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Increased PUFA content and 5-lipoxygenase pathway activity are associated with subcutaneous adipose tissue inflammation in obese women

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Abstract

Background/Objectives: White adipose tissue inflammation plays an important role in the development of insulin resistance and type 2 diabetes mellitus (T2DM). We have previously shown that obese women with T2DM have more inflammation in their subcutaneous adipose tissue than age and BMI similar obese women with normal glucose tolerance (NGT). The aim of the current study was to investigate whether adipose tissue fatty acids and/or oxylipins are associated with the enhanced inflammatory state in adipose tissue of the T2DM women.

Subjects/Methods: Fatty acid profiles were measured by GC-MS in both subcutaneous (sWAT) and visceral adipose tissue (vWAT) of 19 obese (BMI>40kg/m^2) women with NGT and 16 BMI-and age-comparable women with T2DM. Oxylipin levels (in total 49 lipid mediators) were measured in sWAT of all women.

Results: Arachidonic acid (AA) and docosahexaenoic acid (DHA) percentages were higher in sWAT but not vWAT of the T2DM women and correlated positively (trend for DHA) to *CD68* gene expression levels. For the oxylipins there were tendencies for higher concentrations of the leukotrienes, LTD_4, 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4, in sWAT of T2DM women. Gene expression of *ALOX5, ALOX5AP* and *DPEP2* (involved in the 5-LOX leukotriene biosynthesis pathway) was significantly higher in sWAT of T2DM women.

Conclusions: In conclusion, AA and DHA content were higher in sWAT of T2DM women and correlated to the inflammatory state in the tissue. The increased AA content was accompanied by an up-regulation of the 5-LOX pathway and this seems to have led to a modest increase in the conversion of AA into pro-inflammatory leukotrienes in the subcutaneous adipose tissue.

Introduction

Obesity is closely associated with insulin resistance, type-2 diabetes mellitus (T2DM), dyslipidemia, hypertension and cardiovascular disease. Expanding adipose tissue plays an important role in the patho-physiology of obesity-associated disorders as it responds to the energy overload with stress signals which in turn can elicit local immune responses and inflammation [1]. Although the majority of obese individuals (~80%) will eventually develop metabolic disorders associated with a reduced life expectancy, there seems to be a subset of obese individuals that remains relatively insulin sensitive and metabolically healthy throughout life [2, 3]. The reason why these individuals are unaffected is still not completely understood**.** We have previously shown that subcutaneous white adipose tissue (sWAT) from obese women with T2DM contained a larger number of crown-like-structures (CLS) than sWAT of similarly obese women with normal glucose tolerance (NGT) [4]. In a parallel study, we analyzed the transcriptome in adipose tissue samples by RNA deep sequencing in the same cohort of women and observed an up-regulation of genes in inflammatory pathways and a down-regulation of genes in metabolic pathways in WAT of the women with T2DM [5]. Our data thus indicate that metabolically healthy and unhealthy obese women can be differentiated by the inflammatory status of their adipose tissue.

Adipose tissue inflammation involves the accumulation of macrophages in CLS around adipocytes that are stressed or dying due to cellular lipid overload [6]. The enlarged stressed adipocytes exhibit an increased release of pro-inflammatory adipocytokines and chemokines which attract immune cells into the adipose tissue [7]. Hypertrophic adipocytes also store and release increased levels of fatty acids which are known to mediate inflammatory processes as well [8]. Not only the amount of fatty acids released, but also the type of fatty acid stored in adipose tissue has been associated to systemic inflammation [9] and T2DM [10, 11]. For example, an increased percentage of saturated fatty acids or their metabolites has been suggested to induce inflammation by activating Toll-like receptor 4, which in turn leads to an up-regulation of the ceramide biosynthesis pathway [12]. Ceramides activate the NLPR3 inflammasome which is an important contributor to obesity induced inflammation and insulin resistance [13]. Other fatty acids that are involved in inflammation are the polyunsaturated fatty acids (PUFAs). n-3 and n-6 PUFAs can induce pro- and anti-inflammatory pathways respectively [14] via a variety of different mechanisms including signaling via GPR120. PUFAs can be converted to inflammatory lipid mediators called oxylipins. Oxylipin synthesis usually occurs via the cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 (CYP) pathways [15]. All three pathways can metabolize both n-3 and n-6 PUFAs, but the affinity for these substrates differs as does the pro- or anti-inflammatory potency of the different resulting products. For example the 2-series prostaglandins, synthesized from arachidonic acid (AA; C20:4n-6) via the COX pathway, induce mainly pro-inflammatory effects; while the 3-series prostaglandins, synthesized from eisosapentaenoic acid (EPA; 20:5n-3) via COX, induces less potent inflammatory effects [16] and the epoxy metabolites derived from n-3 PUFAs via the

CYP pathway induce anti-inflammatory effects [17]. PUFA-derived oxylipin synthesis can be rapidly induced when triggered by inflammasome signaling [18]. A wide range of oxylipins has been identified in both human [19] and mouse WAT [20]. Oxylipins have been shown to play a role in WAT inflammation, mostly in rodent studies [8]. In the current study we hypothesized that the adipose fatty acid composition is linked to the enhanced inflammatory state in adipose tissue of obese women with T2DM. To test this hypothesis, we determined fatty acid profiles by GC-MS and oxylipin profiles by LC-MS/MS in white adipose tissue of both obese women with T2DM and obese women with NGT.

Subjects and methods

Subjects

The study group consisted of 19 obese women with normal fasting glucose (i.e. normal glucose tolerant (NGT)) and 16 women with T2DM. The women were part of a clinical trial of which the research methods and design have been described elsewhere [21]. The groups were comparable for age and BMI (see Table 1). All women had been morbidly obese (mean BMI=43.4 ± 3.8 kg/m^2) for at least five years. Women who reported the use of weight loss medications within 90 days prior to enrolment in the study were excluded. Body weight of all women had been stable for at least 3 months prior to inclusion. All women were non-smokers, had no signs of any infections nor had any history of auto-immune diseases. The women underwent bariatric surgery (gastric bypass or banding). Within 1h after opening the abdominal wall adipose tissue specimens were taken from the epigastric region of the abdominal wall (subcutaneous sWAT) and from the major omentum (visceral vWAT). These samples were used for determination of fatty acid composition and oxylipin profiles. The study (ClinicalTrials.gov: NTC01167959) was approved by the p5. All subjects gave informed consent to participate in the study.

Medication

For obvious reasons we could not restrict obese individuals to not using any type of medication. All T2DM women were treated with oral medication only (metformin or sulfonylurea derivatives). Participants were allowed to use cholesterol lowering statins and antihypertensive medication. The use of drugs such as statins and antihypertensive drugs was slightly higher in the T2DM women. The patients were not using any anti-inflammatory agents (i.e. NSAIDS, thiazolidinediones or steroids (prednisone)).

Analysis of number of crown like structures and adipocyte size

In a previous study the number of crown like structures in the adipose tissue of our participants was determined by immunohistochemistry and the adipocyte size by direct microscopy [4].

Fatty acid composition of WAT by GC-MS

FA composition analysis of sWAT and vWAT was carried out as described recently by Kloos *et al.* [22]. Briefly: approximately 10 mg WAT was weighed from the obese women. 1 ml of water, 3 ml of methanol and 1 ml of 10M NaOH were added, the samples flushed with argon and hydrolyzed for 1 h at 90 °C. After acidification with 2 ml of 6M HCl, 10 µl of an internal standard solution ([$^2H_{31}$]palmitic acid and ergosterol 10 µg/ml each) was added. The

samples were extracted twice with 3 ml *n*-hexane and the combined organic extracts were dried under a gentle stream of nitrogen. Dried samples were derivatized using 25 µl of *N*-tert.-butyldimethylsilyl-*N*-methyltrifluoroacetamide (Sigma Aldrich, Schnelldorf, Germany) for 10 min at 21 °C, subsequently 25 µl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (Thermo Scientific, Waltham, MA, USA) and 2.5 µl of pyridine were added and the sample was heated for 15 min to 50 °C. Next, 947.5 µl of *n*-hexane, containing 10 µg/ml octadecane (C18) as system monitoring component, was added.

Samples were analyzed in SIM mode on a Scion TQ GC-MS (Bruker, Bremen, Germany) equipped with a 15 m × 0.25 mm × 0.25 mm BR5MS column (Bruker). The injection volume was 1 µl, the injector was operated in splitless mode at 280 °C and the oven program was as follows: 90 °C kept constant for 0.5 min, then ramped to 180 °C with 30 °C/min then to 250 °C with 10 °C/min then to 266 °C with 2 °C/min and finally to 300 °C with 120 °C/min, kept constant for 2 min. Helium (99.9990%, Air Products, The Netherlands) was used as carrier gas. For data analysis a total area correction was applied.

Oxylipin measurements in WAT by LC-MS/MS

Oxylipin analysis was carried out as described elsewhere ([23],[24]) with some modifications. Approximately 100 mg of tissue were cut using a razor blade on a glass plate, transferred into a 2 ml Eppendorf tube and accurately weighed. Three µl of an internal standard solution containing 50 ng/ml each of PGE_2-d4, LTB_4-d4, 15-HETE-d8 and DHA-d5 was added. Subsequently 3-5 stainless steel beads, 500 µl of methanol and 2 µl of a 20 mg/ml butylated hydroxyl toluene solution were added. Next, the samples were homogenized in a bead beater for 4 min centrifuged at 16100 g for 3 min. 400 µl of the supernatant were transferred into a 12 ml glass tube. The samples were re-extracted using 500 µl of methanol by shaking for 5 min. The combined organic extracts were diluted with approximately 9 ml of water, acidified with 6M HCl and further cleaned up using solid phase extraction (employing 100 mg SPE columns) as described elsewhere [25] and finally reconstituted in 150 µl 40% methanol before LC-MS/MS analysis.

Statistics

Data are expressed as mean ± SD or as median and range of the values. Differences between NGT and T2DM were analyzed using unpaired non-parametric t-test's. Linear regression was used to analyze correlations using the F-test in Graphpad Prism 6 (GraphPad Software, CA, USA).

Results

Characteristics of participants

Characteristics of the participants are shown in Table 1. Fasting plasma glucose and LDL-cholesterol levels were significantly higher in T2DM than in NGT women. HOMA-IR index and triglyceride levels tended to be higher in the T2DM women. The gene expression of *CD68* (a macrophage marker) and the number of CLS per area adipose tissue on immunohistochemistry slides was used as an index for the extent of adipose tissue inflammation. The sWAT but not the vWAT of the T2DM women had a significantly higher gene expression of *CD68* and contained more CLS. Adipocyte sizes did not differ between NGT and T2DM women both for sWAT and vWAT.

Table 1: Characteristics of the NGT and T2DM women. Crown like structures (CLS) were determined by immunohistochemistry of CD68 and expressed as number (#) of CLS per area of adipose tissue (AT) section on the slide. Adipocyte size was expressed as mean adipocyte diameter in µm. CLS counts and adipocyte sizes have been published previously [4]. Gene expression was determined by RNA deep sequencing in a previous study [5] and expressed as log transformed normalized gene expression levels (relative units [RU]: log2-scale). Data are expressed as mean ± SD.

	NGT	T2DM	P-value t-test
N	19	16	
BMI (kg/m^2)	43.4 ±3.3	43.4±4.5	NS
Age (y)	47±7	52±6	NS
Weight (kg)	121±8	127±12	NS
HOMA-IR	2.7±2.2	4.0±3.0	0.08
Fasting glucose (mmol/l)	5.0±0.6	9.0±2.6	<0.01
Fasting insulin (mU/l)	10.9±7.7	13.3±7.3	NS
Total cholesterol (mmol/l)	4.7±1.1	4.2±0.8	NS
HDL cholesterol (mmol/l)	1.1±0.3	1.1±0.3	NS
LDL cholesterol (mmol/l)	3.0±1.0	2.2±0.6	0.03
Triglycerides (mmol/l)	1.5±0.7	2.0±0.7	0.08
CRP (mg/l)	7.8±7.5	8.2±6.3	NS
CD68 gene expression in sWAT	8.2±0.4	8.9±0.7	0.003
CD68 gene expression in vWAT	8.2±0.4	8.4±0.5	NS
# of CLS in sWAT (no/AT section)	2.3±1.4	12.3±7.1	0.05
# of CLS in vWAT (no/AT section)	1.9±1.2	2.7±1.0	NS
Adipocyte size in sWAT (µm)	122±16	126±15	NS
Adipocyte size in vWAT (µm)	112±16	118±8	NS

Differences in adipose tissue content of saturated fatty acids and PUFAs between NGT and T2DM obese women

The fatty acid composition of sWAT and vWAT samples from the NGT and T2DM obese women was determined by GC-MS. For both sWAT and vWAT for the T2DM women, we found lower percentages of C10:0 (Decanoic acid) and C12:0 (Lauric acid) (Fig. 1A). The MUFAs did not show large differences between the NGT and T2DM women (Fig. 1B). For the PUFAs in vWAT of the T2DM women, a lower percentage of C18:3n-6 (gamma-linolenic acid) was seen. In the sWAT of the T2DM women, differences in both n-6 and n-3 PUFAs were found. For the n-6 PUFAs higher percentages of C20:4n-6 (Arachidonic acid (AA)) and C22:4n-6 (Adrenic acid (AdA)) were found (Fig. 1C), whereas for the n-3 PUFAs higher percentages of C20:3n-3 (Eicosatetraenoic acid (ETA)), C20:5n-3 (Eicosapentaenoic acid (EPA)), C22:5n-3 (Docosapentaenoic acid (DPA)) and C22:6n-3 (Docosahexaenoic acid (DHA)) were found (Fig. 1D).

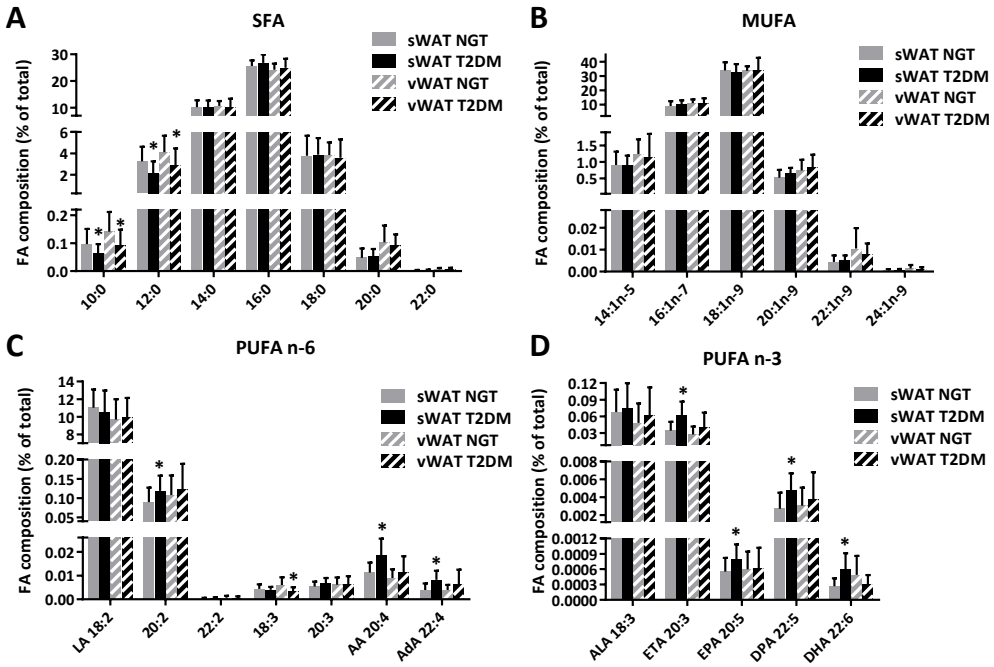

*Figure 1: Fatty acid composition of sWAT and vWAT for NGT versus T2DM individuals. A) Saturated fatty acids. B) Mono-unsaturated fatty acids. C) n-6 and D) n-3 poly-unsaturated fatty acids. Data are expressed as mean ± SD. *p<0.05 for NGT vs T2DM.*

Arachidonic acid and docosahexaenoic acid correlated to CD68 expression in sWAT

The percentage AA of the total fatty acid pool correlated to the gene expression level of *CD68* in sWAT and a trend was visible for DHA. For the correlation of AA with *CD68* expression the goodness-of-fit was r=0.42, p=0.029 (Fig. 2A). For the correlation of DHA with *CD68* expression the goodness-of-fit was r=0.34, p=0.087 (Fig. 2B). There were no significant correlations between any other fatty acid percentage and *CD68* expression in sWAT.

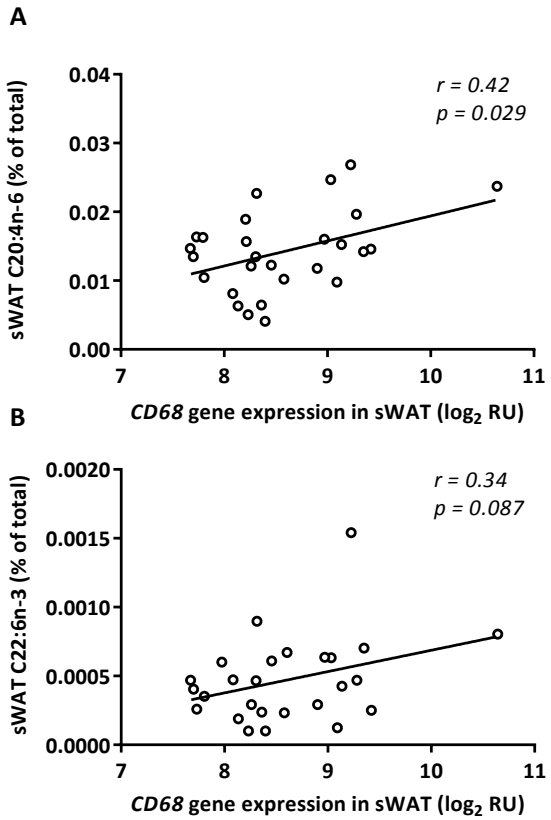

Figure 2: CD68 expression versus PUFA percentage in sWAT. Linear regression of sWAT gene expression of the macrophage marker CD68 with the sWAT percentage of C20:4n-6 (AA) and C22:6n-3 (DHA). Data are log transformed normalized gene expression levels (relative units [RU]: log2-scale). Data are expressed as mean ± SD.

Oxylipin levels in sWAT of women with NGT and T2DM

Table 2 shows the list of the oxylipins that were measured and the levels that were detected per mg of sWAT of the women with NGT and with T2DM. Many of the oxylipins measured, in particular the resolvins, were below the detection limit (see Table 2). There was a large variation in oxylipin concentrations between the women. Of the detectable oxylipins there was a tendency for higher content of some leukotrienes; i.e. LTD_4, 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4 in sWAT of women with T2DM (See Fig. 3A).

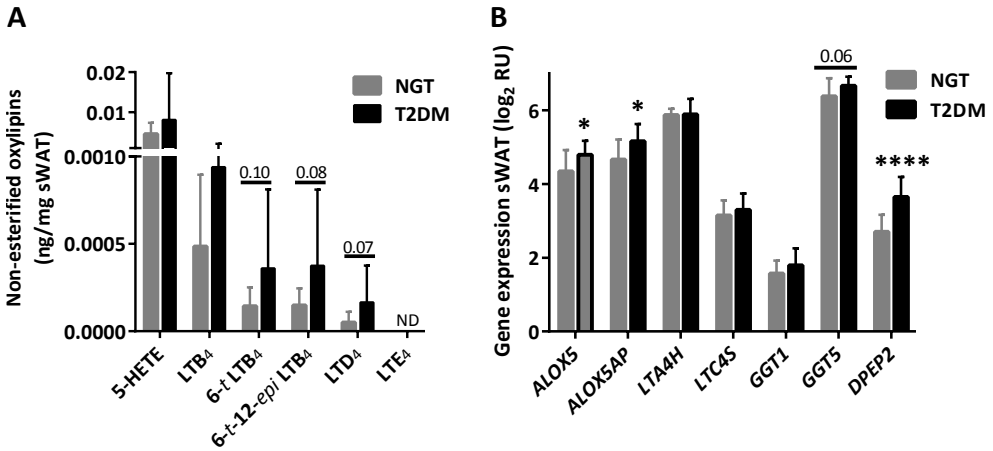

*Figure 3: 5-LOX oxylipins in sWAT. A) Non-esterified oxylipins derived from arachidonic acid via the 5-LOX pathway in sWAT of NGT vs T2DM obese individuals. LTE_4 was below the detection limit (ND=not detectable). LTA_4, LTC_4 and LTF_4 were not included in the LC-MS/MS method. B) Gene expression of genes involved in the leukotriene biosynthesis pathway in sWAT of NGT versus T2DM individuals (ALOX5, ALOX5AP, LTA4H, LTC4S, GGT1, GGT5 and DPEP2). Data are log transformed normalized gene expression levels (relative units [RU]: log2-scale). Data are expressed as mean ± SD. *p<0.05 and ****p<0.0001 for NGT vs T2DM.*

*Table 2: Oxylipins in sWAT of NGT versus T2DM obese women. NGT vs T2DM values were compared using non-parametric t-test. ND= non-detectable. NS= non-significant. *= Compound not in calibration line. P-value calculated from area ratios compared to internal standard. #= Detected, but only in fewer than five individuals in total. Data are expressed as median and range of the concentrations.*

	NGT		T2DM		NGT vs T2DM
Oxylipin (ng/mg sWAT)	**Range**	**Median**	**Range**	**Median**	**P-value**
5-HETE	7.91E-04 - 3.19E-02	3.70E-03	1.47E-03 - 5.07E-02	5.01E-03	NS
8-HETE	4.53E-04 - 1.83E-02	2.28E-03	5.56E-04 - 2.24E-02	2.86E-03	NS
11-HETE	3.58E-04 - 1.46E-02	1.55E-03	3.90E-04 - 1.84E-02	1.89E-03	NS
12-HETE	1.63E-03 - 8.36E-02	1.06E-02	2.77E-03 - 7.90E-02	1.17E-02	NS
15-HETE	7.36E-04 - 3.20E-02	3.49E-03	8.13E-04 - 4.62E-02	4.04E-03	NS
15-HEPE	9.23E-05 - 3.13E-03	4.36E-04	1.54E-04 - 2.62E-03	5.72E-04	NS
18-HEPE	1.25E-04 - 4.94E-03	5.55E-04	2.40E-04 - 3.91E-03	5.04E-04	NS
7-HDHA	*		*		NS
10-HDHA	*		*		NS
17-HDHA	0 - 3.17E-03	5.34E-04	0 - 3.23E-03	1.14E-03	NS
LTB_4	6.42E-05 - 2.69E-03	4.10E-04	4.66E-05 - 4.17E-03	4.24E-04	NS
6-*trans*-12-*epi* LTB_4	2.97E-05 - 3.22E-04	1.29E-04	5.98E-05 - 1.71E-03	1.90E-04	0.08
6-*trans* LTB_4	3.91E-05 - 4.36E-04	1.14E-04	6.39E-05 - 1.86E-03	1.57E-04	0.10
20-OH LTB_4	ND		ND		
LTD_4	0 - 2.08E-04	3.80E-05	0 - 7.75E-04	7.74E-05	0.06
LTE_4	ND		ND		
PGD_2	5.79E-05 - 1.14E-03	2.26E-04	3.93E-05 - 2.65E-03	2.68E-04	NS
PGE_2	7.06E-05 - 2.12E-03	5.95E-04	7.68E-05 - 3.58E-03	4.76E-04	NS
$PG_{F2}\alpha$	0 - 2.12E-03	6.94E-04	1.02E-04 - 2.53E-03	5.11E-04	NS
TxB_2	0 - 1.24E-02	1.54E-03	4.49E-05 - 2.05E-02	1.59E-03	NS
LXA_4	0 - 2.49E-04	0	0 - 1.53E-03	1.52E-05	NS
LXB_4	ND		ND		
AT LXA_4	ND		ND		
8-*iso* PGE_2	ND		ND		
8-*iso* $PGF_{2\alpha}$	0 - 1.83E-04	4.50E-05	0 - 7.64E-04	4.39E-05	NS
15-*keto* PGE_2	1.69E-05 - 3.62E-04	8.60E-05	6.63E-06 - 1.21E-03	6.35E-05	NS
13,14-dihydro-15-*keto* $PGF_{2\alpha}$	0 - 5.05E-03	0	0 - 4.60E-03	0	NS
RvD1	ND		ND		
RvD2	ND		ND		
AT RvD1	ND		ND		
RvE1	ND		ND		
RvE2	#		#		
18S-RvE3	ND		ND		
18R-RvE3	ND		ND		

	NGT		T2DM		NGT vs T2DM
Oxylipin (ng/mg sWAT)	**Range**	**Median**	**Range**	**Median**	**P-value**
7,17-DiHDPA	0 - 1.68E-03	7.39E-05	0 - 8.76E-04	0.00E+00	NS
19,20-DiHDPA	0 - 3.53E-04	9.56E-05	5.99E-05 - 4.30E-04	1.11E-04	NS
10S,17S-diHDHA (PDX)	0 - 1.97E-04	4.00E-05	0 - 2.44E-04	3.93E-05	NS
MaR1	#		#		
7S-MaR1	ND		ND		
5,15-diHETE	0 - 2.08E-02	2.20E-03	0 - 8.71E-02	3.43E-03	NS
14,15-diHETE	0 - 6.32E-04	2.21E-04	0 - 2.15E-03	1.85E-04	NS
8S,15S-diHETE	4.00E-05 - 1.32E-03	1.47E-04	0 - 1.63E-03	1.85E-04	NS
9-HoDE	7.46E-02 - 9.24E-01	3.17E-01	1.27E-01 - 8.32E-01	2.59E-01	NS
13-HoDE	4.74E-02 - 5.27E-01	1.61E-01	6.52E-02 - 4.71E-01	1.42E-01	NS
9-HoTrE	3.41E-03 - 4.90E-02	2.08E-02	3.89E-03 - 3.74E-02	1.49E-02	NS
13-HoTrE	4.45E-02 - 5.02E-01	2.85E-01	5.68E-02 - 3.96E-01	1.95E-01	NS
8(9)EET	*		*		NS
11(12)EET	*		*		NS
14(15)EET	*		*		NS

Differential expression of ALOX5, ALOX5AP and DPEP2 in sWAT between NGT and T2DM women

In a previous study, the adipose tissues of 15 of the NGT and 15 of the T2DM women included in the current study were used for transcriptome analysis by RNA deep sequencing [5]. From the gene expression profiles obtained in that study we could extract gene expression levels of the genes involved in the leukotriene biosynthesis pathway (i.e. *ALOX5*, *ALOX5AP*, *LTA4H*, *LTC4S*, *GGT1*, *GGT5* and *DPEP2*). See Fig. 4 for a scheme of the leukotriene biosynthesis pathway and the genes involved therein. Of these genes *ALOX5*, *ALOX5AP* and *DPEP2* were significantly higher expressed and *GGT5* tended (p=0.06) to be higher expressed in sWAT of T2DM women (see Fig. 3B).

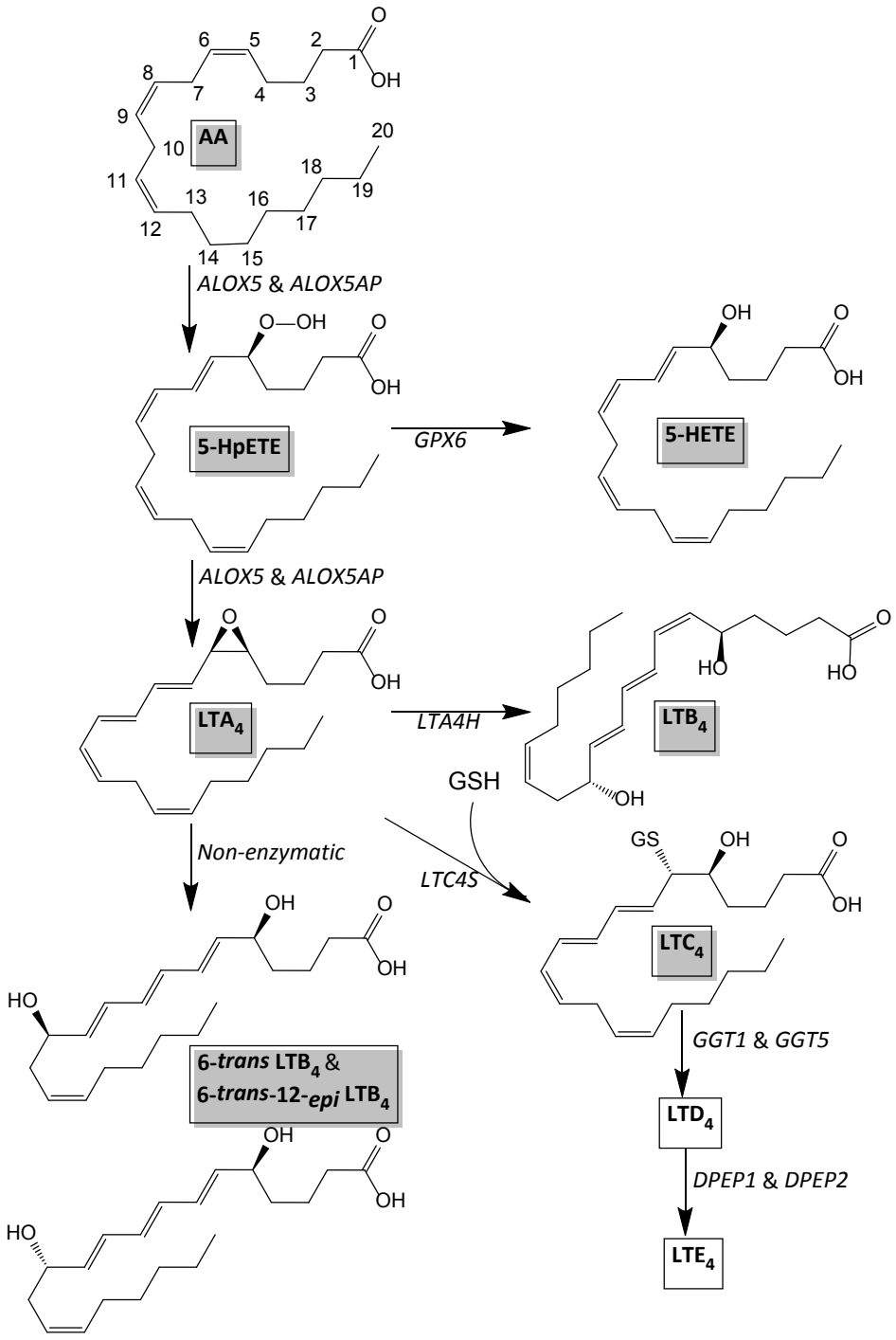

Figure 4: Leukotriene biosynthesis pathway based on Murphy et al. [26]

Discussion

We have previously shown that the macrophage content in the sWAT but not the vWAT of obese women with T2DM was higher. In the current study we investigated whether this increased inflammatory state was linked to a higher percentage of fatty acids that mediate inflammatory processes such as the saturated fatty acids or the PUFAs. We found that the higher inflammatory state in the sWAT of T2DM women was not associated with a higher percentage of saturated fatty acids. The saturated fatty acid percentages of C10:0 and C12:0 were even lower in T2DM women, both in sWAT and vWAT. Interestingly, the sWAT of T2DM women showed increased percentages of both n-3 and n-6 long-chain PUFAs (including AA and DHA) which was not seen in vWAT. AA percentage in sWAT correlated positively with gene expression of the macrophage marker *CD68* (trend for DHA) indicating that PUFAs were associated with the macrophage infiltration in the adipose tissue.

The content of PUFAs in adipose tissue is dependent on several processes including uptake of PUFAs into the cell via fatty acid transporters or passive transport, the biosynthesis of PUFAs within a cell, the degradation of PUFAs and the release of PUFAs from a cell. Previously, we analyzed the transcriptome of adipose tissue by RNA sequencing in the same individuals as included in this study [5]. By using a network-based approach to analyze gene expression in NGT versus T2DM women, we identified the down-regulation of the complete acetyl-CoA metabolic network in the adipose tissue of the T2DM women. This network included a down-regulation of fatty acid biosynthesis, fatty acid degradation as well as fatty acid release. Thus, although there seem to be clear differences in gene expression of PUFA metabolism, we cannot determine the net flux of PUFAs in the adipose tissue based on this gene expression analysis. Further research is required to determine the PUFA mass balance *in vivo* in the adipose tissue to be able to explain the increased PUFA content in the T2DM individuals.

Although most of the fatty acids in adipose tissue are stored in triglycerides within the adipocytes (99%) there is a small fraction of the fatty acids present in phospholipids. We could not determine which of the fatty acid based lipid species in the adipose tissue contributed to the difference in PUFAs in T2DM women. In addition, the (immune) cells of the stromal vascular fraction in adipose tissue contribute to the fatty acid pool and can produce fatty acid-derived mediators. Immune cells are the main producers of oxylipins [27]. It is possible that the observed difference in FA and oxylipin content are due to differences in the cellular composition of the adipose tissues. Additional research is necessary to determine whether the cellular composition of adipose tissue contributed to the observed differences in FA and oxylipin content.

Both AA and DHA can be metabolized into oxylipins with pro or anti-inflammatory properties. We detected several oxylipins in human sWAT, derived either from AA, DHA or EPA. The levels of most of these oxylipins did not differ between NGT and T2DM women, but there were tendencies for higher concentrations of some of the AA derived leukotrienes (LTD_4, 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4). For the leukotriene biosynthesis pathway (see Fig. 4),

AA is metabolized by 5-lipoxygenase (5-LOX) in the presence of an integral nuclear membrane protein, 5-LOX activating protein (also called FLAP). In this reaction, AA is metabolized into the sequential intermediates 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and LTA_4. LTA_4 is conjugated with reduced glutathione by LTC_4 synthase (LTC4S) and released from the cell for extracellular conversion to LTD_4 and LTE_4. Alternatively, cytosolic LTA_4 hydrolase (LTA4H) converts LTA_4 to LTB_4. LTA_4 is also hydrolyzed non-enzymatically into 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4. To further study the leukotriene biosynthesis pathway we analyzed gene expression of genes involved in this pathway. We found a significant higher expression of *ALOX5* (5-LOX), *ALOX5AP* (FLAP) and *DPEP2* (Dipeptidase 2), indicating an overall up-regulation of the 5-LOX pathway in sWAT of T2DM women. The fact that both LTD_4 and the non-enzymatically hydrolyzed LTB_4's tended to be higher (and not only one particular leukotriene) is in line with this notion. Subcutaneous adipose tissue expression of *ALOX5AP* has previously been shown to be positively associated to body weight and insulin resistance as determined by HOMA-IR index [28]. The 5-LOX pathway has also been shown to be increased in obese adipose tissue [29]. Mouse and human adipocytes produce leukotrienes *in vitro* and this production is increased in hypertrophic adipocytes in obesity in mice [30]. Taken together, previous studies and our study suggest that the 5-LOX pathway may provide a link between adipose tissue, inflammation and insulin resistance.

The results obtained in this study differ from those of a previous study by Lieb *et al.* [31] who measured fatty acids and some downstream oxylipins in obese non-diabetic and T2DM individuals. They did not observe any differences in AA or DHA content between the subjects groups neither in sWAT nor vWAT. Instead, they observed an up-regulation of *ALOX12* expression and its metabolite 12(S)-hydroxyeicosatetraenoic acid in vWAT but not sWAT of the T2DM individuals and suggested that the ALOX12 pathway may have a critical role in adipose tissue inflammation. Differences in the subject groups (their study also included males) may explain the discrepancies between their and our study.

Most of the studies on lipid signaling and oxylipins in adipose tissue have been performed in rats or mice. We have performed a study in humans and have made a thorough analysis of several types of oxylipins and concentrations thereof in adipose tissue. We did not detect resolvins in adipose tissue apart from 10S,17S-diHDHA (PDX) and in fewer than five women we could detect some Resolvin E2 and Maresin 1. This is in contrast with several mouse studies that detected more resolvins in adipose tissue and suggested a role for these mediators in counteracting adipose tissue inflammation [32],[33],[34]. Apart from the fact that there may be species specific differences between the studies it is also possible that discrepancies can be explained by the stage of adipose tissue inflammation studied. Most of the mouse studies examine adipose tissue inflammation during a high fat diet feeding, thus during progressive adipose tissue expansion and inflammation. Our obese women had been obese for a relatively long period of time and their adipose tissue inflammation was likely fairly established. Further research is required to determine whether species-specificity or the stage and duration of obesity is responsible for the observed differences. Additionally,

the analysis of oxylipins in adipose tissue is a challenging task due to the large amounts of triglycerides and other hydrophobic matrix constituents. In the presented study we adapted a published protocol for the analysis of adipose tissue, however it has to be mentioned that the recoveries particularly for the internal standards 15-HETE-d8 and DHA-d5 were rather low (< 40%), suggesting that an improvement of the described method for further studies might be of importance. In conclusion, AA and DHA content were higher in sWAT of T2DM women and associated with the inflammatory state in the tissue. The increased AA content was accompanied by an up-regulation of the genes in the leukotriene pathway and this seems to have led to a modest increase in the conversion of AA into pro-inflammatory leukotrienes in the subcutaneous adipose tissue of individuals with T2DM.

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Supplemental data

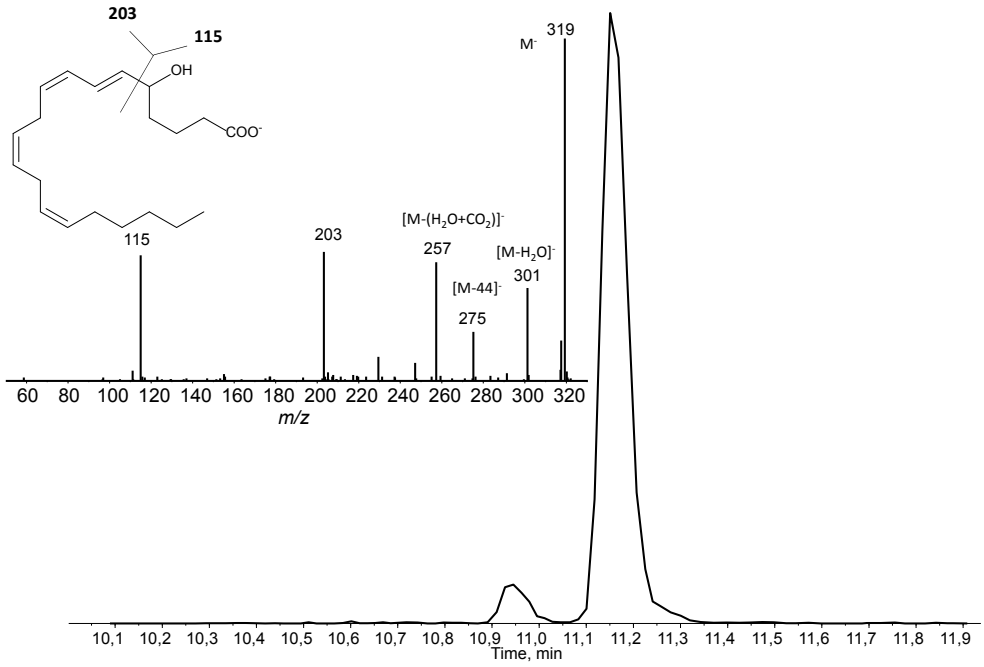

Figure S1: SRM transition m/z 319 -> 115 showing 5-HETE at RT=11.2 min and its two isomers. Upper left corner characteristic MS/MS spectrum and fragmentation of 5-HETE.

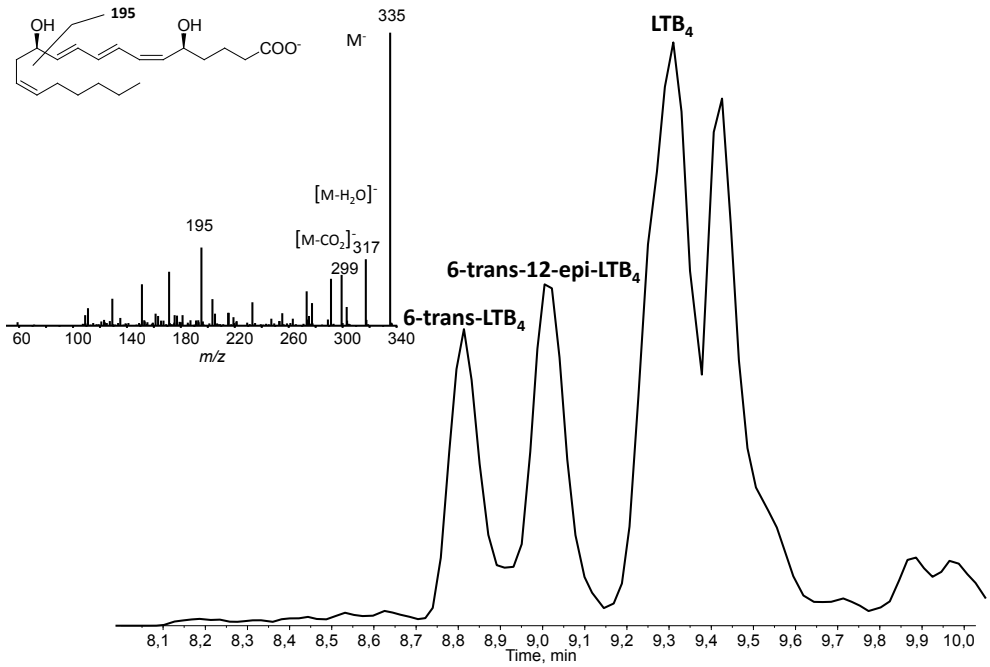

Figure S2: SRM transition m/z 335 -> 195 showing LTB_4 at RT=9.3 min and its two isomers. Upper left corner characteristic MS/MS spectrum and fragmentation of LTB_4.

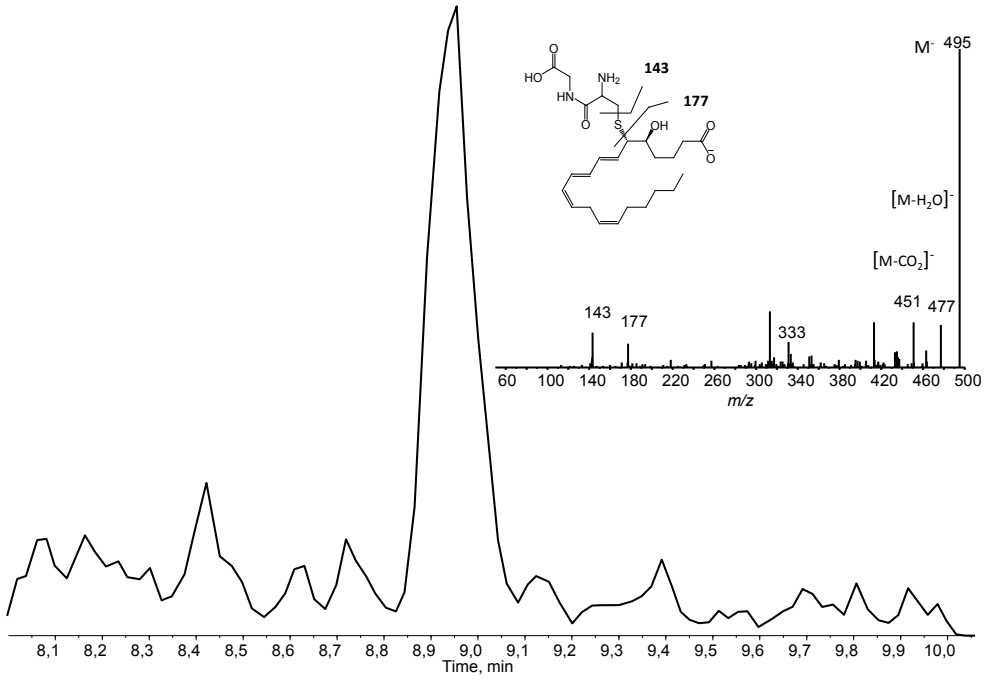

Figure S3: SRM transition m/z 495 -> 177 showing LTD_4 at RT=8.9 min. Upper right corner characteristic MS/MS spectrum and fragmentation of LTD_4.

Table S1: Multiple Reaction Monitoring setup for ion transitions of the target compounds. Symbols in bold refer to internal standards. RT=retention time, Q1=quadrupole 1 ion selection, Q3=quadrupole 3 ion selection, EP=entrance potential, CE=collision energy, CCEP=collision cell exit potential. HODEs, HOTrEs, HETEs, HEPEs, diHETEs and diHDPAs are given without chiral descriptors. Internal standards are indicated in grey.

Symbol	Lipid Maps ID	RT (min)	Q1 (m/z)	Q3 (m/z)	DP (Volts)	EP (Volts)	CE (Volts)	CCEP (Volts)
RvE1	LMFA03070019	4.0	349.1	195.0	-95	-10	-22	-13
20-hydroxy LTB_4	LMFA03020018	4.4	351.1	195.0	-60	-10	-24	-17
8-*iso*-$PGF_{2\alpha}$	LMFA03110001	5.1	353.1	193.0	-135	-10	-34	-11
15-*keto*-PGE_2	LMFA03010030	5.1	349.0	234.9	-65	-10	-20	-13
TxB_2	LMFA03030002	5.2	369.1	169.0	-55	-10	-24	-15
8-*iso*-PGE_2	LMFA03110003	5.3	351.1	271.0	-5	-10	-24	-19
PGE_2-d_4	LMFA03010008	5.6	355.1	193.0	-50	-10	-26	-17
PGE_2	LMFA03010003	5.7	351.2	271.1	-50	-10	-22	-21
PGD_2	LMFA03010004	5.8	351.1	233.0	-30	-10	-16	-13
LXB_4	LMFA03040002	6.0	351.1	220.9	-60	-10	-22	-13
$PGF_{2\alpha}$	LMFA03010002	6.1	353.1	193.0	-80	-10	-34	-11
RvD2	LMFA04000007	6.2	375.1	277.1	-60	-10	-18	-15
LXA_4	LMFA03040001	6.5	351.1	114.8	-40	-10	-20	-11
13,14-dihydro-15-*keto*-$PGF_{2\alpha}$	LMFA03010027	6.6	353.1	195.0	-110	-10	-32	-11
AT-RvD1	LMFA04000074	6.7	375.0	215.0	-50	-10	-26	-11
RvD1	LMFA04000006	6.7	375.1	215.0	-50	-10	-26	-11
AT-LXA_4	LMFA03040003	6.8	351.1	114.9	-20	-10	-22	-11
RvE2	LMFA03070036	7.8	333.1	114.9	-35	-10	-18	-15
18S-RvE3	LMFA03070048	8.8	333.1	245.2	-25	-10	-16	-17
6-*trans*-LTB_4	LMFA03020013	8.9	335.1	194.9	-105	-10	-22	-11
8S,15S-diHETE	LMFA03060050	8.9	335.1	207.9	-55	-10	-22	-17
5,15-diHETE	LMFA03060010	9.2	`335.0	173.1	-55	-10	-20	-11
8(9)-EET	LMFA03080003	11.4	319.0	155.0	-60	-10	-10	-13
11(12)-EET	LMFA03080004	11.4	319.0	167.0	-90	-10	-18	-19
14(15)-EET	LMFA03080005	11.2	319.0	219	-5	-10	-16	-55
LTD_4	LMFA03020006	9.0	495.1	177.0	-70	-10	-28	-19
6-*trans*-12-*epi*-LTB_4	LMFA03020014	9.1	335.1	194.9	-80	-10	-22	-25
10S,17S-diHDHA (PDX)	LMFA04000047	9.2	359.1	153.0	-70	-10	-22	-9
18R-RvE_3	LMFA03070049	9.2	333.1	245.0	-55	-10	-18	-23
7S-MaR1	n.a.	9.3	359.1	249.9	-20	-10	-20	-19
MaR1	LMFA04000048	9.4	359.2	250.2	-65	-10	-20	-13
LTB_4-d_4	LMFA03020030	9.4	339.1	196.9	-70	-10	-22	-19
LTB_4	LMFA03020001	9.4	335.1	195.0	-65	-10	-22	-21
14,15-diHETE	LMFA03060077	9.5	335.1	207.0	-65	-10	-24	-21
7,17-diHDPA	n.a.	9.5	361.1	198.9	-45	-10	-26	-23

Symbol	Lipid Maps ID	RT (min)	Q1 (m/z)	Q3 (m/z)	DP (Volts)	EP (Volts)	CE (Volts)	CCEP (Volts)
LTE_4	LMFA03020002	9.6	438.1	333.1	-55	-10	-26	-15
19,20-diHDPA	LMFA04000043	10.2	361.1	273.0	-55	-10	-22	-15
9-HOTrE	LMFA02000024	10.2	293.0	170.9	-75	-10	-20	-15
13-HOTrE	LMFA02000051	10.3	293.0	195.0	-45	-10	-24	-19
18-HEPE	LMFA03070038	10.4	317.1	259.0	-5	-10	-16	-7
15-HEPE	LMFA03070009	10.5	317.1	219.0	-65	-10	-18	-19
13-HODE	LMFA02000228	10.8	295.0	194.9	-110	-10	-24	-21
9-HODE	LMFA02000188	10.8	295.0	171.0	-130	-10	-22	-7
15-HETE-d_8	LMFA03060080	10.9	327.2	226.0	-85	-10	-18	-11
15-HETE	LMFA03060001	11.0	319.1	219.1	-55	-10	-18	-9
11-HETE	LMFA03060003	11.1	319.1	167.0	-70	-10	-22	-15
7-HDHA	n.a.	11.3	343.1	141.1	-85	-10	-18	-23
10-HDHA	n.a.	11.2	343.1	153.0	-25	-10	-20	-15
17-HDHA	LMFA04000072	11.1	343.1	245.0	-65	-10	-16	-15
12-HETE	LMFA03060007	11.2	319.1	179.0	-65	-10	-20	-23
8-HETE	LMFA03060006	11.2	319.1	154.9	-70	-10	-20	-19
5-HETE	LMFA03060002	11.3	319.1	115.0	-65	-10	-18	-11
ALA	LMFA01030152	12.4	277.0	233.0	-90	-10	-22	-29
EPA	LMFA01030759	12.4	301.0	202.9	-125	-10	-18	-21
DHA-d_5	LMFA01030762	12.4	332.0	288.1	-75	-10	-16	-13
DHA	LMFA01030185	12.7	327.1	229.2	-115	-10	-18	-11
AA	LMFA01030001	12.7	303.0	205.1	-155	-10	-20	-11
LA	LMFA01030120	12.8	279.0	261.0	-115	-10	-28	-13
DPA n-3	LMFA04000044	13.0	329.1	231.1	-50	-10	-20	-17
AdA	LMFA01030178	13.1	331.1	233.0	-130	-10	-22	-11