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# Metabolomics in Hind limb and Heart Muscle of a Mouse Model after a High-fat Diet

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## Abstract

**Objective:** Dysregulation of FA metabolism in heart muscle is a major problem associated with the metabolic syndrome.

**Methods:** C57Bl/6 mice were fed for 40 days a high-fat diet (+0.25% cholesterol 45% energy from bovine lard). High Performance Thin Layer Chromatography (HPTLC) was used to measure Triacylglycerol's (TG) and Free Cholesterol (FCh) in hearts and hind limb muscle of mice. With LC-MS techniques we measured Cholesterol esters (ChE), lysophosphatidyl-cholines (LPC), phosphatidylcholine (PC), sphingomyelin (SPM) and Triacylglycerol's (TG) in hind limb and heart muscle after 40 days of a fatty diet.

**Results:** This study was designed to give more insight in the lipid composition of the heart muscle in comparison to hind limb muscle. Only in the heart muscle cholesterol esters (ChE) were observed. This was confirmed with LC-MS techniques. Especially the 20:3-ChE are significantly increased in the fatty heart with 1317%. The LC-MS techniques gave no clear picture for Lysophosphatidyl-cholines (LPC), Sphingomyelin (SPM) in hind limb and heart muscle after 40 days of a fatty diet. Only for Phosphatidylcholine (PC) the most remarkable observation was that 36:1-PC rises in heart muscle with 4000%. Twenty-three triglycerides were measured in hind limb muscle but no effect was observed after 40 days of high-fat diet. In contrast, in the heart muscle four types of TG increased dramatically after a 40 days fatty diet ( 54:2-TG (+394%), 54:3-TG (+452%), 56:3-TG (+297%) and 56:5-TG (+213%). Based on Principal Component Analysis with different lipid compounds like LPC, SPM, TG, PC and ChE, a separation can be made for high-fat diet fed animals and control diet fed animals both for hind limb muscle and heart muscle. After elimination of the TG and the ChE and a new PCA run we see that four groups can be clearly separated: Control-heart, Fat-heart, Control-hind limb muscle and Fat-hind limb muscle.

**Conclusions:** These analyses provide potential biomarkers for the diagnosis of diet-induced lipid accumulation in heart and hind-limb muscle tissue.

**Keywords**: Cardiac steatosis; Triacylglycerol's; Biomarker; Mice; Biochemistry; Lipid composition

## Introduction

Myocardial energy is predominantly derived from reducing equivalents generated by the mitochondrial ß oxidiation of FA. However, the uptake of FA by the heart is not always tightly controlled in relation to FA oxidation and *vice versa*, which may lead to excessive storage of TG in cardiac myocytes. The myocardial Triacylglycerols (TG) stores by themselves are not inert, and TG are continuously involved in hydrolysis-re-esterification cycles, yielding fatty acids, fatty acyl-CoA esters, diacylglycerol and ceramide as intermediates [1,2].

Increasing evidence exists that accumulation of these FA intermediates underlie cardiac dysfunction and cell damage by inducing inflammation, insulin resistance, mitochondrial dysfunction and apoptosis. A crucial role may be played by increased activity of inflammatory cascades through profound effects of FA intermediates on gene expression of inflammatory proteins, a mechanism which is comparable with lipotoxicity in steato-hepatitis [3-7]. Interestingly, an excessive susceptibility to ischemia due to TG accumulation is present in liver steatosis with a shift from an apoptotic form of cell death to necrosis [8].

Our initial hypothesis is that high fat feeding may lead to triglyceride accumulation in cardiac myocytes but not in hind limb muscle.

To determine the consequences of a high-fat diet on muscle lipid accumulation, we set out to measure the lipid content of hearts from mice exposed to a high-fat diet with High Performance thin layer chromatography (HPTLC) and make a comparison with hind limb muscle. In addition, we will use reversed phase Liquid Chromatography Coupled to Mass Spectrometry (LC-MS) to quantify and qualify the rearrangement and repartitioning of fat stores in the heart muscle of this mice model.

## Methods

### Experimental animals

Mice were housed in a temperature-controlled room (23°C) on a 10-hour dark/14-hour light cycle. Purebred male wild-type C57bl6 mice (age 8-12 weeks), obtained from Charles River (Maastricht, The Netherlands) were used. Animal experiments were approved by the animal experimentation committee of the Leiden University Medical Centre (The Netherlands).

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## Tissues

In this research the 'whole heart tissue' is referred to the cell suspension of all different types of cells and all vessels in the heart. Hind limb muscle was removed from the back leg below the gluteus. A tissue homogenate (10% wet weight/ vol) in PBS (phosphate-buffered saline) was made by stirring the tissue in a closed tube with small glass globules.

## Diet

Mice were fed a standard lab chow (RM3, Special Diet Services, and Witham, UK) containing about 12 energy percent fat. The fatty diet contained 24% protein, 39% carbohydrates, 24% fat, 6% fibers and 7% water (weight-percentages). Before the experiment started the animals were provided unrestricted amounts of food and water. During the experiment the Control mice were fed ad libitum. To standardize the metabolic rate of the Co-group they were fasted 4 hours before the start of the experiment.

## High Performance Thin Layer Chromatography (HPTLC)

Protein content of all samples was determined according to the Lowry assay [9]. Lipids were extracted according to Bligh and Dyer [10]. Briefly, a solution of 200 µg protein in 800 µl of MilliQ was mixed with 3 ml Methanol/Chloroform (2:1), after which 500 µl Chloroform, 100 µl Internal Standard and 1 ml MilliQ water was added. All were mixed and centrifuged for 10 minutes at 300 rpm. After the centrifugation the chloroform layer was collected and dried under nitrogen. The pellets were dissolved in 50 µl chloroform and transferred to a HPTLC plate for separation of TG, FC and CE [11]. The lipids were separated using High-Performance Thin-Layer Chromatography (HPTLC) on silica gel plates as described before [11] and subsequent analysis was performed by TINA2.09 software [12] (Rayest Isotopen Meβgeräte GmbH, Straubenhardt, Germany).

## Mass spectrometry

Fifty micro liter of the well mixed tissue homogenate (hind limb or heart muscle) was mixed with 1000 µl IPA containing 4 internal standards. Samples were placed in an ultrasonic bath for 5 minutes. Thereafter samples were centrifuged at 10000 rpm for 3 minutes followed by injection of 10 µl on the LC-MS Instrument (Thermo Electron, San Jose, USA). A Thermo LTQ is a linear ion-trap LC-MS instrument (Thermo Electron, San Jose, USA).

## Calculations and statistics

For all measured parameters given in Tables 1-12, the mean value of the control mice group was compared to the mean value of the starvation mice group. Statistics were performed via SAS (Statistical Analyzing Software) using a one-way ANOVA for differences between control and starvation groups. $P \leq 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov-Smirnov and $F_{max}$ tests, respectively. Principal Component Analysis (PCA) was carried out on the parameters of lipid metabolism measured via reversed phase liquid chromatography coupled to mass spectrometry. This type of analysis allows one to simultaneously examine the relative state of individuals according to three or more variables. We used Principal Component Analysis (PCA) statistical methods, which are specially developed, for application in

| Compound | Control (Mean ± SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **14:0-LPC** | 0.061 ± 0.011 | 0.071 ± 0.020 | P ≤ 0.261 | 117.23 % |
| **38:5-PC** | 1.091 ± 0.402 | 0.651 ± 0.425 | P ≤ 0.082 | 59.67 % |
| **52:2-TG** | 5.394 ± 3.824 | 3.958 ± 4.306 | P ≤ 0.537 | 73.38 % |

**denotes significant difference P ≤ 0.001

**Table 1:** Lipid compounds found only in hindlimb muscle determined with mass spectrometry: Co-group versus fatty group.

| Compound | Control (Mean ± SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **16:1-ChE** | 0.011 ± 0.005 | 0.063 ± 0.037 | P ≤ 0.009** | 576.96 % |
| **18:1-ChE** | 0.036 ± 0.007 | 0.140 ± 0.076 | P ≤ 0.011 | 384.34 % |
| **18:2-ChE** | 0.170 ± 0.096 | 0.306 ± 0.218 | P ≤ 0.171 | 179.98 % |
| **18:3-ChE** | 0.011 ± 0.006 | 0.033 ± 0.016 | P ≤ 0.011** | 307.76 % |
| **20:3-ChE** | 0.007 ± 0.005 | 0.097 ± 0.071 | P 0.016** | 1316.95 % |
| **20:4-ChE** | 0.068 ± 0.039 | 0.309 ± 0.238 | P ≤ 0.037* | 451.43 % |
| **20:5-ChE** | 0.026 ± 0.014 | 0.026 ± 0.018 | P ≤ 0.975 | 98.93 % |
| **22 :6-ChE** | 0.025 ± 0.013 | 0.082 ± 0.063 | P ≤ 0.054 | 329.83 % |
| **38 :3-PC** | 0.070 ± 0.029 | 0.294 ± 0.162 | P ≤ 0.010** | 421.13 % |
| **54:1-TG** | 0.064 ± 0.097 | 0.079 ± 0.049 | P ≤ 0.741 | 123.27 % |
| **56:6-TG** | 0.058 ± 0.039 | 0.077 ± 0.035 | P ≤ 0.395 | 131.69 % |

**: denotes significant difference P ≤ 0.001

**Table 2:** Lipid compounds found only in heart muscle determined with mass spectrometry: Co-group versus fatty group.

| Compound | Control Hindlimb Muscle (Mean ±SD) | Control Heart Muscle (Mean ± SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **16:0-LPC** | 1.377 ± 0.536 | 0.511 ± 0.345 | P ≤ 0.009** | 37.08 % |
| **16:1-LPC** | 0.098 ± 0.02 | 0.031 ± 0.008 | P ≤ 0.000*** | 31.55 % |
| **18:0-LPC** | 0.134 ± 0.051 | 0.353 ± 0.242 | P ≤ 0.078 | 262.86 % |
| **18:1-LPC** | 0.092 ± 0.023 | 0.076 ± 0.032 | P ≤ 0.339 | 82,52 % |
| **18:2-LPC** | 0.08 ± 0.024 | 0.128 ± 0.050 | P ≤ 0.071 | 159.34 % |
| **20:0-LPC** | 0.003 ± 0.001 | 0.008 ± 0.003 | P ≤ 0.009** | 251.02 % |
| **20:1-LPC** | 0.006 ± 0.001 | 0.010 ± 0.004 | P ≤ 0.073 | 172.76 % |

**: denotes significant difference P ≤ 0.001

**Table 3:** Lipid profiles of lysophosphatidyl-cholines (LPC) in the Co-group of hindlimb *versus* heart muscle tissue determined with mass spectrometry.

| Compound | Control Hindlimb Muscle (Mean ±SD) | Control Heart Muscle (Mean ± SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **32:0-PC** | 0.870 ± 0.156 | 0.824 ± 0.214 | P ≤ 0.676 | 94.63 % |
| **34:0-PC** | 0.071 ± 0.020 | 0.153 ± 0.042 | P ≤ 0.003** | 216.28 % |
| **34:1-PC** | 3.197 ± 0.784 | 1.458 ± 0.451 | P ≤ 0.002** | 45.60 % |
| **34:2-PC** | 3.195 ± 0.953 | 1.554 ± 0.603 | P ≤ 0.007** | 48.66 % |
| **34:3-PC** | 0.349 ± 0.116 | 0.058 ± 0.027 | P ≤ 0.001** | 16.76 % |
| **36:1-PC** | 0.218 ± 0.058 | 0.477 ± 0.154 | P ≤ 0.007** | 219.28 % |
| **36:2-PC** | 0.645 ± 0.204 | 0.957 ± 0.381 | P ≤ 0.116 | 148.51 % |
| **36:3-PC** | 0.881 ± 0.285 | 0.389 ± 0.183 | P ≤ 0.007** | 44.17 % |
| **36:4-PC** | 1.465 ± 0.503 | 0.377 ± 0.188 | P ≤ 0.002** | 25.71 % |
| **36:5-PC** | 0.167 ± 0.063 | 0.044 ± 0.018 | P ≤ 0.004** | 26.24 % |
| **38:2-PC** | 0.022 ± 0.008 | 0.049 ± 0.019 | P ≤ 0.013** | 225.96 % |
| **38:4-PC** | 0.192 ± 0.07 | 0.452 ± 0.220 | P ≤ 0.033** | 235.56 % |
| **38:6-PC** | 3.395 ± 1.311 | 1.781 ± 1.060 | P ≤ 0.042** | 52.46 % |
| **40:6-PC** | 0.352 ± 0.162 | 1.718 ± 1.025 | P ≤ 0.022** | 488.68 % |
| **40:7-PC** | 0.167 ± 0.084 | 0.101 ± 0.057 | P ≤ 0.152 | 60.82 % |

**: denotes significant difference P ≤ 0.001
***: denotes significant difference P ≤ 0.0001

**Table 4:** Lipid profiles of phosphatidylcholines (PC) in hindlimb muscle tissue determined with mass spectrometry.

| Compound | Control Hindlimb Muscle (Mean ±SD) | Control Heart Muscle (Mean ± SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **14:0-SPM** | 0.004 ± 0.002 | 0.002 ± 0.001 | P ≤ 0.475 | 114.53 % |
| **15:0-SPM** | 0.003 ± 0.001 | 0.005 ± 0.001 | P ≤ 0.037* | 152.54 % |
| **16:0-SPM** | 0.104 ± 0.024 | 0.144 ± 0.035 | P ≤ 0.050* | 138.03 % |
| **16:1-SPM** | 0.004 ± 0.002 | 0.007 ± 0.003 | P ≤ 0.050* | 171.67 % |
| **18:0-SPM** | 0.419 ± 0.096 | 0.080 ± 0.017 | P 0.0003*** | 19.00 % |
| **23:0-SPM** | 0.014 ± 0.004 | 0.041 ± 0.012 | P ≤ 0.002** | 290.16 % |
| **23:1-SPM** | 0.006 ± 0.004 | 0.023 ± 0.006 | P ≤ 0.0002*** | 357.88 % |

*: denotes significant difference P ≤ 0.05
**: denotes significant difference P ≤ 0.001

**Table 5:** Lipid profiles of sphingomyelin (SPM) in hindlimb muscle tissue determined with mass spectrometry.

| Compound | Control Hindlimb Muscle (Mean ±SD) | Control Heart Muscle (Mean ± SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **46:1-TG** | 0.018 ± 0.005 | 0.042 ± 0.041 | P ≤ 0.214 | 233.60 % |
| **46:2-TG** | 0.009 ± 0.002 | 0.018 ± 0.017 | P ≤ 0.237 | 204.85 % |
| **48:0-TG** | 0.047 ± 0.016 | 0.130 ± 0.114 | P ≤ 0.135 | 278.70 % |
| **48:1-TG** | 0.177 ± 0.094 | 0.255 ± 0.273 | P ≤ 0.532 | 144.22 % |
| **48:2-TG** | 0.125 ± 0.059 | 0.155 ± 0.170 | P ≤ 0.701 | 123.59 % |
| **48:3-TG** | 0.023 ± 0.007 | 0.034 ± 0.034 | P ≤ 0.455 | 150.63 % |
| **50:0-TG** | 0.104 ± 0.090 | 0.063 ± 0.047 | P ≤ 0.350 | 60.53 % |
| **50:1-TG** | 0.890 ± 0.565 | 0.632 ± 0.643 | P ≤ 0.477 | 71.00 % |
| **50:2-TG** | 1.543 ± 0.932 | 0.804 ± 0.849 | P ≤ 0.182 | 52.12 % |
| **50:3-TG** | 0.387 ± 0.145 | 0.322 ± 0.357 | P ≤ 0.694 | 83.29 % |
| **50:4-TG** | 0.044 ± 0.008 | 0.055 ± 0.057 | P ≤ 0.658 | 125.16 % |
| **52:1-TG** | 0.351 ± 0.218 | 0.296 ± 0.322 | P ≤ 0.737 | 84.30 % |
| **52:3-TG** | 2.511 ± 1.341 | 0.992 ± 0.993 | P ≤ 0.052 | 39.50 % |
| **52:4-TG** | 0.410 ± 0.059 | 0.419 ± 0.415 | P ≤ 0.960 | 102.20 % |
| **52:5-TG** | 0.063 ± 0.024 | 0.074 ± 0.072 | P ≤ 0.722 | 118.34 % |
| **54:2-TG** | 0.975 ± 0.711 | 0.240 ± 0.224 | P ≤ 0.052 | 24.57 % |
| **54:3-TG** | 2.732 ± 1.985 | 0.513 ± 0.498 | P ≤ 0.040** | 18.80 % |
| **54:4-TG** | 0.732 ± 0.407 | 0.470 ± 0.443 | P ≤ 0.310 | 64.13 % |
| **54:5-TG** | 0.228 ± 0.051 | 0.274 ± 0.232 | P ≤ 0.657 | 119.88 % |
| **56:2-TG** | 0.117 ± 0.094 | 0.021 ± 0.016 | P ≤ 0.053 | 17.71 % |
| **56:3-TG** | 0.524 ± 0.400 | 0.064 ± 0.047 | P ≤ 0.037** | 12.23 % |
| **56:4-TG** | 0.250 ± 0.175 | 0.065 ± 0.044 | P ≤ 0.048** | 25.98 % |
| **56:5-TG** | 0.187 ± 0.121 | 0.053 ± 0.038 | P ≤ 0.041 | 28.29 % |

**: denotes significant difference P ≤ 0.001

**Table 6:** Lipid profiles of Triacylglycerols (TG) in a Control group of hindlimb *versus* heart muscle tissue determined with mass spectrometry.

| Compound | Control (Mean ±SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| | | | | |
| **16:0-LPC** | 1.377 ± 0.536 | 0.893 ± 0.452 | P ≤ 0.112 | 65.0 % |
| **16:1-LPC** | 0.098 ± 0.02 | 0.082 ± 0.026 | P ≤ 0.232 | 83.5 % |
| **18:0-LPC** | 0.134 ± 0.051 | 0.128 ± 0.051 | P ≤ 0.833 | 95.4 % |
| **18:1-LPC** | 0.092 ± 0.023 | 0.119 ± 0.049 | P ≤ 0.230 | 129.0 % |
| **18:2-LPC** | 0.08 ± 0.024 | 0.031 ± 0.010 | P ≤ 0.002** | 38.1 % |
| **20:0-LPC** | 0.003 ± 0.001 | 0.002 ± 0.001 | P ≤ 0.073 | 63.8 % |
| **20:1-LPC** | 0.006 ± 0.001 | 0.005 ± 0.001 | P ≤ 0.310 | 86.5 % |

**: denotes significant difference P≤ 0.001

**Table 7:** Lipid profiles of lysophosphatidyl-cholines (LPC) in hindlimb muscle tissue determined with mass spectrometry.

| Compound | Control (Mean ±SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **16:0-LPC** | 0.511 ± 0.345 | 0.537 ± 0.152 | P ≤ 0.867 | 105.18 % |
| **16:1-LPC** | 0.031 ± 0.008 | 0.035 ± 0.010 | P ≤ 0.378 | 114.45 % |
| **18:0-LPC** | 0.353 ± 0.242 | 0.555 ± 0.179 | P ≤ 0.127 | 157.04 % |
| **18:1-LPC** | 0.076 ± 0.032 | 0.224 ± 0.084 | P ≤ 0.003** | 294.89 % |
| **18:2-LPC** | 0.128 ± 0.050 | 0.084 ± 0.037 | P ≤ 0.111 | 66.03 % |
| **20:0-LPC** | 0.008 ± 0.003 | 0.004 ± 0.001 | P ≤ 0.016** | 47.18 % |
| **20:1-LPC** | 0.010 ± 0.004 | 0.011 ± 0.004 | P ≤ 0.650 | 110.90 % |

**: denotes significant difference P ≤ 0.001

**Table 8:** Lipid profiles of lysophosphatidyl-cholines (LPC) in heart muscle tissue determined with mass spectrometry.

| Compound | Control (Mean ±SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **32:0-PC** | 0.870 ± 0.156 | 0.379 ± 0.164 | P ≤ 0.0002*** | 43.53 % |
| **34:0-PC** | 0.071 ± 0.020 | 0.056 ± 0.023 | P ≤ 0.259 | 79.85 % |
| **34:1-PC** | 3.197 ± 0.784 | 3.332 ± 1.652 | P ≤ 0.852 | 104.23 % |
| **34:2-PC** | 3.195 ± 0.953 | 1.377 ± 0.633 | P ≤ 0.004** | 43.11 % |
| **34:3-PC** | 0.349 ± 0.116 | 0.132 ± 0.074 | P ≤ 0.004** | 37.79 % |
| **36:1-PC** | 0.218 ± 0.058 | 0.426 ± 0.186 | P ≤ 0.025** | 195.67 % |
| **36:2-PC** | 0.645 ± 0.204 | 0.747 ± 0.347 | P ≤ 0.523 | 115.95 % |
| **36:3-PC** | 0.881 ± 0.285 | 1.077 ± 0.623 | P ≤ 0.475 | 122.28 % |
| **36:4-PC** | 1.465 ± 0.503 | 1.284 ± 0.788 | P ≤ 0.628 | 87.66 % |
| **36:5-PC** | 0.167 ± 0.063 | 0.120 ± 0.076 | P ≤ 0.249 | 71.95 % |
| **38:2-PC** | 0.022 ± 0.008 | 0.029 ± 0.016 | P ≤ 0.339 | 131.70 % |
| **38:4-PC** | 0.192 ± 0.07 | 0.242 ± 0.138 | P ≤ 0.418 | 126.28 % |
| **38:6-PC** | 3.395 ± 1.311 | 1.496 ± 1.017 | P ≤ 0.017** | 44.09 % |
| **40:6-PC** | 0.352 ± 0.162 | 0.253 ± 0.171 | P ≤ 0.308 | 71.85 % |
| **40:7-PC** | 0.167 ± 0.084 | 0.136 ± 0.098 | P ≤ 0.0563 | 81.88 % |

**: denotes significant difference P ≤ 0.001
***: denotes significant difference P ≤ 0.0001

**Table 9:** Lipid profiles of phosphatidylcholines (PC) in hindlimb muscle tissue determined with mass spectrometry.

| Compound | Control (Mean ±SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **32:0-PC** | 0.824 ± 0.214 | 0.457 ± 0.151 | P ≤ 0.007** | 55.47 % |
| **34:0-PC** | 0.153 ± 0.042 | 0.170 ± 0.063 | P ≤ 0.569 | 111.38 % |
| **34:1-PC** | 1.458 ± 0.451 | 3.465 ± 1.226 | P ≤ 0.004** | 237.68 % |
| **34:2-PC** | 1.554 ± 0.603 | 0.967 ± 0.516 | P ≤ 0.091 | 62.24 % |
| **34:3-PC** | 0.058 ± 0.027 | 0.052 ± 0.027 | P ≤ 0.694 | 89.57 % |
| **36:1-PC** | 0.477 ± 0.154 | 1.923 ± 0.725 | P ≤ 0.002** | 4002.83 % |
| **36:2-PC** | 0.957 ± 0.381 | 1.603 ± 0.731 | P ≤ 0.071 | 167.44 % |
| **36:3-PC** | 0.389 ± 0.183 | 0.893 ± 0.437 | P ≤ 0.023** | 229.49 % |
| **36:4-PC** | 0.377 ± 0.188 | 0.819 ± 0.367 | P ≤ 0.021 | 217.53 % |
| **36:5-PC** | 0.044 ± 0.018 | 0.044 ± 0.022 | P ≤ 0.999 | 99.97 % |
| **38:2-PC** | 0.049 ± 0.019 | 0.117 ± 0.049 | P ≤ 0.010** | 237.98 % |
| **38:4-PC** | 0.452 ± 0.220 | 1.280 ± 0.543 | P ≤ 0.006** | 283.04 % |
| **38:6-PC** | 1.781 ± 1.060 | 1.629 ± 0.733 | P ≤ 0.774 | 91.45 % |
| **40:6-PC** | 1.718 ± 1.025 | 1.770 ± 0.750 | P ≤ 0.920 | 103.02 % |
| **40:7-PC** | .0101 ± 0.057 | 0.260 ± 0.116 | P ≤ 0.011 | 256.51 % |

**: denotes significant difference P ≤ 0.001
***: denotes significant difference P ≤ 0.0001

**Table 10:** Lipid profiles of phosphatidylcholines (PC) in heart muscle tissue determined with mass spectrometry.

biomedical research [13,14] using TNO IMPRESS, EQUEST and WINLIN software.

Principal components analysis (PCA) is a technique used to reduce multidimensional data sets to lower dimensions for analysis. The

| Compound | Control (Mean ±SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **14:0-SPM** | 0.002 ± 0.001 | 0.002 ± 0.0001 | P ≤ 0.614 | 91.58 % |
| **15:0-SPM** | 0.003 ± 0.001 | 0.004 ± 0.002 | P ≤ 0.815 | 105.44 % |
| **16:0-SPM** | 0.104 ± 0.024 | 0.077 ± 0.020 | P ≤ 0.049* | 73.67 % |
| **16:1-SPM** | 0.004 ± 0.002 | 0.005 ± 0.003 | P ≤ 0.522 | 120.66 % |
| **18:0-SPM** | 0.419 ± 0.096 | 0.260 ± 0.116 | P ≤ 0.021** | 62.11 % |
| **23:0-SPM** | 0.014 ± 0.004 | 0.011 ± 0.003 | P ≤ 0.150 | 76.72 % |
| **23:1-SPM** | 0.006 ± 0.004 | 0.008 ± 0.004 | P ≤ 0.380 | 129.84 % |

*: denotes significant difference P ≤ 0.05
**: denotes significant difference P ≤ 0.001

**Table 11:** Lipid profiles of sphingomyelin (SPM) in hindlimb muscle tissue determined with mass spectrometry.

| Compound | Control (Mean ±SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **14:0-SPM** | 0.002 ± 0.001 | 0.004 ± 0.002 | P ≤ 0.040* | 180.99 % |
| **15:0-SPM** | 0.005 ± 0.001 | 0.006 ± 0.002 | P ≤ 0.400 | 118.86 % |
| **16:0-SPM** | 0.144 ± 0.035 | 0.138 ± 0.041 | P ≤ 0.782 | 95.82 % |
| **16:1-SPM** | 0.007 ± 0.003 | 0.010 ± 0.004 | P ≤ 0.138 | 143.01 % |
| **18:0-SPM** | 0.080 ± 0.017 | 0.155 ± 0.038 | P ≤ 0.001** | 194.54 % |
| **23:0-SPM** | 0.041 ± 0.012 | 0.028 ± 0.008 | P ≤ 0.061 | 69.96 % |
| **23:1-SPM** | 0.023 ± 0.006 | 0.026 ± 0.009 | P ≤ 0.570 | 110.27 % |

*: denotes significant difference P ≤ 0.05
**: denotes significant difference P ≤ 0.001

**Table 12:** Lipid profiles of sphingomyelin (SPM) in heart muscle tissue determined with mass spectrometry.

applications include exploratory data analysis data and for generating predictive models. PCA involves the computation of the eigenvalue decomposition or Singular value decomposition of a data set, usually after mean centering the data for each attribute. The results of a PCA are usually discussed in terms of scores and loadings. The score and loading vectors give a concise and simplified description of the variance present in the dataset.

A principal component is a linear combination of the original variables (lipid concentrations) and the magnitude of its eigenvalue is a measure of the explained variance. Typically only a few principal components are required to explain >90% of the total variance in the data. In other words PCA is a dimension reduction method, e.g. from >100 lipid attributes in the data to only a 4 principal components, which simplifies data visualization.

## Results

### HPTLC techniques

We observed a significant 3-fold increase of TG in heart tissue of mice after high-fat diet feeding for approximately 40 days (P ≤ 0.004). Also we observed under these conditions a significant increase of FC (P 0.024). Although there was a tendency for an accumulation of TG in the high-fat diet group in hind-limb muscle this observation was not significantly different (P ≤ 0.40). Furthermore no significant differences were observed for FC in hind limb muscle tissue between the animals from the high fat diet experiment and their control group (Figures 1-7).

### Comparison hearts versus hind limb muscle

In hind limb muscle (control and fat) of this mouse model (Table 1) several compounds were found which were not detected in heart muscle: One triacylglycerol (TG) (52:2-TG), one Lysophosphatidyl-Choline (C-14-0-LPC) and one phosphatidyl-choline (38:5-PC). The 52:2-TG and 38:5-PC both drop in the fatty diet group while the 14:0-LPC increases non significantly in the fatty group. In heart muscle (control and fat) of this mouse model (Table 2) several compounds were found which were not detected in hind limb muscle: One Phosphatidyl-Choline (PC) (38:3PC), mainly Cholesterol esters (16:1-ChE, 18:1-ChE, 18:2-ChE, 18:3-ChE, 20:3-ChE, 20:4-ChE, 20:5-ChE, 22:6-ChE) and two triglycerides (54:1-TG and 56:6-TG). Especially the Cholesterol esters rise significantly in the fatty heart with 20:3-ChE

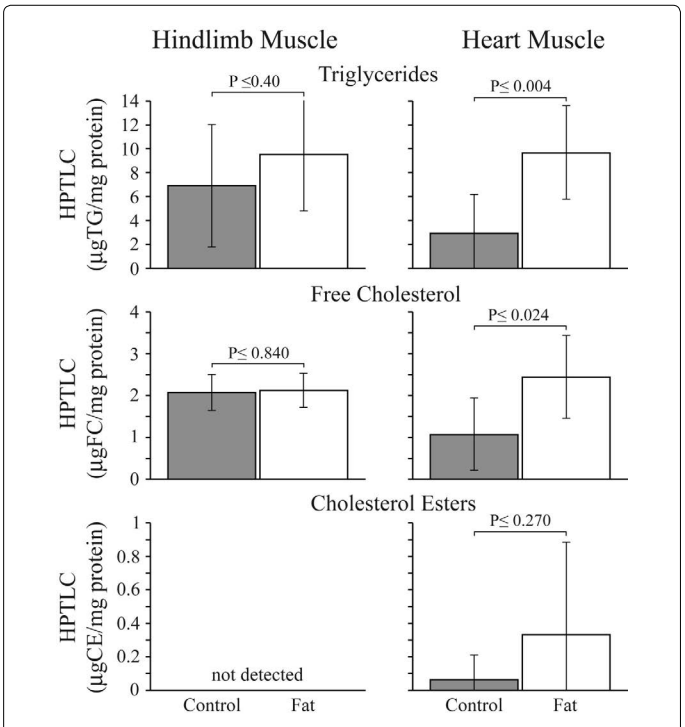


**Figure 1:** Lipid compounds detected with High Performance Thin Layer Chromatography (HPTLC) determined in hindlimb- and heart muscle homogenates of a Control (n=6) and a 24 hours starvation (n=6) mouse group and a Control (n=6) and a fat diet (n=7) mouse group. Mean ± SD is given. P ≤ 0.05 denotes significantly difference.

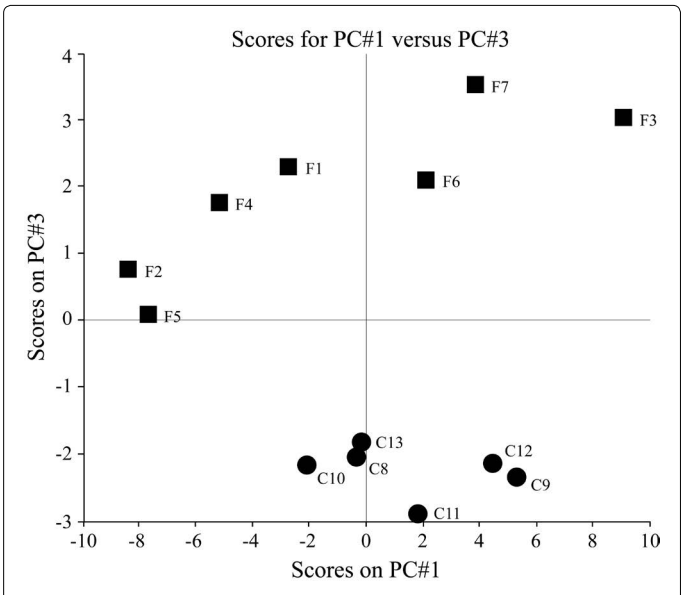


**Figure 2:** Principal component analysis on Hindlimb muscle of a control mice group [C] and a mice group exposed for 40 days to a fatty diet [ F].

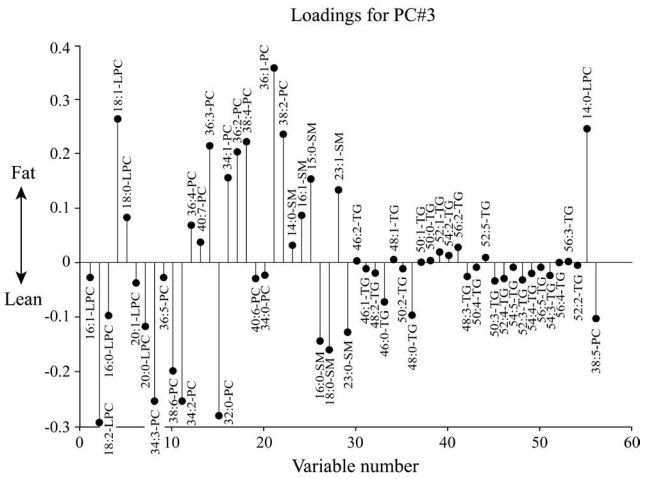


**Figure 3:** Factor spectrum: Top, chemical compounds in hindlimb muscle higher in the mice group exposed for 40 days to a fatty diet. Bottom, chemical compounds in hindlimb muscle higher in the mice control group.

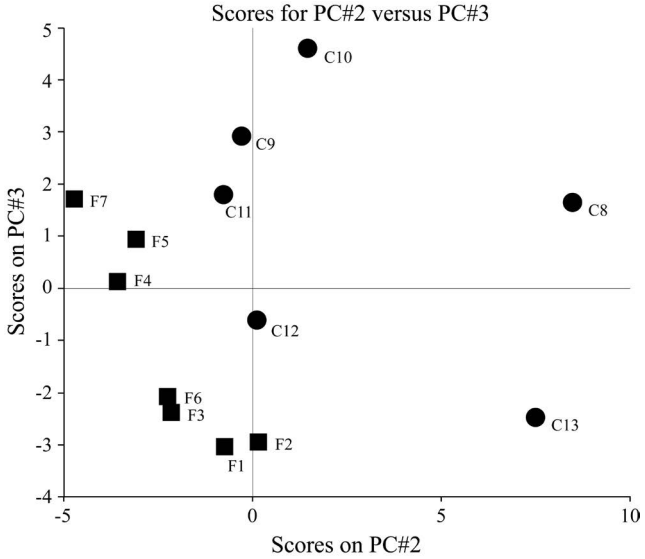


**Figure 4:** Principal component analysis on Heart muscle of a control mice group [C] and a mice group exposed for 40 days to a fatty diet [ F].

with 1317%. This compound if found in blood plasma can maybe be used as a biomarker.

## Comparison of the Control-heart group versus Control-hind limb muscle group

Two Lysophosphatidyl-Cholines (16:0-LPC, 16:1-LPC) were significantly lower in heart muscle in comparison to hindlimb muscle (Table 3). In contrast of this chemical group 20:0-LPC was significantly higher in heart muscle (Table 3). The phosphatidyl cholines also give not a uniform pattern. Some are significantly higher in the Control-heart muscle group (34:0-PC, 36:1-PC, 38:2-PC, 38:4-PC, and the PC with the highest level in Co-heart tissue was 40:6-PC with a change of 489% in comparison to Co-hind limb muscle (Table 4). The sphingomyelines give in general the highest values in Co-heart muscle (15:0-SPM, 16:0-SPM, 16:1-SPM, 23:0-SPM and 23:1-SPM) (Table 5). An exception is 18:0-SPM which is significantly lower in control-heart muscle (Table 5)

In the comparison Co-heart muscle *versus* Co-hind limb muscle there are in general no significant changes for the triglycerides. Only 54:3-TG, 56:3-TG, 56:4-TG and 56:6-TG are significantly higher in hind limb muscle (Table 6).

## Control versus fatty diet group for hindlimb muscle and heart muscle

In the comparison control versus fatty diet for hindlimb muscle there is only a significant decrease for 18:2-LPC in the fatty diet group (Table 7). For the lysophosphatidyl-cholines 18:1-LPC shows a strong significant increase in heart muscle with 295% and can possibly use as a biomarker if this observation is also the case in bloodplasma

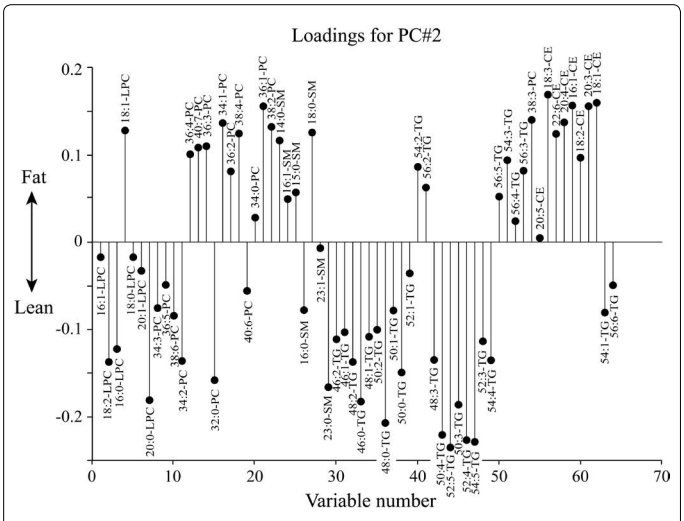


**Figure 5:** Factor spectrum: Top, chemical compounds in heart muscle higher in the mice group exposed for 40 days to a fatty diet. Bottom, chemical compounds in heart muscle higher in the mice control group.

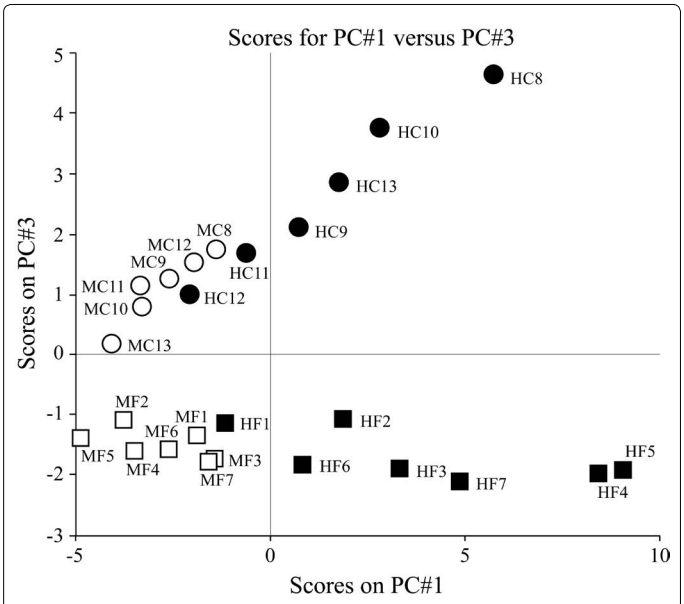


**Figure 6:** Principal component analysis on Heart (H) and Hindlimb muscle (M) of a control mice group [C] and a mice group exposed for 40 days to a fatty diet [ F]. Clearly visible is the separation between the two muscle groups and the Control and fatty animals. Triglycerides and Cholesterol Esters were eliminated from the data set.

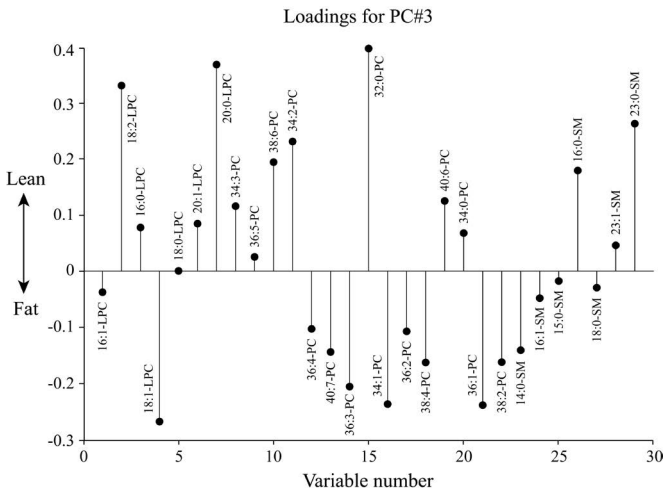


**Figure 7:** Factor spectrum: Top, chemical compounds in heart and hindlimb muscle higher in the mice group exposed for 40 days to a fatty diet. Bottom, chemical compounds in heart muscle and hindlimb muscle higher in the mice control group. Triacylglycerols and Cholesterol Esters were eliminated from the data set.

(Table 8). In contrast 20:0-LPC drops significantly (Table 8). The phosphatidylcholines (34:2-PC, 34:3-PC and 38:6-PC), in hindlimb muscle show a significant drop after a 40 days fatty diet while only 36:1-PC shows a significant increase (Table 9). In heart muscle five phosphatidylcholines (34:1-PC, 36:1-PC and 36:3-PC, 38:2-PC, 38:4-PC) show a significant increase in the fatty diet group (Table 10). The most remarkable observation is that 36:1-PC rises in heart muscle with 4000% (Table 10). This is possibly a candidate for a biomarker is this trend is also performed in blood plasma. In heart muscle only one phosphatidylcholine (32:0-PC) drops significantly in the fatty diet group (Table 10).

Of the seven Sphingomyelines (SPM) in hind limb muscle two (16:0-SPM, 18:0-SPM) decreased significantly after a 40 days fatty diet (Table 11) while two SPM increased in heart muscle (14:0-SPM, 18:0-SPM) in the 40 days fatty diet group (Table 12).

Twenty-three triglycerides were measured in hindlimb muscle of this mouse model. No significant changes were observed after a 40 days fatty diet in this muscle type (Table 13). In contrast in the heart muscle type four TG increased dramatically after a 40 days fatty diet (54:2-TG (+394%), 54:3-TG (+452%), 56:3-TG (+297%) and 56:5-TG (+213%) (Table 14). These are also possible candidates for biomarkers if the same tendency is observed in blood plasma.

## Principal component analysis

Principal Component Analysis (PCA) was carried out on a total of 56 measured compounds. In Figure 2 the score plot (PC1 and PC3) for hind limb muscle is shown. Principal components 1 and 3 accounted for 58% of the total variance. Clearly visible is that the fatty hind limb muscle is on the top part of the figure while the Control hind limb muscle is on the bottom of the figure. In Figure 4 the score plot (PC2 and PC3) for heart muscle is shown. Principal components 1 and 3 accounted for 30% of the total variance (64 compounds). Clearly visible is that the fatty heart muscle is situated on the left part of the graph while the Control heart muscle is on the right part of the figure. In Figure 6 the score plot (PC1 and PC3) for heart and hind limb muscle is shown. Principal component 1 and 3 accounted for 62% of the total variance (n=29 compounds).

After elimination of the TG and the ChE and a new PCA run we see that the groups are clearly separated. The Control Heart group animals are in the right-top part of the graph, the Fatty Heart group animals are in the right-bottom part of the graph. While for hind limb muscle the Control animals are in the left upper part and the Fatty animals in the left bottom part (Figure 6).

The loadings (factor spectra, Figures 3 and 5) of hind limb and heart muscle respectively show a number of corresponding compounds mainly lysophosphatidylcholines, sphingomyelines, and cholesterol esters which are tissue specific.

## Discussion

Some very important observations were made in this study. First, clearly tissue specific differences were observed between heart and hindlimb muscle in the Control groups. These consider mainly ChE (Table 2) LPC (Table 3), PC (Table 4) and SPM (Table 5). See also Figure 5 for visualization. In heart tissue cholesterol esters (ChE) were observed. Especially the C20-3-ChE is significantly increased in the fatty heart (Table 2).

Secondly in the comparison of both control groups the LPC (Table 3), PC (Table 4), SPM (Table 5), TG (Table 6) shows no general pattern. Sometimes these compounds were significantly higher or lower in hind limb- or heart muscle.

Third, this study clearly demonstrates that a fatty diet has an effect on heart tissue muscle but not on hind limb muscle (Figure 5). Especially Triacylglycerol's were increased in the fatty hearts. PC (Table 10) and TG (Table 14) rise significantly. Remarkable for the Tg

| Compound | Control (Mean ± SD) | 40 days fat diet (Mean ±SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **46:1-TG** | 0.018 ± 0.005 | 0.015 ± 0.009 | P ≤ 0.492 | 83.30 % |
| **46:2-TG** | 0.009 ± 0.002 | 0.008 ± 0.004 | P ≤ 0.525 | 87.89 % |
| **48:0-TG** | 0.047 ± 0.016 | 0.034 ± 0.019 | P ≤ 0.209 | 72.11 % |
| **48:1-TG** | 0.177 ± 0.094 | 0.141 ± 0.143 | P ≤ 0.602 | 79.87 % |
| **48:2-TG** | 0.125 ± 0.059 | 0.101 ± 0.078 | P ≤ 0.539 | 80.71 % |
| **48:3-TG** | 0.023 ± 0.007 | 0.021 ± 0.008 | P ≤ 0.642 | 91.57 % |
| **50:0-TG** | 0.104 ± 0.090 | 0.080 ± 0.098 | P ≤ 0.649 | 76.6 % |
| **50:1-TG** | 0.890 ± 0.565 | 0.677 ± 0.737 | P ≤ 0.568 | 76.10 % |
| **50:2-TG** | 1.543 ± 0.932 | 1.177 ± 1.154 | P ≤ 0.540 | 76.27 % |
| **50:3-TG** | 0.387 ± 0.145 | 0.337 ± 0.186 | P ≤ 0.600 | 87.14 % |
| **50:4-TG** | 0.044 ± 0.008 | 0.045 ± 0.014 | P ≤ 0.825 | 103.23 % |
| **52:1-TG** | 0.351 ± 0.218 | 0.285 ± 0.312 | P ≤ 0.665 | 81.22 % |
| **52:2-TG** | 5.394 ± 3.824 | 3.958 ± 4.306 | P ≤ 0.367 | 136 % |
| **52:3-TG** | 2.511 ± 1.341 | 1.954 ± 1.473 | P ≤ 0.491 | 77.82 % |
| **52:4-TG** | 0.410 ± 0.059 | 0.421 ± 0.125 | P ≤ 0.827 | 102.91 % |
| **52:5-TG** | 0.063 ± 0.024 | 0.078 ± 0.034 | P ≤ 0.367 | 123.87 % |
| **54:2-TG** | 0.975 ± 0.711 | 0.747 ± 0.876 | P ≤ 0.615 | 76.62 % |
| **54:3-TG** | 2.732 ± 1.985 | 1.829 ± 2.044 | P ≤ 0.437 | 66.96 % |
| **54:4-TG** | 0.732 ± 0.407 | 0.569 ± 0.427 | P ≤ 0.494 | 77.63 % |
| **54:5-TG** | 0.228 ± 0.051 | 0.231 ± 0.063 | P ≤ 0.940 | 101.06 % |
| **56:2-TG** | 0.117 ± 0.094 | 0.093 ± 0.106 | P ≤ 0.671 | 79.36 % |
| **56:3-TG** | 0.524 ± 0.400 | 0.377 ± 0.437 | P ≤ 0.541 | 71.99 % |
| **56:4-TG** | 0.250 ± 0.175 | 0.189 ± 0.169 | P ≤ 0.536 | 75.53 % |
| **56:5-TG** | 0.187 ± 0.121 | 0.145 ± 0.103 | P ≤ 0.513 | 77.30 % |

*: denotes significant difference P ≤ 0.05
**: denotes significant difference P ≤ 0.001
***: denotes significant difference P ≤ 0.0001

**Table 13:** Lipid profiles of Triacylglycerols (TG) in hindlimb muscle tissue determined with mass spectrometry.

| Compound | Control (Mean ±SD) | 40 days fat diet (Mean ± SD) | P-value | Change in (%) |
|---|---|---|---|---|
| **46:1-TG** | 0.042 ± 0.041 | 0.058 ± 0.021 | P ≤ 0.401 | 139.56 % |
| **46:2-TG** | 0.018 ± 0.017 | 0.026 ± 0.008 | P ≤ 0.367 | 139.69 % |
| **48:0-TG** | 0.130 ± 0.114 | 0.087 ± 0.028 | P ≤ 0.409 | 67.11 % |
| **48:1-TG** | 0.255 ± 0.273 | 0.347 ± 0.156 | P ≤ 0.488 | 136.08 % |
| **48:2-TG** | 0.155 ± 0.170 | 0.192 ± 0.077 | P ≤ 0.634 | 124.29 % |
| **48:3-TG** | 0.034 ± 0.034 | 0.042 ± 0.007 | P ≤ 0.586 | 124.20 % |
| **50:0-TG** | 0.063 ± 0.047 | 0.064 ± 0.020 | P ≤ 0.978 | 100.94 % |
| **50:1-TG** | 0.632 ± 0.643 | 0.954 ± 0.436 | P ≤ 0.327 | 151.00 % |
| **50:2-TG** | 0.804 ± 0.849 | 1.145 ± 0.516 | P ≤ 0.417 | 142.32 % |
| **50:3-TG** | 0.322 ± 0.357 | 0.280 ± 0.118 | P ≤ 0.790 | 86.83 % |
| **50:4-TG** | 0.055 ± 0.057 | 0.029 ± 0.011 | P ≤ 0.321 | 52.80 % |
| **52:1-TG** | 0.296 ± 0.322 | 0.506 ± 0.274 | P ≤ 0.239 | 170.87 % |
| **52:3-TG** | 0.992 ± 0.992 | 1.260 ± 0.573 | P ≤ 0.575 | 127.09 % |
| **52:4-TG** | 0.419 ± 0.415 | 0.174 ± 0.074 | P ≤ 0.211 | 41.52 % |
| **52:5-TG** | 0.074 ± 0.072 | 0.021 ± 0.008 | P ≤ 0.127 | 27.72 % |
| **54:2-TG** | 0.240 ± 0.224 | 0.945 ± 0.585 | P ≤ 0.019** | 394.38 % |
| **54:3-TG** | 0.513 ± 0.498 | 2.319 ± 1.289 | P ≤ 0.009** | 451.76 % |
| **54:4-TG** | 0.470 ± 0.443 | 0.484 ± 0.242 | P ≤ 0.946 | 103.02 % |
| **54:5-TG** | 0.274 ± 0.232 | 0.099 ± 0.043 | P ≤ 0.125 | 36.23 % |
| **56:2-TG** | 0.021 ± 0.016 | 0.055 ± 0.040 | P ≤ 0.074 | 263.12 % |
| **56:3-TG** | 0.064 ± 0.047 | 0.190 ± 0.121 | P ≤ 0.035** | 297.31 % |
| **56:4-TG** | 0.065 ± 0.044 | 0.114 ± 0.057 | P ≤ 0.107 | 175.25 % |
| **56:5-TG** | 0.053 ± 0.038 | 0.113 ± 0.053 | P ≤ 0.038** | 212.94 % |

*: denotes significant difference P ≤ 0.05
**: denotes significant difference P ≤ 0.001
***: denotes significant difference P ≤ 0.0001

**Table 14:** Lipid profiles of Triacylglycerols (TG) in heart muscle tissue determined with mass spectrometry.

is that this increase can only be ascribed to three Tg: 54:2-TG, 54:3-TG and 56:3-TG.

Fourth, a potential biomarkers was observed, a C36-1-PC which rises in the heart with 4000%. If this compound also rises in blood plasma it can be used as a biomarker for insulin resistance. Our first thought was that ceramide (which also contains 36 C atoms) could be associated with this tremendous rise in the heart muscle. In addition ceramides are often associated with insulin resistance in human muscle cells [15] and there even exists a kit for early detection of heart disease based on the detection of ceramide (United States Patent 6534322, [16]). But closer LC-MS investigation indicated that C36:1-PC was a phosphatidylcholine and no ceramide.

Type 2 Diabetes Mellitus (DM) is associated with an excess cardiac mortality [17]. In addition to macro- and microvascular cardiac disease, the etiology includes pathophysiological changes in multiple metabolic pathways which in the absence of coronary heart disease are ascribed to diabetic cardiomyopathy [18]. In normal conditions myocardial energy is predominantly derived from β-oxidation of fatty acids (FA). It is known that obesity and type-2 diabetes leads to an increase in circulating FA-concentration that is known to influence regulation of glucose by insulin but also ectopic fat storage. In this pathogenesis a mismatch exists between uptake and utilisation of FFA which may lead to intracellular storage of triacylglycerol's in adipocytes but also in liver, skeletal muscle and heart muscle. But not like in this study is demonstrated in hind limb muscle. In recent animal models of obesity and diabetes we observed increased cardiac myocyte triacylglycerol (TG) stores. Evidence is emerging that these myocardial lipid accumulations contribute to contractile dysfunction, cardiac arrhythmias and heart failure, involving necrotic and apoptotic pathways. As already mentioned the concept of myocardial lipotoxicity has arisen from animal studies.

Excessive accumulation of TG is found in hearts of obese *ob/ob* mice [19], as well as in obese Zucker Diabetic Fatty rats [20]. In the latter model of obesity, TG accumulation in cardiac myocytes was found to be directly related to cardiac dysfunction as characterized by an increase in the left ventricular end-diastolic diameter and a significant reduction in cardiac contraction [20].

Excessive accumulation of TG was also found in hearts of obese mice [21]. Furthermore insulin-resistant rats had a twofold increase in cardiac TG content compared with lean controls [22].

Increased TG stores were also found in cardiac myocytes of streptozotocin-induced diabetic rats [23] and cholesterol-fed hyperlipidemic rats [24]. Furthermore, it has been shown that TG preferentially accumulates in ischemic zones [25] and in the hypertrophic heart [26].

This could also be an indication for insulin resistance and/or diabetic mellitus type 2 as well as hyperinsulinemia in plasma. Thus, the mice from high-fat diet feeding could be insulin resistant and prone to TG accumulation in the myocardium. Data from our mice group (unpublished results) show a mean insulin level 0.25 ± 0.07 μg/l while our mice group exposed for 40 days to the fatty diet had a mean insulin level 1.09 ± 0.89 μg/l so they have become insensitive to insulin. The accumulation of the intracellular triacylglycerol (TG) is highly correlated with muscle insulin resistance [22].

However LC-MS techniques, as demonstrated in this study, show more in detail biochemical differences in lipid compounds (Lisophosphatidyl choline (LPC), Phosphatidyl choline (PC, Sphingomyelin (SPM), Cholesterol esters (ChE) and Triglycerides (TG) and unravel the molecular compounds in the myocytes. So far, little is known about the pathophysiolocal and molecular mechanisms underlying the association between TG accumulation in cardiac myocytes and cardiac dysfunction.

Subsequent hydrolysis of this expanded intracellular store of TG could eventually lead to high tissue FFA levels in the diabetic heart. These levels of FFA in tissue and blood may have adverse electrophysiological, biochemical and mechanical effects on the heart [27].

With high-fat diet feeding, high TG content in the heart has a negative effect on the hearts function, because elevated cardiac TG content had been associated with depressed contractility, arrhythmias, hypertrophy, heart failure and apoptosis [22]. Although FFA provide up to 75% of the hearts energy requirements in the resting state, excess FFA have detrimental effects on cardiac function and are more likely to develop arrhythmias and infarction [28].

It has also been suggested that FA may induce apoptotic cell death in myocytes through mechanisms that involve alterations in the phospholipid membrane [29].

In addition, recent studies have shown that fatty acids can initiate myocardial apoptosis through enhanced synthesis of ceramide, which subsequently may contribute to profound loss of cardiac function [20]. As earlier said the 4000% rise of the C36:1 PC found in this study can be ascribed to a phosphatidylcholine and not a ceramide. FA and their acyl-CoA derivatives may also have profound effects on gene expression profiles in cardiac myocytes through binding and activation of transcription factors like peroxisome proliferator-activated receptor α (PPARα) [26].

So far, little is known about the pathophysiolocal and molecular mechanisms underlying the association between TG accumulation in cardiac myocytes and cardiac dysfunction.

Accumulation of TG in cardiac myocytes results in the induction of molecular markers of apoptosis in Zucker Diabetic rats [20]. In these rats, treatment with thiazolidinediones reduces TG accumulation in cardiac myocytes considerably, associated with a reduction in apoptotic markers [20]. This induction of apoptosis by intracellular TG accumulation may be an important metabolic determinant for the cardiac reaction to ischemic injury, because border zones of overt histologically infarcted myocardium are characterized by apoptotic myocardium [30].

An important area in which metabolomics has great potential is the discovery of biomarkers related to a metabolic process like hepatic steatosis or a cardiac disease. Using LC-MS, molecular weight and retention time are useful analytical parameters to separate different compounds (for a detailed description of analytical and statistical techniques *vide* [13,14]. In this respect molecular mediators of hepatic steatosis and myocardium injury are important to study because they can serve as biomarkers to trace people vulnerable and susceptible for the pathogenesis of metabolic syndrome [31]. Recently we found in a mouse model after 24 hours of starvation in liver tissue a C49:4 triglycerides with an odd number of C atoms which could be used as a biomarker [32].

Recently B-type natriuretic peptide (BNP) and N-terminal pro B-type natriuretic peptide (NT-proBNP) have been successfully used to aid the diagnosis and monitor the management of heart failure [33]. These studies clearly demonstrate that the natriuretic peptides are released during ischemia [34-37]. As such BNP and NT-proBNP may have utility in assessing patients with acute coronary syndrome (ACS). However we still need a tool to diagnosis low-risk patients with stable coronary heart disease. The biomarkers from the lipid fraction we found in this study (36:1-PC, 54:2-TG, 54:3-TG, 56:3-TG and 56:5-TG) may be valuable for this group of patients.

In animal studies [38] demonstrated that TG measurement *in vivo* with H-1-MRS in the beating heart of a mouse model is possible.

Several authors like Szcecepaniak et al. [39] and McGavock et al. [40] have demonstrated using innovative 1H-MRS, that short-term increases in serum FA induce cardiac TG accumulation together with diastolic dysfunction, thus supporting the concept of lip toxicity in humans.

We propose two strategies to cope with the 'adiposity of the heart tissue' problem:

First, look for biomarkers in the blood which can predict if a person is vulnerable for TG accumulation in the myocardium. From this study the following chemical compounds were interesting candidates:

The cholesterol esters which were only observed in heart muscle. Especially the C20-3-ChE rise significantly in the fatty heart with 1317%.

From the phosphatidycholines the most remarkable observation is that C36-1-PC rises in heart muscle with 4000%.

In heart muscle type four TG increased dramatically after a 40 days fatty diet ( C54-2-TG (+394%), C54-3-TG (+452%), C56-3-TG (+297%) and C56-5-TG (+213%). These can be possible candidates for biomarkers if they can be detected in blood plasma. Furthermore if diet intervention would be possible it is an interesting hypothesis to eliminate these TG from food stuffs in order to prevent TG accumulation in the myocardium.

Furthermore it is recommended in future studies to investigate whether lip toxicity in patients with Diabetes-2 is amendable to therapeutic interventions, including diet and pharmacological interventions like oral antidiabetic drugs like sulfonylurea derivatives or metformin.

## Acknowledgements

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