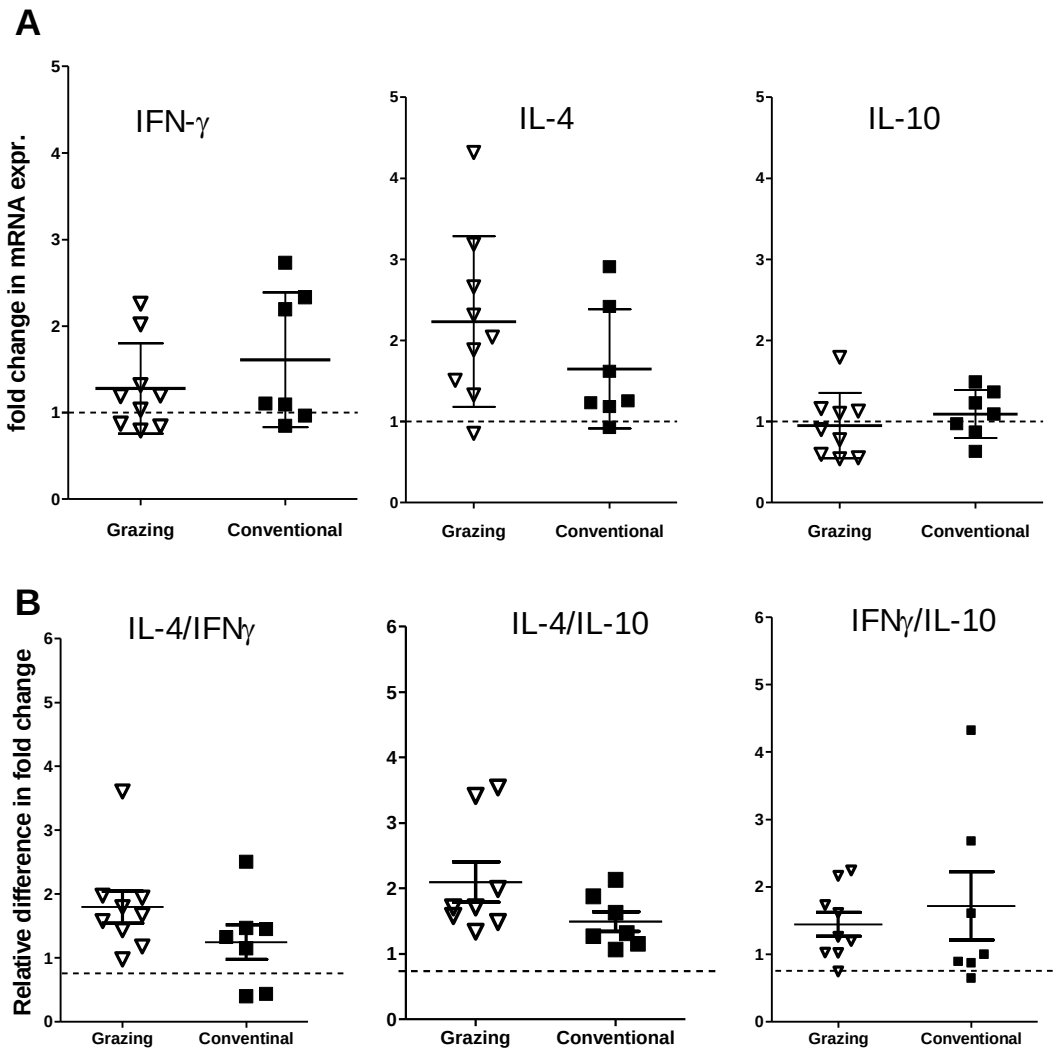

Figure 2. The effect on CD3+ T-cell cytokine profiles of 12-weeks intervention with two butters differing in PA content. A; The relative mRNA expression of IFNG, IL-4 and IL-10 in anti-CD3/CD28-activated CD3+ T-cells after 12-weeks intake of butter from either grazing (n=9) or conventionally fed (n=7) cows. Data are presented as the fold change of cytokine expression levels in cells after versus before the intervention, with each dot representing data from one individual. **B**; Ratios of IL-4/IFN-γ, IL-4/IL-10 and IFN-γ/IL-10 in activated CD3+ T-cells calculated from data in A. Data were tested for statistically significant differences in T-cell responses between the two butter groups by unpaired t-test and MANOVA.

A tendency towards increases in IL-4 levels, lower levels of IFN-γ, and consequently an increase in the IL-4/IFN-γ-ratio (P=0.16, Figure 2B) was observed for individuals receiving butter from grazing

cows, perhaps due to a marginal greater increase in PA levels during the intervention. This skewing of the type of T-cell response could be due to PA, as suggested by our working hypothesis, but as only minor changes were seen within the plasma PA levels in the two butter groups, it may be of greater relevance to look for an overall butter effect on T-cell cytokine profiles instead of subdividing into butter groups. When combining the data from the two butter groups, we observed a statistically significant increase in IL-4 (P=0.0009) and IFN-γ (P=0.0106) expression in activated T-cells, but not in IL-10 levels, after the 12 weeks butter intervention, compared to the expression before the intervention (Figure 3).

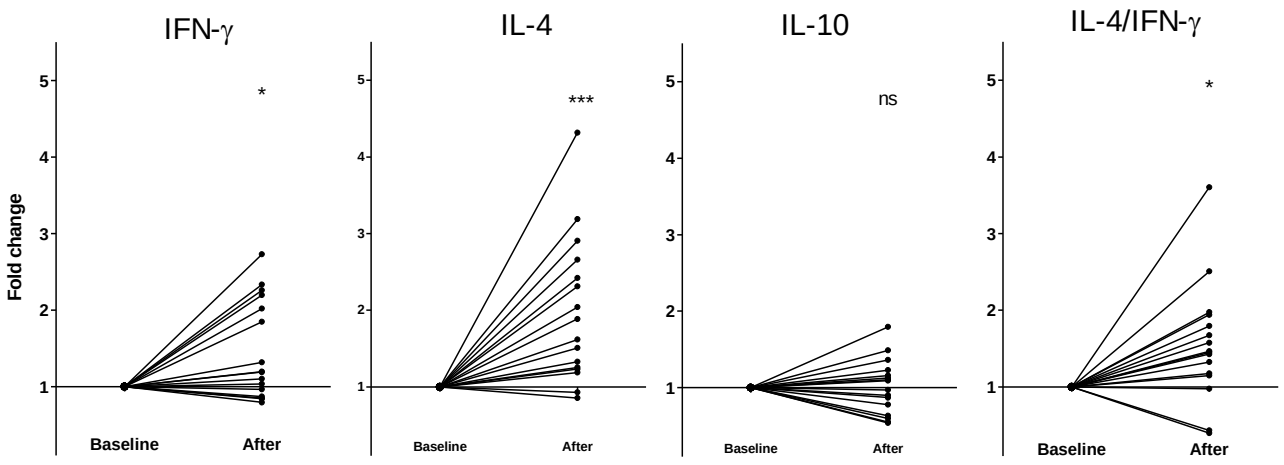

Figure 3. Overall effects of 12-weeks butter intervention on cytokine expression levels in CD3+ T-cells. Gene expression levels of IFNG, IL-4 and IL-10 were measured by qPCR in triplicate cultures of activated CD3+ T-cells, at baseline and after 12 weeks butter intervention (n=16). The ratio of IL-4/IFNG was calculated based on the qPCR values. * indicates statistically significant difference compared to baseline (*: P<0.05, ***: P<0.001), and tested with paired t-test.

Since the fold-increase in IL-4 during the intervention, was higher than both the increase in IFN-γ and in IL-10, this resulted in a significant increase in the IL-4/IFN-γ ratio (mean change: 1.6 fold, 95% CI:1.1-2.0, p for change during intervention = 0.01; Fig 3D) as well as the IL-4/IL-10 ratio (mean

change: 2.2 fold, 95%CI: 1.3 – 3.1, p for change during intervention =0.01, not shown) during the butter intervention. Thus, based on the overall findings, it seems that daily intake of 39 g butter fat from grazing or conventionally fed cows during a 3 month period result in changes in the IL-4/IFN-γ and the IL-4/IL-10 ratios in *ex vivo* activated CD3+ T-cells derived from peripheral blood, indicating polarization of T-cells towards a Th2 profile due to dairy fat intake.

Effect of increased extracellular PA levels on T-cell polarization

As average plasma PA levels were raised for both butter groups during the intervention, we hypothesized that the Th2-shift in the activated T-cells might be caused by plasma PA interacting with the peripheral blood CD3+ T-cells. Therefore we also studied the influence of PA in an *in vitro* cell culture system based on human-blood derived CD3+ T-cells that were incubated with PA or palmitic acid (C16:0), as a control FA. Upon overnight incubation with FAs, the T-cells were activated for 12 hours by CD3/CD28-stimulation. In this study, we measured the expression of IFN-γ (Th1), IL-4 (Th2), IL-10, and also included IL-17 as a marker for Th17 cells. T-cells were incubated overnight with 5 µM PA or palmitic acid to ascertain extracellular PA concentrations resembling those obtained in the plasma during the butter intervention. The T-cells were then activated for 12 hours, followed by analysis for gene expression levels of IFN-γ, IL-4, IL-10, and IL-17. We found that PA incubation of T-cells did not cause any effects on the cytokine profiles that could be related to PA (Figure 4A and 4B). Therefore, based on the data from the *in vitro* incubation with PA versus palmitic acid in physiological relevant concentrations, it seems unlikely that the butter-induced increases in plasma PA levels account for the observed shifting in T-cell cytokines upon the butter intervention, at least not solely. This inference was further supported by a subsequent correlation analysis between changes in plasma PA and gene expression levels in the activated T-cells from

the butter intervention. Here, we found no correlations between changes in PA levels and any of the measured cytokine levels in activated T-cells (data not shown).

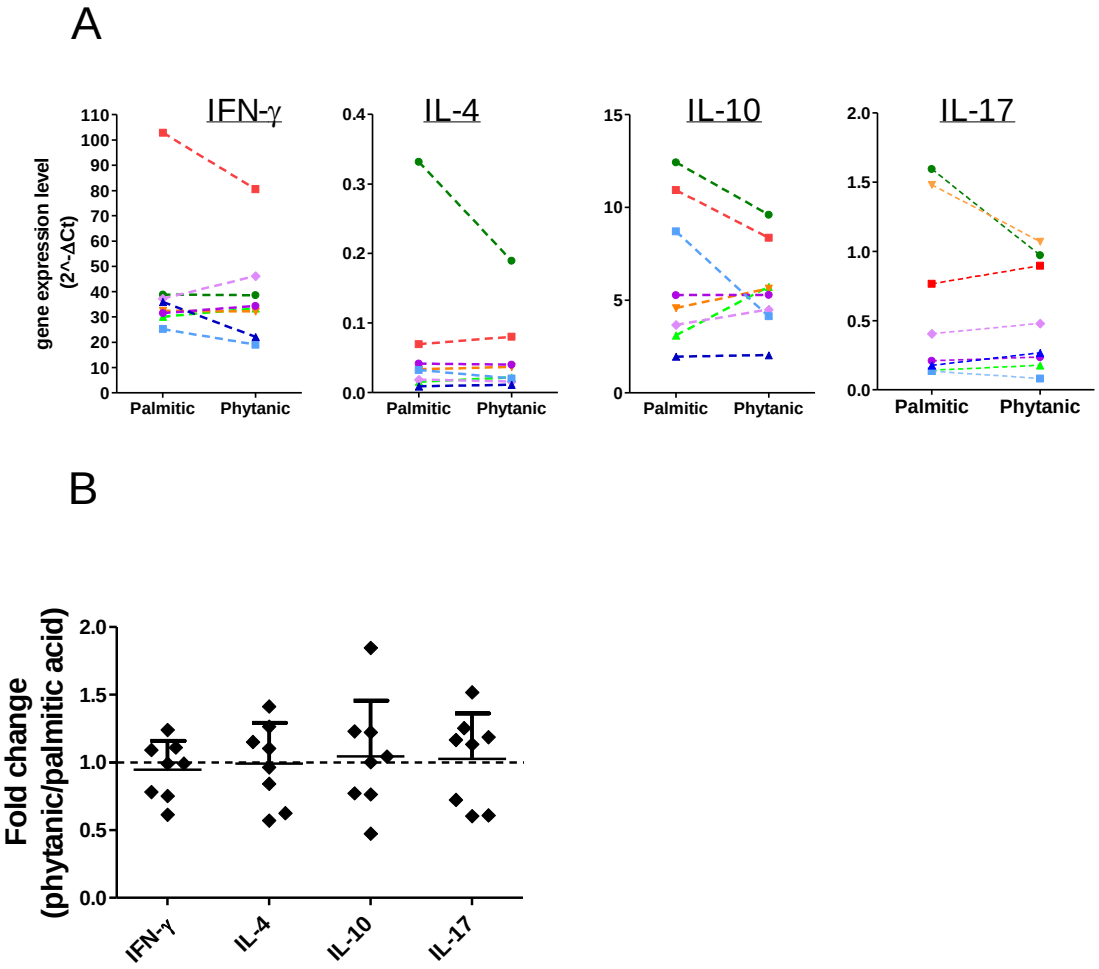

Figure 4. Cytokine expression levels in CD3+ T-cells upon *in vitro* phytanic acid incubation. A; Gene expression of IFNγ, IL-4, IL-10, and IL-17a in anti-CD3/CD28-activated CD3+ T-cells after 20 hours incubation with 5µM BSA-bound phytanic acid or palmitic acid. Each point represents the mean cytokine level in cells from one donor, stimulated in triplicates and measured by qPCR (n=8). Statistical significance was tested by paired t-test, with no statistical significance between the two FAs. **B**; Fold change in individual CD3+ T-cell responsiveness upon 20 hours of phytanic acid versus palmitic acid incubation.

4 Discussion

The results from the intervention study show that three month increased intake of dairy fat from grazing cows did not enhance neither plasma PA levels nor the cytokine-response from *ex vivo* activated CD3+ T-cells isolated from the study participants, when compared with the effects in the control-group consuming dairy fat from conventionally fed cows. However, when analysing the entire study-population for effects of the butter intervention, it was very evident that the intake of approximately 40g butter/day for 12 weeks had pronounced effects on the cytokine profile in CD3+ T-cells in response to CD3/CD28 stimulation. This was in particular manifested as a shift towards relatively higher IL-4 induction, compared with both IFN-γ and IL-10, which is recognized as a hallmark of a more Th2-propagated type of response. Concomitant with the shift in T-cell response was the increase in circulating levels of PA observed in both butter groups, although this only reached significance in the subjects consuming the butter from the grazing group. Thus, the results from the butter intervention study do not support the hypothesis that the concentration of chlorophyll-containing plant material in the cows diet (i.e. the grazing group) will give rise to enhanced plasma PA levels, as compared to conventional butter, that in turn then can affect the CD3+ T-cell polarization pattern in the consumers of the dairy-fat. Instead our results confirm our earlier results that an overall increase in butter intake, independent of the cows diet, result in increased levels of circulating PA in the consumer(Werner *et al.*, 2011), and now furthermore suggests that such plasma PA increase is associated with a Th2-skewing of the CD3+ T-cell response. However, our further *in vitro* studies did not substantiate that increased extracellular PA levels, as a sole factor, affects the CD3+ T-cell response in a Th2 direction. In these studies we selected palmitic acid as a control FA due to the fact that it has identical chain-length as PA, but lacks the methyl-branches. Hence, we are controlling for the effect of the methyl-branches that

signifies PA compared to the common dietary, un-branched, fatty acids. Furthermore, we observed no differences in plasma palmitic acid levels during the butter intervention (data not shown), thus excluding an in vivo effect of this FA on the CD3+ T-cell response. The results clearly show, that PA in concentrations similar to what is found in plasma, do not have specific effects on the cytokine-induction in the activated CD3+ T-cells. Hence, we find it unlikely that the observed effect of the butter-intervention is explained by the increase in plasma PA-concentration, rather other more complex interactions may play a role for the in vivo-propagated Th2 shifting. Alternatively, the manner by which the T-cells are taken up the extracellular PA is different in the in vitro incubation system as opposed to in vivo exposure in peripheral blood, thereby giving rise to the divergence in T-cell cytokine responses upon rises in extracellular PA.

Although it is well known that dietary fatty acid composition can modulate the T-cell response, this research has primarily focused on the role of n-6 and n-3 polyunsaturated FA (PUFA) (Kim *et al.*, 2010), while much less is known on the effect of other fatty acids that are characteristic for dairy fat. However, reports on the effects of conjugated linoleic acid (CLA) and its metabolic precursor vaccenic acid (C18:1 n-7*t*), which both are considered to be potentially bioactive fatty acids in milk fat, do exist. Specifically, in obese JCR:LA-cp rats the feeding of vaccenic acid for three weeks was shown to increase IL-10 production in mitogen-stimulated spleenocytes and mesenteric lymph nodes (Blewett *et al.*, 2009), while spleenocytes from obese Zucker-rats fed either the dairy-derived CLA isomer t10,c12-CLA or c9,t12-CLA produced less IL-4 than the control rats when stimulated with concanavalin A or phorbol-myristate acetat and ionomycin (Ruth *et al.*, 2008). Thus, the increase in the ratio in IL4/IFN-γ and IL-4/IL-10 expression we observed in our present study, cannot be explained by published effects of these two fatty acids in rodents. During our human butter intervention, the participants replaced about 15E% of their habitual fat intake

with the intervention fat (Werner et al 2011b, submitted). This change to butter fat is expected to reduce their intake of unsaturated FA, while enhancing their intake of saturated FA. To the best of our knowledge, there exist no reports on the effect of increased intake of saturated fat on polarization of peripheral CD3+ T-cells in humans. In mice models, it has been shown that high fat feeding with saturated FA cause a substantial increase in IFN-γ/IL-4 production in spleenocytes compared to feeding with either n-6 or n-3 PUFA (Wallace *et al.*, 2001; Verwaerde *et al.*, 2006). Thus, available data from rodent studies indicates another shift in the T-cell cytokine balance upon increased intake of saturated fat than the Th2-skewing we observe after increased butter-intake in humans. Dairy fat is also characterized by the high content of short-chained FAs (SCFAs). SCFAs constitute up to 12 mole% of dairy fat and the specific SCFA receptor GPR43, have been showed to be highly expressed on leukocytes and to be important for an appropriately balanced immune response (Maslowski *et al.*, 2009; Oh & Lagakos, 2011). However, little is known on the role of GPR43 in modulating the cytokine-profile of activated CD3+ T-cells, and of the importance of dietary SCFA intake for leukocyte GPR43-regulation, and therefore presently it is not possible to estimate if the increased intake of SCFA from butter can partly explain the increased expression-ratio of IL-4/IFN-γ in ex vivo-activated T-cells.

Considering the physiological relevance of the observed skewing in the CD3+ T-cell cytokine-profile, these are not easily interpreted. Although a Th2-skewing of the T-cell response, relative to Th1 or regulatory T-cell type of responses, are considered to increase the risk of developing atopic diseases (Jutel & Akdis, 2011), to what extent the observed changes, in a healthy and non-atopic population represent an actual risk-factor for development of atopic diseases, will require further comprehensive studies.

Conclusion

The feeding regimen of the dairy-cows producing the milk-fat used for butter production did not affect expression of fundamental markers of Th1, Th2 and also Treg in ex vivo-activated CD3+ T-cells derived from subjects eating about 40 g butter/day for 12 weeks. However, compared to the response at baseline, the butter-intervention gave rise to upregulation in IL-4 ($P<0.001$) and IFN-γ ($P<0.05$) expression. Since the upregulation of IL-4 expression was relatively higher than the increase in IFN-γ expression, this resulted in a significantly increased activation-induced expression ratio of IL-4/ IFN-γ as well as IL-4/IL-10 after the intervention. This indicates that butter intake has the ability to enhance polarization of T-cells towards a Th2 phenotype. From the in vitro PA-incubation study of human peripheral blood CD3+ T-cells, we conclude that PA in physiological relevant concentrations are not able to affect the expression of fundamental markers of Th1, Th2 or Th17 cells, compared to palmitic acid.

Hence, the observed shift towards increased Th2-propagation in activated T-cells is most likely caused by other butter-derived factors than PA.

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5 References

Reference List

Blewett HJ, Gerdung CA, Ruth MR, Proctor SD & Field CJ (2009) Vaccenic acid favourably alters immune function in obese JCR:LA-cp rats. *British Journal of Nutrition* **102**, 526-536.

Cunard R, Ricote M, DiCampli D, Archer DC, Kahn DA, Glass CK & Kelly CJ (2002) Regulation of cytokine expression by ligands of peroxisome proliferator activated receptors. *Journal of Immunology* **168**, 2795-2802.

Dawson HD, Collins G, Pyle R, Key M & Taub DD (2008) The Retinoic Acid Receptor-alpha mediates human T-cell activation and Th2 cytokine and chemokine production. *Bmc Immunology* **9**.

Dubrac S, Elentner A, Schoonjans K, Auwerx J & Schmuth M (2011) Lack of IL-2 in PPAR-alpha-deficient mice triggers allergic contact dermatitis by affecting regulatory T cells. *European Journal of Immunology* **41**, 1980-1991.

Folch J., Lees M. & Stanley G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497-509.

Gloerich J, van den Brink DM, Ruiter JPN, van Vlies N, Vaz FM, Wanders RJA & Ferdinandusse S (2007) Metabolism of phytol to phytanic acid in the mouse, and the role of PPAR alpha in its regulation. *Journal of Lipid Research* **48**, 77-85.

Hamilton S & Hamilton RJ (1992) Extraction of lipids and derivative formation. In *Lipid analysis - a practical approach*: Oxford University Press, Oxford.

Hansen RP (1966) Occurrence of Phytanic Acid in Rumen Bacteria. *Nature* **210**, 841-&.

Hwang SS, Kim YU, Lee W & Lee GR (2009) Differential Expression of Nuclear Receptors in T Helper Cells. *Journal of Microbiology and Biotechnology* **19**, 208-214.

Jones DC, Ding XH & Daynes RA (2002) Nuclear receptor peroxisome proliferator-activated receptor a (PPAR alpha) is expressed in resting murine lymphocytes - The PPAR alpha in T and B lymphocytes is both transactivation and transrepression competent. *Journal of Biological Chemistry* **277**, 6838-6845.

Jutel M & Akdis CA (2011) T-cell Subset Regulation in Atopy. *Current Allergy and Asthma Reports* **11**, 139-145.

Kim W, Khan NA, McMurray DN, Prior IA, Wang N & Chapkin RS (2010) Regulatory activity of polyunsaturated fatty acids in T-cell signaling. *Progress in Lipid Research* **49**, 250-261.

Kitareewan S, Burka LT, Tomer KB*, et al.* (1996) Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. *Molecular Biology of the Cell* **7**, 1153-1166.

Lee JW, Bajwa PJ, Carson MJ, Jeske DR, Cong Y, Elson CO, Lytle C & Straus DS (2007) Fenofibrate represses interleukin-17 and interferon-gamma expression and improves colitis in interleukin-10-deficient mice. *Gastroenterology* **133**, 108-123.

Lemotte PK, Keidel S & Apfel CM (1996) Phytanic acid is a retinoid X receptor ligand. *European Journal of Biochemistry* **236**, 328-333.

Lough AK (1977) Phytanic Acid Content of Lipids of Bovine Tissues and Milk. *Lipids* **12**, 115-119.

Maslowski KM, Vieira AT, Ng A*, et al.* (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* **461**, 1282-U119.

Oh DY & Lagakos WS (2011) The role of G-protein-coupled receptors in mediating the effect of fatty acids on inflammation and insulin sensitivity. *Current Opinion in Clinical Nutrition and Metabolic Care* **14**, 322-327.

Ruth MR, Taylor CG, Zahradka P & Field CJ (2008) Abnormal immune responses in fa/fa Zucker rats and effects of feeding conjugated linoleic acid. *Obesity* **16**, 1770-1779.

Stephensen CB, Rasooly R, Jiang XW, Ceddia MA, Weaver CT, Chandraratna RAS & Bucy RP (2002) Vitamin A enhances in vitro Th2 development via retinoid X receptor pathway. *Journal of Immunology* **168**, 4495-4503.

Tholstrup T, Hellgren LI, Petersen M, Basu S, Straarup EM, Schnohr P & Sandstrom B (2004) A solid dietary fat containing fish oil redistributes lipoprotein subclasses without increasing oxidative stress in men. *J Nutr* **134**, 1051-1057.

Verwaerde C, Delanoye A, Macia L, Tailleux A & Wolowczuk I (2006) Influence of high-fat feeding on both naive and antigen-experienced T-cell immune response in DO10.11 mice. *Scandinavian Journal of Immunology* **64**, 457-466.

Vetter W & Schroeder M (2010) Concentrations of phytanic acid and pristanic acid are higher in organic than in conventional dairy products from the German market. *Food Chemistry* **119**, 746-752.

Vlaeminck B, Fievez V, Cabrita ARJ, Fonseca AJM & Dewhurst RJ (2006) Factors affecting odd- and branched-chain fatty acids in milk: A review. *Animal Feed Science and Technology* **131**, 389-417.

Wallace FA, Miles EA, Evans C, Stock TE, Yaqoob P & Calder PC (2001) Dietary fatty acids influence the production of Th1-but not Th2-type cytokines. *Journal of Leukocyte Biology* **69**, 449-457.

Werner LB, Hellgren LI, Raff M, Jensen SK, Petersen RA, Drachmann T & Tholstrup T (2011) Effect of dairy fat on plasma phytanic acid in healthy volunteers - a randomized controlled study. *Lipids in Health and Disease* **10**.

Zomer AWM, van der Burg B, Jansen GA, Wanders RJA, Poll-The B & van der Saag PT (2000) Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferater-activated receptor alpha. *Journal of Lipid Research* **41**, 1801-1807.

Appendix D

Article for study D:

"Effect of dairy fat on plasma phytanic acid in healthy volunteers - A randomized controlled study"

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LIPIDS IN HEALTH AND DISEASE

RESEARCH Open Access

Effect of dairy fat on plasma phytanic acid in healthy volunteers - a randomized controlled study

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Abstract

Background: Phytanic acid produced in ruminants from chlorophyll may have preventive effects on the metabolic syndrome, partly due to its reported RXR and PPAR- α agonist activity. Milk from cows fed increased levels of green plant material, contains increased phytanic acid concentrations, but it is unknown to what extent minor increases in phytanic acid content in dairy fat leads to higher circulating levels of phytanic acid in plasma of the consumers.

Objective: To investigate if cow feeding regimes affects concentration of plasma phytanic acid and risk markers of the metabolic syndrome in human.

Design: In a double-blind, randomized, 4 wk, parallel intervention study 14 healthy young subjects were given 45 g milk fat/d from test butter and cheese with 0.24 wt% phytanic acid or a control diet with 0.13 wt% phytanic acid. Difference in phytanic acid was obtained by feeding roughage with low or high content of chlorophyll.

Results: There tended to be a difference in plasma phytanic acid (P = 0.0730) concentration after the dietary intervention. Plasma phytanic acid increased significantly within both groups with the highest increase in control group (24%) compared to phytanic acid group (15%). There were no significant effects of phytanic acid on risk markers for the metabolic syndrome.

Conclusions: The results indicate that increased intake of dairy fat modify the plasma phytanic acid concentration, regardless of cows feeding regime and the minor difference in dietary phytanic acid. Whether the phytanic acid has potential to affects the risk markers of the metabolic syndrome in human still remain to be elucidated.

Trial Registration: ClinicalTrials.gov: NCT01343576

Keywords: Phytanic acid, cow-feeding regime, absorption, fatty acid, total cholesterol, LDL cholesterol, HDL cholesterol, C-reactive protein, insulin, glucose

Background

Phytanic acid is a multi branched-chain fatty acid formed through the metabolism of phytol, which is released from the chlorophyll molecule in the rumen of ruminant animals [1]. Hence, dairy-products, ruminant meat and also some marine fats are the only major dietary sources of phytanic acid or its metabolic precursor, phytol [2]. Dietary intake of phytanic acid have been suggested to be involved in both health- and disease promoting processes, thus some researchers have suggested that it can prevent diabetes and metabolic diseases, while others have suggested that it promotes development of prostate cancer [3,4].

The potential health-promoting properties is based on the fact that animal and in vitro studies have shown that phytanic acid might have preventive effects on metabolic dysfunctions, since it in animal studies increase expression of genes involved in fatty acid oxidation, enhance glucose uptake and metabolism in hepatocyte and potentially reduce metabolic efficacy through

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increased differentiation of brown adipocyte differentiation and expression of uncoupling protein-1 [5-7]. These effects have partly been explained by the PPAR and RXR agonist activity of phytanic acid [6,8-10]. Hence, phytanic acid may be an interesting, though so far overlooked, bioactive FA in dairy products with potential positive effects on metabolic function. However, the suggested involvement of phytanic acid in development of prostate cancer also needs to be taken into consideration. To be able to evaluate the potential risks and advantages of a dietary intake of phytanic acid, we need more data on how intake of phytanic acid containing foodstuffs affects levels of phytanic acid, as well as data on effects on metabolic markers in humans. Although earlier observational studies have found a significant correlation between dairy fat intake and plasma levels of phytanic acid [3,11,12] this is to over knowledge, the first controlled human intervention study in which the relation between dairy fat intake and plasma phytanic acid are directly established.

It is well known that the FA composition of milk fat is markedly influenced by the diet, especially by the amount and FA profile of fat in the cattle fodder [13-16]. Since phytanic acid is formed from metabolites of chlorophyll degradation, the content in milk fat is strongly dependent on the amount of chlorophyll-containing material in the feed [17,18], and will also change over the year depending on the content of green plant material in the cows diet. A study has shown that the phytanic acid concentration in milk fat increased from 0.15 mass% to 0.45 mass%, in cows fed grass only compared to a group fed a mixture of rye grass and maize silage [18] and it was recently shown that the concentration of phytanic acid is higher in organic than conventional dairy products [19].

In this study the test fat was made from milk from one herd divided into two groups that were fed either "green" silage aiming after a moderate content of phytanic acid (0.24 wt%) and "yellow" silage aiming after a low content of phytanic acid (0.13 wt%). Thus, we have investigated how the intake of two different butter and cheese-types, affect plasma phytanic acid concentration in humans. In addition, we investigate if intake of milk fat from the green fed cows, affects the risk markers of the metabolic syndrome differently from milk fat from cows fed predominantly non-green feed.

Subjects and Methods

Study design

We conducted a 4 week, double-blind, randomized, parallel intervention study. In the intervention period subjects were supplied with diets that differed in FA composition, especially phytanic acid, by the Department of Human Nutrition. Participants were stratified according to baseline phytanic acid concentration in plasma and sex into two treatment groups receiving either a diet with a higher content of phytanic acid, phytanic acid group, than the control diet with a low content of phytanic acid, control group. We assessed all outcome variables at the start and end of the intervention period.

Subjects

Male and female subjects, age 20 to 42, were recruited by advertising in local newspapers. The baseline characteristics of the 14 subjects who completed the study were not different between the two groups (Table 1). Exclusion criteria were: BMI > 30 kg/m^2, current or previous chronic disease, regular use of medication, or drug and alcohol abuse. All participants were apparently healthy as indicated by a medical and lifestyle questionnaire. They all agreed to refrain from taking any dietary supplements, from donating blood 2 months before and during the study, and from taking any medication that might interfere with study measurements. All subjects were instructed to maintain the same level of physical activity throughout the study. The subjects completed a 3-day weighed-food record before the intervention and after two weeks of intervention, in order to assess potential differences in dietary intake between the groups before and during the intervention. All records were coded before being evaluated by a clinical dietician, who also calculated energy intake and dietary composition using a national database (Dankost; National Food Agenty, Søborg, Denmark). Mean habitual energy intakes were 10.3 MJ/d (range: 6.7-15 MJ/d); 30.9%

Table 1 Baseline characteristics for the 14 healthy subjects participating in the 4 week intervention[1]

	Treatment	
	Control group (n = 5)	Phytanic acid group (n = 9)
Women, *n*	2	6
Age, *y*	31.6 ± 10.3 (20-42)	28.3 ± 5.2 (21-36)
Height, *cm*	174.6 ± 12.8 (158-190)	171.5 ± 6.9 (162.5-181.5)
Weight, *kg*	68.7 ± 10.9 (56.1-80.25)	65.5 ± 9.5 (54.20-82.95)
BMI, *kg/m2*	22.4 ± 1.7 (20.36-24.5)	22.2 ± 2.1 (19.7-26.2)
Phytanic acid, *μM*	3.35 ± 1.49 (1.75-5.41)	2.93 ± 0.85 (1.89-4.12)
Glucose, *mmol/L*	5.53 ± 0.6 (5.11-6.58)	5.29 ± 0.33 (4.81-5.88)
Insulin, *pmol/L*	22.68 ± 11.88 (7.2-39.1)	29.86 ± 24.58 (7.2-86.6)
TAG, *mmol/L*	0.76 ± 0.35 (0.44-1.31)	0.88 ± 0.22 (0.63-1.32)
Total CH, *mmol/L*	4.22 ± 1.01 (3.14-5.38)	4.23 ± 0.83 (2.92-5.87)
LDL CH, *mmol/L*	2.39 ± 0.72 (1.67-3.24)	2.35 ± 0.54 (1.7-3.55)
HDL CH, *mmol/L*	1.41 ± 0.3 (1.04-1.83)	1.47 ± 0.3 (0.91-1.91)
CRP, *mg/L*	0.54 ± 0.96 (0,05-2.26)	1.25 ± 0.86 (0.05-2.62)

[1]Values are mean ± SD; range in parentheses. CPR, C-reactive protein; CH, Cholesterol; TAG, Triacylglycerol. There were no significant differences between baseline values in the 2 groups (ANCOVA).

(23.7-37.8%) of energy was from fat, 15.1% (11.8-18.1%) was from protein, and 52.2% (42.6-63.7%) was from carbohydrates. There were no significant different between the 2 groups in habitual dietary intake. The protocol and aims of the study were fully explained (orally and in writing) to the participants, who gave written informed consent. The Scientific-Ethical Committees for Copenhagen and Frederiksberg approved the research protocol (H-B-2009-052).

Production of test milk

Milk for the human study was produced by the experimental organic herd at Aarhus University by 56 Danish Holstein cows with an average daily milk production of 39 kg with 4.46% fat. The cows were divided into two groups and fed a concentrate consisting of 1:1 mixture of oat grain and rapeseed cake with 11% fat. This concentrate constituted 40% of the diet. The remaining 60% of the diet consisted of two types of silage, a "green" silage aiming after a high content of phytanic acid and a "yellow" silage aiming after a low content of phytanic acid. The green silage consisted of a mixture of white clover grass and alfalfa silage, while the "yellow" silage consisted of a mixture of corn silage, pea-barley whole crop silage with a small proportion of white clover grass silage. Feeding of the cows was initiated in November 2008 and milk was collected from both groups over one week in February. The milk was transported to Thise Dairy (Roslev, Denmark), where it was processed into butter and cheese. The higher intake of chlorophyll-containing "green" silage resulted in a phytanic acid content of 0.24 wt% in the butter and cheese used for the phytanic acid group compared to the control group were the "yellow" silage resulted in a phytanic acid content of 0.13 wt%.

Diets and test fats

During the intervention period part of the diet of the subjects was replaced with test foods, butter and cheeses with differing FA compositions, provided by the Department of Human Nutrition (Table 2). The test food was butter and cheese with different concentration of phytanic acid. The fatty acid (FA) composition of the test fats in butter are presented in table 2. The FA composition in the two test fats differed only slightly and phytanic acid differed with 0.11 wt%. The test butter with the highest content of phytanic acid, also had the highest content of α-linolenic acid and a lower n-6:n-3 ratio of about 1.8. This is in agreement with the higher proportion of clover and grass in the green feeding regime. The butter and cheese were incorporated into buns and each day during the intervention the subjects were provided with 3 buns each day; two buns with butter and one with cheese. The buns contained 4.1 MJ of energy, with 10 E % from protein, 51 E % from carbohydrates, and 39 E % from fat (47.9 g). All buns contained 13 g of butter each; in addition the cheese bun also contained 32 g of 45+ cheese. This amount of butter and cheese yielded a fat intake from the test diet of 45 g a day (17.5 MJ), which corresponded to approximately 50% of the maximum recommended daily intake of fat for a subject with a daily energy requirement of 10 MJ. To avoid drop outs we decided to use test fat from both butter and cheese.

Table 2 Fatty acid composition of the two test butter

	Butter	
	Control (wt%)	Phytanic acid (wt%)
6:0	1.52	1.51
8:0	1.04	1.2
10:0	2.35	2.31
12:0	2.66	2.60
14:0	9.89	10.07
14:1	0.66	0.62
15:0	0.89	0.91
16:0	22.55	24.80
16:1n-7	1.15	1.16
Phytanic acid	0.13	0.24
17:0	0.47	0.50
17:1	0.20	0.20
18:0	15.39	16.00
18:1n-9 *trans*	2.59	2.25
18:1n-9 *cis*	25.67	25.17
18:1n-7	0.92	1.31
18:2 n-6 *trans*	0.49	0.47
18:2 n-6 *cis*	1.53	1.48
20:0	0.35	0.27
18:3 n-6	0.00	0.00
18:3 n-3	0.48	0.83
≥20:0	0.69	0.29

Compliance

We used the concentration of pentadecanoic acid (15:0) in plasma as an indicator of milk fat intake, and thus of compliance, because 15:0 in serum is considered as a valid marker for intake of milk fat in humans [20-22]. In addition dietary records were used to reinforce the dietary advice and strengthen compliance, and the results of the last records were used in the calculation of dietary changes during the study.

Blood sampling and analysis

After a 12-h overnight fast, venous blood was collected before the intervention period (day 1) and at the end of the intervention (day 28). Blood for FA analysis was collected into tubes containing EDTA, which were kept on ice, and the samples were centrifuged at 4°C and 2200 × g for 15 min.

All samples were stored at -80°C until the samples were analysed. All samples were analyzed at the Department of Human Nutrition in Copenhagen, except from the samples of phytanic acid and FA, which were analyzed at the Technical University of Denmark (DTU).

Blood lipids

We assessed serum LDL and HDL cholesterol by enzymatic colorimetric procedure (ABX Pentra LDL Direct CP and ABX Pentra HDL Direct CP respectively) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France). Total cholesterol was assessed and analyzed by enzymatic photometric procedure (CHOD-PAP from ABX Pentra Cholesterol CP) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France). The concentration of TAG was assessed and analyzed by enzymatic colorimetric procedure (ABX Pentra Triglycerides CP) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France).

Fatty acid analysis in lipids and test fats

Total lipids were extracted and analyzed from blood plasma samples as described by Tholstrup *et al* [23] but using TAG C19:0 as internal standard. The test fats/butters lipids were extracted according to the method of Folch [24], while preparation of FAME was performed as described earlier [23]. Response factor was calculated for methyl esters of phytanic acid and short-chained fatty acid, based on the response of palmitic acid methyl ester (C16:0). The butter FAME were separated on a 60-m Supelco SP-2380 column (Sigma-Aldrich AS, Brøndby, Denmark) in a HP 6890 gas Chromatograph (GC), in split mode using He as carrier gas. GC settings were: Injector temperature 260°C, split ratio 20: 1, carrier flow 1.2 ml/min, detector temperature 300°C, air flow in detector 300,0 ml/min, hydrogen flow 35 ml/min. FAME were separated using a temperature program starting at 50°C and rising to 160°C at 15°C/min; this temperature was kept for 0 min, hereafter the temperature was raised to 182° C at 1°C/min, and directly the temperature was raised to 200°C at 10°C/min, and the oven was kept at 200°C for 15 min before the temperature was raised to 225°C. The final temperature was kept for 12 min (total runtime 61.97 min). FAME was identified with authentic standards, and FA masses were determined based on the peak areas, compared with the internal standard. The commercial standard for phytanic acid was partially separated into two peaks, based on the stereoisomeric properties of phytanic acid. Both peaks were identified in the butter, as well as in the plasma. The separation was however not good enough to quantify the distribution between the isomers. The phytanic acid metabolite pristanic acid, was also separated in the method, this fatty acid did however not reach above the detection limit in any sample analyzed.

C-reactive protein

Blood for analysis of C-reactive protein was collected into dry tubes; after samples were centrifuged at 2200 × g for 15 min at 20°C. Serum was stored at -80°C until the samples were analysed. The CRP concentrations were measured by using latex immunoturbidimetry (ABX Pentra CRP CP) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France).

Glucose and Insulin

Blood for analyses of insulin and glucose concentrations was collected into dry tubes and fluoride citrate respectively; after coagulation, the samples were centrifuged at 2200 × g for 15 min at 20°C. The samples were stored at -80°C until the there were analysed. Insulin concentrations were measured in serum with a chemiluminescent immunometric assay on DPC Immulite 1000 (Siemens Medical Solutions Diagnostics, USA). The intra-assay CV% for insulin was 4.73%. Glucose concentration was measured in plasma and analyzed by enzymatic colorimetric procedure (ABX Pentra Glucose HK CP) on ABX Pentra 400 Clinical Chemistry Analyzer (HORIBA ABX, Montpellier, France).

Statistical analysis

We used an analysis of covariance (ANCOVA) to compare effect of the two diets. The respective baseline values were used as covariates, and the analyses were thus adjusted for the baseline values of each response variable. When necessary values were log transformed to normalize the distribution of residual variance and to obtain variance homogeneity. Statistical tests were performed on the transformed data. Transformation was necessary to ascertain the concentrations of HDL cholesterol and CRP. SAS statistical software (version 9.2; SAS institute Inc, Cary, NC) was used for all statistical analyses. Data describing the characteristics of the participants are summarized as mean ± SD, and data on outcome variables are expressed as least squares (LS) means ± SEM, adjusted for baseline values. We tested baseline, age, BMI, phytanic acid and 15:0 for influence on the results, but all these parameters were not include in the statistical model, because no influence on results were found.

Results

Dietary intake and body weight

No significant changes in body weight and habitual dietary intake were observed during and after the intervention period. We excluded 4 food records (2 from each group) from wk 2 of the intervention from the statistical

analysis. The distribution (% energy) of protein (P = 0.7180), carbohydrates (P = 0.7157), and total fat (P = 0.0774) did not differ between the groups.

Compliance

The increase of serum15:0 was highest in the control group in which an average increase of 31% ($p < 0.01$ vs. baseline) was observed compared to an increase of 20% ($p < 0.05$ vs. baseline) in the phytanic acid group. Althoug, there was no difference in 15:0 between the two groups (p = 0,139), the higher increase could indicate a higher consumption of dairy products during the intervention in the control group.

Blood samples

There tended to be a slight difference in plasma phytanic acid (P = 0.073) between the two groups (Table 3). The study was not designed to compare baseline and treatment, however, due to the explorative approach we considered important to report that the results showed a significant increase ($P < 0.05$) of plasma phytanic acid within both groups. Noteworthingly, the increase was highest in the control group where an average increase of 24% was observed compared to an increase of 15% in the phytanic acid group. There were no effects of the two treatments on serum total (P = 0.700), LDL (P = 0.274) and HDL (P = 0.475) cholesterol, the triacylglycerol (P = 0.202), CRP (P = 0.4043), serum insulin (P = 0.450) and serum glucose (P = 0.8126) (Table 3).

Discussion

In the current study we investigated to what extent milk fat from cows fed either “green” or “yellow” silage affects the concentration of phytanic acid in plasma in healthy young men and female. The intake of “green” silage resulted in a phytanic acid content of 0.24 wt% of total test fat compared to 0.13 wt% in the control group. The test fat was produced to mimic the variation we have seen in dairy fat on the Danish market, with low concentration of phytanic acid during the winter and highest concentrations late in the grazing season.

Table 3 Effects of the 4-wk dietary intervention[1]

	Treatment	
	Control group (n = 5)	Phytanic acid group (n = 9)
Phytanic acid, *μM*	4.16 ± 0.24	3.56 ± 0.18
Glucose, *mmol/L*	5.37 ± 0.10	5.34 ± 0.08
Insulin, *pmol/L*	33.05 ± 5.69	28.11 ± 4.22
Triacylglycerol, *mmol/L*	1.15 ± 0.15	0.9 ± 0.11
Total cholesterol, *mmol/L*	4.46 ± 0.2	4.56 ± 0.15
LDL cholesterol, *mmol/L*	2.59 ± 0.14	2.82 ± 0.10
HDL cholesterol, *mmol/L*	1.28 ± 1.10	1.41 ± 1.07
CRP, *mg/L*	0.26 ± 2.05	0.58 ± 1.77

[1] All values are LSmeans ± SEM. CRP, C-reactive protein. ANCOVA; baseline values were used as covariates.

The main finding of this study was a significant increase of plasma phytanic acid within both groups, regardless of cows feeding regime and test diet phytanic acid content. The higher increase in plasma phytanic acid in the control group compare to the phytanic acid group is opposed to what we have expected. This could be due to different compliance and/or random differences in phytanic acid metabolism between the groups. Since phytanic acid is not produced endogenously in human [25], the presence in the human body is of exogenous origin and ingested from the diet almost exclusively as preformed phytanic acid [26]. The exogenous origin of phytanic acid in human makes it reasonable to assume, that the increased concentration of phytanic acid found after the intervention, was due to an increase intake in this period. When the concentrations of phytanic acid in serum from 250 healthy humans were analyzed, it ranged from 0.04 to 11.5 μM [27]. The large variation probably may illustrate that dietary habit are determining the concentration of phytanic acid. However, genetic factors in regard to the efficacy by which phytanic acid is metabolized may also play a role [11,18]. Because the test diet substituted only a part of the habitual diet the phytanic acid content in the test diets cannot be regarded as being the sole source of dietary phytanic acid. The low degree of control of exposure to additional dietary phytanic acid intake could potentially also have influenced the plasma phytanic acid concentration in the subjects. Indeed, it may have contributed to the surprisingly high increase of plasma phytanic acid within the control group compare to the phytanic acid group. This is supported by the higher average increase of 15:0 in the control group which seems to indicate a higher consumption of dairy products during the intervention compare to the phytanic acid group. In addition, the control group had a higher mean phytanic acid and 15:0 concentrations at baseline (data not shown), indicating a higher habitual dairy fat intake, which also could explain the higher concentration of phytanic acid after the intervention. A second source for phytanic acid is marine food, but the diet protocols of the subjects did not indicate any difference in habitual intake or during the intervention.

Although we in this study demonstrate that intake of dairy products with a low concentration of phytanic acid increase plasma phytanic acid concentrations, there was unfortunately no significant difference between the two groups. This may be related to the small number of subjects, higher baseline phytanic acid levels in the control group, higher consuming of dairy fat in the non-test component of the diet in the control group or a combination of the above. The modest difference in phytanic

acid concentration in the test diets may not be considered to be very high but the differences are relevant since it is similar to the seasonal variation occurring in Danish dairy products. The current study does not give indication of any possible effects of plasma phytanic acid on the metabolic variables and potential risk of developing the metabolic syndrome.

Although the two test butters also differed in the n-3/n-6 ratio, due to a higher content of α-linolenic acid in the phytanic acid rich butter, we do not consider this differences relevant for the outcome of the intervention, since intake of linoleic and α-linolenic acid from the butters only contributed minimally to the test subjects total intake of these fatty acids. Thus, intake of the control butter contributed with only about 675 mg linoleic and 215 mg α-linolenic acid per day and the phytanic acid rich butter with the same amount of linoleic acid but with 380 mg α-linolenic acid. Since a typical daily Danish intake of polyunsaturated fatty acids for men and women are 14 and 10 g/day, respectively [28], the contribution from the test-butters have only marginally affected the total intake.

In the present study population, the plasma phytanic acid concentration varied between 1.75 and 6.13 μM. The EC_{50} for transactivation of all three RXR - isoforms by phytanic acid have been determined to be between 2.3 and 4 μM [9,10]. This indicate that plasma phytanic acid concentration in this study population occur in a concentration in which it has physiological relevance as a modulator of RXR-activity. Finally, the issue on the association between prostate cancer risk and phytanic acid intake needs to be taken into consideration. It has been hypothesized that phytanic acid could be associated with increased risk of prostate cancer [3]. However, no conclusive adverse effects of phytanic acid intake and concentrations within the normal range in healthy humans have been established and there is no data in the literature indicating that the modest changes in phytanic acid that are induced in present study, would have negative impact on human health [29]

The strengths of our study include its controlled and randomized design, follow-up of dietary compliance during the study, the use of validated dietary marker to monitor the dairy fat intake, the test fat was modified from milk originating from dairy cattle from the same herd, and the differences between the two types of test fat was obtained through natural reproducible cow feeding regimes. Furthermore the controlled cow feeding procedure is also considered to be an advantage of the study design. However, since the feeding regimes were designed to mimic what is possible to perform in Danish high-yielding herds, it was not possible to keep the cows on pure green feeding-regime. It would therefore be interesting to study the effect of dairy fat that are derived from cows fed pure green material (e. g. grazing cows), which would result in substantially higher concentration of phytanic acid, as well as other bioactive fatty acids. Among the limitations were the relatively high intakes of butter during the intervention. However, the increase in dietary fat during intervention did not differ between the two groups, and neither did the body weight after the intervention. It is also possible that difference in random habitual dietary habits and compliance such as and higher increase in serum 15:0, indicating higher dairy intake, in the control group after the intervention may have affected the results.

It would be relevant to use pure phytanic acid, but this has not yet been possible due to high costs required to produce purified synthetic phytanic acid products for human intervention studies. There is a general lack of evidence to support the results obtained in the study as research on phytanic acid effect in human is missing. Further research must elucidate if increase intake of dietary phytanic acid might play a part in improving the metabolic syndrome and increase the risk of prostate cancer in human.

Conclusion

This study indicate that increased intake of dairy fat modify the plasma phytanic acid concentration, regardless of cows feeding regime and the minor difference in dietary phytanic acid content after 4 weeks. Further feeding-studies, in which phytanic acid is used in physiological relevant concentration, is essential before it is possible to conclude whether increased intake of phytanic acid have preventive effect on the metabolic syndrome in human.

Abbreviations

RXR: retinoid-X receptor; PPAR-α: peroxisome proliferator-activated receptor-α; UCP 1: uncoupler protein 1; FA: fatty acid; 15:0: pentadecanoic acid; CRP: C-reactive protein

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Authors' contributions

LBW, MR and RAP carried out the practical work with the human study. LBW preformed the statistical analysis, and drafted the manuscript. LIH and TD

analyzed the data and SKJ provided the milk for the project. TT designed the research.
All authors have read and approved the final manuscript.

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

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References

1. Hellgren LI: **Phytanic acid–an overlooked bioactive fatty acid in dairy fat?** *Ann N Y Acad Sci* 2010, **1190**:42-49.
2. Brown JP, Guam Mei, Gibberd FB, Burston D, Mayne PD, Jane EM: **Margaret Sidey: Diet and Refsum disease. The determination of phytanic acid and phytol in certain foods and the application of this knowledge to the choice of suitable convenience foods for patients with Refsum disease.** *Journal of human nutrition and dietetics* 1993, 6.
3. Xu J, Thornburg T, Turner AR, Vitolins M, Case D, Shadle J, Hinson L, Sun J, Liu W, Chang B, Adams TS, Zheng SL, Torti FM: **Serum levels of phytanic acid are associated with prostate cancer risk.** *Prostate* 2005, **63**:209-214.
4. McCarty MF: **The chlorophyll metabolite phytanic acid is a natural rexinoid–potential for treatment and prevention of diabetes.** *Med Hypotheses* 2001, **56**:217-219.
5. Gloerich J, van Vlies N, Jansen GA, Denis S, Ruiter JPN, van Werkhoven MA, Duran M, Vaz FM, Wanders RJA, Ferdinandusse S: **A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPAR{alpha}-dependent and -independent pathways.** *Journal of Lipid Research* 2005, **46**:716-726.
6. Heim M, Johnson J, Boess F, Bendik I, Weber P, Hunziker W, Fluhmann B: **Phytanic acid, a natural peroxisome proliferator-activated receptor (PPAR) agonist, regulates glucose metabolism in rat primary hepatocytes.** *FASEB J* 2002, **16**:718-720.
7. Schluter A, Barbera MJ, Iglesias R, Giralt M, Villarroya F: **Phytanic acid, a novel activator of uncoupling protein-1 gene transcription and brown adipocyte differentiation.** *Biochem J* 2002, **362**:61-69.
8. Ellinghaus P, Wolfrum C, Assmann G, Spener F, Seedorf U: **Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/sterol carrier protein x-deficient mice.** *J Biol Chem* 1999, **274**:2766-2772.
9. Kitareewan S, Burka LT, Tomer KB, Parker CE, Deterding LJ, Stevens RD, Forman BM, Mais DE, Heyman RA, McMorris T, Weinberger C: **Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR.** *Mol Biol Cell* 1996, **7**:1153-1166.
10. Lemotte PK, Keidel S, Apfel CM: **Phytanic acid is a retinoid X receptor ligand.** *Eur J Biochem* 1996, **236**:328-333.
11. Allen NE, Grace PB, Ginn A, Travis RC, Roddam AW, Appleby PN, Key T: **Phytanic acid: measurement of plasma concentrations by gas-liquid chromatography-mass spectrometry analysis and associations with diet and other plasma fatty acids.** *Br J Nutr* 2008, **99**:653-659.
12. Price AJ, Allen NE, Appleby PN, Crowe FL, Jenab M, Rinaldi S, Slimani N, Kaaks R, Rohrmann S, Boeing H, Pischon T, Benetou V, Naska A, Trichopoulou A, Palli D, Sieri S, Tumino R, Vineis P, Bueno-de-Mesquita HB, Donate I, Gonzalez CA, Sanchez MJ, Chirlaque MD, Ardanaz E, Larranaga N, Khaw KT, Rodwell S, Gallo V, Michaud DS, Riboli E, Key TJ: **Plasma phytanic acid concentration and risk of prostate cancer: results from the European Prospective Investigation into Cancer and Nutrition.** *Am J Clin Nutr* 2010, **91**:1769-1776.
13. Dewhurst RJ, Fisher WJ, Tweed JK, Wilkins RJ: **Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate.** *J Dairy Sci* 2003, **86**:2598-2611.
14. Grummer RR: **Effect of feed on the composition of milk fat.** *J Dairy Sci* 1991, **74**:3244-3257.
15. Jenkins TC, McGuire MA: **Major advances in nutrition: impact on milk composition.** *J Dairy Sci* 2006, **89**:1302-1310.
16. Lock AL, Bauman DE: **Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health.** *Lipids* 2004, **39**:1197-1206.
17. Lough AK: **The phytanic acid content of the lipids of bovine tissues and milk.** *Lipids* 1977, **12**:115-119.
18. Leiber F, Kreuzer M, Nigg D, Wettstein HR, Scheeder MR: **A study on the causes for the elevated n-3 fatty acids in cows' milk of alpine origin.** *Lipids* 2005, **40**:191-202.
19. Walter Vetter, Markus Schröder: **Concentrations of phytanic acid and pristanic acid are higher in organic than in conventional dairy products from the German market.** *Food Chemestry* 2011, **119**:746-752.
20. Smedman AE, Gustafsson IB, Berglund LG, Vessby BO: **Pentadecanoic acid in serum as a marker for intake of milk fat: relations between intake of milk fat and metabolic risk factors.** *Am J Clin Nutr* 1999, **69**:22-29.
21. Wolk A, Vessby B, Ljung H, Barrefors P: **Evaluation of a biological marker of dairy fat intake.** *Am J Clin Nutr* 1998, **68**:291-295.
22. Wolk A, Furuheim M, Vessby B: **Fatty acid composition of adipose tissue and serum lipids are valid biological markers of dairy fat intake in men.** *J Nutr* 2001, **131**:828-833.
23. Tholstrup T, Hellgren LI, Petersen M, Basu S, Straarup EM, Schnohr P, Sandstrom B: **A solid dietary fat containing fish oil redistributes lipoprotein subclasses without increasing oxidative stress in men.** *J Nutr* 2004, **134**:1051-1057.
24. Folch J, Lees M, Stanley GHS: **A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues.** *Journal of Biological Chemistry* 1957, **226**:497-509.
25. Baxter JH: **Absorption of chlorophyll phytol in normal man and in patients with Refsum's disease.** *J Lipid Res* 1968, **9**:636-641.
26. van den Brink DM, Wanders RJ: **Phytanic acid: production from phytol, its breakdown and role in human disease.** *Cell Mol Life Sci* 2006, **63**:1752-1765.
27. Al-Dirbashi OY, Santa T, Rashed MS, Al-Hassnan Z, Shimozawa N, Chedrawi A, Jacob M, Al-Mokhadab M: **Rapid UPLC-MS/MS method for routine analysis of plasma pristanic, phytanic, and very long chain fatty acid markers of peroxisomal disorders.** *J Lipid Res* 2008, **49**:1855-1862.
28. Pedersen AN, Fagt S, Groth MV, Christensen T, Biltoft-Jensen A, Matthiessen J, Andersen NL, Kørup K, Hartleopp H, Ygil KH, Hinsch HJ, Saxholt E, Trolie E: **Dietary habits in Denmark 2003-2008.** Rosendahls - Schultz Grafisk A/S; 2010, 1-196.
29. Tang XH, Suh MJ, Li R, Gudas LJ: **Cell proliferation inhibition and alterations in retinol esterification induced by phytanic acid and docosahexaenoic acid.** *Journal of Lipid Research* 2007, **48**:165-176.

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Appendix E

Manuscript for study E:

"Effects of milk derived from mountain-pasture grazing cows on risk markers of the metabolic syndrome compared to conventional Danish milk"

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Effects of milk derived from mountain-pasture grazing cows on risk markers of the metabolic syndrome compared to conventional Danish milk

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Effects of milk derived from mountain-pasture grazing cows on risk markers of the metabolic syndrome compared to conventional Danish milk

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Short running head: Phytanic acid and CVD risk markers

Trial registration**:** ID number, NCT01343589; http://www.clinicaltrials.gov

Abstract

Background: There is considerable interest in dairy products from low-input system, such as mountain-pasture grazing cows, because of the belief that they are healthier than those from high-input conventional systems. However, the effects of such products on human health have been poorly investigated.

Objective: To compare the effect of milk from mountain-pasture grazing cows (green fodder milk/GFM) on risk markers of the metabolic syndrome and type-2 diabetes with the effect of conventional Danish milk (control fodder milk/CFM). The study aims to investigate the importance of phytanic acid content for any apparent effects.

Design: In a double-blind, randomized, 12-week, parallel intervention study, 38 healthy subjects replaced part of their habitual dietary fat intake with 39 g fat from test butter made from milk from mountain-pasture grazing cows from Norway or Danish cows fed conventional winter fodder. Insulin sensitivity was tested by an oral glucose tolerance test before and after the intervention, and blood was sampled on these occasions.

Results: No difference in blood lipids, lipoproteins, CRP, insulin and glucose were observed. However, green fodder resulted in a higher LDL cholesterol and insulin concentration compared with habitual diet. Surprisingly strong correlations between phytanic acid at baseline and total- ($P<0.0001$) and LDL-cholesterol ($P=0.0001$) were observed.

Conclusions: We did not find that butter produced from milk from mountain-pasture grazing cows is more beneficial for health. It is possible that phytanic acid may increase total and LDL cholesterol and have a negative effect on insulin-sensitivity in human.

Introduction

Increased consumer awareness of the link between diet and health has led to research focused on altering the fatty acid (FA) composition of cows' milk to achieve a FA profile consistent with consumer perceptions of a healthy diet and with public health recommendations. However, modification of the FA content of milk fat in dairy cows is affected significantly by the extensive metabolism of lipids that occurs in the rumen and requires an understanding of the interrelationship between dietary supply of lipids, rumen fermentation, and mammary synthesis of milk fat. Important targets include reducing the amount of long-chain saturated fatty acids (SFA´s) such as C12:0-C16:0, enhancing oleic acid to reduce cardiovascular risk, and generally increasing concentrations of mono- and poly-unsaturated fatty acid (MUFA and PUFA) (1-9).

It is well known that the FA composition of milk fat is markedly influenced by the diet, especially by the amount and FA profile of fat in the cattle fodder (10-13). The addition of forage, especially of fresh grass, to fodder has been found to enhance the proportion of unsaturated FAs in milk fat (14-16), and to elevate the concentration of conjugated linoleic acid (CLA) (16;17). Unlike commercial CLA, which has proved to be a biologically active fatty acid with beneficial effects on diseases such as cancer, diabetes, and obesity in animal models (18-20), the major CLA isomer in ruminant fat, *c*-9, *t*-11 CLA, appears to be neutral. The majority of human studies of *c*-9, *t*-11 CLA isomer general find no significant effects on CVD risk markers, such as blood lipids (21-25), inflammatory markers (24;25), and adhesion molecules (24;25). However, the markedly higher intake of chlorophyll-containing green fodder in grazing cows also results in a high phytanic acid content in the milk (26;27). Phytanic acid may be an interesting, though so far overlooked, bioactive FA with potential positive effects on metabolic function. Phytanic acid is a multi-branched fatty acid with reported retinoid X receptor (RXR) and peroxisome proliferatoractivated receptor-α (PPAR-α) agonist activity (28-33). It has been suggested that phytanic acid, because of due the reported RXR and

PPAR-α agonist activity (28-30;34) and its ability to induce glucose uptake in hepatocytes, may prevent metabolic dysfunctions related to the development of metabolic syndrome (19).

In the present study the milk derived from mountain-pasture grazing cows from Norway (GFM) or from conventionally fed Danish cows (CFM). Fat in the GFM had a lower content of cholesterol-raising SFA´s (C12:0-C16:0) and higher content of *c*-9, *t*-11 CLA, alpha-linolenic acid (C18:3 n-3) phytanic acid, and *trans*-vaccenic acid (C18:1 *t*-11) than the CFM and thus tended to produce a more favourable FA profile. These impacts of feeding are well described and are considered advantageous from nutritional point of view. However, the actual effects on human health of milk from pasture grazing cows have been poorly investigated. The objective of the present study was to investigate the effects on risk markers of the metabolic syndrome and type-2 diabetes in healthy human of a diet containing milk delivered from mountain-pasture grazing cows (green fodder milk/GFM) with that of a diet containing milk fat of typical Danish composition (control fodder milk/CFM). No data exists on the effects of phytanic acid in a normal diet so we have focussed on this particular fatty acid.

Subjects and Methods

Study design

We conducted a 12 week, double-blind, randomized, parallel intervention study. In the intervention period subjects were supplied with diets that differed in FA composition by the Department of Human Nutrition. Participants were stratified according to baseline blood glucose level, gender, and waist circumference, into two treatment groups receiving either GFM or CFM. Insulin sensitivity was tested by an oral glucose tolerance test (OGTT) before and after the intervention and blood samples were taken on these occasions.

Subjects

Male and female subjects, age 50 to 70, were recruited by advertising in local newspapers. The baseline characteristics of the 38 subjects who completed the study are given in **Table 1**. There tended to be a significant difference in HDL cholesterol (P=0.056) between the two groups at baseline. No other differences were observed. Exclusion criteria were: BMI > 30 kg/m^2, current or previous chronic disease, regular use of medication, or drug and alcohol abuse. All participants were apparently healthy as indicated by a medical and lifestyle questionnaire. They all agreed to refrain from taking any dietary supplements, from donating blood 2 months before and during the study, and from taking any medication that might interfere with study measurements. All subjects were instructed to maintain the same level of physical activity throughout the study. The subjects completed a 3-day weighed-food record before the intervention and after six weeks of intervention, in order to assess potential differences in dietary intake between the groups before and during the intervention. All records were coded before being evaluated by a clinical dietician, who also calculated energy intake and dietary composition using a national database (Dankost; National Food Agency, Søborg, Denmark). Mean habitual energy intakes before the intervention were 8.6 MJ/d (range: 5.6-15.8 MJ/d); 33.5% (24.2-48.2%) of energy was from fat; 16.3% (11.6-22.8%) was from protein; and 45.7% (30-58.3%) was from carbohydrates. Carbohydrate intake in the GFM group (43.86 ± 5.47 E%) was reported to be lower than in the CFM group (47.84 ± 5.27 E%) (P=0.029) when expressed as E%, but not when expressed as g/d. There were no other differences in habitual dietary intake between the two groups. The protocol and aims of the study were fully explained (orally and in writing) to the participants, who gave written informed consent. The Scientific Ethics Committee of the Copenhagen and Frederiksberg approved the research protocol (H-B-2009-052).

Production of test milk

Milk fat for the human study was derived from milk from paster grazing cows in Norway or conventionally fed Danish cows. The two types of milk produced differed in FA composition. The markedly higher intake of chlorophyll-containing green fodder in the grazing cows resulted in a high phytanic acid content in the GFM compared to the conventional Danish milk. The conventional Danish milk was provided by Arla Food and originated from 47 farms, where all the milk was collected in October. Cattle feeding routines were traditional and farmers selected fodder ingredients and made their own mixtures. Conventional Danish milk cattle fodder consists of a mixture of corn silage (35%), wheat-barley silage (25%), clover grass silage (20%), soybean meal or rapeseed cake (10%), and a small proportion of molasses or beet pellets, straw, vitamins and minerals. Both types of milk were transported to Tine Dairies (Oslo, Norway) where the fat was processed into butter.

Diets and test fats

During the intervention period part of the diet of the subjects was replaced with test foods, butters with differing FA compositions, provided by the Department of Human Nutrition (**Table 2)**. The FA composition in the GFM had a 1.3-fold lower content of the cholesterol-raising lauric, myristic and palmitic acid (C12:0; C14:0 and C:16:0) (36 vs. 45 wt%), a 1.3-fold higher content of stearic acid (C18:0) (11 vs. 15 wt%), a 1.4-fold higher content of *trans*-vacceninc acid (C18:1 *t*-11) (1.7 vs. 2.4 wt%), 1.2-fold higher content of oleic acid (C18:1 *c*-9) (26 vs. 22 wt%), and a 1.5-fold higher content of α-linolenic acid (C18:3 n-3) (0.9 vs. 0.6 wt%) compared to CFM butter. Furthermore, the n-6/n-3 ratio was 1.8 fold lower in the GFM butter (1.6 vs. 2.9). The butter was incorporated into buns and during the intervention subjects were provided with 3 buns each day.

The buns contained 3.5 MJ of energy, with 7.5 E% from protein, 56 E% from carbohydrates, and 36.4 E% from fat. All buns contained 13 g of butter each, which yielded a fat intake from the test diet of 39 g a day (15 MJ), corresponding to approximately 15 E% of the subjects daily intake of fat.

Compliance

We used the concentration of pentadecanoic acid (C15:0) in plasma as an indicator of milk fat intake, and thus of compliance, because C15:0 in serum is considered as a valid marker for intake of milk fat in humans (35-37). In addition dietary records were used to reinforce the dietary advice and strengthen compliance, and the results of the last records were used in the calculation of dietary changes during the study.

Blood sampling and analysis

After a 12-h overnight fast, venous blood was collected before the intervention period (day 1) and at the end of the intervention (day 84). Blood for FA analysis was collected into tubes containing EDTA, which were kept on ice, and the samples were centrifuged at 4 °C and 2200 × g for 15 min. All samples were stored at -80 °C until the samples were analysed. All samples were analyzed at the Department of Human Nutrition in Copenhagen, except the FA-analyses, which were performed at the Technical University of Denmark.

Blood lipids

We assessed serum LDL and HDL cholesterol by enzymatic colorimetric procedure (ABX Pentra LDL Direct CP and ABX Pentra HDL Direct CP respectively) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France). Total cholesterol was assessed and analyzed by enzymatic photometric procedure (CHOD-PAP from ABX Pentra Cholesterol CP) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France). The concentration of TAG was assessed and analyzed by enzymatic colorimetric procedure (ABX Pentra Triglycerides CP) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France).

Fatty acid analysis in lipids and test fats

Total lipids were extracted and analyzed from blood plasma samples as described by Tholstrup *et al* (38) but using TAG C19:0 as internal standard. The test fats/butters lipids were extracted according to the method of Folch (39), while preparation of FAME was performed as described earlier (38). Response factor was calculated for methyl esters of phytanic acid and short-chained fatty acid, based on the response of palmitic acid methyl ester (C16:0). The butter FAME were separated on a 60-m Supelco SP-2380 column (Sigma-Aldrich AS, Brøndby, Denmark) in a HP 6890 gas Chromatograph (GC) in split mode using He as carrier gas. GC settings were: Injector temperature 260 °C, split ratio 1:20, carrier flow 1.2 mL/min, detector temperature 300 °C, air flow in detector 300,0 mL/min, hydrogen flow 35 mL/min. FAME were separated using a temperature program starting at 50 °C and rising to 160 °C at 15 °C /min; this temperature was kept for 0 min, hereafter the temperature was raised to 182 °C at 1 °C /min, and directly the temperature was raised to 200 °C at 10 °C /min, and the oven was kept at 200 °C for 15 min before the temperature was raised to 225 °C. The final temperature was kept for 12 min (total runtime 61.97 min). FAME was identified with authentic standards, and FA masses were determined based on the peak areas compared with the internal standard.

C-reactive protein

Blood for analysis of C-reactive protein (CRP) was collected into dry tubes; after samples were centrifuged at 2200 × g for 15 min at 20 °C. Serum was stored at -80 °C until the samples were analysed. The CRP concentrations were measured by using latex immunoturbidimetry (ABX Pentra CRP CP) on ABX Pentra 400 Chemistry Analyzer (HORIBA ABX, Montpellier, France).

Glucose, insulin and C-peptide

Blood for analysis of glucose, insulin and C-peptide concentrations was collected into respectively dry tubes and tubes with fluoride citrate; for both serum and plasma preparation the samples were centrifuged at 2200 × g for 15 min at 20 °C after coagulation. The samples were stored at -80 °C until analysis. Insulin and C-peptide concentrations were measured in serum with a chemiluminescent immunometric assay on DPC Immulite 1000 (Siemens Medical Solutions Diagnostics, USA). Glucose concentration was measured in plasma and analyzed by enzymatic colorimetric procedure (ABX Pentra Glucose HK CP) on ABX Pentra 400 Clinical Chemistry Analyzer (HORIBA ABX, Montpellier, France).

Oral glucose tolerance test

A standard 75-g OGTT was administered at baseline and after 12 weeks of the study, with blood sampled for glucose and C-peptide at 0, 15, 30, 60, 90, 120 and 180 minutes (40). Three-hour C-peptide and glucose area under the curve (AUC) were calculated from the OGTT data, in millimol per minute per liter for glucose, and picomol per minute per liter for C-peptide. Glucose and C-peptide AUCs are the sum of the area of each time segment by C-peptide or glucose concentration. C-peptide AUC is approximate measures of C-peptide secretion in response to a standard oral glucose load.

Statistical analysis

We used an analysis of covariance (ANCOVA) to compare the effects of the two diets and used the respective baseline values as covariates. The analyses were thus adjusted for the baseline values of each variable. When necessary values were log transformed to normalize the distribution of residual variance and to obtain variance homogeneity. Statistical tests were performed on the transformed data. Transformation was necessary to allow parametric statistical analyses of the concentrations of

glucose, triacylglycerol (TAG), CRP, insulin, C-peptide AUC and glucose AUC. SAS statistical software (version 9.2; SAS institute Inc, Cary, NC) was used for all statistical analyses. Data describing the characteristics of the participants are summarized as mean ± SDs, and data on outcome variables are expressed as least squares (LS) means ± SEM, adjusted for baseline values. We tested baseline, smoking, age, and BMI for influence on the results, but these parameters were not included in the statistical model because no effects were found. A minimum level of $p < 0.05$ was used to establish significant differences between groups. To analyze data from the OGTT, repeated measures analysis of variance were used to assess effects over time, differences between the experimental fats, and interaction between effects of time and type of milk fat during the 3 hours period of OGTT. If a significant interaction between effects of time and type of fat is found, it means that the mean difference between the two fats varies with time. Graphs of the time course were used to illustrate the means difference. In the case of a significant interaction the tests for type of fat and time effects are not meaningful, and therefore not reported. If, however, there is no significant interaction, it means that the two time courses can be regarded as parallel, and the effects of time and type of fat can be tested separately. We used an ANCOVA to compare the AUC for glucose and AUC for C-peptide. Pearson coefficients (r) and the corresponding probabilities of the statistical significance of correlations (p) were calculated between baseline values.

Results

Compliance

There was no difference in plasma C15:0 after the intervention, and the average increase in C15:0 from baseline was around 20% in both groups, which indicates the consumption of a similar amount of dairy products in both groups during the intervention (data not shown).

Dietary intake and bodyweight

Both groups had a minor weight gain during the intervention, (0.93 ± 0.78 and 0.89 ± 1.39 kg for CFM and GFM respectively), but the weight gain did not differ between the groups when expressed as kg body weight (P=0.800) or BMI (P=0.547). A dietician evaluated all dietary records. One record from the GFM group was incomplete and therefore excluded from the statistical analysis. The energy and macronutrient content in the participants´ daily diet during the intervention were calculated from the dietary records (**Table 3**). The distribution (% energy) of protein (P=0.89), carbohydrates (P=0.69), and total fat (P=0.40) did not differ between the two groups.

Blood samples

No significant differences were observed between GFM and CFM in serum total cholesterol (P=0.44), LDL cholesterol (P=0.49), HDL cholesterol (P=0.45), TAG (P=0.39), CRP (P=0.65), insulin (P=0.77), C-peptide (P=0.91), plasma glucose (P=0.53) or plasma phytanic acid (P=0.18) (**Table 4**). There was no significant difference between the AUCs for either glucose (P=0.89) or C-peptide (P=0.65) after the test diets (**Figure 1**). There were no significant differences in the time course of glucose and C-peptide concentrations after the two different test fats, or in the mean glucose and C-peptide concentrations, according to the repeated-measures analysis of variance (**Figure 1**).

The study was not designed to compare baseline and treatment. However, assessment of diet prior to intervention allowed us to compare the effect of treatment and habitual values. Due to the explorative approach we consider it relevant to report the results, which show that GFM resulted in a higher increase in total-, LDL-, HDL-cholesterol and insulin concentrations than CFM. GFM resulted in 5.5% (0.34 mmol/L) higher total cholesterol (P=0.006); 6.5% (0.23 mmol/L) higher LDL cholesterol (P=0.003); 5.2% (0.08 mmol/L) higher HDL cholesterol (P=0.016); 9.3% (3.15 pmol/L) higher

insulin (P=0.039); and 18% (0.66 mmol/L) higher phytanic acid (P<0.0001) than habitual diet. No other significant differences were observed (data not shown). CFM resulted in 4.2% (0.24 mmol/L) higher total cholesterol (P=0.016); 4.7 % (0.16 mmol/L) higher LDL cholesterol (P=0.053); 3.7% (0.05 mmol/L) higher HDL cholesterol (P=0.045); and 12% (0.4 mmol/L) higher phytanic acid (P=0.033) than habitual diet. No other significant differences were observed (data not shown). There was no difference between the effect of diets on total cholesterol/HDL ratio (GFM compared to CFM: P=0.923); (CFM compared to habitual diet: P=0.664); (GFM compared to habitual diet: P=0.711).

There were substantial differences in the FA composition of the two test diets, but this did not lead to any significant differences between groups in the erythrocyte FA composition (data not shown). There were strong correlations between plasma phytanic acid at baseline and total cholesterol (r^2=0.42, P<0.0001), LDL cholesterol (r^2=0.35, P=0.0001). The correlation between plasma C15:0 and both total cholesterol and LDL cholesterol was weaker (r^2=0.14, P=0.02 and r^2=0.11, P=0.04, respectively), although there was a strong correlation between plasma C15:0 and phytanic acid (r^2=0.44, P<0.0001) (**Table 5**).

Discussion

This study was designed to compare the effect of butter produced from milk from mountain-pasture grazing cows with conventional Danish butter on risk markers of the metabolic syndrome and type-2 diabetes in healthy humans. The FA composition of the GFM had a lower content of the cholesterol-raising SFAs, lauric, myristic and palmitic acid, and a substantially lower n-6/n-3 ratio than CFM, while the content of stearic- ,oleic- and *trans*-vaccenic acid was significantly higher in the GFM. However, despite the differences in FA composition, there were no differences between diets in

effect on the metabolic parameters or on CRP. However, GFM resulted in a higher increase in total, LDL, and HDL cholesterol than CFM.

A previous study where 16% of the cholesterol-raising SFA, especially palmitic acid, was replaced by mainly oleic and stearic acid, did not result in any changes in LDL cholesterol (41), which is in line with the present findings. However, this is in contrast to the findings of Noakes *et al* (42), who concluded that modifying the fat composition of dairy products through the substitution of cholesterol-raising SFAs with oleic, linoleic and linolenic acids results in a decrease of 0.28 mmol/L in total plasma cholesterol. The control diets in the study by Noakes *et al* and in our study were similar, but the absolute palmitic and myristic acid content was lower, and oleic acid and PUFA higher (42). Calculated according to the Mensink and Katan equation (5) the change in total serum cholesterol in our study when changing from the CFM to GFM would be -0.53 mmol/L (the energy from vaccenic acid was not included in the equation), but we did not find this to be the case. A reasonable explanation, reported by results from studies by Kris-Etherton´s group, could be that the cholesterol-lowering effect of MUFA is obscured when C12:0, C14:0 and C16:0 is high (8). This may indicate a threshold value for the ratio between cholesterolemic and beneficial FA which has to be passed to obtain reduction in plasma total cholesterol. This might not have been the case in our study and in Tholstrup *et al* (41), in which a substitution of cholesterolemic SFA with oleic and stearic acid did not result in a decrease in LDL cholesterol. However, a combination of a relative lower SFA and substantially higher linoleic and oleic acid in Noakes *et al* (42) compared to our study, could be one of the reasons why the hypocholesterolemic effects of oleic and linoleic acid were successfully “expressed”.

An increase in ruminant *trans* FA (rTFA) was a side effect of manipulating the cattle fodder. Two recent clinical studies found an increase in plasma LDL cholesterol in conjunction with high daily rTFA intakes: 10-12 g/d, representing 3.6-5% of energy (43;44), but not at 4.2 g/d, representing 1.5%

of energy intake (43). Another study observed that the consumption of a rTFA-enriched butter (3.6 g/d) lowered total and HDL cholesterol by 6% and 9% respectively, compared to a butter low in rTFA and high in SFA. The authors concluded that these effects may have been partly attributable to the higher MUFA and lower SFA content in the rTFA-enriched butter, rather than to the effects of rTFA alone (45). We also consider it unlikely that rTFA increased LDL cholesterol concentrations in this study, because no effect of daily rTFA intakes of 4.2 g/d (43) compared with 1g/d and 0.7 g/d from test food was observed in the current study.

The intake of fresh grass also elevated the concentration of CLA in the milk (16;17). CLA has been reported to reduce aortic atherosclerosis in animal studies (46;47). One study in which the CLA content was enriched by rumen technology to provide 1.42 g/d of *c*-9, *t*-11 CLA in dairy products failed to affect LDL cholesterol (25). A similarly produced modified butter that provided substantial amounts of CLA (4.22g/d) also failed to affect LDL cholesterol (21), which is in line with results from another study with a daily intake of 5 g of milk CLA. (24). We also consider it unlikely that CLA could increase LDL cholesterol concentrations in this study.

A focus of the present study was to investigate whether milk fat with a high phytanic acid concentration affects risk markers of the metabolic syndrome and the risk of type-2 diabetes. We found a significant increase in plasma phytanic acid in both groups, verifying that an intake of dairy products with relatively low concentrations of phytanic acid increases circulating phytanic acid concentration, which is in agreement with results from another recent study conducted by our group (accepted). In the present study we found surprisingly strong correlations between phytanic acid at baseline, total cholesterol (r^2=0.42, P<0.0001) and LDL cholesterol (r^2=0.35, P=0.0001). The baseline phytanic acid level probably reflects habitual dairy fat intake, which means that the strong correlations could be due to the hypercholesterolemic effect of dairy fat. This interpretation is supported by the fact that plasma phytanic acid and plasma C15:0 corresponds very strongly at

baseline (r^2=0.44, P<0.0001). However, the correlations between plasma C15:0 and both total cholesterol (r^2=0.14, P=0.02) and LDL cholesterol (r^2=0.11, P=0.04) are substantially weaker than those observed for phytanic acid. This suggests that phytanic acid increases LDL-cholesterol beyond levels that can be explained solely from dairy fat intake. It might therefore also be suggested that the higher increase in total and LDL cholesterol in the GFM group could be due to the higher phytanic acid concentration of this diet, giving rise to a higher average increase in plasma phytanic acid in this group.

We observed no differences in effects on glucose or insulin concentration. However, it is particularly noteworthy that GFM caused a significant increase in fasting insulin, which was not seen with the CFM. Prior to the present study, the available literature supported the hypothesis that phytanic acid might have preventive effects on metabolic dysfunctions related to the development of the metabolic syndrome (28-30;34). In particular the RXR-activating ability of phytanic acid is of interest, since RXR is activated by concentrations at the same level as the phytanic acid concentrations reached in plasma in present study (21). It has been shown that drugs that activate RXR and PPAR-α have a marked impact on whole body metabolism and act as insulin-sensitizers or as hyperlipidemic agents (31;32), while it is not clear to what extent dietary changes in natural agonist concentrations are of physiological importance in humans. The significantly higher increase in insulin and the higher concentration of phytanic acid in the GFM group could, however, indicate that phytanic acid at physiological concentrations has a negative effect on insulin sensitivity in humans rather than a positive effect.

The strengths of our study include its controlled and randomized design, the long intervention period, follow-up of dietary compliance during the study, and the use of a validated dietary marker to monitor the dairy fat intake. The study was also designed to investigate dietary changes caused by an increased intake of dairy products in a realistic setting, and the plasma phytanic acid concentration in

this study population occurred in a concentration which has physiological relevance as a modulator of RXR-activity. Among the limitations were the relatively high intakes of butter during the intervention. Our data cannot determine whether the higher increase in LDL cholesterol and insulin in the GFM group was causally related to the increased concentration of phytanic acid in the diet or whether other constituents of the butter may have played a role. Further research is needed to elucidate whether dietary phytanic acid has a negative or a positive effect in humans.

In conclusion, we did not find that milk derived from mountain-pasture grazing cows was more beneficial to human health conventionally produced Danish milk. One possible reason for this may be that any decrease in LDL cholesterol arising from the substituting of palmitic acid with oleic acid and stearic acid was obscured by the relatively high content of cholesterolemic SFAs in milk fat. Our results also suggest that the higher content of phytanic acid might be an additional explanation for the lack of effect. It could be suggested that phytanic acid increases total and LDL cholesterol and have a negative effect on insulin sensitivity in human. Additional studies will be necessary to clarify the effect of phytanic acid on risk markers of the metabolic syndrome.

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Reference List

1. Bonanome A, Grundy SM. Effect of dietary stearic acid on plasma cholesterol and lipoprotein levels. N Engl J Med 1988;318:1244-8.

2. Denke MA, Grundy SM. Comparison of effects of lauric acid and palmitic acid on plasma lipids and lipoproteins. Am J Clin Nutr 1992;56:895-8.

3. Grundy SM, Florentin L, Nix D, Whelan MF. Comparison of monounsaturated fatty acids and carbohydrates for reducing raised levels of plasma cholesterol in man. The American Journal of Clinical Nutrition 1988;47:965-9.

4. Mensink RP, Katan MB. Effect of a diet enriched with monounsaturated or polyunsaturated fatty acids on levels of low-density and high-density lipoprotein cholesterol in healthy women and men. N Engl J Med 1989;321:436-41.

5. Mensink RP, Katan MB. Effect of dietary fatty acids on serum lipids and lipoproteins. A meta-analysis of 27 trials. Arterioscler Thromb 1992;12:911-9.

6. Temme EH, Mensink RP, Hornstra G. Comparison of the effects of diets enriched in lauric, palmitic, or oleic acids on serum lipids and lipoproteins in healthy women and men. The American Journal of Clinical Nutrition 1996;63:897-903.

7. Tholstrup T, Marckmann P, Jespersen J, Sandstrom B. Fat high in stearic acid favorably affects blood lipids and factor VII coagulant activity in comparison with fats high in palmitic acid or high in myristic and lauric acids. Am J Clin Nutr 1994;59:371-7.

8. Yu S, Derr J, Etherton TD, Kris-Etherton PM. Plasma cholesterol-predictive equations demonstrate that stearic acid is neutral and monounsaturated fatty acids are hypocholesterolemic. Am J Clin Nutr 1995;61:1129-39.

9. Zock PL, de Vries JH, Katan MB. Impact of myristic acid versus palmitic acid on serum lipid and lipoprotein levels in healthy women and men. Arterioscler Thromb 1994;14:567-75.

10. Lock AL, Bauman DE. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids 2004;39:1197-206.

11. Jenkins TC, McGuire MA. Major advances in nutrition: impact on milk composition. J Dairy Sci 2006;89:1302-10.

12. Grummer RR. Effect of feed on the composition of milk fat. J Dairy Sci 1991;74:3244-57.

13. Dewhurst RJ, Fisher WJ, Tweed JK, Wilkins RJ. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. J Dairy Sci 2003;86:2598-611.

14. Schroeder GF, Delahoy JE, Vidaurreta I, Bargo F, Gagliostro GA, Muller LD. Milk fatty acid composition of cows fed a total mixed ration or pasture plus concentrates replacing corn with fat. J Dairy Sci 2003;86:3237-48.

15. Couvreur S, Hurtaud C, Lopez C, Delaby L, Peyraud JL. The linear relationship between the proportion of fresh grass in the cow diet, milk fatty acid composition, and butter properties. J Dairy Sci 2006;89:1956-69.

16. Bargo F, Delahoy JE, Schroeder GF, Baxter JH, Muller L. supplementing total mixed rations with pasture increase the content og conjugated linoleic acid in milk. Animal Feed Science and Technology 2006;131:226-40.

17. Kraft J, Collomb M, Mockel P, Sieber R, Jahreis G. Differences in CLA isomer distribution of cow's milk lipids. Lipids 2003;38:657-64.

18. Ryder JW, Portocarrero CP, Song XM et al. Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. Diabetes 2001;50:1149-57.

19. Ip C, Jiang C, Thompson HJ, Scimeca JA. Retention of conjugated linoleic acid in the mammary gland is associated with tumor inhibition during the post-initiation phase of carcinogenesis. Carcinogenesis 1997;18:755-9.

20. Houseknecht KL, Vanden Heuvel JP, Moya-Camarena SY et al. Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat. Biochem Biophys Res Commun 1998;244:678-82.

21. Desroches S, Chouinard PY, Galibois I et al. Lack of effect of dietary conjugated linoleic acids naturally incorporated into butter on the lipid profile and body composition of overweight and obese men. Am J Clin Nutr 2005;82:309-19.

22. Raff M, Tholstrup T, Basu S, Nonboe P, Sorensen MT, Straarup EM. A diet rich in conjugated linoleic acid and butter increases lipid peroxidation but does not affect atherosclerotic, inflammatory, or diabetic risk markers in healthy young men. J Nutr 2008;138:509-14.

23. Sluijs I, Plantinga Y, de RB, Mennen LI, Bots ML. Dietary supplementation with cis-9,trans-11 conjugated linoleic acid and aortic stiffness in overweight and obese adults. Am J Clin Nutr 2010;91:175-83.

24. Tholstrup T, Raff M, Straarup EM, Lund P, Basu S, Bruun JM. An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 conjugated linoleic acid in postmenopausal women. J Nutr 2008;138:1445-51.

25. Tricon S, Burdge GC, Jones EL et al. Effects of dairy products naturally enriched with cis-9,trans-11 conjugated linoleic acid on the blood lipid profile in healthy middle-aged men. Am J Clin Nutr 2006;83:744-53.

26. Leiber F, Kreuzer M, Nigg D, Wettstein HR, Scheeder MR. A study on the causes for the elevated n-3 fatty acids in cows' milk of alpine origin. Lipids 2005;40:191-202.

27. Lough AK. The phytanic acid content of the lipids of bovine tissues and milk. Lipids 1977;12:115-9.

28. Heim M, Johnson J, Boess F et al. Phytanic acid, a natural peroxisome proliferator-activated receptor (PPAR) agonist, regulates glucose metabolism in rat primary hepatocytes. FASEB J 2002;16:718-20.

29. Kitareewan S, Burka LT, Tomer KB et al. Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. Mol Biol Cell 1996;7:1153-66.

30. Lemotte PK, Keidel S, Apfel CM. Phytanic acid is a retinoid X receptor ligand. Eur J Biochem 1996;236:328-33.

31. Mukherjee R, Davies PJ, Crombie DL et al. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Nature 1997;386:407-10.

32. Neve BP, Fruchart JC, Staels B. Role of the peroxisome proliferator-activated receptors (PPAR) in atherosclerosis. Biochem Pharmacol 2000;60:1245-50.

33. Wolfrum C, Ellinghaus P, Fobker M et al. Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein. J Lipid Res 1999;40:708-14.

34. Ellinghaus P, Wolfrum C, Assmann G, Spener F, Seedorf U. Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/ sterol carrier protein x-deficient mice. J Biol Chem 1999;274:2766-72.

35. Wolk A, Vessby B, Ljung H, Barrefors P. Evaluation of a biological marker of dairy fat intake. Am J Clin Nutr 1998;68:291-5.

36. Wolk A, Furuheim M, Vessby B. Fatty acid composition of adipose tissue and serum lipids are valid biological markers of dairy fat intake in men. J Nutr 2001;131:828-33.

37. Smedman AE, Gustafsson IB, Berglund LG, Vessby BO. Pentadecanoic acid in serum as a marker for intake of milk fat: relations between intake of milk fat and metabolic risk factors. Am J Clin Nutr 1999;69:22-9.

38. Tholstrup T, Hellgren LI, Petersen M et al. A solid dietary fat containing fish oil redistributes lipoprotein subclasses without increasing oxidative stress in men. J Nutr 2004;134:1051-7.

39. Folch J., Lees M., Stanley G.H.S. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 1957;226:497-509.

40. World Health Organization Study Group. Diabetes mellitus: Report of a WHO Study Group. Geneva: World Health Organization 1985;99.

41. Tholstrup T, Sandstrom B, Hermansen JE, Holmer G. Effect of modified dairy fat on postprandial and fasting plasma lipids and lipoproteins in healthy young men. Lipids 1998;33:11-21.

42. Noakes M, Nestel PJ, Clifton PM. Modifying the fatty acid profile of dairy products through feedlot technology lowers plasma cholesterol of humans consuming the products. Am J Clin Nutr 1996;63:42-6.

43. Motard-Belanger A, Charest A, Grenier G et al. Study of the effect of trans fatty acids from ruminants on blood lipids and other risk factors for cardiovascular disease. Am J Clin Nutr 2008;87:593-9.

44. Chardigny JM, Destaillats F, Malpuech-Brugere C et al. Do trans fatty acids from industrially produced sources and from natural sources have the same effect on cardiovascular disease risk factors in healthy subjects? Results of the trans Fatty Acids Collaboration (TRANSFACT) study. Am J Clin Nutr 2008;87:558-66.

45. Tholstrup T, Raff M, Basu S, Nonboe P, Sejrsen K, Straarup EM. Effects of butter high in ruminant trans and monounsaturated fatty acids on lipoproteins, incorporation of fatty acids into lipid classes, plasma C-reactive protein, oxidative stress, hemostatic variables, and insulin in healthy young men. Am J Clin Nutr 2006;83:237-43.

46. McLeod RS, LeBlanc AM, Langille MA, Mitchell PL, Currie DL. Conjugated linoleic acids, atherosclerosis, and hepatic very-low-density lipoprotein metabolism. Am J Clin Nutr 2004;79:1169S-74S.

47. Nicolosi RJ, Rogers EJ, Kritchevsky D, Scimeca JA, Huth PJ. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery 1997;22:266-77.

TABLES

Table 1. Baseline characteristics for the 38 healthy subjects participating in the 12 week intervention[1]

	CFM	GFM
Subjects, (*n)*	18	20
Women, (*n,%)*	55.6 %	65 %
Age, (*y)*	60.7 ± 5.9 (50-69)	61.9 ± 4.9 (52-69)
Height, (*cm)*	173.2 ± 7.5 (159.8-188)	170.5 ± 8.4 (158-187)
Weight, (*kg)*	79.9 ± 15.5 (61.35-122.6)	73.6 ± 8.4 (56.3-93)
BMI, (*kg/m2)*	26.5 ± 3.6 (21.22-34.69)	25.4 ± 2.7 (20.81- 30.55)
Glucose, *(mmol/L)*	5.7 ± 0.47 (5.14-6.57)	5.6 ± 0.51 (5.02-7.14)
Insulin, *(pmol/L)*	41.39 ± 35.04 (7.2-127)	30.59 ± 20.05 (7.2-90.75)
C-peptide, *(pmol/L)*	611.78 ± 287.82 (298-1299.5)	566.23 ± 224.03 (349.5-1065.5)
TAG, (*mmol/L*)	1.27 ±0.59 (0.73-2.74)	1.17 ± 0.46 (0.76-2.62)
Total-cholesterol, *(mmol/L)*	5.51 ± 0.86 (3.98-7)	5.82 ± 1.15 (3.95-7.53)
LDL-cholesterol, *(mmol/L)*	3.25 ± 0.59 (2.45-4.21)	3.29 ± 0.83 (1.46-4.53)
HDL-cholesterol, *(mmol/L)*	1.29 ± 0.31 (0.75-1.73)	1.46 ± 0.29 (1.02-2.06)
CRP, (*mg/L)*	1.02 ± 0.93 (0.05-3.11)	1.18 ± 1.46 (0.05-5.42)
Phytanic acid, (*μM)*	3.01 ± 0.61 (2.3-4.28)	3.02 ± 0.69 (1,98-4,76)
Total:HDL-cholesterol	4.27 ± 2.77 (5.31-4.05)	3.99 ± 3.97 (3.87-3.65)

[1] Values are mean ± SD; range in parentheses. CPR, C-reactive protein; TAG, Triacylglycerol. There were no significant differences between baseline values in the interventions groups (mixed model analysis of covariance. ANCOVA).

Table 2. Fatty acid composition of the two test butter.

% wt	Control fodder milk	Green fodder milk
C4:0	0.80	1.04
C6:0	1.36	1.32
C8:0	1.08	0.96
C10:0	2.79	2.17
C12:0	3.43	2.58
C14:0	11.0	9.43
C14:1	0.98	0.71
C15:0	1.08	0.88
C16:0	31	24.4
C16:1 n-7	1.58	1.15
Phytanic acid	0.17	0.37
C17:0	0.51	0.60
C17:1	0.21	0.24
C18:0	11.1	15.1
C18:1 *t*-11	1.71	2.45
C18:1 n-9	21.5	25.8
C18:1 n-7	0.94	0.39
c-9, *t*-11 CLA	0.58	0.73
C18:2 n-6	1.79	1.52
C18:3 n-3	0.61	0.93

Table 3. Macronutrient intake after 6 weeks of the intervention diets[1]

% of energy	CFM	GFM
Protein	14.3 ± 0.4	14.3 ± 0.4
Carbohydrates	46.7 ± 1.3	47.5 ± 1.3
Total Fat	35.1 ± 0.9	34.0 ± 0.9
Saturated FAs	15.8 ± 0.7	16.0 ± 0.7
Monounsaturated FAs	10.6 ± 0.4	10.2 ± 0.4
Polyunsaturated FAs	3.6 ± 0.2	4.0 ± 0.2

[1] All values are means ± SEM. FAs: Fatty acids. Values were obtained from 3-day weighed food records. One dietary record was incomplete and therefore excluded from the statistical analysis. No significant differences between the two groups were found (mixed model analysis of covariance. ANCOVA; mean values adjusted for baseline values which were used as covariates).

Table 4. Effects of the 12-wk dietary intervention[1]

	CFM	GFM
Glucose, *(mmol/L)*	5.7 ± 1.01	5.6 ± 1.01
Insulin, *(pmol/L)*	31.7 ± 1.09	32.9 ± 1.08
C-peptide, *(pmol/L)*	581.3 ± 1.04	577.7 ± 1.04
TAG, *(mmol/L)*	1.1 ± 1.06	1.2 ± 1.06
Total-cholesterol, (*mmol/L*)	5.9 ± 0.09	6.0 ± 0.08
LDL-cholesterol, *(mmol/L)*	3.4 ± 0.21	3.5 ± 0.2
HDL-cholesterol, *(mmol/L)*	1.4 ± 0.03	1.5 ± 0.03
CRP, (*mg/L)*	0.6 ± 1.28	0.8 ± 1.26
Phytanic acid, *(μM)*	3.4 ± 0.14	3.7 ± 0.13
Total:HDL-cholesterol	4.3 ± 4.48	4 ± 4.3

[1] All values are LSmeans ± SEM. TAG, Triacylglycerol; CRP, C-reactive protein. (mixed model analysis of covariance. ANCOVA; baseline values were used as covariates).

Table 5. Pearson´s correlations coefficients between baseline variables[1]

	Phyt	HDL	LDL	Total CH	Glucose	Insulin	CRP	TAG	C15:0
Phyt	1.0	0.248	0.595[2]	0.645[3]	0.163	0.013	0.283	0.292	0.666[4]
HDL		1.0	0.250	0.539[8]	-0.361[8]	-0.448[8]	-0.266	-0.462[8]	0.113
LDL			1.0	0.926[7]	-0.142	-0.110	0.156	0.213	0.335[5]
Total CH				1.0	-0.163	-0.195	0.065	0.152	0.379[6]
Glucose					1.0	0.402[8]	0.485[8]	0.594[7]	0.406[8]
Insulin						1.0	0.122	0.460[8]	-0.025
CRP							1.0	0.462[8]	0.359[8]
TAG								1.0	0.331[8]
C15:0									1.0

[1] CRP, C-reactive protein; TAG, Triacylglycerol; CH, cholesterol; Phyt, Phytanic acid; C15:0, Plasma pentadecanoic acid. n = 38.

[2] Significantly correlated *P=0.0001*

[3] Significantly correlated *P<0.0001*

[4] Significantly correlated *P<0.0001*

[5] Significantly correlated *P=0.04*

[6] Significantly correlated *P=0.02*

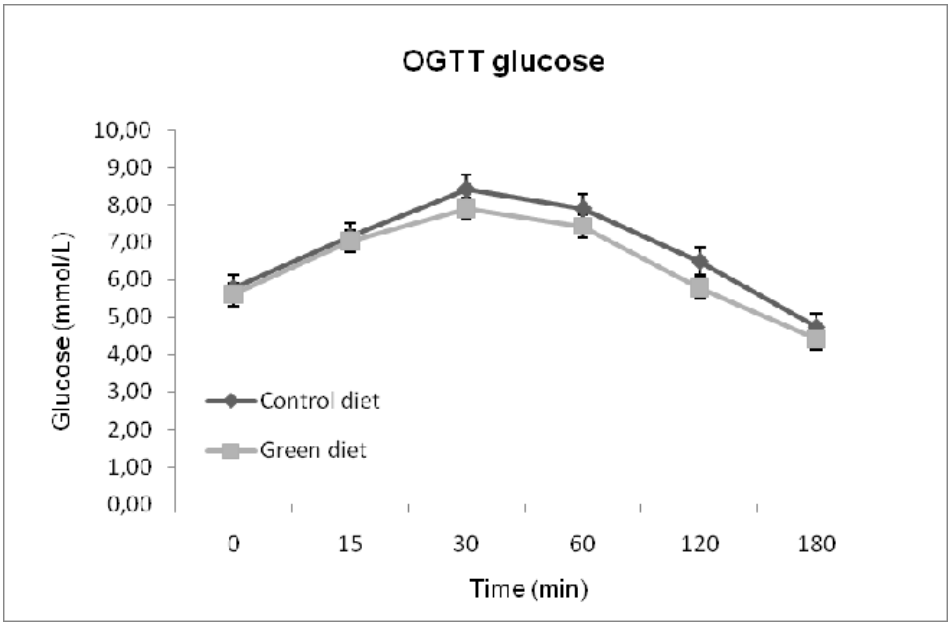
[7] Significantly correlated *P<0.0001*

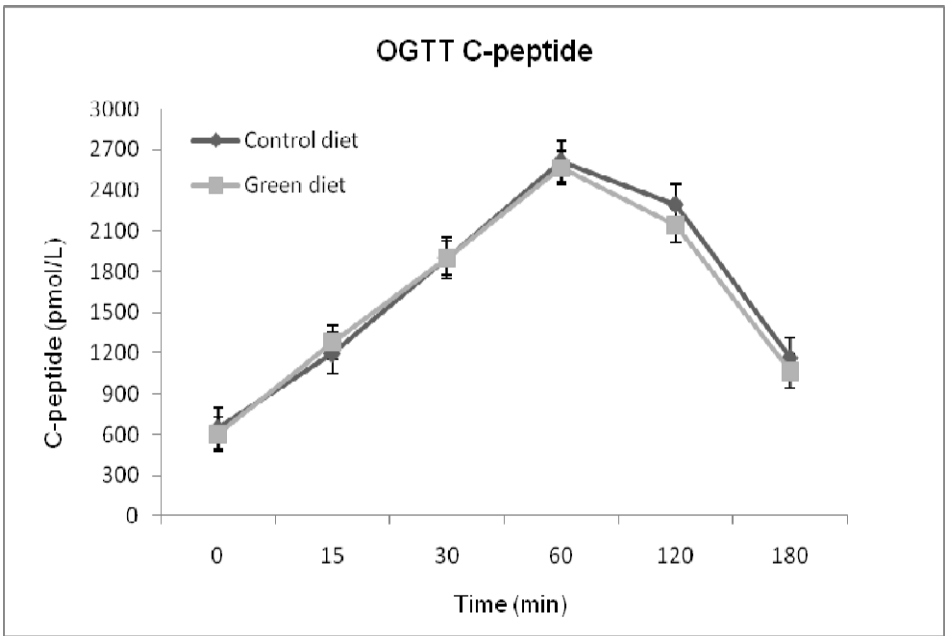
[8] Significantly correlated *P<0.05*

FIGURE LEGENDS

Figure 1. Oral glucose tolerance test (OGTT) measurements performed after 12 weeks. All values are mean ± SEM. Glucose tolerance was measured using a standard 75 g oral glucose-tolerance test. There was no significant difference in the time course of glucose and C-peptide concentration after the two different test fats or in the mean glucose and C-peptide concentration according to the repeated-measures analysis of variance. There was no significant difference between AUCs.

Figure 1.


OGTT glucose
Glucose (mmol/L)
10,00
9,00
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
Control diet
Green diet
0
15
30
60
120
180
Time (min)


OGTT C-peptide
C-peptide (pmol/L)
3000
2700
2400
2100
1800
1500
1200
900
600
300
0
Control diet
Green diet
0
15
30
60
120
180
Time (min)