Contribution of glycerophospholipids and sphingomyelin to the circulating NEFA

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Highlights

- There is a lack of knowledge about the nonesterified fatty acids sources in the postprandial state, which prevails most of the day in humans in modern societies.
- An association between serum nonesterified fatty acids and circulating phospholipids is proposed
- Fatty acid composition in serum samples of 19 post-gestational women and 20 controls is analyzed by liquid chromatography coupled to triple quadrupole mass spectrometry and gas chromatography.
- Glycerophospholipids might contribute long-chain polyunsaturated fatty acid, while sphingomyelin might contribute very long-chain fatty acids to the circulating NEFA in both groups.

Abstract

Background

Serum nonesterified fatty acids (NEFA) are known to be associated with the development of insulin resistance. Recently, differences in the NEFA profile were found in subjects with history of gestational diabetes (postGDM) and healthy controls. Little is known about the NEFA sources in the postprandial state, which prevails most of the day in humans in modern societies. In the present study, we aimed to explore the potential contributions of glycerophospholipid (GPL) and sphingomyelin (SM) fatty acids to the circulating NEFA.

Methods

Serum-samples of 19 postGDM women and 20 controls were obtained in fasting state (t0) and 90 minutes (t90) after an oral glucose tolerance test. Fatty acid composition of NEFA and SM were analyzed with liquid chromatography coupled to triple quadrupole mass spectrometry and GPL by gas chromatography.

Results

The ratio of individual NEFA at t90 vs. t0 (t90/0-ratio) showed no difference between the two groups but increased with chain-length (7% for C16:1, 82% for C26:3). Only NEFA 10:0 was found with lower concentration at t0 and t90 in postGDM. At t90, long-chain polyunsaturated fatty acid correlated closely between NEFA and GPL in postGDM (20:5, 22:4, 22:5 and 22:6) and controls (20:3, 20:4 and 20:5). Very long-chain fatty acid 24:0 correlated significantly between NEFA and SM in postGDM and controls. Saturated and monounsaturated fatty acids correlated less between NEFA and GPL or SM.

Conclusions

The NEFA composition varied highly between fasting and fed state in both groups. GPL appeared to contribute long-chain polyunsaturated fatty acid, while SM appeared to contribute very long-chain fatty acids to the NEFA pool.

1 **Running title:** Contribution of glycerophospholipids and sphingomyelin to the circulating NEFA

2 **Abbreviations:** BMI, body-mass index; CE, cholesteryl esters; FA, fatty acids; FA GPL, fatty acids
3 derived from glycerophospholipids; FA SM, fatty acids derived from sphingomyelins; GC, gas
4 chromatography; GPL, glycerophospholipids; interquartile range, ir; LC-FA, long-chain fatty acids;
5 LC-MS/MS, liquid chromatography coupled triple quadruple mass spectrometry; LC-PUFA, long-
6 chain polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; NEFA, nonesterified fatty
7 acids; OGTT, oral glucose tolerance test; PUFA, polyunsaturated fatty acids; PostGDM, women after
8 gestational diabetes; SFA, saturated fatty acids; SM, sphingomyelin; TAG, triacylglycerol; t90/0-ratio,
9 ratio of FA concentration at t90 divided by concentration at t0; VLC-PUFA, very long-chain
10 polyunsaturated fatty acids;

11 **Key words:** Nonesterified fatty acids; phospholipids; sphingolipids; long-chain polyunsaturated fatty
12 acids; very long-chain fatty acids; postprandial metabolism.

13 ## Introduction

14 Nonesterified fatty acids (NEFA) are associated with metabolic disorders such as insulin resistance
15 and obesity (1). In patients with insulin resistance, polyunsaturated fatty acids (PUFA) were found to
16 be higher, whereas lower concentrations were found for saturated fatty acids (SFA) (2).
17 While the adipose tissue is generally considered as the major source of NEFA (3, 4), information of
18 minor contributors such as the enzymatic degradation of other lipid fractions by cell surface anchored
19 lipases, bloodstream sn-1-, sn-2 lipases and esterases is limited (5-7). Since the postprandial state
20 prevails most of the day when lipolysis in the adipose tissue is down-regulated, the relative
21 contribution of these sources increases and might therefore impact on the development of insulin
22 resistance.
23 Lipid fractions comprise phospholipids (37.7 to 54.6%), cholesteryl esters (CE; 23.6 to 32.4%),
24 triacylglycerols (TAG; 15.4 to 35.8%) and further minor components (8). Phospholipids are
25 compromised dominantly of glycerophospholipids (GPL) and a smaller fraction of sphingomyelins
26 (SM), which accounts for 16-21% of serum phospholipids (9). In GPL, fatty acids (FA) are esterified
27 at position sn-1 and sn-2 of the glycerol backbone. Palmitic acid (16:0), stearic acid (18:0), oleic acid
28 (18:1), linoleic acid (18:2), arachidonic acid (20:4) and docosahexaenoic acid (22:6) are the main FA
29 in GPL (10, 11). Hydrolysis of blood phospholipids contributes primarily the FA 16:0 and 18:1 (12)
30 and the long-chain polyunsaturated FA (LC-PUFA) 20:4 (13). In contrast, FA 12:0, 18:0 and 24:0
31 present the major amid-linked FA at the C2-atome of sphingosine (14).
32 Recently, in the Prediction, Prevention and Subclassification of Type 2 Diabetes Study (PPS-Diab.
33 Study, Diabetes Research Group, Munich) differences in the NEFA profile were found in subjects
34 with high risk for the development of insulin resistance such as women with history of gestational
35 diabetes in comparison to controls (15). In the present study, we aimed to explore different sources of
36 FA, which contribute to the NEFA pool in the fasted and non-fasted state in a subset of the PPS-Diab
37 study population.

38 **Materials and Methods**

39 Ethics Statement

40 This study was approved by the Ethics Committee of the Medical Faculty, Ludwig Maximilians
41 University of Munich (300-11). All participants gave their informed written consent before entering
42 the study.

43 Subjects

44 Serum samples of participants of the Prediction, Prevention and Subclassification of Type 2 Diabetes
45 Study were collected between November 2011 and December 2013 (PPS-Diab. Study, Diabetes
46 Research Group, Munich).
47 Details about the study design and the participating subjects have been published previously (16).
48 Briefly, 39 women, previously enrolled in the PPS-Diab study were studied at 9±3 months after
49 delivery. Twenty women were healthy controls, whereas 19 subjects had been diagnosed during
50 pregnancy with gestational diabetes mellitus (postGDM). The women's length, body weight, and body
51 fat mass (Bioelectrical impedance analysis, Tanita BC-418, Tanita Corporation, Tokyo, Japan) were
52 measured and BMI calculated. Hip and waist circumferences were assessed by tape measurements.

53 Blood sample collection and analysis

54 After overnight fasting, blood samples were drawn before (t0) and 90 minutes (t90) after oral glucose
55 administration (1.75 g/kg Dextro-OGTT solution, Hoffmann-La Roche, Grenzach-Wyhlen, Germany).
56 Blood samples were collected into serum tubes, separated by centrifugation after 30 minutes of
57 coagulation time and subsequently stored at -80°C for LC-MS/MS and GC analysis. All samples were
58 thawed and analyzed in spring 2014. Serum insulin was measured with chemiluminescence
59 technology (CLIA, DiaSorin LIAISON systems, Saluggia, Italy). To measure the relationship between
60 insulin sensitivity and first-phase insulin secretion the disposition index was determined. Glucose
61 concentrations were measured using a glucose analyzer with the glucose oxidase method (Glucose HK
62 Gen.3, Roche Diagnostics, Mannheim, Germany). The Matsuda Index and HOMA-Index were
63 calculated (17, 18).

64 LC-MS/MS analysis

65 NEFA were analyzed by LC-MS/MS (4000 QTRAP, AB Sciex, Darmstadt, Germany) as described
66 previously (19). In short, proteins were precipitated by adding 200 µl isopropanol to 20 µl serum in a
67 96-deepwell plate. After centrifugation, 10 µl of the supernatant were injected for each sample with an
68 eluent flow rate of 700 µl/min. Gradient elution was performed with eluent A (5mM ammonium
69 acetate and 2.1 mM acetic acid) and eluent B (acetonitrile with 20% isopropanol) on Pursuit UPS
70 Diphenyl column (1.9 µm, 100 x 3.0 mm; Varian, Darmstadt, Germany) at 40°C.
71 Analysis of SM species was performed by precipitating proteins of 10 µl serum with 500 µl methanol.
72 After centrifugation, 200 µl of the supernatant were mixed with 700 µl methanol and 30 µl of the
73 mixture were injected and measured by LC-MS/MS.

74 GC analysis

75 The analysis of GPL derived FA was performed by GC (10). 100 µl serum were combined with 100 µl
76 of an internal standard (1.2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol) and
77 600 µl methanol. GPL of the supernatant were re-esterified with sodium methoxide to form FA methyl
78 ester which have been analyzed by using an Agilent Technologies 7890A GC equipped with a BPX 70
79 column (50 m x 0.22 mm, 0.25 µm film, SGE, Weiterstadt, Germany) with an optimized temperature
80 and pressure program.

81 Statistical analysis

82 Data analysis was performed with Microsoft Excel 2010 (Microsoft Inc., Redmond, WA) and SPSS
83 version 22.0 (SPSS Inc., Chicago, IL, USA). Mann-Whitney-U tests were used to detect differences
84 between postGDM and controls. Statistical significance between groups was accepted after correction
85 for multiple testing according to Bonferroni ($p < 0.001$). Correlations coefficients (r) were evaluated
86 using Spearmen´s Correlation. Results were expressed as median with 1st quartile and 3rd quartile in
87 µmol/l or as percentages (% mol/mol).
88 FA were grouped by carbon chain length: medium-chain FA were categorized by chain-length less
89 than thirteen carbons, long-chain FA (LC-FA) between 14 and 22 and very long-chain FA (VLC-FA)

90 more than 23 carbons. Quality control samples were performed for each lipid fraction. FA were

91 excluded from further evaluation, if the coefficient of variation was higher than 20%.

92 **Results**

93 Study population

94 Samples of 39 women (19 postGDM, 20 healthy controls) were obtained and analyzed. Since the
95 major aim of this work was to associate NEFA to other lipid fractions in plasma, we excluded outliers,
96 which would affect data analysis. Two subjects were excluded due to a small difference between
97 fasting and postprandial state with a total NEFA t90/0-ratio of 41% and 49%, respectively (mean
98 NEFA t90/0-ratio was 14±4%, the two outliers were above the 98.5 percentile, shown in Fig. S1).
99 Data of the remaining 37 subjects (17 postGDM, 20 healthy controls) were included for statistical
100 analysis. Their characteristics are shown in Table 1. Age and time interval since delivery were similar
101 in both groups. The postGDM group had significantly higher BMI, WHR and body fat. Insulin and
102 glucose were higher in postGDM at both t0 and at t90, and Matsuda Index, HOMA Index and
103 Disposition Index differed significantly, whereas HbA1c was similar.

104 Differences of individual NEFA between groups

105 Most FA showed no significant difference between postGDM and controls in NEFA, GPL and SM
106 (Table S1, S2, S3). FA 10:0 showed significant lower concentrations in postGDM at both timepoints.
107 FA 26:3 showed higher concentrations in postGDM at t90, however was not significant when
108 corrected with Bonferroni.

109 T90/0-ratio of individual NEFA

110 The ratio of the FA concentration at t90 to t0 (t90/0) was measured for postGDM, controls and the
111 total study population (Figure 1). Mann-Whitney U test showed no significant differences between
112 postGDM and controls. Considering the total study population, 14% of the total NEFA content was
113 not affected by the glucose-mediated down regulation of the adipose tissue release (column 1 of
114 Figure 1). The ratio between t90 and t0 varied strongly between the distinct NEFA species with a
115 minimum of 6% for 16:1 and a maximum of 81% for 26:3. The t90/0-ratio was low in FA with shorter
116 chain length (about 5% in C10) and higher with increasing FA chain length (e.g. about 50-80% in
117 C26) (Fig. 1). In most cases, the degree of unsaturation did not have an apparent influence on the

118 t90/0-ratio. However, relative to 26:0 its unsaturated derivatives showed a continuous increase in the
119 t90/0-ratio with higher degree of unsaturation (Fig. 1). The highest t90/0-ratio was found for VLC-
120 PUFA (26:2, 26:3 and 26:4). T90/0-ratios greater than 40% were also found for VLC-FA (24:0, 24:1)
121 and PUFA (20:4, 24:3, 24:4, 24:5, 24:6). T90/0-ratios below 20% were found for monounsaturated FA
122 (MUFA, 14:1, 15:1, 17:1, 18:1, 19:1, 20:1), most saturated FA (SFA, 10:0, 12:0, 14:0, 15:0,16:0) and
123 the essential FA 18:2 and 18:3.

124 GPL FA

125 In GPL, 17 FA were quantified at t0 and t90 (Table 2). Concentration of GPL FA decreased slightly
126 after glucose intake. The highest amounts were found for 16:0, followed by 18:2, 18:0 and 18:1 at both
127 time points in postGDM and controls. At t0, FA 15:1, 20:5 and 22:6 showed significant correlations
128 for the total study population and the stratification analysis. FA 16:1 showed significant correlation
129 within the total study population as well as in postGDM, but not in the controls. At t90, LC-PUFA
130 (20:3, 20:4, 20:5, 22:5, 22:4 and 22:6) showed significant correlations in the total study population.
131 LC-PUFA 20:3, 20:4 and 20:5 were also significant in the controls, 22:5 and 22:6 in postGDM.

132 SM FA

133 In SM, 23 FA were quantified at t0 and t90 (Table 3). The FA concentrations showed no differences at
134 t0 and t90 in both groups. The highest concentrations were observed for 16:0, followed by 24:1, 20:0,
135 24:2, 22:0, 20:1 and 24:0. At t0, FA 15:1 and 24:0 showed significant correlations in the total study
136 population and FA 24:0 in postGDM. At t90, 24:0 showed high correlation coefficients in the total
137 study population, in postGDM and controls. Also 22:1 showed significant correlations in the total
138 study population and the controls.

139 **Discussion**

140 High levels of serum NEFA are found in insulin resistance and obesity (20-23). Therefore, it has been
141 proposed to measure the NEFA concentration routinely as a risk marker (3). A systematic review of
142 the literature explored the relationship between obesity and plasma NEFA concentration in 43 studies
143 (20). The relationship between the fasting NEFA concentrations was unrelated to the body fat mass,
144 even though the BMI was considered in quintiles for a more detailed phenotyping. Thus, the authors
145 proposed that elevated NEFA concentrations are not necessarily associated with obesity and insulin
146 resistance. They suggested a reevaluation of the relationships between adiposity, fatty acids and
147 insulin resistance (20).

148 Accumulating data in the recent years suggested a more complex role of NEFA (24, 25), e.g. fatty acid
149 metabolites like oxylipins and endocannabinoids are thought to influence the insulin resistance and
150 dysfunction of the adipose tissue (26). The variation in the NEFA composition could have a major
151 impact on biological effects, as well as their metabolic sources. So far, their sources have received
152 surprisingly little attention. The present study assessed a subset of the PPS-Diab Study (16).
153 Previously, the PPS-Diab Study assessed differences in the NEFA composition of 62 women with
154 postGDM and 49 healthy controls during fasting (15). The present study focused on the FA
155 contribution of different sources to the NEFA pool in the fasted and non-fasted state. A comparison
156 between the postGDM and controls showed only decanoic acid (10:0) with significant differences at t0
157 and t90. In an earlier study decanoic acid was found as a modulating ligand of peroxisome proliferator
158 activated receptors (PPARs) (27). High amounts of decanoic acid improved glucose sensitivity and
159 lipid profiles without weight gain in diabetic mice. This mouse model is interesting as in our study
160 decanoic acid was significant higher in controls at both timepoints and similar effects could play in
161 humans. Yet this effect has not been studied in humans and should be considered carefully due to
162 various other PPAR modulating ligands (27) and due to our small study cohort.

163 No differences were found in the t90/0-ratio between postGDM and controls for all other NEFA
164 species, however the individual NEFA species showed vast differences in their behavior after glucose
165 intake. This led us to consider the quality of the individual fatty acids and the derivation of the
166 individual NEFA species more closely.

167 Contribution of phospholipids

168 We aimed to explore if the NEFA composition is affected by the contribution of FA from
169 phospholipids. Since we did not find significant differences in the NEFA, FA GPL and FA SM
170 concentrations between postGDM and controls, we focused on results of the total study population.
171 Fourteen percent of the fasted NEFA concentration remained at t90, which could reflect a residual
172 lipase activity and/or the contribution of other sources. Of interest, only a relatively small decrease of
173 the t90/0-ratio occurred in FA with longer chain length (e.g. LC-FA and LC-PUFA). Therefore, we
174 assumed that these FA are less contributed by the release from adipose tissue. The t90/0-ratio varied
175 widely from 6% for 16:1 up to 81% for 26:3 (Fig. 1), which leads us to conclude that the relative
176 contribution of individual FA from adipose tissues and from other sources varied markedly.

177 Contribution of LC-PUFA from GPL

178 LC-PUFA 20:4 showed a t90/0-ratio of 42% and a significant correlation (r=0.527) between NEFA
179 and GPL at t90. In previous studies, low correlations between adipose tissue and NEFA were observed
180 for 20:4 (4, 28, 29). Thus, other sources than the adipose tissue might contribute 20:4 to the NEFA
181 pool. LC-PUFA 22:6 showed strong correlations between NEFA and GPL at t0 and t90. The
182 decreasing t90/0-ratio of 27% suggested a possible contribution of other sources to the NEFA 22:6.
183 Chen et al. demonstrated that endothelial lipase plays a major role in the 22:6 releases from GPL (30).
184 Endothelial lipase is known to be specific for GPL and the hydrolysis of amid-linked FA, preferably
185 22:6. However, for 22:6 also a close correlation between the adipose tissue and NEFA was
186 demonstrated (4, 29). We conclude that GPL contributes 22:6 particularly in postprandial state, while
187 in the fasted state 22:6 is mainly released from adipose tissue. LC-PUFA 20:5 showed the highest
188 correlation between GPL and NEFA at t0 and t90 and thus could be derived from GPL by $sPLA_2$
189 activity (31). The sources of 20:5 and 22:6 may be of high interest as their amount correlates with
190 increased cardiovascular risk, especially in red blood cells (11). Literature and our data suggest that
191 GPL might contribute an amount of LC-PUFA, which is most detectable in postprandial state (Fig. 2).

192 Contribution of VLC-FA from SM

193 VLC-FA 24:0 showed the highest correlation coefficient of all FA between SM and NEFA (r=0.417)
194 whereas no correlation was found between NEFA and GPL. This agreed with previously reported low
195 correlation coefficients for 24:0 between NEFA and GPL and between NEFA and subcutaneous
196 adipose tissue as well as between NEFA and visceral adipose tissue (r=0.217/r=0.300) (4). As
197 described above, 24:0 is primarily amid-linked in SM (14, 32). We assume that 24:0 is mainly derived
198 from SM cleavage (Fig. 2).

199 Strengths and limitations

200 The hydrolysis of other lipid fractions especially TAG significantly contribute to serum NEFA.
201 However, to perform an adequate TAG fatty acid spectrum analysis is challenging, particularly with
202 small sample volume. Relevant fatty acids would have been missed due to impurities by the thin-layer
203 plate. A high background by these impurities resulted in less precision of most FA. Thus, in this study
204 we focused on the contribution of phospholipids only. In a mouse model using radioactively labeled
205 FA, lipolysis of LDL-TAG affected the NEFA composition (33). No specific FA was determined and
206 the contribution of TAG derived FA varied significantly between 5% and 80-90% (34). In another
207 study, a separate contribution of the lipoprotein lipase was determined to be 25-30% on average, and
208 therefore dietary fat was assumed to contribute one-third to the NEFA composition (35). Serum TAG
209 has high contents of 18:1, 16:0 and 18:2 (9) and thus is a potentially major source for these FA (Fig.
210 2). Cholesterol esterase is involved in lipoprotein metabolism and also liberates esterified FA. CE
211 constitutes up to 40% of LDL (9). The FA 18:2 is the predominant FA esterified with cholesterol.
212 Minor components are 16:0 and 18:1 (9). In our data, 18:2 correlations between NEFA and GPL and
213 between NEFA and SM were poor at t0 and at t90. Hence, CE may be an important contribution to
214 NEFA18:2 (Fig. 2).
215 The NEFA pool is constantly influenced by an input of the adipose tissue, lipid fractions and other
216 sources, but also by the liberation to the prostaglandin synthesis, β-oxidation and membrane
217 integration (Fig. 2).

218 One might argue that even better insights could be delineated from studies using FA labeled with
219 radioisotopes or stable isotopes, but it would be challenging to design an ethically acceptable
220 experiment in humans were individual depots e.g. adipose tissue triglycerides could be labeled for an
221 informative experiment. We used standardized conditions, highly sensitive and precise methodology
222 to measure a broad spectrum of individual FA in NEFA and PL. However, some single FA were not
223 detectable in all lipid fractions, e.g. 24:0 was found in SM whereas its concentration was not
224 detectable in GPL. While OGTT is a widely accepted standardized challenge condition, results cannot
225 be extrapolated to a mixed meal providing dietary fats.
226 Some correlations between LC-PUFA of GPL and NEFA were found to be significant in the total
227 study population but not within both subgroups. The power for the subgroup analysis might be limited
228 due to the small sample size. However, an association for LC-PUFA between GPL and NEFA can be
229 assumed in both groups.

230 **Conclusion**

231 This study investigated differences in the NEFA profile in women with a history of gestational
232 diabetes and controls and the potential contribution of phospholipids to the NEFA composition. The
233 NEFA composition varied highly between fasting and fed state in both groups. GPL appeared to
234 contribute LC-PUFA and SM appeared to contribute VLC-FA.

235 **Conflict of interest**

236 The authors confirm that this article content has no conflicts of interest.

237 **Acknowledgement**

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240 **Author Contributions**

241 Conceived and designed the experiments: OU CH HD BK. Performed the experiments: FM OU HD
242 MF CH. Analyzed the data: FM OU HD CH. Wrote the paper: FM OU MF HD CH BK AL.

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Tables

Table 1. Clinical parameters of the study cohort. Values are represented as median [1st quartile; 3rd quartile]. Fat percentage and mass are measured with the BIA-scale.

	postGDM (n=17)	**controls (n=20)**	**p-value**
age [years]	35 [32;37,5]	36 [34;38]	0.218
height [cm]	167.86 [163.5;173]	167.75 [164;169]	0.956
weight [kg]	86.12 [69.2;100.9]	63.64 [57.5;65.5]	<0.001
BMI [kg/m^2]	30.8 [24.45;35.4]	22.5 [20.52;23.72]	<0.001
hip size [cm]	115.84 [101;130]	95.60 [91.5;98.5]	<0.005
waist size [cm]	94.23 [84;105]	75.15 [69.7;80.5]	<0.005
waist-hip ratio	0.812 [0.76;0.85]	0.788 [0.73;0.84]	0.308
time after delivery [months]	9.7 [8.25;11.65]	8.4 [7.07;9.3]	0.281
fat percentage [%]	39.02 [30.45;45.05]	28.02 [23.4;33.15]	<0.005
fat mas [kg]	35.15 [21.1;45.5]	18.38 [13.35;21.8]	<0.005
insulin at t0 [µU/ml]	13.3 [9.8;15.7]	4.4 [3.47;5.7]	<0.005
insulin at t90 [µU/ml]	97.3 [58.45;88]	32 [21.2;39.1]	<0.005
glucose at t0 [mg/dl]	92 [89;95]	84 [81.5;88.5]	<0.005
glucose at t90 [mg/dl]	128 [106.5;157,5]	93 [79;100]	<0.005
Matsuda Index	3.064 [2.56;3.71]	10.252 [8.05;10.44]	<0.005
HOMA Index	3.051 [2.2;3.66]	0.916 [0.74;1.16]	<0.005
Disposition Index	222.19 [171;285]	354.37 [263.24;445.31]	<0.005
HbA1c [%]	5.44 [5.3;5.65]	5.26 [5.07;5,4]	0.069
HbA1c [mmol/mol]	36.01 [34.4;38.2]	34.02 [31.92;35.5]	0.592

Table 2. Correlation coefficients (r) of fatty acids derived from glycerophospholipids (FA GPL) to the corresponding nonesterified fatty acid (NEFA). Correlations are calculated at t0 and t90 for the total study population, postGDM cases and healthy controls and shown with p-value (* for $p<0.05$; ** for $p<0.01$).

	t0						**t90**					
	total population (r, p-value)		**postGDM (r, p-value)**		**controls (r, p-value)**		**total population (r, p-value)**		**postGDM (r, p-value)**		**controls (r, p-value)**	
SFA												
14:0	0.184	0.290	0.339	0.216	0.074	0.757	0.333	0.051	0.114	0.685	0.358	0.121
16:0	0.095	0.589	0.329	0.232	-0.032	0.895	0.028	0.874	-0.204	0.467	0.140	0.556
17:0	0.250	0.148	0.425	0.114	0.297	0.203	0.146	0.403	0.136	0.630	0.182	0.443
18:0	0.220	0.205	0.246	0.376	0.059	0.806	0.136	0.437	0.311	0.259	0.029	0.902
MUFA												
15:1	0,451**	0.006	0.582*	0.023	0.459*	0.042	0.259	0.133	0.300	0.277	0.360	0.119
16:1	0.495**	0.002	0.679**	0.005	0.357	0.123	0.359*	0.034	0.354	0.196	0.347	0.133
18:1	0.102	0.559	0.243	0.383	0.065	0.787	-0.255	0.139	-0.266	0.337	-0.132	0.580
20:1	-0.047	0.788	0.225	0.420	-0.217	0.359	-0.234	0.176	0.093	0.742	-0.313	0.179
Essential FA												
18:2	0.122	0.486	0.346	0.206	0.029	0.905	-0.021	0.906	0.289	0.296	-0.110	0.645
18:3	0.125	0.476	0.396	0.143	0.023	0.925	0.328	0.054	0.446	0.095	0.229	0.332
LC-PUFA												
20:2	0.116	0.505	0.404	0.136	-0.259	0.271	0.207	0.233	0.422	0.117	0.153	0.519
20:3	0.335*	0.049	0.300	0.277	0.360	0.119	0.467**	0.005	0.161	0.567	0.642**	0.002
20:4	0.270	0.116	0.354	0.196	0.208	0.380	0.527**	0.001	0.396	0.143	0.647**	0.002
20:5	0.683**	0.000	0.829**	0.000	0.502*	0.024	0.835**	0.000	0.889**	0.000	0.839**	0.000
22:4	0.197	0.257	0.568*	0.027	0.150	0.529	0.404*	0.016	0.704**	0.003	0.283	0.227
22:5	0.253	0.142	0.404	0.135	0.204	0.389	0.511**	0.002	0.868**	0.000	0.439	0.053
22:6	0.597**	0.000	0.685**	0.005	0.555*	0.011	0.491**	0.003	0.850**	0.000	0.308	0.186

Table 3. Correlation coefficients (r) of fatty acids derived from sphingomyelin (FA SM) to the corresponding nonesterified fatty acid (NEFA). Correlations are calculated at t0 and t90 for the total study population, postGDM cases and healthy controls and shown with p-value (* for p<0.05; ** for p<0.01).

	t0						**t90**					
	total population (r, p-value)		**postGDM (r, p-value)**		**controls (r, p-value)**		**total population (r, p-value)**		**postGDM (r, p-value)**		**controls (r, p-value)**	
SFA												
12:0	0.394*	0.014	0.653**	0.003	0.236	0.316	0.455**	0.005	0.331	0.195	0.436	0.055
14:0	0.352*	0.030	0.392	0.107	0.178	0.452	0.283	0.090	-0.039	0.881	0.435	0.056
15:0	0.361*	0.026	0.482*	0.043	0.299	0.200	0.280	0.093	0.168	0.519	0.364	0.115
16:0	0.231	0.163	0.214	0.395	0.217	0.359	0.074	0.662	0.179	0.492	-0.027	0.910
17:0	0.324*	0.047	0.302	0.223	0.334	0.150	0.131	0.440	0.324	0.205	0.027	0.910
18:0	0.124	0.457	-0.201	0.423	0.396	0.084	0.130	0.443	0.336	0.187	-0.073	0.760
20:0	0.183	0.272	0.184	0.465	0.023	0.925	0.020	0.906	0.105	0.687	-0.137	0.565
22:0	0.067	0.691	0.263	0.291	-0.131	0.582	0.399*	0.014	0.366	0.148	0.459*	0.042
MUFA												
14:1	0.295	0.072	0.187	0.458	0.319	0.171	0.169	0.318	-0.113	0.667	0.388	0.091
15:1	0.436**	0.006	0.418	0.084	0.412	0.071	-0.109	0.522	-0.234	0.366	0.078	0.743
16:1	0.311	0.057	0.288	0.247	0.340	0.142	0.154	0.364	0.103	0.694	0.272	0.245
18:1	0.110	0.512	-0.149	0.556	0.338	0.145	0.110	0.512	-0.232	0.371	0.114	0.634
20:1	0.180	0.280	-0.129	0.610	0.396	0.084	-0.281	0.092	-0.505*	0.039	-0.241	0.307
22:1	0.240	0.147	0.029	0.909	0.482*	0.032	0.425**	0.009	0.455	0.066	0.458*	0.042
Essential FA												
18:2	0.292	0.075	0.181	0.473	0.368	0.110	0.171	0.312	0.054	0.837	0.278	0.235
LC-PUFA												
22:2	0.097	0.562	0.092	0.717	-0.033	0.890	0.222	0.186	0.446	0.073	0.128	0.591
22:3	0.110	0.512	0.024	0.926	0.011	0.965	0.387*	0.018	0.444	0.074	0.327	0.160
VLC-FA												
24:0	0.458**	0.004	0.681**	0.002	0.215	0.363	0.417*	0.010	0.528*	0.030	0.498*	0.025
24:1	0.326*	0.046	-0.080	0.754	0.538*	0.014	0.023	0.893	-0.189	0.468	0.163	0.492
24:2	0.303	0.065	0.412	0.090	0.355	0.125	-0.011	0.949	0.163	0.532	0.254	0.279
24:5	0.412*	0.010	0.441	0.067	0.424	0.062	0.211	0.209	0.153	0.557	0.307	0.188
26:0	0.269	0.102	0.273	0.272	0.250	0.287	0.138	0.414	0.032	0.903	0.157	0.508
26:2	-0.051	0.763	0.362	0.140	-0.408	0.075	-0.231	0.170	-0.078	0.765	-0.352	0.128

Figures

Figure 1. Postprandial nonesterified fatty acids (NEFA) species relative to fasted state. Ratio of NEFA t90 to t0 is described in percentage (%) for the total study population, cases with postGDM and controls. FA species ordered according to chain length and double bonds.

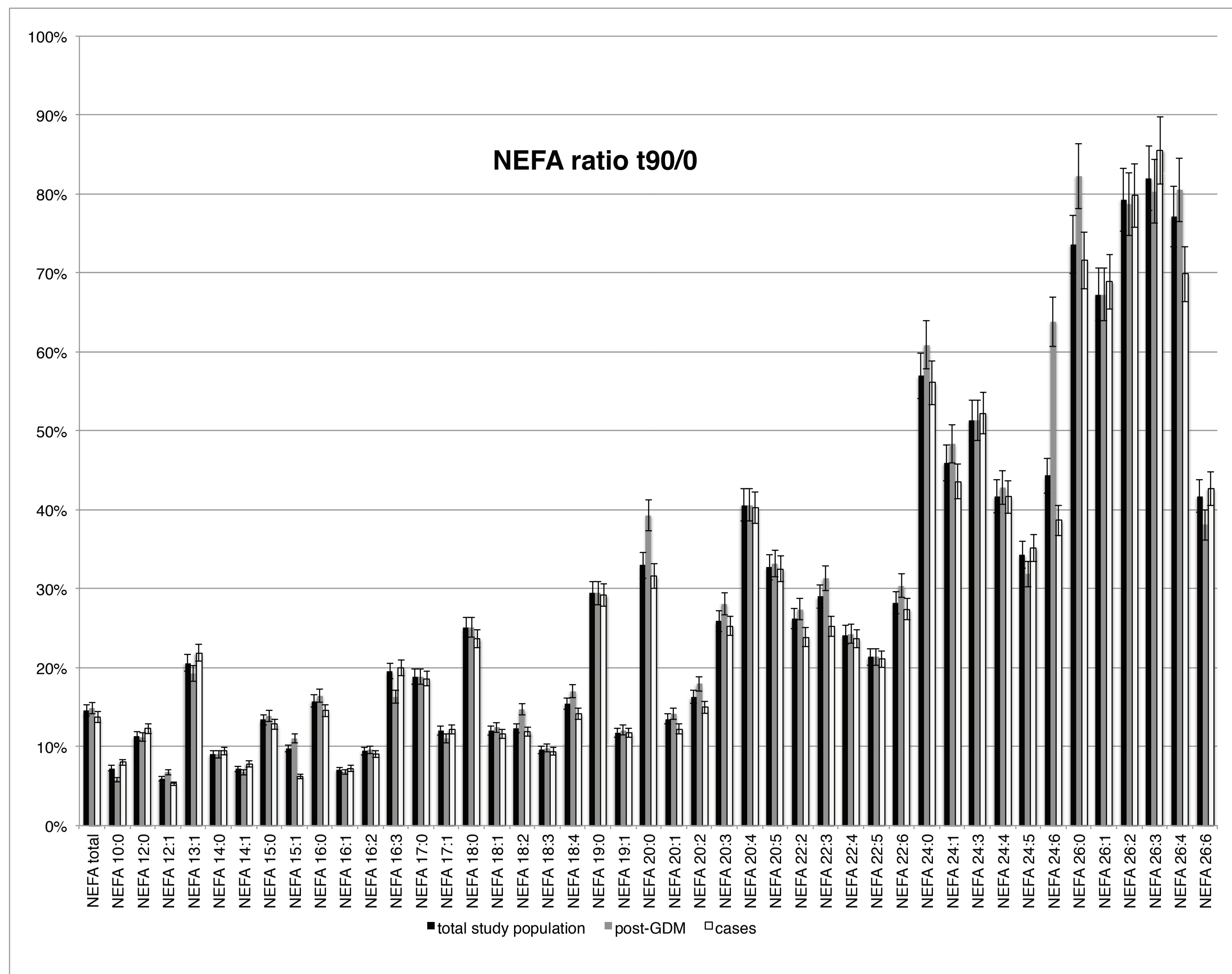
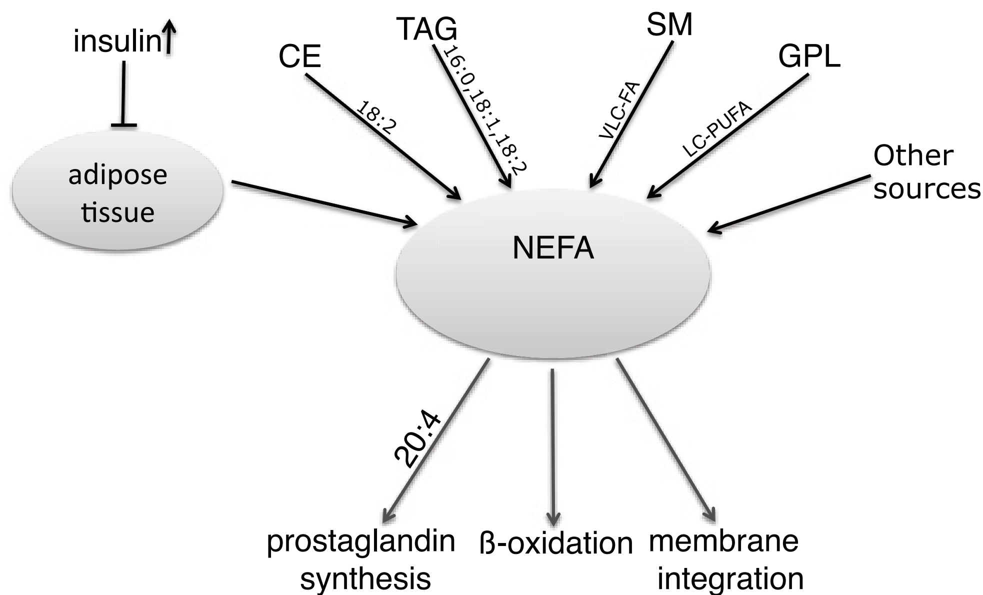
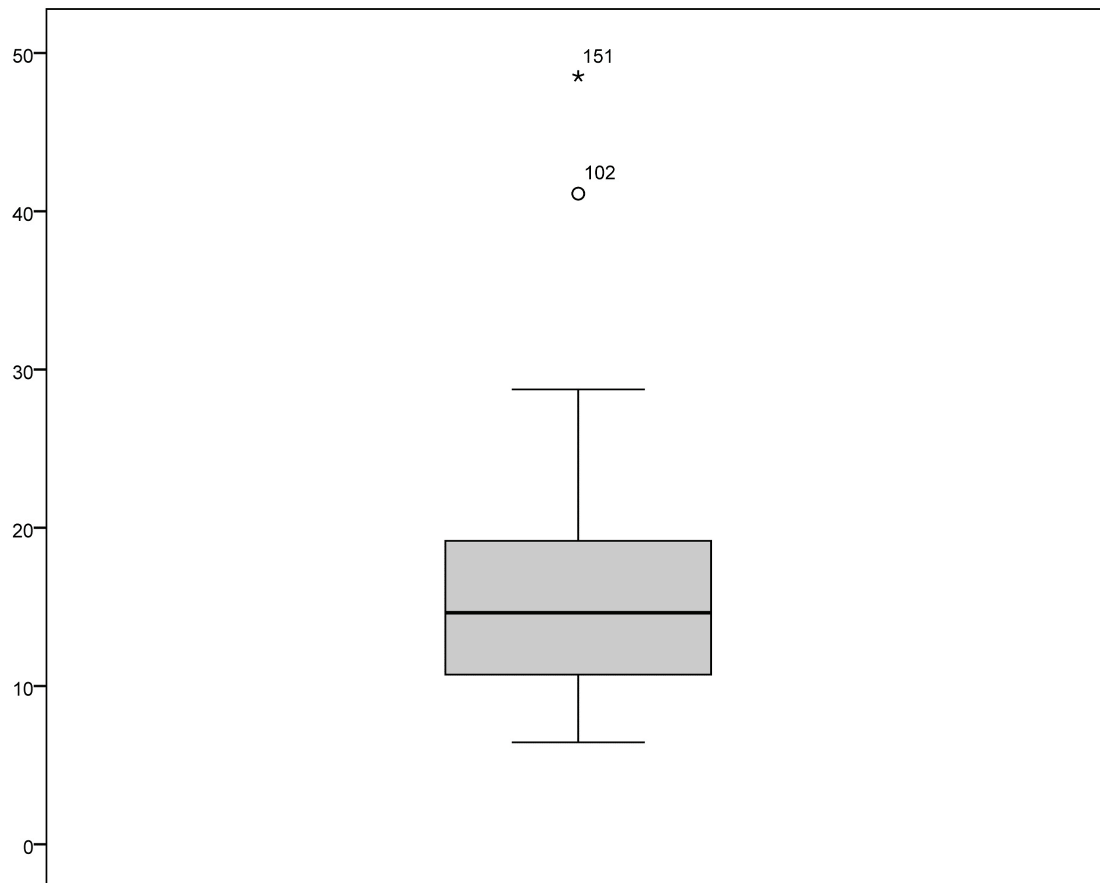


Figure 2. Contribution of serum lipid fractions to the nonesterified fatty acids (NEFA) composition in postprandial state. In postprandial state, insulin down regulates the release of fatty acids (FA) from adipose tissue and the relative contribution of other sources to the NEFA composition increases. Long-chain polyunsaturated fatty acids 20:3; 20:4, 20:5, 22:5 and 22:6 (LC-PUFA) are derived by the hydrolysis of glycerophospholipids (GPL), very long-chain FA 24:0 (VLC-FA) by sphingomyelin (SM). Cholesteryl ester (CE) may contribute 18:2, serum triacylglycerols (TAG) 16:0, 18:1 and 18:2. Other sources and consuming processes like the prostaglandin synthesis, β-oxidation and membrane integration influence the NEFA pool as well.



Supplementary

Supplementary Figure S1. Description of outliers. The boxplot of the ratio t90/0 shows two outliers with the number 151 (>3. IQR of the 75th percentile) and 102 (>1.5 IQR).



Supplementary Table S1. Median concentrations with 1st and 3rd quartiles and ratio of t90/0 (%) of nonesterified fatty acids (NEFA). Concentrations in µmol/l. The fatty acids (FA) are systematically presented as saturated (SFA), monounsaturated (MUFA), essential FA, long-chain and very long-chain polyunsaturated FA (LC-PUFA, VLC-PUFA).

FA	t0		t90		t90/0 ratio
total NEFA	538.6	[433.4; 633.0]	75.0	[57.1; 91.4]	14%
SFA					
10:0	1.0	[0.7; 1.2]	0.069	[0.0; 0.1]	7%
12:0	5.2	[3.3; 5,6]	0.71	[0.3; 0,8]	14%
14:0	17.2	[11.9; 18.9]	1.5	[0.9; 1.7]	9%
15:0	3.4	[2.0; 3.4]	0.34	[0.23; 0.46]	10%
16:0	134.0	[108.0; 159.0]	21.5	[15.9; 26.2]	16%
17:0	2.9	[2.3; 3.2]	0.55	[0.38; 0.69]	19%
18:0	43.9	[39.0; 49.5]	11.1	[8.5; 12.1]	25%
19:0	0.30	[0.21; 0.31]	0.073	[0.059; 0.092]	24%
20:0	0.31	[0.22; 0.37]	0.11	[0.074; 0.123]	35%
MUFA					
12:1	1.0	[0.6; 1.1]	0.067	[0.029; 0.099]	7%
13:1	0.27	[0.15; 0.29]	0.045	[0.035; 0.057]	17%
14:1	3.5	[2.4; 3.9]	0.25	[0.16; 0.30]	7%
15:1	0.30	[0.2; 0.3]	0.021	[0.009; 0.031]	7%
16:1	31.6	[20.5; 36.9]	2.0	[1.3; 2.4]	6%
17:1	2.0	[1.5; 2.3]	0.22	[0.15; 0.25]	11%
18:1	205.2	[167.0; 245.5]	24.1	[17.8; 29.7]	12%
19:1	0.75	[0.60; 0.86]	0.082	[0.052; 0.107]	11%
20:1	1.9	[1.4; 2.3]	0.24	[0.19; 0.28]	13%
Essential FA					
18:2	59.3	[46.1; 73.9]	7.6	[5.6; 9.2]	13%
18:3	6.3	[4.6; 7.4]	0.65	[0.45; 0.85]	10%
LC-PUFA					
14:2	0.27	[0.19; 0.36]	0.049	[0.037; 0.062]	18%
16:2	0.38	[0.27; 0.50]	0.038	[0.023; 0.042]	10%
16:3	0.13	[0.12; 0.17]	0.025	[0.02; 0.03]	19%
18:4	0.13	[0.09; 0.15]	0.019	[0.012; 0.026]	15%
20:2	1.0	[0.8; 1.4]	0.16	[0.13; 0.19]	16%
20:3	1.1	[0.9; 1.4]	0.28	[0.23; 0.33]	25%
20:4	3.8	[3.3; 4.5]	1.6	[1.2; 1.8]	43%
20:5	0.42	[0.27; 0.55]	0.13	[0.093; 0.146]	30%
22:2	0.051	[0.029; 0.045]	0.008	[0.007; 0.010]	17%
22:3	0.052	[0.043; 0.064]	0.014	[0.011; 0.018]	27%
22:4	0.48	[0.37; 0.59]	0.1	[0.083; 0.118]	22%
22:5	0.91	[0.71; 1.2]	0.18	[0.13; 0.23]	20%
22:6	2.1	[1.6; 2.6]	0.58	[0.42; 0.72]	27%
VLC-FA					
24:0	0.16	[0.11; 0.15]	0.070	[0.061; 0.077]	43%
24:1	0.17	[0.15; 0.19]	0.075	[0.062; 0.085]	45%
24:3	0.008	[0.007; 0.01]	0.004	[0.004; 0.005]	50%
24:4	0.026	[0.03; 0.05]	0.010	[0.008; 0.013]	40%
24:5	0.037	[0.027; 0.031]	0.012	[0.007; 0.016]	34%
24:6	0.031	[0.022; 0.038]	0.015	[0.009; 0.018]	48%
26:0	0.022	[0.011; 0.017]	0.010	[0.008; 0.012]	45%
26:1	0.029	[0.026; 0.034]	0.019	[0.017; 0.022]	65%
26:2	0.042	[0.035; 0.047]	0.033	[0.027; 0.036]	79%
26:3	0.016	[0.012; 0.019]	0.013	[0.010; 0.015]	81%
26:4	0.012	[0.011; 0.014]	0.009	[0.001; 0.002]	75%
26:6	0.018	[0.014; 0.021]	0.008	[0.005; 0.012]	44%

Supplementary Table S2. Concentrations of fatty acids in Glycerophospholipids (FA GPL) at timepoint t0 and t90. Values are represented as median [1st quartile; 3rd quartile] of the total population.

FA	t0		t90	
total FA GPL	4,331.2	[3,915.1; 4,637.5]	4,107.5	[3,733.1; 4.351.1]
SFA				
14:0	26.1	[18.3; 28.5]	23.3	[16.2; 25.7]
16:0	1,304.0	[1181.3; 1375.2]	1,236.9	[1,130.7; 1,265.9]
17:0	17.2	[15.3; 18.9]	16.4	[14.9; 18.5]
18:0	615.3	[548.3; 674.6]	585.2	[535.3; 661.5]
MUFA				
15:1	3.9	[2.8; 4.7]	3.6	[2.8; 4.4]
16:1	36.9	[29.6; 40.7]	36.9	[28.0; 40.8]
18:1	541.1	[479.5; 583.2]	512.3	[448.8; 556.0]
20:1	6.6	[5.9; 7.3]	6.3	[5.5; 7.0]
Essential FA				
18:2	969.4	[821.5; 1081.5]	903.6	[775.3; 993.3]
18:3	17.6	[14.2; 19.3]	15.9	[12.6; 16.5]
LC-PUFA				
20:2	13.9	[12.1;15.1]	13.5	[12.1; 14.6]
20:3	150.2	[133.1; 165.1]	145.4	[130.7; 153.1]
20:4	415.2	[363.5; 446.4]	400.4	[335.5; 437.4]
20:5	34.6	[24.0; 42.5]	33.1	[23.8; 39.6]
22:4	11.8	[9.2; 13.3]	11.5	[9.2; 13.8]
22:5	43.8	[38.4; 47,5]	42.5	[36.2; 47.2]
22:6	123.5	[103.6; 144.8]	120.2	[98.1; 141.9]

Supplementary Table S3. Concentrations of fatty acids in Sphingomyelin (FA SM) at timepoint t0 and t90. Values are represented as median [1st quartile; 3rd quartile] of the total population.

FA	t0		t90	
total FA SM	598.3	[506.8; 479.3]	585.8	[479.3; 662.3]
SFA				
12:0	0.888	[0.601; 1.071]	0.87	[0.63; 1.04]
14:0	16.0	[12.7; 18.3]	15.5	[13.2; 17.6]
15:0	10.2	[8.4; 12.2]	10.2	[8.5; 11.9]
16:0	166.4	[141.7; 189.2]	162.9	[133.5; 181.7]
17:0	5.0	[3.9; 6.0]	4.9	[4.0; 5.5]
18:0	31.1	[24.6; 36.7]	30.7	[24.0; 34.8]
20:0	52.7	[42.8; 60.3]	51.2	[39.9; 59.9]
22:0	32.7	[25.6; 37.8]	32.15	[24.7; 37.3]
MUFA				
14:1	1.4	[1.1; 1.7]	1.3	[1.1; 1.7]
15:1	0.45	[0.33; 0.52]	0.43	[0.33; 0.47]
16:1	26.1	[21.2; 30.1]	25.4	[20.4; 29.0]
18:1	16.9	[13.1; 19.7]	16.9	[12.6; 19.2]
20:1	29.7	[24.1; 34.5]	28.9	[22.3; 32.9]
22:1	50.1	[42.6; 55.3]	49.4	[40.9; 54.6]
Essential FA				
18:2	1.3	[1.1; 1.5]	1.3	[1.1; 1.5]
LC-PUFA				
22:2	16.5	[12.8; 20.9]	14.7	[11.6; 16.1]
22:3	3.9	[2.9; 4.7]	3.8	[2.6; 4.5]
VLC-FA				
24:0	29.7	[24.3; 34.3]	29.2	[22.5; 32.5]
24:1	63.5	[50.5; 72.2]	63.3	[50.0; 74.6]
24:2	37.5	[30.4; 43.8]	37.1	[31.0; 43.6]
24:5	4.5	[3.3; 5.7]	4.4	[3.5; 5.4]
26:0	0.13	[0.09; 0.17]	0.12	[0.09; 0.16]
26:2	0.63	[0.39; 0.90]	0.59	[0.37; 0.40]

1 **Contribution of glycerophospholipids and sphingomyelin to the circulating NEFA**

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26 **Running title:** Contribution of glycerophospholipids and sphingomyelin to the circulating NEFA

27 **Abbreviations:** BMI, body-mass index; CE, cholesteryl esters; FA, fatty acids; FA GPL, fatty acids
28 derived from glycerophospholipids; FA SM, fatty acids derived from sphingomyelins; GC, gas
29 chromatography; GPL, glycerophospholipids; interquartile range, ir; LC-FA, long-chain fatty acids;
30 LC-MS/MS, liquid chromatography coupled triple quadruple mass spectrometry; LC-PUFA, long-
31 chain polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; NEFA, nonesterified fatty
32 acids; OGTT, oral glucose tolerance test; PUFA, polyunsaturated fatty acids; PostGDM, women after
33 gestational diabetes; SFA, saturated fatty acids; SM, sphingomyelin; TAG, triacylglycerol; t90/0-ratio,
34 ratio of FA concentration at t90 divided by concentration at t0; VLC-PUFA, very long-chain
35 polyunsaturated fatty acids;

36 **Key words:** Nonesterified fatty acids; phospholipids; sphingolipids; long-chain polyunsaturated fatty
37 acids; very long-chain fatty acids; postprandial metabolism.

38 **Introduction**

39 Nonesterified fatty acids (NEFA) are associated with metabolic disorders such as insulin resistance
40 and obesity (1). In patients with insulin resistance, polyunsaturated fatty acids (PUFA) were found to
41 be higher, whereas lower concentrations were found for saturated fatty acids (SFA) (2).
42 While the adipose tissue is generally considered as the major source of NEFA (3, 4), information of
43 minor contributors such as the enzymatic degradation of other lipid fractions by cell surface anchored
44 lipases, bloodstream sn-1-, sn-2 lipases and esterases is limited (5-7). Since the postprandial state
45 prevails most of the day when lipolysis in the adipose tissue is down-regulated, the relative
46 contribution of these sources increases and might therefore impact on the development of insulin
47 resistance.
48 Lipid fractions comprise phospholipids (37.7 to 54.6%), cholesteryl esters (CE; 23.6 to 32.4%),
49 triacylglycerols (TAG; 15.4 to 35.8%) and further minor components (8). Phospholipids are
50 compromised dominantly of glycerophospholipids (GPL) and a smaller fraction of sphingomyelins
51 (SM), which accounts for 16-21% of serum phospholipids (9). In GPL, fatty acids (FA) are esterified
52 at position sn-1 and sn-2 of the glycerol backbone. Palmitic acid (16:0), stearic acid (18:0), oleic acid
53 (18:1), linoleic acid (18:2), arachidonic acid (20:4) and docosahexaenoic acid (22:6) are the main FA
54 in GPL (10, 11). Hydrolysis of blood phospholipids contributes primarily the FA 16:0 and 18:1 (12)
55 and the long-chain polyunsaturated FA (LC-PUFA) 20:4 (13). In contrast, FA 12:0, 18:0 and 24:0
56 present the major amid-linked FA at the C2-atome of sphingosine (14).
57 Recently, in the Prediction, Prevention and Subclassification of Type 2 Diabetes Study (PPS-Diab.
58 Study, Diabetes Research Group, Munich) differences in the NEFA profile were found in subjects
59 with high risk for the development of insulin resistance such as women with history of gestational
60 diabetes in comparison to controls (15). In the present study, we aimed to explore different sources of
61 FA which contribute to the NEFA pool in the fasted and non-fasted state in a subset of the PPS-Diab
62 study population.

63 **Materials and Methods**

64 Ethics Statement

65 This study was approved by the Ethics Committee of the Medical Faculty, Ludwig Maximilians
66 University of Munich (300-11). All participants gave their informed written consent before entering
67 the study.

68 Subjects

69 Serum samples of participants of the Prediction, Prevention and Subclassification of Type 2 Diabetes
70 Study were collected between November 2011 and December 2013 (PPS-Diab. Study, Diabetes
71 Research Group, Munich).
72 Details about the study design and the participating subjects have been published previously (16).
73 Briefly, 39 women, previously enrolled in the PPS-Diab study were studied at 9±3 months after
74 delivery. Twenty women were healthy controls, whereas 19 subjects had been diagnosed during
75 pregnancy with gestational diabetes mellitus (postGDM). The women's length, body weight, and body
76 fat mass (Bioelectrical impedance analysis, Tanita BC-418, Tanita Corporation, Tokyo, Japan) were
77 measured and BMI calculated. Hip and waist circumferences were assessed by tape measurements.

78 Blood sample collection and analysis

79 After overnight fasting, blood samples were drawn before (t0) and 90 minutes (t90) after oral glucose
80 administration (1.75 g/kg Dextro-OGTT solution, Hoffmann-La Roche, Grenzach-Wyhlen, Germany).
81 Blood samples were collected into refrigerated serum tubes, separated by centrifugation after 30
82 minutes of coagulation time and subsequently stored at -80°C for LC-MS/MS and GC analysis. All
83 samples were thawed and analyzed within the same LC-MS/MS and GC run in spring 2014. Serum
84 insulin was measured with chemiluminescence technology (CLIA, DiaSorin LIAISON systems,
85 Saluggia, Italy). To measure the relationship between insulin sensitivity and first-phase insulin
86 secretion the disposition index was determined. Glucose concentrations were measured using a
87 glucose analyzer with the glucose oxidase method (Glucose HK Gen.3, Roche Diagnostics,
88 Mannheim, Germany). The Matsuda Index and HOMA-Index were calculated (17, 18).

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Gelöscht: analyzed

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Gelöscht: Serum samples of each participant were

91 LC-MS/MS analysis

92 NEFA were analyzed by LC-MS/MS (4000 QTRAP, AB Sciex, Darmstadt, Germany) as described
93 previously (19). In short, proteins were precipitated by adding 200 µl isopropanol to 20 µl serum in a
94 96-deepwell plate. After centrifugation, 10 µl of the supernatant were injected for each sample with an
95 eluent flow rate of 700 µl/min. Gradient elution was performed with eluent A (5mM ammonium
96 acetate and 2.1 mM acetic acid) and eluent B (acetonitrile with 20% isopropanol) on Pursuit UPS
97 Diphenyl column (1.9 µm, 100 x 3.0 mm; Varian, Darmstadt, Germany) at 40°C.
98 Analysis of SM species was performed by precipitating proteins of 10 µl serum with 500 µl methanol.
99 After centrifugation, 200 µl of the supernatant were mixed with 700 µl methanol and 30 µl of the
100 mixture were injected and measured by LC-MS/MS.

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Gelöscht: analyses

101 GC analysis

102 The analysis of GPL derived FA was performed by GC (10). 100 µl serum were combined with 100 µl
103 of an internal standard (1.2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol) and
104 600 µl methanol. GPL of the supernatant were re-esterified with sodium methoxide to form FA methyl
105 ester which have been analyzed by using an Agilent Technologies 7890A GC equipped with a BPX 70
106 column (50 m x 0.22 mm, 0.25 µm film, SGE, Weiterstadt, Germany) with an optimized temperature
107 and pressure program.

108 Statistical analysis

109 Data analysis was performed with Microsoft Excel 2010 (Microsoft Inc., Redmond, WA) and SPSS
110 version 22.0 (SPSS Inc., Chicago, IL, USA). Mann-Whitney-U tests were used to detect differences
111 between postGDM and controls. Statistical significance between groups was accepted after correction
112 for multiple testing according to Bonferroni ($p < 0.001$). Correlations coefficients (r) were evaluated
113 using Spearmen´s Correlation. Results were expressed as median with 1st quartile and 3rd quartile in
114 µmol/l or as percentages (% mol/mol).
115 FA were grouped by carbon chain length: medium-chain FA were categorized by chain-length less
116 than thirteen carbons, long-chain FA (LC-FA) between 14 and 22 and very long-chain FA (VLC-FA)

118 more than 23 carbons. Quality control samples were performed for each lipid fraction. FA were

119 excluded from further evaluation, if the coefficient of variation was higher than 20%.

120 **Results**

121 Study population

122 Samples of 39 women (19 postGDM, 20 healthy controls) were obtained and analyzed. Since the
123 major aim of this work was to associate NEFA to other lipid fractions in plasma, we excluded outliers,
124 which would affect data analysis. Two subjects were excluded due to a small difference between
125 fasting and postprandial state with a total NEFA t90/0-ratio of 41% and 49%, respectively (mean
126 NEFA t90/0-ratio was 14±4%, the two outliers were above the 1.5 percentile, shown in Fig. S1).
127 Data of the remaining 37 subjects (17 postGDM, 20 healthy controls) were included for statistical
128 analysis. Their characteristics are shown in Table 1. Age and time interval since delivery were similar
129 in both groups. The postGDM group had significantly higher BMI, WHR and body fat. Insulin and
130 glucose were higher in postGDM at both t0 and at t90, and Matsuda Index, HOMA Index and
131 Disposition Index differed significantly, whereas HbA1c was similar.

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Gelöscht: for the study cohort,

132 Differences of individual NEFA between groups

133 Most FA showed no significant difference between postGDM and controls in NEFA, GPL and SM
134 (Table S1, S2, S3). FA 10:0 showed significant lower concentrations in postGDM at both timepoints.
135 FA 26:3 showed higher concentrations in postGDM at t90, however was not significant when
136 corrected with Bonferroni.

137 T90/0-ratio of individual NEFA

138 The ratio of the FA concentration at t90 to t0 (t90/0) was measured for postGDM, controls and the
139 total study population (Figure 1). Mann-Whitney U test showed no significant differences between
140 postGDM and controls. Considering the total study population, 14% of the total NEFA content was
141 not affected by the glucose-mediated down regulation of the adipose tissue release (column 1 of
142 Figure 1). The ratio between t90 and t0 varied strongly between the distinct NEFA species with a
143 minimum of 6% for 16:1 and a maximum of 81% for 26:3. The t90/0-ratio was low in FA with shorter
144 chain length (about 5% in C10) and higher with increasing FA chain length (e.g. about 50-80% in
145 C26) (Fig. 1). In most cases, the degree of unsaturation did not have an apparent influence on the

147 t90/0-ratio. However, relative to 26:0 its unsaturated derivatives showed a continuous increase in the
148 t90/0-ratio with higher degree of unsaturation (Fig. 1). The highest t90/0-ratio was found for VLC-
149 PUFA (26:2, 26:3 and 26:4). T90/0-ratios greater than 40% were also found for VLC-FA (24:0, 24:1)
150 and PUFA (20:4, 24:3, 24:4, 24:5, 24:6). T90/0-ratios below 20% were found for monounsaturated FA
151 (MUFA, 14:1, 15:1, 17:1, 18:1, 19:1, 20:1), most saturated FA (SFA, 10:0, 12:0, 14:0, 15:0,16:0) and
152 the essential FA 18:2 and 18:3.

153 GPL FA

154 In GPL, 17 FA were quantified at t0 and t90 (Table 2). Concentration of GPL FA decreased slightly
155 after glucose intake. The highest amounts were found for 16:0, followed by 18:2, 18:0 and 18:1 at both
156 time points in postGDM and controls. At t0, FA 15:1, 20:5 and 22:6 showed significant correlations
157 for the total study population and the stratification analysis. FA 16:1 showed significant correlation
158 within the total study population as well as in postGDM, but not in the controls. At t90, LC-PUFA
159 (20:3, 20:4, 20:5, 22:5, 22:4 and 22:6) showed significant correlations in the total study population.
160 LC-PUFA 20:3, 20:4 and 20:5 were also significant in the controls, 22:5 and 22:6 in postGDM.

161 SM FA

162 In SM, 23 FA were quantified at t0 and t90 (Table 3). The FA concentrations showed no differences at
163 t0 and t90 in both groups. The highest concentrations were observed for 16:0, followed by 24:1, 20:0,
164 24:2, 22:0, 20:1 and 24:0. At t0, FA 15:1 and 24:0 showed significant correlations in the total study
165 population and FA 24:0 in postGDM. At t90, 24:0 showed high correlation coefficients in the total
166 study population, in postGDM and controls. Also 22:1 showed significant correlations in the total
167 study population and the controls.

168 **Discussion**

169 High levels of serum NEFA are found in insulin resistance and obesity (20-23). Therefore, it has been
170 proposed to measure the NEFA concentration routinely as a risk marker (3). A systematic review of
171 the literature explored the relationship between obesity and plasma NEFA concentration in 43 studies
172 (20). The relationship between the fasting NEFA concentrations was unrelated to the body fat mass,
173 even though the BMI was considered in quintiles for a more detailed phenotyping. Thus, the authors
174 proposed that elevated NEFA concentrations are not necessarily associated with obesity and insulin
175 resistance. They suggested a reevaluation of the relationships between adiposity, fatty acids and
176 insulin resistance (20).

177 Accumulating data in the recent years suggested a more complex role of NEFA (24, 25), e.g. fatty acid
178 metabolites like oxylipins and endocannabinoids are thought to influence the insulin resistance and
179 dysfunction of the adipose tissue (26). The variation in the NEFA composition could have a major
180 impact on biological effects, as well as their metabolic sources. So far, their sources have received
181 surprisingly little attention. The present study assessed a subset of the PPS-Diab Study (16).
182 Previously, the PPS-Diab Study assessed differences in the NEFA composition of 62 women with
183 postGDM and 49 healthy controls during fasting (15). The present study focused on the FA
184 contribution of different sources to the NEFA pool in the fasted and non-fasted state. A comparison
185 between the postGDM and controls showed only decanoic acid (10:0) with significant differences at t0
186 and t90. In an earlier study decanoic acid was found as a modulating ligand of peroxisome proliferator
187 activated receptors (PPARs) (27). High amounts of decanoic acid improved glucose sensitivity and
188 lipid profiles without weight gain in diabetic mice. This mouse model is interesting as in our study
189 decanoic acid was significant higher in controls at both timepoints and similar effects could play in
190 humans. Yet this effect has not been studied in humans and should be considered carefully due to
191 various other PPAR modulating ligands (27) and due to our small study cohort.

192 No differences were found in the t90/0-ratio between postGDM and controls for all other NEFA
193 species, however the individual NEFA species showed vast differences in their behavior after glucose
194 intake. This led us to consider the quality of the individual fatty acids and the derivation of the
195 individual NEFA species more closely.

Felicitas Maier 29.12.2015 18:24
Gelöscht: However, a

Felicitas Maier 21.12.2015 09:02
Gelöscht: In our study, decanoic acid was significant lower in postGDM at both timepoints. However this result

200 Contribution of phospholipids

201 We aimed to explore if the NEFA composition is affected by the contribution of FA from
202 phospholipids. Since we did not find significant differences in the NEFA, FA GPL and FA SM
203 concentrations between postGDM and controls, we focused on results of the total study population.
204 Fourteen percent of the fasted NEFA concentration remained at t90, which could reflect a residual
205 lipase activity and/or the contribution of other sources. Of interest, only a relatively small decrease of
206 the t90/0-ratio occurred in FA with longer chain length (e.g. LC-FA and LC-PUFA). Therefore, we
207 assumed that these FA are less contributed by the release from adipose tissue. The t90/0-ratio varied
208 widely from 6% for 16:1 up to 81% for 26:3 (Fig. 1), which leads us to conclude that the relative
209 contribution of individual FA from adipose tissues and from other sources varied markedly.

210 Contribution of LC-PUFA from GPL

211 LC-PUFA 20:4 showed a t90/0-ratio of 42% and a significant correlation (r=0.527) between NEFA
212 and GPL at t90. In previous studies, low correlations between adipose tissue and NEFA were observed
213 for 20:4 (4, 28, 29). Thus, other sources than the adipose tissue might contribute 20:4 to the NEFA
214 pool. LC-PUFA 22:6 showed strong correlations between NEFA and GPL at t0 and t90. The
215 decreasing t90/0-ratio of 27% suggested a possible contribution of other sources to the NEFA 22:6.
216 Chen et al. demonstrated that endothelial lipase plays a major role in the 22:6 releases from GPL (30).
217 Endothelial lipase is known to be specific for GPL and the hydrolysis of amid-linked FA, preferably
218 22:6. However, for 22:6 also a close correlation between the adipose tissue and NEFA was
219 demonstrated (4, 29). We conclude that GPL contributes 22:6 particularly in postprandial state, while
220 in the fasted state 22:6 is mainly released from adipose tissue. LC-PUFA 20:5 showed the highest
221 correlation between GPL and NEFA at t0 and t90 and thus could be derived from GPL by $sPLA_2$
222 activity (31). The sources of 20:5 and 22:6 may be of high interest as their amount correlates with
223 increased cardiovascular risk, especially in red blood cells (11). Literature and our data suggest that
224 GPL might contribute an amount of LC-PUFA, which is most detectable in postprandial state (Fig. 2).

225 Contribution of VLC-FA from SM

226 VLC-FA 24:0 showed the highest correlation coefficient of all FA between SM and NEFA (r=0.417)
227 whereas no correlation was found between NEFA and GPL. This agreed with previously reported low
228 correlation coefficients for 24:0 between NEFA and GPL and between NEFA and subcutaneous
229 adipose tissue as well as between NEFA and visceral adipose tissue (r=0.217/r=0.300) (4). As
230 described above, 24:0 is primarily amid-linked in SM (14, 32). We assume that 24:0 is mainly derived
231 from SM cleavage (Fig. 2).

232 Strengths and limitations

233 The hydrolysis of other lipid fractions especially TAG might contribute to serum NEFA with a high
234 amount. TAG analysis is not shown in our study since it can only be performed by thin-layer
235 chromatography, which contains many inaccuracies. Fatty acids with major interest are impure and the
236 TAG pool cannot be represented with an adequate spectrum. In a mouse model using radioactively
237 labeled FA, lipolysis of LDL-TAG affected the NEFA composition (33). No specific FA was
238 determined and the contribution of TAG derived FA varied significantly between 5% and 80-90%
239 (34). In another study, a separate contribution of the lipoprotein lipase was determined to be 25-30%
240 on average, and therefore dietary fat was assumed to contribute one-third to the NEFA composition
241 (35). Serum TAG has high contents of 18:1, 16:0 and 18:2 (9) and thus is a potentially major source
242 for these FA (Fig. 2). Cholesterol esterase is involved in lipoprotein metabolism and also liberates
243 esterified FA. CE constitutes up to 40% of LDL (9). The FA 18:2 is the predominant FA esterified
244 with cholesterol. Minor components are 16:0 and 18:1 (9). In our data, 18:2 correlations between
245 NEFA and GPL and between NEFA and SM were poor at t0 and at t90. Hence, CE may be an
246 important contribution to NEFA18:2 (Fig. 2).
247 The NEFA pool is constantly influenced by an input of the adipose tissue, lipid fractions and other
248 sources, but also by the liberation to the prostaglandin synthesis, β-oxidation and membrane
249 integration (Fig. 2).
250 One might argue that even better insights could be delineated from studies using FA labeled with
251 radioisotopes or stable isotopes, but it would be challenging to design an ethically acceptable

252 experiment in humans were individual depots e.g. adipose tissue triglycerides could be labeled for an
253 informative experiment. We used standardized conditions, highly sensitive and precise methodology
254 to measure a broad spectrum of individual FA in NEFA and PL. However, some single FA were not
255 detectable in all lipid fractions, e.g. 24:0 was found in SM whereas its concentration was not
256 detectable in GPL. While OGTT is a widely accepted standardized challenge condition, results cannot
257 be extrapolated to a mixed meal providing dietary fats.

258 Some correlations between LC-PUFA of GPL and NEFA were found to be significant in the total
259 study population but not within both subgroups. The power for the subgroup analysis might be limited
260 due to the small sample size. However, an association for LC-PUFA between GPL and NEFA can be
261 assumed in both groups.

Felicitas Maier 19.12.2015 19:50
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Felicitas Maier 19.12.2015 19:51
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264 **Conclusion**

265 This study investigated differences in the NEFA profile in women with a history of gestational
266 diabetes and controls and the potential contribution of phospholipids to the NEFA composition. The
267 NEFA composition varied highly between fasting and fed state in both groups. GPL appeared to
268 contribute LC-PUFA and SM appeared to contribute VLC-FA.

269 **Conflict of interest**

270 The authors confirm that this article content has no conflicts of interest.

271 **Acknowledgement**

272 We thank Stefan Stromer (Division of Metabolic and Nutritional Medicine, Dr. von Hauner Children´s
273 Hospital, University of Munich) who supported us with the analysis of glycerophospholipids.

Felicitas Maier 13.1.2016 15:28
Gelöscht: Franca F. Kirchberg

Felicitas Maier 13.1.2016 15:29
Gelöscht: statistical data

274 **Author Contributions**

275 Conceived and designed the experiments: OU CH HD BK. Performed the experiments: FM OU HD
276 MF CH. Analyzed the data: FM OU HD CH. Wrote the paper: FM OU MF HD CH BK AL.

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Figures and Tables

Table 1. Clinical parameters of the study cohort. Values are represented as median [1st quartile; 3rd quartile]. Fat percentage and mass are measured with the BIA-scale.

	postGDM (n=17)	**controls (n=20)**	**p-value**
age [years]	35 [32;37,5]	36 [34;38]	0.218
height [cm]	167.86 [163.5;173]	167.75 [164;169]	0.956
weight [kg]	86.12 [69.2;100.9]	63.64 [57.5;65.5]	<0.001
BMI [kg/m^2]	30.8 [24.45;35.4]	22.5 [20.52;23.72]	<0.001
hip size [cm]	115.84 [101;130]	95.60 [91.5;98.5]	<0.005
waist size [cm]	94.23 [84;105]	75.15 [69.7;80.5]	<0.005
waist-hip ratio	0.812 [0.76;0.85]	0.788 [0.73;0.84]	0.308
time after delivery [months]	9.7 [8.25;11.65]	8.4 [7.07;9.3]	0.281
fat percentage [%]	39.02 [30.45;45.05]	28.02 [23.4;33.15]	<0.005
fat mas [kg]	35.15 [21.1;45.5]	18.38 [13.35;21.8]	<0.005
insulin at t0 [µU/ml]	13.3 [9.8;15.7]	4.4 [3.47;5.7]	<0.005
insulin at t90 [µU/ml]	97.3 [58.45;88]	32 [21.2;39.1]	<0.005
glucose at t0 [mg/dl]	92 [89;95]	84 [81.5;88.5]	<0.005
glucose at t90 [mg/dl]	128 [106.5;157,5]	93 [79;100]	<0.005
Matsuda Index	3.064 [2.56;3.71]	10.252 [8.05;10.44]	<0.005
HOMA Index	3.051 [2.2;3.66]	0.916 [0.74;1.16]	<0.005
Disposition Index	222.19 [171;285]	354.37 [263.24;445.31]	<0.005
HbA1c [%]	5.44 [5.3;5.65]	5.26 [5.07;5,4]	0.069
HbA1c [mmol/mol]	36.01 [34.4;38.2]	34.02 [31.92;35.5]	0.592

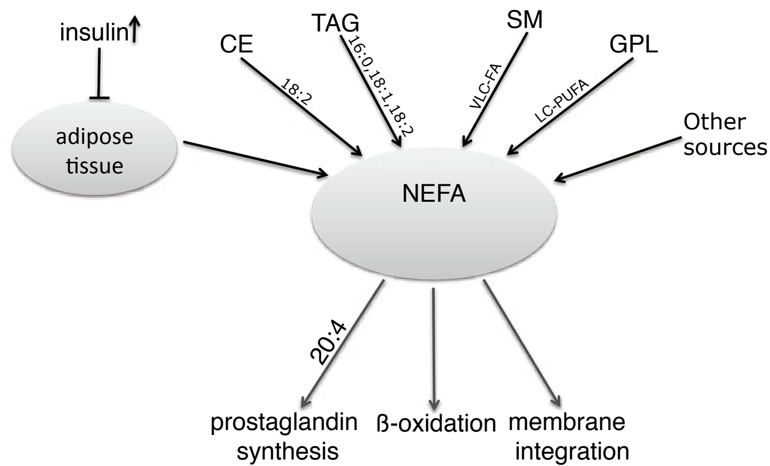
Table 2. Correlation coefficients (r) of fatty acids derived from glycerophospholipids (FA GPL) to the corresponding nonesterified fatty acid (NEFA). Correlations are calculated at t0 and t90 for the total study population, postGDM cases and healthy controls and shown with p-value (* for p<0.05; ** for p<0.01).

	t0						**t90**					
	total population (r, p-value)		**postGDM (r, p-value)**		**controls (r, p-value)**		**total population (r, p-value)**		**postGDM (r, p-value)**		**controls (r, p-value)**	
SFA												
14:0	0.184	0.290	0.339	0.216	0.074	0.757	0.333	0.051	0.114	0.685	0.358	0.121
16:0	0.095	0.589	0.329	0.232	-0.032	0.895	0.028	0.874	-0.204	0.467	0.140	0.556
17:0	0.250	0.148	0.425	0.114	0.297	0.203	0.146	0.403	0.136	0.630	0.182	0.443
18:0	0.220	0.205	0.246	0.376	0.059	0.806	0.136	0.437	0.311	0.259	0.029	0.902
MUFA												
15:1	0,451**	0.006	0.582*	0.023	0.459*	0.042	0.259	0.133	0.300	0.277	0.360	0.119
16:1	0.495**	0.002	0.679**	0.005	0.357	0.123	0.359*	0.034	0.354	0.196	0.347	0.133
18:1	0.102	0.559	0.243	0.383	0.065	0.787	-0.255	0.139	-0.266	0.337	-0.132	0.580
20:1	-0.047	0.788	0.225	0.420	-0.217	0.359	-0.234	0.176	0.093	0.742	-0.313	0.179
Essential FA												
18:2	0.122	0.486	0.346	0.206	0.029	0.905	-0.021	0.906	0.289	0.296	-0.110	0.645
18:3	0.125	0.476	0.396	0.143	0.023	0.925	0.328	0.054	0.446	0.095	0.229	0.332
LC-PUFA												
20:2	0.116	0.505	0.404	0.136	-0.259	0.271	0.207	0.233	0.422	0.117	0.153	0.519
20:3	0.335*	0.049	0.300	0.277	0.360	0.119	0.467**	0.005	0.161	0.567	0.642**	0.002
20:4	0.270	0.116	0.354	0.196	0.208	0.380	0.527**	0.001	0.396	0.143	0.647**	0.002
20:5	0.683**	0.000	0.829**	0.000	0.502*	0.024	0.835**	0.000	0.889**	0.000	0.839**	0.000
22:4	0.197	0.257	0.568*	0.027	0.150	0.529	0.404*	0.016	0.704**	0.003	0.283	0.227
22:5	0.253	0.142	0.404	0.135	0.204	0.389	0.511**	0.002	0.868**	0.000	0.439	0.053
22:6	0.597**	0.000	0.685**	0.005	0.555*	0.011	0.491**	0.003	0.850**	0.000	0.308	0.186

Table 3. Correlation coefficients (r) of fatty acids derived from sphingomyelin (FA SM) to the corresponding nonesterified fatty acid (NEFA). Correlations are calculated at t0 and t90 for the total study population, postGDM cases and healthy controls and shown with p-value (* for p<0.05; ** for p<0.01).

	t0						t90					
	total population (r, p-value)		postGDM (r, p-value)		controls (r, p-value)		total population (r, p-value)		postGDM (r, p-value)		controls (r, p-value)	
SFA												
12:0	0.394*	0.014	0.653**	0.003	0.236	0.316	0.455**	0.005	0.331	0.195	0.436	0.055
14:0	0.352*	0.030	0.392	0.107	0.178	0.452	0.283	0.090	-0.039	0.881	0.435	0.056
15:0	0.361*	0.026	0.482*	0.043	0.299	0.200	0.280	0.093	0.168	0.519	0.364	0.115
16:0	0.231	0.163	0.214	0.395	0.217	0.359	0.074	0.662	0.179	0.492	-0.027	0.910
17:0	0.324*	0.047	0.302	0.223	0.334	0.150	0.131	0.440	0.324	0.205	0.027	0.910
18:0	0.124	0.457	-0.201	0.423	0.396	0.084	0.130	0.443	0.336	0.187	-0.073	0.760
20:0	0.183	0.272	0.184	0.465	0.023	0.925	0.020	0.906	0.105	0.687	-0.137	0.565
22:0	0.067	0.691	0.263	0.291	-0.131	0.582	0.399*	0.014	0.366	0.148	0.459*	0.042
MUFA												
14:1	0.295	0.072	0.187	0.458	0.319	0.171	0.169	0.318	-0.113	0.667	0.388	0.091
15:1	0.436**	0.006	0.418	0.084	0.412	0.071	-0.109	0.522	-0.234	0.366	0.078	0.743
16:1	0.311	0.057	0.288	0.247	0.340	0.142	0.154	0.364	0.103	0.694	0.272	0.245
18:1	0.110	0.512	-0.149	0.556	0.338	0.145	0.110	0.512	-0.232	0.371	0.114	0.634
20:1	0.180	0.280	-0.129	0.610	0.396	0.084	-0.281	0.092	-0.505*	0.039	-0.241	0.307
22:1	0.240	0.147	0.029	0.909	0.482*	0.032	0.425**	0.009	0.455	0.066	0.458*	0.042
Essential FA												
18:2	0.292	0.075	0.181	0.473	0.368	0.110	0.171	0.312	0.054	0.837	0.278	0.235
LC-PUFA												
22:2	0.097	0.562	0.092	0.717	-0.033	0.890	0.222	0.186	0.446	0.073	0.128	0.591
22:3	0.110	0.512	0.024	0.926	0.011	0.965	0.387*	0.018	0.444	0.074	0.327	0.160
VLC-FA												
24:0	0.458**	0.004	0.681**	0.002	0.215	0.363	0.417*	0.010	0.528*	0.030	0.498*	0.025
24:1	0.326*	0.046	-0.080	0.754	0.538*	0.014	0.023	0.893	-0.189	0.468	0.163	0.492
24:2	0.303	0.065	0.412	0.090	0.355	0.125	-0.011	0.949	0.163	0.532	0.254	0.279
24:5	0.412*	0.010	0.441	0.067	0.424	0.062	0.211	0.209	0.153	0.557	0.307	0.188
26:0	0.269	0.102	0.273	0.272	0.250	0.287	0.138	0.414	0.032	0.903	0.157	0.508
26:2	-0.051	0.763	0.362	0.140	-0.408	0.075	-0.231	0.170	-0.078	0.765	-0.352	0.128

Figure 2. Contribution of serum lipid fractions to the nonesterified fatty acids (NEFA) composition in postprandial state. In postprandial state, insulin down regulates the release of fatty acids (FA) from adipose tissue and the relative contribution of other sources to the NEFA composition increases. Long-chain polyunsaturated fatty acids 20:3; 20:4, 20:5, 22:5 and 22:6 (LC-PUFA) are derived by the hydrolysis of glycerophospholipids (GPL), very long-chain FA 24:0 (VLC-FA) by sphingomyelin (SM). Cholesteryl ester (CE) may contribute 18:2, serum triacylglycerols (TAG) 16:0, 18:1 and 18:2. Other sources and consuming processes like the prostaglandin synthesis, β-oxidation and membrane integration influence the NEFA pool as well.



Supplementary

Supplementary Figure S1. Description of outliers. The boxplot of the t90/0-NEFA ratio shows two outliers with the number 151 (>3. IQR of the 75th percentile) and 102 (>1.5 IQR).

Felicitas Maier 29.12.2015 13:49
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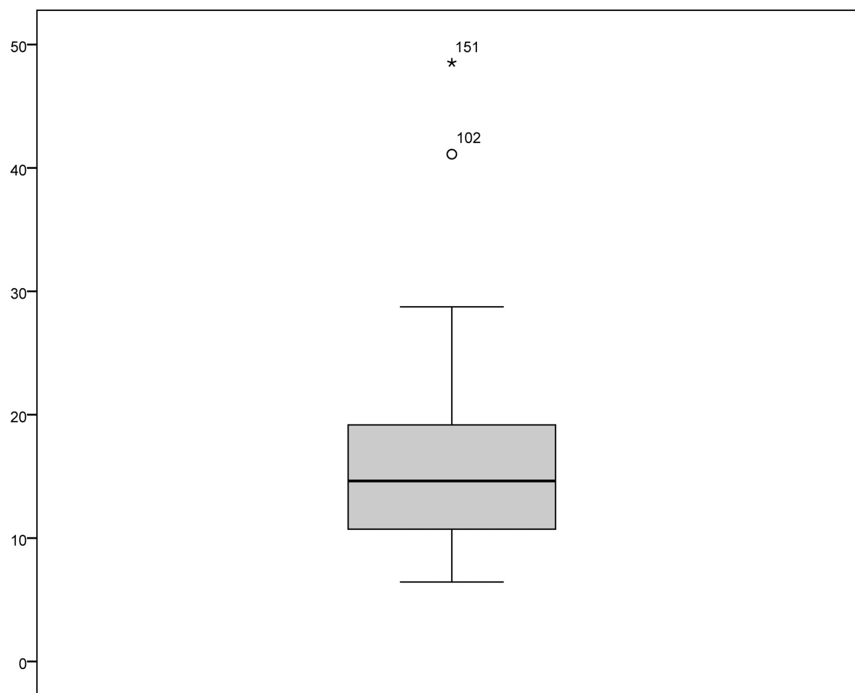

Supplementary Table S1. Median concentrations of nonesterified fatty acids (NEFA) in postGDM and controls at t0 and t90. Concentrations in µmol/l with 1st and 3rd quartiles. Differences between the groups were calculated by Mann-Whitney-U test and significance was accepted with p-values <0.001 after correction according to Bonferroni (marked with *).

FA	t0			t90		
	postGDM (n=17)	control (n=20)	p-value	postGDM (n=17)	control (n=20)	p-value
total NEFA	503.93 [422; 630]	496.79 [430; 611]	0.855	69.62 [60.04; 87.00]	72.29 [50.87; 95.23]	0.927
SFA						
10:0	0.57 [0.42; 0.81]	1.13 [1.00; 1.32]	0.000*	0.04 [0.013; 0.06]	0.09 [0.07; 0.12]	0.000*
12:0	3.44 [2.81; 4.34]	4.59 [3.39; 7.16]	0.040	0.38 [0.32; 0.49]	0.52 [0.41; 1.25]	0.063
14:0	13.60 [10.4; 18.6]	15.40 [12.00; 18.73]	0.279	1.19 [1.1; 1.39]	1.44 [0.90; 1.90]	0.377
15:0	2.37 [1.77; 3.29]	2.53 [1.92; 2.93]	0.819	0.29 [0.25; 0.36]	0.32 [0.22; 0.47]	0.703
16:0	124.00 [102; 154]	131.00 [110; 158]	0.542	19.80 [17.8; 25.2]	19.90 [14.8; 30.18]	0.964
17:0	2.67 [2.01; 3.26]	2.91 [2.36; 3.08]	0.760	0.49 [0.45; 0.59]	0.52 [0.36; 0.73]	0.951
18:0	39.90 [34.3; 43.9]	45.60 [40.58; 51.80]	0.063	8.99 [8.62; 10.4]	10.45 [9.25; 12.88]	0.206
MUFA						
12:1	0.73 [0.56; 1.04]	0.79 [0.70; 1.07]	0.552	0.04 [0.03; 0.10]	0.06 [0.02; 0.11]	0.939
13:1	0.19 [0.12; 0.27]	0.21 [0.15; 0.28]	0.428	0.04 [0.03; 0.05]	0.05 [0.04; 0.07]	0.247
14:1	2.73 [2.41; 3.97]	2.67 [2.39; 3.53]	0.867	0.22 [0.16; 0.25]	0.24 [0.16; 0.33]	0.604
15:1	0.20 [0.15; 0.29]	0.19 [0.17; 0.24]	0.867	0.03 [0.02; 0.03]	0.02 [0.01; 0.02]	0.088
16:1	27.10 [22.7; 37.1]	21.60 [19.1; 34.05]	0.411	1.88 [1.51; 2.29]	1.89 [1.08; 2.45]	0.726
17:1	1.78 [1.45; 2.26]	1.59 [1.39; 2.12]	0.637	0.22 [0.18; 0.23]	0.20 [0.15; 0.26]	0.715
18:1	196.00 [163; 236]	195.00 [169; 249]	0.927	23.40 [20.4; 28.7]	24.05 [15.23; 29.43]	0.637
Essential FA						
18:2	63.70 [45.8; 74.6]	53.10 [46.12; 70.13]	0.879	6.79 [6.11; 9.63]	7.61 [5.20; 8.92]	0.300
18:3	6.13 [4.66; 7.74]	6.06 [4.49; 7.11]	0.703	0.63 [0.49; 0.82]	0.56 [0.40; 0.92]	0.522
LC-PUFA						
16:2	0.32 [0.27; 0.49]	0.30 [0.23; 0.42]	0.419	0.03 [0.03; 0.04]	0.03 [0.02; 0.05]	0.314
16:3	0.15 [0.11; 0.17]	0.11 [0.10; 0.16]	0.156	0.03 [0.02;0.03]	0.02 [0.02; 0.03]	0.464
18:4	0.11 [0.08; 0.13]	0.13 [0.10; 0.15]	0.353	0.02 [0.02; 0.02]	0.02 [0.01; 0.03]	0.503
19:0	0.26 [0.18; 0.26]	0.26 [0.21; 0.29]	0.437	0.07 [0.06; 0.08]	0.07 [0.06; 0.10]	0.411
19:1	0.68 [0.58; 083]	0.70 [0.60; 0.87]	0.855	0.08 [0.06; 0.09]	0.07 [0.05; 0.11]	0.964
20:0	0.23 [0.19; 0.30]	0.32 [0.22; 0.45]	0.088	0.10 [0.07, 0.11]	0.10 [0.08; 0.13]	0.604
20:1	1.63 [1.52; 2.10]	1.74 [1.33; 2.47]	0.879	0.24 [0.21; 0.28]	0.24 [0.15; 0.27]	0.583
20:2	1.05 [0.8; 1.37]	0.88 [0.78; 1.24]	0.532	0.16 [0.14; 0.18]	0.15 [0.10; 0.19]	0.419
20:3	1.03 [0.91; 1.47]	1.00 [0.92; 1.36]	0.681	0.29 [0.25; 0.30]	0.26 [0.22; 0.33]	0.272
20:4	3.78 [3.33; 4.71]	3.54 [3.09; 4.33]	0.353	1.44 [1.1; 1.96]	1.59 [1.15; 1.78]	0.807
20:5	0.31 [0.27; 054]	0.39 [0.33; 0.55]	0.329	0.11 [0.09; 0.13]	0.11 [0.10; 0.15]	0.831
22:2	0.034 [0.03; 0.05]	0.034 [0.03; 0.04]	0.807	0.008 [0.01; 0.01]	0.008 [0.01; 0.01]	0.563
22:3	0.050 [0.04; 0.06]	0.052 [0.04; 0.06]	0.659	0.012 [0.01; 0.02]	0.016 [0.01; 0.02]	0.170
22:4	0.47 [0.37; 0.62]	0.38 [0.36; 0.53]	0.156	0.11 [0.09; 0.12]	0.09 [0.07; 0.11]	0.117
22:5	0.89 [0.7; 1.17]	0.79 [0.69; 1.20|	0.964	0.17 [0.14; 0.23]	0.17 [0.12; 0.25]	0.855
22:6	1.89 [1.56; 2.48]	2.13 [1.52; 2.74]	0.411	0.50 [0.46; 0.59]	0.53 [0.40; 0.75]	0.831
VLC-FA						
24:0	0.11 [0.09; 0.13]	0.14 [0.12; 0.15]	0.026	0.072 [0.06; 0.08]	0.066 [0.06; 0.08]	0.532
24:1	0.15 [0.14; 0.17]	0.17 [0.16; 0.20]	0.053	0.076 [0.06;0.09]	0.073 [0.06; 0.08]	0.437
24:3	0.008 [0.01; 0.01]	0.008 [0.01; 0.01]	0.951	0.004 [0.01; 0.01]	0.005 [0.01; 0.01]	0.053
24:4	0.024 [0.02; 0.03]	0.025 [0.02; 0.03]	0.474	0.010 [0.01; 0.01]	0.012 [0.01; 0.01]	0.152
24:5	0.032 [0.30; 0.40]	0.034 [0.03; 0.05]	0.637	0.011 [0.01; 0.01]	0.009 [0.01; 0.02]	0.161
24:6	0.028 [0.02; 0.04]	0.028 [0.02; 0.03]	0.761	0.010 [0.01; 0.01]	0.013 [0.01; 0.01]	0.065
26:0	0.011 [0.01; 0.02]	0.025 [0.01; 0.02]	0.067	0.011 [0.01; 0.01]	0.010 [0.01; 0.01]	0.345
26:1	0.029 [0.02; 0.03]	0.029 [0.03; 0.03]	0.692	0.019 [0.02; 0.02]	0.021 [0.02; 0.02]	0.300
26:2	0.042 [0.04; 0.05]	0.037 [0.30; 0.40]	0.094	0.034 [0.30; 0.40]	0.029 [0.02; 0.04]	0.072
26:3	0.017 [0.01; 0.02]	0.013 [0.01; 0.02]	0.005	0.015 [0.01; 0.02]	0.010 [0.01; 0.01]	0.007
26:4	0.012 [0.01; 0.01]	0.012 [0.01; 0.01]	0.229	0.009 [0.01; 0.01]	0.012 [0.01; 0.01]	0.692
26:6	0.015 [0.01; 0.02]	0.019 [0.02; 0.02]	0.170	0.007 [0.01; 0.01]	0.009 [0.01; 0.01]	0.279

Supplementary Table S2. Median concentrations of individual fatty acids in glycerophospholipids in postGDM and controls at t0 and t90. Concentrations in µmol/l with 1st and 3rd quartiles. Differences between the groups were calculated by Mann-Whitney-U test and significance was accepted with p-values <0.001 after correction according to Bonferroni (marked with *).

FA	t0			t90		
	postGDM (n=17)	control (n=20)	p-value	postGDM (n=17)	control (n=20)	p-value
total GPL	4007 [3698;4323]	4296 [3953;4532]	0.189	4041 [3708;4236]	3821 [3395;4155]	0.295
SFA						
14:0	23.91 [19.0; 28.1]	27.91 [16.9; 28.9]	0.313	22.6 [17.4; 25.7]	24.27 [16.5; 25.97]	0.456
16:0	1193 [1098; 1269]	1268 [1133;1343]	0.318	1150.9 [1073; 1197]	1190 [1073; 1248]	0.445
17:0	15.17 [13.7; 17.0]	17.23 [15.0; 18.82]	0.025	15.44 [14.2; 17.54]	15.76 [14.23; 17.6]	0.027
18:0	558.9 [504; 616]	606.9 [541; 656]	0.105	550.7 [511; 612]	563.9 [500; 639]	0.220
MUFA						
15:1	3.5 [2.6; 4.2]	3.8 [2.7; 4.5]	0.377	3.39 [2.7; 4.2]	3.46 [2.63; 4.21]	0.490
16:1	36.8 [33.6; 38.6]	33.6 [24.2; 37.1]	0.370	32.41 [25.6; 36.2]	32.18 [24.6; 35.95]	0.268
18:1	483.3 [427; 551]	539.7 [467; 575]	0.096	471.11 [422; 522]	488.4 [426; 531]	0.123
20:1	6.1 [5.16; 6.71]	6.5 [5.78; 7.11]	0.433	6.0 [5.2;6.6]	6.2 [5.44; 6.72]	0.091
Essential FA						
18:2	918.6 [798; 1030]	1007.7 [932; 1101]	0.151	894.0 [779; 988]	907.9 [77; 986]	0.201
18:3	15.3 [12.8; 16.8]	17.8 [14.2; 20.5]	0.194	14.8 [11.9;15.7]	15.2 [12.3;15.8]	0.169
LC-PUFA						
20:2	13.6 [11.5; 14.8]	13.08 [11.6; 13.2]	0.603	12.8 [11.6;13.9]	12.88 [11.7; 13.8]	0.921
20:3	143.4 [130; 159]	143.79 [127; 158]	0.968	136.8 [126;146]	141.4 [127; 148]	0.958
20:4	398.2 [335; 442]	396.10 [355; 425]	0.935	375.2 [312;493]	392.4 [333; 438]	0.774
20:5	31.7 [20.4; 40.1]	36.8 [27.2;45.7]	0.323	32.0 [23.2;35.9]	33.3 [23.6;41.3]	0.350
22:4	11.3 [8.6; 12.3]	11.4 [9.9; 12.6]	0.904	10.9 [8.6;13]	11.6 [9.5; 13.3]	0.863
22:5	39.4 [34.9;44.3]	43.9 [38.5; 47.6]	0.123	40.27 [34.6;43.74]	42.1 [37.4;46.3]	0.176
22:6	114.6 [97;137]	121.5 [100;145]	0.520	113.4 [92; 136]	115.2 [96.6; 134.8]	0.650

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Supplementary Table S3. Median concentrations of individual fatty acids in sphingomyelins in postGDM and controls at t0 and t90. Concentrations in µmol/l with 1st and 3rd quartiles. Differences between the groups were calculated by Mann-Whitney-U test and significance was accepted with p-values <0.001 after correction according to Bonferroni (marked with *).

FA	t0					t90				
	postGDM (n=17)		controls (n=20)		p-value	postGDM (n=17)		controls (n=20)		p-value
total FA SM	587.7	[513; 664]	603.5	[497.6; 677.7]	0.157	605.6	[527.7; 664.5]	614.7	[515.2; 665.5]	0.068
SFA										
12:0	0.879	[0.60; 1.035]	0.904	[0.56; 1.10]	0.019	0.844	[0.62; 1.03]	0.891	[0.66; 1.06]	0.003
14:0	15.6	[12.7; 18.0]	16.11	[12.68; 19.15]	0.019	15.035	[13.06; 17.29]	15.736	[13.27; 17.99]	0.007
15:0	10.25	[8.38; 11.97]	10.47	[8.22; 12.48]	0.035	9.854	[8.53; 11.55]	10.226	[8.55; 12.07]	0.006
16:0	163.3	[141; 189]	168.1	[141.7; 189.4]	0.027	157.819	[131.6; 180.3]	165.458	[139.2; 184.4]	0.012
17:0	5.0	[3.96; 5.92]	5.01	[3.91; 6.10]	0.176	4.751	[4.04; 5.45]	4.860	[4.05; 5.61]	0.077
18:0	30.69	[24.5; 35.8]	31.42	[24.30; 37.37]	0.298	29.897	[23.83; 33.15]	31.030	[24.33; 36.53]	0.177
20:0	51.97	[43.3; 59.3]	53.77	[42.90; 60.87]	0.470	49.906	[39.62; 57.99]	52.008	[39.87; 61.55]	0.299
22:0	31.98	[25.7; 37.1]	32.82	[24.23; 37.95]	0.610	31.189	[24.85; 37.17]	32.740	[24.63; 37.88]	0.189
MUFA										
14:1	1.41	[1.07; 1.66]	1.43	[1.02; 1.73]	0.806	1.350	[1.06; 1.55]	1.384	[1.04; 1.75]	0.509
15:1	0.42	[0.33; 0.50]	0.42	[0.31; 0.54]	0.888	0.423	[20.33; 0.45]	0.433	[0.33; 0.51]	0.319
16:1	25.76	[21.5; 29.6]	26.49	[21.67; 30.82]	0.700	24.706	[19.94; 28.70]	25.864	[20.95; 29.48]	0.296
18:1	16.78	[13.05; 19.6]	17.13	[13.33; 19.89]	0.813	16.594	[12.26; 18.571]	17.137	[12.58; 20.28]	0.592
20:1	29.45	[24.5; 34.0]	30.38	[24.69; 35.33]	0.271	28.232	[21.75; 32.13]	29.586	[23.01; 34.42]	0.116
22:1	49.06	[42.4; 54.9]	50.55	[43.05; 55.49]	0.107	48.116	[39.72; 54.54]	50.165	[41.10; 55.94]	0.049
Essential FA										
18:2	1.34	[1.15; 1.46]	1.33	[1.05; 1.47]	0.846	1.301	[1.07; 1.50]	1.309	[1.06; 1.48]	0.920
LC-PUFA										
22:2	16.3	[12.4; 20.3]	16.82	[11.90; 21.90]	0.596	13.829	[10.09; 15.70]	14.901	[11.57; 17.55]	0.598
22:3	3.9	[2.96; 4.39]	3.89	[2.89; 4.67]	0.670	3.720	[2.78; 4.53]	3.879	[2.49; 4.60]	0.605
VLC-FA										
24:0	29.0	[24.2; 33.6]	30.24	[24.53; 36.00]	0.270	28.045	[22.32; 31.27]	29.865	[24.55; 34.47]	0.088
24:1	62.6	[50.1; 72.0]	63.45	[49.16; 72.24]	0.304	61.748	[49.73; 74.17]	63.483	[49.98; 75.29]	0.178
24:2	37.0	[29.9; 43.3]	37.38	[30.38; 43.81]	0.470	36.085	[30.26; 43.44]	37.042	[30.99; 43.60]	0.268
24:5	4.4	[3.3; 5.7]	4.549	[3.35; 5.82]	0.289	4.276	[3.22; 5.25]	4.461	[3.61; 5.53]	0.374
26:0	0.12	[0.09; 0.16]	0.122	[0.09; 0.17]	0.600	0.123	[0.07; 0.17]	0.132	[0.09; 0.17]	0.135
26:2	0.58	[0.36; 0.75]	0.573	[0.36; 0.77]	0.922	0.612	[0.39; 0.87]	0.610	[0.40; 0.84]	0.884