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1 **Short report**

2 **Dietary n-6 to n-3 fatty acid ratio is related to liver fat content independent of genetic effects:**  
3 **evidence from the monozygotic co-twin control design**

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14

15 **Words:** 1454 including the abstract, excluding references

16 **Keywords:** identical twins; twin study; liver fat; nutrition; omega-6: omega-3 ratio

17 **Abstract**

18 **Background & aim:** Lifestyle changes focusing on diet and exercise remain the cornerstone of the  
19 treatment of non-alcoholic fatty liver disease (NAFLD). The present co-twin control study of  
20 monozygotic (MZ) twin pairs was designed to identify nutritional factors potentially involved in the  
21 pathogenesis of NAFLD.

22 **Methods:** Cross-sectional study of 50 MZ twin pairs (age range: 23-36 years), of which ten pairs were  
23 discordant for liver fat (liver fat percentage of one twin  $\leq$  5% and his/her co-twin  $>$  5% and a  
24 difference between co-twins of  $>$  5%) as determined by magnetic resonance spectroscopy. Nutrient  
25 intake was calculated from 3-day food records.

26 **Results:** Among the ten liver fat-discordant twin pairs, the n-6: n-3 ratio was significantly higher in  
27 the twins with higher liver as compared to their co-twins with lower liver fat (6.6: 1 vs. 3.2: 1,  $p$ -value  
28 = 0.005). In multiple regression analysis of within-pair differences including all 50 twin pairs, a  
29 higher n-6: n-3 ratio was significantly associated with a higher liver fat percentage within MZ twin  
30 pairs after adjustment for body mass index, energy intake and other covariates (standardized beta=  
31 0.43,  $p$ -value = 0.001).

32 **Conclusions:** Our findings suggest that the n-6: n-3 ratio is a promising dietary agent for the  
33 prevention and treatment of NAFLD. Clinical trials are required to better understand causal  
34 relationships and required doses.

35

## 36 **Introduction**

37 Non-alcoholic fatty liver disease (NAFLD), characterized by fat accumulation in more than 5% of  
38 hepatocytes in the absence of excessive alcohol consumption, is the most common chronic liver  
39 disease in the world. It affects up to a quarter of the general population, depending on the country  
40 studied<sup>1</sup>. The disease spectrum includes simple steatosis, steatohepatitis, advanced fibrosis and  
41 cirrhosis which can progress to liver failure<sup>1</sup>.

42 The heritability of NAFLD is low, underscoring the importance of environmental factors in its  
43 etiology<sup>2</sup>. Currently, there are no approved pharmacotherapies for patients with NAFLD. Therefore,  
44 weight reduction and lifestyle changes through diet and exercise form the first-line therapy for  
45 treatment<sup>3</sup>. Diets high in saturated fatty acids and refined carbohydrates could exacerbate NAFLD<sup>4</sup>,  
46 while Mediterranean dietary patterns might reduce liver fat even in the absence of weight loss<sup>5</sup>.  
47 Randomized controlled trials suggest that supplementation with omega-3 (n-3) polyunsaturated fatty  
48 acids (PUFAs) has therapeutic potential for treating patients with NAFLD<sup>6</sup>.

49 Another powerful study design that allows stronger inference about causality than observational  
50 studies of unrelated individuals is the co-twin-control design of disease-discordant monozygotic  
51 (MZ) twin pairs because MZ twins are genetically identical at the DNA sequence level and  
52 discordance within pairs must thus be of environmental origin. Our previous twin study suggests that  
53 metabolic abnormalities in acquired obesity are tightly linked to liver fat content<sup>7</sup>. The present study  
54 of MZ twins was designed to identify nutritional factors potentially involved in the pathogenesis of  
55 NAFLD.

## 56 **Research design and methods**

57 TwinFat study participants were enrolled from two Finnish population-based longitudinal studies of  
58 five consecutive birth cohorts of young adult twins<sup>8</sup>. Participants were invited to the TwinFat study  
59 based on their reported body mass index (BMI) during these studies (at the age of 23 to 27 years)  
60 with the aim to represent a wide range of intra-pair differences in BMI. Participants were then  
61 weighed and their height was measured while barefoot and in light clothing by trained research  
62 nurses. Whole-body fat was measured by dual energy x-ray absorptiometry, abdominal  
63 subcutaneous and intra-abdominal fat by magnetic resonance imaging and liver fat percentage by  
64 magnetic resonance spectroscopy as described earlier<sup>9</sup>. The homeostasis model assessment of  
65 insulin resistance (HOMA-IR) was calculated based on 2-hour oral glucose tolerance tests. Fasting  
66 plasma cholesterol and triglyceride concentrations were determined with enzymatic methods.  
67 Supine blood pressure was measured using an automatic digital sphygmomanometer (mean of three  
68 measurements).

69 Nutrient intake was calculated from 3-day food records by the Diet32 program (Aivo), based on a  
70 national Finnish database (Fineli, [www.fineli.fi](http://www.fineli.fi), National Institute for Health and Welfare, Nutrition  
71 Unit, Helsinki, Finland). The sports activity index was calculated from the Baecke questionnaire<sup>10</sup>.  
72 Out of 107 twin individuals with data on liver fat and food records, one twin individual was excluded  
73 because of missing information on physical activity, two twin individuals because of implausible  
74 energy intakes (one woman reported < 714 kcal/day, and one men > 4200 kcal/day) and another four  
75 twin individuals because of missing co-twin information. Thus, the final analytic sample consisted of  
76 50 MZ twin pairs, of which 10 twin pairs were discordant for liver fat. Liver fat discordance was  
77 defined as the liver fat percentage of one twin  $\leq 5\%$  and his/her co-twin  $> 5\%$  and a difference between  
78 co-twins of  $> 5\%$  resulting in identification of ten such pairs. The study protocol was in accordance  
79 with the Helsinki Declaration and approved by local Ethical Committees. Written informed consent  
80 was given by all twins.

81 Data were reported as median and interquartile ranges. Differences between discordant twin pairs  
82 were analysed using Wilcoxon signed rank test. In all twin pairs, multiple linear regression analysis  
83 was used to relate within-pair differences in log transformed liver fat to within-pair differences in the  
84 omega-6 (n-6) to n-3 ratio adjusted for covariates. Statistical analyses were performed using  
85 Stata version 13 (Stata Corp, College Station, TX).

## 86 **Results**

87 The descriptive characteristics (clinical, behavioral, nutritional and liver fat) of the 100 twin  
88 individuals (mean age 29.9 years (range: 23-36 years) and of the subset of ten liver fat-discordant  
89 twin pairs are shown in **Table 1**. Among these ten pairs, the twins with higher liver fat had more body  
90 fat and a worse metabolic profile than their co-twins with lower liver fat. The twins with higher and  
91 lower liver fat did not differ in physical activity indices and energy intake, or the intake of protein,  
92 fat and carbohydrates. In every single discordant pair, the twin with the higher liver fat percentage  
93 had consistently a higher n-6: n-3 ratio than his or her co-twin with less liver fat (**Supplementary**  
94 **Figure**). The twins with the lower liver fat percentage had a mean n-6: n-3 ratio of 3.2: 1, while the  
95 ratio in the co-twins with higher liver fat percentage was 6.6: 1 ( $p$ -value = 0.005 for the difference  
96 between pairs).

97 The results from the within-pair linear regression on all 50 MZ pairs are presented in **Table 2**. The n-  
98 6: n-3 ratio and BMI were significant predictors and of about equal importance in predicting within-  
99 pair differences in liver fat. The gender interaction (male vs. female pairs) was not significant.  
100 Additional adjustment for intra-abdominal fat (standardized beta= 0.37,  $p$ =0.08) did not change the  
101 effect estimate for the n-6: n-3 ratio (standardized beta= 0.39,  $p$ -value = 0.002), but removed the  
102 influence of BMI (standardized beta= 0.13,  $p$ -value = 0.52). In a model that additionally adjusted for  
103 sucrose, the effect size for sucrose was not significant (standardized beta= -0.12,  $p$ -value = 0.28), and  
104 the effect estimate for the n-6: n-3 ratio remained unchanged (standardized beta= 0.40,  $p$ -value =  
105 0.001).

## 106 **Discussion**

107 To our knowledge, this is the first study that evaluated nutrient intake differences in MZ twin pairs  
108 discordant for liver fat. Among the 10 twin pairs discordant for liver fat, the co-twins with higher  
109 liver fat reported a higher dietary n-6: n-3 ratio than his or her co-twin in every single pair. We  
110 employed the MZ co-twin control design, which is analogue to a case-control study, where the twin  
111 with the higher liver fat is the case and the co-twin with the lower liver fat the control, with the  
112 additional advantage that cases and controls are naturally matched for several factors including age,  
113 sex and genotype.

114 Due to modern agriculture and the popularity of fast foods, Western diets contain excessive amounts  
115 of n-6 PUFAs and low amounts of n-3 PUFAs. An overconsumption of n-6 PUFAs coupled with  
116 under-consumption of n-3 PUFAs may lead to inflammation and pro-inflammatory cytokine  
117 production involved in many disease processes<sup>11</sup>. In addition, n-3 PUFAs may influence the intestinal  
118 microbiota<sup>12</sup> and thereby modulate NAFLD susceptibility. Recent meta-analysis of randomized  
119 controlled trials suggest that n-3 PUFA supplementation can reduce liver fat and glucose control in  
120 patients with NAFLD, at amounts that can be easily obtained through the habitual diet<sup>6</sup>.

121 In the present study of liver-fat discordant MZ twin pairs, a mean n-6: n-3 ratio of 3.2: 1 was  
122 associated with normal liver fat content, while a mean ratio of 6.6: 1 was associated with increased  
123 liver fat content independent of genetic and familial confounding factors. Dietary advice for  
124 preventing and treating NAFLD should focus on shifting consumption away from processed foods

125 (such as refined vegetable oils rich in n-6 fatty acid used for deep frying) towards fresh foods (such  
126 as vegetable oils used in salads), and by increasing fish intake while decreasing meat intake<sup>11</sup>. Our  
127 findings could have important implications for the dietary management of NAFLD and encourage  
128 clinical trials to better understand causal relationships and required doses.

### 129 **Competing interest statement**

130 The authors declare no competing interests.

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**Table 1.** Clinical characteristics, physical activity and macronutrient intakes of the monozygotic twins

	All twins	Twin pairs discordant for liver fat (within-pair difference in liver fat > 5%) <sup>1</sup>		p-value <sup>2</sup>
		Co-twins with liver fat ≤ 5%	Co-twins with liver fat > 5%	
<b>Demographic factors</b>				
Number (n) of individuals <sup>3</sup>	100	10	10	
Males (n)	46	6	6	
Liver fat >5% (n)	22	0	10	
Liver fat (%) <sup>4</sup>	1.0 (0.5, 3.9)	1.1 (0.8, 3.2)	9.4 (7.3, 9.4)	
<b>Clinical parameters<sup>4</sup></b>				
BMI (kg/m <sup>2</sup> )	26.6 (23.7, 29.8)	25.7 (22.9, 27.8)	31.8 (27.6, 35.0)	<b>0.005</b>
Body fat (%)	32.4 (26.9, 40.7)	27.7 (21.2, 38.8)	38.3 (31.9, 43.4)	<b>0.007</b>
Intra-abdominal fat (dm <sup>3</sup> )	807 (422, 1355)	604 (439, 11165)	1709 (1416, 2417)	<b>0.007</b>
Subcutaneous fat (dm <sup>3</sup> )	3727 (2449, 5513)	3263 (2150, 5498)	5768 (3195, 6965)	<b>0.007</b>
LDL-cholesterol (mmol/l)	2.7 (2.3, 3.2)	2.7 (2.5, 2.9)	3.3 (2.7, 3.8)	0.07
HDL-cholesterol (mmol/l)	1.4 (1.2, 1.7)	1.5 (1.3, 1.7)	1.2 (1.1, 1.4)	<b>0.02</b>
Triglycerides (mmol/l)	1.0 (0.7, 1.3)	0.7 (0.5, 1.1)	1.3 (1.0, 2.2)	<b>0.01</b>
HOMA-IR index	1.4 (0.8, 2.0)	1.1 (0.5, 1.8)	2.4 (1.8, 2.8)	<b>0.01</b>
Systolic blood pressure (mmHg)	124 (118, 138)	131 (122, 140)	139 (124, 148)	0.36
Diastolic blood pressure (mmHg)	70 (65, 80)	70 (70, 78)	79 (70, 80)	0.18
<b>Physical activity<sup>4</sup></b>				
Sports activity index	2.8 (2.3, 3.5)	3.3 (2.3, 4)	2.4 (1.8, 3.5)	0.33
Leisure time activity index	2.8 (2.5, 3.3)	2.8 (2.5, 3.0)	2.9 (2.5, 3.3)	>0.99
Work activity index	2.5 (2.0, 3.3)	2.3 (2.1, 3.0)	2.6 (1.9, 2.9)	0.14
Total energy intake (kcal) <sup>4</sup>	2075 (1774, 2551)	2169 (1809, 2382)	2611 (2319, 2800)	0.07
<b>Macronutrient intake (% energy intake)<sup>4</sup></b>				
Carbohydrates	43.1 (38.3, 48.1)	43.5 (37.9, 50.0)	37.4 (33.4, 44.0)	0.09
Protein	16.5 (14.8, 18.8)	18.0 (14.2, 22.9)	16.0 (14.1, 18.9)	0.24
Total fat	35.2 (30.6, 39.0)	32.4 (26.3, 38.9)	39.0 (31.6, 42.8)	0.11
Sucrose	9.7 (6.0, 13.0)	4.8 (3.8, 7.0)	8.8 (7.9, 11.2)	<b>0.04</b>
Saturated fatty acids	13.5 (11.1, 16.2)	11.3 (10.9, 13.8)	13.1 (10.7, 16.3)	0.58
Monounsaturated fatty acids	10.6 (9.0, 12.3)	8.8 (7.4, 12.8)	12.0 (9.6, 13.4)	0.24
Polyunsaturated fatty acids	4.5 (3.8, 5.8)	4.1 (3.5, 5.5)	5.6 (4.8, 5.8)	0.09
Omega-6/omega-3 ratio <sup>4</sup>	4.8 (3.0, 6.1)	3.2 (2.3, 5.4)	6.6 (3.8, 7.4)	<b>0.005</b>

Abbreviations: BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance

<sup>1</sup>Discordance was defined as the liver fat percentage of one twin ≤ 5% and the liver fat percentage of his/her co-twin > 5% and a within-pair difference in liver fat of > 5%. The difference in liver fat percentage between discordant co-twins ranged from 5 to 20%.

<sup>2</sup>P-values are from Wilcoxon signed rank test comparing co-twins discordant for liver fat.

<sup>3</sup>The 100 monozygotic twin individuals are from 50 complete twin pairs of which 10 twin pairs are discordant for liver fat.

<sup>4</sup>Data are median and interquartile ranges.

**Table 2.** Multiple linear regression analysis predicting (log transformed) liver fat within 50 pairs of monozygotic twins

<b>Dependent variable:</b> Within-pair difference ( $\Delta$ ) in log liver fat	Unstandardized beta coefficient	Standard error	Standardized beta coefficient	p-value
Sex (Female twin pairs)	-0.57	0.23	-0.31	<b>0.017</b>
Age of the twin pairs (years)	-0.02	0.02	-0.09	0.42
$\Delta$ Omega-6/omega-3 ratio	0.24	0.06	0.43	<b>0.001</b>
$\Delta$ Total energy intake (per 100 kcal)	0.00	0.02	-0.02	0.83
$\Delta$ Body mass index ( $\text{kg}/\text{m}^2$ )	0.10	0.03	0.42	<b>0.003</b>
$\Delta$ Alcohol intake (% energy intake)	-0.02	0.02	-0.12	0.30
$\Delta$ Sports activity index	-0.14	0.10	-0.17	0.17
<b><math>R^2=0.49</math></b>				

Supplementary Figure. Omega-6/omega-3 ratio in ten monozygotic twin pairs discordant for liver fat

