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PREFACE AND ACKNOWLEDGEMENTS

The scope of the present PhD thesis was i) to review the observational evidence for an association between intake of *trans* fat and risk of coronary heart disease, including the evidence to distinguish between the effects of *trans* fat of industrial and ruminant origin; and ii) to examine if dietary *trans* fat from hydrogenated vegetable oil affects traditional and emerging cardiovascular risk markers in a vulnerable study population.

The thesis is based on a systematic review and meta-analysis of observational studies as well as a dietary intervention study, called COBRA (Copenhagen Obesity Risk Assessment Study) conducted at the Department of Human Nutrition, University of Copenhagen in the time period from September 2007 to September 2010, which has provided the following four manuscripts:

I. Bendsen NT, Christensen R, Bartels EM & Astrup A. Consumption of industrial and ruminant *trans* fatty acids and risk of coronary heart disease: A systematic review and meta-analysis of cohort studies (*Submitted)*.

II. Bendsen NT, Chabanova C, Thomsen HS, Larsen TM, Newman JW, Stender S, Dyerberg J, Haugaard SB & Astrup A. Effect of *trans* fatty acid intake on abdominal and liver fat deposition and blood lipids - a randomized trial in overweight postmenopausal women (*Submitted)*.

III. Bendsen NT, Haugaard SB, Larsen TM, Chabanova C, Stender S & Astrup A. Effect of *trans* fatty acid intake on insulin sensitivity and intramuscular lipids – a randomized trial in overweight postmenopausal women (*Submitted)*.

IV. Bendsen NT, Stender S, Szecsi PB, Pedersen SB, Basu S, Hellgren LI, Newman JW, Larsen TM, Haugaard SB & Astrup A. *Trans* fatty acid intake induces low-grade systemic inflammation: evidence from a randomized, controlled trial in overweight postmenopausal women (*draft)*.

The intervention study was carried out as a part of the research program of the Danish Obesity Research Centre (DanORC, see www.danorc.dk). DanORC is supported by the Danish Council for Strategic Research (Grant 2101-06-0005). The study was also supported by the Danish Council for Independent Research | Medical Sciences (Grant 271-08-0715), and the Danish Diabetes Association. The test fats were kindly provided by AarhusKarlshamn Denmark A/S. The meta-analysis was kindly co-sponsored by Arla Foods Amba.

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Nathalie Tommerup Bendsen,

Frederiksberg, September 2010

SUMMARY

Consumption of trans fatty acids (TFA), which form part of partially hydrogenated edible oils, is detrimental to human health. An average daily intake of just ~5 grams of TFA increases the risk of coronary heart disease (CHD) by more than 20%, according to data from prospective cohort studies. Associations between TFA intake and incidence of type 2 diabetes have also been reported. Several studies have attempted to elucidate the causal physiological mechanisms of action. However, the documented effects of TFA intake on cardiovascular risk markers, such as blood lipids, can explain less than 50% of the increase in risk of CHD found in observational studies, leaving a so-called "explanatory gap".

The present thesis examines whether there is a scientific basis for differentiating between TFA of industrial and ruminant origin in terms of health risk, and whether the TFA-associated increase in CHD risk may be explained by detectable effects on established as well as emerging risk factors for lifestyle diseases. To address these issues, the thesis combines a review of the scientific literature with i) a systematic review and meta-analysis of published and unpublished prospective cohort studies assessing the association between TFA consumption and risk of CHD (Paper I); and ii) results of a dietary intervention study called COBRA, in which 52 abdominally obese, postmenopausal women for 16 weeks were to consume 15 g/day of TFA from partially hydrogenated soybean oil or a control fat high in oleic and palmitic acid (Papers II, III and IV).

The thesis attends to the following questions:

Question (Q): Is TFA intake associated with increased CHD risk – and may TFA from ruminant and industrial sources be regarded as equally harmful?
Answer (A): The systematic review of cohort studies confirms that TFA intake is associated with an increased risk of CHD of the magnitude previously reported (~20% per 5 g/day TFA). However, it also highlights the scarcity of this type of studies, which may be due to the difficulties in appropriately assessing TFA intake in observational studies or to publication bias. Nevertheless, evidence from case-control studies, using biomarkers of TFA intake, collectively support the notion that TFA intake is associated with increased risk of CHD.

Based on data from the cohort studies, it appears that the risk associated with TFA intake may be ascribable to industrially produced, and not ruminant TFA, possibly due to low intake levels of the latter. However, a very limited number of studies prohibits any firm conclusions concerning whether the source of TFA is important. Biomarker studies do not lend support to the notion that R-TFA should be less harmful than IP-TFA; neither do intervention studies examining the effect on blood lipids. Yet, there is no evidence to support that ruminant TFA increases the risk of CHD in the amounts usually consumed.

Q: Does TFA consumption affect blood lipids?
A: TFA consumption increases LDL-cholesterol and decreases HDL- cholesterol concentrations and thereby induces a more atherogenic blood cholesterol profile than all other fatty acid classes. This has been confirmed by numerous intervention studies and appears to apply to TFA of both ruminant and industrial origin, at least at high intake levels. The observed increase in the ratio of LDL-cholesterol to HDL-cholesterol of 34% ($P<0.001$) in the COBRA study, can therefore be considered an indication of good compliance with the study diets. An increase of the size found in the COBRA study may translate into an increase in heart disease risk of ~6% per 5 g/day TFA. In addition, there is evidence to suggest that TFA intake induces a modest increase

in triglyceride and lipoprotein (a) concentrations and gives rise to production of smaller LDL particles.

Q: Does TFA consumption affect markers of low-grade inflammation and endothelial dysfunction?
A: The current evidence for an effect of TFA consumption on low-grade systemic inflammation and endothelial dysfunction mainly stem from cross-sectional studies. Yet, with the finding of a 12% increased production of tumor necrosis factor α (P=0.002) and concomitant increases in its soluble receptors, the COBRA study supports the notion that dietary TFA promote low-grade inflammation. TFA consumption has been found to modestly increase CRP production in a few studies, but such an effect could not be detected in the COBRA study.

Results from the COBRA study tend to confirm prior reports of an E-selectin-increasing effect of TFA, indicating that TFA may promote endothelial dysfunction. However, only one study (out of two) has pointed towards a negative effect of TFA on more direct measures of endothelial function.

Q: Does TFA consumption affect body composition and fat deposition?
A: A few large prospective cohort studies have suggested that TFA intake increases the risk of abdominal obesity or weight gain in general, and a 6-year study in monkeys supports these findings. A substantial TFA consumption for 4 months in the COBRA study did not affect the abdominal fat accumulation in postmenopausal women, but weak trends towards a greater whole-body fat deposition was observed in women exposed to TFA (P=0.12). Whereas the evidence for an effect of TFA on body adiposity in rodents is highly conflicting, an increased accumulation of fat in the liver has been an almost consistent finding. The COBRA study failed in the attempt to verify this effect in humans. The null-finding may, however, be due to study limitations, such as a high degree of within-subject variability in liver fat content and a lack of standardization of the study diets.

Q: Does TFA exposure affect insulin sensitivity?
A: Most animal studies suggest that TFA intake impairs insulin sensitivity compared with other FA classes, whereas evidence from studies in humans is highly conflicting. Whether this discrepancy is due to the longer duration of TFA exposure in animal studies or simply reflects metabolic differences between species is unknown. The few indices of a detrimental effect of TFA intake on insulinemia in humans come from studies in metabolically vulnerable study populations. However, TFA exposure did not result in changes in insulin sensitivity and markers of lipolysis in older overweight women in the COBRA study.

Q: What may be concluded from the COBRA study?
A: In brief, with the findings of a pro-inflammatory effect of TFA in the COBRA study, our research has provided evidence to explain part of the aforementioned "explanatory gap", although it is difficult to estimate what this effect may translate into in terms of heart disease risk. In contrast, induction of ectopic fat deposition or insulin resistance could not be confirmed as explanatory factors.

SAMMENDRAG

Indtag af transfedtsyrer (TF), som findes i delvist hærdede spiseolier, er skadeligt for helbredet. Et gennemsnitligt dagligt indtag af TF på bare ~5 gram øger risikoen for at udvikle hjertesygdom med mere end 20%, ifølge data fra prospektive kohortestudier. Indtag af TF er ligeledes blevet kædet sammen med øget risiko for type 2 sukkersyge. En række interventionsstudier har undersøgt, hvilke fysiologiske virkningsmekanismer der kan tænkes at ligge til grund for den øgede sygdomsrisiko. Dokumenterede effekter af TF, såsom påvirkning af blodets fedtstoffer, kan imidlertid forklare mindre end 50% af den øgede risiko for hjertesygdom fundet i befolkningsundersøgelser. Dermed står vi tilbage med et såkaldt "forklaringsunderskud".

Nærværende PhD-afhandling undersøger, hvorvidt der er videnskabeligt belæg for at skelne mellem TF, som stammer fra industriel hærdning og TF, som stammer fra mikrobiel hærdning i vommen hos drøvtyggere, med hensyn til risiko for livsstilssygdomme. Desuden undersøges, om den øgede sygdomsrisiko kan forklares ved at indtag af TF påvirker en række etablerede samt nyere risikofaktorer for hjertesygdom og type 2 sukkersyge. Afhandlingen kombinerer en gennemgang af den eksisterende litteratur med i) et systematisk review og meta-analyse af publicerede og ikke-publicerede prospektive kohortestudier, som undersøger sammenhængen mellem indtag af TF og forekomsten af hjertesygdom (Artikel I), og ii) resultaterne fra et kostinterventionsstudie, kaldet COBRA, hvor 52 postmenopausale kvinder med mavefedme blev bedt om at indtage 15 g/dag TF fra delvist hærdet sojabønneolie eller et kontrolfedtstof med højt indhold af olie- og palmitinsyre i 16 uger.

Afhandlingen adresserer følgende spørgsmål:

Spørgsmål (SP): Er indtag af TF forbundet med øget risiko for hjertesygdom – og kan TF fra industriel og mikrobiel hærdning betragtes som lige skadelige?
Svar (SV): Det systematiske review af kohortestudier bekræfter, at indtag af TF er forbundet med øget risiko for hjertesygdom i samme størrelsesorden som tidligere rapporteret (~20% pr. 5 g/dag TF). Det fremhæver imidlertid også, at der findes ganske få studier af denne type, hvilket kan skyldes publicerings-skævhed eller vanskelighederne forbundet med at estimere TF-indtag i befolkningsundersøgelser. Ikke desto mindre bliver disse resultater underbygget af data fra case-kontrol-studier, som har undersøgt sammenhængen mellem biomarkører for indtag af TF og hjertesygdom.

På baggrund af data fra kohortestudier synes den TF-associerede forøgelse af risiko for hjertesygdom at være forårsaget af indtag af industrielt hærdet, ikke mikrobiel hærdet, TF, hvilket muligvis skyldes, at sidstnævnte indtages i mindre mængder. Et meget sparsomt antal studier forhindrer imidlertid klare konklusioner om, hvorvidt kilden til TF er afgørende. Hverken biomarkør-undersøgelser eller interventionsstudier har kunnet bekræfte, at TF fra mikrobiel hærdning skulle være mindre skadelig end TF fra industriel hærdning. Der er dog ingen indikationer af, at TF fra mikrobiel hærdning øger risikoen for hjertesygdom i de mængder, der normalt indtages.

SP: Påvirker indtag af TF blodets fedtstoffer?
SV: Indtag af TF øger blodets koncentration af LDL-kolesterol og sænker koncentrationen af HDL-kolesterol. Dermed medfører TF en mere sygdomsfremkaldende blod-fedtprofil end alle andre typer fedtsyrer. Dette er blevet bekræftet i talrige interventionsundersøgelser og forekommer at gælde for TF fra såvel industriel som mikrobiel hærdning, i hvert fald ved indtag af en vis mængde TF. Den observerede 34% stigning i ratioen mellem LDL- og HDL-kolesterol i COBRA studiet kunne derfor betragtes som et tegn på god efterlevelse af kostinterventionen fra

forsøgsdeltagernes side. Den fundne stigning i LDL/HDL-kolesterol menes at svare til en ~6% øget risiko for hjertesygdom pr. 5 g/dag TF. Desuden tyder resultater fra flere undersøgelser på, at indtag af TF medfører en svag øgning af triglycerid og lipoprotein (a) i blodet, ligesom produktionen af små LDL partikler stiger.

SP: Påvirker indtag af TF markører for systemisk inflammation og endothel dysfunktion?
SV: Størstedelen af den foreliggende evidens for en sammenhæng mellem indtag af TF og mild systemisk inflammation og endothel dysfunktion stammer fra tværsnitsundersøgelser. Med fundet af en 12% stigning i koncentrationen af TNFα (P=0.002), samt en stigning i koncentrationen af TNF-receptorer, understøtter COBRA studiet imidlertid formodningen om, at indtag af TF fører til øget inflammation. Indtag af TF har desuden i enkelte studier vist at medføre let øget produktion af CRP, men en sådan effekt kunne ikke påvises i COBRA studiet

Resultater fra COBRA studiet gik i retning af at bekræfte tidligere fund af en øget E-selectin koncentration som følge af TF-indtag, hvilket indikerer, at indtag af TF medfører endothel dysfunktion. Imidlertid har kun et enkelt studie (ud af to) peget på, at TF påvirker mere direkte mål for endothelfunktion i en negativ retning.

SP: Påvirker indtag af TF kropssammensætning og fedtdeponering?
SV: Enkelte store befolkningsundersøgelser har peget på, at indtag af TF øger risikoen for mavefedme og generel vægtforøgelse, og disse fund støttes af et 6-årigt interventionsstudie i aber. Et betragteligt indtag af TF over 4 måneder i COBRA studiet havde ingen indvirkning på deponering af fedt på maven hos postmenopausale kvinder, men der var svage tegn på en stigning i kroppens samlede fedtindhold (P=0.12). Dyrestudier har vist modstridende resultater, med hensyn til om TF medfører øget kropsvægt eller fedmegrad, men de peger samstemmende på en øget deponering af fedt i leveren. I COBRA studiet kunne en sådan effekt ikke bekræftes hos mennesker. Det negative resultat kan dog skyldes begrænsninger i studiets design, såsom høj variabilitet i deltagernes leverfedtindhold og manglende standardisering af deltagernes kost.

SP: Påvirker indtag af TF insulinfølsomhed?
SV: De fleste dyrestudier har peget på, at indtag af TF påvirker insulinfølsomheden negativt, mens resultater fra undersøgelser i mennesker er modstridende. Det er uvist, om denne divergens skyldes det faktum, at dyreforsøg generelt er af længere varighed, eller om den er et resultat af artsforskelle. De få indikationer af en negativ effekt af TF på insulinæmi i mennesker stammer fra studier med metabolisk vulnerable forsøgspersoner. TFA eksponering førte imidlertid ikke til ændringer i markører for lipolyse eller insulinfølsomhed hos ældre, overvægtige kvinder i COBRA studiet.

SP: Hvad kan konkluderes på baggrund af COBRA studiet?
SV: Med opdagelsen af en pro-inflammatorisk effekt af TF i COBRA studiet har vores forskning bidraget til at mindske det føromtalte "forklaringsunderskud", selvom det er vanskeligt at estimere, hvor stor betydning denne effekt har på risikoen for hjertesygdom. Derimod fremstod hverken insulinresistens eller ektopisk fedtdeponering som bidragende forklaringsfaktorer.

LIST OF ABBREVIATIONS

8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin-$F_{2\alpha}$
AT	adipose tissue
AUC	area under the curve
BMI	body mass index
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
COBRA	Copenhagen Obesity Risk Assessment Study
CRP	C-reactive protein
CTP-1	carnitine pamitoyl transferase 1
CTR	control
DEXA	dual-energy x-ray absorptiometry
g/d	grams per day
FA	fatty acid(s)
FFA	free fatty acid(s)
FMD	flow mediated vasodilation
HDL-C	high density lipoprotein cholesterol
HOMA-IR	homeostasis model assessment index of insulin resistance
IAAT	intra-abdominal adipose tissue
IGT	impaired glucose tolerance
IL	interleukin
IP-TFA	industrially produced *trans* fatty acid(s)
ISR	Insulin secretion rate
IVGTT	intravenous glucose tolerance test
LCAT	lecithin:cholesterol acyltransferase
LDL-C	low density lipoprotein cholesterol
MCP-1	monocyte chemoattractant protein
MI	myocardial infarction
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MTP	microsomal triglyceride transfer protein
MUFA	monounsaturated fatty acid(s)
NAFLD	non-alcoholic fatty liver disease
NF-κB	nuclear factor κB
NGT	normal glucose tolerance
OGTT	oral glucose tolerance test
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid(s)
RBC	red blood cell(s)
RR	relative risk or risk ratio
R-TFA	ruminant *trans* fatty acid(s)
SAT	subcutaneous adipose tissue
sE-selectin	soluble E-selectin
SFA	saturated fatty acid(s)
sICAM-1	soluble intercellular adhesion molecule 1
SREBP-1	sterol regulatory element binding protein 1
sTNF-R1	soluble tumor necrosis factor receptor 1
sTNF-R2	soluble tumor necrosis factor receptor 2
sVCAM-1	soluble vascular cell adhesion molecule 1
Total-C	total cholesterol
TFA	*trans* fatty acid(s)
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
VOI	volume of interest

CONTENTS

1 INTRODUCTION

During the last decades, much attention has been paid to the presumed adverse health effects of dietary *trans* fatty acids (TFA). It has been estimated that a TFA intake of merely ~5 grams per day (g/d) (or 2% of energy (E%)) increases the risk of coronary heart disease (CHD) by more than 20%[1]. A single fast food meal could readily contain more than 15 g of TFA in 2006[2].

The general belief has been that this increase in risk of CHD should be ascribed to TFA derived from industrial production (IP-TFA), whereas the evidence for a detrimental effect of those produced by ruminants (R-TFA) is less strong[3-5]. In addition, TFA intake has been positively associated with greater risk of type 2 diabetes (T2D)[6,7] and weight gain[8,9].

Many intervention studies have been conducted to elucidate the underlying metabolic effects of dietary TFA. It has been known for decades now that IP-TFA intake increases plasma low-density lipoprotein cholesterol concentrations (LDL-C) as well as the ratio between LDL-C and high-density lipoprotein cholesterol (HDL-C), as summarized in several meta-analyses[1,10-13]. However, these effects on blood cholesterol levels may only explain a cardiovascular risk increase of ~5% per 2 E% TFA[14]. Even when taking into account the less well established effects of TFA intake on other cardiovascular risk markers, such as lipoprotein a (Lp(a)), the ratio of apolipoprotein (Apo)B to ApoA-1 and C-reactive protein (CRP), a considerable explanatory gap (of up to ~50%) persist between the risk increase found in observational studies and that which can be explained by the assessed cardiovascular risk factors[11]. Limited evidence suggests that TFA intake may induce low-grade systemic inflammation[15-19], insulin resistance[20,21] and/or ectopic fat deposition[22], but evidence from controlled intervention studies is scarce.

Despite the fact that the explanatory mechanisms responsible for the health risk associated with dietary TFA have not been fully elucidated, the evidence against TFA has by many governmental agencies worldwide been considered sufficient to recommend that dietary sources of TFA should be reduced as much as possible. Extensive campaigns have been conducted in several countries to eliminate TFA from the food supply based on the arguments that i) TFA can be considered nutritionally unnecessary and ii) most food products (except those of ruminant origin) can be produced without TFA[23].

Whereas some countries, such as Denmark and Switzerland, have implemented legislation restricting the use of TFA in fats destined for human consumption[23], others, such as the USA, Canada, Argentina and Brazil, have enforced limits to the TFA content in restaurant foods or/and introduced obligatory labeling of TFA containing foods[24]. In June 2010, the European Parliament voted for a mandatory labeling of TFA content[25], but this act has not yet been approved by the European Commission. These legislative actions, together with voluntary reformulations by industry, have drastically decreased the TFA availability in most foods sold in Western countries[23,24].

In most countries, including Denmark[23], the TFA intake seems to have followed a downward trend during the last decades (reviewed by Craig-Schmidt & Rong[26]). In 1989-91, the mean TFA intake in the US population was 5.3 g/day (2.6 E%) and 10% of the population was esti-

mated to have an intake above 9.4 g/d[27]. More recent mean TFA intake estimates from 2002-2004 range from 1 to 3 E%[26]. Estimates of TFA intake in Europe has generally been somewhat lower with mean intakes in the range of 2.4 to 5.4 g/d (28-79% from R-TFA) in most northern European and Nordic countries in 1995-99[28], with the 90th percentiles generally being 50-70% above the mean values. In Denmark, the R-TFA is believed to constitute almost all of the TFA consumed after the *trans* fat law went into force in 2004, and intake is likely to have remained constant since 1999 where the median R-TFA intake was 1.8 g/d (80% central range 0.9-2.9)[29].

Unfortunately, updated reports of population TFA intakes are lacking. It should be kept in mind, that mean intake values tend to hide the fact that subpopulations may have considerably higher intakes. In 2006, it was possible to obtain an intake of TFA beyond 15 g in a single serving of fast food from international fast food restaurants in selected countries[2]. Since then, McDonald's claim to have substantially reduced the TFA content in their products sold in the US, Australia and Europe[30] and products from Kentucky Fried Chicken now allegedly contain a maximum of 1 g of TFA per serving[31]. A recent assessment of the TFA content in popular foods in Europe, confirmed that meals from these two fast food chains were indeed free of TFA in 2009. Yet, in Eastern European countries, some snack products, such as cakes and popcorn, contained more than 5 g TFA per 100 g (Stender *et al*. 2010, *submitted*).

While the access to TFA has decreased considerable in many countries, populations living in some developing countries, may still be exposed to high TFA-foods, because partially hydrogenated vegetable oils represent inexpensive and stable sources of dietary fat[32]. Examples are Iran, where the mean TFA intake in 2001-03 was estimated to ~12.3 g/d (4.3 E%)[33] and India where the intake of vanaspati, a hydrogenated vegetable oil high in TFA, has increased during recent years[24].

The fact that some populations or sub-groups may still be exposed to considerable amounts of IP-TFA, together with the fact that R-TFA cannot be removed from meat and dairy products, makes it pertinent to fully understand the risk associated with intake of TFA from these two sources on lifestyle diseases. We found it timely to systematically review all the available observational evidence on the association between TFA intake and CHD, with specific emphasis on differentiating between TFA from ruminant and industrial sources.

Furthermore, since TFA intake appears to be associated with greater risk or CHD than can be explained by established risk markers, and since causal relationships can only be assessed in well-designed controlled studies, we performed such a study with the aim to examine the effect of a substantial daily intake of TFA on established as well as emerging risk factors for lifestyle diseases, including blood lipids, ectopic fat deposition, insulin sensitivity, and markers of endothelial dysfunction and low-grade inflammation.

In the subsequent chapters, the body of evidence linking dietary TFA with risk of CHD and T2D will be discussed and results of the following research will be included where appropriate:

i. A systematic review and meta-analysis of published and unpublished analyses from prospective cohort studies examining the association of dietary TFA from both industrial and ruminant sources on the risk of developing CHD (**Paper I**).

ii. A randomized controlled trial (RCT) carrying the acronym COBRA (Copenhagen Obesity Risk Assessment Study), which included 52 abdominally obese, postmenopausal women with body mass index (BMI) between 25 and 32 kg/m^2. The intervention period lasted 16 weeks, during which the women were exposed to high daily amounts of TFA (~15 g/d

corresponding to ~7 E%) from partially hydrogenated soybean oil or a control fat consisting of a blend of palm oil and high oleic sunflower oil. With this choice of control fat, TFA were compared to a mix of 23% palmitic (16:0), 67% oleic (*cis*18:1n-9) and 10% linoleic acid (*cis*18:2n-6). Before and after the intervention, as well as after a subsequent 12-week weight loss program, a range of measurements were performed as depicted in **Figure 1**. Nineteen normal-weight women (BMI 19 to 24 kg/m^2) served as lean references and underwent baseline examinations only.

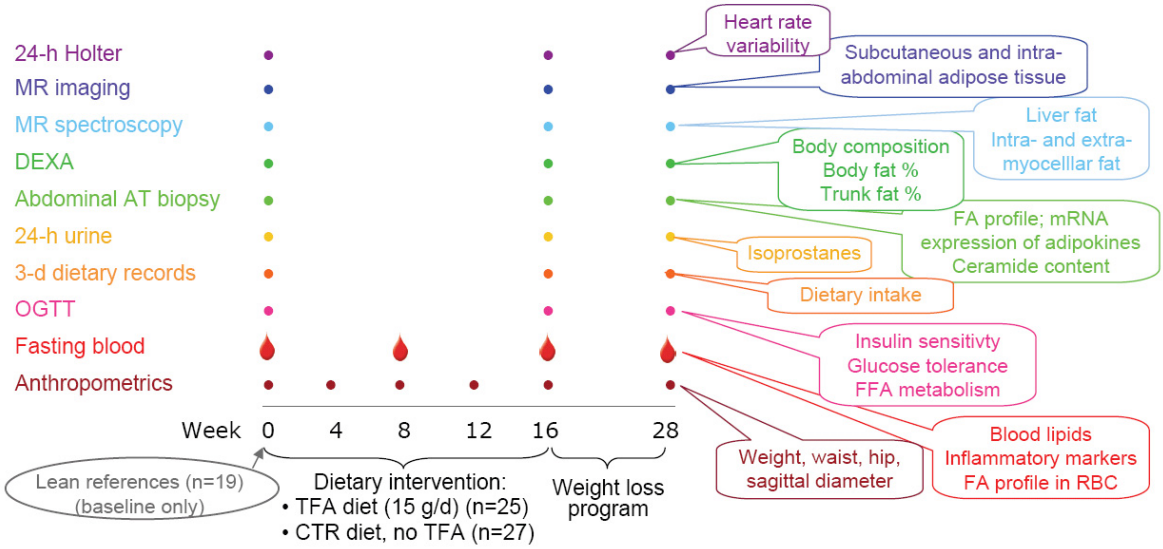

Figure 1: Design of the COBRA study. Fifty-two abdominally obese, overweight but otherwise healthy postmenopausal women were randomized to the dietary intervention; 25 to the trans fat (TFA) and 27 to the control (CTR) group (24 and 25, respectively, completed the intervention). Nineteen lean women underwent baseline examinations only. AT; adipose tissue; DEXA; dual-energy x-ray absorptiometry; FA, fatty acids; FFA, free fatty aids; MR, magnetic resonance; OGTT, oral glucose tolerance test; RBC, red blood cells.

The endpoints presented to the right in Figure 1 are covered in **Paper II** (*blood lipids, fatty acid (FA) profile in red blood cells (RBC), anthropometrics, body composition, liver fat, subcutaneous and intraabdominal adipose tissue (AT), and dietary intake*); **Paper III** (*insulin sensitivity, glucose tolerance and markers of lipolysis*); and **Paper IV** (*systemic markers of inflammation and endothelial dysfunction, subcutaneous AT FA profile, ceramide content and mRNA expression of cytokines, and urinary isoprostanes*), respectively.

The intention with the weight loss program was to provide the overweight women with an incentive to participate in the TFA intervention as well as to compensate for the putative negative TFA-induced health effects. The results of the examinations performed after the weight loss period will not be covered in this thesis; neither will data obtained from measurement of heart rate variability.

2 THE BASICS ABOUT TRANS FAT

According to the European Food Safety Authority, the term "*trans* fat", or more correct "*trans* fatty acids" (TFA), refers to any unsaturated fatty acid (FA) with at least one double bond in the *trans* configuration. The United States Food and Drug Administration and the Codex Alimentarius employ a more exclusive definition, excluding FAs with conjugated double bonds, i.e. double bonds not interrupted by at least one methylene group[34,35].

In the *trans* configuration, the hydrogen atoms on either side of the FA's double bond are positioned on opposite sides of the carbon chain, resulting in an almost straight molecule, which resembles that of a saturated fatty acid (SFA). Since animals and most plants lack the enzymatic capacity to synthesize double bonds with *trans* configuration, most naturally occurring unsaturated FAs are *cis*-FAs. Around *cis* double bonds, the hydrogen atoms are located on the same side of the carbon chain imposing a kink in the molecule (**Figure 2**). Due to this difference in geometry, TFA have higher melting points than their *cis*-isomers, although not as high as the SFA of equal chain length[36]. The structural differences between *cis* and *trans* FAs affect their physiological properties and metabolic effects.

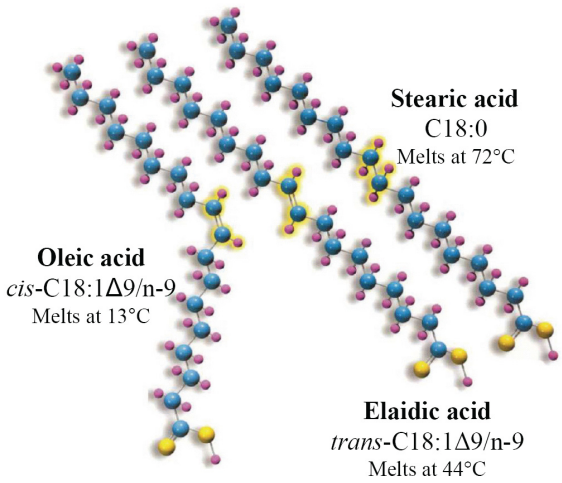

Figure 2: Structure of c*is* and *trans* monounsaturated C18 fatty acids and the corresponding saturated fatty acid. The Δ nomenclature denotes the position of the double bond counted from the carboxylic end of the fatty acid, whereas the "n-" nomenclature counts from the methyl end. Adapted and modified from Stender & Dyerberg[36].

FROM WHERE DO WE GET TRANS FAT?

TFA in the human food supply come mainly from two sources: One is industrial hydrogenation of unsaturated FAs in edible oils, the other biohydrogenation of unsaturated FAs in the rumen of ruminants. The first type of hydrogenation gives rise to IP-TFA, the other to R-TFA. Whereas IP-TFA are mostly found in processed food products such as margarine/shortenings, fried fast foods, snacks and bakery products, R-TFA are naturally incorporated into the fat from ruminants (cows, sheep and goats), and is therefore present in dairy and fatty meat products.

The two main fat sources of TFA have distinct characteristics. First of all, the concentration of TFA can be much higher in partially hydrogenated oils (up to 60%)[37], than in ruminant fat (normally up to 6%)[29,38], although the concentration in the latter may be elevated to 20% by manipulating the ruminants' feed[39]. Secondly, the TFA isomer distributions differ. Vaccenic acid, *trans*18:1n-7 (or *trans*11-18:1), is by far the most predominant isomer in ruminant *trans* fat,

constituting up to 50% of total TFA[38,39]. In contrast, industrially produced *trans* fat encompasses a wide range of positional *trans*-18:1 isomers with a more even distribution of the Δ8/n-10 to Δ11/n-7 isomers (**Figure 3**)[37]. Both sources of TFA also contain some TFA with a chain-length of 16 carbon atoms as well as some TFA with two double bonds (*trans*18:2), although these FA isomers seldom constitute great proportion of total TFA (<10% each) [29,38,40].

In addition, minor amounts of dietary TFA may originate from other processes where unsaturated FAs are heated in the presence of hydrogen, such as deodorization (or refining) of vegetable oils and oil-frying of foods[41]. However, whereas the first process gives rise to quantitatively negligible amounts of TFA (0.1-0.3% in cold pressed vegetable oils) and TFA are produced at the low rate of 0.3% per hour when frying potatoes in soybean oil at 180˚C[41], a considerable amount of TFA may be produced if highly unsaturated frying oil is reused and if frying temperature is high (>250˚C)[42].

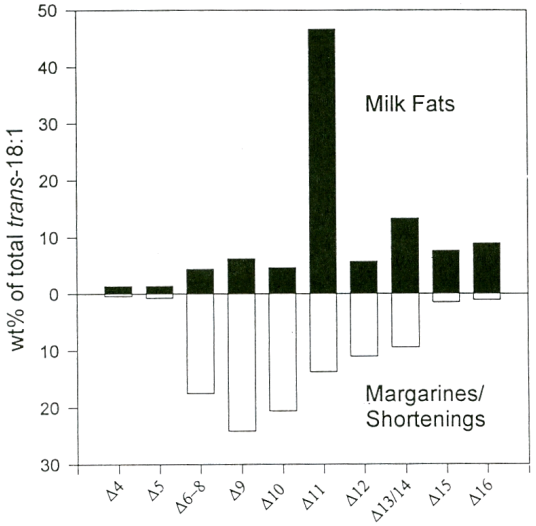

Figure 3: Typical *trans*18:1 isomer distribution in *trans* fat of ruminant (milk fat) and industrial (margarines/shortening) origin. From Wolff *et al*.[37].

HOW THE STORY OF TRANS FAT BEGAN

As described in **Chapter 1,** it is now broadly recognized that dietary IP-TFA are detrimental to human health. However, when hydrogenation of edible oils, and thereby *trans* fat, was first introduced more than a hundred years ago, it was considered a great new invention.

In the 1890s, the French chemist Paul Sabatier discovered that metal catalysts could be used to precipitate hydrogenation reactions. In the beginning of the 20th century, the German chemist Wilhelm Normann expanded these findings and in 1903 he received a patent for the hydrogenation of liquid fat. In this process, the unsaturated FAs are converted to a mix of SFA and TFA. Since food products containing unsaturated FAs are susceptible to oxygenation and therefore have relatively short shelf lives, this new technique was soon adopted by food manufacturers[26,41]. The first food product developed that contained partially hydrogenated vegetable oil, and thereby TFA, was Crisco shortening, introduced in 1911 by Procter & Gamble Company[43].

In the 1950s and 1960s the use of hydrogenation to stabilize the shelf life of food products grew rapidly. During the 1960s, margarine became viewed as the healthy alternative to butter because it was lower in SFA, and by the 1980s, many food manufacturers and restaurants had replaced tallow and lard with TFA-rich products[26,44].

However, already in the late 1970s some researchers began to question the widespread use of TFA. In 1979, Kummerow pointed to the facts that heart disease prevalence was higher in countries with high TFA intake and that margarines high in TFA might increase blood cholesterol levels[45]. Inspired by this work among others, Willet and colleagues, in 1980, initiated the collection of dietary TFA data in the large prospective *Nurses' Health Study* to investigate the association between dietary factors, and among them TFA consumption, and risk of life style

diseases[44]. In 1993, after 10 years of follow-up, Willet *et al.* could report a positive association with risk of CHD across increasing quintiles of TFA intake[5].

Since then much research has been undertaken to confirm these findings and to explore putative mechanisms of action of TFA in increasing the risk of CHD and other lifestyle diseases.

3 TRANS FAT INTAKE AND RISK OF CORONARY HEART DISEASE – EVIDENCE FROM OBSERVATIONAL STUDIES

Since Willet and coauthors' first report on the association between TFA intake and incident CHD in the *Nurses' Health Study*[5], several observational analyses on this subject have been published. Generally, studies with a prospective design are regarded as superior to studies with a case-control design due to i) the recall bias associated with reporting of dietary information after a myocardial infarction (MI); ii) the bias introduced when it is the more health-conscious controls who chose to participate in a study, and ii) the fact that in a case-control study of CHD, usually only non-fatal cases are included, whereas the important fatal cases are not[44]. Some of these shortcomings may be overcome by using biomarkers of TFA intake.

ASSOCIATION OF TRANS FAT INTAKE WITH RISK OF CORONARY HEART DISEASE IN PROSPECTIVE COHORT STUDIES

Following the 1993 publication from the *Nurses' Health Study*[5], similar analyses have been published from four other prospective cohort studies: the American *Health Professionals' Follow-up Study*[3], the Finnish *Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study*[4], the Dutch *Zutphen Elderly Study*[46], and the Native American *Strong Heart Study*[47]. Positive associations between TFA intake and risk of CHD events or fatal CHD[4,5,46,48] or trends towards the same[3] were found in most of the studies, but not all[47] after adjustment for various confounding factors.

In a variance weighted pooled analysis of the four earliest studies, including data from more than 146,000 subjects who experienced a total of 3,170 coronary events, Oomen *et al*.[46] estimated that an isocaloric substitution of 2 E% of carbohydrates with TFA would correspond to an increase of 25% (95% confidence interval (CI): 11 to 40) in the risk of coronary events. An updated inverse-variance weighted estimate of 23% (CI: 11 to 37) after more years of follow-up in two of the included studies (total number of events 4,965), was later provided by Mozaffarian *et al*.[1] Recently, based on the same few cohort studies, it was estimated that if TFA replaced 2 E% of SFA, monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) the corresponding risk increases would be 20% (CI: 7 to 34), 27% (CI: 14 to 42) and 32% (CI: 17 to 49), respectively[11,49].

Even fewer observational studies have provided risk-analyses stratified for the source of TFA. Whereas two of the cohort studies[4,5] together with data from a case-control study[50] suggested that IP-TFA, rather than R-TFA, intake was responsible for the increased risk associated with TFA intake, a third cohort study found no difference in risk between the two sources[46]. Recently, the risk of CHD associated with intake of R-TFA was assessed in the Danish MONICA cohort showing no association in men and a trend towards a negative association in women[51]. However, no pooled estimate of the cardiovascular risk associated with R-TFA and IP-TFA intake, respectively, has yet been published.

For this reason, and because a very limited number of studies has been included in prior meta-analyses of the association between TFA from any source (total-TFA) and CHD, we found it timely to conduct a systematic review and meta-analysis of the evidence from cohort studies of an association between intake of TFA and the risk of fatal and/or non-fatal CHD. Our work had a specific emphasis on stratifying the results according to industrial or ruminant origin of the TFA (**Paper I**).

Our literature search only identified one published study[47] that had not been included in the meta-analyses by Oomen *et al*.[46] and Mozaffarian *et al*.[1] However, by contacting principal investigators of five prospective cohort studies, who had not published data on the association between TFA intake and CHD, despite having collected relevant data, we identified one unpublished analysis from the *Finnish Mobile Clinic Health Examination Survey* and an analysis of data from the *Iowa Womens' Health Study* was performed on our request (**Paper I**).

Thereby, we identified at total of five published and two unpublished prospective cohort studies, assessing the association between TFA intake and incidence of fatal and/or non-fatal CHD. A total of 150,000 subjects experiencing 4,649 CHD events and 105,796 subjects with 2,998 fatal MIs, were included in the pooled analysis of total and fatal CHD, respectively. None of the unpublished analyses were stratified for the origin of the TFA, whereby only four and three analyses could be pooled in meta-analyses of the risk associated with intake of ruminant or industrial TFA, respectively.

The pooled relative risk estimates for comparison of extreme quintiles of total-TFA intake (corresponding to intake increments ranging from 2.8 to ~10 g/d) were 1.22 (CI: 1.08 to 1.38; P = 0.002, I^2=15%) for CHD events and 1.24 (CI: 1.07 to 1.43; P = 0.003, I^2=0%) for fatal CHD [**Figure 4**]. Thereby our estimate of the risk of CHD events was in accordance with that of a prior analysis, also comparing extreme quintiles of intake[52]; whereas the inclusion of the two unpublished analyses resulted in a slightly lower risk estimate for fatal CHD, compared to that found previously (relative risk (RR)=1.32 (CI: 1.08 to 1.61))[52].

Besides the prospective cohort studies included in the meta-analysis, a few case-control studies have examined the association between TFA intake and CHD[50,53,54] [*see* **Appendices A, B and C** for details]. In one study, an increased risk of first MI was evident only among subjects in the top quintile of TFA intake (6.5 g/d)[50], whereas two other studies (with TFA intakes of 1.4 and 5.5 g/d in the top quintiles) did not show any association between TFA intake and MI[53,54].

Is the source of TFA important?

In our analysis [Figure 4], R-TFA intake (increments ranging from 0.5 to 1.9 g/d) was not significantly associated with risk of CHD [RR = 0.92 (CI: 0.76 to 1.11); P = 0.36], and neither was IP-TFA intake (increments ranging from ~1.3 to 5.0 g/d), although there was a trend towards a positive association [RR = 1.21 (CI: 0.97 to 1.50); P = 0.09].

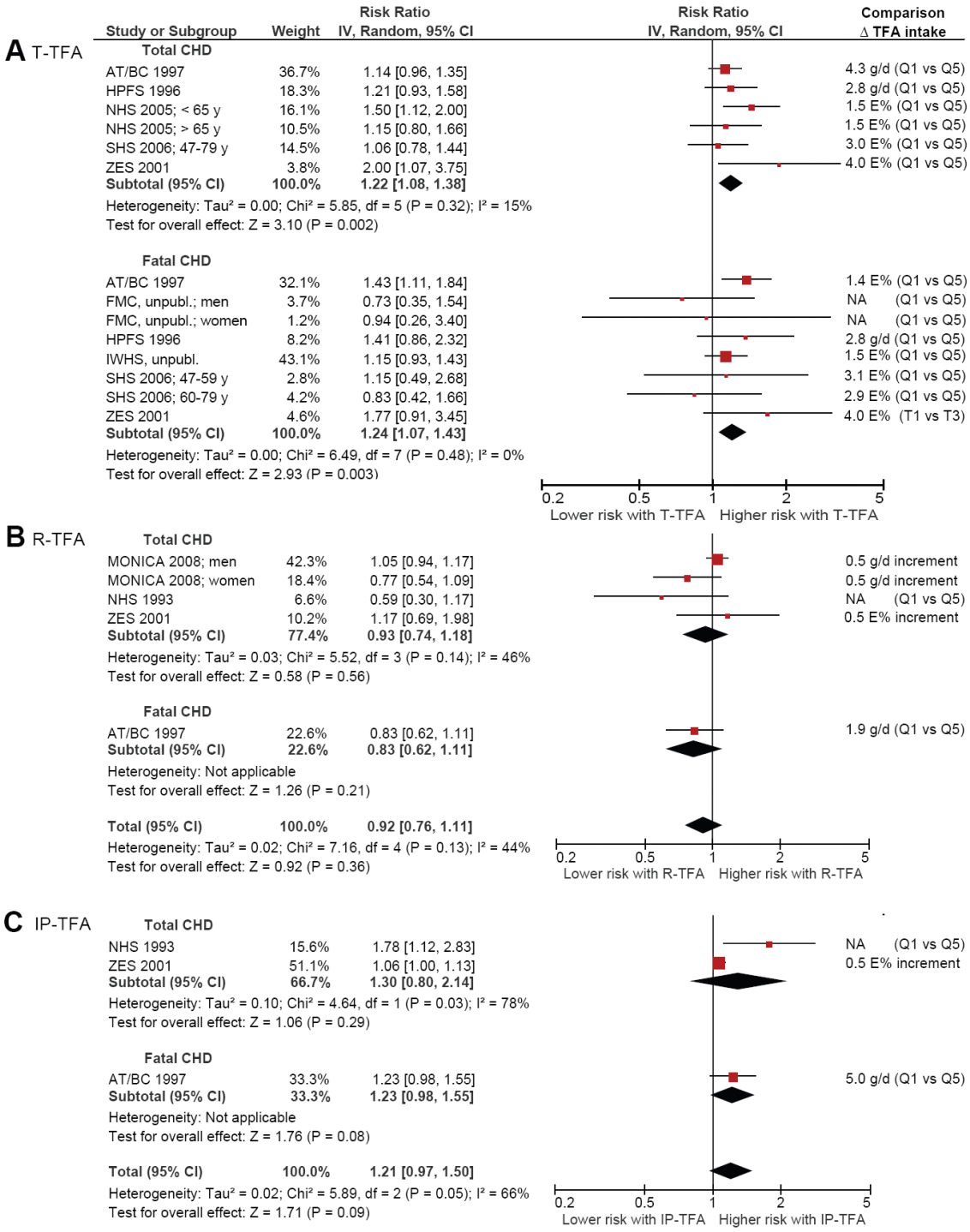

Figure 4: Risk ratios and 95% confidence intervals for fully adjusted random-effects models examining the associations of intake of trans fatty acids from any source (T-TFA) with total coronary heart disease (CHD) events and fatal CHD (Figure A); for studies examining the associations of intake of ruminant trans fatty acids (R-TFA) with total CHD events and fatal CHD (Figure B) and for studies examining the associations of intake of industrially produced trans fatty acids (IP-TFA) with total CHD events and fatal CHD (Figure C). AT/BC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study[4]; FMC, Finnish Mobile Clinic Health Examination Survey (*unpubl.*); HPFS, The Health Professionals' Follow-up Study[3]; IWHS, the Iowa Womens' Health Study (*unpubl.*); MONICA[51]; NHS, The Nurses' Health Study[5,48]; Q, quintile; T, tertile; SHS, The Strong Heart Study[47]; ZES, The Zutphen Elderly Study[46]. Modified from **Paper I**.

Since very few prospective cohort studies have investigated the association between R-TFA or IP-TFA intake and CHD, data from other types of studies may add important evidence. Results from the case-control study by Ascherio *et al*.[50] are in support of the estimate from our pooled analyses by indicating that IP-TFA was associated with increased risk, but only at intake levels above 3.3 g/d, whereas R-TFA appeared to be neutral (when comparing intakes up to 1.8 g/d). A negative association of R-TFA with undiagnosed CHD was found among men (n=4,490) in a cross-sectional analysis of the *Scottish Heart Health Study*, while total-TFA and IP-TFA were neutral, after adjustment for relevant confounders. Among women (n=5,170), no linear dose-response was found between any source of TFA and CHD, despite a wide range of intakes (mean intakes in the first/fifth quintile in men: 1.5/4.9 g/d R-TFA and 1.0/8.8 g/d IP-TFA)[55].

In summary, the observational evidence suggests that in contrast to dietary IP-TFA, R-TFA intake does not affect the risk of CHD or may even be slightly protective. However, this may be ascribed to either the different FA profile or the fact that R-TFA generally is consumed in much lower quantities than IP-TFA.

Limitations of TFA intake data

In epidemiological studies, the assessment of intake of TFA is potentially affected by substantial random measurement error due to a number of factors: i) participant recall bias; ii) problems with identifying and quantifying hidden fat and fats used for cooking; iii) insufficiently updated values in local food composition tables; iv) considerable variation of TFA content within foods from the same food category; and v) substantial changes in the TFA content of foods over time.

As described in **Chapter 2**, margarine was perceived as the healthy alternative to butter during the 1960s[44] and in the following decades tallow and lard were widely replaced by TFA-containing products as these were thought to be healthier due to their lower content of SFA. However, during the 1990s the food industry in many countries made efforts to reduce the TFA content of margarines and shortenings, and in recent years a drastic decline in the industrial TFA content of most foods in Western countries has been reported[26]. Thereby, values for TFA content in food composition tables are outdated quickly.

Part of these changes over time can be accounted for by performing repeated assessment of dietary intake; yet of the studies included in our meta-analysis only one[48] performed repeated dietary assessments. However, even though food databases may be adequately updated, the fact that similar foods, even from the same manufacturer, may have substantially different TFA contents, compromises the accuracy of TFA intake estimates. Innis *et al*.[56]demonstrated a large variability in the content of TFA in Canadian food products from the same category; e.g. in 17 brands of crackers the TFA content varied from 1 to 13 g TFA per 100 g. Moreover, using minimum and maximum values for TFA within a given food category, the estimated daily TFA intake could range from 1.4 to 25.4 g/d. Also, Stender *et al*.[2] documented how the TFA content in the same fast food meal from the same chain of fast food restaurants sampled from 26 countries around the world varied from 0.3 to 24 g.

Furthermore, a problem of 'confounding by indication' may arise in dietary surveys, and this is difficult to correct for: A subject with symptoms of CHD or family history of MI may be more aware of consuming a healthy diet, which in the 1980s meant replacing butter with margarine. Thereby subjects at higher risk may have increased their margarine and with this their IP-TFA consumption while reducing their butter, and R-TFA, consumption.

BIOMARKERS OF TRANS FAT INTAKE AND RISK OF CORONARY HEART DISEASE

Considering the difficulties in assessing TFA intake in free-living populations, biomarkers of TFA intake, such as proportions of TFA in blood or adipose tissue (AT), may provide more reliable estimates of individual TFA exposure, although this will not correct for the above-mentioned confounding by indication. As TFA cannot be synthesized in the human body, the proportions of TFA in plasma, red blood cells (RBC) and AT are thought to reflect the integrated dietary intake during the last weeks, months and year, respectively[26]. Assessment of tissue TFA does not allow for separation of the TFA isomers into R-TFA and IP-TFA (since most isomers are present in both ruminant and industrial sources). Instead it is possible to separate the exposure data into *trans*16:1, *trans*18:1 and *trans*18:2 isomers.

A number of case-control studies have found increased cardiovascular risk associated with blood or AT biomarkers of TFA intake[57-62] , whereas many others have not[54,63-67]. Jakobsen & Overvad[68] provided a review of the evidence from case-controls studies, suggesting that biomarkers of *trans*18:2 intake are associated with increased risk of CHD whereas those of *trans*18:1 are not. However, since many of the published studies include a limited number of subjects, they may be underpowered to detect significant associations. A pooled analysis might shed more light on this subject.

We therefore extracted and pooled the data from all the identified case-control studies reporting an odds ratio (OR) for the odds of being a case in the highest quantile of tissue TFA compared to the lowest (n =12)[54,58-60,62-67,69,70] and included data from two additional case-control studies published after the literature search by Jakobsen & Overvad[57,61] [*see* study characteristics and individual risk estimates in Appendices A, B and C]. The concentration of the sum of all TFA isomers (total-TFA) in AT and blood was positively associated with increased risk of CHD, although the heterogeneity was considerable [OR=1.33; CI: 1.01 to 1.74; P=0.04, I^2= 76%; *see* Forest plot in **Appendix D**]. The level of *trans*18:1 in AT and blood was also significantly associated with risk of CHD [OR=1.16; CI: 1.04 to 1.29; P=0.008, I^2= 75%], and the level of *trans*18:2 even more so [OR=1.30; CI: 1.14 to 1.47; P<0.0001, I^2= 68%]. However, caution should be taken in concluding too strongly on a meta-analysis compiling data with different end points (such as sudden cardiac death and non-fatal MI).

Yet, if the risk of CHD associated with intake of *trans*18:2 isomers is indeed greater than for *trans*18:1, this may help to explain why the prospective studies indicated a higher risk associated with IP-TFA intake compared to R-TFA; the content of *trans*18:2 is generally higher in IP-TFA compared to R-TFA. Alternatively, the tendency for a risk reduction seen with R-TFA, may relate to the fact that vaccenic acid (the predominant TFA isomer in R-TFA) may be converted endogenously to the conjugated linoleic acid (CLA) isomer *cis*9, *trans*11-18:2[71]. The adipose tissue content of this CLA isomer has been shown to be inversely associated with MI risk[72]. It is also possible that R-TFA intake may simply reflect milk fat intake just as the FAs 15:0 and 17:0. The content of these in plasma phospholipids was recently shown to correlate negatively to the risk of MI[73].

Of all the case-control studies, only three presented analyses for specific positional *trans*18:1 isomers. Lemaitre *et al*.[60] found no association of the RBC proportions of *trans*18:1n-7 (vaccenic acid), *trans*18:1n-8 or *trans*18:1n-9 with risk of sudden cardiac death, whereas Sun *et al*.[62] showed that *trans*18:1n-7, *trans*18:1n-9 and *trans*18:1n-12 were all significantly positively associated to risk of CHD after adjusting for potential confounders. Clifton *et al*.[53,74] observed that

AT *trans*18:1n-7 was an independent predictor of first MI. Taken together; these studies do not support a weaker association between vaccenic acid and risk of heart disease than estimated for other *trans*18:1 isomers. However, since vaccenic acid is also present in IP-TFA, it may be an invalid marker of R-TFA intake, as suggested recently[75].

In our pooled analysis of case-control studies, the *trans*16:1 concentration in AT and blood was not significantly associated with incident CHD, but few studies were included in this analysis, and tissue concentrations were low [OR = 1.09; CI: 0.82 to 1.46; P=0.55, I^2= 52%]. It must be acknowledged that the results from half of the case-control studies were not isomer-specific which would have complicated the interpretation of any significant finding reported in these studies[59,65], e.g. the *trans*16:1n-7 isomer is thought to reflect the intake of R-TFA and has been shown to correlate with dairy and red meat intake, the *trans*16:1n-9 isomer correlates with margarine intake[75].

SUMMARY: TRANS FAT INTAKE AND RISK OF CORONARY HEART DISEASE

The evidence against TFA as a risk factor for CHD from prospective studies seems strong, although causality cannot be inferred from this type of study. It is however noteworthy that a very limited number of prospective cohort studies have published analyses of the association between TFA intake and incident CHD. Publication bias may be a contributory factor. Yet, studies examining the association of biomarkers of TFA intake with risk of CHD collectively confirm that TFA exposure is associated with an increased risk of CHD. This appears to be the case for TFA with one or two double bonds, though the latter may be associated with higher CHD risk.

The pooled analysis of prospective cohort studies suggests that the risk associated with TFA intake may be ascribable to IP-TFA, not R-TFA. However, the very limited number of studies prohibits any firm conclusions concerning whether the source of TFA is important. The biomarker studies do not lend support to the notion that R-TFA should be less harmful than IP-TFA. This subject will be further addressed in **Chapter 5**.

4 TRANS FAT INTAKE AND RISK OF TYPE 2 DIABETES – EVIDENCE FROM OBSERVATIONAL STUDIES

Very few studies have reported on the association between dietary TFA and risk of T2D. Data from the *Nurses' Health Study* suggest that TFA intake is positively associated with incidence of T2D. In an analysis including 84,204 women with 2,507 incident cases of T2D during 14 years of follow-up, Salmeron *et al*.[7] reported a dose-response related increase in risk across quintiles of TFA intake, with women in the highest quintile having a 31% (CI: 10 to 56) greater risk of T2D than those in the lowest quintile after adjustment for various confounders; the corresponding estimate was 40% after 16 years of follow-up[6]. Furthermore, Salmeron *et al*.[7] estimated that replacement of 2 E% TFA by PUFA would decrease the risk of T2D by 40% (CI: 25-52). Moreover, the association between TFA intake and T2D risk was strongest among obese and physically inactive women[7]. In support of these findings, an Iranian cross-sectional study in 486 women found a positive association between intake of partially hydrogenated oil (generally containing 25-35% TFA) and insulin resistance (assessed by homeostasis model assessment index of insulin resistance (HOMA-IR) after adjustment for relevant confounders[76].

However, no association with risk of T2D was found in two other large prospective cohort studies. After 12 years of follow-up in 42,504 men (1,321 incident cases) in the *Health Professionals' Follow-up Study*, there was a risk increase across quintiles of TFA intake in multivariate analysis, but it disappeared after adjustment for other dietary variables and BMI [RR for extreme quintiles: 0.90 (CI: 0.74 to 1.10); P for trend = 0.33]. After 11 years of follow-up in 35,988 older women in the *Iowa Womens' Health Study* (n=1,890 incident cases), intake of TFA was inversely related to T2D risk in multivariate analysis, but not after additional adjustment for other dietary variables [RR for extreme quintiles: 0.92 (CI: 0.75 to 1.11); P for trend = 0.20][77]. Importantly, however, in the two latter studies the median TFA intakes were slightly lower (~1.3 and 1.6 E%, respectively) compared to the *Nurses' Health Study* (2 E%). Also, in the *Iowa Womens' Health Study* repeated dietary measurements were not obtained and the self-reported T2D incidence was invalid in one third of the cases[77].

In a recent study, TFA intake in the first trimester of pregnancy and incidence of gestational diabetes or glucose intolerance (assessed by oral glucose tolerance tests) were not associated in a sample of 1,733 women. The odds ratio was 0.87 (CI: 0.51 to 1.49) for developing gestational diabetes and 0.93 (CI: 0.66 to 1.31) for glucose intolerance per 1 E% increase in TFA intake[78].

SUMMARY: TRANS FAT INTAKE AND RISK OF TYPE 2 DIABETES

Collectively, the observational evidence connecting TFA intake to risk of T2D must be considered weak. However, the strong association seen in the large high-quality *Nurses' Health Study* calls for more analyses in other cohorts.

5 DIETARY TRANS FAT & BLOOD LIPIDS

The causality of the observational associations between dietary TFA and risk of CHD, and the putative association with T2D, has been investigated in a number of randomized controlled trials (RCTs) examining the effect of TFA intake on various disease risk markers. TFA may affect several steps in the atherosclerotic process, which ultimately leads to heart disease, either by acting directly on the endothelium, or indirectly by affecting metabolism and signaling in several tissues, such as muscle, liver and adipose tissue (AT).

ROLE OF LIPOPROTEINS IN THE ATHEROSCLEROTIC PROCESS

Lipoprotein particles play an important role in the atherosclerotic process. In early plaque formation, immune cells and lipid droplets accumulate in the intima, which is the innermost layer of the artery. The concentration of LDL in the circulation as well as the permeability of the endothelium is believed to accelerate the development of atherosclerosis[79].

Infiltration and retention of LDL particles in the arterial intima causes activation of the endothelium[80]. In the intima, LDL particles undergo oxidative modifications, whereby a mixture of heterogeneously modified particles is formed. The mechanism by which LDL is oxidized is not clear, but oxidative stress-induced lipid-peroxidation as well as enzymatic modification of both lipid and protein moieties may be involved[81]. The modifications give rise to production and release of oxidized phospholipids and possibly other lipid mediators, which initiate an inflammatory response in the arterial wall. The modified LDL particles are taken up by macrophages, whereby foam cells loaded with cholesterol ester containing droplets are formed and cytokine release is stimulated[82-84] (*see* **Chapter 6** for more detail).

In contrast, HDL particles, and particularly large HDL particles, are thought to be anti-atherogenic due to their reverse transport of cholesterol from the artery and peripheral tissues to the liver, and possibly also due to anti-oxidative and anti-inflammatory properties[85].

LDL- and HDL-cholesterol - risk factors or risk markers?

In a major meta-analysis including 68 long-term prospective trials and more than 300,000 subjects, the HDL-C and the non-HDL-C concentrations were independent predictors of CHD, whereas the triglyceride concentration was not. An increase in the HDL-C concentration of 0.39 mmol/l (corresponding to 1 standard deviation) was associated with a hazard ratio for CHD of 0.78 (CI: 0.74 to 0.82) and an increase in non-HDL-C concentration of 1.11 mmol/l with a hazard ratio of 1.50 (CI: 1.39 to 1.61) after controlling for standard risk factors. The risk associated with LDL-C (only measured in some of the included studies; n=44,234) was similar to that of non-HDL-C. Moreover, the HDL-C and non-HDL-C associated risk estimates were largely independent from each other[86]. Another comprehensive meta-analysis (61 trials and 900,000 subjects) largely supported these findings, and additionally indicated that the ratio of total-

cholesterol (total-C) to HDL-C was the strongest predictor of CHD mortality (40% more informative than non-HDL-C and more than twice as informative as total-C)[14].

A beneficial effect of lowering non-HDL-C, or LDL-C, has been firmly established, as both primary and secondary prevention trials with LDL-C-lowering statins consistently have shown that LDL-C reductions are accompanied by a decreased risk of CHD events[87]. In contrary, the general view of HDL-C as an anti-atherogenic factor[85] has been questioned, since HDL-increasing therapy has failed to decrease CHD mortality independently of LDL-C levels, as shown in a recent meta-analysis of RCTs[88].

TRANS FAT INTAKE AND BLOOD CHOLESTEROL

Today, ample evidence to substantiate a pro-atherogenic effect of TFA on blood lipid levels exists. A handful of metabolic studies conducted in the 1960s and 1970s examined the effect of partially hydrogenated fats on serum cholesterol levels. These studies were of varying quality and produced conflicting results as reviewed by Katan *et al*.[89]

However, when instead cholesterol content in different lipoprotein classes was measured, first in the beginning of the 1980s by Laine *et al*.[90] and later in 1990 by Mensink and Katan[91], detrimental effects of TFA became evident. The latter study, in which 59 subjects were fed 3 different diets for 3 weeks each, showed that TFA (11 E%) compared to oleic acid (18:1n-9) increased serum LDL-C and decreased HDL-C. Thereby, TFA had an even more unfavorable effect on the lipoprotein profile than SFA (a mix of lauric (12:0), myristitic (14:0) and palmitic acid (16:0))[91].

Since then, a number of human intervention studies have investigated the effect of TFA from hydrogenated vegetable or fish oils on blood lipid levels, collectively confirming that in comparison to all other FA classes, TFA dose-dependently increase the concentration of LDL-C and decrease HDL-C[92-109]. Several meta-analyses, based on 8 to 13 of these studies, have been published within the last two decades[1,10-12,110]. Mozaffarian & Clarke[11] performed the most recent and inclusive of these, and the main results are presented in **Table 1**.

Isocaloric replacement of 1 E% of MUFA with TFA was estimated to increase the ratio of total-C to HDL-C by 0.054 units (with smaller and greater estimated changes ensuing from replacement of SFA and PUFA, respectively). An increase of 0.054 may translate into an increased risk of CHD of approximately 2.0-2.5%[14]. Or in other terms, an increase in TFA intake of 2 E%, in exchange for MUFA, may increase the risk of CHD of ~5% based on the impact on blood cholesterol levels alone.

Table 1: Changes in blood cholesterol (C) concentrations associated with replacement of 1 E% intake of industrially produced trans fatty acids (IP-TFA) with saturated (SFA), monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA).

	Δ Total-C/HDL-C	Δ Total-C (mmol/l)	Δ LDL-C (mmol/l)	Δ HDL-C (mmol/l)
TFA replaces SFA (per E%)	0.031* (0.007)	-0.006 (0.006)	0.008 (0.005)	-0.013* (0.002)
TFA replaces MUFA (per E%)	0.054* (0.009)	0.032* (0.008)	0.038* (0.007)	-0.010* (0.003)
TFA replaces PUFA (per E%)	0.067* (0.009)	0.047* (0.008)	0.051* (0.007)	-0.013* (0.003)

Values are changes (SE), calculated from a meta-analysis of 13 randomized controlled dietary trials including 41 diets, adjusted for the effects of the other types of fat, dietary cholesterol, protein intake, duration of diet, total calories, weight and age. *Indicates corresponding P-value is <0.05. Adapted from[11].

In the COBRA study, we found that intake of 7 E% TFA decreased plasma HDL-C by 10% and increased LDL-C by 18% (**Paper II**). The mean difference between diet groups in the ratio of total-C to HDL-C of 0.45 (CI: 0.24 to 0.65; P<0.001), was somewhat larger than what could have been expected from the meta-analysis by Mozaffarian & Clarke[11]. We used a blend of palm oil and high oleic sunflower oil as a control fat, whereby 7 E% from TFA in the TFA group was compared to an intake of 1.6 E% from SFA, 4.7 E% from MUFA and 0.7 E% from PUFA in the CTR group. According to the estimates proposed by Mozaffarian & Clarke (Table 1) a difference in the total-C to HDL-C ratio of 0.35 between the TFA and CTR diet groups was expected.

The effect of TFA on blood lipids in the COBRA study was also considerably greater than that observed in our previous 8-wk study with similar design but in normal-weight young men[111]. The fact that a more metabolically vulnerable study population of moderately overweight, abdominally obese and older women (> 45 y), was enrolled in the COBRA study may help to explain the more pronounced effect of TFA on plasma cholesterol.

The adverse change in blood lipids after consumption of ~7 E% TFA, observed in the COBRA study, may translate into an increase in the risk of CHD of approximately 20%[14]. A TFA intake this high (15 g/d) is likely to be consumed by very few people in Western countries today. However, as described in **Chapter 1**, the TFA intake may very well still reach this level among subgroups in some developing countries[32].

EFFECT OF TRANS FAT ON BLOOD LIPIDS AND LIPOPROTEINS BEYOND CHANGES IN LDL-C AND HDL-C

Besides affecting the cholesterol-content of the different lipoprotein classes, TFA intake has also been shown to affect the number of lipoprotein particles, as reflected by increased ApoB (reflecting number of ApoB-containing particles) and decreased ApoA1 (reflecting HDL particle number) concentrations in a number of studies[91,93,95,99,103,106,112]. These effects are attenuated, but not eliminated, when adjusting for the total-C to HDL-C ratio[11]. A clear association between the ApoB to A1 ratio and risk of MI has been shown[113] although this ratio may not add substantial information beyond that obtained from the total-C to HDL-C ratio[114].

In addition, TFA intake has been shown to increase fasting plasma Lp(a) in several studies[92,93,99,102,103,115], whereas in other studies such an effect was only observed in subgroups of participants with high baseline Lp(a) concentrations[96,116] or not at all[98,112]. The Lp(a) particle is a lipoprotein similar to LDL that has an extra glycoprotein apoprotein(a). Plasma Lp(a) has been shown to be an independent marker of CHD risk with a ~50 mg/l higher Lp(a) concentration corresponding to a RR of 1.7 (CI: 1.4 to 1.9)[117]. In the meta-analysis by Mozaffarian & Clarke[11] a change in the TFA intake of 1 E% was estimated to increase the concentration of Lp(a) by 3.8, 1.4 and 1.1 mg/l when compared to SFA, MUFA and PUFA, respectively, suggesting that replacement of TFA by SFA would more favorably affect Lp(a) concentrations than replacement by MUFA or PUFA. Neither Apo-lipoprotein, nor Lp(a) concentrations were measured in the COBRA study.

Whereas the effect of TFA on fasting triglyceride concentrations was not significantly different from that of SFA in the same meta-analysis, a change in the TFA intake of 1 E% was estimated to modestly increase the concentration by 0.014 -0.015 mmol/l when replacing either MUFA or PUFA[11]. Using these estimates, a difference between diet groups of 0.08 mmol/l in triglyceride concentrations could have been expected in the COBRA study. We found a non-significant in-

crease of 0.11 ± 0.06 mmol/l (P=0.09) from baseline in the TFA group, but also a slight increase of 0.05 ± 0.07 mmol/l (P=0.54) in the CTR group, resulting in no significant difference between diet groups (**Paper II**).

A few studies have examined the effect of TFA intake on lipoprotein particle sizes. TFA decreased the LDL particle size in a dose-dependent, but subtle, manner in one study[118] and a tendency towards the same was observed in another[119]. It has been suggested that smaller LDL particles may be more susceptible to oxidation[120] and thereby more atherogenic. However, TFA intake was found not to increase the vulnerability of LDL to oxidative change[102,119].

DO ALL TRANS FATTY ACID ISOMERS ELICIT THE SAME EFFECT ON BLOOD LIPIDS?

Since all of the above-mentioned effects of TFA have been observed in studies where subjects were supplied with elaidic acid (*trans*18:1n-9) or mixtures of TFA isomers ensuing from hydrogenation of different vegetable oils (mainly *trans*18:1), very little is know about the effects of individual TFA isomers. Moreover, the effect of TFA with more than one double bond remains elusive. A study by Vermunt *et al*.[121] showed that low amounts of *trans*18:3 (1.4 g/d or < 1 E%) caused an increase in the LDL-C to HDL-C ratio of more than 8%, suggesting a greater effect of this TFA isomer compared to equivalent intakes of TFA from hydrogenated oils. Also, it appears that TFA from hydrogenated fish oil, containing C20 and C22 TFA isomers, may be more harmful than those from hydrogenated vegetable oils[92,101,112].

The effect of TFA from ruminant sources has long been unknown. A study in hamsters suggested that the effect of vaccenic acid, the predominant isomer in R-TFA, was comparable to, or slightly worse, than that of elaidic acid, the isomer typical of IP-TFA, when evaluating different blood lipid parameters[122]. Recently, a few intervention studies comparing industrial and ruminant sources of TFA have been published shedding some more light on this issue in humans[107,123,124].

Motard-Belanger *et al*.[107] showed that when men consumed R-TFA in high amounts (3.7 E%) the effect on blood lipids was comparable to the effects of IP-TFA, increasing the LDL-C and decreasing the HDL-C concentrations in plasma. However, when R-TFA was consumed in moderate amounts (1.5 E%) the effect on blood lipids was not significantly different from that of a control diet with low TFA content (0.8 E% from any source). In accordance, Tardy *et al*.[124] found no difference in LDL-C or HDL-C after consumption of diets with ~2 E% TFA from either of the two sources, when compared to a control fat (TFA replaced by oleic and palmitic acid) in overweight women[124].

A study by Chardigny *et al*.[123] indicated that women may respond differently to TFA from the two sources, while men do not. Concentrations of both HDL-C and LDL-C were higher in women after intake of 5 E% R-TFA compared to equivalent intakes of IP-TFA, resulting in no difference in the total-C to HDL-C ratio. No significant differences were observed in men[123]. In this regard it is noteworthy, than we in our meta-analysis (**Paper I**) found that in analyses stratifying for sex, a negative association of R-TFA with CHD, was present in women [RR=0.73 (CI: 0.54 to 0.99); P = 0.05], but not in men [RR=1.01 (CI: 0.87 to 1.17); P = 0.91]. However, the sample size may be inadequate for these stratified analyses, since only two and three cohort studies reported separate analysis for women and men, respectively,.

In summary, these studies indicate that adverse effects of TFA on blood lipids are related to the amount of TFA rather than to the dietary source. The suggested sex difference in response to high intakes of TFA call for confirmation in future studies.

MECHANISMS BY WHICH TRANS FATTY ACIDS MAY AFFECT BLOOD LIPID METABOLISM

Different classes of FA may differently influence blood lipid levels by affecting various steps in lipid metabolism, including LDL-receptor production, abundance and activity; hepatic very-low-density lipoprotein (VLDL) secretion; and catabolism of triglyceride-rich lipoproteins.

Lipoprotein production versus catabolism

The elevation in circulating levels of cholesterol seen after TFA exposure was shown to be accompanied by a rise in endogenous cholesterol synthesis in one study [108] but this was not the case in others[119,125].

A study in hamsters suggested that increases in LDL-C were due to suppression of hepatic LDL receptor activity[126]. In support of this notion, the catabolic rate of LDL was decreased in humans after consumption of TFA from hydrogenated soybean oil compared to un-hydrogenated oil. Also, the catabolic rate of HDL was increased, while the production rates of neither HDL nor LDL were affected[127]. An increased catabolism of HDL was likewise seen in a study in cibus monkeys fed elaidic acid[128]. Moreover, one study indicated that TFA-induced increases in LDL-C concentrations were not caused by changes in the conversion rate of triglyceride-rich lipoproteins to intermediate-density lipoproteins (IDL) or IDL to LDL. LDL clearance appeared to be the only explanatory factor[127].

CETP enzyme activity

The effect of TFA intake on LDL-C and HDL-C concentrations likely involves cholesteryl ester transfer protein (CETP), a protein in human plasma that transfers esterified cholesterol from HDL to lipoproteins of lower density in exchange for triglycerides. *In vitro*, an increased transfer of HDL-C to LDL due to increased activity of CETP was shown in response to elaidic acid but not oleic acid[129]. An increased postprandial transfer of cholesterol from high to low density lipoproteins[130] and an increased fasting activity of CETP was subsequently confirmed in some[131-133] but not all[109] human TFA feeding studies as well as in Cibus monkeys[128]. However, no effect on CETP activity was seen, despite significant TFA-induced reduction in HDL-C, when compared to a myristitic/palmitic acid-rich baseline diet in a study by Aro *et al*.[93]. Also, results by Lichtenstein *et al*.[133] indicated that the observed changes in HDL-C were unlikely due to changes in CETP activity alone.

Additional putative mechanisms

Increased phospholipid transfer protein (PLTP) activity in response to TFA intake was observed in two studies[93,133]. However, the role of PLTP in lipoprotein modulation in humans appears to be dual. On one hand, PLTP increases the production of atherogenic apoB-containing lipoproteins, on the other, PLTP is thought to be anti-atherogenic by contributing to the formation of new HDL-particles, thereby increasing reverse-cholesterol transport[134]. It is therefore somewhat

surprising that the increase in PLTP activity was accompanied by a concomitant decrease in HDL-C in the two TFA intervention studies[93,133].

Matthan and colleagues[135] have explored additional pathways by which TFA may affect lipoprotein levels, and they suggested that the observed decrease in plasma concentrations of acylation-stimulated protein (a determinant of the rate of adipocyte triglyceride uptake) and a concomitant increase in free FAs (FFA) after TFA feeding, would cause a higher flux of FFA to the liver and subsequent increased production of VLDL[135]. Also, an observed decrease in the esterification of cholesterol was suggested to partly explain the lowering of HDL, since HDL is the predominant site for this esterification via lecithin:cholesterol acyltransferase (LCAT). However, this interpretation seems to contradict the null-effect on the esterification rate of cholesterol in HDL observed in a larger sample of subjects from the same study[133].

The cellular mechanisms behind the effect of TFA on blood lipids remain elusive, but the proposed effect of TFA on LDL receptor activity may occur via modulation of membrane fluidity. Also, the fact that different FAs have different ligand affinities for various nuclear transcription factors may explain other metabolic effects. These subjects will be further discussed in **Chapter 9**.

SUMMARY: TRANS FAT & BLOOD LIPIDS

Is has been firmly established that TFA consumption increases LDL-C and decreases HDL-C concentrations and thereby induces a more atherogenic blood lipid profile than all other FA classes. This appears to apply to TFA of both ruminant and industrial origin, at least at high intake levels. The observed changes in blood lipids in the COBRA study could therefore be considered an indication of good compliance with the study diets. TFA may induce a modest increase in triglyceride levels compared to MUFA and PUFA, but this is not a consistent finding, maybe owing to lack of power in individual studies. TFA intake also appears to increase Lp(a) and may give rise to production of smaller LDL particles.

In the COBRA study, we did not examine putative mechanism by which TFA may exert these changes. The available evidence suggests that TFA intake neither affects the synthesis of cholesterol nor the production rate of LDL or HDL particles. Instead, TFA may decrease the activity of the LDL-receptor and thereby the catabolic rate of LDL. In parallel, HDL particles may be catabolized at a higher rate. A stimulating effect of TFA on CETP activity likely explains part of the increase in LDL-C and decrease in HDL-C, whereas putative effects on other enzyme activities have not been substantiated. Moreover, the cellular mechanism by which TFA intake may convey these effects, have not been established.

6 DIETARY TRANS FAT & OXIDATIVE STRESS, ENDOTHELIAL DYSFUNCTION AND CHRONIC LOW-GRADE INFLAMMATION

It has been suggested that TFA intake may increase the risk of CHD and T2D via induction of oxidative stress, endothelial dysfunction and systemic inflammation[18,19,136-140]. These disturbances are tightly implicated in development of insulin resistance and in the atherosclerotic process. The initiation and progression of atherosclerosis has been reviewed by Libby & Hansson[83,141] and will not be covered in detail in this thesis. However, an understanding of some key steps is important for the appreciation of how TFA might affect the process. The involvement of blood lipids in the progression of atherosclerosis has been dealt with in **Chapter 5**; in this chapter, the concepts of oxidative stress, endothelial dysfunction, and chronic low-grade inflammation will be briefly described.

ATHEROSCLEROSIS AS AN INFLAMMATORY DISEASE

As already described, infiltration and retention of LDL particles in the arterial intima causes activation of the endothelium[80]. Cytokines, such as tumor necrosis factor α (TNFα) and interleukin (IL)1β, induce increased expression of leukocyte adhesion molecules on the surface of the activated endothelial cells[84,142,143]. Selectins (such as E-selectin) on endothelial cells are essential for the initiation of leucocyte rolling along the endothelium, and adhesion molecules, (vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1)), cause the blood cells to adhere at the site of activation[143]. The blood cells are stimulated to migrate into the subendothelial space by adhesion molecules and chemokines, such as monocyte chemoattractant protein 1 (MCP-1), produced in the intima and by vascular cells, and here they differentiate into macrophages[141]. The modified LDL particles are taken up by scavenger receptors or toll-like receptors (TLR) of the macrophages, with ensuing development of foam cells, stimulation of cytokine release[82-84] and secretion of reactive oxygen species[144]. T-cells are attracted and activated by antigens presented by macrophages and dendritic cells and they progressively infiltrate the atheroma. The activated T-cells produce cytokines (e.g. TNFα and interferon-γ) which again activates macrophages and vascular cells, initiating a potent inflammatory cascade[83].

Initially, the activated immune cells produce TNFα and IL1β, which induce the production of IL6. IL6 may then be released into the circulation and stimulate production of the acute phase reactants CRP and fibrinogen in the liver[141]. The pro-inflammatory cytokines and proteases released from the activated immune cells can reduce collagen formation and attack the fibrous cap that covers the atheroma. This weakens the stability of the plaque and makes it more vulnerable to rupture[83].

MARKERS OF LOW-GRADE SYSTEMIC INFLAMMATION

The identification of an exact cause of a rise in the systemic level of a given inflammatory marker is not straightforward, as they may stem from processes in the vascular endothelium, but also from local inflammatory processes in adipose tissue, muscle and/or liver. Also, cytokines may have different effects in different tissues or in different conditions[145,146]. However, systemic cytokine or CRP concentrations are thought to be reflective of spontaneously ongoing inflammatory responses *in vivo* and thereby mirror the overall inflammatory state of an individual[147].

As described above, it is progressively recognized that inflammatory processes are involved in the initiation and progression of atherosclerosis[83,141] and insulin resistance[148-150]. Furthermore, obesity is a disorder characterized by chronic mild inflammation, with increased adipose tissue expression, production and release of various cytokines, including TNFα, IL6 and MCP-1 as well as a decrease in adiponectin. This is thought to be partly due to macrophage infiltration as well as the hypoxia ensuing from tissue mass expansion[151].

TNFα and TNF receptors

TNFα is a pro-inflammatory cytokine, which is ubiquitously expressed in multiple cell types. TNFα is elevated in obesity and is thought to be one of many causal factors in obesity-induced insulin resistance[152,153]. As already described, TNFα in also highly involved in the atherosclerotic process by triggering vascular inflammation via activation of specific membrane-bound receptors[146]. TNFα triggers the production of IL6[154] and suppresses that of adiponectin *in vitro*[155]. The cytokine is short-lived in the circulation and concentrations may reflect local TNFα activation or mirror overall systemic inflammation[156].

TNF receptors are shed as a consequence of exposure to TNFα and other cytokines[156]. Soluble TNF receptors are thought to have a buffering function, either as inhibitors or as carrier proteins for TNFα[156]. An endotoxin-stimulated increase in TNFα has been shown to be followed by a prolonged rise in both TNF receptor 1 and 2 (TNF-R1 and TNF-R2)[157].

Rather than exerting pro-inflammatory effects in themselves, the soluble receptors are thought to be more easily measurable markers of TNF system activity than systemic TNFα, since the latter is present in plasma in very low concentrations. Also, the natural variability over time is higher for TNFα than for TNF receptors[158].

TNFα and TNF-receptor concentrations are elevated in heart failure patients, as reviewed by Bozkurt *et al.*[159], and high systemic TNFα concentration was shown to increase the risk of recurrent coronary events after a MI[160]. However, adjustment for lipid risk markers may attenuate the association of TNF receptors with risk of CHD[161].

IL6

IL6 is another cytokine reported to be elevated in obese subjects[153]. The physiological role of IL6 is, however, controversial. Some investigators have argued for a pro-inflammatory and insulin resistance-inducing effect, largely based on evidence from observational or animal studies[153,162]. Based on data from trials in humans, others[154] have argued that IL6 has insulin sensitizing and anti-inflammatory properties and that high systemic levels of IL6 simply reflect a stimulation by locally produced TNFα. In humans, IL6 has been shown to stimulate anti-inflammatory cytokines and suppress TNFα production[154]. Nevertheless, the systemic concentration of IL6 has been shown to be an independent predictor of cardiovascular events[163].

Adiponectin

Adiponectin is an adipokine produced by adipocytes and present in the circulation in high concentrations[164]. In contrast to other adipokines, adiponectin secretion and circulating levels are inversely proportional to body fat content[165]. Adiponectin increases hepatic insulin sensitivity, decreases vascular inflammation and antagonizes many effects of TNFα[164]. Furthermore, TNFα gene expression is inversely related to that of adiponectin in human AT[166]. Adiponectin levels may not independently predict cardiovascular risk[167] but they are inversely associated with risk of T2D[168].

CRP

The bulk of circulating CRP is produced by hepatocytes largely under regulatory control of IL6 and IL1, but CRP may also be produced in the atherosclerotic lesion by smooth muscle cells and macrophages[169,170]. As recently reviewed by Devaraj *et al.*[169], evidence is now emerging to support the notion that CRP is indeed an active factor and not just a marker of atherogenesis, although the function of CRP in human physiology remains uncertain. Effects of CRP are thought to include inhibition of fibrinolysis, promotion of endothelial dysfunction (reduction of endothelial nitric oxide and elevated levels of cellular adhesion molecules), increased production of pro-inflammatory cytokines by macrophages and monocytes and release of reactive oxygen species[169]. Systemic concentrations of CRP have been found to predict the risk of cardiovascular events independent of other risk markers[114].

TFA intake and markers of low-grade systemic inflammation – evidence from observational studies

A few cross-sectional analyses in healthy subjects and heart failure patients have reported increasing concentrations of markers of low-grade systemic inflammation, such as CRP, TNFα and soluble TNF-receptors with higher intake of TFA[18,19,136,137], or partially hydrogenated vegetable oils[139][**Table 2**].

In healthy women from the *Nurses' Health Study*, the concentrations of sTNF-R1 and sTNF-R2 were 9% and 12% higher in the highest quintile of TFA intake compared to the lowest (interquintile range ~2.1 g/d TFA)[19]. Also in heart failure patients, the proportion of TFA in RBC membranes or intake of TFA has been associated with increased concentrations of TNFα and its soluble receptors[137, 136].

Positive associations between intake of TFA (or partially hydrogenated oil) and IL6 and CRP were found in populations of moderately overweight women[18,139]. In one study, positive associations were only seen in women with higher BMI[19], whereas in another, stratification by BMI showed similar associations in women with BMI higher or lower than the median value[18]. Thereby, limited data support the hypothesis proposed by Mozaffarian *et al.*[19] that the effect of TFA intake of IL6 and CRP production is modified by body weight.

Table 2: Cross-sectional studies examining the association between *trans* fatty acid (TFA) intake and markers of endothelial dysfunction and inflammation

Reference	Subjects	Comparison	Effect of TFA							
			TNFα	TNF-R1	TNF-R2	CRP	IL6	E-Se-lectin	I-CAM	V-CAM
Mozaffarian *et al*. (2004)[19]	823 F from NHS; healthy, 61 y; BMI 24.6 kg/m^2	Quintiles of TFA intake (FFQ) [1] Mean TFA intake: 2.7 g/d)		↑ 9%	↑ 12%	↔[2]	↔[2]			
Mozaffarian *et al*. (2004)[137] [3]	86 F+M; heart failure; ~53 y; BMI ~30 kg/m^2	1% diff in TFA in RBC membranes [4] Mean TFA in RBC: 1.8% (range: 0.7-2.9%)	↑ 249%	↑ 41%	↑ 247%	↓ -48%	↑ 123%			
Lennie *et al*. (2005)[136]	42 F+M; heart failure; 61 y; no BMI data.	High vs. low TFA intake, cut-off 3.3 g/d (4d food records) Mean TFA intake: 4.0 g/d)	↑ 51%	↔	↔		↔			
Lopez-Garcia *et al*. (2005)[18]	730 F from NHS; healthy; 56 y; BMI ~26 kg/m^2	Quintiles of TFA intake (FFQ) [5] Mean TFA intake: 2.4 g/d			↑ 5%	↑ 73%	↑ 17%	↑ 20%	↑ 10%	↑ 10%
Esmaillzadeh *et al*. (2008)[139]	486 F; healthy; 40-60 y; BMI ~27 kg/m^2	Quintiles of PHVO intake (FFQ) [6] Mean PHVO intake: 23 g/d, or ~7.5 g TFA	↑ 56%			↑ 29%	↑ 59%	↔	↑ 16%	↔

↑ and ↓ represent higher and lower concentration of this marker with increasing TFA intake. ↔, no significant effect of TFA. NHS, the Nurses' Health Study; PHVO, Partially hydrogenated vegetable oil. [1] adjusted for age; BMI; smoking; physical activity; intake of energy, alcohol, SFA, PUFA, protein and fiber; use of aspirin and anti-inflammatory drugs, [2]Positively associated with TFA intake only in women with higher body mass index; [3] TFA was also positively associated with interleukin (IL)1β, IL1 receptor antagonist, IL10, monocyte chemoattractant protein 1, and brain natriuretic peptide; [4] adjusted for age, sex, BMI, smoking, ejection fraction, New York Heart Association class, ischemic etiology, statin use and serum glucose; [5] adjusted for age, BMI, physical activity, smoking, intake of alcohol, MUFA, PUFA, SFA and postmenopausal hormone therapy; [6] adjusted for, age, smoking, physical activity, estrogen use, dietary intakes, BMI, plasma glucose and lipids and other confounders.

The findings from these cross-sectional analyses have not been substantiated by prospective analysis looking at associations between changes in TFA intake and markers of inflammation. Moreover, supporting evidence from RCTs is sparse [**Table 3**].

TFA intake and inflammatory markers - Intervention studies

Han *et al*.[171] showed increased production of TNFα and IL6 in stimulated mononuclear cells of mildly hypercholesterolemic, older and overweight subjects after 32 days of 7 E% TFA intake from stick margarine compared to soybean oil. In contrast, in two studies in young, lean and healthy subjects, supplementation with a mix of *trans*18:1n-7 and *trans*18:1n-6 (~3 E%) for 6 weeks had no effect on plasma concentrations of TNFα, IL6, IL8 or adiponectin[172], and 5 weeks consumption of 10 E% TFA from hydrogenated soybean oil did not affect the serum TNFα or IL6 concentration when compared to palm stearin or high oleic palm olein[17].

Table 3: Intervention studies examining the effect of *trans* fatty acid (TFA) intake on markers of endothelial dysfunction and inflammation

Reference	Subjects	Design	Diets	Measured in	Effect of TFA			
					TNFα	CRP	IL6	E-Selectin
Han *et al.* (2002)[171]	19 F+M (hyperchol., mean age 65 y, mean BMI 29 kg/m^2)	Cross-over (32d)	Isoenergetic, 30 E% from fat. 1) Stick marg. (7 E%TFA); 2) Soybean oil; 3) Butter (1 E% TFA)	LPS stimulated PBMCs	↑ 58%[1]		↑ 36%[1]	
Lichtenstein *et al.* (2003)[173]	36 F+M (hyperchol., mean age 63 y, mean BMI 27 kg/m^2)	Cross-over (35d)	Isoenergetic, 30 E% from fat. 1) Soft marg. (3 E% TFA); 2) Shortening (4 E% TFA); 3) Stick marg. (7 E% TFA); 4) Soybean oil; 5) Semi-liquid marg.; 6) Butter (1 E% TFA)	Plasma		↔		
Baer *et al.* (2004)[15]	50 M (healthy, mean age 42 y, mean BMI 26 kg/m^2)	Cross-over (5wk)	Isoenergetic, 39 E% from fat. 1) Carbohydrate (31E% from fat); 2) OA; 3) SA; 4) TFA (8 E%); 5) 50/50 SA+TFA 6) Medium-chain SFA	Plasma		↑ 21%[2]	↔[3]	↑ 14%[4]
Kuhnt *et al.* (2007)[172]	24 F+M (healthy, mean age 25 y, BMI 20-22 kg/m^2)	Parallel (6wk)	Isoenergetic, 30 E% from fat. 1) TFA (t11/t12-18:1; 3 E%) 2) Palm/rapeseed oil (PUFA)	Plasma	↔		↔[5]	
Motard-Belanger *et al.* (2008)[107]	38 M (healthy, mean age 33 y, mean BMI 24 kg/m^2)	Cross-over (4wk)	Isoenergetic, 37 E% from fat. 1) R-TFA (~4 E%); 2) R-TFA (1.5 E%); 3) IP-TFA (~4 E%); 4) Butter/canola/peanut oil (OA)	na		↔		
Tardy *et al.* (2009)[124]	57 F (healthy, 18-50 y, mean BMI 32.5 kg/m^2, abd. obese)	Parallel (5wk)	Isoenergetic, 42-45 E% from fat. 1) IP-TFA (~2 E%); 2) R-TFA(~2 E%); 3) Palm oil (PA/OA)	Plasma		↔		
Vega-Lopez *et al.* (2009)[174]	30 F (hyperchol., mean age 64 y, mean BMI 26 kg/m^2)	Cross-over (35d)	Isoenergetic, 25-26 E% from fat. 1) Part. hydr. soybean oil (4 E% TFA); 2) Corn oil (PUFA)	Plasma		↔		
Teng *et al.* (2010)[17]	41F+M (healthy, mean age 29 y, mean BMI 22 kg/m^2)	Cross-over (5wk)	Isoenergetic, 32-34 E% from fat. 1) Part. hydr. soybean oil (10 E% TFA); 2) Palm stearin (PA/OA); 3) High OA palm oil (OA/LA/ALA)	Serum	↔	↑ 22%[6]	↔[7]	

↑ and ↓ represent higher and lower concentration of this marker with increasing TFA intake. ↔, no significant effect of TFA. ALA, alpha-linolenic acid; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid; [1]Compared to soybean oil, not different from butter diet; [2] Compared to OA or carbohydrate, not different from SA or medium-chain SFA; [3] Lower after OA compared to all other diets; [4] Compared to OA, 6-9 % higher compared to all other diets; [5] Also no effect on IL1β, IL8, IL10, IL12, leptin or adiponectin; [6] Compared to palm steartin, 25% compared to high OA palm oil. [7] Also no effect on IL1β, but lower IL8 after TFA compared to palm stearin diet.

It is possible that the TNF system is more susceptible to TFA-induced activation in older and overweight subjects. This would explain why we in the COBRA study were able to show a 12% (CI: 5 to 20; P=0.002) higher TNFα concentration in TFA-exposed abdominally obese postmenopausal women compared to controls (**Paper IV**). We substantiated our findings by showing concomitant increases in sTNF-R1 and sTNF-R2 plasma concentrations in the TFA group of 22 ± 7% and 14 ± 3%, respectively [**Figure 5**]. The TNFα concentration was significantly correlated with both sTNF-R1 (r=0.31, P=0.03) and sTNF-R2 (r=0.48; P<0.001) concentrations after the intervention. Also, it is worth noting that we, in accordance with others[175], observed higher TNF receptor concentrations in the overweight women compared to the lean references, supporting the notion that these receptors may be markers of an overall dysmetabolic state and low-grade inflammation.

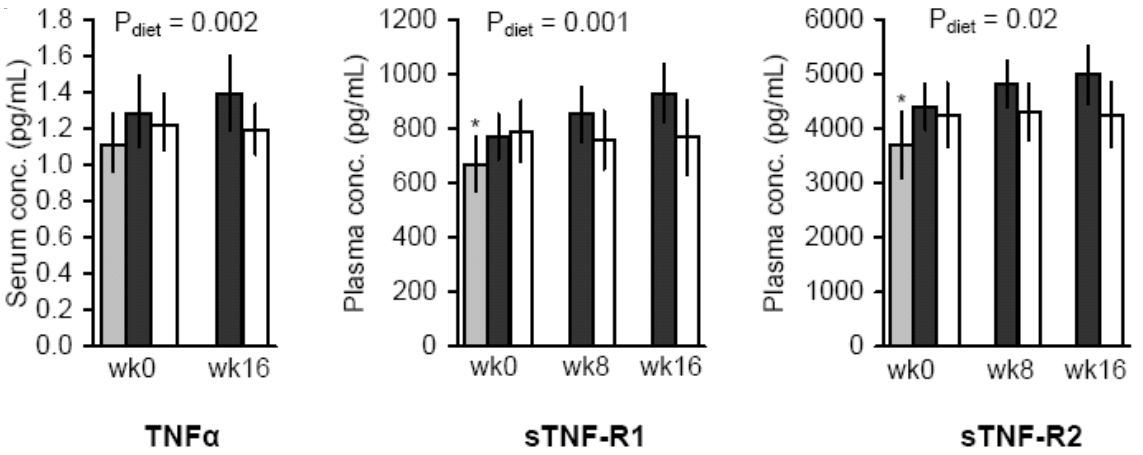

Figure 5: Serum concentration of tumor necrosis factor (TNF)α assessed by ELISA and plasma concentrations of soluble tumor necrosis factor receptors 1 and 2 (sTNF-R1 and sTNF-R2) assessed by Bead array technology before and after 8 and 16 weeks (wk) of supplementation with 15.7 g/d trans fatty acids (dark grey bars; n=24) or a control oil (white bars; n=25), and in lean references (light grey bars; n = 19). From **Paper IV**.

It would be interesting to assess whether subjects with high incorporation of TFA into RBC membranes were more likely to experience a high TNFα response. Unfortunately, due to methodological shortcomings, the RBC FA composition analyses did not allow for exact quantification of RBC TFA content (**Paper II**), rendering such analyses impossible.

Although TFA intake has been associated with IL6 concentrations in cross-sectional analysis[18,139], no RCT has been able to demonstrate an effect of TFA intake on systemic concentrations of IL6[15,17,172]. In the COBRA study, we did not see an effect of the intervention on circulating IL6 either, although IL6 is stimulated by TNFα. This may be due to the fact that the natural variability in circulating levels of IL6 is significantly higher than that of TNFα, and considerably higher than that of TNF receptors[158]. Thereby, TFA-induced changes, if present, may only be detected in stimulated cells[171]. However, *in vitro* results indicate that TFA indeed affects TNFα production more so than that of IL6. Incubation of murine Kupffer cells with *trans*18:1 or *trans*18:2 isomer-containing medium resulted in higher lipopolysaccharide-stimulated TNFα, but not IL6, production compared to incubation with the corresponding *cis* isomers[176].

Previous intervention studies examining the effect of TFA on systemic CRP concentrations are limited and contradictory. In studies providing healthy subjects with 8 or 10 E% TFA, the con-

centration of CRP increased compared to provision of carbohydrate or oleic acid[15] or compared to oleic acid/PUFA or palmitic/oleic acid[17]. In contrast, CRP was unaffected by dietary TFA in doses of 3.6 to 5.2 E%[173, 174, 107] [**Table 3**]. It is possible that very high dietary TFA levels are needed before CRP is affected, or more likely, that most studies have simply been underpowered to detect the small changes induced by TFA. In the two crossover studies showing significant effects of TFA on CRP, the difference between diets was only 0.12 mg/l (n=41)[17] and 0.20 mg/l (n=50)[15], respectively. In the COBRA study, the baseline-adjusted mean CRP concentrations were 1.93 (CI: 1.53 to 2.43) and 1.91 (CI: 1.50 to 2.42), in the TFA and CTR groups, respectively. Consistent with the width of these confidence intervals, we were not able to detect a difference between diet groups of the size previously reported.

Few studies have examined the effect of TFA consumption on adiponectin levels. Consumption of hydrogenated soybean oil (4 E% TFA) resulted in a significant increase of ~2% in adiponectin levels in postmenopausal women compared to corn oil[109]. In contrast, the serum adiponectin concentration was not affected in the COBRA study [mean relative difference between diet groups: 11% (CI: -32 to 15); P= 0.37] (**Paper IV**). The evidence from rodent studies is equally conflicting, with one study showing an adiponectin-increasing effect of TFA compared to MUFA[177], and another finding the exact opposite when TFA was compared to PUFA[178].

TFA intake and mRNA expression of inflammatory markers in adipose tissue

The higher circulating concentrations of TNFα and its soluble receptors after TFA consumption in the COBRA study was not reflected by increased mRNA expression in subcutaneous abdominal AT (SAT) (**Paper IV**). In accordance, no effect of 6 years of TFA-feeding was seen on AT TNFα protein concentration in monkeys[22].

In the COBRA study, neither serum TNFα, IL6 nor adiponectin concentrations were correlated with mRNA expressions in SAT (**Paper IV**). This is no too surprising, given that TNFα and IL6 are also produced by other tissues/cells, e.g. the vascular endothelium and skeletal muscle[83,179], and the fact that transcript and protein turnover is different in various tissues.

The fact that serum TNFα but not AT mRNA was affected by TFA intake in the COBRA study, may also be due to limited incorporation of TFA into AT during the 16 weeks of intervention. The AT content of the two predominant TFA isomers increased two-fold in the TFA group, but still only constituted ~1% of total FA. This low degree of TFA incorporation is not unexpected given the half life of SAT FA of 6-18 months[180-182].

However, as the intra-abdominal AT (IAAT) depot FA turnover is higher than that of the SAT[183] and since meal FA uptake is relatively larger in the former depot[184], more TFA may have been incorporated here. Previously, the TFA concentration was shown to be higher in IAAT compared to SAT[185]. Also, the SAT depot may not adequately reflect an effect of TFA on AT inflammation; in overweight subjects, the mRNA of TNFα was found to be considerable higher in IAAT compared to SAT[186].

In the COBRA study, we did not see a difference in AT mRNA expression of TNFα or adiponectin between the overweight women (BMI ~28 kg/m^2) and lean references (BMI ~21 kg/m^2). Again, this may be due to the sampling of SAT and not IAAT. Previously, higher TNFα and lower adiponectin expression in IAAT was observed in overweight (BMI 28 kg/m^2) compared to lean subjects. In contrast, no difference in adiponectin mRNA was seen in SAT, whereas TNFα mRNA was even higher in lean subjects in this depot[186].

The evidence from rodent studies looking at effects of TFA-feeding on cytokine or adipokine mRNA expression is somewhat conflicting [**Appendix E**]. In rats fed TFA-diets until 120 days-of-age, the mRNA expression of TNFα was higher and the expression of adiponectin lower in retroperitoneal AT compared to PUFA-fed rats[178]. In another study in rats, AT mRNA expression of adiponectin was decreased by SFA, but not TFA[187]. Also, TFA-feeding of rats and mice did not affect hepatic expression of TNFα and IL6[176,188], but expression of IL-1β was higher than in mice fed SFA[188].

MARKERS OF ENDOTHELIAL DYSFUNCTION

Disturbance of the normal function of the vessel endothelium, so-called endothelial dysfunction, triggers the initiation of atherogenesis, as described in the beginning of this chapter. Endothelial dysfunction is characterized by an activation of endothelial cells leading to increased adhesion of leucocytes to the activated endothelium, but also by a disturbed regulation of vessel tone and blood pressure due to a disturbed balance in the release of relaxing and constricting factors[189].

Despite clear roles for adhesion molecules in the pathogenesis of atherosclerosis, the association between plasma levels of these molecules and cardiovascular risk is less apparent. Whereas levels of soluble ICAM-1 (sICAM-1) have been associated with risk of cardiovascular disease in large observational studies[190-193], the findings for soluble VCAM-1 (sVCAM-1), and soluble E-Selectin (sE-selectin)[190,191] are inconsistent. It has been proposed that sICAM-1 is the most powerful predictor of events in healthy populations, whereas sVCAM-1 may be a prognostic marker in patients with established atherosclerotic disease[194]. One study suggested that the combination of high levels of both sICAM-1 and sVCAM-1 might be associated with the development of CHD, independent of other CHD risk factors[195].

TFA intake and endothelial dysfunction

In a cross-sectional analysis in overweight women, concentrations of sE-selectin, sICAM-1 and sVCAM-1 were positively associated with TFA intake[18], while only sICAM-1 was associated with intake of hydrogenated vegetable oil in another study[139] [**Tabel 2**]. Again, the supporting evidence from randomized studies is limited. An increase in E-selectin after 5 weeks of 8 E% TFA consumption (compared to carbohydrate, stearic acid, oleic acid and a mix of lauric, myristitic and palmitic acid) has been reported in a crossover study in 50 men[15]. In the COBRA study, we confirmed these findings by showing increases in serum sE-selectin of 19 ± 4% in the TFA group (P= 0.004 for within-group change). The effect of diet reached significance when sE-selectin was measured in plasma [mean difference 10% (CI: 1 to 21); P=0.04], but not in serum [8% (CI: -2 to 19); P=0.13]. In contrast, there was no effect of diet on sICAM-1 and sVCAM-1 (**Paper IV**). Of note, only sE-selectin, not sICAM-1 and sVCAM-1, was lower in overweight compared to lean subjects, indicating that the former may be a better marker of obesity-related endothelial disturbances.

Given the questionable validity of these systemic markers of endothelial dysfunction in predicting disease risk in healthy subjects, more direct measures of endothelial dysfunction, such as flow-mediated vasodilation (FMD), may convey more useful information. In 29 lean young subjects, high TFA consumption (9 E% or 23 g/d) for 3-4 weeks was shown to negatively affect FMD of the brachial artery compared to a diet high in lauric, myristitic and palmitic acid (FMD: 4.4 ± 2.3% after TFA compared to 6.2 ± 3.0% after SFA; P=0.02)[196]. However, this finding could not be confirmed in a study in 75 lean and young men consuming TFA (6 E% or 20 g/d),

palmitic acid or fish oil (12 g/d) for 8 weeks[111]. The power of the latter study was lower, due to a parallel study design, but no trends in either direction were observed.

A few *in vitro* experiments support the notion that TFA may more potently induce endothelial dysfunction than the corresponding *cis* FA. Human aortic endothelial cells exposed to *trans*18:2 significantly increased the expression of endothelial adhesion molecules, including ICAM-1, compared to *cis*18:2[197]. Moreover, the *trans*18:2 isomer increased the release of MCP-1 and resulted in increased adhesion of monocytes to cell monolayers[198]. In another study, elaidic acid was shown to maintain the TNFα-stimulated level of ICAM-1 and VCAM-1 in human bone marrow endothelial cells, whereas oleic acid suppressed the expression[199]. Finally, incorporation of TFA into endothelial cell membrane phospholipids has also been shown to suppress the secretion of prostacyclin, which is a potent vasodilator and inhibitor of platelet aggregation and leukocyte adhesion[200].

MARKERS OF OXIDATIVE STRESS

Oxidative stress occurs when the body experiences potentially deleterious distress due to formation of reactive radical species in levels that exceed those that can be controlled by the body's antioxidant defense systems. Lipid peroxidation can occur via enzymatic or non-enzymatic pathways. Arachidonic acid (20:4n-6) can be enzymatically oxidized via cyclooxygenase and lipooxygenase mediated pathways to form bioactive leukotrienes, prostaglandins and lipoxins, which are highly involved in inflammatory pathways[201]. Non-enzymatic oxidation is induced by free radicals, and the ensuing oxidized lipids, so-called isoprostanes, can modify the cell membrane fluidity and induce inflammation in neighboring cells[202]. Urinary 8-iso-prostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), which is the product of free radical catalyzed peroxidation of arachidonic acid, is a validated marker of non-enzymatic lipid peroxidation and overall *in vivo* oxidative stress[203]. Elevated levels are found in many conditions, such as chronic inflammation, diabetes and atherosclerosis (reviewed by Basu *et al.*[202]).

TFA intake and markers of oxidative stress

Intake of vaccenic acid or a mixture of vaccenic acid and *trans*18:1n-6 has been shown to increase the urinary excretion of the isoprostane 8-iso-$PGF_{2\alpha}$[71,138], although this was not found after intake of vaccenic acid-rich butter[204]. However, a mixture of TFA isomers from partially hydrogenated vegetable oils, as provided to the subjects in the COBRA study, had no detectable effect on the urinary concentration of 8-iso-$PGF_{2\alpha}$ (**Paper IV**). Whether consumption of vaccenic acid *per se* may induce oxidative stress or whether the observed effect in prior studies should rather be ascribed to the endogenous conversion of vaccenic acid to CLA[71], is not known. Supplementation with CLA has consistently been shown to cause elevated urinary isoprostane excretion[205-207].

Yet, there is some evidence suggesting that TFA, as such, may increase oxidative stress. TFA intake was positively correlated with urinary 8-iso-$PGF_{2\alpha}$ in a 5-year follow-up of 1,610 women[140], and increased plasma levels of F_2-isoprostanes were found after hydrogenated vegetable oil-feeding in mice[208]. Also, human aortic endothelial cells incubated with *trans*18.2 showed signs of oxidative stress as indicated by increased nitrosylation of proteins[197].

It has been proposed that induction of oxidative stress may be an intermediary step by which dietary FA may adversely affect insulin sensitivity. A negative effect of CLA supplements on insulin sensitivity was abolished when adjusting for concomitant increases in urinary excretion

of isoprostanes[205]. In the COBRA study, the changes in urinary isoprostane excretion did not correlate with changes in insulin sensitivity, as assessed by oral glucose tolerance tests (**Paper III**).

MECHANISMS BY WHICH TRANS FATTY ACIDS MAY AFFECT INFLAMMATORY PROCESSES

The molecular mechanisms implicated in the apparent pro-inflammatory effect of TFA remain speculative. First, TFA incorporation into phospholipids may affect membrane properties[209], and thereby the function of membrane-bound receptors that mediate the transduction of inflammatory stimuli, such as TNF-receptors and TLR[210]. Second, TFA may interfere with the metabolism of essential FAs and thereby the production of various pro- or anti-inflammatory lipid mediators[201]. Finally, TFA may stimulate the expression of pro-inflammatory cytokines by activating TLR signaling, as seen for SFA[211,212]. This will be covered in more detail in **Chapter 9**.

SUMMARY: DIETARY TRANS FAT & SYSTEMIC INFLAMMATION, ENDOTHELIAL DYSFUNCTION AND OXIDATIVE STRESS

The evidence for an effect of TFA consumption on low-grade systemic inflammation, endothelial dysfunction and oxidative stress must be considered limited. Our findings of an increased production of TNFα and its soluble receptors after TFA intake do, however, lend convincing support to the notion that dietary TFA promote low-grade inflammation. If TFA consumption affects CRP production, the effect appears to be modest, which may explain why we failed to detect it in the COBRA study. Reports on the effect of TFA intake on IL6 or adiponectin production are few and inconsistent. Our research showed that TFA does not affect the mRNA expression of TNFα, IL6 and adiponectin in subcutaneous adipose tissue within 16 weeks. Thereby, it remains to be answered which tissue(s) may be responsible for the increase in systemic TNFα.

Furthermore, the evidence for a TFA-induced adverse effect on oxidative stress and endothelial dysfunction is far from convincing. Results from our study tended to confirm prior reports of a sE-selectin-increasing effect of TFA, indicating that TFA may indeed promote endothelial dysfunction. However, only one study has provided evidence for a negative effect of TFA on the more direct measure of endothelial function, flow-mediated vasodilation; a finding that could not be confirmed by others.

7 DIETARY TRANS FAT & FAT DEPOSITION

Increased BMI and especially increased abdominal fat deposition, as reflected by increased waist circumference, are positively associated with risk of T2D[213] and CHD[113,214]. In women, a waist circumference of ≥76.2 cm was associated with a more than 2-fold higher risk of CHD, after adjustment for other established risk factors[214].

A few years ago, a 15 year prospective analysis in 3,031 young adults showed that fast food consumption was strongly associated to weight gain[215]. Frequent fast food consumption might simply reflect a generally unhealthy lifestyle, or fast food may cause obesity due to high energy density and large serving sizes. But it has also been suggested, that the high content of TFA in fast food contributes to this obesogenic effect.

TRANS FAT AND OBESITY – EVIDENCE FROM OBSERVATIONAL STUDIES

Observational studies have indicated that TFA intake is positively associated with obesity indices, such as weight, waist circumference, skinfold thickness and percent body fat [8,9,216,217], although this is not a consistent finding[185,218].

In cross-sectional baseline analyses from the *Nurses' Health study* (n=31,940), the strongest positive association between intake of a single nutrient and BMI (adjusting for age and energy intake) was found for TFA[219]. When using prospective data from the same cohort (n=41,518), Field *et al*.[8] showed that change in TFA intake, was stronger associated to weight gain than change in any of the other FA classes. Moreover, the association was particularly strong among overweight women; for every 1% increase in E% from TFA, they gained an additional 1.0 kg (CI: 0.8 to 1.3) during 8 years of follow-up. Data from the *Health Professional's Follow-up Study* (n=16,587) indicated that TFA intake may specifically increase abdominal obesity. A 2% increment in energy intake from TFA, isocalorically substituted for PUFA or carbohydrates, was associated with a 0.77 cm waist gain in multivariate analyses during 9 years of follow-up. This association was only modestly reduced after adjustment for BMI. Furthermore, in an analysis adjusting for measurement error, it was calculated that isocaloric replacement of 2 E% of TFA with PUFA would result in a 2.7 cm decrease in waist circumference over 9 years ($P < 0.001$)[9].

In contrast, observational studies with much smaller study populations have not been able to confirm these findings. In a case-control study of 34 obese Spanish children and 20 lean controls, neither TFA intake nor plasma TFA concentration differed between obese and lean children[220]. Likewise, in a cross-sectional study among 617 Canadians, intake of TFA was not associated with waist-to-hip ratio[218], yet TFA intake was quite low in both populations (<1 g/d). Also, in Brazilian subjects there was no difference in AT TFA concentration between non-obese (BMI<30 kg/m^2) and obese (BMI>40 kg/m^2) subjects, despite very high AT TFA levels (6-9% of FA)[185]. A recent cross-sectional study in 1,785 subjects suggested that individual TFA isomers may have divergent effects on adiposity. Whereas the concentration of *trans*18:2 in AT

was positively associated with BMI, waist circumference and skinfold thickness, inverse associations were seen for *trans*18:1 in multivariate analyses[217].

Two studies in American women have suggested that high TFA intakes may be associated with increased maternal postpartum weight or body fat percentage[216,221]. In 902 women, the odds ratio of retaining at least 5 kg at 1 year postpartum was 1.32 (CI: 1.08 to 1.62) per 0.5 E% TFA increment in an analysis adjusting for pre-pregnancy BMI, gestational weight gain and a range of other putatively explanatory variables[221]. Likewise, in 96 mothers, the odds of having >30% body fat at three months postpartum was 5.81 (CI: 1.05 to 32.32) in women consuming >4.5 g/d TFA compared to those consuming less in multivariate analysis. Maternal TFA intake also tended to predict 3-month-old infant adiposity, but not after adjusting for possible confounders[216].

TRANS FAT AND BODY FAT DEPOSITION

A few years ago, a long-term TFA-feeding study in non-human primates received considerable attention as it was the first dietary intervention to document a fattening effect of TFA intake in a non-rodent animal model. Despite the fact that energy supply was targeted at body weight maintenance (and based on baseline body weight), African green monkeys fed a diet enriched with 8 E% TFA for 6 years (equivalent to ~15 years in humans) gained significantly more body weight (7.2 ± 2.7% increase) than a control group (1.8 ± 2.0% increase) whose diet was rich in *cis*-MUFA (P=0.05). Moreover, monkeys fed TFA had deposited more fat in both the subcutaneous and the intra-abdominal regions (non-significant (ns)) and had a significantly higher ratio of intra-abdominal AT (IAAT) to subcutaneous abdominal AT (SAT) after adjusting for body weight (P=0.02)[22].

With the COBRA study we are the first to examine the effect of TFA consumption on fat deposition in a human intervention. We observed weak trends towards greater increases in waist circumference and total fat mass (assessed by DEXA) in the TFA group compared to controls independent of body weight changes. Waist circumference tended to increase more in the TFA group [baseline-adjusted mean difference between diet groups: 1.1 cm (CI: -0.1 to 2.4); P=0.08] and so did body fat: The baseline-adjusted change in body fat mass was 1.2 kg (CI: 0.7 to 1.8) in the TFA group and 0.8 kg (CI: 0.3 to 1.3) in the CTR group with an estimated 0.5 kg (CI: -0.1 to 1.2; P=0.16) difference between diet groups at the end of the intervention (P=0.12 in an analysis adjusting for weight change).

However, these trends were not reflected in the observed changes in abdominal fat deposition (assessed by single-slice magnetic resonance imaging (MRI)). After the intervention, the total abdominal AT volume was an estimated 2 cm^3 lower (CI: -25 to 21; P=0.85) in the TFA group compared to controls; the SAT volume was 3 cm^3 lower (CI: -15 to 9; P=0.58), and the IAAT volume was 7 cm^3 higher (CI: -12 to 26; P=0.46), with no effect of adjusting for weight change [**Figure 6**]. The width of these confidence intervals suggests that we would have been able to detect differences between diets corresponding to ~5% of the baseline AT, 7% of the SAT and 9% of the IAAT volume. The observed within-group changes were of somewhat smaller magnitude; the increase in total AT was 2.9 ± 1.9% in the TFA group and 4.4 ± 1.9% in the CTR group, with corresponding increases in IAAT of 2.1 ± 2.4% and 3.2 ± 2.2%, and in SAT of 4.9 ± 2.8% and 5.9 ± 4.6%, in the two groups respectively.

Thereby, our data did not show any trend towards confirming the effect of TFA on the ratio of IAAT to SAT, as seen in monkeys[22]. The difference between diet groups in this ratio was 0.01 (CI: -0.06 to 0.07; P=0.87) in our study (**Paper II**). However, others have observed considerable changes in abdominal AT as a consequence of altering the dietary fat composition. After consumption of a diet high in PUFA for 5 weeks, the SAT volume (assessed by single-slice MRI) was ~30% lower compared to after a diet high in SFA, despite no differences in body weight in 17 men and women[222].

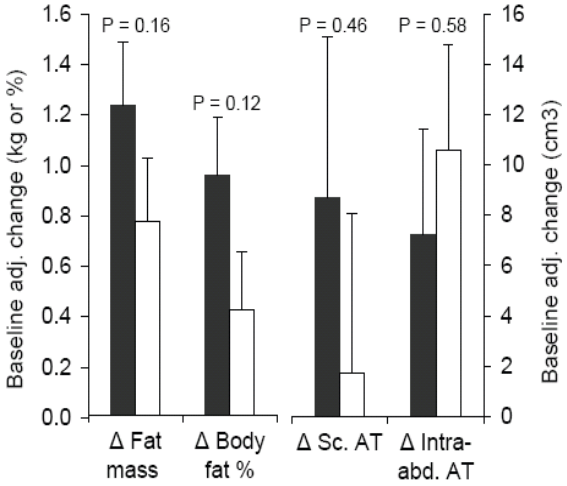

Figure 6: Changes in whole-body fat mass and percentage body fat assessed by DEXA and changes in subcutaneous (sc.) and intra-abdominal (abd.) adipose tissue (AT) during 16 weeks in the TFA group (black bars) and in the CTR group (white bars). Bars represent baseline-adjusted means and SEM. P-values were obtained by baseline-adjusted ANCOVA.

It cannot be discounted that our lack of control of the diets in the COBRA study may have limited the strength of the study to detect TFA-related changes in fat deposition. Our subjects were given two bread rolls per day, containing a total of 26 g of fat (TFA or CTR) and 2500 kJ, which they were instructed how to substitute isocalorically for food items from their habitual diets. The rest of their diets was not controlled. However, results of 3-day food records from the week before the last MRI indicated no significant differences between diet groups in intake of either energy or any of the macronutrients. The only differences were a higher TFA intake in the TFA group and a higher MUFA intake in the controls (**Paper II**). Yet, minor differences may not have been picked up by this dietary assessment.

It may also be considered a limitation of the COBRA study that AT volumes were assessed by single trans-axial images (single-slice MRI), and not by examination of the entire abdominal region. We obtained images at the level of the third lumbar vertebrae, which has been shown to be the single slice volume (or area) to correlate best with the IAAT of the whole abdominal region in women (R^2 = 0.94-0.97)[16,223,224]. For SAT, the correlation between single- and multiple-slice MRI is high regardless of the position of the single-slice measurement[224]. However, single-slice methods may be less likely to detect small changes in abdominal adiposity. One study estimated that ~6% more women would be needed in a trial using single-slice as compared to multiple-slice measurements to reach the same power[223] and another showed that the variability in exercise-induced changes in IAAT was somewhat larger when measured with the former compared to the latter method[225].

Apart from methodological limitations, the fact that we did not see an effect of the TFA intervention on abdominal fat deposition may be due to the relatively short intervention period. It is likely that 16 weeks of exposure was too short a period of time to detect changes in abdominal fat independent of body weight even with high daily TFA intakes. Indeed, body weight gain may be an intermediary step leading to TFA induced increases in abdominal fat deposition, which we may not have been able to detect with our isocaloric study design. In monkeys fed a weight-maintenance diet with 8 E% TFA, the abdominal fat deposition was 31% greater than in monkeys fed *cis*MUFA and the weight gain was ~5.4% after 6 years (when subtracting the

weight gain in the control group)[22]. This would correspond to a weight gain of only 0.24 kg in an 80 kg woman during 16 weeks. Moreover, the difference in weight between diet groups in the study in monkeys did not appear to emerge before after one year[22].

Yet, as mentioned above, 5 weeks of intervention was long enough to show significant diet-induced changes in abdominal AT deposition in 17 subjects[222], and CLA supplementation of 4.5 g/d has been shown to significantly decrease fat mass (assessed by DEXA) in postmenopausal women within 16 weeks on iso-caloric diets[226]. This confirms that abdominal fat distribution can be highly affected by dietary fat manipulations within the timeframe of our study.

TFA and fat deposition in rodent models

Due to the limited data available from human studies on the effect of TFA on adiposity, data from rodent models may shed some more light on this subject. However, results from studies in rabbits, rats and mice are highly conflicting [**Appendix E**] and may not predict effects in humans. Most rodent studies examining the metabolic effect of a high TFA intake as part of high-fat diets have found no effect on body weight compared to stearic acid, SFA, MUFA or PUFA[177,227-231] or even lower body weight compared to a mixture of other FAs[188], oleic acid[232] or SFA [233]. Whereas, the lower body weight after TFA-feeding was followed by lower adiposity in some studies[232-234], the opposite was seen in one study where epididymal AT weight (mimicking IAAT in humans) was 86% higher in TFA-fed rats compared to rats fed a MUFA/PUFA mix[227].

It appears that the fat load may modify the effect of TFA feeding on adiposity in rodents. In rats fed TFA-enriched diets with moderate fat content (~10-16 E%), body weight, fat mass, and liver fat were significantly elevated compared to rats fed SFA, MUFA or PUFA, but not palmitic acid [235,236]. In one study, abdominal fat was preferentially deposited intra-abdominally after consumption of a low-fat TFA-enriched diet, and liver fat accumulation was comparable to that in rats fed high-fat SFA diets[235]. An increased accumulation of triglyceride in the liver after TFA feeding, compared to SFA, MUFA or PUFA, has been observed in several other rodent studies, despite no effect on body weight[176,227-229,231,234].

TRANS FAT AND LIVER FAT DEPOSITION

In humans, liver fat accumulation is considered the hepatic manifestation of metabolic dysfunction[237,238] and is associated with intra-abdominal fat deposition[239] and insulin resistance[240-242]. Liver fat may be a more important risk factor for obesity-related metabolic disorders than IAAT[241,243]. Based on data from healthy normal weight (BMI<25 kg/m2) subjects in the *Dallas Heart Study*, an upper limit of normal liver fat content of 5.5% was established. Using this cut-off, the prevalence of hepatic steatosis (also called non-alcoholic fatty liver disease (NAFLD)) in the general population (of whom 76% had a BMI >25 kg/m^2 and 43% had a BMI >30 kg/m^2) was estimated to be as high as 33.6%[244]. NAFLD is linked to an increased risk of cardiovascular disease in otherwise healthy subjects, independently of underlying cardiometabolic risk factors[245].

Few studies in humans have investigated the impact of dietary fat quality on liver fat deposition. Intake of SFA has been associated with increased liver fat[242], and long-chain n-3 PUFA supplements have been shown to reduce liver fat deposition[246,247].

With the COBRA study, we were the first to examine the effect of a high TFA intake on liver fat deposition in humans and we did not see a significant effect. After the intervention, the baseline-adjusted liver fat content was 7% lower (CI: -61 to 120; P=0.87) in the TFA group compared to controls (**Paper II**). Given the geometric mean liver fat concentration of approximately 2.5% at baseline, these confidence limits may translate into an absolute mean decrease in liver fat of 1.5% or an absolute increase of 3.5%. Therefore, our results cannot discount the possibility that TFA intake may exert effects on liver fat smaller than this, prohibiting firm conclusions on minor changes. However, no detectable trend in liver fat was observed in either direction in either diet group and plasma liver enzymes remained unchanged, arguing that if TFA induced changes in liver fat concentration, they were small indeed.

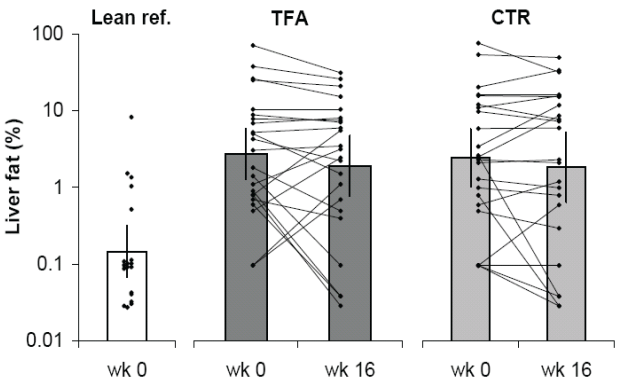

Figure 7: Liver fat % in the trans fat group (TFA; n=23) and in the control group (CTR; n=23) before and after 16 wk of dietary intervention and in lean references (n=19) obtained by ^{1}H MR spectroscopy. Bars represent geometric means (95% confidence intervals). P=0.87 for baseline-adjusted differences between diet groups at wk 16, by ANCOVA. Liver fat % was significantly lower in lean references than in overweight subjects (diet groups combined); P<0.01. From **Paper II**.

We measured liver fat by proton magnetic resonance spectroscopy (^{1}H-MRS), which is the most accurate non-invasive technique available[248]. However, as seen in **Figure 7**, the between-subject variability in baseline liver fat was large (range 0.1 to 73.0%), which also has been found in other populations of overweight subjects [241,249]. It is questionable if subjects with very high liver fat content will be susceptible to further increases due to dietary manipulations. In retrospect, a screening for liver fat content should have been considered, since a modest diet-induced change may be more easily detected in a study population with more homogeneous liver fat levels.

Also, it may be a limitation of the COBRA study that we only measured liver fat in a single volume of interest (VOI). A voxel size of 11x11x11 mm was found to be optimal in the COBRA study as the scanner was sufficiently sensitive to obtain a good signal from this volume. It was anticipated that a larger voxel size (as the 20 or 30 mm used by others[244,249]) would cause uncertainty in water/fat ratio due to presence of large blood vessels in the VOI. However, a single small VOI may not adequately reflect the average change in liver fat. Differences in fat content of up to 50% between different regions of the liver have been observed in some subjects[249], but modest within-liver coefficients of variation (CV) of 11.0-14.5% have also been reported[244,248]. Moreover, reproducibility of single-voxel measurements is acceptable, with CVs of 4.5-8.5% for measurements performed within the same day[244,248,249] and of 9.5% for measurements performed weeks apart without standardization of diet or exercise[248].

In the COBRA study we aimed to standardize the liver fat assessments by controlling alcohol intake, physical activity and diet from the evening before the examination. However, the fact that the diets were not controlled for longer periods before the scans may have limited our ability to detect small TFA-induced-changes in liver fat. Recently, it was found that the total dietary fat intake during the prior ten days, and not that of the previous day, is correlated to liver fat content[240]. It has been documented that liver fat deposition in humans can increase as much as 100% (from 2 to 4% and from 11 to 22%) after 3-4 days on hyper-caloric high-fat diets[250,251].

Also, an increase in liver fat of 35% (from 10 to 14%) was seen after 14 days on an eu-caloric high-fat diet (56 E% from fat)[252]. The mean fat intake was 34-37 E% in the COBRA study, assessed by 3-day food records (**Paper II**). It can be speculated that high-fat diets would have been required for us to show effects of TFA on liver fat deposition, as indicated by some[176], but not all[229,235], rodent studies.

MECHANISMS BY WHICH TRANS FATTY ACIDS MAY AFFECT BODY FAT DEPOSITION

There is very limited data from studies in humans to elucidate how TFA may affect body weight and fat deposition. Neither effects on appetite nor energy expenditure could be detected in an acute study comparing meals enriched in MUFA, PUFA or TFA (57 E%) in overweight human subjects[253]. The effect of long-term TFA exposure on appetite and energy expenditure has not been assessed. A few studies have suggested that TFA may be more prone to oxidation than to storage compared to other FA, and thereby less likely to lead to obesity. Fat oxidation was higher after a 4-week diet enriched with 9 E% TFA (31.4 ± 1.5 g/d) compared to MUFA (26.0 ± 1.5 g/d)[100], and a meal study showed that elaidic acid is oxidized to a greater degree than oleic, palmitic, stearic and linoleic acid for 9 hours postprandially[254]. In contrast, no difference in the total fat oxidation was observed for 8 hours after high-fat meals enriched with 10 E% TFA or *cis*-MUFA in an acute meal study[21].

Generally, if in fact TFA has a long-term impact on body adiposity in humans, this effect may be mediated through modulation of the expression of genes involved in the regulation of fat metabolism in a number of tissues. Given the seemingly reducing effect of n-3 long-chain PUFA on adiposity and liver fat deposition[246,247,255], one may hypothesize that the effect of TFA on expression of some of these genes, opposes that observed for n-3 long-chain PUFA. For example, TFA-feeding has been shown to stimulate the hepatic mRNA expression of steroyl regulatory element binding protein (SREBP)-1 in mice[256], which may result in increased transcription of lipogenic genes as well as increased cholesterol synthesis in the liver. In contrast, PUFA have been shown to suppress SREBP-1[257].

The etiology of fatty liver disease is complex and not fully understood. Liver fat accumulation results from an imbalance between lipid availability (from circulating lipid uptake or *de novo* lipogenesis) and lipid disposal (via β-oxidation of fatty acids or VLDL secretion)[258], and both sides of the equation may be affected by dietary fat intake. A study in rats found a decreased hepatic expression of microsomal triglyceride transfer protein (MTP), suggesting a diminished transfer of triglycerides to nascent ApoB particles and thereby impaired VLDL assembly and secretion[234]. However, no effect on hepatic MTP expression was found in mice[176].

These issues will be further discussed in **Chapter 9**.

SUMMARY: DIETARY TRANS FAT & FAT DEPOSITION

In summary, there is limited data available to assess whether TFA consumption is associated to increased risk of obesity. However, if in fact TFA intake increases the risk of abdominal obesity or general weight gain, as suggested by a few large prospective cohort studies, this is likely to occur gradually over a long time-span, as also seen in monkeys. This field of research in strongly hampered by the lack of RCTs, possibly due to the difficulties in conducting intervention studies with sufficiently long-term TFA exposure. Even very high TFA consumption for 4

months does not appear to affect the abdominal fat deposition in postmenopausal women. However, our research cannot exclude that whole-body fat accumulation may be slightly stimulated.

Whereas the evidence for an effect of TFA on body adiposity in rodents is highly conflicting, an increased accumulation of fat in the liver has been an almost consistent finding. To what extent these findings can be translated into humans, remains to be answered. Our attempt to verify this effect in humans failed. This may be due to i) a high degree of within-subject variability in liver fat content in our study population, ii) a lack of standardization of the study diets, or iii) a true lack of an effect of TFA exposure on liver fat deposition in humans.

8 DIETARY TRANS FAT & INSULIN SENSITIVITY

Impaired insulin sensitivity is the underlying abnormality in most people who develop T2D. As a compensation for insulin resistance (defined as a smaller than expected biological response to a given dose of insulin), the pancreatic β-cells increase the secretion of insulin to maintain blood glucose homeostasis. If the compensatory secretion is insufficient, blood glucose increases in the fasting and/or postprandial state[259]. Insulin resistance in AT, and thereby a diminished ability of insulin to suppress lipolysis, results in increased plasma FFA concentrations, which in the long term cause insulin resistance in muscle and liver and impair insulin secretion. An elevated FFA concentration is also thought to be a causal factor in excess hepatic and muscle lipid accumulation[258], which again is positively associated with hepatic[237] and peripheral[260] insulin resistance.

Thereby, increased levels of insulin, glucose and FFA both in the fasted state and after glucose challenges, as well as indices of insulin-mediated glucose disposal, insulin secretion and tissue insulin resistance, measured by more invasive techniques (e.g. clamps), may be used as early markers of an increased risk of developing T2D[259].

TRANS FAT AND INDICES OF INSULIN RESISTANCE – INTERVENTION STUDIES

Very few controlled intervention studies have examined if the positive association between intake of TFA and risk of T2D observed in the *Nurses' Health Study*[7] may be explained by adverse effects on measures of glucose homeostasis and insulin sensitivity. Moreover, the results are inconclusive [**Table 4**]. In lean and healthy young subjects, a high intake of industrial TFA (5-9 E%) during 4 weeks does not seem to impair insulin and glucose metabolism measured by the intravenous glucose tolerance test (IVGTT)[100,261] and TFA of ruminant or industrial origin does not affect fasting insulin or glucose concentrations[173,204]. Recently, it was shown that ~2 E% of TFA (ruminant as well as industrial) during 5 weeks did not affect peripheral insulin sensitivity, assessed by hyperinsulinemic euglycemic clamps, in moderately overweight women either[124].

In contrast, in overweight subjects and type 2 diabetic patients, meals with high TFA contents have been shown to produce higher postprandial insulin and C-peptide concentrations than similar meals with *cis* FAs[20,21]. Christiansen *et al*.[20] showed a 59% increase in postprandial insulin area under the curve (AUC) (240 min) and a 42% increase in C-peptide after consumption of a diet with 20 E% TFA for 6 weeks compared to MUFA. The authors hypothesized that since the intervention had no effect on postprandial glycemia, the insulinemia most likely reflected an increased peripheral insulin resistance and not an impairment of β-cell function. However, the design of the study did not allow for interpretation of whether the changes in postprandial insulinemia were due to an acute meal effect of TFA as found by others[21], or an effect of an increased TFA intake for the prior 6 weeks. In any case, it is somewhat surprising that no effect of the quite substantial dietary intervention was seen on blood lipid levels in this study[20].

Table 4: Intervention studies examining the effect of *trans* fatty acid (TFA) intake on insulin resistance or related outcomes

Reference	Subjects	Design	Diets	Method	Effect of TFA on insulin resistance and other outcomes	
Louheranta *et al*. (1999)[261]	14 F; healthy; mean age 23 y; mean BMI 20.6 kg/m^2	Crossover (4 wk)	Isoenergetic, 36 E% from fat. 1) Marg. w. TFA (5 E%); 2) Olive oil (OA)	IVGTT	↔	Fasting insulin ↑ ~9% after TFA (P=0.09); Fasting glucose ↔; Insulin sensitivity ↔; Glucose effectiveness ↔; Acute insulin response ↔; total to HDL-cholesterol ↑ after TFA.
Lovejoy *et al*. (2002)[100]	25 M+F; healthy; mean age 28 y,; mean BMI 23.5 kg/m^2	Crossover (4 wk)	Isoenergetic, 28 E% from fat. 1) TFA (~9 E%); 2) OA; 3) PA	IVGTT	↔	Fasting insulin and glucose ↔; Insulin sensitivity ↔; Glucose effectiveness ↔; Acute insulin response ↔; Disposition index ↔; total-, LDL- and HDL-cholesterol ↔ vs. OA; Insulin sensitivity ↓ 11% (ns) for n=6 with BMI>25 after TFA vs. OA.
Tardy *et al*. (2009)[124]	57 F; healthy, 18-50 y; mean BMI 32.5 kg/m^2;	Parallel (5 wk)	Isoenergetic, 42-45 E% from fat. 1) IP-TFA (~2 E%); 2) R-TFA(~2 E%); 3) Palm oil (PA/OA)	Hyperinsulemic euglyc. clamp	↔	Fasting insulin, glucose and HOMA ↔; Steady state glucose infusion rate ↔; Insulin sensitivity ↔; HDL-C ↓ on all diets.
Christiansen *et al*. (1997)[20]	25 M+ F; T2D; mean age 55 y; mean BMI 33.5 kg/m^2	Crossover (6 wk)	Isoenergetic, 30 E% from fat. 1) TFA (20 E%); 2) MUFA; 3) SFA	4h meal test	↑	4h insulin AUC ↑ 59% and C-peptide AUC ↑ 27% after TFA meal/diet compared to MUFA but not SFA; 4h glucose AUC ↔ : fasting insulin and C-peptide ↔; LDL-, HDL- and LDL/HDL- C ↔.
Lefevre *et al*. (2005) [21]	22 M + F; healthy; mean age 40y; mean BMI 25.7 kg/m^2	Crossover (meal)	Breakfast meal with 40% of daily energy, 50 E% from fat. 1) TFA (10E%); 2) MUFA	8h meal test	↑	Insulin * glucose at each time point and 8h AUC ↑ and 8-h insulin AUC ↑ after TFA compared to MUFA; 8-h glucose AUC ↔
Lichtenstein *et al*. (2003)[173]	36 F+M; hyperchol.; mean age 63 y; mean BMI 27 kg/m^2	Crossover (35d)	Isoenergetic, 30 E% from fat. 1) Soybean oil; 2) Semi-liquid marg.; 3) Soft marg. (3 E% TFA); 4) Shortening (4 E% TFA); 5) Stick marg. (7 E% TFA); 6) Butter (1 E% TFA)	fasting	↔	Minor diff. between diets in glucose, insulin and HOMA and neither increased with increasing TFA
Tholstrup *et al*. (2006)[204]	42 M; healthy, mean age 25 y; mean BMI 23 kg/m^2	Parallel (5 wk)	Isoenergetic, 42-44 E% from fat. 1) Butter enriched in R-TFA (5 g/d) 2) Butter without TFA	fasting	↔	Fasting insulin and glucose ↔.
Vega-Lopez *et al*. (2006)[109]	15 F+ M; hyperchol.; mean age 64y; mean BMI 26 kg/m^2	Crossover (5 wk)	Isoenergetic, 28-30 E% from fat. 1) Part. hydr. soybean oil (TFA, 4 E%); 2) Soybean oil (LA/ALA); 3) Palm oil (PA/OA)	fasting	↑	HOMA and insulin, but not glucose, ↑ 20-28% after TFA compared to soybean, but not palm oil; total- and LDL-cholesterol ↑ compared to soybean, but not palm oil, total/HDL- cholesterol ↔.

↑ and ↓ represent higher and lower level of insulin resistance (IR)-related outcomes with increasing TFA intake. ↔, no significant effect of TFA. ALA, alpha-linolenic acid; IP-TFA, industrially produced TFA; IVGTT, intravenous glucose tolerance test; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; R-TFA, ruminant TFA; SA, stearic acid.

It appears that the effect of TFA may be modified by an underlying predisposition to insulin resistance. In support of this notion, intake of partially hydrogenated soybean oil (4 E% TFA) for 5 weeks resulted in increased fasting insulin and HOMA-IR compared to soybean or canola oil in overweight, hypercholesterolemic and older women (mean age 64 y)[109]. Also, in the study by Lovejoy *et al*.[100] a decrease in insulin sensitivity (IVGTT) of 11% (ns) after TFA consumption compared to MUFA was seen in a sub-group of overweight women (n=6) only.

These results led us to hypothesize that the failure of previous studies in showing an effect of TFA on insulin sensitivity was either due to i) too short study durations not allowing time for TFA to be incorporated into myocellular membrane lipids with possible influence on insulin sensitivity[262], ii) too low TFA intake levels, and/or iii) too young and healthy study populations.

However, in the COBRA study we failed to detect an effect of the 7 E% TFA intervention on insulin sensitivity, β-cell effectiveness and markers of lipolysis (all assessed by oral glucose tolerance test) in abdominally obese, postmenopausal women (**Paper III**).

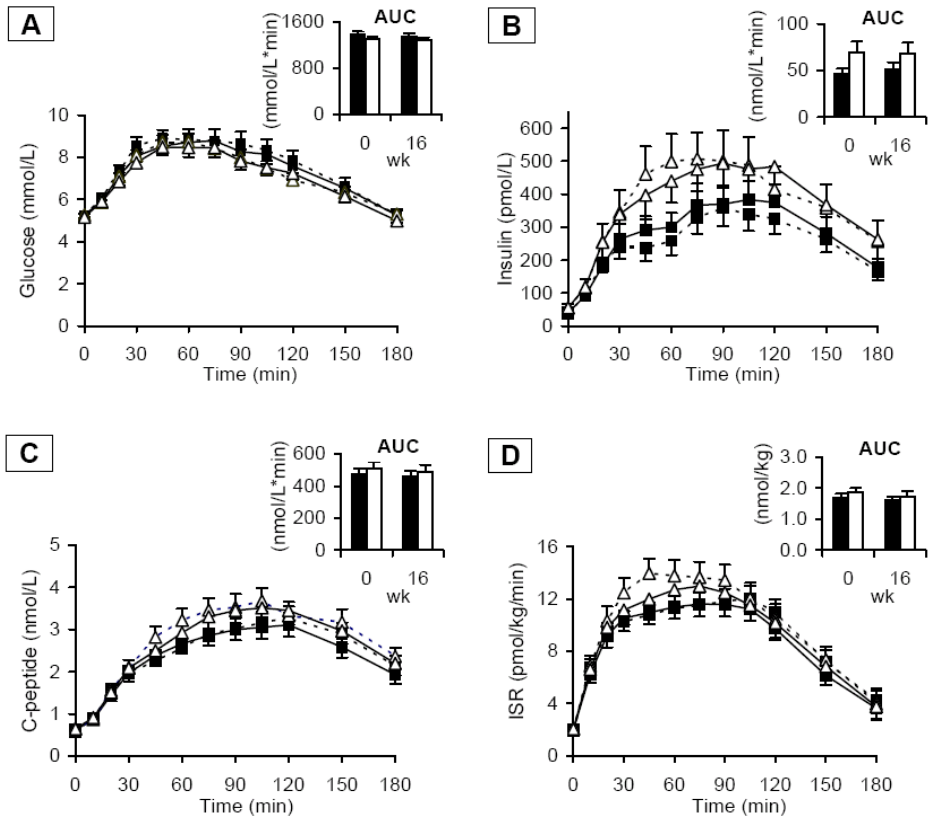

Figure 8: Glucose and insulin metabolism during oral glucose tolerance test. Mean (±SEM) glucose (A), insulin (B) and C-peptide (C) concentrations and insulin secretion rates (ISR) during the 3-hour OGTT performed at baseline (week 0; dotted lines) and after the dietary intervention (week 16; solid lines), in subjects who consumed 15.7 g/d trans fatty acids (TFA; n=24; black squares); and a control oil (CTR; n=25; white triangles). The corresponding areas under the curves (AUC) are also depicted (black bars = TFA; white bars = control. From **Paper III**.

This could be due to lack of power. Indeed, after the dietary intervention, the relative mean difference between diet groups in insulin sensitivity index was -4% (CI: -23 to 20), indicating that we would not have been able to detect TFA-induced changes smaller than ~20%. However, the

glucose, insulin and C-peptide responses to a glucose challenge were practically unaltered in both diet groups after the 16-weeks of intervention (**Figure 8**), strongly suggesting that a larger sample size would not have led to different conclusions. It is however possible, that the higher intake of palmitic acid in the CTR group may have attenuated putative differences between diet groups, as palmitic acid has been shown to induce insulin resistance in muscle[263].

Yet, the null-findings in the COBRA study regarding insulin sensitivity are supported by the fact that we, in contrast to Christiansen *et al*.[20] were able to detect substantial negative effects of the TFA intervention on cholesterol levels (**Paper II**). This, along with the TFA-induced increase in TNFα levels (**Paper IV**), confirms that our intervention indeed was capable of provoking a negative metabolic impact in our study population.

Subgroup analysis

As stated above, it may be that TFA intake induces insulin resistance in subjects with a predisposition to T2D only. Of the 49 women who completed the COBRA study, 30 had normal glucose tolerance (NGT) and 19 had impaired glucose tolerance (IGT) or T2D (based on the baseline 2-hour glucose values and using the criteria proposed by The American Diabetes Association[264]). When excluding the women with NGT, the insulin sensitivity index was 15% higher (CI: -22 to 68; P=0.46) in the TFA group compared to controls after the intervention, leaving no indication of a more negative effect of TFA in these women. However, this analysis included only 13 and 6 women in the TFA and CTR groups, respectively.

Does dietary fat quality affect insulin sensitivity?

Maybe it is not too surprising that we did not see an effect of TFA on insulin sensitivity in the COBRA study. In general, there is sparse evidence from high quality human RCTs to suggest an impact of dietary fat quality on insulin sensitivity, even in large scale studies. The KANWU multicentre study showed a decrease in insulin sensitivity of 10% (by IVGTT) after 3 months of high SFA consumption (18 E% SFA, 13 E% MUFA) compared to high MUFA (21 E% MUFA, 10 E% SFA) in 162 healthy subjects. However, the difference between diets only reached significance in subjects consuming diets with < 37 E% fat[265]. These results are in accordance with recent data from the large multicentre study LIPGENE that included more than 100 subjects with the metabolic syndrome in each study arm. This study failed to show a significant effect of diets high in SFA (16 E% SFA, 12 E% MUFA) compared to MUFA (20 E% MUFA, 8 E% SFA) for 12 weeks on insulin sensitivity (by IVGTT), whereas in subjects with low habitual fat intake, SFA intake decreased insulin sensitivity[266]. Also recently, in the multi-centre study RISCK, 24-week diets high in SFA (16E% SFA, 11.5 E% MUFA, n=79 completers) were compared to diets high in MUFA (16 E% MUFA, 10 E% SFA, n=103), and no difference in insulin sensitivity (by IVGTT) was detected[267].

Long-term exposure may be required

It cannot be ruled out, that TFA intake only will exert adverse effects on glucose metabolism at unrealistically high intake levels (>10 E%) and only when compared to MUFA (or PUFA), not SFA, as seen in the study by Christiansen *et al*.[20] Alternatively, very long exposures may be required. After 6 years on a diet with 8 E% TFA, increased fructosamine concentrations, increased postprandial insulin concentrations as well as decreased insulin-activation of muscle Akt, were found in green monkeys, compared to monkeys fed MUFA[22]. Since Akt (a kinase) mediates the signal transduction from the insulin receptor to translocation of glucose transporter 4 (GLUT4) to the muscle cell membrane, these findings indicate that TFA may impair post-

receptor signaling. A decreased phosphorylation status of Akt was also found in livers of mice[176] but not in rat myotubes[230] after TFA exposure.

TFA and insulin sensitivity in rodent models

Generally, rodent models allow for examination of longer durations of TFA exposure, at least relative to life span, and most studies indicate that long-term TFA intake impairs insulin sensitivity [**Appendix E**]. Ibrahim and colleagues showed that feeding rats 3 E% TFA for 3 months had multiple adverse effects, such as i) decreased insulin-stimulated glucose transport in both diaphragm muscle[268] and epididymal adipocytes; ii) decreased anti-lipolytic effect of insulin in adipocytes; and iii) increased insulin AUC after an oral glucose load (ns) when compared to PUFA or a mix of palmitic and oleic acid[269]. Moderate intake levels of TFA (4-5 E%) has also been shown to decrease the whole-body glucose disposal rate (assessed by clamp) compared to SFA in rats[235], whereas insulin sensitivity (assessed by intra-peritoneal glucose tolerance tests) was not different compared to MUFA[230]. In mice, high TFA intakes (12-20 E%) have been found to increase fasting insulin and insulin resistance[188,234]. Finally, a few studies in rodents indicate that early life TFA-exposure (intra-uterine and/or during lactation) may predispose to insulin resistance in adulthood[270-272] although this is not a consistent finding[273].

MECHANISMS BY WHICH TRANS FATTY ACIDS MAY AFFECT INSULIN SENSITIVITY

Not much is known about how TFA exposure may affect insulin sensitivity. Since the FA composition of phospholipids of skeletal muscle has been shown to be an important predictor of insulin sensitivity, with higher degree of unsaturation being associated with increased sensitivity[262,274-276], it has been hypothesized that FA-induced changes in membrane properties are of importance. Insulin signaling and translocation of GLUT4 to the skeletal muscle cell membrane are both membrane-associated events; therefore membrane characteristics are likely to influence insulin sensitivity[269].

Also, insulin resistance has been associated with accumulation of body fat, particularly intramyocellularly[260]. Dietary fat load has been shown to affect the amount of lipid deposited here. In humans, three days of high-fat feeding (45-60 E% from fat) was shown to increase the intramyocellular lipid deposition in muscle by ~25-50%[277,278], and a lipid infusion-stimulated increase in intramyocellular lipid deposition was shown to inversely correlate to insulin sensitivity [277]. A study in rats indicated that TFA may indeed affect glucose metabolism via stimulation of intramuscular lipid accumulation (in diaphragm muscle, in which insulin-stimulated glucose uptake supposedly reflects whole-body insulin sensitivity)[268]. In contrast, this was not confirmed in another rat study examining the lipid deposition in *tibialis* and *soleus* muscle[235]. In the COBRA study lipid deposition in *psoas major* muscle was not affected by TFA (**Paper III**), and no other human studies appear to have examined the effect of FA quality on this endpoint. However, in growing pigs, 12 weeks of supplementation with CLA (1.2 weight%) was shown to significantly increase the intramuscular fat content in *longissimus dorsi* by 19%[279]. This indicates that relatively small amounts of specific FA may affect muscle lipid accumulation, at least during growth; unfortunately insulin sensitivity was not assessed in this study.

Triglyceride accumulation in muscle cells does, however, not seem to be detrimental *per se*, but may serve as a surrogate marker for the build-up of other lipid species within the muscle. FA intake appears to determine the FA composition of the intramyocellular triglyceride pool and

thereby the identity of intramyocellular lipid metabolites. Palmitate and stearate stimulate the synthesis of diacylglycerol (DAG) and ceramide that have been suggested to play roles as primary mediators in lipid-induced insulin resistance[263,280]. In rats, TFA consumption decreased whole-body glucose disposal and increased the DAG content in *soleus* muscle 2-fold, but the glucose disposal did not appear diminished in this specific muscle[235].

Finally, TFA may affect insulin sensitivity via induction of inflammation. TNFα and pro-inflammatory lipid mediators are known to be involved in development of insulin resistance[152,201,212] (see **Chapter 9** for more detail).

SUMMARY: DIETARY TRANS FAT & INSULIN SENSITIVITY

Most animal studies suggest that TFA intake impairs insulin sensitivity compared with other FA classes, whereas evidence from studies in humans is highly conflicting. Whether this discrepancy is due to the longer duration of TFA exposure in animal studies or simply reflects metabolic differences between species is unknown. The few indices of a detrimental effect of TFA intake on insulinemia in humans come from studies in metabolically vulnerable study populations. Yet, there was an absence of change in any of the glucose homeostatic parameters measured in older overweight women in the COBRA study, also when analyzing a subgroup with impaired glucose tolerance at baseline.

In the future, it would, however, be interesting to see if an effect of TFA intake may be detected in a larger sample from a more homogenous study population, i.e. glucose intolerant subjects only. In addition, such a study could include skeletal muscle biopsies to identify changes in insulin signal transduction and membrane phospholipid FA composition.

9 PUTATIVE CELLULAR MECHANISMS BY WHICH DIETARY TRANS FATTY ACIDS MAY AFFECT METABOLIC PATHWAYS

The precise molecular mechanisms by which TFA exert the documented metabolic effects have yet to be unraveled. Although not much is known about the cellular actions of TFA *per se*, they may involve some of those, which have been elucidated for other dietary FA. Therefore, in this chapter, the primary ways in which dietary FAs may modulate cell function in general will be briefly discussed. FAs are known to affect cell function i) by determining the FA composition of phospholipids in cell membranes and thereby the membrane properties and function of membrane-bound proteins; ii) by influencing essential FA metabolism and thereby formation of a variety of lipid mediators; iii) by binding to and modulating nuclear receptors that regulate gene transcription; and iv) by activating cell-surface receptors such as TLR.

Membrane properties

Since individual FAs differ by spatial configuration and thus chemical property, they may differently affect the fluidity and rigidity of the cell membrane that they make up[281]. The efficiency of molecular signal transduction is highly dependent on the orientation and positioning of various proteins within the cell membrane. Thereby, the FA composition of cellular membranes, and of lipid rafts within the membrane, may for instance play pivotal roles in insulin signaling and transduction of inflammatory stimuli.

TFA are readily incorporated into phospholipids in cell membranes. Their *trans* double bonds result in a more rigid packing than for *cis* residues, conveying membrane properties more similar to those of SFA[209]. Thereby, incorporation of TFA into the phospholipid bilayer of cell membranes induces a structural change that may alter cellular function[210,281]. The activity of rhodopsin (a prototypical member of the G protein-coupled receptor family) was decreased by incorporation of TFA into model membrane phospholipids compared to MUFA[281]. Activation of membrane-spanning G protein-coupled receptors is the initial event in various signaling cascades.

However, *in vivo* evidence in support of this hypothesis is limited. Whereas TFA-feeding (3 E%) led to decreased membrane fluidity and impaired insulin-stimulated glucose transport in rat adipocytes, irrespective of dietary linoleic acid level[269], no effect of erythrocyte membrane fluidity was seen in humans after 5 weeks intake of 6.6 E% TFA[282]. In monkeys, 18-weeks of TFA-feeding resulted in decreased insulin receptor number and increased insulin binding affinity, but membrane fluidity of erythrocytes was not affected despite incorporation of 11% *trans*18:1[283]. Yet, it is possible that local (lipid raft), more than global, membrane fluidity is important for receptor functions.

Formation of lipid mediators

Changes in membrane phospholipid FA composition may also affect the production of a range of lipid mediators, which are involved in cell signaling processes. Essential FA may be converted to lipid mediators, such as eicosanoids, and depending on the precursor FA the lipid mediator may have pro- or anti-inflammatory properties. Arachidonic acid can be enzymatically oxidized (by enzymes such as lipooxygenase and cyclooxygenase) to form bioactive leukotrienes, prostaglandins and lipoxins, while essential long-chain n-3 PUFA may be converted to anti-inflammatory protectins and resolvins[201]. The overall pattern of lipid-derived compounds may be modulated by dietary fats.

It has been suggested that TFA interfere with essential FA metabolism, and thereby the production of lipid mediators. TFA-feeding has been shown to decrease the ratio of arachidonic to linoleic acid in AT of mice[269] and rats[269] and in the phospholipids of piglet arterial cells[284], suggesting that TFA inhibit the metabolic conversion of linoleic acid to arachidonic acid. In accordance, incorporation of TFA into endothelial cell membrane phospholipids appeared to inhibit the synthesis of linoleic acid to arachidonic acid and lowered the secretion of prostacyclin[200].

Transcription factors

It is possible that TFA, like other dietary FAs, bind to nuclear receptors that regulate the transcription of various genes involved in numerous cellular processes. FAs are known to be natural ligands of peroxisome proliferator-activated receptors (PPARs), SREBP-1 and liver X receptor α (LXRα). PPARs act as key regulators of the expression of genes involved in both lipid and glucose metabolism and inflammatory pathways, and SREBP-1 and LXRα are mainly implicated in transcription of genes implicated in hepatic lipid metabolism.

However, not much is known about how, or if, TFA affects these transcription factors. Whereas elaidic and oleic acid showed similar potency to bind to and activate PPARα as compared to PUFA *in vitro*, they were both poorer ligands for PPARβ and PPARγ than PUFA[285]. TFA may, however, also affect transcription factors by modulating the production of lipid mediators, as described above. Several of these act as transcription factor ligands[286].

There is some evidence to suggest that the metabolic effects of TFA involve a change in the expression level of some of these transcription factors, both in liver and AT, although results are somewhat conflicting. An increase in the mRNA expression of SREBP-1 and PPARγ in livers of TFA-fed mice was suggested as a an explanatory factor for the observed increase in hepatic lipid accumulation[176]. SREBP-1 and PPARγ are known to stimulate the transcription of lipogenic genes, such as fatty acid synthase and acetyl-CoA carboxylase, and these were indeed expressed in excess compared to mice fed *cis*-unsaturated FA[176]. In contrast, acetyl-CoA carboxylase and diacylglycerol acyltransferase, the enzyme mediating triglyceride formation, were not affected in rats fed TFA in another study[228].

Activation of hepatic PPARα results in increased uptake and oxidation of FFA through mitochondrial and peroxisomal β-oxidation partly via activation of carnitine palmitoyl transferase I (CTP-I), which is the main control point for mitochondrial β-oxidation of FAs[258]. A decrease in the activity of CTP-I was seen in livers of rats fed TFA[228] and the activity of the lipogenic citrate carrier enzyme was increased[228]. However in mice, TFA-feeding did not affect the hepatic expression of PPARα and CTP-1[176].

Different effects have been observed in AT. Here, PPARγ plays a role in increasing insulin sensitivity as well as in promoting fatty acid uptake into adipocytes, via regulation of lipoprotein lipase (LPL)[287]. Thereby, activation of PPARγ in AT results in an increased triglyceride storage in subcutaneous adipocytes and a reduced FFA delivery to the liver[258]. In TFA-fed rats, PPARγ and LPL expression was decreased in AT, but AT mass was not affected, compared to rats fed soybean oil[178], palm oil or groundnut oil[187]. Furthermore, increased body weight and fat accumulation in TFA-fed rats, compared to rats fed a mix of MUFA and PUFA, could not be explained by differences in LPL activity in another study[236].

Overall, the evidence from rodent studies of an effect of TFA-feeding on expression of transcription factors, and the enzymes they regulate, is far from coherent.

Cell-surface receptors

FAs are capable of inducing pro-inflammatory cytokine expression in macrophages and adipocytes via activating TLR4[212]. TLR4 activation triggers a downstream signaling cascade, leading to activation of the nuclear factor κB (NF-κB) pathway, which then promotes the transcription of many genes that encode pro-inflammatory molecules including cytokines and chemokines. Recently, it was shown that the stimulatory effect of high-fat diets rich in SFA on expression of pro-inflammatory cytokines in AT and liver, was diminished in TLR4 knock-out mice (*abstract*)[288]. In addition, TLR4 knock-out mice appeared to be protected from the increase in liver fat deposition seen in wild-type mice.

TLR4 mediated activation of NF-κB may also be involved in FFA-induced insulin resistance in adipocytes and *in vivo* after high-fat diets[212,288]. NF-κB activation impairs insulin signaling by increasing the inhibitory serine phosphorylation of insulin receptor substrates and thus decreasing phosphatidylinositol 3-kinase activity and Akt phosphorylation. Lower Akt phosphorylation after TFA-feeding has been shown in mice[176] and monkeys[22] but not in myocytes after incubation with TFA[230,289]

However, the potency of TFA to activate TLR4 or NF-κB is uncertain. Whereas palmitate potently activated the NF-κB transcription factor and induced IL6 and TNFα expression in adipocytes and macrophages, the TFA isomers elaidic and vaccenic acid had only minor stimulatory effects on macrophage IL6 mRNA expression *in vitro*[212]. In contrast, physiological concentrations of elaidic acid was shown to activate NF-κB in cultured skeletal muscle cells although this did not result in decreased insulin-stimulated glucose uptake or translocation of GLUT4[289]. However, if TFA exposure induces insulin resistance, this may occur via stimulation of TNFα, which also activates the NF-κB pathway[146].

10 CONCLUSIONS & PERSPECTIVES

Based on our systematic review and meta-analysis of cohort studies (**Paper I**) and review of the additional scientific literature, the following may be concluded:

- Our meta-analysis of published and unpublished prospective cohort studies suggested that TFA intake is associated with an increased risk of heart disease of the magnitude previously reported (~20% increased risk per 2 E% TFA). However, it also highlighted the scarcity of this type of studies, which may be due to the difficulties in appropriately assessing TFA intake in observational studies or to publication bias. Nevertheless, the available case-control studies, using biomarkers of TFA intake, collectively supported the findings from the pooled analysis of prospective studies.
- From the pooled analysis it appeared that the risk of CHD associated with TFA intake may be ascribable to industrial, not ruminant, TFA, possibly due to low intake levels of the latter. Of note, however, a very limited number of studies addressing differential effects of industrial and ruminant TFA prohibited any firm conclusions concerning the importance of the source of TFA in risk prediction. The biomarker studies did not lend support to the notion that R-TFA should be less harmful than IP-TFA; neither did intervention studies examining the effect on blood lipids.
- There is, however, no evidence to support that R-TFA increases the risk of CHD in the amounts usually consumed.

In addition, a number of conclusions may be drawn from our intervention study COBRA:

- We confirmed, that induction of dyslipidemia may partly explain the TFA-associated increase in the risk of cardiovascular disease estimated from the meta-analysis. The observed increase in the total-C to HDL-C ratio may translate into an increase in heart disease risk of ~6% per 2 E% TFA. The greater increase, compared to that seen in previous studies after TFA consumption, suggests that older and overweight populations may be more susceptible to TFA-induced metabolic changes than young and lean ones, at least when it comes to adverse effects on lipoproteins.
- Our findings of an increased production of TNFα and its soluble receptors after TFA exposure support the notion that dietary TFA promote low-grade inflammation. It is, however, difficult to estimate what this effect may translate into in terms of heart disease risk.
- Results from our study tended to confirm prior reports of a sE-selectin-increasing effect of TFA, indicating that TFA consumption may promote endothelial dysfunction.
- Even a high TFA consumption for 4 months does not appear to affect the abdominal fat deposition in postmenopausal women; yet whole-body fat accumulation may be slightly increased.
- We did not see an increase in liver fat deposition in response to TFA exposure, although increased liver triglyceride accumulation has been shown consistently in rodent models. Whether this null-finding should be ascribed to limitations of the study, including a high

degree of within-subject variability in liver fat content as well as lack of standardization of diets, or if it reflects a true lack of an effect of TFA exposure on liver fat deposition in humans, is, however, not clear.

- Finally, we found no evidence for a detrimental effect of TFA intake on insulin sensitivity, β-cell function and markers of lipolysis within 4 months in a metabolically vulnerable study population.

With the findings of a pro-inflammatory effect of TFA in the COBRA study, we have provided evidence that narrows the explanatory gap within the TFA research area, as presented in Chapter 1. For the curious scientist, some unresolved issues could be pursued in the future:

- The trend towards a greater whole-body fat deposition after TFA consumption in the COBRA study merits further investigations. A larger sample size as well as a fully controlled study diet may improve the chances of detecting a significant effect. Yet, great challenges lie in conducting long-term trials with highly controlled diets.
- Further characterization of the lipoproteins after TFA exposure, i.e. in terms of content of oxidized lipids, may provide new insights into the pathway by which TFA affects inflammatory processes.
- The indication of a sex-difference in the physiological response to TFA of ruminant and industrial origin is interesting, and this should be further examined.
- The evidence for a TFA-mediated induction of endothelial dysfunction is still weak, and should be substantiated. Assessment of flow-mediated vasodilation would have cast more light on whether the increased sE-selectin production after TFA consumption in the COBRA study was reflective of impaired endothelial function. Measurement of endothelial nitric oxide production, as reflected by plasma nitrite/nitrate (NOx), could also be considered in future studies.

Yet, for the pragmatic scientist, research funding is probably better spent on resolving other issues of greater relevance to public health. It is no longer possible to argue for a lack of evidence of the adverse health effects associated with TFA consumption. Thereby, it is hard to question that action towards eliminating TFA from the food supply worldwide should be taken. Our research highly supports this notion, and the elimination of TFA fortunately appears to be a work in progress.

The low risk of achieving high daily intakes of TFA from ruminant sources when consuming normal foods, along with the difficulties in removing ruminant TFA from natural food sources, gives less priority to reduction efforts in this field.

It is worrisome, however, that alternatives to the inexpensive hydrogenated vegetable oils may not be readily available in developing countries where TFA intake is likely to be high. On a positive note, industry appears to be working with, not against, the out-facing of TFA. Thereby, future challenges lie in resolving the difficulties associated with substituting the world supply of partially hydrogenated fats with appropriate alternatives low in saturated fat. Interesterified fats are being developed as alternatives to hydrogenated oils, but the health effects associated with consumption of these still remain largely unknown; this should be the subject of future dietary investigations.

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APPENDICES & PAPERS

Appendix A: Characteristics of case-control studies examining the association between *trans* fatty acid (TFA) intake or biomarkers of intake on risk of coronary heart disease (CHD)

Appendix B: Relative risk estimates for the association of TFA intake or biomarkers or intake with risk of CHD in case-control studies

Appendix C: Confounders adjusted for in individual case-control studies

Appendix D: Risk ratios and 95% confidence intervals for fully adjusted random-effects models examining the associations of biomarkers of total-TFA (A); *trans*18:1 (B); *trans*18:2 (C) and *trans*16:1(D) and risk of CHD

Appendix E: TFA-feeding studies in animals

Papers:

I. Bendsen NT, Christensen R, Bartels EM & Astrup A. Consumption of industrial and ruminant *trans* fatty acids and risk of coronary heart disease: A systematic review and meta-analysis of cohort studies (*Submitted)*.

II. Bendsen NT, Chabanova C, Thomsen HS, Larsen TM, Newman JW, Stender S, Dyerberg J, Haugaard SB & Astrup A. Effect of *trans* fatty acid intake on abdominal and liver fat deposition and blood lipids - a randomized trial in overweight postmenopausal women (*Submitted)*.

III. Bendsen NT, Haugaard SB, Larsen TM, Chabanova C, Stender S & Astrup A. Effect of *trans* fatty acid intake on insulin sensitivity and intramuscular lipids – a randomized trial in overweight postmenopausal women (*Submitted)*.

IV. Bendsen NT, Stender S, Szecsi PB, Pedersen SB, Basu S, Hellgren LI, Newman JW, Larsen TM, Haugaard SB & Astrup A. *Trans* fatty acid intake induces low-grade systemic inflammation: evidence from a randomized, controlled trial in overweight postmenopausal women (*draft)*.

Appendix A: Characteristics of case-control studies examining the association between trans fatty acid (TFA) intake or biomarkers of intake on risk of coronary heart disease (CHD)

Reference	Study design	Study year	Country of origin	No of cases	No of controls	Participant eligibility criteria	Controls matched for	Exposure	Outcome	TFA level
Ascherio *et al*. 1994 [50]	Retrospective	1982-83	USA	239	282	White men and women. Excluded if: age > 75 y; history of MI, angina, diabetes, high cholesterol.	Age, sex, town of residence.	TFA intake previous year (116 item FFQ)	First AMI	Median TFA intake: 4.4 g/d
Aro *et al*. 1995 [63]	Multicentre retrospective (ERAMIC)	1991-92	9 European countries	671	717	Men. Excluded if: age > 70 y; change of diet for health reasons; weight loss> 5kg during previous year; history of alcohol or drug abuse; major psychiatric disorders, institutionalization.	Age, catchment area.	TFA in AT	AMI	Median AT conc. in controls: t18:1 = 1.59%
			Finland	*57*	*59*					Median AT conc. in controls: t18:1 = 1.51%
			Germany	*56*	*95*					Median AT conc. in controls: t18:1 = 1.38%
			Israel	*50*	*58*					Median AT conc. in controls: t18:1 = 2.15%
			Netherlands	*67*	*58*					Median AT conc. in controls: t18:1 = 2.43%
			Norway	*98*	*100*					Median AT conc. in controls: t18:1 = 2.15%
			Russia	*87*	*94*					Median AT conc. in controls: t18:1 = 1.68%
			UK	*58*	*41*					Median AT conc. in controls: t18:1 = 2.22%
			Spain, Granada	*54*	*52*					Median AT conc. in controls: t18:1 = 0.43%
			Spain, Malaga	*91*	*96*					Median AT conc. in controls: t18:1 = 0.47%
			Switzerland	*53*	*64*					Median AT conc. in controls: t18:1 = 1.72 %

(*Continued*)

Appendix A (*continued*)

Reference	Study design	Study year	Country of origin	No of cases	No of controls	Participant eligibility criteria	Controls matched for	Exposure	Outcome	TFA level
Roberts *et al*. 1995 [67]	Retrospective	1990-91	UK	66	286	Men. Excluded if: age > 64 y; history of CHD.	Age, sex, general practitioner.	TFA in AT	SCD	Median AT conc. in controls: Total-TFA = 2.9%; t18:1 =2.3%; t18:2 = 0.6%
Pedersen *et al*. 2000 [66]	Retrospective	1996	Norway	100	98	Men and postmenopausal women. Excluded if: age <45 y and > 75 y; history of MI, diabetes, cancer, major psychiatric disease, abuse of alcohol or drugs; weight change > 5 kg during previous year; use of hypolipidemic drugs.	Age, sex, geografic area.	TFA in AT	First AMI	Median AT conc. in controls: total-TFA = 4.1 %, t18:1 = 2.6%.
Lemaitre *et al*. 2002 [60]	Retrospective	1988-99	USA	179	285	Married men and women. Excluded if: age <25 y and > 74 y; history of CHD or life-threatening co-morbidities; use of fish oil supplements.	Age, sex, community.	TFA in blood (RBC PL)	Primary cardiac arrest	Median RBC conc. in controls: total-TFA = 2.0 %, t16:1 = 0.17%; t18:1 = 1.64%; t18:2 = 0.20%.
Baylin *et al*. 2003 [69]	Retrospective	1994-98	Costa Rica	482	482	Men and women. Excluded if: age ≥ 75 y; died during hospitalization; previous hospital admission related to CVD; unable to answer questionnaire.	Age, sex, area of residence	TFA in AT	First non-fatal MI	Median AT conc. in controls: t161 =0.08%
Clifton *et al*. 2004 [53]	Retrospective	1995-97	Australia	209	174	Men and women. Excluded if: age ≥ 75 y; previous diagnosis of MI, angina, hypercholesterolemia, hypertriglyceridemia or diabetes.	Age, sex, postal code.	TFA intake (300-item FFQ)	first MI	Median TFA intake: 3.0 g/d

(*Continued*)

Appendix A (*continued*)

Reference	Study design	Study year	Country of origin	No of cases	No of controls	Participant eligibility criteria	Controls matched for	Exposure	Outcome	TFA level
Colon-Ramos *et al*. 2006, I [58]	Retrospective	1994-99	Costa Rica	477	477	Men and women. Excluded if: age ≥ 75 y; died during hospitalization; previous hospital admission related to CVD; unable to answer questions.	Age, sex, area of residence	TFA in AT	First non-fatal AMI	Median TFA intake: 4.1 g/d. Median AT conc. in entire population: Total-TFA = 2.99%; t18:1 =1.61%; t18:2 = 1.20%
Colon-Ramos *et al*. 2006, II [58]	Retrospective	2000-03	Costa Rica	1320	1320	,,	,,	TFA in AT	First non-fatal AMI	Median AT conc. in entire population: Total-TFA = 2.57%; t18:1 =1.29%; t18:2 = 1.05%
Lemaitre *et al*. 2006 [65]	Prospective; nested in CHS; mean follow-up 3 y	1992-93	USA	214	214	Men and women, age ≥ 65 y at baseline. Excluded if: died in nursing home; use of fish oil supplements.	Age, sex, cohort, clinic site, time of blood draw (± 90 days); presence of absence of CVD at time of blood draw; follow-up duration.	TFA in blood (plasma PL)	Fatal IHD	Median RBC PL conc. in controls: t16:1 = 0.28%; t18:1 =2.01%; t18:2 = 0.30%
Harris *et al*. 2007 [64]	Retrospective	2001-02	USA	94	94	Men and women; "younger age" (mean age 46 y).	Age, sex, race.	TFA in blood (whole blood)	Acute coronary syndrome	Mean whole blood conc. in controls: total-TFA = 1.98%; t18:1 =1.63%; t18:2 = 0.35%

(*Continued*)

Appendix A (*continued*)

Reference	Study design	Study year	Country of origin	No of cases	No of controls	Participant eligibility criteria	Controls matched for	Exposure	Outcome	TFA level
Lopes *et al*. 2007 [54]	Retrospective	NA	Portugal	49 (AT); 297 (intake)	49 (AT data); 301 (intake data)	Men. Excluded if: age<40 y and if ≥ 65 y in combination with low mental score; change of food habits during prior year; unable to answer questionnaire; history of MI.	Catchment area	TFA intake (82-item FFQ)and TFA in AT	First AMI	Median TFA intake in controls: 1.2 g/d: Median AT conc. in controls: total-TFA = 0.79%
Sun *et al*. 2007a [62] & Sun *et al*. 2007b [70]	Prospective; nested in NHS, mean follow-up 6 y	Baseline: 1989-90	USA	166	327	Women;Excluded if: diagnosed with cancer or CVD	Age, smoking status, date of blood draw, fasting status.	TFA in RBC	Incident CHD	Mean RBC conc. in controls: total-TFA = 1.66%; t16:1n-7 = 0.14%; t18:1 =1.16%; t18:2 = 0.36%
Ghahremanpour *et al*. 2008 [59]	Retrospective	NA	Iran	105	68	Men and women. Excluded if: ≥ 75 y; previous hospital admission for CVD; previous diagnosis of angina, hyperchol. or diabetes.	Age, sex.	TFA in AT	CAD	Mean AT conc. in controls: total-TFA = 8.02%; t16:1 = 0.50%; t18:1 =6.01%; t18:2 = 1.50%
Block *et al*. 2008 [57]	Retrospective	2004-05	USA	768	768	Men and women. History of CHD did NOT exclude cases or controls.	Age, sex, race.	TFA in blood cells	Acute coronary syndrome	Mean AT conc. in controls: total-TFA = 3.34%; t16:1n-7 = 0.70%; t18:1 =2.48%; t18:2 = 0.15%
Park *et al*. 2009 [61]	Retrospective	2006-08	South Korea	50	50	Men and women. Excluded if: history of CHD, cancer, hyperlipidemia or diabetes.	Age, sex.	TFA in RBC	Non-fatal AMI	RBC conc. in controls: total-TFA = 1.01%

[1] The study by Baylin *et al*.[69] and the study by Colon-Ramos *et al*. [58] are partly based on the data from the same subjects and can therefore not be considered independent. From Baylin *et al*.[69] we extracted results for *trans*16:1 only, as these are not presented in Colon-Ramos *et al*.[58]. [2] The study by Colon-Ramos *et al*.[58] reported results from examination performed between 1996-1999 and 2001-2003 (before and after reduction of TFA in foods) separately, whereby the results from the two different time periods can be considered independent and thereby regarded as two separate studies, Colon-Ramos I and II. AMI; acute myocardial infarction; AT, adipose tissue; CAD, coronary artery disease; CHD, coronary heart disease; CHS, Cardiovascular Health Study; CVD, cardiovascular disease; FFQ, food frequency questionnaire; ICH, ischemic heart disease; NA, not available; NHS, Nurses' Health Study; RBC, red blood cells; SCD, sudden cardiac death; t, *trans*.

Appendix B: Relative risk estimates for the association of TFA intake or biomarkers or intake with risk of CHD in case-control studies

Reference	End point	Exposure	OR	95%CI		Comparison	Comparison span	Unit of measurement
Case-control studies with intake of TFA								
Ascherio *et al*. 1994 [50]	First MI	Total-TFA	2.03	0.98	4.22	Q1 (1.7 g/d) vs Q5 (6.5 g/d)	4.8 g/d	Energy-adjusted g/d
”	First MI	R-TFA	1.02	0.43	2.41	Q1 (0.5 g/d) vs Q5 (1.8 g/d)	1.3 g/d	Energy-adjusted g/d
”	First MI	IP-TFA	1.94	0.93	4.04	Q1 (0.8g/d) vs Q5 (5.0 g/d)	4.2 g/d	Energy-adjusted g/d
Clifton *et al*. 2004 [74]	First MI	Total-TFA	2.25	1.16	4.32	Q1 (1.6 g/d) vs Q5 (5.5 g/d)	3.9 g/d	g/d
”	First MI	Total-TFA	0.98	NA	NA	Q1 (1.6 g/d) vs Q5 (5.5 g/d)	3.9 g/d	g/d adj. for energy and SFA
Lopes *et al*. 2007 [54]	First MI	Total-TFA	0.81	0.48	1.37	Q1 (0.3 g/d) vs Q4 (1.4 g/d)	1.1 g/d	g/d
Case-control studies with total TFA, t16:1, t18:1, or t18:2 in adipose tissue								
Aro *et al*. 1995 [63]	First MI	*Trans*18:1	0.97	0.56	1.67	Q1 (0.5%) vs Q4 (2.5%)	2.0% point	% of FAs in sc AT (buttock)
Norway	*First MI*	*Trans18:1*	*5.4*	*1.5*	*13.1*	*Q1 vs Q4*	*1.24% point*	*% of FAs in sc. AT (buttock)*
Finland	*First MI*	*Trans18:1*	*5.0*	*1.3*	*19.6*	*Q1 vs Q4*	*1.49% point*	*% of FAs in sc. AT (buttock)*
Germany	*First MI*	*Trans18:1*	*1.8*	*0.7*	*5.2*	*Q1 vs Q4*	*0.71% point*	*% of FAs in sc. AT (buttock)*
UK	*First MI*	*Trans18:1*	*1.6*	*0.4*	*6.6*	*Q1 vs Q4*	*1.29% point*	*% of FAs in sc. AT (buttock)*
Switzerland	*First MI*	*Trans18:1*	*1.5*	*0.5*	*4.9*	*Q1 vs Q4*	*0.98% point*	*% of FAs in sc. AT (buttock)*
Israel	*First MI*	*Trans18:1*	*0.8*	*0.2*	*3.2*	*Q1 vs Q4*	*1.78% point*	*% of FAs in sc. AT (buttock)*
Netherlands	*First MI*	*Trans18:1*	*0.8*	*0.2*	*2.6*	*Q1 vs Q4*	*2.02% point*	*% of FAs in sc. AT (buttock)*
Spain, Malaga	*First MI*	*Trans18:1*	*0.3*	*0.1*	*1*	*Q1 vs Q4*	*0.52% point*	*% of FAs in sc. AT (buttock)*
Russia	*First MI*	*Trans18:1*	*0.2*	*0.1*	*0.7*	*Q1 vs Q4*	*1.36% point*	*% of FAs in sc. AT (buttock)*
Spain, Granada	*First MI*	*Trans18:1*	*0.2*	*0*	*0.6*	*Q1 vs Q4*	*0.51% point*	*% of FAs in sc. AT (buttock)*
Baylin *et al*. 2003 [69]	First AMI	*Trans*16:1	2.58	1.22	5.43	Q1 (0.0%) vs Q5 (0.1%)	0.1% point	% of FAs in sc. AT (buttock)
Colon-Ramos *et al*. 2006, I [58]	First AMI	*Total*-TFA	3.28	1.68	6.42	Q1 (1.9%) vs Q5 (4.4%)	2.5% point	% of FAs in sc. AT (buttock)
“	First AMI	*Trans*18:1	1.75	0.97	3.15	Q1 (0.9%) vs Q5 (2.5%)	1.6% point	% of FAs in sc. AT (buttock)
“	First AMI	*Trans*18:2	4.76	2.24	10.11	Q1 (0.8%) vs Q5 (2.0%)	1.2% point	% of FAs in sc. AT (buttock)
Colon-Ramos *et al*. 2006, II [58]	First AMI	Total-TFA	1.03	0.75	1.42	Q1 (1.8%) vs Q5 (3.4%)	1.6% point	% of FAs in sc. AT (buttock)
“	First AMI	*Trans*18:1	1.02	0.75	1.37	Q1 (0.9%) vs Q5 (1.9%)	1.0% point	% of FAs in sc. AT (buttock)
“	First AMI	*Trans*18:2	1.15	0.8	1.64	Q1 (0.7%) vs Q5 (1.4%)	0.7% point	% of FAs in sc. AT (buttock)
Ghahremanpour *et al*. 2008 [59]	IHD	Total-TFA	1.41	1	1.8	Q1 vs Q5	13.7% point	% of FAs in sc. AT (buttock)
”	IHD	*Trans*16:1	0.8	0.3	1.9	Q1 vs Q5	1.5% point	% of FAs in sc. AT (buttock)
”	IHD	*Trans*18:1	1.32	1	1.8	Q1 vs Q5	11.3% point	% of FAs in sc. AT (buttock)
”	IHD	*Trans*18:2	1.85	0.6	4.8	Q1 vs Q5	4.6% point	% of FAs in sc. AT (buttock)

(*Continued*)

Appendix B (*continued*)

Reference	End point	Exposure	OR	95%CI		Comparison	Comparison span	Unit of measurement
Lopes *et al*. 2007 [54]	First AMI	Total-TFA	0.04	0.01	0.32	T1 (0.6%) vs T3 (0.9%)	0.3% point	% of FAs in sc. AT (buttock)
Pedersen *et al*. 2000 [66]	First AMI	Total-TFA	1.49	0.47	4.69	Q1 (2.9%) vs Q5 (5.5%)	2.6% point	% of FAs in sc. AT (buttock)
Roberts *et al*. 1995 [67]	SCD	Total-TFA	0.63	0.22	1.84	Q1 (≤2.3%) vs Q5 (≥3.4%)	>1.1% point	% of FAs in sc. AT (buttock)
”	SCD	*Trans*18:1	0.59	0.19	1.83	Q1 (≤1.8%) vs Q5 (≥2.8%)	>1.0% point	% of FAs in sc. AT (buttock)
”	SCD	*Trans*18:2	0.99	0.35	2.84	Q1 (≤0.5%) vs Q5 (≥0.7%)	>0.2% point	% of FAs in sc. AT (buttock)
Case-control studies with total TFA, t16:1, t18:1, or t18:2 in blood								
Block *et al*. 2008 [57]	ACS	Total-TFA	1.31	1.11	1.55	1 SD increase	~1.4% point	% of FAs in whole blood
”	ACS	*Trans*18:1	1.24	1.06	1.45	1 SD increase	~0.8% point	% of FAs in whole blood
”	ACS	*Trans*18:2	1.1	0.93	1.3	1 SD increase	~0.1% point	% of FAs in whole blood
Harris *et al*. 2007 [64]	ACS	Total-TFA	0.87	0.55	1.37	1 SD increase	~0.8% point	% of FAs in whole blood
”	ACS	*Trans*18:1	0.8	0.51	1.25	1 SD increase	~0.7% point	% of FAs in whole blood
”	ACS	*Trans*18:2	1.41	0.84	2.39	1 SD increase	~0.2% point	% of FAs in whole blood
Lemaitre *et al*. 2002 [60]	SCD	Total-TFA	1.47	1.01	2.13	Interquintile range	0.9% point	% of FAs in erythrocytes
”	SCD	*Trans*18:1	0.77	0.48	1.24	Interquintile range	0.8% point	% of FAs in erythrocytes
”	SCD	*Trans*18:2	3.05	1.71	5.44	Interquintile range	0.1% point	% of FAs in erythrocytes
Lemaitre *et al*. 2006 [65]	Fatal IHD	Total-TFA	0.94	0.65	1.34	Interquintile range	1.39% point	% of FAs in plasma
”	Fatal IHD	*Trans*16:1	0.95	0.64	1.42	Interquintile range	0.13% point	% of FAs in plasma
”	Fatal IHD	*Trans*18:1	0.38	0.17	0.86	Interquintile range	NA	% of FAs in plasma
”	Fatal IHD	*Trans*18:2	1.68	1.21	2.33	Interquintile range	0.13% point	% of FAs in plasma
Park *et al*. 2009 [290]	non-fatal MI	Total-TFA	72.7	6.7	790.7	T1 (<0.06%) vs T3 (>0.45%)	~0.4% point	% of FAs in erythrocytes
”	non-fatal MI	*Trans*18:1	50.5	5.3	482.7	T1 (<0.22%) vs T3 (>0.35%)	~0.2% point	% of FAs in erythrocytes
”	non-fatal MI	*Trans*18:2	3.8	0.96	15.4	T1 (<0.19%) vs T3 (>0.29%)	~0.1% point	% of FAs in erythrocytes
Sun *et al*. 2007a [62]	IHD	Total -FA	3.3	1.5	7.2	Q1 (1.2%) vs Q4 (2.2%)	1.0% point	% of FAs in erythrocytes
”	IHD	*Trans*18:1	3.1	1.5	6.7	Q1 (0.8%) vs Q4 (1.6%)	0.8% point	% of FAs in erythrocytes
”	IHD	*Trans*18:2	2.8	1.2	6.3	Q1 (0.3%) vs Q4 (0.5%)	0.2% point	% of FAs in erythrocytes
Sun *et al*. 2007b [70]	IHD	*Trans*16:1	0.98	0.53	1.83	Q1 (0.1%) vs Q4 (0.2%)	0.1% point	% of FAs in erythrocytes

AMI; acute myocardial infarction; AT, adipose tissue; ICH, ischemic heart disease; NA, not available; RBC, red blood cells; sc., subcutaneous; SCD, sudden cardiac death.

Appendix C: Confounders adjusted for in individual case-control studies

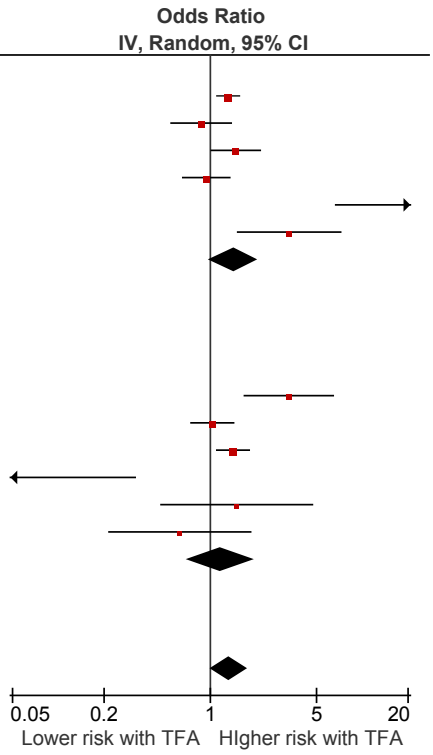
Reference	Age	AT α-linolenic acid	AT linoleic acid	AT arachidonic acid	AT oleic acid	AT n-3 log-chain PUFA	Blood linoleic acid	Blood n-6 PUFA	Blood n-3 log-chain PUFA	Blood lipids	BMI	Education	Diabetes status	Familiy history of MI	History of high blood cholesterol	History of hypertension	Hypertension	Intake of alcohol	Intake of cholesterol	Intake of energy	Intake of fiber	Intake of fruit and vegetables	Intake of linoleic acid	Intake of MUFA	Intake of protein	Intake of SFA	Menopausal status	Multivitamin supplements	Physical activity	Profession	Sex	Smoking status	Study center	Use of aspirin	Use of postmenopausal hormones	Vitamin E supplements	Vitamin supplements	Waist-to-hip ratio	Other confounders
Ascherio *et al*. 1994 [50]	•										•			•		•		•	•											•	•							
Aro *et al*. 1995 [63]	•		2	2	2						•																				•	•						
Roberts *et al*. 1995 [67]	•		•		•								•			•														•							
Pedersen *et al*. 2000 [66]	•	•												•																•	•						•	
Lemaitre *et al*. 2002 [60]	•								•			•	•	•			•											•		•							3
Baylin *et al*. 2003 [69]		•									4		•			•		•		•	4					•		•			•				4		Income, years living in the house
Clifton *et al*. 2004 [74]																				•						•												
Colon-Ramos 2006, I&II [58]	•	•										5	•				•	•	5	•	5				5	•			•		•	•						Income; vit E intake
Lemaitre *et al*. 2006 [65]	•								•			•	•																	•	•						CVD; clinic site; time of blood draw; congestive heart failure; history of stroke. [3]
Harris *et al*. 2007 [64]	•									6	•	•	•					•												•							Race; history of MI or revascularization
Lopes *et al*. 2007 [54]	•									7	•	•	7	•			7			•								•			•						
Sun *et al*. 2007a [62]	•						•	•	•		•		•	•	•	•		•			8	8				•		•			•			•	8		
Sun *et al*. 2007b [70]	•							•			•		•	•	•	•		•			9	9				•		•			•		•	•	9		Total TFA in RBC
Block *et al*. 2008 [57]									•	10 10	•	•	•	•			•	•													•		•				Personal history of CHD, use of statins and antiplatelet drugs.
Ghahremanpour *et al*. 2008 [59]																	•														•							Plasma triglycerides; AT PUFA
Park *et al*. 2009 [290]	•									•			•	•			•													•	•						Blood glucose

[1]Further adjustment for these variables and marital status, personality type and intakes of carotene, Vit E and Vit C did not materially change the estimates; [2]Further adjustment for these variables and socioeconomic status did not materially change the estimates; [3] Blood t18:1 and t18:2 were assessed simultaneously; [4]Further adjustment for these variables (plus waist-to-hip ratio and intake of Vit E and folate) did not materialy change the estimates; [5]Further adjustment for these variables (plus weight, height, waist-to-hip ratio, other AT FA, intake of folate, margarine, fish and dairy products) did not materialy change the estimates; [6] The adjustment for blood lipids did not materially change the estimates; [7]Further adjustment for these variables (plus angina pectoris) did not materially change the estimates; [8]Further adjustment for these variables (plus folate intake and erythrocyte SFA, MUFA and α-linolenic acid) did not materially change the estimates; [9]Further adjustment for these variables (plus intake of folate, calcium and Vit D) did not materially change the estimates. [10]Excluding blood lipids and blood n-3 fatty acids did not change the estimates.

Appendix D: Risk ratios and 95% confidence intervals for fully adjusted random-effects models examining the associations of biomarkers of total-TFA (A); *trans*18:2 (B); *trans*18:1 (C) and *trans*16:1(D) and risk of CHD

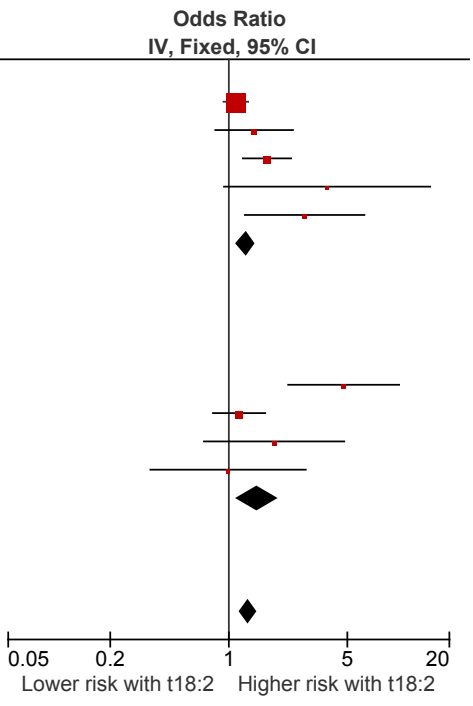
A Total-TFA

Study or Subgroup	Weight	Odds Ratio IV, Random, 95% CI
TFA in blood		
Block 2008	14.1%	1.31 [1.11, 1.55]
Harris 2007	10.6%	0.87 [0.55, 1.37]
Lemaitre 2002	11.7%	1.47 [1.01, 2.13]
Lemaitre 2006	11.9%	0.94 [0.66, 1.34]
Park 2009	1.2%	72.68 [6.68, 790.95]
Sun 2007a	6.8%	3.30 [1.51, 7.20]
Subtotal (95% CI)	**56.2%**	**1.42 [0.98, 2.07]**
Heterogeneity: Tau² = 0.14; Chi² = 22.94, df = 5 (P = 0.0003); I² = 78%		
Test for overall effect: Z = 1.83 (P = 0.07)		
1.1.2 TFA in adipose tissue		
Colon-Ramos 2006; I	7.9%	3.28 [1.67, 6.43]
Colon-Ramos 2006; II	12.4%	1.03 [0.75, 1.42]
Ghahremanpour 2008	13.3%	1.41 [1.10, 1.80]
Lopes 2007	1.6%	0.04 [0.00, 0.32]
Pedersen 2000	4.1%	1.49 [0.47, 4.69]
Roberts 1995	4.6%	0.63 [0.22, 1.84]
Subtotal (95% CI)	**43.8%**	**1.16 [0.69, 1.95]**
Heterogeneity: Tau² = 0.25; Chi² = 22.29, df = 5 (P = 0.0005); I² = 78%		
Test for overall effect: Z = 0.56 (P = 0.57)		
Total (95% CI)	**100.0%**	**1.33 [1.01, 1.74]**
Heterogeneity: Tau² = 0.13; Chi² = 45.24, df = 11 (P < 0.00001); I² = 76%		
Test for overall effect: Z = 2.01 (P = 0.04)		
Test for subgroup differences: Chi² = 0.01, df = 1 (P = 0.90), I² = 0%		

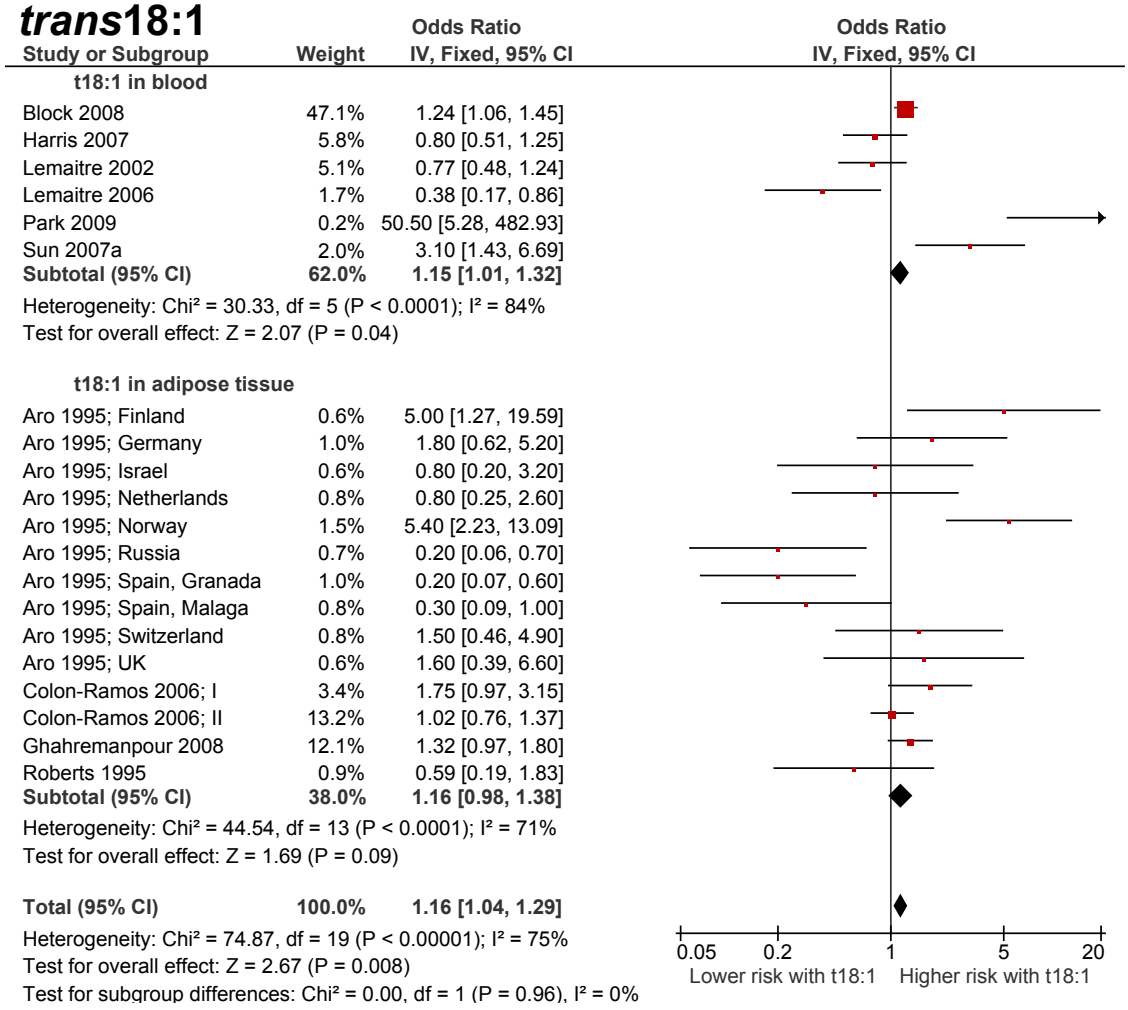


B *trans*18:2

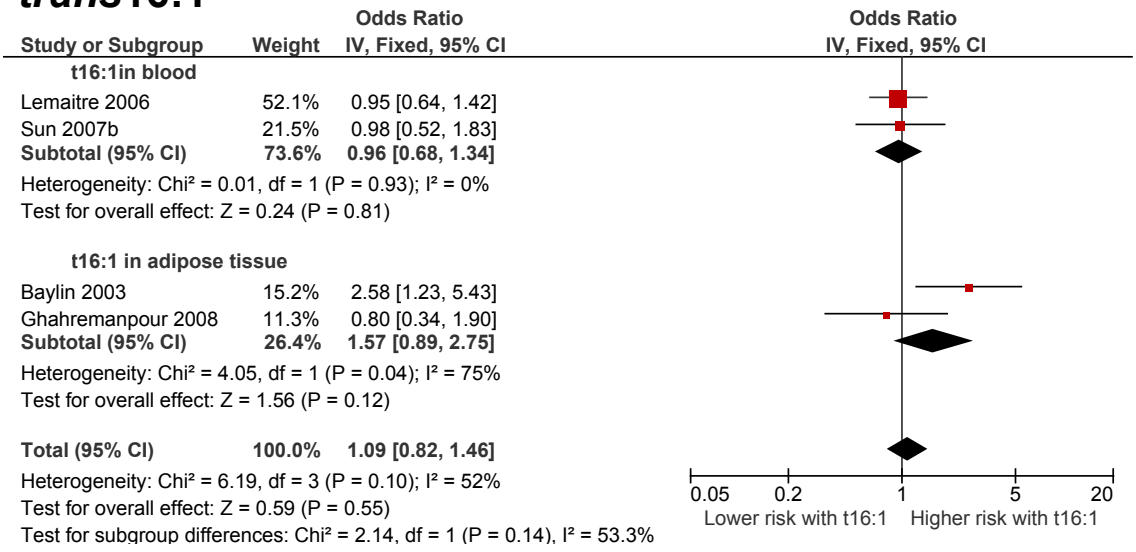
Study or Subgroup	Weight	Odds Ratio IV, Fixed, 95% CI
t18:2 in blood		
Block 2008	57.5%	1.10 [0.93, 1.30]
Harris 2007	5.7%	1.41 [0.83, 2.39]
Lemaitre 2006	14.9%	1.68 [1.21, 2.33]
Park 2009	0.8%	3.80 [0.94, 15.40]
Sun 2007a	2.4%	2.80 [1.24, 6.31]
Subtotal (95% CI)	**81.3%**	**1.26 [1.09, 1.45]**
Heterogeneity: Chi² = 11.83, df = 4 (P = 0.02); I² = 66%		
Test for overall effect: Z = 3.23 (P = 0.001)		
t18:2 in adipose tissue		
Colon-Ramos 2006; I	2.8%	4.76 [2.24, 10.10]
Colon-Ramos 2006; II	12.7%	1.15 [0.81, 1.64]
Ghahremanpour 2008	1.8%	1.85 [0.71, 4.79]
Roberts 1995	1.4%	0.99 [0.34, 2.84]
Subtotal (95% CI)	**18.7%**	**1.47 [1.10, 1.97]**
Heterogeneity: Chi² = 11.96, df = 3 (P = 0.008); I² = 75%		
Test for overall effect: Z = 2.60 (P = 0.009)		
Total (95% CI)	**100.0%**	**1.30 [1.14, 1.47]**
Heterogeneity: Chi² = 24.68, df = 8 (P = 0.002); I² = 68%		
Test for overall effect: Z = 4.03 (P < 0.0001)		
Test for subgroup differences: Chi² = 0.90, df = 1 (P = 0.34), I² = 0%		



C *trans*18:1



D *trans*16:1



Appendix E: TFA-feeding studies in animals

Reference	Species	Duration	Diets	Diet composition	Body weight	Fat (pad) mass or TG content	Hepatic TG	Results
Kavanagh *et al*. (2007) [22]	Adult male green monkeys (n=21 per group; 17 for AT volumes; 8 y old)	6 y	a) TFA (8E%) from PH soybean oil b) MUFA	35 E% fat (17 wt%) Amount of feed based on body weight at baseline. Intended to maintain weight.	TFA>MUFA	TFA>MUFA	-	• TFA: body weight ↑ 7% vs 2 % in MUFA →diff. 0.45 kg at 6 y. • TFA: IAAT ↑ 33% and VAT↑ 29% vs MUFA (ns) • TFA: IAAT/SAT ↑ vs. MUFA (p=0.02), adj. for body weight. • TFA: ↑ 18% fasting insulin (ns) • TFA: ↑ 9% fasting glucose (ns) • TFA: ↑ 24% HOMA (ns) • TFA: ↑ 27% fructosamine • TFA: ↑ > 300% insulin at 3h postprandially (p=0.02; n=6+6) • TFA: Muscle insulin-stimulated Akt phosphorylation ↓ ~300% (n=3 vs 6 in MUFA) • AT Akt activation ↔ • Muscle or AT IR activation ↔ • AT TNFα protein ↔ (not detectable in muscle)
Colandre *et al*. (2003) [227]	Male Wistar rats (n=6 per group; weight: 120 g)	30 d	a) TFA (30 wt% of fat) from PH corn oil b) SA from hydr. corn oil c) OA+LA from corn oil	38.5%E fat Isoenergetic, ad libitum	↔	TFA/SA > OA+LA	TFA > SA > OA+LA	• TFA: Epid. AT weight 87% ↑ vs OA+LA. • TFA: Liver weight 26% ↑ vs OA+LA. • TFA: Liver TG 126 % ↑ vs. OA+LA and 56 % ↑ vs. SA (ns) • Carcass fat slightly ↓ on TFA/SA vs OA+LA (p=0.05) • Serum cholesterol ↔
Giudetti *et al*. (2003) [228]	Male Wistar rats (n=12 per group; 6 wk old)	14 d	a) TFA (48 wt% of fat) from PH soybean oil b) SA from hydr. soybean oil/olive oil c) OA from olive oil	20 wt% fat Isoenergetic, ad libitum	↔	-	OA > TFA > SA	• Liver weight ↔ • TFA: feed intake ↓ vs OA/SA • TFA: Serum cholesterol ↓ • TFA vs OA/SA: hepatic enzyme activity: citrate carrier (lipogenic) ↓ (vs SA); CPT-I (FA oxidation)↓; 3-HAD (FA oxidation) ↓

(*Continued*)

Appendix E (*continued*)

Reference	Species	Duration	Diets	Diet composition	Body weight	Fat (pad) mass or TG content	Hepatic TG	Results
Tetri *et al*. (2008) [231]	Male C57BL/6 mice (n=10 per group; 5-6 wk old)	16 wk	a) TFA (30 wt% of fat) from PH soybean oil b) SFA+MUFA from lard	45E% fat + high-fructose corn syrup	↔	-	TFA> SFA+MUFA (ns)	• Feed intake ↔ • Plasma cholesterol ↔ • TFA: liver weight ↑ • TFA: liver TG 78% ↑ but ns. • TFA: degree of hepatic steatosis ↑. • TFA: degree of micro-vesicular steatosis ↑. • Glucose tolerance ↔
Huang *et al*. (2009) [177]	Male Wistar rats (n=10 per group; 22-25 d old)	16 wk	a) TFA (4.5wt% of feed) from margarine b) CTR: Corn oil	16.5 wt% fat	↔	-	-	• TFA: Serum total and HDL cholesterol ↓ • TFA: Plasma adiponection ↑ resistin ↑ leptin. ↓ • Plasma glucose and insulin sensitivity index ↔
Koppe *et al*. (2009)[188]	Male AKR/J mice (n=4-5 per group;9-10 wk old)	8 wk	a) TFA (20E%) from shortening b) CTR: TFA substituted by SFA:MUFA:PUFA (30:20:50) from lard	45 E% fat; ad libitum	TFA<CTR	-	↔	• TFA: Serum ALT ↑ • Degree of hepatic steatosis ↔ • Serum cholesterol ↔ • TFA: Leptin ↓ • TFA: higher fasting insulin • TFA: insulin resistance (QUICKI) ↑ • TFA: serum leptin ↓ • TFA: hepatic expression of IL-1β ↑, TNFα ↔, IL6 ↔; IL10 ↔
Atal *et al*. (1994) [232]	Male C57B1/6J mice (n=4-6 per group; 0 days old)	2 y	a) TFA (25 wt% of fat) shortening b) OA from olive and corn oil	10 wt% fat Isoenergetic, ad libitum. Fed to mothers during lactation and to pubs after 21d	OA > TFA	OA > TFA	-	• TFA: epid. and perirenal fat pad mass ↓. • TFA: AA ↓and higher LA ↑ in AT • TFA: Adipose cell size ↓

(*Continued*)

Appendix E (*continued*)

Reference	Species	Duration	Diets	Diet composition	Body weight	Fat (pad) mass or TG content	Hepatic TG	Results
Machado *et al*. (2010) [234]	Male C57BL/6J LDLr-KO mice (n=13-14 per group; post-weaning)	16 wk	a) TFA (36wt% of fat) from PH soybean oil b) PUFA (More OA + LA + ALA, less SA compared to TFA diet) from sunflower and canola oil c) SFA (More PA + OA + LA, and less SA compared to TFA diet) from mostly palm oil	40 E% fat, ad libitum	TFA<SFA<PUFA	TFA<SFA<PUFA	TFA>SFA/PUFA	• Feed intake ↔ • TFA: 36 % ↓ SAT vs. PUFA and SFA • TFA: Epid. fat pad content ↓ • TFA: liver weight > 60% ↑ vs. PUFA and SFA • TFA: LDL and VLDL cholesterol ↑ • TFA: Macrovesicular hepatic steatosis and inflammatory infiltrate vs. very mild microvesicular steatosis and minimal inflammatory process in SFA and PUFA • TFA vs SFA/PUFA: hepatic gene expression: SREBP-1c ↑ PPARγ ↑ (lipid synthesis); MTP↓ (vs PUFA; TG export); PPARα and CTP-1 ↔ (FA oxidation) • TFA: Fasting glucose ↑ vs. PUFA, but not SFA • Fasting insulin ↔ • TFA: Higher HOMA vs PUFA and SFA
Tardy *et al*. (2008) [230]	Male wistar rats (n=10-14 per group; 12 wk old)	8 wk	a) IP-TFA (4 E%) from PHVO b) R-TFA (4 E%) from enriched butter b) OA from mix of palm stearin/rapeseed/sunflower oils	25 E% fat	↔	↔	-	• Liver weight and energy intake ↔ • Muscle oxidative capacity ↔ • TFA from either source: Body weight and adipose tissue weight slightly ↑ vs. OA, but ns. • Insulin and glucose AUC after intraperitoneal glucose tolerance test ↔ • TFA: Akt activation in myotubes not different from OA, but higher than PA
Faulconnier *et al*. (2006) [233]	Male NZ white rabbits (n=6 per group; 10 wk old)	12 wk	a) TFA, t18:1Δ10 (15 wt% of fat) from enriched butter b) SFA (12:0, 14:0, PA) from butter	13 wt% fat Isoenergetic, ad libitum	SFA > TFA	SFA > TFA	↔	• Feed intake ↔ • TFA vs SFA: AT enzyme activity: G3PDH ↓; G6PDH ↓; LPL ↓(ns) (lipogenic) • No effect on hepatic enzyme activity: G3PDH ↔; G6PDH ↔; FAS ↔ (lipogenic)

(*Continued*)

Appendix E (*continued*)

Reference	Species	Duration	Diets	Diet composition	Body weight	Fat (pad) mass or TG content	Hepatic TG	Results
Lessa *et al*. (2010) [229]	Male Wistar rats (n=10 per group)	7 wk	a) TFA (5 E%) from PHVO b) PUFA (LA + ALA) from soybean oil	12 wt% fat. Ad libitum. Fed to mothers during gestation and lactation and to pubs from day 23 to wk 7	↔	-	TFA>PUFA	• TFA: Liver fat deposition in newborns and after weanling ↑, but not significantly so in young adult rats (p=0.06). • TFA: Fasting glucose during young adulthood ↑ • TFA: Liver/body weight ratio during young adulthood ↑
Obara *et al*. (2010) [176]	Femal C57BL/6Njcl mice (n=6 per group; 8-10 wk old)	24 wk	a) TFA (29 % of fat), low fat (LF) b) MUFA/PUFA, LF c) TFA (29 % of fat), high fat (HF) d) MUFA/PUFA, HF	LF: 12E% fat; HF: 64 E% fat. Ad libitum.	TFA>MUFA/PUFA on HF diets only	-	TFA>MUFA/PUFA on HF diets only	• TFA HF: Liver weight ↑ compared to all others • TFA HF: Hepatic expression of: SREBP-1 ↑ PPARγ1 ↑ FAS ↑ ACC ↑ • TFA LF: Hepatic expression of SREBP-1 ↑ • Hepatic expression of PPARα ↔, CTP-1 ↔, MTP-1 ↔, TNFα ↔ and IL6 ↔
Silva *et al*. (2006) [236]	Male Wistar rats (n=6 per group; 0 d old)	45 d	a) TFA (15% of fat) from PHVO b) PA from palm oil c) OA from canola oil d) PUFA from soybean oil	7 wt% fat Isoenergetic, ad libitum Fed to mothers during lactation and to pubs from day 21 to 45	TFA/PA > OA/PUFA	PA >TFA> OA/PUFA	-	• TFA: Feed intake vs all others ↓ • TFA: Body weight ↑ vs. OA/PUFA • TFA: De novo lipogenesis rate in epid. AT ↑ vs. OA/PUFA but ↓ vs SA • TFA: Lipid content in epid. AT ↑ vs. OA/PUFA but ↓ vs PA • TFA: level of PUFA in AT ↓. • TFA: serum cholesterol ↑, HDL-C ↓ • LPL activity ↔
Duque-Guimaraes *et al*. (2009) [178]	Female Wistar rats	17 wk	a) TFA (15wt% of fat) from PHVO b) Soybean oil (PA+LA) c) Fish oil	7 wt% fat; Ad libitum	TFA>PA +LA/fish oil		Fish oil< TFA/PA+ LA	• TFA: feed intake ↓ vs two other groups until wk 7 • TFA: Body weight ↑ vs other groups from wk 5-13, not from wk 13-17 • TFA: total/HDL-C ↑ • TFA: Insulin ↑ • TFA: Plasma adiponectin ↓ • TFA: AT expression of resistin ↑; TNFα ↑; adiponectin ↓; PPARγ ↓ vs. the two other groups

(*Continued*)

Appendix E (*continued*)

Reference	Species	Duration	Diets	Diet composition	Body weight	Fat (pad) mass or TG content	Hepatic TG	Results
Dorfman *et al*. (2009) [235]	Male Sprague-Dawley rats (n= 8-11 per group; 8 wk old)	6 wk (8 wk for IS)	a) TFA (4.6 E%) from addition of EA to soy-bean oil b) SFA (PA+SA) from lard and soybean oil	10E% fat; Ad libitum	TFA>SFA	TFA>SFA	TFA>SFA	• TFA: Energy intake ↑ • TFA: whole body AT gain 50% ↑ vs SFA • TFA: primarily gain in IAAT • TFA: 6-fold ↑ in hepatic fat • Intramuscular fat ↔ • TFA: LA and DHA ↓ in AT, liver, muscle • TFA: whole-body glucose disposal rate ↓ (clamp) • Whole body insulin action (clamp) ↔ • TFA: hepatic glycogenesis ↑ • Liver glycogen content ↔ • Hepatic insulin action ↔ • TFA: 2-fold ↑ DAG in soleus muscle
Cassagno *et al*. (2005) [208]	C57B1/6J mice (n= 10 per group; 8 wk old)	7 wk	a) TFA (11 wt% of fat from PHVO) b) Rapeseed oil (OA + LA)	10 wt% fat	-	-	-	• Total-/HDL-C ↔ • TFA: plasma triglyceride ↑ • TFA: hepatic enzyme expression: FAS ↑ SREBP-1↑ (lipogenic) MTP ↑ apoB100 (TG export), LDL-R ↔, cholesteryl 7α-hydroxylase ↔ and LXR ↔ • Plasma LCAT activity ↔ • Reverse cholesterol transport ↔ • TFA: 18 % ↑ plasma F_2-isoprostanes

(*Continued*)

Appendix E (*continued*)

Reference	Species	Duration	Diets	Diet composition	Body weight	Fat (pad) mass or TG content	Hepatic TG	Results
Ibrahim *et al*. (2005) [269]	WNIN rats (n=8 per group; weanling)	3 mo	a) TFA (3 E% from Vanaspati, high LA (4E%)) b) TFA (3 E% from Vanaspati, low LA (2E%)) c) PA+OA (palmolein) d) PUFA (groundnut oil)	10 wt% fat	↔	↔	-	• TFA: Membrane fluidity ↓ • TFA: Fasting insulin ↑ 17% • TFA: Anti-lipolytic effect of insulin in adipocytes ↓ vs. PUFA and PA+OA • TFA: insulin-stimulated glucose transport in adipocytes ↓ vs. PUFA and PA+OA • TFA: HDL-cholesterol ↓ • Body weight and epid. fat mass ↔ • Insulin and glucose AUC after oral glucose tolerance test ↔ • No. effect of varying the amount of LA.
Natarajan *et al*. (2005) [268]	Same study as above							• TFA: FA in diaphragm phospholipids: LA ↑, AA ↓ , ratio of AA to LA ↓, and total PUFA ↓ • TFA: Diaphragm intramyocellular triglycerides ↑ 65% vs PUFA and 46% vs. PA+OA • TFA: Insulin-stimulated glucose transport in diaphragm ↓ 30% vs. PUFA and PA+OA. • No. effect of varying the amount of LA.
Saravanen *et al*. (2005) [187]	Same study as above							• TFA: mRNA expression in AT: PPARγ ↓ LPL ↓ vs. PUFA and PA+OA, resistin ↓ vs. PUFA • mRNA expression of adiponectin in AT was decreased by SFA but not TFA

ACC, acetyl CoA carboxylase; ALA, alpha-linolenic acid; epid., epididymal; CTP-1, carnitine pamitoyl transferase 1; DHA, docosahexaenoic acid; FAS, fatty acid synthase; G3PDH, glycerol-3-phosphate dehydrogenase; G6PDH, glycerol-6-phosphate dehydrogenase; IAAT, intra-abdominal adipose tissue; IR, insulin receptor; LA, linoleic acid; LCAT, lecithin cholesterol acyl transferase; LDLr-KO, LDL receptor knockout; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; OA, oleic acid; PA, palmitic acid; PH, partially hydrogenated; PHVO, partially hydrogenated vegetable oil; PPAR, peroxisome proliferator-activaeted receptor; SA, stearic acid; SAT, subcutaneous adipose tissue; SREBP-1c, sterol regulatory element binding protein-1c; TG, triglyceride; wt, weight.

PAPER I

PAPER I

CONSUMPTION OF INDUSTRIAL AND RUMINANT TRANS FATTY ACIDS AND RISK OF CORONARY HEART DISEASE: A SYSTEMATIC REVIEW AND META-ANALYSIS OF COHORT STUDIES

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Submitted

ABSTRACT

Background and Aims: The aim of this systematic review and meta-analysis was to summarize the evidence from observational studies assessing the association between intake of *trans* fatty acids (TFA) and the risk of coronary heart disease CHD), with a specific emphasis on distinguishing between TFA of industrial and ruminant origin.

Methods: By searching five bibliographic databases, six published and two unpublished prospective cohort studies, assessing the association of intake of TFA with fatal and/or non-fatal CHD, were identified. Four and three studies reported separate associations for intake of ruminant or industrial TFA, respectively.

Results and Conclusions: The pooled relative risk estimates for comparison of extreme quintiles of total-TFA intake (corresponding to intake increments ranging from 2.8 to ~10 g/d) were 1.22 (95% confidence interval (CI): 1.08 - 1.38; P = 0.002) for CHD events and 1.24 (CI: 1.07 - 1.43; P = 0.003) for fatal CHD. Ruminant TFA intake (increments ranging from 0.5 to 1.9 g/d) was not significantly associated with risk of CHD (RR = 0.92; CI: 0.76 - 1.11; P = 0.36), and neither was industrial TFA intake, although there was a trend towards a positive association (RR = 1.21; CI: 0.97 - 1.50; P = 0.09]. In conclusion, our analysis suggests that industrial TFA is positively related to CHD, whereas ruminant TFA is not, but the limited number of available studies prohibits any firm conclusions concerning whether the source of TFA is important. The null association of ruminant TFA with CHD risk may be due to lower intake levels.

INTRODUCTION

Dietary TFA have long been suspected of increasing the risk of coronary heart disease (CHD), especially at high intake levels. No randomized intervention studies evaluating the effect of dietary TFA on hard endpoints for CHD have been conducted. However, as several epidemiological studies have shown a strong positive association between the intake of TFA and risk of CHD[1-3], the suspicion of a harmful effect of TFA seems justified. It has been estimated that a two percent increase in energy intake from TFA is associated with a 23% increased risk of CHD in a previous pooled analysis of four prospective cohort studies with nearly 140,000 subjects[4].

However, TFA is not just TFA. TFA in food originate from two main sources; industrial partial hydrogenation of edible oils, and bacterial hydrogenation of unsaturated fatty acids in the rumen of ruminants. Today, the industrially produced TFA (IP-TFA) are primarily found in snacks and fast food products whereas the ruminant TFA (R-TFA) constitute a part of the fat in meat and dairy products. The isomer distribution of the TFA from these two sources is dissimilar. In R-TFA the predominant isomer is vaccenic acid (*trans*18:1n-7) whereas IP-TFA generally has a Gaussian distribution of *trans*18:1 isomers with highest levels of elaidic acid (*trans*18:1n-9)[5].

There has been debate as to whether R-TFA is equally harmful as IP-TFA. Some results from epidemiological studies of intake of R-TFA and risk of CHD have indicated that intake of R-TFA is innocuous or even protects against CHD[6].

We found it fitting and timely to conduct a systematic review and meta-analysis to assess the empirical evidence of an association between intake of TFA and the risk of fatal and/or non-fatal CHD, with a specific emphasis on stratifying results according to industrial or ruminant origin of the TFA. We aimed for inclusion of all the available prospective cohort studies which have assessed the association between intake of TFA and incident CHD in adult populations.

SUBJECTS AND METHODS

We conducted this review in accordance with the PRISMA Statement for Reporting Systematic Reviews and Meta-Analyses of Studies That Evaluate Health Care Interventions[7] on the basis of a predefined protocol, which was made publicly available and distributed among interested parties.

Study selection

With this work we aimed to review all prospective cohort studies describing the association between intake of TFA (industrial and/or ruminant) and the incidence of fatal and non-fatal CHD. We conducted a systematic literature search of MEDLINE via Pubmed from 1950 to March 2010; EMBASE via Ovid from 1980 to March 2010; Food Science and Technology Abstracts via Ovid from 1969 to March 2010; Web of Science from 1900-14 to March 2010, and SciFinder Scholar for studies describing the association between TFA intake and incidence of CHD. Two search themes were combined: TFA intake and CHD mortality and morbidity [for detailed description of the search strategy please refer to Supplemental Appendix A]. The search strategy had no language or study design restrictions. Instead, prospective cohort studies were identified from titles and abstracts of the retrieved references. Additionally, we screened the reference lists of all identified relevant studies, and of review articles, in order to identify possi-

ble studies of interest. We contacted authors of included studies for references to studies not identifiable by our other searches, including unpublished studies.

Two reviewers (NTB, investigator, and EMB, Senior Researcher in Biophysics and Research Librarian D.B.) independently identified articles eligible for further review by screening of identified titles and abstracts. Articles were considered for inclusion in the meta-analysis if they reported data from an original prospective cohort study with an assumed healthy study population of adult men and/or women at study baseline. Studies had to assess intake of one or all of IP-TFA, R-TFA and TFA from any source (total-TFA), and one of the outcomes had to be the incidence of fatal MI, non-fatal MI, or total CHD-related deaths. Studies were included for further screening if one or both reviewers decided the study to be possibly eligible. The second screening was based on reading of the full-text versions. Any disagreement between reviewers was resolved by consensus.

Data extraction

The included studies were scrutinized and reviewed without blinding of reviewers. One investigator (NTB) was responsible for the extraction of data, which was then checked by another reviewer (RC). Data was extracted using a customized data extraction form. Relevant data included the first author's name, year of publication, baseline year, name of cohort, country of origin, number of participants (men and women) at baseline, participant eligibility criteria, age of participants, duration of follow-up, dietary assessment method and validity of the method, number of dietary assessments, TFA mean or median intake and extreme quantiles of intake, outcome assessment method, number of events, unit of measurement, confounders adjusted for in the statistical analysis, relative risks of CHD (from the most adjusted analysis) comparing extreme quantiles of TFA intake or per unit of TFA intake, and corresponding 95% confidence intervals (CI).

Statistical analysis

The most adjusted Risk Ratio (RR) was used as a measure of the association between TFA and CHD. Summary estimates were obtained using inverse variance random-effects meta-analysis, with the DerSimonian and Laird estimate of the variance τ^2 as a measure of heterogeneity between trials[8]. We applied random-effects, rather than fixed-effects models to estimate pooled RRs in order to take into account the heterogeneity, however small, of the risk estimates and thereby be more conservative. Meta-analyses were performed by using Review Manager 5.0 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark; http://www.cc-ims.net/RevMan). We assessed homogeneity of effects across studies using the *Q*-test and quantified by I^2 index[9], which represents the percentage of total variation across studies that is attributable to inconsistency (heterogeneity) rather than chance[10].

In accordance with the protocol we performed secondary analyses for intake of R-TFA and IP-TFA, respectively. Sample size was inadequate for analysis stratifying for sex.

We examined the influence of an individual study on the pooled estimate of RR by excluding each study in turn. We specified sensitivity analyses with the aim to evaluate the influence of study quality on effect estimates as follows: i) An analysis stratifying for the dietary assessment tool (diet records, validated food frequency questionnaire (FFQ), non-validated FFQ, diet history, 24-h recall) where the former was rated as better, ii) A meta-regression-analysis to examine if the number of confounders adjusted for in each study affected the effect size; and iii) A

meta-regression-analysis to examine if the number of stars obtained in the Newcastle-Ottawa Scale quality assessment affected the effect size. The Newcastle-Ottawa Scale is one of the more comprehensive instruments for assessing the quality of non-randomized studies in meta-analyses[11]. The 8-item instrument consists of three subscales, namely, selection of subjects (4-item), comparability of subjects (1-item) and assessment of outcome/exposure (3-item). For each item each study got a score of zero, one or two stars, with a maximal total score of nine stars.

RESULTS

A total of 257 unique references were identified through the literature search and an additional 12 from reference lists, giving a total of 269. Of these, only 8 met the selection criteria as shown in **Figure 1**. Three publications presented results from the *Nurses' Health Study* (NHS) after 8, 14 and 20 years of follow-up, respectively[1,12,13]. Since only the first publication[13] presented analysis of the association of IP-TFA and R-TFA intake with CHD, we extracted these data from this publication. For results on the association of total-TFA intake with CHD we extracted data from the publication with the longest follow-up of 20 years[1]. One of the selected studies included subjects who were diagnosed with diabetes at baseline and adjusted for diabetes status in the statistical analyses[14]. We contacted the authors of all the selected studies to request updated analysis or additional information. The author of one study was able to respond to our request[6].

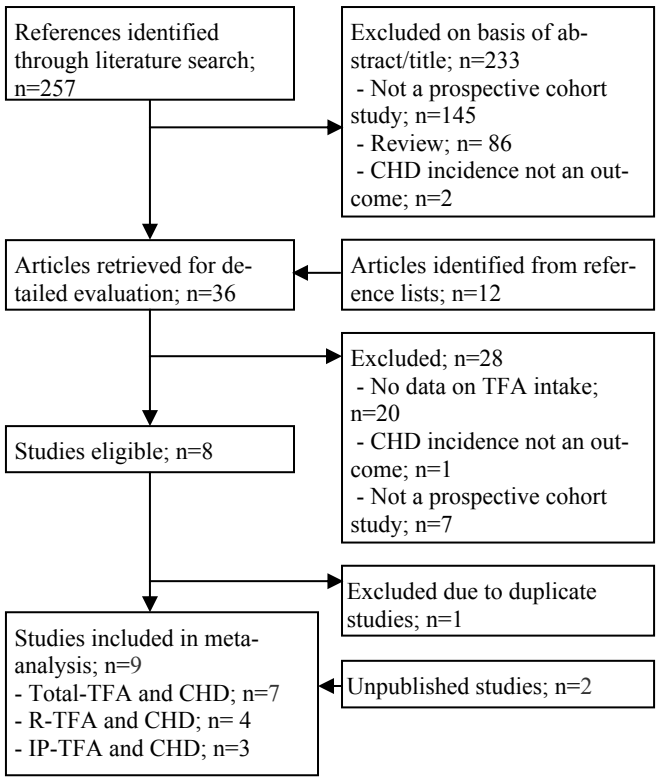

Figure 1: Study selection

In addition, we contacted the principal investigators (PI) of five prospective cohort studies who had not published data on the association between TFA intake and CHD despite having collected relevant data. The PI of the *Västerbotten Intervention Program*[15] and the *Womens' Health Study*[16] could not accommodate our request and the reporting of TFA intake data was judged to be of inadequate quality by the PI of the *Atherosclerosis Risk in Communities Study*[17]. The *Iowa Womens' Health Study* (IWHS) performed a relevant analysis on our request (Personal Communication with Dr. Kim Robien) and the PI of the *Finnish Mobile Clinic Health Examination Survey* (FMC) provided us with the results of an unpublished analysis (Personal Communication with Dr. Paul Knekt). Thereby, data from seven peer-reviewed articles were included in this review[1-3,6,13,14,18] as well as unpublished data from two prospective cohort studies.

Table 1: Study characteristics of the prospective cohort studies of trans fatty acid (TFA) intake and coronary heart disease (CHD)

Study	Baseline year	Country of origin	No of participants at baseline	Participant eligibility criteria	Age at baseline	Average duration of follow-up	Person years of follow-up	TFA intake	Outcome	Outcome assessment method	Dietary assessment method	No of dietary assessments	Validity of the dietary assessment method
NHS, 1993 [13]	1980	USA	85,095 women; of these 69,181 did not report changes in their margarine intake for 10 y (used in subanalyses of R-TFA and IP-TFA)	Female nurses. Excluded if: left>10 items blank on FFQ, improbable energy intake; history of angina, MI or stroke; high serum cholesterol or diabetes. Subanalyses of R-TFA and IP-TFA also excluded women reporting marked changes in margarine intake within previous 10 y.	34-59 y	8 y	661,996 for entire cohort	Mean: 4.0 g/d in entire cohort	Incident CHD [non-fatal MI + CHD death]	Medical records, hospital records, autopsy or death certificate; WHO criteria.	FFQ 61 food items (1 y). Specific questions about type of margarine and type of fat used for cooking	1 (at baseline)	Validated for this study. TFA intake was correlated to TFA in adipose tissue (r=0.5; n=115)
HPFS, 1996 [18] *HPFS, 2006* [4 1]	1986	USA	43,757 men (*38 461 men in updated analysis*)	Male health professionals. Excluded if: left>70 items blank on FFQ, improbable energy intake; previous diagnosis of MI, angina, coronary artery surgery, stroke, transient ischemic attack, peripheral arterial disease, or diabetes.	40-75 y	6 y (*14 y in updated analysis*)	236,782	Median: 2.7 g/d	Total MI [non-fatal MI + fatal CHD] & Fatal CHD	Medical records, necropsy reports or death certificate;WHO criteria	FFQ with 131 food items (1 y)	1 (at baseline; *updated dietary information in updated analysis*)	Validated against two 7-d food records (n=127); TFA intake was correlated to TFA in adipose tissue, r= 0.29
AT/BC, 1997 [3]	1985-1988	Finland	21,930 men	Male smokers. Excluded if: history of cancer or other serious disease; prior diagnosis of MI, angina, stroke or diabetes; use of vitamin supplements in excess of predefined doses; treatment with anticoagulating agents; missing cardiovascular risk factors; exercise-related chest pain.	50-69 y	6.1 y (median)	129,389 (deaths); 126,969 (events)	Median: 2.0 g/d	Major coronary event [first non-fatal MI + CHD death] & CHD death	Hospital discharge register; death certificates; ICD-8/9 codes 410-414	FFQ w/ 276 food items/dishes (1 y). Specific questions about fat on bread and type of fat used for cooking	1 (at baseline)	Validated against 24-d food records (n=190) in a pilot study prior to the trial

(*Continued*)

Table 1 (*continued*)

Study	Baseline year	Country of origin	No of participants at baseline	Participant eligibility criteria	Age at baseline	Average duration of follow-up	Person years of follow-up	TFA intake	Outcome	Outcome assessment method	Dietary assessment method	No of dietary assessments	Validity of the dietary assessment method
ZES, 2001 [2]	1985	Nethe rlands	667 men	Excluded if: previous diagnosis of MI or angina.	64-84 y	10 y	NA	Median: 3.9 E%	Incident CHD [fatal CHD + non-fatal MI] & Fatal CHD	Municipal registries; Statistics Netherlands; hospital discharge data; general practitioners; ICD (codes 410-414)	Cross-check dietary history method (1mo); interview verified against checklist of foods bought per week	3 (but only baseline data used in analyses)	Assessment of reproducibility after 3 and 12 months
NHS, 2005 [1]	1980	USA	78,778 women	Female nurses. Excluded if: left>10 items blank on FFQ, implausible energy intake; history of angina, MI, stroke, other CV disease; cancer; high serum cholesterol or diabetes.	34-59 y	20 y	NA	Median: 1.9 E%	Incident CHD [non-fatal MI + fatal CHD]	Medical records, hospital records, autopsy or death certificate; WHO criteria.	FFQ (1 y); 61 food items in 1980; 116 food items in subsequent. Specific questions about type of margarine and type of fat used for cooking.	6 (in 1980; 1984, 1986, 1990, 1994, 1998)	Validated for this study. TFA intake was correlated to TFA in adipose tissue (r=0.5; n=115)
SHS, 2006 [14]	1993-95	USA	2,938 men and women	American Indians; Excluded if: prior MI or CHD, dialysis treatment, kidney transplant or liver cirrhosis; improbable energy intake; age>79y	47-79 y	7.2 y	21,101	Mean: 2.4E%	Incident CHD [fatal CHD + first non-fatal MI] & Fatal CHD & Non-fatal CHD	Medical records, fatal CHD confirmed by review committees.	24-h dietary recall (1 d)	1 (at baseline)	NA

(*Continued*)

Table 1 (*continued*)

Study	Baseline year	Country of origin	No of participants at baseline	Participant eligibility criteria	Age at baseline	Average duration of follow-up	Person years of follow-up	TFA intake	Outcome	Outcome assessment method	Dietary assessment method	No of dietary assessments	Validity of the dietary assessment method
MONICA, 2008 [6]	1974-1993	Denmark	3,686 (1837 men)	Excluded if: previous diagnosis of CHD or diabetes; missing data for confounders; implausible dietary assessment results.	30-71 y	18 y	NA	Median R-TFA: Men: 1.8 g/d; Women 1.5 g/d	Incident CHD [Fatal + non-fatal CHD]	Cause of Death Registry and National Patient Registry; Review of medical files; ICD-8 (codes 410-414) until ultimo 1994; ICD-10 (codes I20-I25) thereafter.	7-d food record (94%) or dietary history interview (1 m; 6%)	1 (at baseline)	The two methods have been validated against each other
FMC (unpubl.)	1967-72	Finland	3738 (1956 men and 1782 women)	Excluded if: CHD at baseline	40-69 y	15 y	NA	Mean: Men: 2.6 g/d; Women: 1.9 g/d	Fatal CHD [Fatal CHD + CHD events]	Cause of Death Registry; ICD-8 (codes 410-414)	Dietary history interview, structured via 100-item questionnaire	1 (baseline)	Assessment of short-term (4-8 m) and long-term (4-7 y) reproducibility
IWHS (unpubl.)	1986	USA	32766 women	Postmenopausal women. Excluded if: diabetes or CHD at baseline, improbable energy intake.	55-69 y	21 y	NA	Median: 1.6 E%	Fatal CHD	Cause of Death Registry; ICD-9 (codes 410-414 or 429.2)	FFQ with 127 food items (1 y)	1 (baseline)	Validated against five 24-h dietary recall surveys, but not specifically for TFA.

[1] An updated analysis after 14 y follow-up with updated dietary data was published in a review in 2006. The principal investigator of the Health Professionals' Follow-up Study could not provide us with more detailed information about this analysis. [2] Data were kindly provided by P. Knekt, A. Reunanen, R. Seppänen, R. Järvinen and A. Aromaa. [3] Data were kindly provided by K. Robien and S. Motzinger. Abbreviations: AT/BC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; E%, energy %; FFQ, food frequency questionnaire; FMC, the Finnish Mobile Clinic Health Examination Survey, HPFS, The Health Professionals' Follow-up Study; ICD, International Classification of Diseases; IP-TFA, industrially produced trans fatty acids; IWHS, the Iowa Womens' Health Study; MI, myocardial infarction; MONICA, The 1914 cohort + The 1936 cohort + MONICA I and III; NA, not available; NHS, The Nurses' Health Study; R-TFA, ruminant trans fatty acids; SHS, The Strong Heart Study; ZES, The Zutphen Elderly Study.

Table 2: Relative risk estimates for the association of trans fatty acid (TFA) intake with risk of coronary heart disease (CHD)

Study	Outcome	N, event	N, total	Sex, age	Exposure	RR	95%CI		P for trend	Comparison	Comparison span
NHS, 1993 [13]	Total CHD	356	69181	F	IP-TFA [1]	1.78	1.12	2.83	0.009	Q1 vs Q5	NA
	Total CHD	356	69181	F	R-TFA [1]	0.59	0.3	1.17	0.23	Q1 vs Q5	NA
HPFS, 1996 [18]	Total CHD	734	43757	M	Total-TFA [1]	1.21	0.93	1.58	0.2	Q1 (1.5 g/d) vs Q5 (4.3 g/d)	2.8 g/d
	”	”	”	”	”	1.13	0.81	1.58	NA	2 E% increment	2 E%
	Fatal CHD	229	43757	M	Total-TFA [1]	1.41	0.86	2.32	0.42	Q1 (1.5 g/d) vs Q5 (4.3 g/d)	2.8 g/d
	”	”	”	”	”	0.93	0.52	1.69	NA	2 E% increment	2 E%
HPFS, 2006 [4 2]	Total CHD	1702	38461	M	Total-TFA	1.26	0.99	1.61	NA	2 E% increment	2 E%
AT/BC, 1997 [3]	Total CHD	1399	21930	M	Total-TFA [1]	1.14	0.96	1.35	0.16	Q1 (1.3 g/d) vs Q5 (5.6 g/d)	4.3 g/d
	Fatal CHD	635	21930	M	Total-TFA [1]	1.43	1.12	1.84	0.004	Q1 (0.6 E%) vs Q5 (2.0 E%)	1.4 E%
	Fatal CHD	635	21930	M	IP-TFA [1]	1.23	0.97	1.55	0.004	Q1 (0.1 g/d) vs Q5 (5.1)	5.0 g/d
	Fatal CHD	635	21930	M	R-TFA [1]	0.83	0.62	1.11	0.035	Q1 (0.6 g/d) vs Q5 (2.5)	1.9 g/d
ZES, 2001 [2]	Total CHD	98	667	M	Total-TFA [1]	2.00	1.07	3.75	0.03	T1 (2.4 E%) vs T3 (6.4 E%)	4 E%
	”	”	”	”	”	1.28	1.01	1.61	NA	2 E% increment	2 E%
	Total CHD	98	667	M	IP-TFA (C18:1)	1.05	0.94	1.17	NA	0.5 E% increment	0.5 E%
	Total CHD	98	667	M	IP-TFA (other)	1.07	0.99	1.15	NA	0.5 E% increment	0.5 E%
	Total CHD	98	667	M	IP-TFA (All) [1,3]	1.06	1.0	1.13	NA	0.5 E% increment	0.5 E%
	Total CHD	98	667	M	R-TFA [1]	1.17	0.69	1.98	NA	0.5 E% increment	0.5 E%
	Fatal CHD	49	667	M	Total-TFA	1.33	0.96	1.86	NA	2 E% increment	2 E%
	Fatal CHD	49	667	M	Total-TFA [1, 4]	1.77	0.91	3.45	NA	T1 (2.4 E%) vs T3 (6.4 E%)	4 E%
NHS, 2005 [1]	Total CHD	1766	78778	F	Total-TFA	1.33	1.07	1.66	0.01	Q1 (1.3 E%) vs Q5 (2.8 E%)	1.5 E%
	Total CHD	1111	NA	F, < 65 y	Total-TFA [1]	1.5	1.13	2	0.01	Q1 vs Q5	NA
	Total CHD	655	NA	F, > 65 y	Total-TFA [1]	1.15	0.8	1.66	0.49	Q1 vs Q5	NA
SHS, 2006 [14]	Total CHD	436	2938	F+M	Total-TFA [1]	1.06	0.78	1.44	0.88	Q1 (0.9 E%) vs Q4 (3.9 E%)	3.0 E%
	Fatal CHD	46	1659	F+M, 47-59 y	Total-TFA [1]	1.15	0.49	2.68	0.66	Q1 (0.9 E%) vs Q4 (4.0 E%)	3.1 E%
	”	”	”	”	”	1.73	0.57	5.25	NA	5 E% increment	5 E%
	Fatal CHD	92	1279	F+M, 60-79 y	Total-TFA [1]	0.83	0.42	1.66	0.54	Q1 (1.0 E%) vs Q4 (3.9 E%)	2.9 E%
	”	”	”	”	”	1.34	0.48	2.46	NA	5 E% increment	5 E%
	Non-fatal CHD	298	2938	F+M	Total-TFA	1.21	0.85	1.74	0.41	Q1 (0.9 E%) vs Q4 (3.9 E%)	3.0 E%

(*Continued*)

Table 2 (*continued*)

Study	Outcome	N, event	N, total	Sex, age	Exposure	RR	95%CI		P for trend	Comparison	Comparison span
MONICA, 2008 [6]	Total CHD	374	3686	F+ M	R-TFA	1.05	0.92	1.19	NA	0.5 g/d increment	0.5 g/d
	Total CHD	253	1837	M	R-TFA [1]	1.05	0.94	1.17	NA	0.5 g/d increment	0.5 g/d
	Total CHD	121	1849	F	R-TFA [1]	0.77	0.55	1.09	NA	0.5 g/d increment	0.5 g/d
FMC (unpubl.) [5]	Fatal CHD	216	1956	M	Total-TFA [1]	0.73	0.35	1.54	NA	Q1 vs Q5	NA
	Fatal CHD	72	1782	F	Total-TFA [1]	0.94	0.26	3.40	NA	Q1 vs Q5	NA
IWHS (unpubl.) [6]	Fatal CHD	1875	32766	F	Total-TFA [1]	1.15	0.92	1.43	0.31	Q1 (1.0 E%) vs Q4 (2.5 E%)	1.5 E%

[1] Data included in meta-analysis (Figure 2). [2] These data are from a review providing an updated analysis of data from the Health Professionals' Follow-up Study after 14 y follow-up with updated dietary data. The principal investigator of the study could not accommodate our request for an updated analysis comparing extreme quintiles of intake. [3] Pooled RR calculated from RRs for IP-TFA (C18:1) and IP-TFA(other). [4] RR calculated based on RR for 2E% increments and intake levels in extreme quintiles. [5] Data were kindly provided by P. Knekt, A. Reunanen, R. Seppänen, R. Järvinen and A. Aromaa. [6] Data were kindly provided by K. Robien and S. Motzinger. Abbreviations: AT/BC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; F, females; E%, energy %; FMC, the Finnish Mobile Clinic Health Examination Survey, HPFS, The Health Professionals' Follow-up Study; IP-TFA, industrially produced trans fatty acids; IWHS, the Iowa Womens' Health Study; M, males; MONICA, The 1914 cohort + The 1936 cohort + MONICA I and III; NA, not available; NHS, The Nurses' Health Study; R-TFA, ruminant trans fatty acids; SHS, The Strong Heart Study; ZES, The Zutphen Elderly Study.

Table 3: Confounders adjusted for in the included studies

Study	Age	Blood lipids	Blood pressure	BMI	Diabetes status	Education	Familiy history of MI	History of high blood cholesterol	History of hypertension	Hypertension	Intake of alcohol	Intake of cholesterol	Intake of energy	Intake of fat	Intake of fiber	Intake of foods containing IP-TFA	Intake of fruit and vegetables	Intake of linoleic acid	Intake of marine n-3 fatty acids	Intake of MUFA	Intake of protein	Intake of PUFA	Intake of SFA	Intake of α-linolenic acid	Menopausal status	Physical activity	Sex	Smoking status	Use of aspirin	Use of postmenopausal hormones	Vitamin E supplements	Vitamin supplements	Other confounders	**Total no of confounders**
NHS, 1993 [13]	•			•			•			•	•	[1]	•		[1]			•		•			•		•	[1]		•		•		•		**13**
HPFS, 1996 [18]	•			•			•	•	•		•		•		•										•		•					Profession	**11**
AT/BC, 1997 [3]	•		•	•		•					•	[2]	•		•			[2]					[2]		•		•					Treatment.group	**10**
ZES, 2001 [2]	•			•							•	•	•		•					•		•	•				•				•		**11**
NHS, 2005 [1]	•			•			•		•		•	•	•		•		•		•	•	•	•	•	•	•	•	•	•	•	•	•		**22**
SHS, 2006 [14]	•	•		•	•					•	•		•								•					•	•						Study center	**11**
MONICA, 2008 [6 3]	•		•	•		•	•				•	•	•		•	•				•	•	•	•		•	•	•					Cohort ID	**18**
FMC (unpubl.) [4]	•	•	•	•																•		•	•				•							**8**
IWHS (unpubl.) [5]	•			•		•					•	•	•		•				•	•	•	•	•			•	•		•					**15**

[1] There was no adjustments for physical activity or fiber and cholesterol intake in the extracted sub-analyses of R-TFA and IP-TFA intake. However these covariates did not change the estimates in analysis of total-TFA in the whole cohort. [2] Further adjustments for intake of cholesterol, linoleic acid and SFA did not materially change the estimates. [3] A confounder was included if it changed the beta-coefficient for the R-TFA variable 10% or more. [4] Data were kindly provided by P. Knekt, A. Reunanen, R. Seppänen, R. Järvinen and A. Aromaa. [5] Data were kindly provided by K. Robien and S. Motzinger. Abbreviations: AT/BC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; FMC, the Finnish Mobile Clinic Health Examination Survey, HPFS, The Health Professionals' Follow-up Study; IP-TFA, industrially produced trans fatty acids; IWHS, the Iowa Womens' Health Study; MONICA, The 1914 cohort + The 1936 cohort + MONICA I and III; NHS, The Nurses' Health Study; R-TFA, ruminant trans fatty acids; SHS, The Strong Heart Study; ZES, The Zutphen Elderly Study.

The study design characteristics of the nine studies are presented in **Table 1.** Seven studies evaluated the association of total-TFA intake with fatal- and/or total CHD[1-3,14,18](+IWHS and FMC, *unpublished*); four examined the association of R-TFA with fatal- or total CHD[2,3,6,13] and three considered the association of IP-TFA with fatal- or total CHD[2,3,13]. The studies included from 667 to 78,778 participants who were followed for periods ranging from 6 to 21 years. Three studies included only men, two studied only women and three studies both men and women. The published studies' quality was given a rating of 6 to 8 (out of 9) when assessed by the Newcastle-Ottawa Scale [Supplemental Table 1]. Different methods for assessing dietary TFA were applied in the included studies: five used validated FFQs, one used 7-d weighed food records, two used the dietary history method, and one study used single 24-h recalls. Only one study performed repeated dietary assessments[1]. The risk estimates for the most fully adjusted analysis for the individual studies are presented in **Table 2,** and the covariates adjusted for in **Table 3**. The number of covariates ranged from 10 to 22.

We pooled the individual studies' risk estimates for comparison of extreme quintiles of total-TFA intake (**Figure 2A**) and found that total-TFA intake was associated with an increased risk of CHD events of 22% (RR = 1.22; 95% confidence interval (CI): 1.08 - 1.38; P = 0.002) and an almost similar risk of fatal CHD (RR = 1.24; CI: 1.07 - 1.43; P = 0.003). The risk estimates for the individual studies corresponded to variable intake spans of total-TFA ranging from 2.8 g/d to 4 E% (or ~10 g/d), as shown in Table 2. Yet, there was no indication of heterogeneity between the studies; the I^2 value was below 15% for both analyses. When omitting the two unpublished studies, the risk estimate for fatal CHD changed to RR = 1.37 (CI: 1.13 - 1.68; P = 0.002), and when omitting the study that included subjects diagnosed with diabetes at baseline[14], the effect estimates increased modestly to 1.25 (CI: 1.09 - 1.45; P = 0.002) and 1.26 (CI: 1.09 - 1.47; P = 0.003) for total and fatal CHD, respectively.

There was no significant association between R-TFA intake and risk of CHD events when pooling all available estimates for risk associated with increasing R-TFA intake (one study compared extreme quintiles of intake[13], and two studies reported estimates for increments of 0.5 g/d[6] or 0.5 E%[2]) (RR = 0.93; CI: 0.74 - 1.18; P = 0.56; **Figure 2B**). Inclusion of one study evaluating the risk of fatal CHD (comparing intake quintiles[3]) did not change this estimate (RR = 0.92; CI: 0.76 - 1.11; P = 0.36). The test for heterogeneity was not significant and the risk estimates for the individual studies corresponded to in R-TFA intake increments ranging from 0.5 to 1.9 g/d [Table 2].

Only three studies assessed the CHD risk associated with intake of IP-TFA. The pooled effect estimate suggested that IP-TFA intake increases the risk of CHD, although this association did not reach statistical significance (RR = 1.21; CI: 0.97 - 1.50; P = 0.09; **Figure 2C**). There was an indication of heterogeneity between the studies in this analysis (I^2 = 66%; P = 0.05), which was reduced when omitting the study of women only (I^2 = 29%; P = 0.23) whereby also the effect estimate changed (RR = 1.09; CI: 0.98 - 1.22; P = 0.12). The risk estimates for the individual studies corresponded to IP-TFA intake increments ranging from ~1.3 g/d (0.5 E%) to 5.0 g/d [Table 2].

To address whether one single study carried most of the effect, we examined the influence of an individual study on the pooled RR estimate by excluding each study in turn. When the *Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study* (AT/BC) was omitted from the pooled analysis of the risk of fatal CHD associated with intake of total-TFA, the risk estimate decreased and was no longer significant, RR = 1.16 (CI: 0.97 - 1.38; P = 0.10). No other study had substantial influence on the pooled estimates.

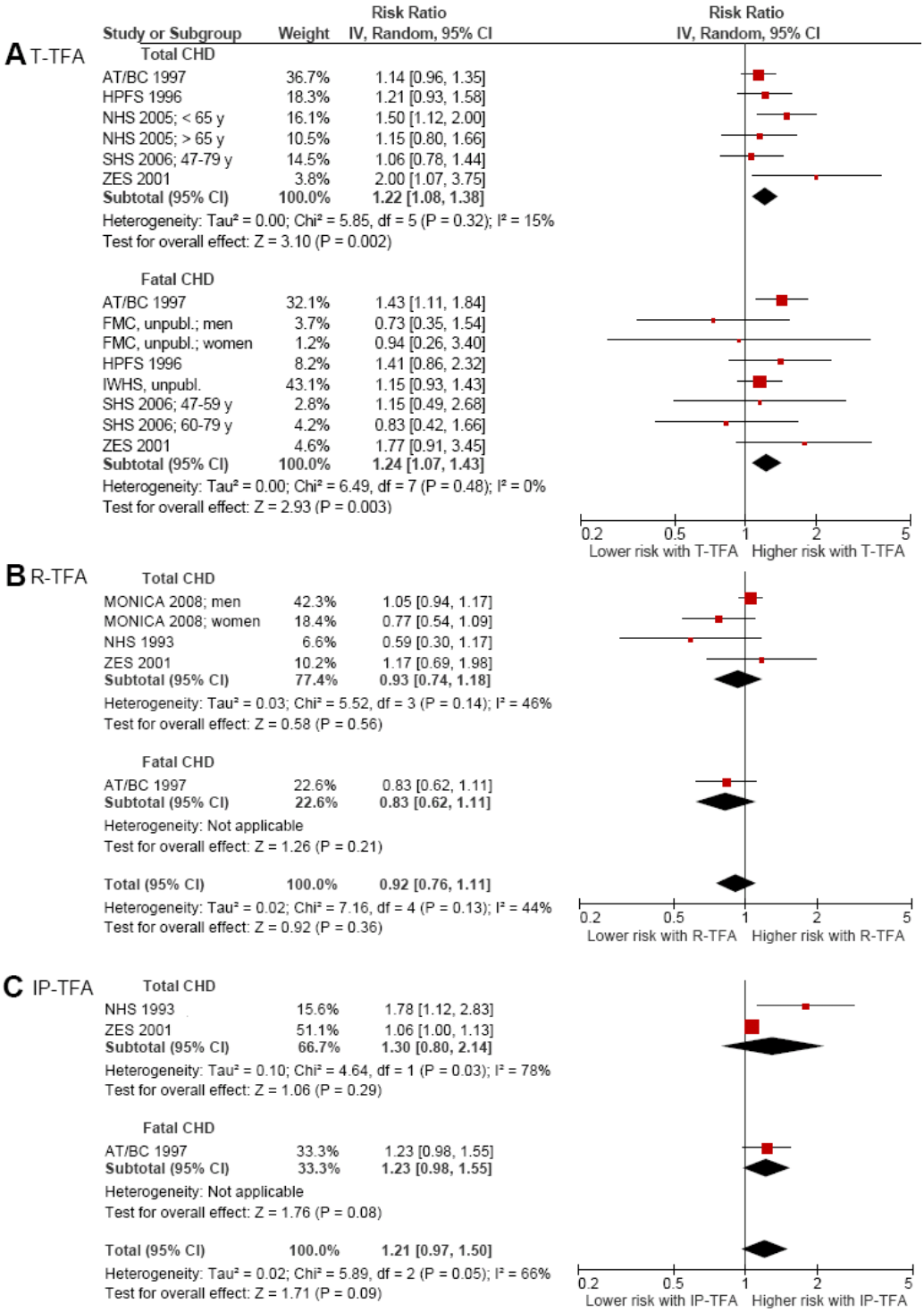

Figure 2: Risk ratios and 95% confidence intervals for fully adjusted random-effects models examining the associations of intake of trans fatty acids from any source (T-TFA) with total coronary heart disease (CHD) events and fatal CHD (Figure 2A); for studies examining the associations of intake of ruminant trans fatty acids (R-TFA) with total CHD events and fatal CHD (Figure 2B) and for studies examining the associations of intake of industrially produced trans fatty acids (IP-TFA) with total CHD events and fatal CHD (Figure 2C). Abbreviations: AT/BC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study[3]; FMC, the Finnish Mobile Clinic Health Examination Survey, HPFS, The Health Professionals' Follow-up Study[18]; IWHS, the Iowa Womens' Health Study; MONICA, The 1914 cohort + The 1936 cohort + MONICA I and III[6]; NHS, The Nurses' Health Study[1,13]; SHS, The Strong Heart Study[14]; ZES, The Zutphen Elderly Study[2].

When restricting the analysis to studies assessing TFA intake by means of validated FFQs or food records, the effect estimate for the association of total-TFA intake with CHD events or fatal CHD did not change considerably (RR = 1.21; CI: 1.07 - 1.37; P = 0.002 and RR = 1.28; CI: 1.09 - 1.49; P=0.002 for total and fatal CHD, respectively), neither did the association of R-TFA with CHD (RR = 0.88; CI: 0.71 - 1.09; P = 0.25). Due to the limited number of identified studies, the relatively low degree of heterogeneity and the fact that the studies were rated almost equally by means of the New-Castle-Ottawa Scale, we did not find the data suitable for further sensitivity analysis.

DISCUSSION

The present systematic review addresses the question of whether we in year 2010 have enough available evidence from prospective cohort studies to evaluate if intake of IP-TFA and R-TFA, respectively, affects the risk of CHD. Our comprehensive literature search of published and unpublished results identified limited new data, whereby data from a total of only eight prospective cohort studies could be pooled.

In accordance with a previous analysis[19], we found that total-TFA intake is associated with an increased risk of fatal and total CHD of more than 20% when comparing extreme quintiles of intake. Inclusion of unpublished data from two cohort studies resulted in a slightly lower risk estimate of fatal CHD (RR = 1.24), compared to that found previously (RR = 1.32)[19]. We did not calculate a risk estimate for CHD events associated with incremental TFA intake, since our literature search did not identify new data that would complement recent meta-analyses estimating that an increased TFA intake of 2 E% is associated with an RR of CHD events of 1.23 (CI: 1.11 - 1.37) based on data from NHS, AT/BC, the *Health Professionals' Follow-up Study* (HPFS) and the *Zutphen Elderly Study* (ZES)[4,19].

Our pooled analyses of the risk associated with R-TFA and IP-TFA intake, respectively, were compromised by a very limited number of available studies, which indeed prohibits any firm conclusions about whether the source of TFA is important. The pooled estimates suggested that while dietary IP-TFA increases the risk of CHD, R-TFA intake doses not. Two studies showed strong positive association of total-TFA with CHD: the ZES which included elderly men with a very high TFA intake[2] and the NHS[1]. Whereas the association could be ascribed to IP-TFA intake in the NHS, the ZES did not indicate that R-TFA is less harmful than IP-TFA.

The validity of any meta-analysis highly depends on the quality of the included studies. Both the quality of the dietary assessment and the confounders adjusted for in the statistical analysis are central features. The lack of an effect in the *Strong Heart Study* (SHS)[14] and the FMC (*unpublished*) could be due to the fact that blood lipids were adjusted for in the analyses from these studies. It is indeed problematic when potential metabolic effects of TFA intake are adjusted for, as the effects of intake of TFA on risk of CHD may be mediated through the effect of these intermediate factors. In the study by Ascherio *et al.*[18], fiber intake was shown to be an important confounder eliminating an otherwise positive association between TFA intake and CHD. Therefore it is positive that all studies but the FMC and the SHS[14] took this dietary factor into account. Whereas all studies adjusted for smoking, age, body mass index and intake of energy and alcohol, only five of the included studies adjusted for other subclasses of dietary fat[1,2,6](+IWHS and FMC, *unpublished*).

Of the included studies, the Nurses' Health Study had the most powerful design with repeated dietary assessments using a FFQ which specifically addressed the type and brand of margarine and a continuously updated food composition database specifically constructed for assessment of TFA composition of foods[20]. In contrast, the validity of single 24-h recalls to estimate usual intakes of TFA is questionable and may explain why no association of TFA intake with CHD was found in the SHS[14].

In epidemiological studies, the assessment of intake of TFA is potentially affected by substantial random measurement error due to a number of factors such as i) participant recall bias; ii) insufficiently updated values in food composition tables and iii) substantial changes in the TFA content of foods over time[21]. During the 1960s margarine became viewed as the healthy alternative to butter because it was lower in SFA[20] and many food manufacturers and restaurants replaced tallow and lard with TFA based products. However, during the 1990s the food industry in many countries made efforts to reduce the TFA content of margarines and shortenings and in recent years a drastic decline in the industrial TFA content of most foods in Western countries has been reported[22]. These changes can only be accounted for by performing repeated assessment of dietary intake.

Also, a problem of confounding by indication may arise in dietary surveys, and this is difficult to correct for: A subject with symptoms of CHD or family history of MI may be more aware of consuming a healthy diet, which in the 1970s meant replacing butter with margarine. Thereby subjects at higher risk may have increased their margarine and with this their IP-TFA consumption while reducing their butter, and R-TFA, consumption.

Considering the difficulties in assessing TFA intake and the fact that very few prospective cohort studies have investigated the association between R-TFA or IP-TFA intake and CHD, data from other types of studies may add important evidence. The results from a case-control study by Ascherio *et al.*[23] are in support of the results from the pooled analyses of cohort studies by indicating that IP-TFA was associated with increased risk, but only at intake levels above 3.3 g/d, whereas R-TFA was neutral (when comparing intakes up to 1.8 g/d).

Whereas IP-TFA intake has consistently been shown to adversely affect risk markers for CHD in controlled trials[4], very few intervention studies have examined the effect of R-TFA on cardiovascular risk markers. One study showed that when men consumed R-TFA in high amounts (3.7 E% or 10.2 g/d), the effect on blood lipids was comparable to the effects of IP-TFA by increasing the low-density lipoprotein cholesterol (LDL-C) and decreasing the high-density lipoprotein cholesterol (HDL-C) concentrations in plasma. However, when R-TFA was consumed in moderate amounts (1.5E% or 4.2 g/d) the effect on blood lipids was not significantly different from that of a control diet with low TFA content (0.8 E% or 2.2 g/d from any source)[24]. Recently, an intervention study also found no difference in the effect of IP-TFA and R-TFA on blood lipids or insulin sensitivity at low intake levels (5 g/d) in overweight women[25]. A third intervention study suggested that R-TFA intake could affect women and men differently. Among women, total cholesterol, HDL-C and LDL-C were higher after intake of 5 E% (~11-12 g/d) R-TFA compared to equivalent intakes of IP-TFA, whereas only minor differences were observed in men[26]. In accordance no significant difference between the effects of TFA from the two sources (P=0.37) on the LDL- to HDL-C ratio was found in the quantitative review by Brouwer *et al.*[27] who compiled the evidence from 29 and 6 treatments with IP-TFA and R-TFA intake, respectively.

In summary, the observational evidence suggests that in contrast to dietary IP-TFA, R-TFA intake does not affect the risk of CHD or may even be slightly protective. However, this could be ascribed to the fact that R-TFA generally is consumed i) in much lower quantities than IP-TFA, and ii) together with dairy products, which may be heart protective[28]. Alternatively, the weak tendency for a risk reduction seen with R-TFA, may relate to the fact that vaccenic acid may be converted endogenously to the conjugated linoleic acid isomer *cis*9, *trans*11-18:2[29], the adipose tissue content of which has been shown to be inversely associated with MI risk[30].

Denmark introduced legislation, effective from 1 January 2004, restricting the use of TFA to a maximum of 2% in oils and fats destined for human consumption. R-TFA were excluded from this legislation. The results of this systematic review support the notion that TFA intake is detrimental to the heart. However, the limited number of available studies prohibits any firm conclusions concerning whether the source of TFA is important. Any legislative discrimination between TFA from the two sources must therefore be the result of a pragmatic decision based on: i) the low risk of achieving high daily intakes of R-TFA when consuming normal foods vs. the risk of consuming considerable amount of IP-TFA with high intakes of certain food products such as fast food and snacks (or with hydrogenated vegetable oil in non-Western countries[31]); ii) the difficulties in removing R-TFA from natural food sources vs. the achievable elimination of IP-TFA from most foods[32]; and iii) the belief that R-TFA-containing foods are often otherwise healthy, whereby the consumption of these should not be restricted vs. the notion that IP-TFA are nutritionally unnecessary.

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CONFLICTS OF INTEREST

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Supplemental Appendix A: Search Strategy

For searches in MEDLINE and EMBASE the following search strategy was be applied: 1) trans fatty acid*; 2) elaidic acid; 3) vaccenic acid*; 4) ruminant fat*; 5) animal fat*; 6) hydrogenated fat*; 7) hydrogenated oil* (1-7 all as free text, and where applicable as exploded MESH/Key Words); 8) 1 or 2 or 3 or 4 or 5 or 6 or 7; 9) myocardial infarction; 10) cardiovascular disease; 11) Sudden death; 12) Ischemic heart disease; 13) Coronary heart diseas* (9-13 as free text and where applicable as exploded MESH/Key Words); 14) 9 or 10 or 11 or 12 or 13; 15) 8 and 14.

A similar search strategy was applied for Food Science and Technology Abstracts (FSTA), with use of Key Words where applicable. In Web of Science we searched for the following terms (trans fatty acid* or elaidic acid or vaccenic acid* or ruminant fat* or animal fat* or hydrogenated oil* or hydrogenated fat*) AND (myocardial infarction* or cardiovascular diseas* or vascular diseas* or ischemic heart diseas* or sudden death). In SciFinder Scholar we searched for relevant references by using the phrase “trans fatty acids and cardiovascular disease”. The search was refined by specifying the document type (Book, Clinical trial, Conference, Dissertation and Journal) and by using the phrase “human not animal”.

Supplemental Table 1: Assessment of risk of bias in published prospective cohort studies according to the New-Castle/Ottawa Scale.

	NHS, 1993 [13]	**HPFS, 1996** [18]	**AT/BC, 1997** [3]	**ZES, 2001** [2]	**NHS, 2005** [1]	**SHS, 2006** [14]	**MONICA, 2008** [6]
Selection [one mark in each section]							
1. Representativeness of the intervention cohort							
a) truly representative of *the average free-living adult subject* *				*			*
b) somewhat representative of *the average free-living adult subject* *							
c) selected group of subjects	√ (Nurses)	√ (Health profs)	√ (Male smokers)		√ (Nurses)	√ (American Indians)	
d) no description of the derivation of the cohort							
2. Selection of the non intervention cohort							
a) drawn from the same community as the intervention cohort *	*	*	*	*	*	*	*
b) drawn from a different source							
c) no description of the derivation of the non intervention cohort							
3. Ascertainment of intervention							
a) secure record (*diet records*) *							* 1
b) structured interview (*validated FFQ)* *	*	*	*		*		
c) written self report (*non-validated FFQ*)							
d) other / no description (*e.g. diet history / 24-hour recall*)				√		√	
4. Demonstration that outcome of interest was not present at start of study							
a) yes *	*	*	*	*	*	*	*
b) no							
Comparability [one or two marks as appropriate]							
1. Comparability of cohorts on the basis of the analysis							
a) study controls for *age, sex, BMI, energy intake* *	*	*	*	*	*	*	*
b) study controls for *Intake of SFA, MUFA, PUFA, fiber* *	* 1			*	*		*

(*Continued*)

Supplemental Table 1 (*continued*)

	NHS, 1993 [13]	HPFS, 1996 [18]	AT/BC, 1997 [3]	ZES, 2001 [2]	NHS, 2005 [1]	SHS, 2006 [14]	MONICA, 2008 [6]
Outcome [one mark in each section]							
1. Assessment of outcome							
a) independent blind assessment (e.g. link to health records) *	*	*	*	*	*	*	*
b) record linkage (e.g. ICD codes on database records) *							
c) self report							
d) other / no description							
2. Was follow up long enough for outcomes to occur							
a) yes, if median duration of follow-up >= *6 years* *	*	*	*	*	*	*	*
b) no, if median duration of follow-up < *6 years*							
3. Adequacy of follow up of cohorts							
a) complete follow up: all subjects accounted for *							
b) subjects lost to follow up unlikely to introduce bias (number lost <= *20%,* or description of those lost suggesting no different from those followed) *	* (98% on deaths)	* 2		*		* (99.8% on deaths)	
c) follow up rate < *80%* and no description of those lost							
d) no statement			√		√		√
Total no of stars	8	7	6	8	7	6	8

[1] (in 94% of subjects). [2] 94% of subjects returned questionnaire in each two year follow-up cycle. Abbreviations: AT/BC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; FMC, the Finnish Mobile Clinic Health Examination Survey, HPFS, The Health Professionals' Follow-up Study; IWHS, the Iowa Womens' Health Study; MONICA, The 1914 cohort + The 1936 cohort + MONICA I and III; NHS, The Nurses' Health Study; SHS, The Strong Heart Study; ZES, The Zutphen Elderly Study.

PAPER II

PAPER II

EFFECT OF TRANS FATTY ACID INTAKE ON ABDOMINAL AND LIVER FAT DEPOSITION AND BLOOD LIPIDS - A RANDOMIZED TRIAL IN OVERWEIGHT POSTMENOPAUSAL WOMEN

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Submitted

ABSTRACT

Background: Intake of industrially produced trans fatty acids (TFA) is, according to observational studies, associated with an increased risk of cardiovascular disease, but the causal mechanisms have not been fully elucidated. Besides inducing dyslipidemia, TFA intake is suspected of promoting abdominal and liver fat deposition.

Objective: We examined the effect of a high intake of TFA as part of an isocaloric diet on whole-body, abdominal and hepatic fat deposition and blood lipids in postmenopausal women.

Methods: In a 16-wk double-blind parallel intervention study, 52 healthy overweight postmenopausal women were randomized to receive either partially hydrogenated soybean oil providing 15.7 g/d of TFA or a control oil with mainly oleic and palmitic acid. Before and after the intervention, body composition was assessed by dual-energy x-ray absorptiometry, abdominal fat by magnetic resonance (MR) imaging, and liver fat by ^{1}H MR spectroscopy.

Results: Compared to the control fat, TFA intake decreased plasma HDL-cholesterol by 10%, increased LDL-cholesterol by 18%, and resulted in an increased LDL/HDL-cholesterol ratio [baseline adjusted mean (95%CI) difference between diet groups 0.41 (0.22; 0.60); P<0.001]. TFA tended to increase body fat [0.46 (-0.20; 1.17) kg; P = 0.16] and waist circumference [1.1 (-0.1; 2.4) cm; P=0.08] more than the control fat, whereas neither abdominal nor liver fat deposition was affected by TFA.

Conclusion: The adverse effect of dietary TFA on cardiovascular disease risk involve induction of dyslipidemia, and perhaps body fat, whereas weight gain-independent accumulation of ectopic fat could not be identified as a contributory factor during short-term intake.

INTRODUCTION

A high intake of industrially produced *trans* fatty acids (TFA) has in observational studies consistently been shown to be associated to an increased risk of coronary heart disease (CHD). In a meta-analysis of four prospective cohort studies it was estimated that that an isocaloric substitution of two percent of the total energy intake (E%) of carbohydrates with TFA corresponds to a 23% increase in the risk of cardiovascular events[1].

It has been well established that TFA increase the ratio of total cholesterol (TC) to HDL-cholesterol (HDL-C) in the blood. A recent meta-analysis estimated that an absolute increase of two E% in the intake of TFA is associated with an increase in the TC/HDL-C ratio of ~0.1 unit, when replacing *cis* monounsaturated fatty acids (MUFA)[2]. An increase of this size is estimated to increase the risk of CHD by ~5%[3], suggesting that TFA exert harmful effects beyond those affecting cholesterol concentrations. Even when taking into account the possible unfavorable effects of TFA on lipoprotein (a), triglycerides, apolipoprotein (Apo) B/ApoAI ratio and C-reactive protein a considerable explanatory gap persists[2]. We hypothesize that this gap might be partly explained by adverse effects of TFA on body fat deposition.

Abdominal fat deposition is an established risk factor for cardiovascular disease and emerging evidence suggests that liver fat should be considered one as well[4]. TFA intake was positively associated with a modest increase in waist circumference during nine years of follow-up in the Health Professional's Follow-up Study, even after adjustment for body mass index (BMI)[5], and with weight change during 8 years of follow-up in the Nurses' Health Study[6]. Moreover, a recent intervention study in non-human primates suggested that TFA might stimulate intra-abdominal fat deposition irrespective of weight gain when consumed in considerable amounts over a long period of time[7], and rodent studies have shown that a high TFA intake may lead to increased deposition of fat in the liver[8,9]. In humans, it has yet to be examined whether TFA intake affects abdominal or liver fat deposition.

We conducted a long-term dietary intervention study with the objective to examine the effect of a high intake of industrially produced TFA on lipid deposition in liver and in the abdominal region in TFA-depleted overweight postmenopausal women. Also, we wanted to confirm previous findings of the effect of TFA on blood lipids.

SUBJECTS AND METHODS

The present work examined the effect of a high intake of TFA from partially hydrogenated soybean oil on risk markers for cardiovascular disease, with the primary endpoints being LDL-cholesterol (LDL-C)/HDL-C ratio and liver fat deposition.

Study design

We conducted a 16-wk double-blind, parallel dietary intervention study, where 52 women were randomized to two test diets stratified by waist circumference. The cut-off waist circumference was set at 96 cm for separation into strata. For each stratum a randomization sequence with random permuted blocks of four was computer generated by a third party prior to initiating the randomization. A coding list was given to the department's kitchen staff. The investigators

responsible for seeing the study subjects allocated the next available number on entry into the trial, and each woman collected her experimental diet directly from the kitchen. Thereby the code was kept undisclosed for the investigators until after study completion.

All study personnel and participants were blinded to the treatment assignment for the duration of the study. Information about which diet was assigned to which group was not disclosed to the researcher responsible for the statistical analyses before completion of the analyses.

The women visited the department for 4 examinations during the study; at screening (1-8 weeks prior to baseline), baseline (wk 0), mid-intervention (wk 8), and at end of treatment (wk 16). In addition, the subjects attended the department for control weighing at wk 4 and wk 12. The habitual activity level during the last year was assessed at baseline using the questionnaire by Baecke et al.[10] and subjects were instructed to maintain their habitual activity level throughout the dietary intervention period. Subsequent to the dietary intervention period the subjects participated in a 12-wk dietary weight loss program after which they came in for follow-up examinations. Data from these examinations will be reported elsewhere.

Subjects attended all visits after an overnight fast of at least 10h. The subjects were requested to avoid vigorous exercise and alcohol consumption on the day before and on the day of examinations at wk 0, 8 and 16. Also, subjects were instructed to consume similar evening meals on the evening before each of the visits at wk 0 and 16.

Subjects

The participating women were recruited from Copenhagen and the surrounding areas by advertisements posted on university web pages and in the local media. Subject inclusion criteria were: healthy; moderately overweight (BMI between 25 and 32 kg/m^2); waist circumference > 80 cm; postmenopausal (self reported) for the last 12 months; and aged 45-70 y, extremes included. The exclusion criteria were diabetes or other chronic diseases (active cancer, psychiatric disorders, moderate to severe rheumatologic disorders, active gastrointestinal disorders, chronic lung disease); history of cardiovascular disease; smoking (current and within six months prior to screening); hypertension (>160/100 mmHg); fasting plasma triglycerides >3 mmol/L; fasting plasma LDL-C >6 mmol/L; fasting plasma glucose >7 mmol/L; use of hormones or anti-hypertensive, anti-lipidemic or anti-cholesterolemic drugs; weight changes >3 kg for two months prior to screening; participation in strenuous physical activity >10 h/week; and abnormalities in routine biochemical and haematological tests. The subjects were not allowed to donate blood during the study and three months prior to screening or to use fish oil as dietary supplements during the study and one month prior to commencement.

To assess how adversely the metabolism of the intervention group was affected by their elevated weight, the study included 19 lean reference subjects (BMI = 19-24 kg/m^2; waist circumference ≤ 80 cm), who underwent baseline examinations only. These reference subjects fulfilled all other inclusion and exclusion criteria as the intervention subjects.

The subjects were given both verbal and written information, whereupon all gave written consent. The study was carried out at the Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark, between April 2008 and March 2009 and was approved by the Municipal Ethical Committee of The Capital Region of Denmark in accordance with the Helsinki-II declaration (H-B_2007-089). Subjects received ~900 US$ as compensation on completion of all the tests. Lean reference subjects received ~500 US$. The trial was registered at clinicaltrials.gov as NCT00655902.

Study diets

In this study, the physiological effect of consuming 15.7 g/d of industrially produced TFA from 26 g/d partially hydrogenated soybean oil was assessed and a 50/50% mix of palm oil and high oleic sunflower oil was used as the control. Both test fats were supplied by AarhusKarlshamn, Denmark. The fatty acid (FA) composition of the two test fats was analyzed chemically as described below for red blood cell FA composition. The two fats mainly differed in the content of TFA, palmitic (16:0), oleic (cis18:1n-9) and linoleic acid (cis,cis18:2n-6) (Table 1). The test fats were incorporated into carrot bread rolls, baked in the department's metabolic kitchen by experienced kitchen staff. The study participants were instructed to consume two bread rolls per day providing 26 g/d of test fat and a total of 2500 kJ (approximately 7 E% protein, 41 E% fat and 51 E% carbohydrate, calculated using the Dankost 3000® dietary assessment software (Danish Catering Center, Herlev, Denmark)). Frozen rolls were handed out to the women every 1-4 weeks from the department for consumption at home.

The intervention was not intended to promote weight gain; hence a clinical dietician instructed the participants how to let the bread rolls iso-calorically substitute food items from their habitual diets. The subjects were weighed every 4 weeks during the dietary intervention. They were offered an additional dietary session if their weight increased more than 2 kg from baseline; three subjects from the TFA group and two from the control (CTR) group accepted the offer.

Table 1: Fatty acid composition of the two test fats

Fatty acid (w/w%)	Control fat	TFA fat
C14:0	0.7	0.2
C16:0	26.7	12.7
C16:1-*trans* (n-7) [1]	< 0.1	< 0.1
C16:1-*cis* (n-7)	< 0.1	< 0.1
C17:0	< 0.1	0.1
C18:0	3.8	6.2
Total C18:1-*trans*	< 0.7	59.0
trans (n-12) / *trans* (n-11) / *trans* (n-10)	< 0.1	9.6
trans (n-9)	< 0.1	12.7
trans (n-8)	< 0.1	12.7
trans (n-7)	< 0.1	10.7
trans (n-6)	< 0.1	7.8
trans (n-5)	< 0.1	3.9
trans (n-4)	< 0.1	1.6
Total C18:1-*cis*	61.4	19.6
cis (n-12) / *cis* (n-11) / *cis* (n-10)	< 0.1	2.9
cis (n-9)	60.2	6.8
cis (n-8)	< 0.1	3.2
cis (n-7)	0.6	3.4
cis (n-6)	0.6	3.2
C18:2-*trans, trans* (n-6)	< 0.1	1.4
C18:2-*cis*, *cis* (n-6)	6.6	0.2
C20:0	0.4	0.4
C22:0	0.4	0.3
Total *trans* fatty acids	< 0.7	60.4

[1] Positions of double bonds are counted from the methyl end of the fatty acid.

Compliance

At baseline, the study subjects were handed a diary in which they were to report whether or not both test rolls were consumed each day. Also, they were to weigh back and report amounts not eaten. As an objective compliance measure the incorporation of FA into phospholipids of red blood cells (RBC) was assessed at wk 0, 8, and 16. For determination of FA composition, saline washed RBCs were spiked with tri-heptadecenoylglyceride, solubilized in 9:1 methanol/toluene, and centrifuged. Fatty acid methyl esters in supernatant aliquots were prepared by sequential treatment with anhydrous methanolic sodium methoxide and methanolic hydrochloric acid[11]. Fatty acid methyl esters were back extracted into hexane, enriched with methyl tricosanoate (23:0) as an internal standard, and quantified by gas chromatography (GC) on high polarity cyano-silica columns and a flame ionization detector (FID) (see also Supplemental Method Description).

Due to co-chromatography of interference with variable relative retention times on the two chromatographic systems used during this study, an accurate quantitative assessment of TFA

concentrations could unfortunately not be made. However, a qualitative ranking of the presence and abundance of the TFA profile relative to *cis*18:1n-7 was possible (Supplemental Figure 1).

Dietary intake

The dietary intake was assessed by 3-day weighed food records before the intervention and in the last week of the intervention. The records were coded, and the energy intake and macronutrient composition were calculated by using a national food database (National Food Agency, Søborg, Denmark[12]) with the Dankost 3000® dietary assessment software (Danish Catering Center, Herlev, Denmark).

Anthropometrics and blood pressure

Body weight and waist and hip circumference were measured at all visits. Body weight was measured in kilograms with one decimal by a Lindeltronic 8000 scale (Sweden). Height was assessed at screening to the nearest 0.5 cm using a Seca stadiometer (Hultafors, Sweden). Waist circumference was measured at the midpoint between the lower part of the last rib and the top of the hip, and hip circumference at the maximal width over the greater trochanters, using a non-extendable linen tape measure to the nearest 0.5 cm. Sagittal abdominal diameter (SAD) was measured in the supine position as the maximal distance between the top of the examination table and a horizontally placed spirit level placed above the abdomen at the level of the iliac crest (in expiration phase).

Blood pressure measurements were performed in the seated position after 10 minutes of rest, with an automatically inflated cuff (UA-787, A & D Co Ltd, Saitama, Japan) at wk 0, 8, and 16.

Body composition was measured by dual-energy x-ray absorptiometry (DXA) using a GE Lunar Prodigy Advance DXA scanner (GE Medical Systems Lunar, Madison, WI, USA). A diagonal line passing through the middle of the femoral neck separated the lower-body and trunkal regions for determination of trunk fat in percentage of total tissue mass.

Magnetic resonance scans

We assessed total, subcutaneous and intra-abdominal adipose tissue (AAT) volumes by magnetic resonance (MR) imaging technique and measured lipid content of the liver by ^{1}H MR spectroscopy. MR-examinations were performed on all subjects at wk 0 and wk 16 one to three days after the other examinations (but at a similar day and time of day for each subject at both visits). The subjects were instructed to refrain from alcohol consumption and vigorous physical activity for 24 h prior to each scan. At the baseline visit, subjects recorded all food and drinks consumed the evening before the scan and on the morning of the day of the scan. At the subsequent visit this intake was to be replicated.

All MR measurements were performed using Achieva 3.0 T MR imaging system (Philips Medical Systems, Best, the Netherlands) and a sense cardiac coil. In the beginning of the MR examination T2w TSE coronal and axial slices through the abdomen were acquired for positioning the spectroscopy volumes of interest (VOI). The spectroscopy VOI (11 mm x 11 mm) was positioned within liver avoiding major blood vessels and intrahepatic bile ducts. A single voxel PRESS sequence with respiratory triggering was used for acquisition. The MR scanner's software was used to fit the acquired spectrum to relative content of water and lipid. Thereby, hepatic lipid content was expressed as lipid relative to water (%)[13].

A fast T1w TFE MR sequence in transverse plane was used for obtaining data for estimating adipose tissue volumes. A transverse slice with 10 mm thickness was acquired for all subjects in the middle of the third lumbar vertebra (L3). Image analysis to determine the total and the intra-AAT fat volumes was carried out on Philips ViewForum workstation using "segmentation tool" in "volume analysis".

Blood sampling and analysis

Fasting blood was sampled from an antecubital vein after minimum 10 min of rest. Routine haematological and biochemical analyses including blood lipids were performed at the Department of Clinical Biochemistry, Gentofte University Hospital, Denmark. Fasting plasma TC, HDL-C, and triglycerides were measured by dry chemistry slides on Vitros 5.1 FS (Johnson & Johnson, Rochester, NY, USA). The coefficients of variation (CV%) were 4.4, 6.0 and 9.6 for TC, HDL-C, and triglycerides, respectively. All tests were performed in accordance with ISO 15189 accreditation. Plasma LDL-C concentration was estimated by the Friedewald equation[14].

Statistical analyses

The study size was estimated based on the effect of TFA on the LDL-C/HDL-C ratio. Based on data from two meta-analyses we assumed a within-subject standard deviation of 0.6 for this ratio[1,15]. We estimated that a minimum of 48 subjects was required to detect an absolute difference of 0.5 in the LDL-C/HDL-C ratio between diet groups with a statistical power of 80%, and a two-sided significance level of 5%. Assuming a standard deviation for liver fat of 6.5% in moderately obese women[16] a difference between diet groups of approximately 6% point liver fat could be detected with at statistical power of 80%, a two-sided significance level of 5% and 24 expected completers in each group. To allow for an estimated 10% drop out rate, 52 participants were recruited.

Data were analyzed using Statistic Analysis Package, SAS© version 9.1 (SAS Institute, Cary, NC, USA). The primary analysis included participants who completed the intervention (n=49). The statistical significance level is defined as $P<0.05$.

At baseline, characteristics of participants in the two diet groups were compared with the use of unpaired t-tests or Kruskal-Wallis tests for skewed data. Baseline values for the overweight intervention subjects were compared to values for the lean reference subjects by similar tests.

Analysis of covariance (ANCOVA) was used to assess the baseline-adjusted difference between diet groups for variables measured at wk 0 and 16, i.e. the baseline value was included as a covariate. For variables measured at wk 0, 4, 8, 12 and 16 and wk 0, 8 and 16, respectively, a mixed model of repeated measures examining the effect of diet and time (wk 4, 8, 12, and 16) and their interactions was applied, again with the baseline value as a covariate, and with “subject” treated as a random effect. Variance homogeneity and normality were investigated by residual plots, histograms and Shapiro-Wilk’s test and data were log transformed when needed. Analyses were performed with and without adjustment for changes in body weight.

RESULTS

49 subjects (24 in the TFA group and 25 in the CTR group) completed the intervention (**Figure 1**). One subject withdrew immediately after baseline examinations (she felt medicalized by the study) and two subjects dropped out after a few weeks due to lack of time.

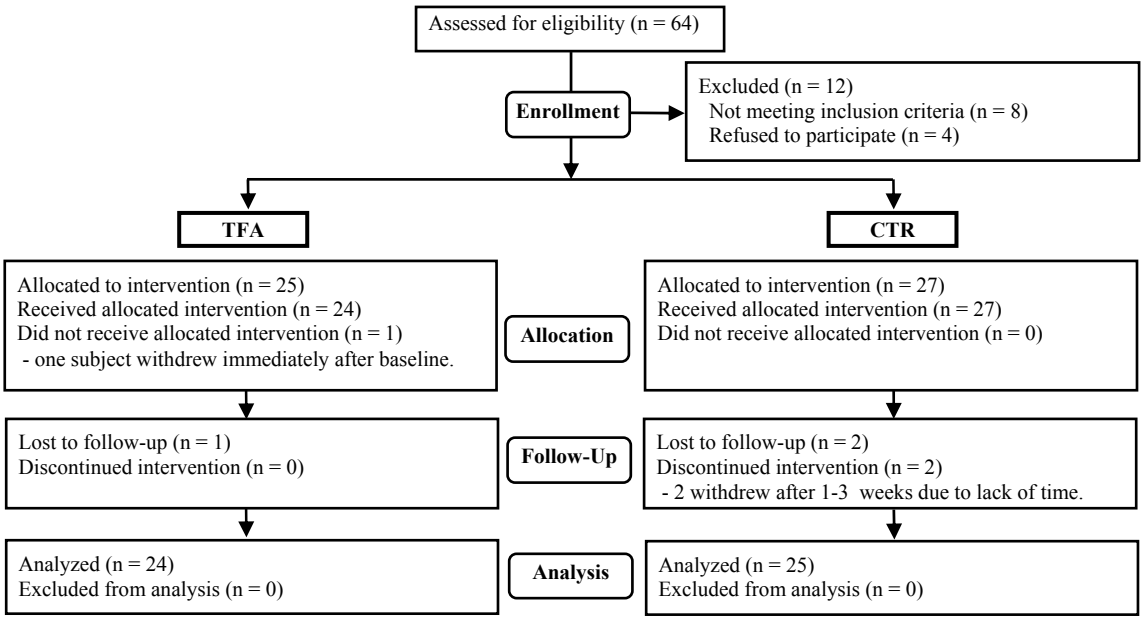

Figure 1: CONSORT flow diagram displaying subjects recruited into the dietary intervention. Subjects were randomly assigned into two diet groups: a trans fatty acid (TFA) group and a control (CTR) group.

Baseline characteristics of the subjects in the two diet groups were comparable and the lean reference subjects were adequately matched for age and height (**Table 2**).

Table 2: Baseline characteristics for subjects in the trans fat (TFA; n=24) and control (CTR; n=25) groups and for lean references (n=19) [1]

	TFA	CTR	Lean references	P[2]
Age (y)	58.5 ± 4.6	58.8 ± 5.5	60.1 ± 5.9	ns
Height (cm)	165.3 ± 5.5	166.9 ± 5.2	166.7 ± 4.3	ns
Weight (kg)	78.7 ± 7.1	78.4 ± 8.6	59.4 ± 4.8	<0.001
BMI (kg/m^2)	28.8 ± 1.7	28.1 ± 2.2	21.3 ± 1.4	<0.001
Waist circumference (cm)	97.1 ± 7.3	95.5 ± 6.8	74.9 ± 3.8	<0.001
Blood pressure				
Systolic (mmHg)	118 ± 12	118 ± 11	120 ± 14	ns
Diastolic (mmHg)	81 ± 8	82 ± 6	80 ± 10	ns
Physical activity index[10]	7.5 ± 1.4	7.9 ± 1.6	8.6 ± 1.4	0.02

[1] Values are means ± SD, completers only. [2] P value refers to difference between overweight intervention subjects (diet groups combined) and lean reference subjects by unpaired t-tests (ns, non significant; P>0.05).

Compliance with the study diets

The test diets provided on average 28% of the subjects' energy requirements. Self reported compliance assessed using study diaries showed that 98% of all test bread rolls were consumed, with no difference between diet groups. The presence of elevated *trans*18:1 residues in RBC phospholipids determined by GC/-FID was used as an objective compliance measure. Due to an analytical interference, accurate quantification of *trans*18:1 isomers was not possible. Instead a qualitative intensity scale was established and used to assign a value of TFA enrichment to each sample (Supplemental Figure 1). These results were transformed to ranks and showed that subjects in the TFA group all had elevated *trans*18:1 residue levels at both 8 and 16 wk of intervention, whereas the control subjects did not (**Figure 2**). The intervention modestly decreased the relative abundance of saturated fatty acids (SFA) and increased that of polyunsaturated fatty acids (PUFA) in the RBCs of both diet groups after 16 wk. Oleic acid increased in the control group only (Supplemental Table 1).

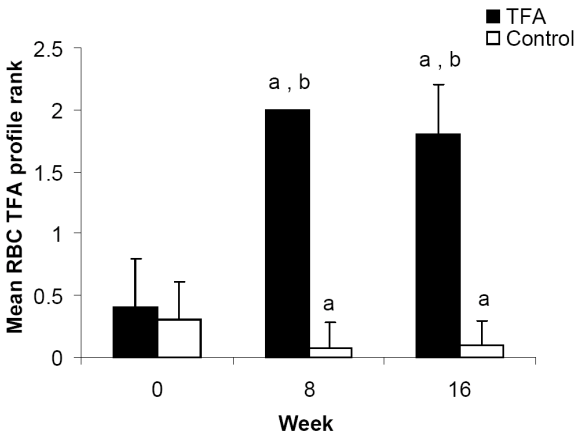

Figure 2: Semi-quantitative assessment of *trans* fatty acids (TFA) in red blood cell (RBC) phospholipids assessed by gas chromatography. Based on semi-quantitative assessments of RBC *trans*18:1 profiles, including the scores 0, 0.5, 1, 1.5 and 2 (where 2 signifies full enrichment) [Supplemental Figure 1], maximal TFA-enrichment was observed by wk 8 and was generally sustained at wk 16 in the TFA group (n=24), whereas the subjects in the control group experienced no TFA-enrichment (n=25). Changes from baseline TFA profiles were assessed by repeated measures ANOVA. Due to the non-normal distribution of the semi-quantitative data, the analyses were performed on ranked data. [a]significantly different from the baseline value ($P<0.001$). [b]significantly different from the control group ($P<0.001$).

Dietary intake

The energy intake in the last week of the intervention (representing the intake during the dietary period) was not different between diet groups (Supplemental Table 2). The only significant dietary differences between diet groups during the intervention were the contributions of energy from MUFA and TFA, indicating that the diets were overall comparable apart from the FA composition. TFA constituted 7% of the energy intake in the TFA group.

Anthropometrics

Contrary to the intention, body weight increased slowly but steadily in both diet groups during the dietary intervention with no difference between groups ($P= 0.002$ for effect of time in baseline adjusted repeated measures analysis testing for interaction between diet and time) (**Figure 3**). At wk 16 mean (± SEM) weight changes from baseline in the TFA and CTR group were 1.1 ± 0.2 kg and 1.1 ± 0.3 kg, respectively. Waist circumference tended to increase more in the TFA group after adjustment for baseline values [mean difference (95% confidence interval (CI) between diets 1.1 cm (-0.1 to 2.4); $P = 0.08$]; adjusting for weight change had no effect on this estimate (Figure 3). There was no difference between groups in hip circumference (data not shown).

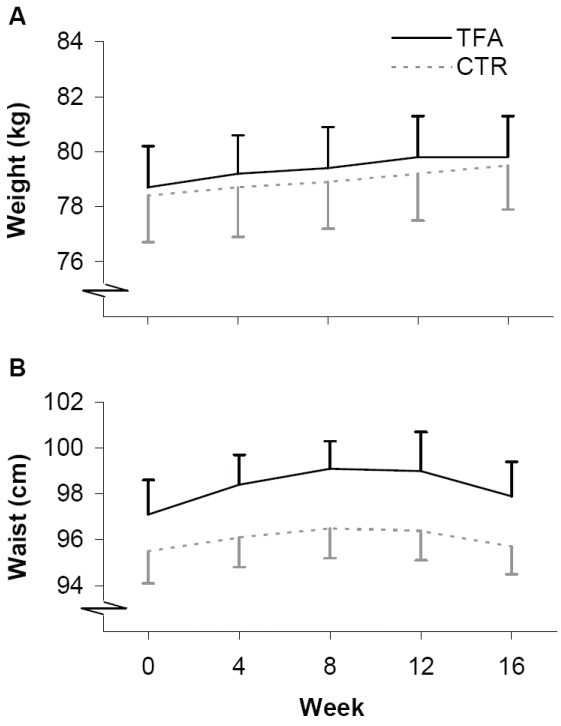

Figure 3: Development in mean (±SEM) body weight (figure 3A) and waist circumference (figure 3B) in the trans fat (TFA; n=24) and control (CTR; n=25) groups during the 16-wk dietary intervention. Baseline values were not significantly different (unpaired t-test, P>0.05). Note that the y-axes have been truncated. In spite of the isocaloric study design, body weight increased significantly in both diet groups (P=0.002 for effect of time in repeated measures analysis of covariance testing for interaction between diet group and time (wk 4, 8, 12, 16) with wk 0 as a covariate; there was no significant interaction between diet and time, nor effect on diet). Waist circumference tended to increase more in the TFA group (P=0.08 for effect of diet; there was no significant interaction between diet and time, nor effect of time).

Fat deposition

The changes in whole body and trunk fat mass assessed by DXA were in line with the trend for waist circumference. The fat gain was numerically greater in the TFA group, although not significantly so when compared to controls (**Table 3**). The baseline adjusted mean difference in body fat between diets at wk 16 was 0.46 kg (CI: -0.20 to 1.17; P = 0.16).

Due to technical reasons, the liver fat was obtained by ^{1}H MR spectroscopy only in 23 subjects in each diet group, and fat distribution was obtained by MR imaging in 19 subjects in each diet group. The dietary intervention did not significantly affect liver fat. The between-subject variability for liver fat changes was large (**Figure 4**), and thereby the estimate of the liver fat concentration being 7% lower in the TFA group at week 16 compared to controls, came with a wide

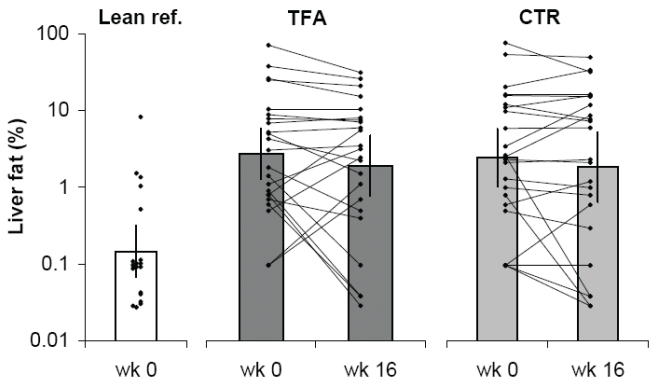

Figure 4: Liver fat percentage in the trans fat group (TFA; n=23) and in the control group (CTR; n=23) before and after 16 wk of dietary intervention and in lean references (n=19) obtained by ^{1}H MR spectroscopy. Bars show geometric means (95% confidence intervals). There was no significant effect of diet, P=0.87 for differences between diet groups at wk 16 with wk 0 as a covariate, by analysis of covariance performed on log transformed data. The liver fat % in lean references was significantly lower than in overweight intervention subjects (diet groups combined) by Kruskal-Wallis test for skewed data; P<0.01.

confidence interval (CI: -61 to 120%; P = 0.87; Table 3). According to MR imaging, the dietary intervention affected neither total, subcutaneous, intra-AAT nor the ratio of intra- to subcutane-

ous AAT (Table 3). As expected, the liver fat percentage, as well as the subcutaneous and intra-AAT volumes, was significantly greater in overweight subjects compared to lean references.

Table 3: Body composition and abdominal fat deposition in the trans fat (TFA) and control (CTR) groups and in lean references [1]

	Week 0 [2]	Week 16	P_{diet} [3]
Fat mass (kg)			0.16
TFA (n=24)	33.4 ± 1.0	34.6 ± 1.1	
CTR (n=25)	31.8 ± 1.1	32.7 ± 1.0	
Lean references (n=19)	16.8 ± 0.8 [4]	n/a	
Fat free mass (kg)			0.20
TFA (n=24)	45.6 ± 0.7	45.6 ± 0.8	
CTR (n=25)	47.0 ± 1.0	47.3 ± 0.9	
Lean references (n=19)	43.3 ± 0.6 [4]	n/a	
Whole body fat (%)			0.12
TFA (n=24)	42.1 ± 0.7	43.0 ± 0.7	
CTR l (n=25)	40.2 ± 0.9	40.7 ± 0.8	
Lean references (n=19)	27.8 ± 0.9 [4]	n/a	
Trunk fat (%)			0.17
TFA (n=24)	43.2 ± 0.9	44.2 ± 0.8	
CTR (n=25)	41.3 ± 1.0	42.0 ± 0.9	
Lean references (n=19)	25.5 ± 1.1 [4]	n/a	
Sagittal diameter (cm)			0.57
TFA (n=24)	22.2 ± 0.3	22.8 ± 0.4	
CTR (n=25)	22.1 ± 0.3	22.5 ± 0.3	
Lean references (n=19)	17.2 ± 0.2 [4]	n/a	
Total AAT (cm^3) [5]			0.85
TFA (n=19)	446 ± 20	458 ± 20	
CTR (n=19)	390 ± 21	407 ± 21	
Lean references (n=19)	138 ± 10 [4]	n/a	
Subcutaneous AAT (cm^3) [5]			0.46
TFA (n=19)	313 ± 15	317 ± 15	
CTR (n=19)	255 ± 16	261 ± 14	
Lean references (n=19)	106 ± 7.6 [4]	n/a	
Intra-AAT (cm^3) [5]			0.58
TFA (n=19)	133 ± 9	141 ± 10	
Control (n=19)	135 ± 14	146 ± 17	
Lean references (n=19)	33 ± 4 [4]	n/a	
Intra/subcutaneous AAT [5, 6]			0.67
TFA (n=19)	0.45 ± 0.04	0.46 ± 0.05	
CTR (n=19)	0.56 ± 0.08	0.60 ± 0.10	
Lean references (n=19)	0.31 ± 0.03 [4]	n/a	
Liver fat (%) [6]			0.87
TFA (n=23)	2.7 (1.3; 5.7)	1.9 (0.8; 4.6)	
CTR (n=23)	2.4 (1.0; 5.6)	1.8 (0.6; 5.1)	
Lean references (n=19)	0.1 (0.07; 0.3) [4]	n/a	

[1]Values are means ± SEM or geometric means (95% confidence interval) for skewed data, completers only. n/a, not applicable; AAT, abdominal adipose tissue. [2]There were no significant differences in baseline values between diet groups (unpaired t-test). [3]P value refers to differences between diet groups at wk 16 with wk 0 as a covariate, by analysis of covariance (ANCOVA). Adjusting for weight change did not affect the results. [4]Significantly different from overweight intervention subjects (diet groups combined) by unpaired t-test or Kruskal-Wallis test for skewed data; $P<0.05$. [5]Obtained from MR imaging. [6]ANCOVA performed on log10-transformed values.

Plasma lipids

TFA adversely affected blood cholesterol by increasing LDL-C and decreasing HDL-C (**Table 4**). Hereby, the ratio between the two was significantly greater in the TFA diet group at both wk 8 and 16 compared to the control group [baseline adjusted mean (95%CI) difference between diets 0.41 (0.22 to 0.60); P<0.001]. Accordingly, the TC/HDL-C ratio was also significantly increased by TFA [difference between diets 0.45 (0.24 to 0.65); P<0.001]. There was no effect of diet on fasting plasma triglycerides.

Table 4: Blood lipids in the trans fat (TFA; n=24) and control (CTR; n=25) groups and in lean references (n=19) [1]

				ANCOVA [3]		
	Week 0 [2]	Week 8	Week 16	$P_{time*diet}$	P_{diet}	P_{time}
Total cholesterol (mmol/L)				0.16	0.03	0.88
TFA	5.44 ± 0.19	6.07 ± 0.21	5.95 ± 0.19			
CTR l	5.54 ± 0.20	5.77 ± 0.18	5.86 ± 0.21			
Lean references	5.46 ± 0.18	n/a	n/a			
HDL cholesterol (mmol/L)				0.23	0.03	<0.001
TFA	1.54 ± 0.06	1.54 ± 0.05	1.38 ± 0.04			
CTR	1.65 ± 0.07	1.69 ± 0.08	1.59 ± 0.06			
Lean references	1.69 ± 0.05	n/a	n/a			
LDL cholesterol (mmol/L)				0.24	0.002	0.01
TFA	3.39 ± 0.18	3.91 ± 0.19	4.01 ± 0.18			
CTR	3.36 ± 0.20	3.46 ± 0.16	3.72 ± 0.19			
Lean references	3.47 ± 0.17	n/a	n/a			
LDL/HDL-cholesterol ratio				0.23	<0.001	<0.001
TFA	2.31 ± 0.16	2.64 ± 0.17	3.02 ± 0.20			
CTR	2.20 ± 0.20	2.20 ± 0.17	2.45 ± 0.16			
Lean references	2.08 ± 0.11	n/a	n/a			
Total/HDL-cholesterol ratio				0.22	<0.001	<0.001
TFA	3.65 ± 0.19	4.08 ± 0.22	4.45 ± 0.23			
CTR	3.54 ± 0.23	3.60 ± 0.21	3.83 ± 0.20			
Lean references	3.25 ± 0.12	n/a	n/a			
Triglycerides (mmol/L) [4]				0.78	0.39	0.09
TFA	1.13 ± 0.10	1.39 ± 0.15	1.24 ± 0.09			
CTR	1.19 ± 0.10	1.35 ± 0.12	1.23 ± 0.11			
Lean references	0.67 ± 0.04 [5]	n/a	n/a			

[1] Values are means ± SEM, completers only. n/a, not applicable. [2] There were no significant differences in baseline values between diet group (unpaired t-test, P>0.05). [3] P values were derived by repeated measures analysis of co-variance testing for interaction between diet group and time (wk 8, 16) with wk 0 as a covariate. P values for effects of diet and time, respectively, were derived from analyses omitting the interaction term. [4] ANCOVA performed on log10-transformed values. [5] Significantly different from overweight intervention subjects (diet groups combined) by unpaired t-test; P<0.05.

Safety assessments

Blood pressure was stable in both diet groups during the entire study period (data not shown). Hematological (hemoglobin, thrombocytes, leucocytes and erythrocytes) and biochemical (albumin, bilirubin, creatinin, sodium, and potassium) blood parameters did not change during the course of the study. Nor did plasma liver enzymes (alanine aminotransferase, aspartate amino transferase and alkaline phosphatase; data not shown).

DISCUSSION

The present study aimed at decreasing the explanatory gap between the harmful effect of industrially produced dietary TFA seen in observational studies and the affirmed effects on individual risk markers for cardiovascular disease. Due to the Danish legislation, which went into force in 2003 and practically eliminated industrially produced TFA from food products sold in Denmark, the study population was depleted of industrially produced TFA. In metabolically vulnerable women the present intervention study found that a high intake of TFA from partially hydrogenated soybean oil for 16 weeks increased the ratio of plasma LDL- to HDL-C but had no detectable effect on hepatic and abdominal fat deposition.

In dietary interventions examining the effect of a specific FA composition, it is always a challenge to decide on an appropriate control fat. For the present study, a fat containing predominantly MUFA but also SFA and PUFA was chosen, because fast food products and snacks produced without TFA have higher levels of all three FA classes compared to similar products high in TFA[17]. Also, a recent report has documented that when products high in TFA are reformulated to eliminate TFA, the TFA is mainly replaced by unsaturated FA[18].

TFA intake led to an increase in LDL-C as well as a decrease in HDL-C concentrations, resulting in a mean difference between diet groups in the LDL/HDL-C ratio of 0.41, or 0.059 per E% TFA. This is comparable to the results from a recent quantitative review of 23 dietary trials estimating that a 1 E% increase in industrially produced TFA would result in an increase in the LDL/HDL-C ratio of 0.055 when replacing *cis* MUFA[19]. However, in the present study the control fat did not solely consist of *cis* MUFA.

The mean difference between diet groups in the TC/HDL-C ratio of 0.45 was somewhat larger than what could have been expected from a recent meta-analysis by Mozaffarian & Clarke[2] estimating that a replacement of 1 E% TFA by SFA, MUFA and PUFA increases the TC/HDL-C ratio by 0.031, 0.054 and 0.067, respectively[2]. The present study used a blend of palm oil and high oleic sunflower oil as a control fat whereby 7 E% from TFA in the TFA group was compared to an intake of 1.6 E% from SFA, 4.7 E% from MUFA and 0.7 E% from PUFA in the control group. According to the estimates from the study by Mozaffarian & Clarke, a difference in the TC/HDL-C ratio of 0.35 between the TFA and control diet was expected. The effect of TFA on blood lipids in the present study is also considerably greater than that observed in our previous 8-wk study with similar design but with normal weight young men[20]. The fact that only moderately overweight women above the age of 45 y were enrolled may help to explain the more pronounced effect of TFA on plasma cholesterol in the present study. It has previously been suggested that a high TFA intake may be more detrimental towards women than men[21,22].

The TC/HDL-C ratio is twice as informative of cardiovascular risk as TC or LDL-C alone[3]. The increase in the TC/HDL-C ratio in the TFA group of 0.45 compared to the control subjects, may translate into an increase in the risk of CHD of approximately 20%[3]. An intake of 15 g/day of TFA may be a likely scenario in selected communities or subpopulations around the world. E.g., in an Iranian population the mean TFA intake was as high as 12.3 g/day in year 2001-2003[23] and in 1989-91, the mean TFA intake in the US population was 5.3 g/day and 10% of the population was estimated to have an intake above 9.4 g/d[24]. However, although more recent intake estimates are lacking, the TFA intake in the US has most likely declined in recent years as increased trans fat awareness has forced the food industry to limit the content of TFA[25]. Still, it is a matter of concern that one may obtain an intake of TFA beyond 15 g in a single serving of fast food from international fast food restaurants in selected countries, as assessed in 2006[26].

In prospective cohort studies TFA intake has been positively associated with changes in weight and waist circumference[5,6]. In our study, women in both diet groups experienced slight weight gains during the course of the intervention, implying that they did not succeed in isocalorically substituting the test food for food items from their habitual diets. We observed weak trends towards greater increases in waist circumference and total fat mass in the TFA group compared to controls independent of body weight changes. However, these trends were not reflected by the observed changes in abdominal fat deposition. We saw no differences between the diet groups in either intra-abdominal or subcutaneous abdominal fat deposition, nor in the ratio between the two. Thereby, the present study could not substantiate the findings from a study in green monkeys observing an increase in the ratio of intra-to subcutaneous AAT volume irrespective of weight gain after 6 y of isocaloric TFA feeding[7]. It is likely that 16 weeks of exposure was too short a period of time to detect changes in abdominal fat deposition independent of body weight even with high daily TFA intakes. Indeed body weight gain may be an intermediary step leading to TFA induced increases in abdominal fat deposition, which we may not have been able to detect with our isocaloric study design. However, in comparison, conjugated linoleic acid supplementation of 4.5 g/d has been shown to significantly decrease fat mass (assessed by DXA) in postmenopausal women within 16 weeks on iso-caloric diets[27]. Also, isocaloric diets high in PUFA have been found to significantly decrease subcutaneous AT (assessed by single-slice MRI) compared to diets high in SFA within 5 wk, despite no changes in body weight[28]. This confirms that abdominal fat distribution can be affected by dietary fat manipulations within the timeframe of our study.

Since a long-term dietary intervention study providing high daily amounts of TFA is not feasible, it is questionable if an effect on abdominal fat will ever be established in humans. Conversely, potential changes in liver fat due to high intakes of TFA might be detected within a reasonable period of time. Liver fat is thought to be a more sensitive marker of a deleterious fat deposition pattern[29] and is suggested to be a more important risk factor for obesity-related metabolic disorders than intra-AAT[30].

We are the first to report on the influence of a high TFA intake on liver fat deposition in humans. The data from the present study were consistent with liver fat being from 61% lower to 140% higher in the TFA group at wk 16 when compared to controls. Given the geometric mean liver fat concentration of approx. 2.5% at baseline, the confidence limits may translate into an absolute mean decrease in liver fat of 1.5% or an absolute increase of 3.5%. Therefore, our results cannot discount the possibility that TFA intake may exert effects on liver fat smaller than this, prohibiting firm conclusions on minor changes. However, no detectable trend in liver fat, was observed in either direction in either diet group and plasma liver enzymes remained unchanged, arguing that if TFA induced changes in liver fat concentration, they were small indeed.

In several rodent studies, TFA feeding has been shown to increase liver fat by more than 100% compared to animals fed *cis* FA[8,9,31], although this is not a consistent finding[32,33]. Yet, extrapolation from animal studies is problematic. While it has been documented that liver fat deposition in humans is affected by intake of energy and fat (liver fat increased 100% (from 2 to 4% and from 11 to 22%) after 3-4-day hypercaloric high-fat diets[34,35] and 35% (from 10 to 14%) after 14-day eucaloric high-fat diets[36]), the influence of dietary FA composition on liver fat deposition in humans has yet to be evaluated in controlled trials. It has however been documented that intake of specific FAs can affect liver fat accumulation; consumption of n-3 long chain PUFA supplements (2-4 g/d) for 8 - 26 wk has been shown to reduce liver fat in subjects with hepatic steatosis (liver fat contents of at least 5%)[37,38].

Also, liver fat is affected by body weight changes and has been shown to decrease by 30-60% in obese subjects after ~6-11 kg weight loss[39,40]. In accordance, we observed significant decreases of about 40% in liver fat mean values after a 12-wk dietary weight loss period (mean weight loss ~4 kg) subsequent to the dietary intervention (data not shown).

In conclusion, our research confirms that part of the increase in risk of cardiovascular disease seen with increased TFA consumption in observational studies is likely to be due to dyslipidemia. The magnitude of the observed impact of TFA intake on blood cholesterol levels suggests that older and overweight populations may be more susceptible to TFA-induced metabolic changes than young and lean ones. Conversely, weight gain-independent induction of liver or intra-abdominal fat deposition could not be identified as contributory explanatory mechanisms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplemental table 1: Fatty acid composition of phospholipids in red blood cell (RBC) membranes expressed as a mole percent of total quantified lipids with mean intensities >1% in the trans fat (TFA; n=24) and control (CTR; n=25) groups and in lean references (n=19) [1].

				ANCOVA[3]		
	Week 0 [2]	Week 8	Week 16	$P_{time*diet}$	P_{diet}	P_{time}
C16:0				0.99	0.29	<0.001
TFA	40.22 ± 0.71	41.18 ± 0.93	37.84 ± 0.71			
CTR	41.98 ± 1.19	40.44 ± 0.94	37.10 ± 0.71			
Lean references	40.78 ± 0.92	n/a	n/a			
C16:1, n-7 [4, 5]				0.58	0.47	0.002
TFA	0.83 ± 0.07	1.09 ± 0.08	0.82 ± 0.07			
CTR	1.04 ± 0.08	1.18 ± 0.09	1.04 ± 0.15			
Lean references	0.77 ± 0.08	n/a	n/a			
C18:0				0.86	0.04	<0.001
TFA	20.85 ± 0.39	18.98 ± 0.61	17.05 ± 0.40			
CTR	21.04 ± 0.61	19.93 ± 0.52	18.18 ± 0.41			
Lean references	23.56 ± 0.67 [6]	n/a	n/a			
C18:1, n-9				0.63	0.04	<0.001
TFA	17.74 ± 0.65	18.18 ± 0.47	16.25 ± 0.48			
CTR	16.92 ± 0.59	19.36 ± 0.49	16.99 ± 0.38			
Lean references	15.70 ± 0.72	n/a	n/a			
C18:1, n-7				0.38	0.02	0.74
TFA	1.51 ± 0.13	1.33 ± 0.06	1.43 ± 0.09			
CTR	1.30 ± 0.15	1.20 ± 0.08	1.16 ± 0.07			
Lean references	1.24 ± 0.11	n/a	n/a			
C18:2, n-6				0.41	0.04	<0.001
TFA	10.51 ± 0.56	11.25 ± 0.57	13.08 ± 0.41			
CTR	10.23 ± 0.85	10.40 ± 0.46	11.56 ± 0.49			
Lean references	9.68 ± 0.76	n/a	n/a			
C20:3, n-6 [5]				0.20	0.37	<0.001
TFA	0.93 ± 0.10	0.59 ± 0.08	1.08 ± 0.09			
CTR	1.16 ± 0.12	0.79 ± 0.14	1.64 ± 0.20			
Lean references	0.76 ± 0.08	n/a	n/a			
C20:4, n-6 [5]				0.40	0.50	<0.001
TFA	4.95 ± 0.49	4.68 ± 0.56	7.74 ± 0.49			
CTR	4.12 ± 0.48	4.25 ± 0.65	7.68 ± 0.46			
Lean references	4.97 ± 0.48	n/a	n/a			
C22:5, n-3 [7]				0.72	0.90	0.02
TFA	0.79 ± 0.12	0.92 ± 0.09	1.18 ± 0.14			
CTR	0.80 ± 0.11	0.88 ± 0.10	1.22 ± 0.13			
Lean references	0.85 ± 0.09	n/a	n/a			
C22:6, n-3 [5]				1.00	0.98	<0.001
TFA	1.69 ± 0.23	1.83 ± 0.31	3.55 ± 0.36			
CTR	1.40 ± 0.25	1.62 ± 0.28	3.47 ± 0.36			
Lean references	1.65 ± 0.35	n/a	n/a			
∑SFA [5]				0.98	0.97	<0.001
TFA	61.07 ± 0.81	60.15 ± 1.42	54.89 ± 0.74			
CTR	63.02 ± 1.48	60.38 ± 1.24	55.27 ± 0.94			
Lean references	64.34 ± 1.45	n/a	n/a			

(*Continued*)

Supplemental table 1 (*Continued*)

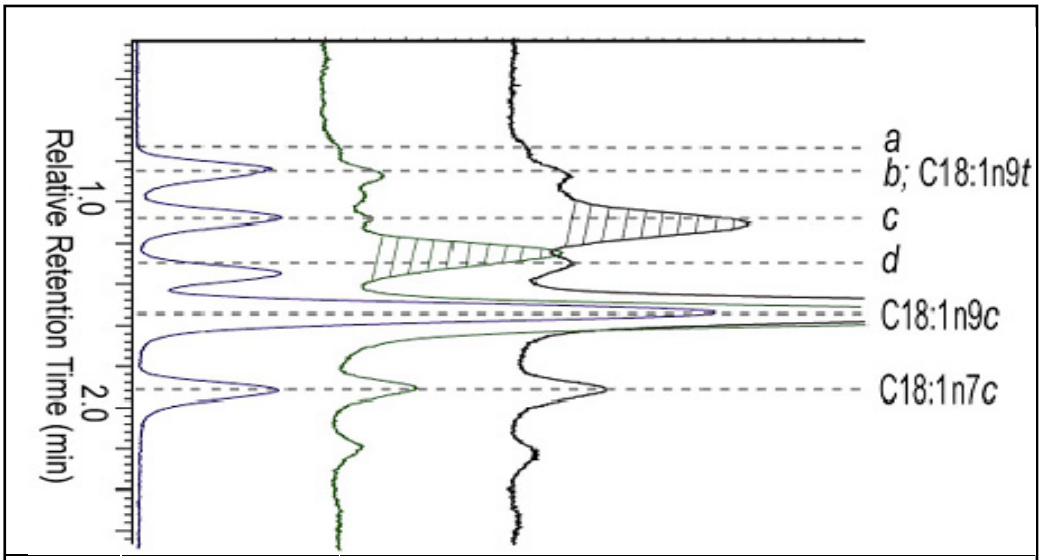
	Week 0 [2]	Week 8	Week 16	ANCOVA[3] $P_{time*diet}$	P_{diet}	P_{time}
∑MUFA				0.66	0.08	<0.001
TFA	20.08 ± 0.71	20.60 ± 0.48	18.50 ± 0.53			
CTR	19.26 ± 0.65	21.74 ± 0.53	19.19 ± 0.46			
Lean references	17.72 ± 0.76 [6]	n/a	n/a			
∑PUFA				0.81	0.56	<0.001
TFA	18.87 ± 0.91	19.27 ± 1.45	26.61 ± 0.90			
CTR	17.71 ± 1.28	17.94 ± 1.40	25.57 ± 0.95			
Lean references	17.92 ± 1.28	n/a	n/a			

[1] Values are means ± SEM, completers only. n/a, not applicable; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, trans fatty acids. Accurate quantitative determination of RBC C18:1 *trans* fatty acids was prevented by the presence of an analytical interference with variable relative retention times on the gas chromatographs used in this study, see Figure 2 and Supplemental Figure 1 for details. [2] There were no significant differences between diet groups at baseline (unpaired t-test; P>0.05). [3] P values were derived by repeated measures analysis of covariance testing for interaction between diet group and time (wk 8 and 16) with wk 0 as a covariate. P values for effects of diet and time, respectively, were derived from analyses omitting the interaction term. [4]All double bonds have *cis* configuration. Positions of double bonds are counted from the methyl end of the fatty acid. [5]ANCOVA analysis performed on log 10 transformed values. [6] Significantly different from overweight intervention subjects (diet groups combined) by unpaired t-test, P<0.05). [7] ANCOVA analysis performed on square root transformed values.

Supplemental Table 2: Dietary intake estimated using 3-d weighed food records in the trans fat (TFA; n=24) and control groups (CTR; n=25) [1]

	Week 0 [2]	Week 16	P[2]
Energy (MJ/d)			ns
TFA	7.35 ± 0.30 [a]	8.30 ± 0.34 [b]	
CTR l	8.26 ± 0.48	8.01 ± 0.43	
Protein (E%)			ns
TFA	18.3 ± 0.6 [a]	14.5 ± 0.4 [b]	
CTR	17.0 ± 0.7	16.0 ± 0.8	
Carbohydrate (E%)			ns
TFA	47.3 ± 1.5 [a]	44.2 ± 1.0 [b]	
CTR	45.0 ± 1.7	44.0 ± 1.6	
Fat (E%)			ns
TFA	31.0 ± 1.2 [a]	37.1 ± 1.0 [b]	
CTR	30.8 ± 1.8	33.9 ± 1.4	
SFA (E%)			ns
TFA	11.3 ± 0.6	11.2 ± 0.5	
CTR	11.3 ± 0.9	11.8 ± 0.6	
*cis*MUFA (E%)			<0.001
TFA	9.8 ± 0.5	10.3 ± 0.4	
CTR	9.7 ± 0.7 [a]	13.9 ± 0.8 [b]	
*cis*PUFA (E%)			ns
TFA	5.0 ± 0.2 [a]	4.5 ± 0.3 [b]	
CTR	5.1 ± 0.3	4.3 ± 0.3	
TFA (E%)			<0.001
TFA	0.4 ± 0.0 [a]	7.0 ± 0.2 [b]	
CTR	0.5 ± 0.1 [a]	0.3 ± 0.0 [b]	
Cholesterol (mg/d)			ns
TFA	331 ± 27 [a]	257 ± 24 [b]	
CTR	310 ± 23	281 ± 32	
Alcohol (g/d)			ns
TFA	8.7 ± 2.0	14.4 ± 4.1	
CTR	19.3 ± 3.3	18.1 ± 3.6	
Fiber (g/d)			ns
TFA	21.2 ± 1.2 [a]	18.6 ± 1.0 [b]	
CTR	21.4 ± 1.6 [a]	18.7 ± 1.6 [b]	

[1] Values are means ± SEM. ns, non significant (P>0.05). Means in a row with superscripts without a common letter differ; P<0.05 (paired t-test). [2] There were no significant differences in baseline values between diet groups (unpaired t-test), except for alcohol (P<0.05). [3] P value is for differences between diet groups at wk 16, unpaired t-test.



Value	*trans*18:1	Assessment Criterion
0	Not present	*a* & *c*, or *a* & *d* not observed
0.5	Indeterminate	*a* & *c,* or *a* & *d* < 3x background & *a* < 0.5 *b*
1	Low	*a* & *c,* or *a* & *d* > 3x background, and *b* < 0.5 18:1n7*c*
2	High	*a* & *c,* or *a* & *d* > 3x background & *b* > 0.5 18:1n7*c*

Supplemental figure 1: Semi-quantitative analysis of red blood cell (RBC) *trans* fatty acid (TFA) profiles. A representative chromatogram showing "high" C18:1 TFA profiles observed in RBC sample extracts of TFA fed subjects run on two different gas chromatographers and an authentic standard. A peak interfering with the *trans*-C18:1 profile (indicated by hatching) impeded quantitative analysis. Rather, four peaks (*a*, *b*, *c*, and *d*) from the *trans* profile were used to indicate the presence or absence of an industrial TFA profile, while the relative abundance to *cis*-C18:1(n-7) was used as an indication of TFA intensity. Peak relative retention times to *cis*-C18:1(n-9) and *cis*-C18:1(n-7) were used as positive identification. Peaks *b* and *c*, respectively co-eluted with *trans*-C18:1(n-9) and *trans*-C18:1(n-7) in authentic standards. A value of 0 was applied if *a* & *c* or *a* & *d* were not observed, indicating that *trans*-C18:1 was not present; a value of 0.5 was applied if *a* & *c,* or *a* & *d* < 3x background and *a* < 0.5 *b,* indicating that the presence of *trans*-C18:1 was indeterminate; a value of 1 was applied if *a* & *c,* or *a* & *d* > 3x background and *b* < 0.5 18:1n7*c* indicating low presence of *trans*-C18:1; a value of 2 was applied if *a* & *c,* or *a* & *d* > 3x background and *b* > 0.5 18:1n7*c* indicating high presence of *trans*-C18:1.

Supplemental Method Description

Fatty acid methyl ester (FAME) analysis gas chromatography conditions

Esterified lipids were separated by gas chromatography (GC) on Agilent 6890 GCs with split/-splitless inlets run in splitless mode with a 0.8 min purge delay. The resolution of eighteen carbon *cis/trans* MUFA isomers was equivalent under these two described chromatographic conditions.

Dietary FAMEs were separated on a 100m x 0.25mm id x 0.25µm CP-Select capillary column (Varian, Inc.; Walnut Creek, CA). The oven temperature gradient was as follows: 2min at 80ºC; 75ºC/min to 160ºC; 25min hold; 1ºC/min to 225ºC; 8 min hold; 75ºC/min to 80ºC; 2min hold (total run time 104 min). The column helium flow gradient was as follows: Initial flow 1.5mL/min, held 10min; 1mL/min² to 0.8L/min, held 77min: 1mL/min² to 1.5mL/min. Co-elution of some *trans* and *cis* C18:1 isomers could not be resolved and therefore the assumption that the 11-*trans*/11-*cis*-ratio established the relative abundance for all *cis/trans* pairs in the *trans* test fat was applied.

RBC FAMEs were separated on a 100m x 0.25mm id x 0.2µm SP-2380 capillary column (Varian, Inc.; Walnut Creek, CA). The oven temperature gradient was as follows: 1 min at 80ºC; 35ºC/min to 150ºC; 25min hold; 2ºC/min to 205ºC; 1ºC/min to 211ºC; 0.7ºC/min to 219ºC; 0.4ºC/min to 223ºC; 32min hold; 35ºC/min to 80ºC; 2min hold (total run time 120 min). The column helium flow gradient was as follows: Initial flow 1.2mL/min, held 4min; 1mL/min² to 0.8L/min, held 60min: 1mL/min² to 1.2mL/min, held 0min.

PAPER III

PAPER III

EFFECT OF TRANS FATTY ACID INTAKE ON INSULIN SENSITIVITY AND INTRAMUSCULAR LIPIDS – A RANDOMIZED TRIAL IN OVERWEIGHT POSTMENOPAUSAL WOMEN

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Submitted

ABSTRACT

Background and aim: Intake of industrially produced trans fatty acids (TFA) has been linked to increased risk of type 2 diabetes in observational studies. We investigated the causality of this association by examining if a high intake of TFA impairs measures of glucose homeostasis and induces intramuscular lipid deposition in abdominally obese women.

Methods: In a double-blind parallel dietary intervention study, 52 healthy but overweight postmenopausal women were randomised to receive either partially hydrogenated soybean oil (15 g/d TFA) or a control oil (mainly oleate/palmitate) for 16 weeks. Three markers of glucose homeostasis and four markers of lipolysis were derived from glucose, insulin, C-peptide, nonesterified fatty acid (NEFA) and glycerol concentrations during a 3-hour frequent sampling oral glucose tolerance test (OGTT). Intramuscular lipids were assessed by magnetic resonance spectroscopy.

Results: Forty-nine women completed the study. Insulin sensitivity (assessed by $ISI_{composite}$), beta-cell function (the disposition index) and the metabolic clearance rate of insulin were not significantly affected by the dietary intervention. Neither was the ability of insulin to suppress plasma NEFA and glycerol during oral glucose ingestion nor the intramuscular lipid deposition.

Conclusions: Our study did not find any effect of high TFA intake on glucose metabolism over 16 weeks. A study population with a stronger predisposition to insulin resistance and/or a longer duration of exposure may be required for insulin sensitivity to be affected by intake of industrial TFA.

INTRODUCTION

Intake of *trans* fatty acids (TFA) is associated to an increased risk of type 2 diabetes mellitus according to data from the prospective Nurses' Health Study. Replacement of 2 energy% (E%) TFA by polyunsaturated fatty acids (PUFA) was estimated to decrease the risk of type 2 diabetes mellitus by 40%[1]. Whereas others have failed to find such an association in large prospective cohort studies with slightly lower median TFA intakes (~1.3 E% vs. 2 E% in the Nurses' Health Study)[2,3], an Iranian cross-sectional study points in the same direction[4].

A few controlled intervention studies have investigated the effect of TFA intake on measures of glucose homeostasis, but the results are inconclusive. In lean and healthy young subjects a high intake of neither ruminant nor industrial TFA seems to impair insulin and glucose metabolism in short-term studies of 4 to 5 weeks duration[5-7]. Recently, it was shown that ~5 g/d of TFA (ruminant as well as industrial) did not affect peripheral insulin sensitivity in moderately overweight women either[8]. In contrast, meals with high TFA contents have been shown to produce higher postprandial insulin and C-peptide concentrations than similar meals with *cis* fatty acids in overweight subjects and type 2 diabetic patients[9,10].

We hypothesized that industrial TFA may affect substrate metabolism in several ways that would increase the risk of developing type 2 diabetes mellitus: A high TFA intake might i) induce peripheral insulin resistance, possibly through increased deposition of intramuscular lipids[11,12]; ii) impair beta-cell function; and iii) increase lipolysis by decreasing the inhibitory action of insulin.

To explore these hypotheses, we conducted a dietary intervention study examining the effect of long-term exposure to high daily amounts of industrial TFA on glucose homeostasis and markers of lipolysis in healthy but abdominally obese postmenopausal women. We anticipated that a 16-week intervention period would be sufficiently long to allow for incorporation of TFA into muscle structural lipids with possible influence on development of insulin resistance[13] and for induction of intramuscular lipid accumulation[11].

MATERIALS AND METHODS

The present work examines the effect of high intakes of industrial TFA on insulin sensitivity, beta-cell function, markers of lipolysis and intramuscular lipids. The measurements reported here were collected at baseline (week 0) and at the end of the dietary intervention (week 16).

Study design

This dietary intervention study had a randomized, double-blind, parallel design. Fifty-two healthy, moderately overweight postmenopausal women were allocated to 26 g/d of partially hydrogenated soybean oil with ~60% TFA or control oil for 16 weeks. The participants were randomized to the two diets stratified by waist circumference. The computer generated randomization sequences with random permuted blocks of four, was kept undisclosed for the investigators until after study completion. All study personnel and participants were blinded to the treatment assignment for the duration of the study.

The subjects were recruited from Copenhagen and the surrounding areas by advertising in the local media. Subject inclusion criteria were: healthy; body mass index between 25 and 32 kg/m^2; waist circumference >80 cm; postmenopausal (self reported) for the last 12 months; and aged 45-70 years. The exclusion criteria were known diabetes or other chronic diseases; history of cardiovascular disease; smoking; hypertension (>160/100 mmHg); fasting plasma triglycerides >3 mmol/L; fasting plasma LDL-cholesterol >6 mmol/L; fasting plasma glucose >7 mmol/L; use of hormones or anti-hypertensive, anti-lipidemic or anti-cholesterolemic drugs; weight changes >3 kg for two months prior to screening; participation in strenuous physical activity >10 h/week; and abnormalities in routine biochemical and haematological tests. The subjects were not allowed to donate blood during the study and three months prior to screening or to use fish oil as dietary supplements during the study and one month before commencement.

All participants gave their informed consent to the study, which was carried out at the University of Copenhagen, Denmark and was approved by the Municipal Ethical Committee of The Capital Region of Denmark (H-B_2007-089), and registered at clinicaltrials.gov as NCT-00655902.

Intervention and compliance

We compared the effect of consuming 15.7 g/d of TFA from partially hydrogenated soybean oil with that of a control oil consisting of a 50/50% mix of palm oil and high oleic sunflower oil. The test fats (kindly supplied by AarhusKarlshamn, Denmark) mainly differed in the content of TFA, palmitic, oleic and linoleic acid. The TFA fat consisted of 60.4% TFA (of which ~98% was *trans*18:1 and 2% *trans*18:2 isomers) 19.6% *cis*18:1, 12.7% C16:0 and 6.2% C18:0, and the control fat consisted of 61.4% *cis*18:1, 26.7% C16:0, 6.6% *cis*18:2 and 3.8% C18:0. Both fats contained minor amounts of C14:0, C20:0 and C22:0.

The fats were incorporated into bread rolls providing a total of 600 kcal/d (41 E% fat) equivalent to 28% of the subjects' energy requirements on average. A clinical dietician instructed the participants how to let the bread rolls iso-calorically substitute food items from their habitual diets.

Dietary intake was measured using 3-day weighed food records at baseline and in the last week of the intervention. The only significant dietary differences between diet groups during the intervention were the contributions of energy from monounsaturated fatty acids (MUFA) and TFA, indicating that the diets were overall comparable apart from the fatty acid composition. The intake of TFA was higher in the TFA group (7.0 ± 0.2 E% (mean±SEM) vs. 0.3 ± 0.0 E%) and the intake of MUFA lower compared to the control (CTR) group (10.3 ± 0.4 E% vs. 13.4 ± 0.8 E%).

Self reported compliance as assessed by means of study diaries showed that 98% of all test bread rolls were consumed with no difference between diet groups. Incorporation of fatty acids into phospholipids of red blood cells (RBC) was used as an objective compliance measure. RBCs from all TFA fed subjects were enriched in *trans*18:1 residues after the dietary intervention, whereas those of control subjects were not (data not shown).

Measurements

At each visit the subjects were told to be 10 h fasted (except for 0.5 l water). They were instructed to avoid vigorous exercise and alcohol consumption on the day before and to con-

sume similar carbohydrate-rich evening meals on the evening before each visit. Body weight and height were measured by standard procedures.

We assessed insulin sensitivity by use of frequent sampling 3 hour glucose tolerance tests (OGTT) where subjects ingested a solution of 75 g glucose dissolved in 300 ml water. Venous blood samples were collected before and during the OGTT for measurement of plasma glucose, and serum insulin and C-peptide at the following time points -10, 0, 10, 20, 30, 45, 60, 75, 90, 105, 120, 150, and 180 min. Samples for measurement of plasma glycerol and non-esterified FA (NEFA) were collected at -10, 0, 30, 60, 90 and 120 min.

Intra- and extra-myocellular lipids (in *psoas major*) were measured by ^{1}H magnetic resonance spectroscopy[14] using Achieva 3.0 T MRI system (Philips Medical Systems) and a sense cardiac coil. A single voxel PRESS sequence with the spectroscopy volumes of 11 mm x 11 mm x 11 mm was used for acquisition. The MR scanner's software was used to fit the acquired spectrum to obtain spectroscopic areas of water, intra- and extra-myocellular lipids. Consequently, content of the lipids in % relative to water was calculated.

Laboratory analyses

Fasting and OGTT plasma glucose concentrations were analysed by a colorimetric assay (Ortho-Clinical Diagnostics, Johnson & Johnson, Birkerød, Denmark) for the Vitros 5.1 FS analyzer (intra-assay CV: 5.1%). Fasting and OGTT serum insulin and C-peptide concentrations were analysed by a solid-phase, enzyme-labeled chemiluminescent immunometric assay and a solid-phase, two-site chemiluminescent immunometric assay, respectively (Siemens Medical Solutions Diagnostics, Ballerup, Denmark) for the IMMULITE 2500 analyzer (intra-assay CVs: 4.2% and 2.6%, respectively).

Fasting and OGTT plasma glycerol concentrations were analyzed by a direct colorimetric assay (Randox, Crumlin, UK) and plasma NEFA concentrations were measured by an enzymatic colorimetric assay (Trichem, Frederikssund, Denmark) both for the Pentra 400 Analyzer (Horiba ABX Diagnostics).

The fatty acid composition of dietary fats as well as of phospholipids in RBC membranes was analyzed by gas chromatography (on an Agilent 6890 GC) of fatty acid methyl esters on high polarity cyano-silica columns (Varian, Inc.; Walnut Creek, CA) and a flame ionization detector.

Calculations

Areas under the curves (AUC) were calculated by means of the trapezoidal rule, and incremental AUC (iAUC) represent AUC above the fasting value.

Three central parameters to describe insulin sensitivity, clearance of insulin and beta-cell function, respectively, during oral glucose ingestion were chosen:

i) Insulin sensitivity was estimated by use of the insulin sensitivity index $ISI_{composite}$ as proposed by Matsuda & DeFronzo, which has been shown to correlate closely with the *M* value of the glucose clamp in individuals displaying a range of glucose tolerance[15]. $ISI_{composite}$ was calculated as = 10 000 / (fasting glucose x fasting insulin x mean $glucose_{t0\text{-}30\text{-}60\text{-}90\text{-}120}$ x mean $insulin_{t0\text{-}30\text{-}60\text{-}90\text{-}120}$)½, where glucose is measured in mg/dl and insulin in μU/ml.

ii) The insulin metabolic clearance rate (MCRi) was estimated as the changes in prehepatic insulin secretion relative to plasma insulin concentration during the OGTT. The calculation of

MCRi has previously been described in detail[16] and is based on the following principle: As C-peptide is co-secreted equimolarly with insulin from the pancreatic beta-cells, but is not absorbed by the liver prior to entering the blood stream, the C-peptide concentrations during the OGTT allows for the estimation of the prehepatic insulin secretion rate (ISR). The estimation was performed by deconvolution of the C-peptide concentration using the computer program ISEC[17], which has been validated to calculate prehepatic ISR during intravenous glucose tolerance tests[18] meal tests[19] and OGTT[20]. The ISR-AUC is an expression of the total amount of prehepatic insulin secreted during the OGTT. Since the insulin-AUC represents both insulin secretion and clearance, the MCRi can be calculated as the ratio of ISR-AUC to insulin-AUC.

iii) Beta-cell function was expressed by the Disposition Index that estimates the beta-cell responsiveness taking into account ambient insulin sensitivity. A detailed description of these endpoints has been given by Haugaard *et al.*[16]. Briefly, the index for beta-cell responsiveness (or insulin secretory capacity) B_{total} was calculated as the change in ISR per unit change in glucose concentration by performing cross-correlation analysis between plasma glucose concentrations and ISR during the OGTT. The Disposition Index was calculated as the product of B_{total} and $ISI_{composite}$.

Four integrated parameters to measure and describe lipolysis during oral glucose ingestion were chosen. First, the plasma concentrations of NEFA and glycerol at their most suppressed period during the OGTT (90 -120 min), $NEFA_{mean,\ 90\text{-}120\ min}$, and $Glycerol_{mean,\ 90\text{-}120\ min}$, were taken as independent markers of remnant lipolysis. Second, the ability of insulin to suppress both NEFA and glycerol during oral glucose ingestion was estimated as the change in NEFA and glycerol, respectively, relative to the integer of changes in insulin concentration during the OGTT ($\Delta NEFA/Insulin_{iAUC}$ and $\Delta Glycerol/Insulin_{iAUC}$). The change in NEFA and glycerol during the OGTT, (ΔNEFA and ΔGlycerol) were calculated as the change from t=0 min to the mean of values at t=90 and 120 min as percentage of t=0 min value.

Statistical analyses

The study size was estimated based on the effect of TFA on the LDL/HDL-cholesterol ratio, which was one of the 3 primary endpoints of this study (the others being hepatic fat and insulin sensitivity). The results for blood lipids and hepatic fat will be reported elsewhere. It was estimated that a minimum of 48 subjects was required to detect an absolute difference of 0.5 in the LDL-C/HDL-C ratio between diet groups with a statistical power of 80%, and a two-sided significance level of 5%. Fifty-two participants were recruited to allow for an estimated drop out rate of 10%.

Data were analyzed using Statistic Analysis Package, SAS© version 9.1 (SAS Institute, Cary, NC, USA). The primary analysis included participants who completed the intervention (n=49). The statistical significance level is defined as $P<0.05$.

At baseline, characteristics of participants in the two diet groups were compared with the use of 2-tailed unpaired t-tests or Kruskal-Wallis' χ^2 tests for skewed data. Analysis of covariance (ANCOVA) was used to assess the baseline-adjusted difference between diet groups for variables measured at week 0 and 16. For variables with repeated measures over time (OGTT data) a mixed model of repeated measures examining the effect of diet and time (with time denoting the measurement time points during the OGTT) and their interactions was applied, again with the baseline value as a covariate. A Gaussian covariance structure was used for all variables.

Variance homogeneity and normality were investigated by residual plots, histograms and Shapiro-Wilk's test and data was log transformed when needed.

RESULTS

Baseline characteristics for the 49 completers of the study (24 out of 25 randomized to the TFA group and 25 out of 27 randomized to the CTR group) are presented in **Table 1**. Body weight increased by 1.1 ± 0.2 kg in both diet groups during the dietary intervention.

Table 1: Subject characteristics at baseline for completers in the trans fat (TFA) and control (CTR) groups.

	TFA	CTR
n	24	25
Age (years)	58.5 ± 4.6	58.8 ± 5.5
Height (cm)	165.3 ± 5.5	166.9 ± 5.2
Weight (kg)	78.7 ± 7.1	78.4 ± 8.6
Body mass index (kg/m^2)	28.8 ± 1.7	28.1 ± 2.2
Waist circumference (cm)	97.1 ± 7.3	95.5 ± 6.8
Systolic blood pressure (mmHg)	118 ± 12	118 ± 11
Diastolic blood pressure (mmHg)	81 ± 8	82 ± 6
No of NFG / IFG / T2D [1]	18 / 6 / 0	20 / 5 / 0
No of NGT / IGT / T2D [2, 3]	11 / 11 / 2	19 / 6 / 0
Fasting blood parameters		
p-Glucose (mmol/l)	5.2 ± 0.6	5.3 ± 0.4
s-Insulin (pmol/l)	24 (16; 37)	37 (25; 54)
s-C-Peptide (pmol/l)	563 (471; 674)	598 (505; 708)
p-Glycerol (pmol/l)	96 ± 38	84 ± 42
p-NEFA (µmol/l)	732 ± 162	693 ± 195
p-Triglycerides (mmol/l)	1.13 ± 0.50	1.19 ± 0.50
p-Cholesterol (mmol/l)	5.4 ± 0.9	5.5 ± 1.0
OGTT derived parameters		
$ISI_{composite}$ [3]	7.3 (5.0; 10.7)	5.3 (3.8; 7.4)
B_{total} [3]	2.3 ± 0.2	3.0 ± 0.3 [4]
Disposition Index [3]	14.9 (9.6; 23.2)	14.6 (11.1; 19.1)
MCRi (l/min) [3]	3.4 ± 0.3	2.7 ± 0.2 [4]
$NEFA_{mean,\ 90\text{-}120\ min}$ (µmol/l)	37 (28; 50)	42 (35; 53)
$\Delta NEFA/Insulin_{iAUC}$ (% min^{-1} $pmol^{-1}$ l)	4.2 (3.2; 5.5)	2.9 (2.1; 4.0) [4]
$Glycerol_{mean,\ 90\text{-}120\ min}$ (pmol/l)	21 ± 3	28 ± 4
$\Delta Glycerol/Insulin_{iAUC}$ (% min^{-1} $pmol^{-1}$ l) [5]	3.7 ± 0.5	2.4 ± 0.3 [4]
Intramuscular lipids		
Intramyocellular lipids (%) [6]	1.6 (1.1; 2.3)	1.5 (1.1; 1.9)
Extramyocellular lipids (%) [6]	5.2 (4.0; 6.7)	4.3 (3.5; 5.4)
Total myocellular lipids (%) [6]	7.3 (6.0; 9.0)	6.0 (5.0; 7.2)

Values are means ± SD or geometric means (95%CI) for skewed data, completers only. [1] NFG, normal fasting glucose (defined as fasting plasma glucose <5.6 mmol/l); IFG, impaired fasting glucose (≥5.6 to 6.9 mmol/l); T2D, type 2 diabetes (fasting glucose ≥7.0 mmol/l) [28]; [2] NGT, normal glucose tolerance (defined as plasma 2-hour glucose <7.8 mmol/l during an oral glucose tolerance test); IGT, impaired glucose tolerance (2h glucose ≥7.8 and <11.1 mmol/l); T2D (2h glucose ≥11.1 mmol/l) [28]. No significant difference between groups by Fisher's Exact Test, $P>0.05$. [3] n = 23 in the TFA group. [4] $P<0.05$ vs. TFA group. [5] n = 22 in the TFA group. [6] Assessed by magnetic resonance spectroscopy of psoas major; n = 23 in the TFA group and n = 22 in the control group due to a few erroneous scans. B_{total}, beta-cell responsiveness; ISI, insulin sensitivity index; MCRi, metabolic clearance rate of insulin; NEFA, non-esterified fatty acids: p, plasma; s, serum.

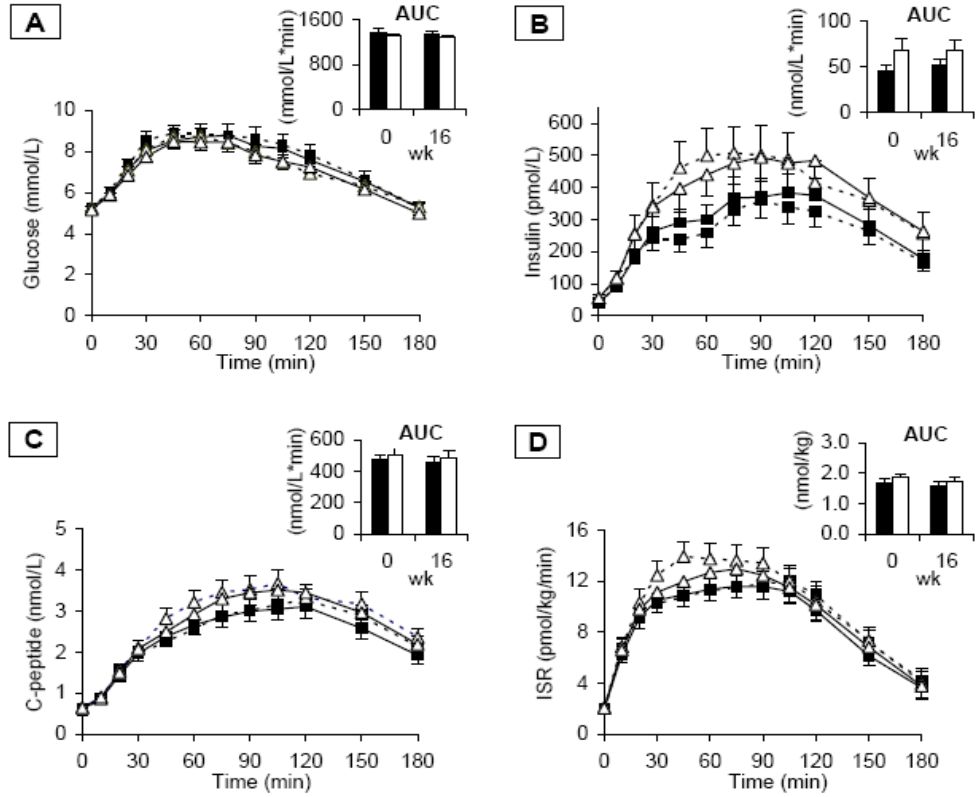

Figure 1: Glucose and insulin metabolism during oral glucose tolerance test (OGTT)
Mean (±SEM) glucose (A), insulin (B) and C-peptide (C) concentrations and insulin secretion rates (ISR) during the 3-hour OGTT performed at baseline (week 0; dotted lines) and after the dietary intervention (week 16; solid lines), in subjects who consumed 15.7 g/d trans fatty acids (TFA; n=24; black squares); and a control oil (control; n=25; white triangles). The corresponding areas under the curves (AUC) are also depicted (black bars = TFA; white bars = control). No diet x time interaction was observed for either glucose (P=0.81), insulin (P=1.00) C-peptide (P=0.98) or ISR (P=0.90) by repeated measures analysis, nor were the AUCs significantly different in baseline-adjusted analyses (P>0.39). The insulin response was slightly higher in the control group at baseline (significant diet x time interaction in repeated measures analysis (P<0.001), with no significant difference between diets at individual time points after Bonferroni correction), however, the AUC value was not statistically different from the TFA group (P= 0.11 by unpaired t-test). There were no significant baseline differences between the two groups for the other variables. Wk, week.

There was no effect of diet on fasting concentrations of glucose, insulin, C-peptide, glycerol and NEFA (**Table 2**). Responses for glucose, insulin, C-peptide and ISR during the 3h OGTT, before and after the intervention, are depicted in **Figure 1**. At week 16 there were neither significant diet by time (in min) interactions, nor significant effects of diet in repeated measures analysis for any of these variables when adjusting for the response at baseline. The AUC values were not significantly different either (Figure 1).

As expected, the baseline relationship between $ISI_{composite}$ and B_{total} fitted a hyperbola (n=30, r=0.52, P=0.004) for subjects with normal glucose tolerance (defined as 2h glucose <7.8 mmol/L) whereas a shift to the left (P=0.06) was seen for subjects with impaired glucose tolerance (2h glucose ≥7.8 mmol/L).

None of the OGTT-derived parameters describing glucose homeostasis or lipolysis changed significantly in either diet group during the 16 weeks of intervention. Thereby, after the intervention there was no difference between diet groups in either $ISI_{composite}$, Disposition Index,

MCRi, nadir concentrations of NEFA/glycerol or the ability of insulin to suppress NEFA/-glycerol, when adjusting for baseline values (Table 2).

Intra- and extra-myocellular lipids did not change during the dietary intervention in either study group (Table 2); neither did the ratio between the two (data not shown).

DISCUSSION

The present study investigated if the positive association between intake of industrially produced TFA and risk of type 2 diabetes mellitus observed in the Nurses' Health Study[1] might be explained by adverse effects on insulin sensitivity and beta-cell function. We found that in metabolically vulnerable postmenopausal women a high intake of TFA from partially hydrogenated soybean oil for 16 weeks had no measurable effect on any of the examined diabetes related end points compared to intake of a control oil where TFA was replaced by mainly oleic but also palmitic and linoleic acid.

We did not find an effect of TFA exposure on insulin sensitivity in this study, despite high self-reported compliance with the intervention diet (98% of bread rolls consumed), reflected by incorporation of TFA isomers in the red blood cell membrane of all subjects in the TFA group. Also, the fact that the TFA group experienced a significant 34% increase in the plasma LDL/HDL-cholesterol ratio (Bendsen et al. 2010, *submitted observations*), is indicative of good compliance. Dietary TFA has consistently been shown to increase this ratio compared to all other FA classes[21]. We assessed insulin sensitivity by use of the OGTT-derived $ISI_{composite.}$ This index of whole-body insulin sensitivity has been shown to correlate well with the rate of insulin-mediated glucose disposal during the euglycemic insulin clamp[15]. An advantage of the OGTT is that it reflects physiological stimulation of insulin secretion. Also, the few studies that have suggested an insulin-raising effect of TFA have employed meal tests[9,10,22] suggesting that the entero-insular axis may be of relevance.

However, it is a limitation of this study that sample size calculations were not based on insulin sensitivity. The relative mean difference between diets in $ISI_{composite}$ was -4% (95% confidence interval: -23 to 20) after the intervention, indicating that we would not have been able to detect TFA-induced changes in $ISI_{composite}$ smaller than 20%. Nevertheless, within-group changes were very small, which strongly indicates that TFA intake do not induce a clinically relevant change in insulin sensitivity in this metabolically sensitive population.

To examine possible effects of TFA on glucose metabolism beyond those on whole-body insulin sensitivity, we calculated additional indices derived from the OGTT data. The metabolic clearance rate of insulin, MCRi, was assessed because a decrease in this measure would reflect a compensatory mechanism to comply with increased insulin resistance[23]; the Disposition Index was calculated, since insulin secretion should be compared to the insulin sensitivity of the individual in order to reflect beta-cell function[24]; and finally, we assessed the ability of insulin to suppress both NEFA and glycerol during oral glucose ingestion as it is suggested to reflect the sensitivity of adipose tissue to the antilipolytic effect of insulin, which has been shown to be impaired in healthy subjects with muscle insulin resistance[25]. Neither of these indices was significantly affected by the dietary intervention.

Table 2: Glucose, insulin and lipid metabolic data and intramuscular lipids in the trans fat (TFA) and control (CTR) groups after the 16-week dietary intervention

	TFA (n=24)		**CTR** (n=25)		**TFA - CTR**	
	Week 16 [1]	**Change from baseline** [2]	**Week 16** [1]	**Change from baseline** [2]	**Mean difference at week 16** [3]	**P** [4]
Fasting blood parameters						
p-Glucose (mmol/l)	5.3 (5.2; 5.4)	0.02 ± 0.05	5.2 (5.1, 5.3)	-0.10 ± 0.07	0.13 (-0.04; 0.30)	0.13
s-Insulin (pmol/l) [5]	32 (25; 39)	0.0 (-5.3; 9.9)	33 (27; 41)	1.1 (-3.6; 8.8)	-4 (-30; 31) %	0.77
s-C-Peptide (pmol/l) [5]	605 (550; 665)	22 (-54; 127)	584 (532; 641)	22 (-63; 108)	4 (-9; 18) %	0.60
p-Glycerol (pmol/l) [5]	73 (61; 88)	-9 (-38; 0)	82 (69; 98)	2 (-25; 19)	-11 (-31; 15) %	0.37
p-NEFA (µmol/l)	626 (550; 702)	-100 ± 31	673 (599; 748)	-25 ± 55	-47 (-153; 60)	0.38
OGTT derived parameters						
$ISI_{composite}$ [5, 6]	5.8 (5.0; 6.8)	-0.5 (-2.0; 0.5)	6.0 (5.2; 7.0)	-0.1 (-0.7; 0.5)	-4 (-23; 20) %	0.72
B_{total} [6]	2.7 (2.3; 3.1)	0.2 ± 0.2	2.4 (2.0; 2.8)	-0.4 ± 0.2	0.3 (-0.3, 0.8)	0.36
Disposition Index [5, 6]	14.2 (11.8; 17.1)	-0.7 (-3.0; 3.0)	12.5 (10.4; 14.9)	-1.1 (-2.7; 1.2)	14 (-12; 47) %	0.31
MCRi (l/min) [5]	2.8 (2.5; 3.0)	-0.5 ± 0.3	2.6 (2.3; 2.8)	-0.2 ± 0.1	0.2 (-0.2; 0.6)	0.35
$NEFA_{mean,\ 90\text{-}120\ min}$ (µmol/l) [5]	41 (31; 56)	10 (-15; 26)	41 (30; 55)	3 (-18; 7)	2 (-34; 56) %	0.94
$\Delta NEFA/Insulin_{iAUC}$ (% min^{-1} $pmol^{-1}$ l) [5]	3.2 (2.7; 3.9)	-0.2 (-1.2; 0.4)	3.4 (2.8; 4.1)	0.1 (-0.7; 0.5)	-5 (-27; 23) %	0.68
$Glycerol_{mean,\ 90\text{-}120\ min}$ (pmol/l)	29 (23; 36)	6.1 ± 4.0	24 (17; 31)	-3.8 ± 4.5	5.5 (-4.5; 15.4)	0.27
$\Delta Glycerol/Insulin_{iAUC}$ (% min^{-1} $pmol^{-1}$ l) [7]	2.4 (1.8; 2.9)	-1.0 ± 0.4	2.9 (2.4; 3.4)	0.2 ± 0.2	-0.5 (-1.2; 0.3)	0.38
Intramuscular lipids						
Intramyocellular lipids (%) [5, 8]	1.8 (1.6; 2.1)	0.0 (-0.9; 1.1)	1.6 (1.4; 1.9)	-0.1 (-0.4; 0.6)	12 (-11; 42) %	0.30
Extramyocellular lipids (%) [5, 8]	4.7 (4.0; 5.6)	-0.2 (-0.8; 1.0)	4.2 (3.5; 5.0)	-0.6 (-1.3; 0.4)	12 (-11; 42) %	0.33
Total myocellular lipids (%) [5, 8]	6.7 (5.8; 7.7)	0.5 (-1.0; 1,3)	6.1 (5.3; 7.0)	-0.9 (-1.4; 0.5)	10 (-10; 35) %	0.33

[1] Values are least squares means (95%CI). [2] Values are means ± SEM or medians (interquartile range) for skewed values, completers only. [3] Mean baseline-adjusted difference (or relative difference) between diet groups at week 16 (TFA – CTR); [4] P value is for differences between diet groups at week 16 with week 0 as a covariate, by ANCOVA. [5] ANCOVA performed on log10-transformed values. [6] n = 23 in the TFA group. [7] n=22 in the TFA group. [8] Assessed by magnetic resonance spectroscopy of *psoas major*; n = 23 in the TFA group and n = 22 in the control group due to a few erroneous scans. B_{total}, beta-cell responsiveness; ISI, insulin sensitivity index; MCRi, metabolic clearance rate of insulin; NEFA, non-esterified fatty acids, p, plasma; s, serum.

Also, we hypothesized that TFA might affect glucose metabolism through induction of ectopic intramuscular lipid accumulation, as previously observed in rats[12]. Intramuscular fat (in combination with diminished mitochondrial lipid oxidation) is thought to be a major contributor to obesity-associated insulin resistance as reviewed by Corcoran *et al*.[11] However, TFA did not increase the deposition of intramuscular lipids in this study. In contrast, 12 weeks of supplementation with conjugated linoleic acid (1.2 wt%) significantly increased the intramuscular fat content in growing pigs' *longissimus dorsi* by 19%[26]. This indicates that relatively small amounts of specific fatty acids may affect muscle lipid accumulation, at least during growth; unfortunately insulin sensitivity was not assessed in this study.

Our results are in agreement with findings from previous studies of shorter duration (≤ 5 weeks) in lean and overweight subjects, finding no effect of industrial TFA on pancreatic insulin secretion or insulin sensitivity[5,6,8]. We hypothesized that the failure of these studies to show an effect was either due to i) too short study durations not allowing time for TFA to be incorporated into myocellular membrane lipids with possible influence on insulin sensitivity[13], ii) too low TFA intake levels, and/or iii) too young and healthy study populations. Lovejoy *et al*.[6] found a trend towards a decrease in insulin sensitivity after TFA intake only in overweight subjects and Vega-Lopez *et al*.[27] reported that TFA consumption modestly increased fasting insulin levels in older and moderately hyperlipidemic subjects.

In accordance, in moderately overweight subjects consumption of a single meal with 10 E% TFA resulted in increased postprandial levels of insulin and C-peptide compared to a meal with *cis* MUFA[9], and the same picture emerged in type 2 diabetic patients after consumption of a diet very high in industrial TFA (20 E%)[10]. In the latter study an increase of 59% in postprandial insulin response was seen after TFA compared to MUFA intake, whereas the response did not differ from that seen after consumption of saturated fatty acids.

This raises the question of whether TFA intake only will exert adverse effects on glucose metabolism at very high intake levels in subjects with a strong underlying predisposition to insulin resistance, and only when compared to MUFA. Alternatively, very long exposures may be required. After 6 years on a diet with 8 E%, TFA-increased postprandial insulin concentrations were found in green monkeys compared to monkeys fed MUFA[22]. However, the question has not previously been sought answered by studies using adequate insulin sensitivity assessment methodology. In the present study we failed to find an effect in abdominally obese, older women after a relatively long period of high TFA exposure employing the validated OGTT-derived $ISI_{composite.}$

As no detrimental effects on glucose metabolism were seen in metabolically vulnerable subjects after 16 weeks of high daily amounts of TFA in the present study, we conclude that either i) unrealistically high intake levels and/or a longer duration of exposure may be required for insulin sensitivity to be affected by intake of industrial TFA or ii) the observational data showing associations to risk of type 2 diabetes are potentially flawed by confounding. It may indeed be difficult to separate the effects of industrial TFA intake *per se* from that of an unhealthy life style with high fast food consumption.

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DECLARATION OF INTERESTS

NTB, SBH, TML, SS and AA designed the study; NTB and EC conducted the data collection and analyses and NTB wrote the manuscript. All authors reviewed the manuscript. None of the authors have competing interests to declare.

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PAPER IV

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TRANS FATTY ACID INTAKE INDUCES LOW-GRADE SYSTEMIC INFLAMMATION: EVIDENCE FROM A RANDOMIZED, CONTROLLED TRIAL IN OVERWEIGHT POSTMENOPAUSAL WOMEN

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Draft

ABSTRACT

Background: Intake of industrial trans fatty acids (TFA) has been positively associated to systemic markers of low-grade inflammation and endothelial dysfunction in cross-sectional studies, but results from intervention studies are inconclusive.

Objective: To examine the effect of a high intake of TFA on systemic biomarkers of inflammation and endothelial dysfunction; adipose tissue expression of adipokines and content of ceramide; and urinary markers of oxidative stress.

Design: A 16-weeks double-blind parallel intervention study where 52 healthy overweight postmenopausal women were randomized to receive either partially hydrogenated soybean oil providing 15.7 g per day of TFA or a control oil where TFA was replaced by mainly oleic and palmitic acid.

Results: After 16 weeks, TFA exposure resulted in an increase in baseline-adjusted serum tumor necrosis factor (TNF) α of 12% (95% confidence interval (CI): 5 – 20; P=0.002) compared to controls. Plasma soluble TNF receptors 1 and 2 were also increased by TFA [155 pg/ml (CI: 63 – 247); P<0.001 and 480 pg/ml (CI: 72 – 887); P=0.02, respectively]. Serum C-reactive protein, interleukin (IL) 6 and adiponectin and subcutaneous abdominal adipose tissue mRNA expression of IL6, IL8, TNFα and adiponectin and ceramide content were not affected by the dietary intervention, neither was the urinary marker of oxidative stress 8-iso-prostaglandin-$F_{2\alpha}$.

Conclusions: This dietary trial shows that the mechanisms linking dietary TFA to cardiovascular disease is likely to involve induction of low-grade systemic inflammation, although this is not mirrored in subcutaneous adipose tissue mRNA expression within 16 weeks.

INTRODUCTION

Intake of industrially produced *trans* fatty acids (TFA) is in cross-sectional studies positively associated to systemic concentrations of various inflammatory markers such as C-reactive protein (CRP), tumor necrosis factor α (TNFα) and interleukin 6 (IL6)[1-3]. Recently, similar associations to inflammatory markers were found with intake of partially hydrogenated vegetable oil, which is the primary source of industrial TFA[4]. Consequently, induction of low-grade systemic inflammation has been suggested as a mechanism by which TFA intake increases the risk of cardiovascular disease (CVD) and type 2 diabetes, as seen in large prospective cohort studies[5,6]. However, the evidence from randomized studies is limited; one study has shown increased production of TNFα and IL6 in stimulated mononuclear cells from humans after high TFA consumption[7] and two studies found increased CRP blood concentrations[8,9], whereas several others have failed to find an effect of dietary TFA on inflammatory markers[10-12].

TFA intake has also been positively associated with markers of endothelial dysfunction, such as E-selectin and adhesion molecules, in a cross-sectional analysis of the Nurses' Health Study[3], a finding which causality was strengthened by a randomized trial showing higher concentrations of plasma E-selectin after TFA consumption[8]. In concordance with this, a study by de Ross et *al*.[13] suggested that TFA decrease flow-mediated vasodilation, which is a more direct measure of endothelial function, although this finding failed to be confirmed in our hands[14].

Furthermore, TFA intake has been suggested to increase oxidative stress in humans, which might accelerate diseases such as CVD and type 2 diabetes[15]. An increase in the urinary concentration of 8-iso-prostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), a marker of the whole-body level of oxidative stress *in vivo*[16], has previously been documented after high intakes of conjugated linoleic acid (CLA), and the TFA isomers *trans*18:1n-7 and *trans*18:1n-6[17-19].

In the present dietary intervention study we examined the effect of a high intake of industrial TFA on systemic and adipose biomarkers of inflammation. Also, we wished to investigate possible effects on markers of endothelial dysfunction and oxidative stress.

SUBJECTS AND METHODS

This work presents data from a dietary intervention study examining the effect of a high intake of TFA on a range of risk markers for CVD and type 2 diabetes. Here we report the results for systemic markers of low-grade inflammation and endothelial dysfunction, urinary markers of oxidative stress as well as adipokine expression and ceramide content in subcutaneous abdominal adipose tissue (AT).

The details of this 16-week (wk) double-blind, parallel dietary intervention study has been reported previously[20]. In brief, 52 women were randomized to two test diets by using a computer-generated randomization sequence. Subjects in the TFA group were given 26 g/d of partially hydrogenated soybean oil (providing 15.7 g/d TFA) incorporated into 2 daily bread rolls (2500 kJ), whereas subjects in the control (CTR) group received bread rolls with a low TFA oil consisting of a 50/50% mixture of palm oil and high oleic sunflower oil. The test fats were kindly supplied by AarhusKarlshamn, Denmark. The fatty acid (FA) composition of the two test fats mainly differed in the content of TFA, palmitic (16:0), oleic (*cis*18:1) and linoleic acid (*cis*18:2) as shown in **Table 1**. The data were collected at wk 0 (baseline), 8 and 16.

Subject were healthy; moderately overweight (body mass index (BMI) 25 - 32 kg/m^2); waist circumference > 80 cm; postmenopausal for the last year; and aged 45-70 years. Criteria for exclusion were diabetes or other chronic diseases; history of CVD; smoking; hypertension; hyperlipidemia, hypercholesterolemia; hyperglycemia; use of hormones or anti-hypertensive, anti-lipidemic or anti-cholesterolemic drugs; recent weight changes; strenuous physical activity; and abnormalities in routine biochemical and haematological tests.

To assess the effect of body weight on the inflammatory markers, the study included 19 lean reference women (BMI = 19-24 kg/m^2; waist circumference ≤ 80 cm) who underwent baseline examinations only. These reference subjects fulfilled the same inclusion and exclusion criteria as the intervention subjects, apart from the limits for BMI and waist.

After having received both verbal and written information, all participants gave written consent. The study was approved by the Municipal Ethical Committee of The Capital Region of Denmark in accordance with the Helsinki-II declaration (H-B_2007-089) and was registered at clinicaltrials.gov as NCT00655902.

Table 1: Fatty acid composition of the trans fat (TFA) and the control fat

Fatty acid (w/w%)	CTR	TFA
C14:0	0.7	0.2
C16:0	26.7	12.7
C18:0	3.8	6.2
C18:1-*trans*	< 0.7	59.0
C18:1-*cis*	61.4	19.6
C18:2-*trans*	< 0.1	1.4
C18:2-*cis*	6.6	0.2
C20:0	0.4	0.4
C22:0	0.4	0.3
Total *trans* fatty acids	< 0.7	60.4

Sample collection and analysis

At all visits subjects were fasted for 10h. After minimum 10 min of rest, blood was sampled from an antecubital vein into Vacutainer lithium heparin gel tubes and silicone coated serum tubes (BD Medical Systems, Franklin Lakes, NJ, USA). Serum was obtained after 30 minutes clotting at room temperature and 10 min centrifugation at 3000 G. Samples were stored at -80˚C before analysis.

C-reactive protein (#6801739) was analyzed on a Vitros 5.1 (Johnson & Johnson, Rochester, NY, USA) with an inter-assay variation coefficient (CV) of 5.0% and a detection limit of 0.1 mg/L (minimum detectable concentration + 2 x standard deviation). Samples (2%) that were below the CRP detection limit (DL) of 0.1 mg/L were defined as 0.05 mg/L. A total of seven CRP values (four at baseline, two at week 8 and one among the reference subjects) were excluded due to CRP concentrations >10 mg/L, indicating acute inflammation. Serum adiponectin (CV: 7.3%) was analyzed by a human enzyme-linked immunosorbent assay (ELISA) kit (B-Bridge International, California, USA). Serum TNFα (CV: 4.7% , DL: 0.11 pg/mL) and IL6 (CV: 9.8%, DL: 0.039 pg/mL) concentrations were also measured by using human ELISA kits (Quantikine HS #HSTA00D & #HS600B, ELISA, R&D Systems, Abingdon, UK).

Concentrations in plasma and serum of soluble TNF receptor 1 (sTNF-R1) (CV:5.3%, DL: 60 pg/ml) and soluble TNF receptor 2 (sTNF-R2) (CV:7.6%, DL:79 pg/ml) were measured by 2-plex immunoassays (#HSCR-32K) using fluorescently labeled microsphere beads kits (Millipore, Billerica, MA, USA) and analysis on BioPlex 200® (Biorad, Hercules, CA, USA); as well as by ELISA (#DRT100 & DRT200, R&D Systems) (CV:2.8%, DL:1.14 pg/mL & CV:2.4%, DL:0.78 pg/mL, respectively). A 3-plex kit (Millipore #HCVDI-67AK) was used for measurement in plasma and serum of soluble E-selectin (sE-selectin) (CV:11.8%, DL:2.1

ng/ml), soluble vascular cell adhesion molecule 1 (sVCAM-1) (CV:4.1%, DL:1.1 ng/ml), and soluble intercellular adhesion molecule 1 (sICAM-1) (CV:7.6%, DL:1.8 ng/ml).

The concentration of free 8-iso-$PGF_{2\alpha}$ was analyzed in 24-hour urine samples, with the use of a highly specific and sensitive radioimmunoassay as previously described by Basu[21], and was adjusted for creatinine value measured with a commercial kit (IL Test; Monarch Instrument, Amherst, NH).

A subcutaneous AT biopsy was taken from the abdominal region under local anaesthesia (1% lidocaine) on the left or right side of the abdomen about 5 cm lateral from the umbilicus using a Hepafix Luer lock syringe (Braun Medical, Bethlehem, PA) and a 2.10 x 80 mm Braun Medical Sterican needle. The biopsy (~500 mg) was washed in physiological saline, divided into 3 sterile cryo tubes, immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

AT (200 mg) were homogenized in TriZol reagent (Gibco BRL, Life Technologies, Roskilde, Denmark) and total RNA was extracted following the manufacturer's protocol. RNA was quantified by measuring absorbance at 260 and 280 nm using a NanoDrop 8000 (NanoDrop products, Bancroft, DE, USA), and there was a ratio ≥1.8. The integrity of the RNA was checked by visual inspection of the two ribosomal RNAs, 18S and 28S, on an agarose gel.

For real-time reverse transcriptase polymerase chain reaction (PCR), complementary DNA was constructed using random hexamer primers as described by the manufacturer (Verso cDNA kit, Abgene, Epsom, UK). Then KAPA SYBR FAST qPCR mastermix (Kapa Biosystems, Inc. Woburn, MA, USA) and the following primer pairs were added:
IL6: AAATGCCAGCCTGCTGACGAAG and AACAACAATCTGAGGTGCCCATGCTAC, TNFα: CGAGTGACAAGCCTGTAGC and GGTGTGGGTGAGGAGCACAT, Adiponectin: CATGACCAGGAAACCACGACT and TGAATGCTGAGCGGTAT, IL8: TTGGCAGCCTTCCTGATTTC and AACTTCTCCACAACCCTCT-G, ß-actin: TGTGCCCATCTACGAGGGGTATGC and GGTACATGGTGGTGCCGCCAGACA.
Real-time quantification of genes was performed using an ICycler from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Each gene was amplified in separate tubes, and the increase in fluorescence was measured in real time. The threshold cycle was calculated, and the relative gene expression was calculated essentially as described in the User Bulletin no. 2, 1997, from Perkin-Elmer. All samples were amplified in duplicate. A similar set-up was used for negative controls, except that the reverse transcriptase was omitted and no PCR products were detected under these conditions.

AT ceramide content was analyzed basically as have been described[22]. In brief, AT was extracted using the Folch procedure, ceramide with C17 long-chained base (LCB) was added during the extraction procedure. Glycerolipids were hydrolyzed using mild alkaline hydrolysis and ceramide was isolated from other sphingolipids using amino-propyl cartridges. The isolated ceramide were hydrolyzed to LCB and free FA, the LCB was derivatized with *o*-phtalaldehyde and analyzed using an HPLC-system equipped with an amino-propyl column. Ceramide content was determined from fluorescence intensity of the endogenous LCB-peaks in relation to the internal standard.

For assessment of AT triglyceride FA composition, lipids were spiked with extraction surrogates and solvent extracted[23]. Triglycerides were isolated from 50-100µg of lipid with amino-propyl solid phase extraction columns using modification of published procedures[24]. Samples were transesterified and isolated FA methyl esters were analyzed by gas chromatography on high polarity cyano-silica columns with flame ionization detection[20].

Statistical analyses

The study size was estimated based on the effect of TFA on the LDL-cholesterol to HDL-cholesterol ratio, as reported previously[20].

Data were analyzed using Statistic Analysis Package, SAS© version 9.1 (SAS Institute, Cary, NC, USA). The analysis included those participants who completed the intervention (n=49). The statistical significance level is defined as $P<0.05$.

Baseline values for the overweight intervention subjects were compared to values for the lean reference subjects by 2-tailed unpaired t-tests or Kruskal-Wallis' χ^2 tests for skewed data.

Analysis of covariance (ANCOVA) was used to assess the baseline-adjusted difference between diet groups for variables measured at wk 0 and 16, i.e. the baseline value was included as a covariate. For variables measured at wk 0, 8 and 16 a mixed model of repeated measures examining the effect of diet and time (wk 8 and 16) and their interactions was applied, again with the baseline value as a covariate and with "subject" as a random effect. Variance homogeneity and normality were investigated by residual plots, histograms and Shapiro-Wilk's test and data was log transformed when needed. Changes within groups from baseline to wk 16 were evaluated by 2-tailed paired t-tests or Signed Rank tests for skewed data.

RESULTS

Baseline characteristics of the 24 completers in the TFA group were comparable to those of the 25 completers in the control group, and lean references were adequately matched for age and height (**Table 2**).

Table 2: Baseline characteristics for subjects in the trans fat (TFA; n=24) and control (CTR, n=25) groups and for lean references (lean ref.; n=19).

	TFA	CTR	Lean ref.
Age (y)	58.5 ± 4.6	58.8 ± 5.5	60.1 ± 5.9
Height (cm)	165.3 ± 5.5	166.9 ± 5.2	166.7 ± 4.3
Weight (kg)	78.7 ± 7.1	78.4 ± 8.6	59.4 ± 4.8 [1]
BMI (kg/m^2)	28.8 ± 1.7	28.1 ± 2.2	21.3 ± 1.4 [1]
Waist circumference (cm)	97.1 ± 7.3	95.5 ± 6.8	74.9 ± 3.8 [1]
Urine 8-*iso*-$PGF_{2\alpha}$ (nmol/mmol creatinine) [2]	0.28 (0.24; 0.32)	0.31 (0.24; 0.43)	0.35 (0.28; 0.41)

Values are means ± SD or geometric means (95%CI) for skewed data, completers only. [1] Significantly different from overweight intervention subjects (diet groups combined) by 2-tailed unpaired t-tests ($P<0.05$). [2] n=18 in lean reference group due to exclusion of one outlier.

Systemic markers of low-grade inflammation

The serum TNFα concentration increased by 10 ± 3% in the TFA group during the intervention ($P= 0.02$ for within-group change) while decreasing by 2 ± 2% in the CTR group (non-significant (ns)), whereby the mean baseline-adjusted TNFα concentration was 12% (95% confidence interval (CI): 5 – 20) higher at week 16 compared to controls ($P=0.002$; **Figure 1A**).

The plasma concentration of sTNF-R1, as assessed by Bead array, increased by 22 ± 7% in the TFA group (P= 0.0003 for within-group change by paired t-test) vs. a decrease of 3 ± 6% (ns) in the CTR group during the 16-wk intervention, resulting in a baseline adjusted mean difference between diets of 155 pg/ml (CI: 63 – 247; P<0.001). The Bead array-assessed plasma concentration of sTNF-R2 also increased more on the TFA diet: 14 ± 3% (P= 0.0007) vs. 2 ± 4% (ns) in the CTR group (mean baseline adjusted difference between diet groups: 480 pg/ml; CI: 72 – 887; P=0.022).

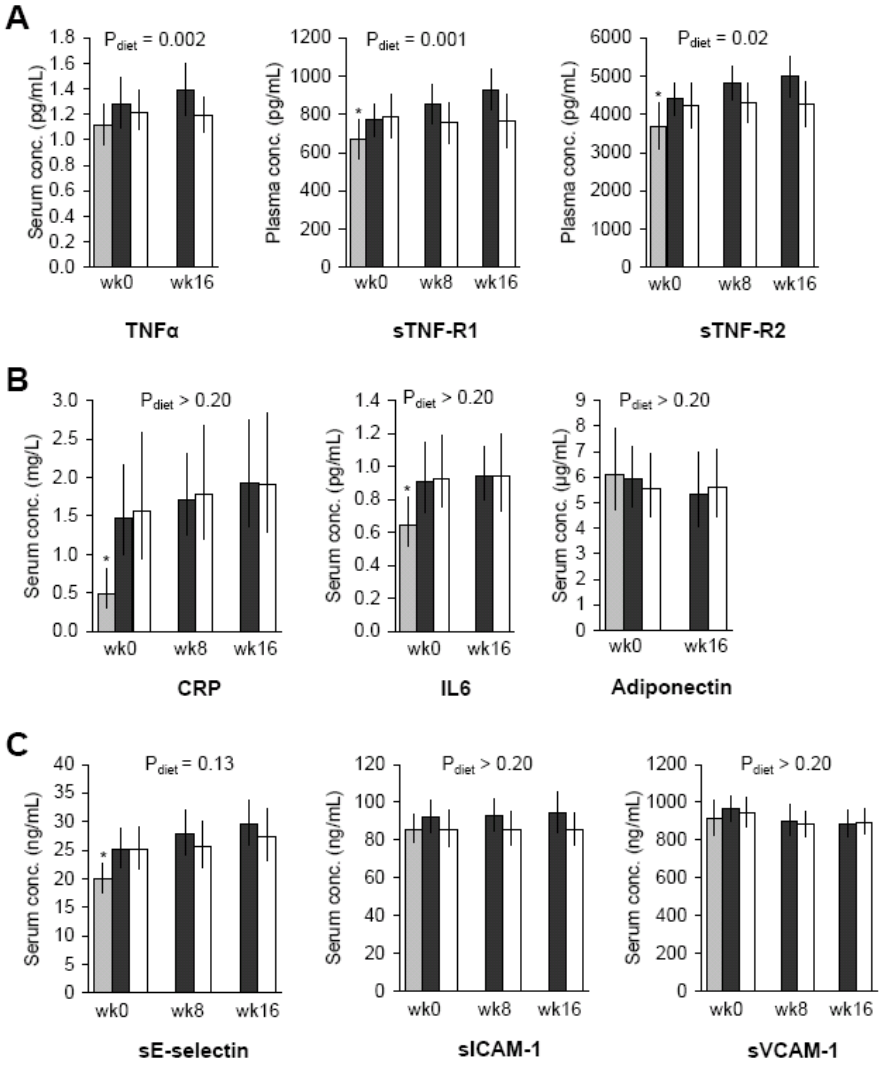

Figure 1: Systemic concentrations of markers of inflammation and endothelial dysfunction before and after 8 and 16 weeks (wk) of supplementation with 15.7 g/d trans fatty acids (TFA; dark grey bars; n=24) or a control oil (CTR; white bars; n=25), and in lean references (light grey bars; n = 19). **Figure 1A**: Tumor necrosis factor (TNF) α assessed by ELISA and soluble tumor necrosis factor receptors 1 and 2 (sTNF-R1 and sTNF-R2) assessed by Bead array technology. **Figure 1B**: C-reactive protein (CRP; n=23, 22 and 18 in the TFA and CTR group and lean references, respectively, due to exclusion of CRP values > 10 mg/l), interleukin 6 (IL6) and adiponectin, assessed by ELISA. **Figure 1C:** Soluble E-selectin (sE-selectin), soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular adhesion molecule 1 (sVCAM-1) assessed by Bead array technology.
Bars represent means (95% confidence intervals (CI)) for TNF-R1 and TNF-R2 and geometric means (CI) for all other variables. P-values are for effect of diet, by baseline-adjusted ANCOVA. *Significantly different from overweight intervention subjects (diet groups combined); P<0.05 by 2-tailed t-tests or Kruskal-Wallis' χ^2 test.

As no randomized study previously has documented this effect of dietary TFA on soluble TNF receptor concentrations in plasma, we decided to further explore these finding by repeating the analyses using antibodies from a different provider and another platform (ELISA, see Methods section). For sTNF-R2 we obtained a similar difference between diets with ELISA, suggesting a robust difference([257 pg/ml; CI: 93 – 421; P=0.003; note that concentration were generally lower with ELISA due to different calibrations). For sTNF-R1 there was a trend in the ELISA-assessed data similar to the that observed with Bead array, but the difference between diets did not remain significant (56 pg/ml; CI: -14 – 125; P=0.11). These differences between diet groups in plasma sTNF receptor concentrations were not present when the same analyses were carried out in serum (**Supplemental Figure 1**).

While serum CRP tended to increase from baseline in both diet groups (P=0.12 in both groups), but with no difference between diets, neither serum adiponectin nor IL6 concentrations were affected by the dietary intervention (**Figure 1B**).

Systemic markers of endothelial dysfunction

Serum sE-selectin increased by 19 ± 4% in the TFA group during the intervention (P= 0.004). However, the baseline-adjusted relative difference between diets at wk 16 was not significant: 8% (CI: -2 – 19; P=0.13; **Figure 1C)**, since the serum sE-selectin concentration also increased in the CTR group (12 ± 5%; P=0.04). In plasma, the difference between diets in sE-selectin reached statistical significance (10%; CI: 1 – 21; P=0.04; data not shown). Serum sVCAM-1 and sICAM-1 concentrations were not affected by the intervention and neither were plasma concentrations.

Oxidative stress

During the 16-wk intervention the concentration of the urinary marker of oxidative stress 8-iso-$PGF_{2\alpha}$ changed little in either diet group (-7 ± 11% and -3 ± 13% in the TFA and CTR groups, respectively) with no significant baseline-adjusted difference between diet groups (P=0.50).

Fatty acid composition, mRNA expression of adipokines and ceramide content in adipose tissue

In the TFA group there was an almost 2-fold increase in the AT content of the sum of the *trans*18:1n-9 and *trans*18:1n-7 isomers (from 0.47 ± 0.03% to 0.97 ± 0.06% of FA), which was not seen in the CTR group (P<0.01). There were no significant differences between diet groups in the sum of saturated, monounsaturated or polyunsaturated FA, respectively, nor were there differences in AT content of individual FA (Supplemental Table 1).

The TFA intervention had no effect on the mRNA expression of IL6, IL8, TNFα or adiponectin in subcutaneous abdominal AT (**Figure 2**). The AT mRNA expressions of IL6, TNFα and adiponectin were not significantly correlated to the respective serum concentrations at baseline in the overweight intervention subjects, nor were the changes in AT and serum over the course of the intervention correlated (P>0.20 for all).

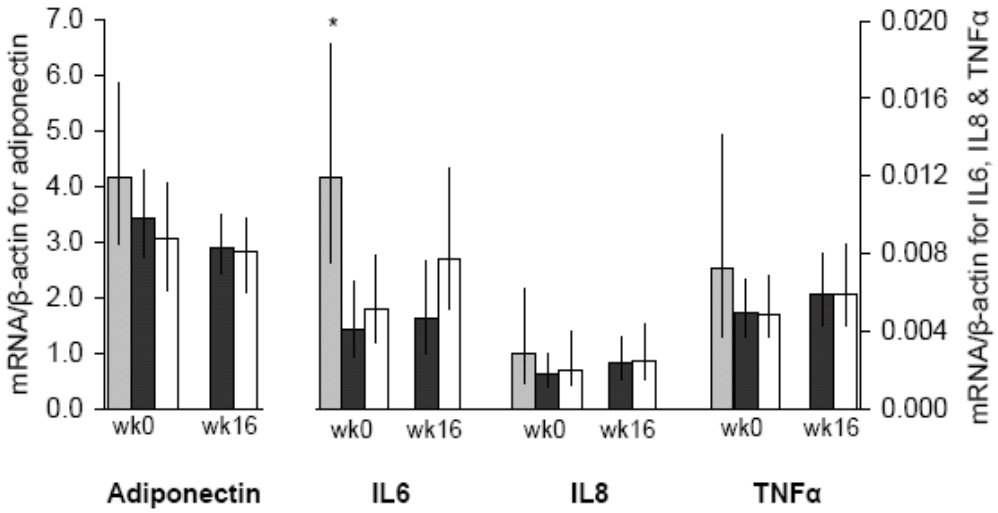

Figure 2: Subcutaneous abdominal adipose tissue mRNA expression before and after 16 weeks (wk) of supplementation with 15.7 g/d trans fatty acids (TFA; dark grey bars; n=24; n= 23 for IL8) or a control oil (CTR; white bars; n=25), and in lean references (light grey bars; n = 17). mRNA expressions are expressed relative to β-actin and bars represent geometric means (95% confidence intervals). There were no differences between diet groups at wk 16 by ANCOVA with baseline value as a covariate (P>0.10). *Significantly different from overweight intervention subjects (diet groups combined); P<0.001 by Kruskal-Wallis' χ^2 test. IL6, interleukin 6; IL8, interleukin 8; TNFα, tumor necrosis factor α.

Baseline AT ceramide content was lower in lean references (176 ± 7 pmol/mg tissue protein) compared to in overweight subjects (320 ± 7 pmol/mg tissue protein). AT ceramide tended to decrease in the TFA group (by 8 ± 5%; P=0.09 for within-group change), but the change was not significantly different from the 1 ± 4% decrease in the CTR group (P=0.23 by baseline-adjusted ANCOVA).

DISCUSSION

In addition to the previously described adverse effects of TFA on plasma cholesterol concentrations[20], we found that high TFA intakes (~7% of energy (E%)) increase the TNF system activity as shown by elevated circulating concentrations of TNFα and its soluble receptors TNF-R1 and TNF-R2. Thereby, the results of our study support the notion that the findings from previous cross-sectional analyses[1-4,25] are causal, and suggest that part of the increased incidence of heart disease related to dietary TFA is due to induction of low-grade systemic inflammation.

We are the first to report increased systemic concentrations of TNFα after TFA supplementation, and we substantiate our findings by showing concomitant increases in the soluble TNF receptors. TNFα, as well as other cytokines, is known to induce shedding of the soluble receptors, and these may thereby be biomarkers of local TNFα activation or mirror overall systemic inflammation[26]. The soluble receptors affect the local and systemic availability of TNFα and appear to act as a buffer system either prolonging or attenuating the biological effects of TNFα[26].

Soluble TNF receptors were positively associated with CHD risk in the Nurses' Health Study, but not after adjustment for lipid and other cardiovascular risk factors[27]. When we adjusted for the TFA-induced 34% increase in the LDL-cholesterol to HDL-cholesterol ratio (as reported previously[20]), the difference between diets in TNFα and sTNF-R1 became more pronounced, suggesting that the effect of TFA was not mediated through blood lipid changes. The effect of diet for sTNF-R2 was attenuated slightly by adjustment for lipid concentrations.

Others have examined the effect of TFA consumption on TNFα with discordant results. Han *et al*.[7] showed increased production of TNFα (and IL6) in stimulated mononuclear cells of mildly hypercholesterolemic, older and overweight subjects after 32 days of 7 E% TFA intake from stick margarine compared to intake of soybean oil. In contrast, in two studies in young, lean and healthy subjects, supplementation with a mix of *trans*18:1n-7 and *trans*18:1n-6 (~3 E%) for 6 weeks had no effect on plasma concentrations of TNFα, IL6, IL8 or adiponectin[10], and 5 weeks consumption of 10 E% TFA from hydrogenated soybean oil did not affect the serum TNFα (or IL6) concentration when compared to palm stearin or high oleic palm olein[9]. It is possible, that the TNF system is more susceptible to TFA-induced activation in older and overweight subjects.

The effect of the TFA-rich diet on sTNF-Rs was visible in heparinized plasma but not in serum. Hypothetically, the incorporation of TFA into cell membranes and ensuing change in cellular lipid rafts may have imposed conformational changes on the extra-cellular domains of membrane-bound TNF receptors[28], which may have persisted after shedding from the membrane. The coagulation cascade occurring during serum formation activates several proteases that may alter the sTNF-R molecules. A difference in configuration between sTNF-Rs exposed to TFA or control fat could affect the extent to which the molecules are modified and thereby their recognition by specific antibodies.

Wong et al. described great differences in cytokines between serum and plasma[29], and hypothesized that this might be due to i) degradation of the cytokines during the clotting process, ii) *ex vivo* degranulation of granulocytes and platelets, and/or iii) non-specific interference related to the protein matrix. For sTNF-R we observed a difference between concentrations in plasma and serum, but more importantly we found that the sTNF-R response to TFA exposure is detectable in plasma but not in serum. Whatever the explanation, our observations suggest that plasma is the material of choice for studying the effect of dietary fats on sTNF-R. Notably, cross-sectional associations between TFA intake and sTNF-Rs were detected in studies analyzing plasma[1-3], but not in one using serum samples[25].

Previous intervention studies examining the effect of TFA on systemic CRP concentrations are limited and contradictory. In studies providing healthy subjects with 8 or 10 E% TFA, the concentration of CRP increased compared to provision of carbohydrate and oleic acid[8] or compared to oleic acid/polyunsaturated FA or palmitic/oleic acid[9]. In contrast, CRP was unaffected by dietary TFA in a study comparing diets with four types of margarine (up to 5.2 E% TFA), butter or soybean oil[11], in a study comparing intake of partially hydrogenated soy bean oil (4 E% TFA) to corn oil[12] and in a study comparing stick margarine intake (3.6 E% TFA) with a butter/canola oil mix[30]. It is possible that very high dietary TFA levels are needed before CRP is affected, or more likely, that most studies have simply been underpowered to detect the small changes induced by TFA; the difference between diets in CRP in the two crossover studies showing significant effects of TFA was only 0.12 mg/l (n=41)[9] and 0.20 mg/l (n=50)[8], respectively. In our study, the baseline-adjusted mean values for CRP at wk 16 in the TFA and CTR groups were 1.93 mg/l (CI: 1.53 – 2.43) and 1.91 mg/l (CI: 1.50 – 2.42), respectively. Consis-

tent with the width of these confidence intervals, we were not able to detect a difference between diet groups of the size previously reported.

We did not see a clear pattern for the effect of TFA on systemic markers of endothelial dysfunction. Serum sE-selectin increased in the TFA group, but not significantly more than in controls. In contrast, sVCAM-1 and sICAM-1 concentrations remained unchanged. An increase in E-selectin after 5 weeks of 8 E% TFA consumption (compared to carbohydrate, stearic acid, oleic acid and a mix of lauric, myristitic and palmitic acid) has been reported in a previous trial[8].

Others have documented that urinary 8-iso-$PGF_{2\alpha}$, a validated marker of oxidative stress *in vivo*, is increased by intake of CLA and the TFA isomers *trans*18:1n-7 and *trans*18:1n-6[17-19]. However, a mixture of TFA isomers, as provided to the subjects in the present study, had no detectable effect on the urinary concentration of 8-iso-$PGF_{2\alpha}$ and we hypothesize that the increase in oxidative stress markers previously seen with *trans*18:1n-7 consumption may be ascribed to endogenous conversion to CLA[18].

The dietary intervention did not affect mRNA expression of inflammatory markers and ceramide content in subcutaneous AT. This may be due to limited incorporation of TFA into AT during the 16 weeks of intervention; the AT content of the two predominant TFA isomers doubled in the TFA group, but still only constituted ~1% of total FA. The low degree of TFA incorporation is not surprising given the half life of subcutaneous AT FA of 6-18 months[31,32]. However, as the visceral depot FA turnover is higher than that of the subcutaneous[33] and since meal FA uptake is relatively larger in the former depot[34], more TFA may have been incorporated here. Previously, the TFA concentration was shown to be higher in visceral compared to subcutaneous AT[35]. Also, the subcutaneous AT depot may not adequately reflect an effect of TFA on AT inflammation; macrophage infiltration is higher in visceral than in subcutaneous AT[36] and in obese subjects, the mRNA of TNFα was found to be considerable higher in visceral compared to subcutaneous AT[37,38].

The facts that cytokines are also produced by other tissues/cells, e.g. skeletal muscle[39], and that transcript and protein turnover is different[40], would also explain why mRNA expression did not correlate with serum levels.

In conclusion, our research indicates that the TFA-associated increase in cardiovascular risk, beyond the adverse effect explained by changes in blood lipids, is likely to be due to induction of systemic low-grade inflammation.

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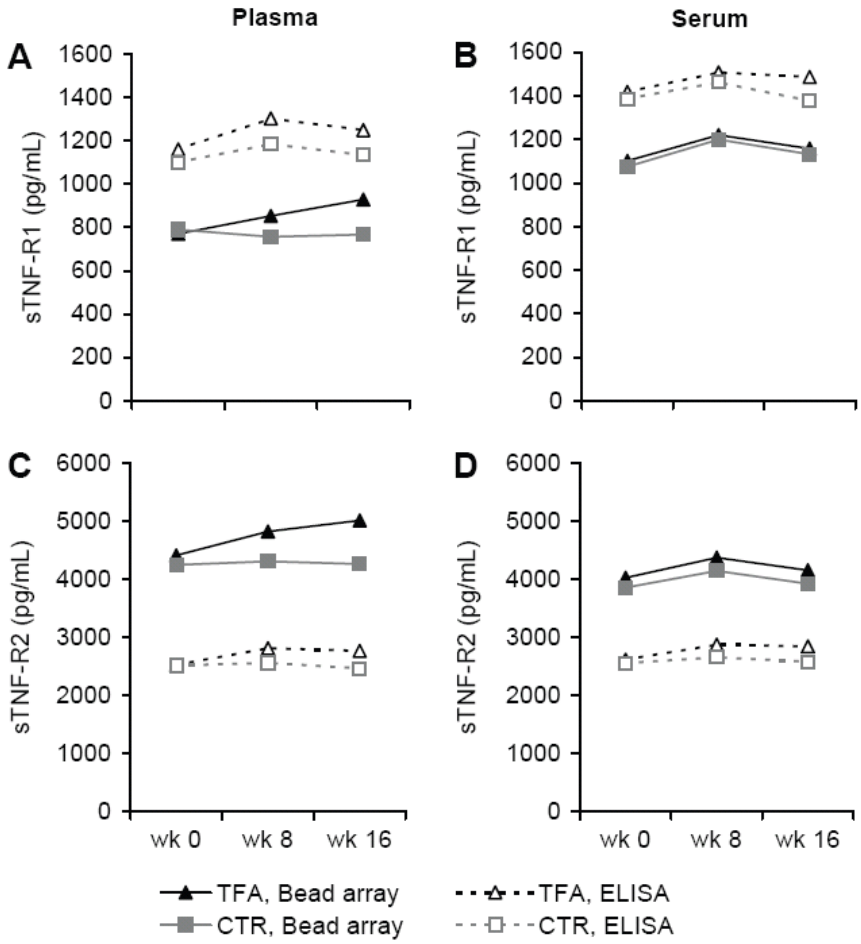

Supplemental Figure 1: Mean concentrations of soluble tumor necrosis factor receptor 1 (sTNF-R1) in plasma (panel A) and serum (panel B) and of soluble tumor necrosis factor receptor 2 (sTNF-R2) in plasma (panel C) and serum (panel D) assessed by Bead array and ELISA technology.

Supplemental Table 1: Fatty acid composition in subcutaneous abdominal adipose tissue (AT) triglycerides in the trans fat (TFA; n=23) and control (n=25) groups and in lean references (n=18).

Fatty acid (nmol/g AT)	Week 0	Week 16	ΔWeek 0-16	Group mean difference (95%CI) [1]
C14:0				-50 (-201; 101)
TFA	773 ± 36	705 ± 49	-71 ± 52	
CTR	732 ± 37	745 ± 56	12 ± 64	
Lean ref.	737 ± 41			
C16:0				-663 (-1742; 415)
TFA	5812 ± 296	5023 ± 279	-739 ± 335	
CTR	5477 ± 219	5606 ± 446	129 ± 474	
Lean ref.	4974 ± 246 [2]			
C18:0				116 (-59; 292)
TFA	525 ± 51	788 ± 58	255 ± 76	
CTR	498 ± 53	670 ± 63	172 ± 81	
Lean ref.	732 ± 65 [2]			
C20:0				0 (-9; 9)
TFA	26 ± 3	30 ± 3	4 ± 4	
CTR	29 ± 3	29 ± 3	0 ± 5	
Lean ref.	43 ± 4 [2]			
C16:1n-7*trans*				5 (-16; 27)
TFA	34 ± 4 [3]	29 ± 3	-6 ± 6	
CTR	25 ± 2	29 ± 9	3 ± 10	
Lean references	19 ± 3 [2]			
C16:1n-7*cis*				-119 (-469; 230)
TFA	1742 ± 172	1457 ± 126	-219 ± 150	
CTR	1655 ± 99	1571 ± 124	-83 ± 165	
Lean ref.	1585 ± 110			
C18:1n-9*trans*				32 (10; 55) [4]
TFA	51 ± 6	96 ± 7	46 ± 11	
CTR	58 ± 7	62 ± 8	4 ± 11	
Lean references	69 ± 6			
C18:1n-7*trans*				32 (9; 54) [4]
TFA	71 ± 6	111 ± 8	41 ± 11	
CTR	74 ± 7	79 ± 8	5 ± 11	
Lean references	80 ± 7			
C18:1n-12*cis*&C18:1n-5*trans*?				27 (9; 45) [4]
TFA	72 ± 6	99 ± 7	27 ± 7	
CTR	79 ± 6	76 ± 7	-3 ± 7	
Lean ref.	79 ± 7			
C18:1n-9*cis*				-537 (-2497; 1422)
TFA	11565 ± 571	9728 ± 615	-1598 ± 619	
CTR	10694 ± 461	10162 ± 728	-532 ± 914	
Lean ref.	10530 ± 455			
C18:1n-7*cis*				-34 (-126; 59)
TFA	566 ± 46	468 ± 25	-76 ± 41	
CTR	514 ± 25	499 ± 37	-15 ± 46	
Lean ref.	446 ± 31 [2]			
C18:2n-6*cis*				49 (-548; 642)
TFA	3403 ± 150	2685 ± 166	-667 ± 195	
CTR	3230 ± 156	2635 ± 234	-595 ± 294	
Lean ref.	3242 ± 169			
C20:3n-6*cis*				2 (-18; 21)
TFA	76 ± 7	61 ± 6	-12 ± 9	
CTR	69 ± 5	59 ± 7	-10 ± 9	
Lean ref.	67 ± 8			

(*Continued*)

Supplemental Table 1 *(continued)*

Fatty acid (nmol/g AT)	Week 0	Week 16	ΔWeek 0-16	Group mean difference (95%CI) [1]
C20:4n-6*cis*				24 (-28; 76)
TFA	174 ± 14	176 ± 17	5 ± 25	
CTR	143 ± 12	149 ± 18	6 ± 18	
Lean ref.	155 ± 14			
C22:4n-6*cis*				-2 (-14; 11)
TFA	70 ± 6	38 ± 3	-30 ± 7	
CTR	61 ± 4	41 ± 5	-21 ± 7	
Lean ref.	38 ± 5 [2]			
C18:3n-3&C20:1n-9*cis*				14 (-101; 128)
TFA	540 ± 29	486 ± 33	-53 ± 31	
CTR	530 ± 29	470 ± 46	-60 ± 54	
Lean ref.	657 ± 51 [2]			
C20:5n-3*cis*				4 (-13; 21)
TFA	58 ± 6 [3]	57 ± 6	0 ± 9	
CTR	79 ± 8	53 ± 5	-26 ± 10	
Lean ref.	78 ± 10			
C22:5n-3*cis*				4 (-25; 33)
TFA	155 ± 11	110 ± 10	-43 ± 16	
CTR	159 ± 13	107 ± 10	-53 ± 14	
Lean ref.	121 ± 13 [2]			
C22:6n-3*cis*				9 (-26; 44)
TFA	188 ± 14	127 ± 14	-60 ± 21	
CTR	215 ± 20	120 ± 11	-95 ± 20	
Lean ref.	160 ± 22			
ΣSFA				-589 (-1910; 733)
TFA	7135 ± 349	6545 ± 348	-551 ± 424	
CTR	6736 ± 278	7050 ± 538	313 ± 583	
Lean references	6486 ± 264			
ΣMUFA-*cis*				-604 (-2974; 1766)
TFA	14100 ± 776	11988 ± 750	-1786 ± 795	
CTR	13098 ± 564	12477 ± 882	-621 ± 1109	
Lean references	12808 ± 582			
ΣPUFAn-6*cis*				76 (-567; 718)
TFA	3724 ± 164	2960 ± 180	-704 ± 220	
CTR	3504 ± 161	2884 ± 251	-620 ± 311	
Lean references	3503 ± 179			
ΣPUFAn-3*cis*				39 (-137; 215)
TFA	942 ± 51	780 ± 58	-156 ± 69	
CTR	983 ± 55	749 ± 65	-234 ± 80	
Lean references	1016 ± 88			
n-6:n-3 PUFA				-0.1 (-0.4; 0.2)
TFA	4.0 ± 0.1 [3]	3.9 ± 0.1	-0.1 ± 0.1	
CTR	3.7 ± 0.1	3.9 ± 0.1	0.2 ± 0.1	
Lean references	3.7 ± 0.2			

Values are means ± SEM, completers only. AT, adipose tissue; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; SFA, saturated fatty acids. [1]Baseline-adjusted least square mean difference (95% CI) between diet groups at week 16. [2]Significantly different from overweight interventions subjects (diet groups combined) by two-tailed t-test (P<0.01). [3]Significantly different from CTR group by two-tailed t-test (P<0.05). [4]Significant difference between diets by baseline-adjusted ANCOVA (P<0.01).