Metabolomic and gene expression analysis to study the effects of dietary saturated and polyunsaturated fats

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Structured abstract

Purpose of review: Give an update on recent dietary intervention studies that have used peripheral blood mononuclear cell gene expression analysis and/or metabolic profiling to understand how intake of polyunsaturated and saturated fat affects and biological pathways linked to cardiovascular disease.

Recent findings: Several studies showed that intake of fish oil and vegetable oil, high in omega-3 fatty acids, reduced expression level of genes involved in inflammation. One intervention study showed that gene transcripts encoding genes involved inflammation and lipid metabolism increased after intake of polyunsaturated fat (mainly omega-6 fatty acids) compared to saturated fat. Additionally, using targeted metabolomics, the concentrations of atherogenic lipoprotein particles and several metabolites including palmitoylcarnitine, myristoylcarnitine, kynurenine were reduced after intake of polyunsaturated fat compared to saturated fat, whereas acetate, acetoacetate were increased. The use of targeted metabolomics showed that overfeeding with polyunsaturated fat reduced the serum concentration of ceramides, dihydroceramides, glucosylceramides and lactosylceramides, while overfeeding with saturated fat increased serum concentration of these metabolites.

Summary: The use of gene expression profiling and metabolomics are promising tools to identify possible new biomarkers linking fat quality to cardiovascular disease risk.

Key words

Dietary intervention, saturated fat, polyunsaturated fat, peripheral blood mononuclear cells, metabolomics

Introduction

We lack a comprehensive understanding of how fat quality may reverse or promote metabolic dysregulation preventing or causing cardiovascular disease (CVD). Dietary fatty acids, in particular omega-6 and omega-3 polyunsaturated fatty acids (PUFAs) are potent signaling molecules acting as ligands for peroxisome-proliferator activated receptors (PPARs) that are transcription factors regulating genes important in lipid metabolism. In addition, activation of PPARs inhibits the expression of a number of pro-inflammatory genes in macrophages probably mediated by inhibiting NF- κ B activity or the Toll-like receptors (TLRs). In contrast, SFAs have been shown to activate TLR4, leading to activation of the NF- κ B signaling pathway (1). To understand further the role of dietary fat on CVD risk in humans, we need a better understanding of how intake of dietary fat affect biological pathways are linked to CVD disease progression. Peripheral blood mononuclear cells (PBMCs) include monocytes and lymphocytes and provide a good model system for studying gene expression of mediators involved in the early development of atherosclerosis (2). Gene expression analysis in these cells have shown that they may act as potential biomarkers reflecting metabolic health changes in the liver (3) and in the adipose tissue (4). A complementary approach is to quantify and identify plasma metabolites and decipher pathways involved in the pathophysiology of CVD (5), and this is also a powerful tool to increase our understand of the impact of diet on health (6).

Use of PBMC gene expression analysis in dietary intervention studies focusing on SFA and PUFA

The very long chain omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are fatty acids known to influence blood pressure, lipid metabolism and inflammation. Many have previously utilized PBMC gene expression analysis in fish oil supplementation studies reviewed by Ulven et al. (7). The most important findings from these earlier studies when using a targeted approach is that fish oil supplementation seem to reduce the gene expression levels of e.g. *PDGF*, *CCL2*, *IL1B*, *IL10* and *IL23A* encoding inflammatory genes important for progression of atherosclerotic lesions (7). When using a whole genome transcriptional approach, annotation and pathways analysis showed that fish oil supplementation modulated the expression of genes involved in inflammatory pathways, such as eicosanoid synthesis, interleukin signaling, oxidative stress response, cell cycle, cell

adhesion, apoptosis, scavenger receptor activity, and DNA damage reviewed by Ulven et al. (7).

Recent studies using fish oil and krill oil have further confirmed and shed more light on the role of omega-3 PUFAs in metabolic regulation in humans. In women with polycystic ovary syndrome (PCOS) changes in expression of genes involved in insulin, lipid and inflammation was studied in a randomized, double blind, placebo-controlled trial 12-week intervention with 180 mg EPA and 120 mg DHA /day in fish oil or placebo (paraffin) was examined. Fish oil, compared to control, significantly increased the gene expression levels of *PPARG*, while the expression levels of *IL1A* and *IL8* were significantly decreased (8)*. In addition, fish oil supplementation did not change the gene expression levels of *TNF*, in line with previous data (9, 10). Fish oil also did not change the expression levels of *TGFB1*, *LPA*, *LDLR*, and *SLC2A1* (8). In a double-blind randomized trial involving *PPARg* ProAla polymorphism genotyped type 2 diabetes patients the effect of DHA supplementation (2.4 g/day) for 8 weeks led to upregulation of gene expression level of *P16INK* (11)*. In line with others, the expression levels of *TNF* and *IL6* were not affected (8-10). Intervention with either krill oil (445 mg EPA+210 mg DHA/day), lean and fatty fish (195 mg EPA + 343 mg DHA/day), or high oleic sunflower oil (control) for 8 weeks in healthy adults (n=36) with slightly elevated triglycerides led to changes in the mRNA levels of *PPARGC1A*, *SCD*, *ABCA1*, and *CD40* between the groups (12)*. Within the krill oil group, the expression levels of genes were down-regulated involved in glucose and cholesterol metabolism and fatty acid β -oxidation (12), whereas the mRNA levels of inflammatory genes, including *TNF* were not altered after krill oil supplementation. Interestingly, the mRNA levels of several inflammatory genes were downregulated in the control group, who received sunflower oil high in oleic acid. These findings are interesting and need to be followed up in order to see if PUFA rich oils such as krill oil, high oleic sunflower oil and fish oil supplementation affect gene expression differently, potentially explaining the different metabolic phenotypes observed after intake of these oils.

Vegetable oils, such as flaxseed oil and camelina sativa oil, are rich in the omega-3 fatty acid alpha-linolenic acid (ALA). Although conflicting evidence exist, ALA may have a beneficial effects on lipid profile, glucose metabolism, and inflammation (13, 14). A few human dietary intervention studies have used a targeted approach to study if ALA can modulate PBMC gene expression profiles. Only the expression level of *IFNG* was decreased after 12-week randomized controlled trial with camelina sativa oil supplementation, containing 10 g

ALA/day, among 72 adults with impaired fasting glucose (15)**. The mRNA expression level of *PPARG* increased, and the expression levels of *LPA*, *IL1A*, and *TNF* decreased after 12-week supplementation with flaxseed oil (800 mg ALA/day) among 60 diabetic patients compared to placebo (paraffin) (16). The mRNA levels of *LDLR*, *IL8*, and *TGF* were not changed during the intervention. The increased expression level of *PPARG* has also been observed after 12-week supplementation with 4g/day of ALA (17). To our knowledge, no whole genome profiling data exist from human dietary intervention study with ALA.

We recently showed that several genes involved in lipid metabolism and inflammation were favorably changed in PBMCs when replacing 6 E % SFA with PUFA (mainly omega-6 fatty acids) during eight-week double blind, randomized controlled dietary intervention trial with subjects with slightly elevated LDL-C (n=99) (18)*. Of particular interest was the upregulation of mRNA levels of *LDLR*, *LXRA*, *ABCG*, *SREBF1*, and *FASN* in the omega-6 PUFA diet group compared to the control group. These changes may explain the reduction in total cholesterol and LDL-C during the intervention (19). In contrast, the mRNA levels *UCP2* and *PPARD* were reduced during the intervention. Among inflammatory genes, the mRNA levels of *IRAK1*, *TNFSF1*, *TLR4*, *GATA3*, *IL2RG* and *CD8A* were increased during the intervention (18). These findings are interesting and needs further investigation to understand the role of increased omega-6 PUFA intake on inflammation in humans.

Metabolic profiling in dietary intervention studies focusing on SFA and PUFA

Comprehensive metabolic profiling has emerged as an important tool to identify novel biomarkers of CVD development (20). Cohort studies using metabolomics have identified a large number of metabolites linked to CVD risk (21-24). Dietary intervention studies focusing on healthy dietary patterns have shown also that the human metabolome responds to changes in diet (25-29). The impact of fat quality on the plasma metabolome however has scarcely been investigated. In a double-blind, parallel-group, randomized trial where 39 young and normal-weight individuals were overfed muffins high in SFAs (palm oil) or omega-6 PUFA (sunflower oil) for seven weeks, Elmsjö et al. showed that lactate, acetate, and 3-hydroxybutyrate significantly differed between SFA and PUFA overfeeding (30). Intake of PUFA was associated with higher levels of lactate, and lower levels of acetate and 3-hydroxybutyrate compared to SFA. Within-group analysis showed that in the PUFA group, the levels of lactate, alanine, and the aromatic amino acids tryptophan, tyrosine and phenylalanine increased, while the levels of acetate and 3-hydroxybutyrate decreased. In the

SFA group, the branched chain amino acids valine, leucine and isoleucine increased and lactate decreased (30). Overfeeding with SFAs and PUFAs caused similar increase in body weight, but SFA increased liver fat and visceral fat compared to PUFAs, and PUFAs caused a threefold increase in lean tissue (31). The increase in total body fat and liver fat correlated with increased plasma levels of the branched chain amino acids, 3-hydroxybutyrate and 3-methyl-2 oxovalerate, and decreased levels of alanine. Decreased lactate levels were also correlated with increased body fat (30). This overfeeding strategy has recently been repeated in overweight subjects (n=61) overfed muffins high in SFAs (palm oil) of omega-6 PUFA (sunflower oil) for eight weeks in a double-blind randomized trial, followed by a four week low-calorie diet period (32)**. Again, body weight gain was the same in the two groups, and SFAs induced higher liver fat content along with liver enzymes and increased LDL-C and apoB, whereas PUFAs overfeeding reduced LDL-C. In addition, the visceral fat content and total body fat was similar, which was in contrast to their previous study with lean healthy subjects. Overfeeding with SFAs increased serum concentration, while PUFAs reduced the serum concentration of ceramides, dihydroceramides, glucosylceramides and lactosylceramides (32). The changes in all species of ceramide groups were directly associated with change in liver fat and correlated to changes in the circulating fatty acid 16:1n-7. Since ceramides have been shown to be involved in pathogenesis of non-alcoholic fatty liver disease (NAFLD), these findings may provide more information about how SFA compared to PUFA may cause NAFLD via ceramide synthesis and *de novo* lipogenesis. In overweight subjects (n=38) overfed (1000 excess calories/day) with either SFA or unsaturated fat, or carbohydrates, the SFA group increased the intrahepatic triglyceride content more compared to the other groups (33)*. In addition, the total plasma ceramide concentration increased after SFA overfeeding compared to the other groups, where no changes in plasma ceramides were observed (33). The increase in total ceramides in the SFA group was due to increases in several long-chain ceramides, which is in accordance to the study by Rosqvist et al. All together these data shows that using more targeted metabolomics will increase knowledge of the effect of SFA versus unsaturated fat regarding NAFLD and liver fat accumulation.

Using a food exchange approach, we obtained an 11 % reduction in LDL-C and 9 % reduction in total cholesterol in the experimental group (PUFA group) compared to the control-diet group (SFA group) exchanging similar food items with improved fat quality (replacing SFA with mostly omega-6 PUFA) (19). In addition to gene expression analysis described above,

we further used multiple metabolomics platforms and measured 129 unique metabolites of which the fasting concentrations of palmitoylcarnitine, myristoylcarnitine, cysteine and kynurenine were reduced whereas acetate, acetoacetate; the amino acids serine, asparagine, proline, and the amino acid metabolite cystathionine were increased in the PUFA group compared to the SFA group (18). No differences between the groups during intervention was observed for other acyl carnitines, total carnitine, or carnitine metabolites, including betaine, choline, and the gut flora metabolite trimethylamine *N*-oxide (TMAO). When we correlate the change in total cholesterol and LDL-C with changes in these metabolites, there was a significant positive correlation between change in total cholesterol and LDL-C and change in valerylcarnitine, propionylcarnitine, palmitoylcarnitine and acetylcarnitine and a negative correlation between change in serine, and asparagine (personal communication). We also performed a comprehensive lipoprotein subclass profiling and showed that the fasting concentrations of atherogenic lipoprotein particles were reduced after intake of PUFA compared to SFA. Phospholipids such as total phosphoglycerides (PG), total cholines, phosphatidylcholines (PC), and sphingomyelins (SM), were also reduced in PUFA group compared to SFA (18). Michelesen et al. has used the same NMR platform to examine the effects of consuming a SFA rich diet, a MUFA rich diet, or a Mediterranean diet for 8 weeks in 47 subjects at risk of metabolic syndrome (34)*. They assessed in total 162 serum metabolites, and 52 of these were differently altered during the intervention. Both the MUFA and the Mediterranean diet decreased the same LDL fractions, including particle number, phospholipid and free cholesterol fractions compared to the SFA diet. In addition, the Mediterranean diet decreased the larger subclasses of VLDL, related VLDL fractions, VLDL-TG, and serum-TG (34). This shows that the intake of Mediterranean diet has an additional effect on lipoprotein subclasses and composition compared to the effect of MUFA alone. Future studies comparing the effect of replacing SFA with omega-6 PUFA, or MUFA on the plasma metabolome would be interesting to perform in order to understand further the role of fat on CVD risk.

Conclusion

Here we show that metabolic profiling may help to identify novel biomarkers associated with development of CVD. Many of the genes regulated by PUFAs in dietary intervention studies are involved in metabolic processes such as inflammation and lipid metabolism, important for atherosclerotic plaque formation. The next step is to combine gene expression analysis with metabolome analysis to unravel the interplay between diet and health, which in the future may

provide us with a better understanding of nutritional impacts on disease. Another future direction is to focus more on postprandial changes in gene expression profiling since postprandial challenges has been suggested be useful tool to identify new health biomarkers (35). It is well known that PUFA, MUFA and SFA differently regulate genes involved in inflammation and lipid metabolism in postprandial test meal studies (36-40), Recently, it was shown that PBMC gene expression time-course analysis during challenge tests delivered more information on the effect of diet than single fasting PBMC gene expression measurements (41). The role of genotype on gene expression changes during dietary intervention studies may also give us more insight to understand why some respond better to intervention than others (10, 42). PBMC gene expression profiles can also be used to differentiate responders and non-responders and understand why some respond better to intervention than others (43, 44).

Key points

- Omega-3 fatty acids reduce the PBMC expression levels of genes involved in inflammation, but *TNF* seems not to be regulated by omega-3 intake.
- Intake of PUFA (mainly omega-6 fatty acids) compared to SFA increase the PBMC expression levels of genes involved in lipid metabolism and inflammation.
- Comprehensive lipoprotein particle profiling can increase our understanding of how PUFA and MUFA reduce risk of CVD.
- Overfeeding studies show that overfeeding with SFAs increase serum concentration of ceramides, while PUFAs reduces the concentration.
- Ceramides are associated with change in liver fat, and these findings can associate SFA intake with increased NAFLD risk.

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Conflicts of interest

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Reference section

1. Georgiadi A, Kersten S. Mechanisms of gene regulation by fatty acids. *Advances in nutrition*. 2012;3(2):127-34.
2. Visvikis-Siest S, Marteau JB, Samara A, Berrahmoune H, Marie B, Pfister M. Peripheral blood mononuclear cells (PBMCs): a possible model for studying cardiovascular biology systems. *Clin Chem Lab Med*. 2007;45(9):1154-68.
3. Bouwens M, Afman LA, Muller M. Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid beta-oxidation: functional role of peroxisome proliferator activated receptor alpha in human peripheral blood mononuclear cells. *The American journal of clinical nutrition*. 2007;86(5):1515-23.
4. O'Grada CM, Morine MJ, Morris C, Ryan M, Dillon ET, Walsh M, et al. PBMCs reflect the immune component of the WAT transcriptome--implications as biomarkers of metabolic health in the postprandial state. *Molecular nutrition & food research*. 2014;58(4):808-20.
5. Basak T, Varshney S, Hamid Z, Ghosh S, Seth S, Sengupta S. Identification of metabolic markers in coronary artery disease using an untargeted LC-MS based metabolomic approach. *Journal of proteomics*. 2015.
6. Hillesheim E, Brennan L. Metabotyping and its role in nutrition research. *Nutr Res Rev*. 2019:1-10.
7. Ulven SM, Myhrstad MC, Holven KB. Marine n-3 Fatty Acids and Gene Expression in Peripheral Blood Mononuclear Cells. *Current cardiovascular risk reports*. 2014;8(11):412.
8. Rahmani E, Jamilian M, Dadpour B, Nezami Z, Vahedpoor Z, Mahmoodi S, et al. The effects of fish oil on gene expression in patients with polycystic ovary syndrome. *European journal of clinical investigation*. 2018;48(3). *This study show that fish oil, compared to control, significantly increased the gene expression levels of *PPARG*, while the expression levels of *IL1A* and *IL8* were significantly decreased. In addition, the study confirm that *TNF* expression is not altered by fish oil.
9. Weaver KL, Ivester P, Seeds M, Case LD, Arm JP, Chilton FH. Effect of dietary fatty acids on inflammatory gene expression in healthy humans. *J Biol Chem*. 2009;284(23):15400-7.
10. Cormier H, Rudkowska I, Lemieux S, Couture P, Vohl MC. Expression and Sequence Variants of Inflammatory Genes; Effects on Plasma Inflammation Biomarkers Following a 6-Week Supplementation with Fish Oil. *Int J Mol Sci*. 2016;17(3):375.
11. Toupchian O, Sotoudeh G, Mansoori A, Abdollahi S, Ali Keshavarz S, Djalali M, et al. DHA-enriched fish oil upregulates cyclin-dependent kinase inhibitor 2A (P16(INK)) expression and downregulates telomerase activity without modulating effects of PPARGgamma Pro12Ala polymorphism in type 2 diabetic patients: A randomized, double-blind, placebo-controlled clinical trial. *Clinical nutrition*. 2018;37(1):91-8. *This study show that fish oil upregulate the gene expression level of *P16INK*, while the expression of *TNF* and *IL6* is not affected.
12. Rundblad A, Holven KB, Bruheim I, Myhrstad MC, Ulven SM. Effects of fish and krill oil on gene expression in peripheral blood mononuclear cells and circulating markers of inflammation: a randomised controlled trial. *Journal of nutritional science*. 2018;7:e10. *This is the first study who show that intake of krill oil down-regulate genes involved in glucose and cholesterol metabolism and fatty acid β -oxidation in humans, while inflammatory genes, including *TNF* was not altered.
13. Su H, Liu R, Chang M, Huang J, Jin Q, Wang X. Effect of dietary alpha-linolenic acid on blood inflammatory markers: a systematic review and meta-analysis of randomized controlled trials. *European journal of nutrition*. 2018;57(3):877-91.
14. Schwab US, Lankinen MA, de Mello VD, Manninen SM, Kurl S, Pulkki KJ, et al. Camelina Sativa Oil, but not Fatty Fish or Lean Fish, Improves Serum Lipid Profile in Subjects with Impaired Glucose Metabolism-A Randomized Controlled Trial. *Molecular nutrition & food research*. 2018;62(4).
15. de Mello VD, Dahlman I, Lankinen M, Kurl S, Pitkanen L, Laaksonen DE, et al. The effect of different sources of fish and camelina sativa oil on immune cell and adipose tissue mRNA expression in subjects with abnormal fasting glucose metabolism: a randomized controlled trial. *Nutr Diabetes*.

- 2019;9(1):1. ** Few studies have investigated the effect of vegetable omega-3 fatty acids. This study showed that the expression level of *IFNG* was decreased after camelina sativa oil supplementation.
16. Hashemzadeh AA, Nasoohi N, Raygan F, Aghadavod E, Akbari E, Taghizadeh M, et al. Flaxseed Oil Supplementation Improve Gene Expression Levels of PPAR-gamma, LP(a), IL-1 and TNF-alpha in Type 2 Diabetic Patients with Coronary Heart Disease. *Lipids*. 2017;52(11):907-15.
 17. Zhao N, Wang L, Guo N. alpha-Linolenic acid increases the G0/G1 switch gene 2 mRNA expression in peripheral blood mononuclear cells from obese patients: a pilot study. *Lipids in health and disease*. 2016;15:36.
 18. Ulven SM, Christensen JJ, Nygard O, Svardal A, Leder L, Ottestad I, et al. Using metabolic profiling and gene expression analyses to explore molecular effects of replacing saturated fat with polyunsaturated fat-a randomized controlled dietary intervention study. *The American journal of clinical nutrition*. 2019;109(5):1239-50. * This study combined PBMC gene expression analysis with targeted metabolomics in a dietary intervention study where polyunsaturated fat replaced saturated fat.
 19. Ulven SM, Leder L, Elind E, Ottestad I, Christensen JJ, Telle-Hansen VH, et al. Exchanging a few commercial, regularly consumed food items with improved fat quality reduces total cholesterol and LDL-cholesterol: a double-blind, randomised controlled trial. *The British journal of nutrition*. 2016;116(8):1383-93.
 20. Shah SH, Kraus WE, Newgard CB. Metabolomic profiling for the identification of novel biomarkers and mechanisms related to common cardiovascular diseases: form and function. *Circulation*. 2012;126(9):1110-20.
 21. Wurtz P, Havulinna AS, Soininen P, Tynkkynen T, Prieto-Merino D, Tillin T, et al. Metabolite profiling and cardiovascular event risk: a prospective study of 3 population-based cohorts. *Circulation*. 2015;131(9):774-85.
 22. Strand E, Pedersen ER, Svingen GF, Olsen T, Bjorndal B, Karlsson T, et al. Serum Acylcarnitines and Risk of Cardiovascular Death and Acute Myocardial Infarction in Patients With Stable Angina Pectoris. *Journal of the American Heart Association*. 2017;6(2).
 23. Zuo H, Ueland PM, Ulvik A, Eussen SJ, Vollset SE, Nygard O, et al. Plasma Biomarkers of Inflammation, the Kynurenine Pathway, and Risks of All-Cause, Cancer, and Cardiovascular Disease Mortality: The Hordaland Health Study. *Am J Epidemiol*. 2016;183(4):249-58.
 24. Ruiz-Canela M, Hruby A, Clish CB, Liang L, Martinez-Gonzalez MA, Hu FB. Comprehensive Metabolomic Profiling and Incident Cardiovascular Disease: A Systematic Review. *Journal of the American Heart Association*. 2017;6(10).
 25. Lankinen M, Schwab U, Kolehmainen M, Paananen J, Nygren H, Seppanen-Laakso T, et al. A Healthy Nordic Diet Alters the Plasma Lipidomic Profile in Adults with Features of Metabolic Syndrome in a Multicenter Randomized Dietary Intervention. *The Journal of nutrition*. 2016.
 26. Lankinen M, Kolehmainen M, Jaaskelainen T, Paananen J, Joukamo L, Kangas AJ, et al. Effects of whole grain, fish and bilberries on serum metabolic profile and lipid transfer protein activities: a randomized trial (Sysdimet). *PloS one*. 2014;9(2):e90352.
 27. Guasch-Ferre M, Zheng Y, Ruiz-Canela M, Hruby A, Martinez-Gonzalez MA, Clish CB, et al. Plasma acylcarnitines and risk of cardiovascular disease: effect of Mediterranean diet interventions. *The American journal of clinical nutrition*. 2016;103(6):1408-16.
 28. Yu E, Ruiz-Canela M, Guasch-Ferre M, Zheng Y, Toledo E, Clish CB, et al. Increases in Plasma Tryptophan Are Inversely Associated with Incident Cardiovascular Disease in the Prevencion con Dieta Mediterranea (PREDIMED) Study. *The Journal of nutrition*. 2017;147(3):314-22.
 29. Toledo E, Wang DD, Ruiz-Canela M, Clish CB, Razquin C, Zheng Y, et al. Plasma lipidomic profiles and cardiovascular events in a randomized intervention trial with the Mediterranean diet. *The American journal of clinical nutrition*. 2017;106(4):973-83.
 30. Elmsjo A, Rosqvist F, Engskog MK, Haglof J, Kullberg J, Iggman D, et al. NMR-based metabolic profiling in healthy individuals overfed different types of fat: links to changes in liver fat accumulation and lean tissue mass. *Nutr Diabetes*. 2015;5:e182.

31. Rosqvist F, Iggman D, Kullberg J, Cedernaes J, Johansson HE, Larsson A, et al. Overfeeding polyunsaturated and saturated fat causes distinct effects on liver and visceral fat accumulation in humans. *Diabetes*. 2014;63(7):2356-68.
32. Rosqvist F, Kullberg J, Stahlman M, Cedernaes J, Heurling K, Johansson HE, et al. Overeating saturated fat promotes fatty liver and ceramides compared to polyunsaturated fat: a randomized trial. *The Journal of clinical endocrinology and metabolism*. 2019.** This overfeeding study showed that overfeeding with SFAs increased serum concentration, while PUFAs reduced the serum concentration of ceramides, dihydroceramides, glucosylceramides and lactosylceramides. The changes in all species of ceramide groups were directly associated with change in liver fat. These findings can be associated with NAFLD risk.
33. Luukkonen PK, Sadevirta S, Zhou Y, Kayser B, Ali A, Ahonen L, et al. Saturated Fat Is More Metabolically Harmful for the Human Liver Than Unsaturated Fat or Simple Sugars. *Diabetes care*. 2018;41(8):1732-9.** This overfeeding study with SFA or unsaturated fat, or carbohydrates, showed that the SFA group increased the intrahepatic triglyceride content more compared to the other groups. The total plasma ceramide concentration increased after SFA overfeeding compared to the other groups.
34. Michielsen C, Hangelbroek RWJ, Feskens EJM, Afman LA. Disentangling the Effects of Monounsaturated Fatty Acids from Other Components of a Mediterranean Diet on Serum Metabolite Profiles: A Randomized Fully Controlled Dietary Intervention in Healthy Subjects at Risk of the Metabolic Syndrome. *Molecular nutrition & food research*. 2019;63(9):e1801095.* This study shows that MUFA and the Mediterranean diet decreased the same LDL fractions, including particle number, phospholipid and free cholesterol fractions compared to the SFA diet. In addition, the Mediterranean diet decreased the larger subclasses of VLDL, related VLDL fractions, VLDL-TG, and serum-TG
35. van Ommen B, Keijer J, Heil SG, Kaput J. Challenging homeostasis to define biomarkers for nutrition related health. *Molecular nutrition & food research*. 2009;53(7):795-804.
36. Bouwens M, Grootte Bromhaar M, Jansen J, Muller M, Afman LA. Postprandial dietary lipid-specific effects on human peripheral blood mononuclear cell gene expression profiles. *The American journal of clinical nutrition*. 2010;91(1):208-17.
37. van Dijk SJ, Mensink M, Esser D, Feskens EJ, Muller M, Afman LA. Responses to high-fat challenges varying in fat type in subjects with different metabolic risk phenotypes: a randomized trial. *PloS one*. 2012;7(7):e41388.
38. Esser D, van Dijk SJ, Oosterink E, Lopez S, Muller M, Afman LA. High fat challenges with different fatty acids affect distinct atherogenic gene expression pathways in immune cells from lean and obese subjects. *Molecular nutrition & food research*. 2015.
39. Myhrstad MC, Narverud I, Telle-Hansen VH, Karhu T, Lund DB, Herzig KH, et al. Effect of the fat composition of a single high-fat meal on inflammatory markers in healthy young women. *The British journal of nutrition*. 2011;106(12):1826-35.
40. Oyri LKL, Narverud I, Bogsrud MP, Hansson P, Leder L, Byfuglien MG, et al. Postprandial changes in gene expression of cholesterol influx and efflux mediators after intake of SFA compared with n-6 PUFA in subjects with and without familial hypercholesterolaemia: secondary outcomes of a randomised controlled trial. *Journal of nutritional science*. 2019;8:e27.
41. van Bussel IPG, Fazlzadeh P, Frost GS, Rundle M, Afman LA. Measuring phenotypic flexibility by transcriptome time-course analyses during challenge tests before and after energy restriction. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2019:fj201900148R.
42. Matualatupauw JC, Radonjic M, van de Rest O, de Groot LC, Geleijnse JM, Muller M, et al. Apolipoprotein E genotype status affects habitual human blood mononuclear cell gene expression and its response to fish oil intervention. *Molecular nutrition & food research*. 2016;60(7):1649-60.
43. Rudkowska I, Paradis AM, Thifault E, Julien P, Barbier O, Couture P, et al. Differences in metabolomic and transcriptomic profiles between responders and non-responders to an n-3 polyunsaturated fatty acids (PUFAs) supplementation. *Genes & nutrition*. 2013;8(4):411-23.

44. Rundblad A, Larsen SV, Myhrstad MC, Ottestad I, Thoresen M, Holven KB, et al. Differences in peripheral blood mononuclear cell gene expression and triglyceride composition in lipoprotein subclasses in plasma triglyceride responders and non-responders to omega-3 supplementation. *Genes & nutrition*. 2019;14:10.