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Current Vascular Pharmacology, **2017,** *15,* **000-000**

RESEARCH ARTICLE

Lipid Metabolism in Patients with End-Stage Renal Disease: A Five Year Follow-up Study

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Abstract: ***Background:*** Patients with end-stage renal disease (ESRD) exhibit high morbidity as well as mortality for atherosclerotic cardiovascular diseases (CVD). Therefore, we investigated differences in individual lipoprotein classes and subclasses in ESRD patients under chronic high volume hemodiafiltration (HV-HDF) in comparison with a control group. We also assessed the prognosis of these patients and analyzed these parameters after 5 years follow-up.

Methods: 57 patients and 50 controls were enrolled. We analysed high density (HDL) and low density (LDL) lipoprotein subfractions using the Quantimetrix Lipoprint[(R)] system. Subfractions were correlated with selected clinical-biochemical parameters including risk factors for atherosclerotic CVD at the beginning of and after 5 years follow-up.

Results: Fourteen patients survived the 5-year follow-up. Follow-up results revealed a shift toward smaller HDL subfractions. In lipoproteins carrying apolipoprotein B, there was a shift of cholesterol from very low density (VLDL) to intermediate density (IDL) lipoproteins and LDLs. Hypolipidaemic therapy did not influence lipoprotein profiles in HV-HDF patients.

Conclusions: **1.** HV-HDF patients exhibit specific lipid profiles with elevated triacylglycerol, low HDL and LDL and higher content of cholesterol in remnant particles (VLDL and IDL) at the expense of large LDL. HDL subfractions were linked to the number of risk factors for CVD in the control group only. **2.** Baseline lipoprotein profiles did not differ between survivors and non-survivors. Non-survivors had higher CRP and lower HDL-C. **3**. During the 5 year follow-up period, cholesterol in HDL particles and lipoproteins carrying apolipoprotein B redistributed in survivors towards smaller particles, thus resembling the profile of control patients.

ARTICLE HISTORY

Received: March 09, 2017
Revised: May 20, 2017
Accepted: May 20, 2017

DOI:
10.2174/1570161115666170530104143

Keywords: ESRD, dyslipidemia, lipoproteins, hemodiafiltration, survival.

INTRODUCTION

End-stage renal disease (ESRD), along with its many socioeconomic consequences, has become a major medical issue. In 2015, the number of patients in the Czech Republic with ESRD on chronic haemodialysis (HD) program reached 6200, representing a prevalence of 600 per million [1]. In Austria, the number reached 510 per million with prevalence of dialysis in the U.S. at 1500 per million [2]. The aetiology of renal failure is multifactorial. Three-year survival is estimated to be 75% with up to half of lethal complications caused by cardiovascular (CV) disease (CVD) complications [2].

One of the causative factors of CV complications in chronic kidney disease (CKD) is dyslipidaemia, first described by Tsaltas in 1968 [3]. However, the CV benefits of lipid-lowering therapy with statins in patients with CKD fades out with the progression of the disease [4] suggesting dyslipidaemia not being the primary risk factor for CV morbidity in ESRD patients, although diabetics on HD could benefit from rosuvastatin therapy [5]. The SHARP study showed that statins with ezetimibe could have an important role in primary prevention of CV events and mortality in patients with early CKD [6], but analyses of data from other studies focused on advanced stages of CKD, such as ESRD patients in AURORA [7] and 4D [8]

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studies did not prove significant effects of statin therapy on CV primary endpoints.

Patients with ESRD exhibit characteristic lipid profiles including higher content of cholesterol in very low density (VLDL) and intermediate density (IDL) lipoproteins. Higher concentrations of triacylglycerols (TAG) are connected with low concentrations of cholesterol in high density lipoproteins (HDL), while HDL particles are richer in TAG. Concentrations of cholesterol in low density lipoproteins (LDL) is normal/lower sometimes in connection with the higher prevalence of small dense LDLs (sdLDL), which are considered to have increased atherogenic potential and are connected with elevated proteinuria [9].

Increased plasma levels of lipoprotein(a) Lp(a) in high volume haemodiafiltration (HV-HDF) patients correlate with the parameters of chronic inflammation (high sensitivity C-reactive protein, interleukin 6) [10], which may provide context for the cholesterol paradox (the negative correlation of cardiovascular mortality with total plasma cholesterol) often seen in HV-HDF patients [11]. Chronic inflammation modifies lipoprotein particles both quantitatively and functionally. The modified HDL particles accumulate oxidized lipids, which leads to further inhibition of antioxidative enzymes localized in HDL, changes in the structure of apolipoprotein A-I (apoA-I) and also leads to mitigation or inactivation of principal HDL function (reverse cholesterol transport) [12]. The presence of dysfunctional HDL particles probably causes different relationship of cholesterol content and protein composition of HDL to CV events in diabetic HD patients [13]. Although the dyslipidaemic features of HV-HDF patients are known, the data on lipoprotein subfractions are scarce. Analysis of the HDL and LDL subfractions, which partly reflect lipoprotein function and metabolism, may shed some more light on the stratification of CV risk in HD patients as well as on the pathophysiological mechanisms underlining dyslipidaemia in ESRD.

The aim of the study was to investigate differences in individual lipoprotein classes and subclasses in patients undergoing chronic HV-HDF in patients with ESRD compared with a control group. Our analyses also covered parameters in those who survived *vs* those who did not survive the 5-year follow-up period. Subfractions and selected clinical/biochemical parameters were compared at the beginning of and 5 years after follow-up.

MATERIALS AND METHODS

Patients and Study Design

A total of 57 patients (35M/22F, aged 62.9 ± 12.7 years) with ESRD were enrolled. All patients were on dialysis [median 2 years (interquartile range: 1 month - 16 years)] as part of the HD programme run by the Dialysis Center Vinohrady Fresenius Medical Care in Prague, Czech Republic. All patients were of stable clinical status without signs of acute cardiac problems. Some of the patients were on hypolipidaemic therapy (30 on statins (atorvastatin), 2 on others (simvastatin)), three had anuria and 28 had type 2 DM (insulin analogues therapy in 13 cases). As a phosphate binder, Renagel® (sevelamer hydrochloride) was used in 13 patients. For the follow-up purposes, only 14 patients were eligible (36 did not survive – 31 due to CV events, and 5 due to oncologic comorbidity; 10 patients were transplanted). Of these, samples from 1 patient could not be analysed because consent was not given for further analyses. All patients were dialysed for 12-15 h/week and dialysis efficacy, expressed as the on-line clearance monitoring (OCM) parameter, reached values of 1.64 ± 0.33. No patient had statistically significant deviations from hydration status.

The exclusion criteria were as follows: serious endocrinopathy, pregnancy and breastfeeding, alcohol abuse and acute inflammatory disease. During the follow-up interval, none of the exclusion criteria emerged in those who survived. We did not exclude individuals on hypolipidaemic (e.g. statin) therapy from the study. According to current recommendations [14], it is suggested that statin treatment be administered to dialysis patients, which was the case in around the half of our HV-HDF group. Indeed, the history of dialysis treatment shows that the majority of the patients had an indication for statin therapy. Our study thus depicts the situation normally encountered with dialysis patients (see comments on the effect of statin therapy on lipoprotein subfraction profiles in Discussion).

The age- and sex- matched control group (32M/18F, age 61.6 ± 7.8 years) consisted of 50 inpatients of the Lipid Clinic at the 4th Department of Medicine, First Faculty of Medicine, Charles University, Prague. None of these patients had any signs of renal insufficiency {eGFR was calculated as follows: eGFR (ml/s/1.73m^2) = 186.3*[(serum creatinine (mmol/l))/88.4]exp(-1.154)*[(age)]exp(-0.203)*1/60*f, where f = 1 for men and f = 0.742 for women [15]}. Patients were treated with cholesterol-lowering drugs (9 on statins, 1 on others - fenofibrate), 20 had hypertension, 17 had type 2 DM (8 treated with metformin), and 3 had manifested CVD. Individuals remained in a stabilised (>1 year) metabolic state without changes in therapy. The patients were also characterised according to the number of conventional risk factors (RF) for CVD, such as: gender (males aged ≥55 years), presence of DM, hypertension, lipid disorder (dyslipidaemia) [LDL-C >1.8 mmol/l and/or TAG >1.7 mmol/l and/or HDL-C <1.0 mmol/l), smoking, and BMI (>25 kg/m^2)]. Stratification of intima/media thickness (IMT) status was carried out according to Belcaro [16] by merging together categories IA, IB, and II to produce 3 groups: normal/thickening wall (IA+IB+II); non-stenosing plaque (III); stenosing plaque (IV).

The study was approved by the Joint Ethics Committee of the First Medical Faculty, Charles University, and the General Faculty Hospital in Prague. All participants received detailed information about the research and gave their informed signed consent.

Methods

Blood samples were obtained after 12 h fasting before dialysis. Total cholesterol (TC), TAG, HDL-C, glucose and uric acid in plasma were assessed using enzymatic colourimetric methods. Other biochemical parameters were analysed using common enzymatic-colourimetric or nephelometric methods. Cholesterol in LDL+IDL particles

(LDL-C) was calculated according to the Friedewald formula [17]. The atherogenic index of plasma was calculated as log[TAG(mmol/l)/HDL-C(mmol/l)] [18] and the difference between non-HDL-C and LDL-C was used to estimate cholesterol in remnant particles.

All dialysed patients underwent the HD procedure on the 5008 CorDiax system **(**Fresenius Medical Care, Bad Homburg, Germany**).** Subfractions of LDL and HDL were analysed using high-performance discontinuous gel electrophoresis with polyacrylamide gel tubes (Lipoprint® LDL/HDL System, Quantimetrix, U.S.). ApoB-containing lipoproteins were separated into a VLDL fraction, 3 IDL (IDL-C, IDL-B, and IDL-A) subfractions, and 7 LDL subfractions (LDL1-LDL7). Subfractions LDL1 and LDL2 represented large (buoyant) particles, whereas LDL3-7 subfractions denoted small dense LDL (sdLDL). The HDL particles were separated using another kit into 10 subfractions: (HDL1-3) large, (HDL4-HDL7) intermediate and small (HDL8-HDL10).

Statistical Analysis

STATISTICA CZ ver.12 (StatSoft Inc., Tulsa, U.S.) software was used. Non-normally distributed variables (TAG, CRP, glucose) were transformed logarithmically or using arcsin (percentage values in HDL and LDL subfractions). The significance of differences between groups was assessed by t-test or ANOVA, within groups with paired t-tests with two-tailed alternative hypothesis.

RESULTS

HD and Control Groups

In Table **1**, a comparison of the haemodialysed (HV-HDF) group with the control (CON) group is presented. Both groups did not differ in basic anthropometric parameters, such as age, body mass or BMI. The dialysed group exhibited higher values of systolic and lower values of diastolic blood pressure (BP). The effect of the presence of DM within groups on studied parameters was negligible. Both groups had similar gender distribution for diabetics and non-diabetics (both $p>0.5$, χ^2 test with Yates' correction). Only concentrations of glucose were different but without no effect on lipoprotein distributions (*data not presented*). Also, DM therapy (metformin in the control group or insulin analogues in the HV-HDF group) did not influence lipoprotein subfraction profiles (data not presented). Although overall gender distribution in both groups was similar, we did observe some minor effects of gender on lipoprotein subfractions. Within the HV-HDF group, males tended to have lower content of large HDL subfractions (48 ± 12 *vs* 42 ± 9% of cholesterol in HDL, F *vs* M; $p = 0.04$). The efficacy of haemodialysis, expressed as the OCM parameter, was slightly higher in women (1.75 ± 0.40 *vs* 1.56 ± 0.23, $p = 0.04$). However, OCM did not correlate with the cholesterol percentage of HDL/LDL subfractions in men or women on HV-HDF (*data not presented*). In the CON group, men had higher content of cholesterol in VLDL (21 ± 8 *vs* 27 ± 5% of cholesterol, $p<0.001$) and sdLDL (1.2 ± 1.2 vs 2.9 ± 2.7% of cholesterol, $p = 0.02$). The effect of hypertension on the observed parameters was not significant.

Table 1. Basic clinical characteristics of the HV-HDF patients and CON groups.

	HV-HDF	CON
Number of participants	57	50
Males n (%)	35 (61%)	32 (64%)^{NS}
Age (years)	62.9 ± 12.7	61.6 ± 7.8^{a}
BMI (kg.m^{-2})	28.1 ± 6.3	29.3 ± 5.0
DM n (%)	28 (49%)	17 (34%)^{NS}
Sum of RF for CVD (0-4/5-7)	34/23	39/11*
Hypertension n%)	29 (51%)	37 (74%)*
CVD n (%)	37 (65%)	3 (6%)***
Dyslipidaemia n (%)	51 (89%)	49 (98%)^{NS}
Statin therapy n (%)	27 (47%)	13 (26%)*
Systolic BP (mmHg)	143 ± 22	135 ± 16*^{b}
Diastolic BP (mmHg)	70 ± 15	88 ± 11***
Optimal body weight (kg)	83 ± 21	86 ± 16
Urea (mmol/l)	17.8 ± 4.7	5.6 ± 1.4***
Creatinine (μmol/l)	681 ± 177	80 ± 18***
Uric acid (μmol/l)	316 ± 63	320 ± 83
Total protein (g/l)	67 ± 5	72 ± 4***
Albumin (g/l)	38 ± 3	46 ± 2***
CRP (mg/l)	4.1 (2.5-9.9)^{c}	2.5 (1.2-5.2)**
TC (mmol/l)	4.4 ± 1.0	5.1 ± 1.0***
TAG (mmol/l)	2.1 ± 1.1	1.5 ± 0.8**
HDL-C (mmol/l)	1.2 ± 0.4	1.4 ± 0.4*
LDL-C (mmol/l)	2.3 ± 0.8	3.1 ± 0.8***
non-HDL-C – LDL-C (mmol)	0.96 ± 0.42	0.66 ± 0.29***
Glucose (mmol/l)	7.5 ± 3.6	5.3 ± 0.8***
AIP (ratio)	0.23 ± 0.34	0.01 ± 0.27***
eGFR (ml/s)	0.13 ± 0.05	1.41 ± 0.25***
VLDL (% of cholesterol)	35.4 ± 5.6	24.8 ± 6.7***
total LDL (% of cholesterol)	39.5 ± 8.5	53.5 ± 7.2***
- IDL (IDL_{A-C})^{d} (% of cholesterol)	30.3 ± 4.6	25.3 ± 5.2***
- large LDL (LDL_{1-2}) (% of cholesterol)	7.5 ± 4.6	25.6 ± 6.2***
- small LDL (LDL_{3-7}) (% of cholesterol)	0.0 (0.0 - 2.4)^{c}	1.4 (0.7 - 3.7)
sum of HDL fractions (% of cholesterol)	24.4 ± 4.9	21.4 ± 6.4*
- large HDL (HDL_{1-3}) (% of HDL cholesterol)	44.5 ± 10.6	21.8 ± 6.9***
- intermediate HDL (HDL_{4-7}) (% of HDL cholesterol)	37.7 ± 7.0	43.1 ± 3.5***
- small HDL (HDL_{8-10}) (% of HDL cholesterol)	17.8 ± 5.3	35.4 ± 8.0***

Legends and abbreviations: AIP – atherogenic index of plasma (= log(TAG(mmol/l)/ HDL-C (mmol/l)), BMI – body mass index, BP – blood pressure, CON – control group, CRP – C-reactive protein, CVD – cardiovascular disease, DM – diabetes mellitus, eGFR – estimated glomerular filtration rate (ml/s), HDL – high density lipoprotein, HDL-C – high density lipoprotein cholesterol, HV-HDF – high volume haemodiafiltration, IDL – intermediate density lipoproteins, LDL – low density lipoproteins, LDL-C –low density lipoprotein cholesterol, RF – risk factor, TAG – triacylglycerols, TC – total cholesterol, VLDL – very low density lipoproteins; ^{a} – data are in average ± S.D. format, ^{b} – *p < 0.05, **p < 0.01, *** p < 0.001 (unpaired t-test), ^{NS} – not significant (χ2 test with Yates correction); ^{c} – median (Q1-Q4), ^{d} - subscripts denote the respective fraction of the lipoprotein class determined using the Lipoprint kit.

Dialysed patients had higher urea, creatinine, glucose and CRP concentrations. Lower total cholesterol in the HV-HDF group, was caused by both lower LDL-C and HDL-C concentrations, whereas TAG levels were higher in the HV-HDF group. Lipoprotein subfraction profiles are presented in Table **1**. We observed significant differences between the HV-HDF and CON groups for apoB-containing lipoprotein particles as well as HDL subfractions. The HV-HDF group had higher cholesterol content in VLDL and IDL particles at the expense of lower content in large LDL particles. Differences in sdLDL subfractions did not reach statistical significance. The distribution of HDL subfractions was also different: the HV-HDF patients had skewed distribution of HDL cholesterol towards large HDL subfractions. In contrast, cholesterol content in medium HDL and in small HDL particles was lower for the HV-HDF group.

Baseline Characteristics of Survivors

After 5 years, only 14 out of 47 patients (excluding those having undergone renal transplantation) survived, which is comparable with the respective data for the Czech Republic [1]. The survivors were characterised (see Table **2**) by higher diastolic BP, lower concentrations of CRP and glucose. Lipid parameters differed only in concentrations of HDL-C and lipoprotein distribution did not reveal significant differences. The age and distribution of females in both groups did not differ. Also, the length of the haemodialysis programme before baseline enrolment was similar for both groups. Our group of survivors was not characterised with more favourable stratified IMT status than non-survivors (Table **2**).

Follow-up Analysis

We analysed 13 paired data points in the survivors´ group. During the follow-up period, we observed (Table **3**) a decrease in magnesium and a decline in phosphorus concentrations. We did not observe trends in urea, creatinine or uric acid concentrations. However, concentrations of β_2-microglobulin increased during follow-up. The decrease in total protein was not accompanied by changes in albumin. Concentrations of CRP increased during the 5-year follow-up period. Changes in lipid parameters involved the lowering of LDL-C. The decreasing trends in TC and TAG concentrations did not prove significant, as was also the case for the AIP parameter. Nevertheless, lipoprotein subfractions profiles were subjects to many changes. We observed significant trends for apoB-containing lipoprotein particles and HDL subfractions. After the follow-up, we found lower content of cholesterol in VLDL and higher fractions of LDL particles, caused by lower content of cholesterol both in large LDL and sdLDL particles. The distribution of HDL subfractions was also different: after the 5-year follow-up, the distribution of HDL cholesterol in HV-HDF patients changed towards smaller and intermediate HDL subfractions. This came at the expense of the large HDL particles, which carried lower cholesterol content.

DISCUSSION

Comparison of the HV-HDF vs the Control Group

The biochemical parameters analysed reveal specific differences for HV-HDF patients including lipid profiles with

Table 2. Comparison of survivors and non-survivors at baseline.

	Survivors	Non-survivors
Number of patients (M/F)	14 (6/8)	33 (22/11)^{NS}
Age (years)	58.7 ± 12.1	65.6 ± 12.4^{a}
BMI (kg.m^{-2})	28.2 ± 6.9	28.3 ± 6.4
DM (n (%))	4 (29)	20 (61)^{NS}
IMT stratification (IA+IB+II/III/IV)	8/5/1	14/12/7^{NS}
Systolic BP (mmHg)	140 ± 20	145 ± 21^{b}
Diastolic BP (mmHg)	76 ± 9	65 ± 14***
Optimal body weight (kg)	85.1 ± 28.9	84.5 ± 18.0
Ca (mmol/l)	2.38 ± 0.28	2.20 ± 0.12*
P inorganic (mmol/l)	1.88 ± 0.48	1.69 ± 0.58
Mg (mmol/l)	1.07 ± 0.19	1.02 ± 0.34
Urea (mmol/l)	16.4 ± 3.9	18.3 ± 5.4
Creatinine (μmol/l)	684 ± 155	660 ± 192
Uric acid (μmol/l)	310 ± 65	322 ± 67
Total protein (g/l)	667 ± 6	67 ± 5
Albumin (g/l)	39 ± 3	38 ± 3
Albumin < 35 g/l n(%)	2(15)	8(24)^{NS}
CRP (mg/l)	3.3 (1.7-4.0)^{c}	7.5 (3.3-15.1)***
TC (mmol/l)	4.9 ± 0.8	4.3 ± 1.1
non-HDL-C – LDL-C (mmol)	0.9 ± 0.6	1.0 ± 0.4
TAG (mmol/l)	2.3 ± 2.2	1.4 ± 1.0
HDL-C (mmol/l)	1.3 ± 0.4	1.0 ± 0.4*
LDL-C (mmol/l)	2.6 ± 0.6	2.3 ± 0.9
Glucose (mmol/l)	5.1 (4.4-6.2)^{c}	7.6 (5.6-10.8)*
AIP (ratio)	0.20 ± 0.31	0.32 ± 0.32
eGFR (ml/s)	0.11 ± 0.13	0.03 ± 0.07
Dialysis time (h/week)	14.04 ± 0.73	13.86 ± 0.73
OCM (ratio)	1.73 ± 0.41	1.59 ± 0.30
HV-HDF init (years)	2.1 (0.5-6.2)^{c}	2.4 (1.4-2.9)
VLDL (% of cholesterol)	35.1 ± 5.2	35.0 ± 5.9
total LDL (% of cholesterol)	39.5 ± 4.5	40.0 ± 2.5
- IDL (IDL_{A-C})^{d} (% of cholesterol)	31.2 ± 5.2	30.2 ± 4.5
- large LDL (LDL_{1-2}) (% of cholesterol)	7.0 ± 4.2	7.8 ± 4.4
- small LDL (LDL_{3-7}) (% of cholesterol)	0.0 (0.0 – 1.9)^{c}	0.6 (0.0 – 2.9)
sum of HDL fractions (% of cholesterol)	24.9 ± 4.7	24.2 ± 5.0
- large HDL (HDL_{1-3}) (% of HDL cholesterol)	44.5 ± 10.0	44.2 ± 9.2
- intermediate HDL (HDL_{4-7}) (% of HDL cholesterol)	37.4 ± 6.5	37.7 ± 4.9
- small HDL (HDL_{8-10}) (% of HDL cholesterol)	18.0 ± 5.3	18.0 ± 5.4

Abbreviations: AIP – atherogenic index of plasma (= log(TAG(mmol/l)/HDL-C(mmol/l)), BMI – body mass index, BP – blood pressure, CRP – C-reactive protein, DM – diabetes mellitus, eGFR – estimated glomerular filtration rate (ml/s), HDL – high density lipoproteins; HDL-C – high density lipoprotein cholesterol, HV-HDF init – duration of HV-HDF before study enrollment, IDL – intermediate density lipoproteins, IMT – intima/media thickness, LDL-C –low density lipoprotein cholesterol, OCM – on-line clearance monitoring parameter, TAG – triacylglycerols, VLDL – very low density lipoproteins; ^{a} – data are in average ± SD format, ^{b} – *p < 0.05, **p < 0.01, *** p < 0.001 (unpaired t-test), ^{c} – median (Q1-Q4), ^{d} - subscripts denote the respective fraction of the lipoprotein class determined using the Lipoprint kit; ^{NS} – not significant (χ^2 test with Yates correction)

Table 3. Characteristic of survivors at the baseline and follow-up.

	Baseline HV-HDF (n = 13)	5 year follow-up (n = 13)
BMI ($kg.m^{-2}$)	28.1 ± 7.1	27.6 ± 8.3
Systolic BP (mmHg)	143.8 ± 19.6	136.2 ± 22.1[b]
Diastolic BP (mmHg)	74.0 ± 9.6	65.6 ± 16.4
Total calcium (mmol/l)	2.41 ± 0.29	2.22 ± 0.24
Inorganic phosphorus (mmol/l)	1.98 ± 0.46	1.56 ± 0.55*
Magnesium (mmol/l)	1.08 ± 0.21	0.86 ± 0.24***
Urea (mmol/l)	16.8 ± 4.1	16.6 ± 4.7
Creatinine (μmol/l)	749 ± 146	822 ± 181
Uric acid (μmol/l)	309 ± 50	260 ± 107
Total protein (g/l)	67.2 ± 6.1	62.2 ± 4.8*
Albumin (g/l)	38.2 ± 2.9	38.0 ± 4.2
CRP (mg/l)	3.3 (1.7-4.0)[c]	9.1 (6.0-13.9)*
TC (mmol/l)	4.8 ± 1.0	4.2 ± 0.7
non-HDL-C – LDL-C (mmol)	1.0 ± 0.5	0.9 ± 0.5
TAG (mmol/l)	2.2 ± 1.5	1.9 ± 1.1
HDL-C (mmol/l)	1.3 ± 0.4	1.3 ± 0.4
LDL-C (mmol/l)	2.5 ± 0.6	2.1 ± 0.7*
Glucose (mmol/l)	5.0 (4.6-6.2)	5.4 (4.7-6.1)
AIP (ratio)	0.18 ± 0.35	0.13 ± 0.35
eGFR (ml/s)	0.11 ± 0.02	0.09 ± 0.03
Dialysis time (h/week)	14.37 ± 1.00	13.74 ± 0.73
OCM (ratio)	1.62 ± 0.31	1.83 ± 0.31*
β_2-microglobulin (mg/l)	16.4 ± 2.4	21.2 ± 2.5***
VLDL (% of cholesterol)	33.9 ± 6.5 [a]	18.4 ± 7.2**
total LDL (% of cholesterol)	42.8 ± 7.1	61.7 ± 10.7**
- IDL (IDL_{A-C}) [d] (% of cholesterol)	32.7 ± 6.8	37.3 ± 11.5
- large LDL (LDL_{1-2}) (% of cholesterol)	6.9 ± 3.9	16.2 ± 7.3**
- small LDL (LDL_{3-7}) (% of cholesterol)	0.0 (0.0 - 2.7)[c]	4.1 (0.0 - 17)*
sum of HDL fractions (% of cholesterol)	24.1 ± 5.2	20.7 ± 5.8*
- large HDL (HDL_{1-3}) (% of HDL cholesterol)	46.5 ± 8.8	29.7 ± 8.6***
- intermediate HDL (HDL_{4-7}) (% of HDL cholesterol)	36.4 ± 6.5	44.9 ± 4.5**
- small HDL (HDL_{8-10}) (% of HDL cholesterol)	17.2 ± 4.0	25.4 ± 11.2*

Abbreviations: AIP – atherogenic index of plasma (= log(TAG(mmol/l)/HDL-C(mmol/l)), BMI – body mass index, BP – blood pressure, CRP – C-reactive protein, DM – diabetes mellitus, eGFR – estimated glomerular filtration rate (ml/s); HDL – high density lipoproteins; HDL-C – high density lipoprotein cholesterol, HV-HDF – high volume haemodiafiltration, IDL – intermediate density lipoproteins, IMT – intima/media thickness, LDL – low density lipoprotein; LDL-C – low density lipoprotein cholesterol, OCM – on-line clearance monitoring parameter, TAG – triacylglycerols, VLDL – very low density lipoproteins; [a] – data are in average ± S.D. format, [b] – *p < 0.05, **p < 0.01, *** p < 0.001 (paired t-test), [c] – median (Q1-Q4), [d] - subscripts denote the respective fraction of the lipoprotein class determined using the Lipoprint kit.

low HDL-C, LDL-C, TC and increased TAG. These changes are usually accompanied by different lipoprotein profiles resulting from impaired clearance and dysfunction and tend to favour subfractions leading to the accumulation of oxidatively modified lipids [19]. Fractionation of lipoprotein subclasses has become possible thanks to various methods, including ultracentrifugation, nuclear magnetic resonance (NMR), gel electrophoresis and high pressure liquid chromatography (HPLC) [20]. For CVD risk evaluation, lipoprotein profile analysis has gained increasing popularity. However, the data on lipoprotein profiles in HV-HDF patients using Lipoprint® method are only emerging [21, 22]. Our group of HV-HDF patients revealed changes similar to the results of these studies that compare HV-HDF patients with controls without statin treatment and signs of CVD. The individuals in our control group had almost no CVD complications (only 3 out of 50). CVD progression in HV-HDF patients is linked to high content of VLDL and IDL particles [23], which is probably caused by decreased catabolism of these particles due to low activity of lipoprotein lipase (LPL) and/or decreased uptake of remnant particles by liver LDL receptors and LDL receptor-related proteins [24]. The lipoprotein pattern in our HV-HDF patients followed the above findings, *i.e.* higher content of cholesterol in VLDL, IDL particles at the expense of large, buoyant LDL particles.

The profile of HDL particles in HV-HDF patients changed for all 3 principal subclasses, but still favoured larger particles. Debate continues with regard to the potential of various HDL subclasses to form a link to CVD, with large HDL being considered atheroprotective [25]. On the other hand, it has been found that small HDL are more efficient acceptors of cholesterol than large, lipid-rich, HDL. Therefore, an important attribute of the HDL particle is also its function, not only its size [26]. Nevertheless, small HDL have been shown to be independent predictors for lethal outcomes in haemodialysis patients [27]. In our patients, the link between the number of conventional RF for CVD and HDL subclasses was observed only in the control group (negative with large HDL content, $r = -0.396$, $p<0.01$, Spearman´s rank order correlation coefficient, and positive with small HDL content, $r = 0.348$, $p<0.05$), whereas we did not observe any respective correlations within the HV-HDF group. The distribution of the sum of RF for CVD was skewed toward higher values (>4) in the HV-HDF group in comparison with the control group. This finding is in accordance with the results of Li *et al*. [28], who observed fading out of the link between the number of traditional RF for CVD and large HDL in groups with an RF number >4.

Statin Therapy and HV-HDF

It is generally agreed that hypolipidaemic treatment influences lipoprotein metabolism. Although these effects are not usually pronounced both in LDL and HDL classes [29], in HV-HDF patients, the issue becomes more complicated. In CKD without HD, stabilisation of lipid profiles during long-term hypolipidaemic treatment is recommended [30], but statin therapy is recommended only in hypercholesterolaemic HD patients [31]. Notably, van den Akker *et al*. [32] failed to find changes in LDL subclasses separated by ultracentrifugation after the treatment with atorvastatin in HD patients. In our previous work regarding hypertriacylglycero-

laemic patients, we did not observe any differences in apoB lipoprotein distribution according to statin treatment [33] both at baseline and after supplementation with polyunsaturated fatty acids of n-3 family (PUFAn-3), which lowered TAG levels. Sorensen *et al.* came to the same conclusion [34] for a group of ESRD patients. Moreover, the effects of statins on sdLDL in CKD patients have recently been evaluated as negligent [35]. In the present study, ANOVA analysis revealed that the effect of the presence of statin treatment is far less potent than the group effect, as the highest observed effects of statin treatment were for large HDL and VLDL subfractions, both reaching only $p<0.05$ compared with the group effect ($p<0.0001$). Since we did not analyse the lipoprotein profiles before the beginning of the statin therapy, we cannot precisely describe the effect of statins on lipoprotein profiles.

Survivors vs Non-Survivors

The non-survivor group had an insignificantly higher ratio of patients with DM (χ^2 with Yates correction, $p = 0.1$), which may led to higher concentrations of glucose in this group and provide context for the higher burden of chronic inflammation in non-survivors, given that CRP concentrations in these patients were higher. The higher diastolic BP observed in non-survivors comply with the established U-shape curve for an association between BP and mortality risk in dialysis patients [36], which, in low-value regions, is caused by heart failure. Except for lower HDL-C in non-survivors, the lipid profiles did not distinguish between survivors and non-survivors. Survival for HV-HDF patients is connected with better nutritional status. Importantly, both groups did not differ in lipoprotein profiles at the baseline. In one 36-month follow-up study, no differences were observed in HDL profiles between the survivors and non-survivors [27].

Follow-up

According to our knowledge, there are no data on the trends in lipoprotein profiles for HD programmes. During the follow-up, lipid profiles did not change except for a decrease in LDL-C; even the values of derived AIP values did not change. Therefore, this parameter lacks distinguishing potential for survival in our HV-HDF patients. It is tempting to speculate that those who survived for more than a median of 2 years on the HV-HDF programme and 5 years of follow-up, were also able to respond to CVD risk factors by rearranging their lipoprotein profiles. This rearrangement of apoB lipoprotein cholesterol from VLDL to LDL particles together with absolute decrease in LDL-C implies that TAG-rich particles were remodeled, even though overall TAG levels did not change. ApoB concentrations were not available for both data points, hence no definitive conclusions could be made regarding the absolute number of apoB lipoprotein particles. Some authors even proposed that sdLDL are not associated with CVD in chronic HV-HDF patients [37]. Some studies indicate that HDL is more important than LDL in the progression of atherosclerosis in CKD patients [38]. During the follow-up phase we observed a lowering of cholesterol content from large HDL (which some consider to be important players in atherosclerosis progression [39]) to medium HDL and small HDL, which are also supposed to have CVD potential [27]. This phenomenon may be part of the complex responses to the metabolism of HDL particles during long-term HD. During CVD pathogenesis, changes in the HDL lipoprotein function/proteome are possible [40]. During the follow-up period, we did not detect progression of CVD and no changes to hyperlipidaemic treatment were necessary. Changes to the parameters that influence the development of CVD, such as plasma Ca, parathyroid hormone and thyroid stimulating hormone [41] were negligible and insignificant. Increased concentration of β_2-microglobulin during the follow-up implies the gradual worsening of residual kidney function (secondary amyloidosis linked to ageing and the HD process). Nevertheless, in our HV-HDF group, this protein did not show any link to lipoprotein subfractions (data not presented). The follow-up interval in this study was 5 years, which is longer than the median duration of the HD programme before baseline enrolment (2.0 years).

This retrospective study is the first follow-up study of HD that focuses on lipoprotein subfractions of HDL and apoB lipoproteins measured using the Lipoprint® system in a homogeneous group of HV-HDF patients. The limitations of the study include the absence of patients in CKD stages 4 and 5 in the control group, the low number of survivors not allowing general conclusions on lipid metabolism and the lack of elucidation on probable trends due to one point follow-up. Due to the small numbers in studied groups, we were not able to prove some differences (though big) to be statistically significant**.** Also, we did not analyse the proteins involved in the metabolism of lipoproteins, such as structural apolipoproteins apoB-100 and B-48, or the activities of enzymes that modify the lipoprotein content (lecithin:cholesterol acyl transferase, lipoprotein lipase, cholesteryl ester transferring protein and hepatic lipase).

In summary, the changes we observed in lipoprotein profiles during follow-up showed signs of convergence to the lipoprotein phenotype in the control group. Either this is a common situation in patients surviving long-time HD, which prevails the progression at least in some aspects of cardiovascular disease, and/or it is the as-not-yet-unknown physical effect of long-term haemodialysis procedures on lipoprotein particles. The problematic pertaining to lipoprotein subfractions during haemodialysis undoubtedly warrant further attention.

CONCLUSIONS

1. HD patients exhibited specific lipid profile accompanied by redistribution of apoB lipoproteins into VLDL and IDL particles and high content of cholesterol in large HDL. HDL subfractions were linked to the number of risk factors for CVD in the control group only.
2. Baseline lipoprotein profiles did not differ between survivors and non-survivors with the exception of HDL-C and were not influenced by intima-media thickness.
3. During the follow-up period in survivors, cholesterol in HDL particles was redistributed towards smaller particles, thus resembling the profiles of control patients. Changes in apoB lipoproteins followed a similar pattern of cholesterol rearrangement from VLDL into LDL par-

ticles. Hypolipidaemic therapy did not influence lipoprotein profiles in these HD patients.

LIST OF ABBREVIATIONS

AIP	=	Atherosclerotic index of plasma
apoA-I	=	Apolipoprotein A-I
apoB	=	Apolipoprotein B
BP	=	Blood pressure
CKD	=	Chronic kidney disease
CON	=	Control group
CV	=	Cardiovascular
CVD	=	Cardiovascular diseases
DM	=	Diabetes mellitus
ESRD	=	End-stage renal disease
HD	=	Haemodialysis
HDL	=	High density lipoproteins
HDL-C	=	Cholesterol in high density lipoproteins
HPLC	=	High pressure liquid chromatography
HV-HDF	=	High volume haemodiafiltration
IDL	=	Intermediate density lipoproteins
IMT	=	Intima/media thickness
LDL	=	Low density lipoproteins
LDL-C	=	Cholesterol in low density lipoproteins
LPL	=	Lipoprotein lipase
Lp(a)	=	Lipoprotein(a)
NMR	=	Nuclear magnetic resonance
OCM	=	On-line clearance monitoring
PUFA	=	Polyunsaturated fatty acids
sdLDL	=	Small dense low density lipoprotein
TAG	=	Triacylglycerol
TC	=	Total cholesterol
VLDL	=	Very low density lipoprotein

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare that the results presented in this paper have not been published previously in whole or part, except in abstract format. The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The study was supported by Research Projects RVO-VFN64165/2012, PROGRES Q25/LF1 UK

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Antioxidant Status and Oxidative Stress Markers in Pancreatic Cancer and Chronic Pancreatitis

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Objectives: Oxidative stress has been implicated in the pathogenesis of chronic pancreatitis (CP) and pancreatic cancer (PC). The study aim was to assess the oxidative stress markers and antioxidant defense system in patients with CP and those with PC.

Methods: Activities of superoxide dismutase 1 (SOD1), catalase (CAT), glutathione peroxidase 1 (GPX1), glutathione reductase (GR), arylesterase (PON1-A) and lactonase (PON1-L) activities of paraoxonase 1 (PON1) and concentrations of reduced glutathione, conjugated dienes in low-density lipoprotein (CD/LDL) and oxidized LDL (ox-LDL/LDL) were assessed in 50 PC and 50 CP patients and 50 age and sex-matched controls.

Results: Comparison of PC and CP groups to controls found the following changes: glutathione peroxidase 1 (GPX1) (−20.2%, −25.5%; $P < 0.001$), glutathione reductase (GR) (−9.5%, −11.9%; $P < 0.05$), SOD1 (+22.9%; $P < 0.01$), CAT (−10.6%; $P < 0.05$), PON1-A (−34.3%, −16.0%; $P < 0.001$), PON1-L (−44.2%; −17.0%; $P < 0.01$), conjugated dienes in LDL (CD/LDL) (+20%, +33.3%; $P < 0.05$) and ox-LDL/LDL (+42.2%, +14.4%; $P < 0.05$). The patients with PC had changed activities and levels of SOD1 (+24.2%), CAT (−10.4); $P < 0.01$), PON1-A (−21.7%), PON1-L (−32.9%), and ox-LDL/LDL (+24.3%); (all $P < 0.01$) compared with the patients with CP.

Conclusions: Reduced antioxidant defense system capacity and increased markers of oxidative stress were found in PC and CP. PON1-L and CAT activities, along with ox-LDL/LDL levels, were the independent factors differentiating the patients with PC from the patients with CP.

Key Words: oxidative stress, oxidative stress markers, antioxidant enzymes, chronic pancreatitis, pancreatic cancer, discriminant analysis

Abbreviations: CAT – catalase, CD - conjugated dienes, CP - chronic pancreatitis, CT - computed tomography, EUS – endoscopic ultrasonography, GPX1 - glutathione peroxidase 1, GR - glutathione reductase, GSH - reduced glutathione, HDL - high-density lipoprotein, HOMA-IR - homeostasis model assessment of insulin resistance, LDL - low-density lipoprotein, MDA - multivariate discriminant analysis, MRCP - magnetic resonance cholangiopancreatography, NRI – Nutritional Risk Index, NT – nitrotyrosine, ox-LDL – oxidized LDL, PC - pancreatic carcinoma, PON1 - paraoxonase 1, PON1-A - PON1 arylesterase, PON1-L – PON1 lactonase, RONS – reactive oxygen and nitrogen species, ROS - reactive oxygen species, SAA - serum amyloid A, SOD1 - Cu-Zn superoxide dismutase

(*Pancreas* 2013;42: 614–621)

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Received for publication April 25, 2012; accepted September 28, 2012.

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This study was supported by the research project RVO-VFN 64165/2012 of the Ministry of Health of the Czech Republic. It was not supported by any of the following organizations: National Institutes of Health (NIH), Wellcome Trust, or Howard Hughes Medical Institute (HHMI).

The authors declare no conflict of interest.

Overproduction of reactive oxygen and nitrogen species (RONS) and oxidative stress have been implicated in the pathogenesis of pancreatitis, both in its acute and chronic form, as well as in the pathogenesis of pancreatic cancer (PC).[1] Chronic pancreatitis (CP) shares risks with PC such as smoking and alcohol abuse as well as being a risk factor per se for PC.[1–3] Among them, cigarette smoking, alcohol abuse, diabetes mellitus, and other insulin resistance (IR) states are connected with increased RONS formation and oxidative stress.[1] Chronic pancreatitis is a progressive inflammatory disease with irreversible damage to the pancreas and the destruction of exocrine and endocrine tissue.[4] The underlying causes of CP seem to be multifaceted, including environmental as well as genetic factors, but its pathogenesis to date has not been completely understood. Although most cases of CP have been attributed to alcohol abuse and/or genetic predisposition, other etiologic risk factors such as enhanced oxidative stress could play an important role.[5,6]

Reactive oxygen and nitrogen species are generated during endogenous oxidative stress that is linked to the pancreatic renin-angiotensin system[7] or exogenous oxidative stress caused by environmental or lifestyle-related xenobiotics, which is connected with the detoxification system.[3] It has been proposed that local oxidative stress and reactive oxygen species (ROS) generation, caused by overexpression of membrane nonmitochondrial nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, is connected with pancreatic (patho)biology such as cell growth regulation and apoptosis, acinar cell inflammation, fibrosis, and disturbed islet microcirculation.[7,8] The inflammatory process is associated with increased production of RONS resulting in local or systemic oxidative stress.[9,10] A number of experimental and clinical studies have demonstrated impaired antioxidant status that may be a contributing factor for increasing oxidative stress in CP. The involvement of oxidative stress in CP has been described both in experimental and clinical studies.[1,3]

Oxidative stress could not only be the cause of CP (and PC) but also a consequence of the underlying disease (CP or PC, respectively). Moreover, increased RONS production and oxidative stress seem to be independent from the etiology of CP.[1,11]

In patients with CP, decreased levels of antioxidant thiols (cysteine, glutathione, and cysteinylglycine), decreased total antioxidant capacity, along with increased carbonylated proteins, thiobarbituric acid–reactive substances, malondialdehyde and 4-hydroxynonenal levels were found.[10,12–14] Similarly, in patients with CP (both alcoholic and tropical), decreased concentrations of glutathione, vitamin C, and zinc in erythrocytes were connected with elevated thiobarbituric acid–reactive substances.[15]

Levels of conjugated dienes (CD) are the most sensitive indicator of lipid peroxidation and can be regarded as a global marker of systemic oxidative stress[16] and also are a marker of minimally oxidatively modified low-density lipoprotein (LDL). On the contrary, oxidized LDL (ox-LDL) reflects concentration of malondialdehyde and 4-hydroxynonenal, the highly reactive

end products of lipid peroxidation that are bound, as adducts, to ε-amino group of lysine in molecule of apolipoprotein B.[17] Increased susceptibility of LDL to oxidation (LDL oxidability) was observed in CP.[18] Concentration of ox-LDL has not yet been studied in human PC.

Among enzymes that regulate RONS, glutathione peroxidase (GPX) and catalase (CAT) play an important role by the reduction of hydrogen peroxide, which is generated by superoxide dismutase (SOD) in the dismutation of superoxide. The main ROS scavenger in the pancreas is supposed to be reduced glutathione (GSH), which is recycled back to its reduced form by glutathione reductase (GR).[1,19] The enzyme paraoxonase 1 (PON1) as high-density lipoprotein (HDL)-associated enzyme is implicated in the anti-inflammatory and antioxidant activities of HDL and impedes oxidative modification of LDL, protects cell membranes from the damage caused by products of lipoperoxidation, and eliminates carcinogenic lipid-soluble radicals.[20–22]

The activities of some antioxidant enzymes in CP were already studied: SOD activity in the studies by Girish et al,[15] Quillot et al,[18] and Szuster-Ciesielska et al[23] and GPX1 activity in the studies by Quillot et al[18] and Szuster-Ciesielska et al.[18,23] These studies show lowered antioxidant capacity in CP.

The aim of the study was to ascertain the importance of lipoperoxidation markers (CD and ox-LDL) in relation to the group of main antioxidant enzymes, such as SOD, CAT, GPX1, GR, and PON1 in patients with CP and PC. Because there are intercorrelations between oxidative stress markers and antioxidant enzymes activities, both in CP and PC groups, we used the multivariate discriminant analysis to differentiate PC from CP as well as to evaluate the discriminative power of different oxidative stress markers and antioxidant enzymes.

MATERIALS AND METHODS

This prospective study was carried out at the Fourth Department of Internal Medicine of General University Hospital from January 2009 to September 2011. The study protocol was approved by the institutional review board and the Ethics Committee of the General University Hospital in Prague. Written informed consent was obtained from all participants.

Patients

The study population consisted of 3 groups: 50 patients with PC, 50 patients with CP, and 50 healthy controls. All groups are age and sex matched.

Diagnosis of PC was confirmed in all of the patients (based on histological examination of pancreatic resection or endoscopic ultrasonography-guided aspiration cytology). The tumor staging was evaluated by the combination of criteria issued by the Union Internationale Contre le Cancer and the American Joint Committee on Cancer (UICC/AJCC 2002).[24] The clinical diagnosis of CP was based on clinical features (abdominal pain, nausea and/or vomiting, anorexia and/or malnutrition, and steatorrhea) confirmed by 2 or more imaging methods (abdominal ultrasonography [USG], contrast-enhanced computed tomography [CT]), endoscopic retrograde cholangiopancreatography, magnetic resonance cholangiopancreatography (MRCP), and endoscopic ultrasonography (EUS). Only patients with definite CP were included. The grade of CP (mild, moderate, or severe) was assessed according to the M-ANNHEIM pancreatic imaging criteria[25] (M-ANNHEIM stands for M, multiple risk factor classification; A, alcohol consumption; N, nicotine consumption; N, nutritional factors; H, hereditary factors; E, efferent pancreatic duct factors; I, Immunological factors; M, miscellaneous and metabolic factors). All the patients were assessed by the combination of EUS and other imaging methods (CT, or USG, or MRCP) because EUS does not differentiate between the moderate and severe grades, and other methods (CT, or USG, or MRCP) cannot differentiate between mild and moderate changes.[25]

Exclusion criteria for all the 3 groups were the following: current antioxidant therapy (eg, vitamin C, vitamin E, allopurinol, *N*-acetylcysteine, supplementation with n-3 polyunsaturated fatty acids), kidney disease (creatinine >150 μmol/L), clinically manifest proteinuria (urinary protein >500 mg/L), and liver cirrhosis, decompensate diabetes mellitus, concomitant malignancies, chronic, immunosuppressive, and anti-inflammatory therapy, as well as chemotherapy. Further criteria for exclusion were the following: endocrine disease, acute pancreatitis, or acute relapse of CP; unstable angina pectoris, stage within 1 year after acute myocardial infarction; coronary aortic bypass grafting, or percutaneous coronary intervention, and stroke. Persons who were operated on in the upper gastrointestinal tract (in the previous year) and subjects after systemic inflammation in the previous 6 months were also excluded. Patients with CP enrolled into the study were reexamined after 2 years to exclude the development of PC and thus to avoid enrollment of patients with initial stages of PC into the study.

Among our 50 patients with PC, there were 22 patients with diabetes. In this group were the following stage distributions: 9 patients with stage II (2 patients with stage IIA and 7 patients with stage IIB) disease, 24 patients with stage III disease, and 17 patients with stage IV disease. Alcoholic CP was diagnosed in 38 patients, obstructive CP in 5 subjects, and idiopathic CP in 7 patients. Among the 50 patients with CP, there were 30 patients with severe grade, 17 patients with moderate, and 3 patients with mild grade changes in pancreatic morphology. Severe exocrine dysfunction (concentration of pancreatic stool elastase 1 <200 ng/g) was found in 29 patients with CP. Complications (ascites, bleeding, obstruction/or stricture ductus choledochus, pancreatic fistula, duodenal stenosis, splenic and/or portal vein thrombosis, and segmental portal hypertension) were found in 28 patients with CP.

Data Collection

Samples from all participants were obtained after overnight fast (at least 10 hours). All study participants' medical history and intake of any medications were documented at study entry. Blood was taken by puncturing a peripheral vein. Concentrations of C-reactive protein (CRP), conjugated dienes (CD/LDL) in precipitated LDL, serum amyloid A (SAA), 3-nitrotyrosine (NT), tumor markers (CA 19-9, CA 72-4, and CEA), albumin, bilirubin, uric acid, calcium, copper, zinc, iron, selenium, vitamins A and E, and lipid parameters, as well as activity of routine biochemical tests (pancreatic amylase, alanine transaminase, aspartate aminotransferase, γ-glutamyltransferase, cholinesterase alkaline phosphatase [data not shown]), PON1 arylesterase (PON1-A), PON1 lactonase (PON1-L), and oxidized-LDL (ox-LDL) were measured in serum. Serum was prepared after coagulation in vacutainer tubes by centrifugation at 3500 rpm at 4°C for 10 minutes. Activities of antioxidant enzymes CAT, GPX1, GR, and Cu-Zn superoxide dismutase (SOD1), as well as the concentration of GSH were measured in hemolyzed erythrocytes. The samples were stored at −80°C until assay. All samples were marked with unique identification numbers made anonymous, and the data were merged only after the assays had been completed.

Laboratory Measurements

Activities of antioxidant enzymes were determined by spectrophotometric kinetic methods, and the concentration of

GSH were assessed spectrophotometrically as previously described by Kodydková et al.[26] The lactonase activity of PON1 was measured according to the modified method described earlier[27] using dihydrocoumarin (final concentration, 1 mmol/L) as a substrate. The increase in absorbance at 270 nm was monitored for 2 minutes. The enzyme activity was calculated from the molar extinct coefficient of the reaction product [3-(2-hydroxyphenyl)-propionate (ε = 1295 dm^3 mol^{-1} cm^{-1})] estimated during the linear phase of reaction.[28] The concentration of CD in precipitated LDL was determined by the Wieland modified spectrophotometric method at 234 nm.[29,30]

The levels of SAA, 3-NT, and ox-LDL were established using sandwich enzyme-linked immunosorbent assay kits (Invitrogen, Camarillo, Calif; Biovendor, Brno, Czech Republic, Czech Republic; and Mercodia, Upsala, Sweden; respectively).

All routine clinical tests were measured at the Institute for Clinical Biochemistry and Laboratory Diagnostics of General University Hospital in Prague. Concentration of CRP was measured by the immunoturbidimetric method using a K-ASSAY CRP kit (Kamiya Biomedical Company, Seattle, Wash) on a Hitachi Modular analyzer (Tokyo, Japan). Tumor markers (CEA, CA 19–9, and CA 72–4) were measured by chemiluminescence assay on ADVIA Centaur analyzer, Siemens (Tarrytown, NY). Selenium, copper, and zinc were measured using atomic absorption spectrometry. Concentrations of total cholesterol and triglycerides were measured by enzymatic-colorimetric methods. High-density lipoprotein cholesterol was determined in the supernatant after precipitation of lipoproteins B by phosphotungstic acid/Mg^{2+} (Boehringer Mannheim, Germany); LDL cholesterol was calculated according to the Friedewald formula. Apolipoprotein B and apolipoprotein A1 were measured by the Laurell rocket electroimmunoassay using standards and specific antibodies (Behringwerke Marburg, Germany). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as HOMA-IR = [fasting serum glucose (mmol/L) × fasting serum insulin (μU/mL)] / 22.5.[31] Malnutrition was categorized into the mild, moderate, and severe forms according to the Nutritional Risk Index (NRI).[32] The NRI was calculated according to the following formula: NRI = (1.519 × albumin + 41.7 × current body weight/usual body weight), and the classification was as follows: normal nutrition, NRI > 100; mild malnutrition, NRI = 97.5–100; moderate malnutrition, NRI = 83.0–97.4; severe malnutrition, NRI < 83.0.

Statistical Analysis

Data are expressed as mean ± SD for parametric and as median and interquartile range (25th–75th percentiles) for nonparametric data. Normality of data distribution was tested with the Shapiro-Wilks *W* test. Differences between the compared groups (PC, CP, and controls) were tested with the one-way analysis of variance with Scheffé and Newman-Keuls post-tests. For nonparametric analysis, the Kruskal-Wallis analysis of variance was used. For correlation analysis, the Spearmen coefficient was used. All previously described statistical analyses were performed using StatSoft Statistica version 9.0 software (2007, Czech Republic). $P < 0.05$ was considered statistically significant.

The multivariate discriminant analysis (MDA) was carried out by using Statistical Analysis System (SAS, Cary, NC), JMP version 9 software. The process of MDA is carried out in a stepwise manner using the minimum Wilks λ (within-groups sum of squares–total sum of squares ratio) as a measure of group discrimination. At each step in the process, the variable, which contains the most discriminating power, is identified and its coefficient is determined. The relative importance of each variable is indicating by so-called approximate F statistic. This is a transformation of Wilks λ, which can be compared with F distribution. The process is stopped when the remaining variables are determined to lack significant discriminating power ($P > 0.05$). We used discriminant models for classification into the groups of PC and CP. Only variables with an appropriate final F statistic ($P < 0.05$) were included in our discriminant functions. The oxidative stress and inflammatory markers (CD/LDL, ox-LDL/LDL, and SAA), the antioxidant enzymes (SOD1, CAT, GPX1, GR, PON1-A, PON1-L), and their combinations as variables were subjected into the discriminant functions.

RESULTS

This study was focused on the antioxidant status in patients with PC and CP. Fifty patients with PC and 50 patients with CP were enrolled in the study. The basic clinical and biochemical data of the studied groups are summarized in Table 1.

As markers of oxidative stress, the levels of CD/LDL, ox-LDL/LDL, and NT were determined. The levels of CD/LDL and ox-LDL/LDL were significantly increased in both the patients with PC and those with CP compared with the controls (Figs. 1A, B). Furthermore, the patients with PC had higher levels of ox-LDL/LDL than the patients with CP ($P < 0.001$). There was an increase in the concentration of 3-NT only in the patients with CP ($P < 0.01$) in comparison with the controls (Fig. 1C).

Erythrocyte activity of SOD1 was increased and CAT activity was decreased in the patients with PC compared with the patients with CP and the controls (Figs. 2A, B). In addition, the serum concentration of SOD1 cofactor Cu was observed as elevated; and the serum concentration of Fe, the CAT cofactor, was decreased in the patients with PC in comparison with the controls (Table 2). The concentration of Zn was increased in the patients with CP compared with the controls. Conversely, decreased erythrocyte activities of GPX1 and GR were found in patients with PC and those with CP as compared with the controls (Figs. 2C, D). The decreased GPX1 activities in both the PC and CP groups were accompanied with lower serum selenium levels compared with the controls (Table 2). The concentration of GSH in erythrocytes in the patients with PC differed from that of the subjects with CP and controls, respectively (Fig. 1D). We have found that serum concentrations of Zn negatively correlate with activity of SOD1 in the entire group ($r = -0.312$; $P < 0.001$), in the patients with PC ($r = -0.357$; $P < 0.05$) and also in the patients with CP ($r = -0.458$; $P < 0.001$). There was a significant correlation between Se and GPX1 in the entire group ($r = 0.319$; $P < 0.01$) and also in the patients with CP ($r = 0.470$; $P < 0.01$).

The PON1-A and PON1-L activities in serum were decreased in the patients with PC and those with CP in comparison with the controls (Figs. 2E, F). Furthermore, decreased activities of these enzymes in the patients with PC compared with the patients with CP (both $P < 0.001$) were observed. Both PON1 activities were significantly correlated in the PC ($r = 0.711$; $P < 0.001$) and CP ($r = 0.811$; $P < 0.001$) groups and in the controls ($r = 0.687$; $P < 0.001$) as well as in all the studied groups ($r = 0.806$; $P < 0.001$). The levels of both PON1 activities correlated negatively with ox-LDL/LDL ($r = -0.309$; $P < 0.001$; and $r = -0.358$; $P < 0.001$; respectively) in the entire group.

Serum amyloid A concentrations were higher in the patients with PC than in the patients with CP and in the controls (both $P < 0.001$; Table 1). The studied groups did not differ in concentrations of PON1 cofactor—calcium. Additionally, we

TABLE 1. Basal Clinical and Biochemical Characteristics

	PC	CP	CON
No. patients (M/F)	50 (40/10)	50 (40/10)	50 (40/10)
Age (range), yrs	63 (56–68)	59 (53–65)	60 (55–65)
Smokers, n (%)	34 (68)	15 (30)	13 (26)
DM, no. patients (M/F)	28 (24/4)	30 (23/7)	–
NRI, kg/m^2	96.4 ± 12.3*	109.1 ± 7.3	–
CRP, mg/L	10.9 (5.8–54.8)***; $^{+++}$	4.5 (2.0–10.3)*	2.1 (1.0–4.9)
SAA, μg/mL	49.0 (21.7–134.2)***; $^{+++}$	14.5 (7.2–49.8)	12.7 (4.6–25.6)
CEA, μg/L	2.75 (1.65–6.45) ***	2.37 (1.44–3.42)***	0.71 (0.5–1.45)
CA 19–9, kU/L	105.2 (24–2301.3)***; $^{+++}$	14.6 (8.5–26.5)*	8.7 (6.1–10.7)
CA 72–4, kU/L	2.32 (1.31–9.70)*; $^{++}$	1.45 (0.97–2.23)	1.44 (1.02–3.86)
Glucose, mmol/L	6.5 (5.2–8.9) ***	6.6 (5.8–7.6)***	5.1 (4.9–5.3)
Glycated hemoglobin, mmol/mol	5.46 ± 1.85***	5.11 ± 1.49***	3.91 ± 0.34
TC, mmol/L	4.7 (3.7–6.6)*	4.9 (4.4–6.0)	5.4 (4.6–5.9)
TG, mmol/L	1.67 (1.26–2.12) ***	1.48 (1.10–2.01)***	1.06 (0.79–1.34)
HDL-C, mmol/L	0.92 (0.73–1.05)***; $^{+++}$	1.42 (1.21–1.59)	1.5 (1.27–1.73)
LDL-C, mmol/L	2.76 (2.12–3.24)*	2.73 (2.34–3.17)*	3.33 (2.72–3.73)
Apo A1, g/L	0.88 ± 0.32***; $^{+++}$	1.61 ± 0.44	1.53 ± 0.30
NEFA, mmol/L	0.71 ± 0.35**	0.62 ± 0.40*	0.48 ± 0.24

Data are expressed as mean ± SD for parametric variables, and as median and interquartile range (IQR, 25th–75th percentile) for nonparametric variables;

PC or CP versus CON: ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; PC versus CP: $^{+++}P < 0.001$, $^{+}P < 0.05$ (one-way analysis of variance (ANOVA) with Newman-Keuls posttest), and $^{++}P < 0.01$.

Apo-A1, apolipoprotein A1; CA, carbohydrate antigen; CEA, carcinoembryonic antigen; CON, control subjects; NEFA, nonesterified fatty acids; TC, total cholesterol; TG, triglycerides.

have observed statistically decreased concentrations of vitamin A, albumin, and uric acid and higher levels of bilirubin in the patients with PC compared with the patients with CP and the controls (Table 2).

Discriminant models for classification into the groups of PC and CP are shown in Table 3. The concentrations of ox-LDL and CD/LDL were the best discriminators (model A) when only oxidative stress and inflammatory markers were entered into the

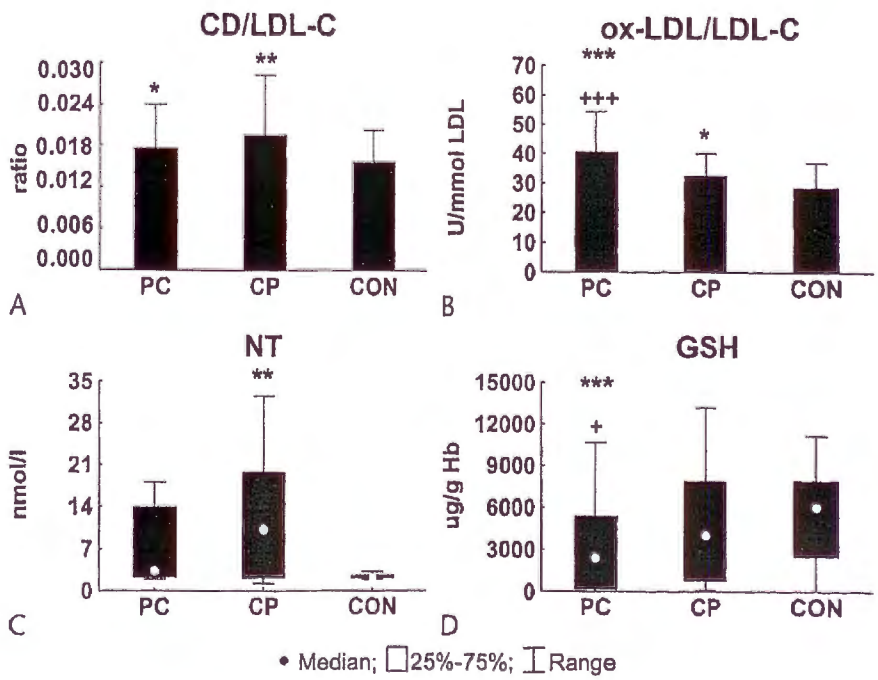

FIGURE 1. Serum concentration of oxidative stress markers and reduced glutathione. Data are expressed as mean ± SD for parametric variables and as median and IQR (25th–75th percentile) for nonparametric variables. PC or CP versus CON: ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; PC versus CP: $^{+++}P < 0.001$ and $^{+}P < 0.05$ (one-way ANOVA with Newman-Keuls posttest or Kruskal-Wallis ANOVA).

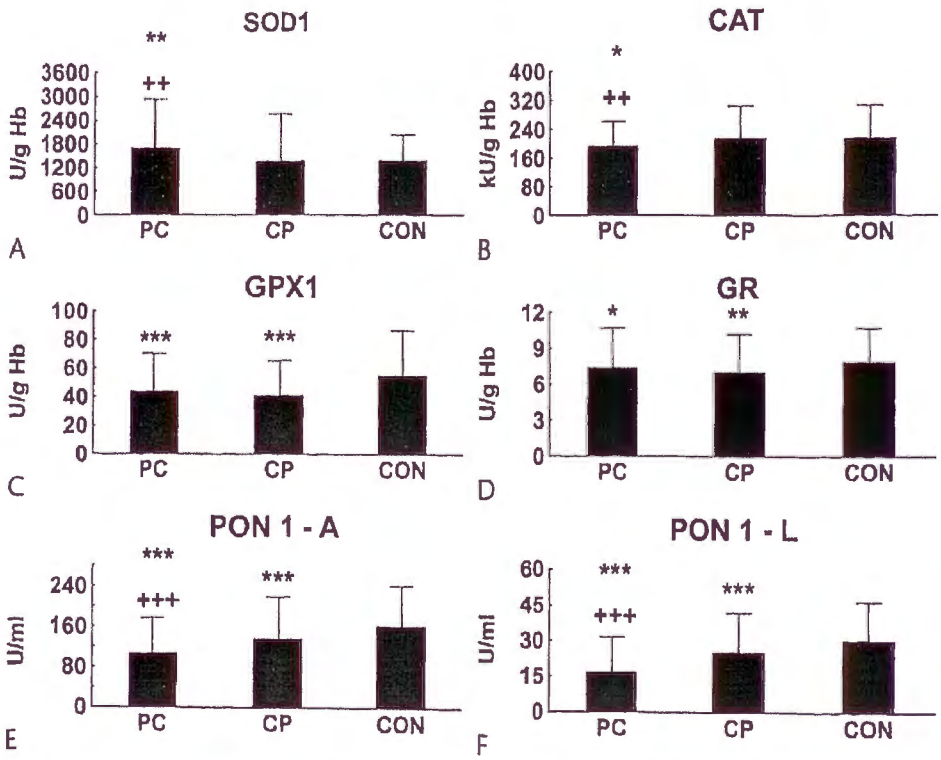

FIGURE 2. Activities of antioxidant enzymes. Data are expressed as mean ± SD; PC or CP versus CON: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; PC versus CP: $^{++}P < 0.01$ (one way ANOVA, Newman-Keuls posttest) and $^{+++}$ and $^{***}P < 0.001$ $P < 0.001$.

MDA. Using only antioxidant enzymes as discriminating variables resulted in 73% of the final correct classification. Variables subjected into the analysis were PON1-L, SOD1, and CAT (model B). A combination of both models resulted in 83% of the final correct classification. The activity of PON1-L, the levels of ox-LDL/LDL, and the activity of CAT, in that order, were found to be the best set of independent factors discriminating PC and CP, with higher values for PC (model C).

Table 2 presents data of nonenzymatic antioxidants and of cofactors of antioxidant enzymes.

DISCUSSION

The present study demonstrates contemporary changes in the levels of selected inflammatory and oxidative stress markers as well as a set of the antioxidant defense system (both enzymatic and nonenzymatic) in the patients with PC and those with CP in comparison with age- and sex-matched controls. The reduced capacity of the antioxidant defense system and an increased oxidative stress in patients with PC and those with CP was confirmed in our study. The antioxidant system seems to be more affected in the patients with PC compared with the patients with CP.

The groups of CP and PC differed statistically significantly in many oxidative stress and antioxidant variables using univariate analysis. Moreover, there were intercorrelations between oxidative stress markers and activities of antioxidant enzymes. Therefore, multivariate discriminant analysis was performed to determine the set of independent oxidative stress

TABLE 2. Nonenzymatic Antioxidants, Cofactors of Antioxidant Enzymes, and Other Parameters of Antioxidant Capacity

	PC	CP	CON
Vitamin A, mg/L	0.51 ± 0.24*; +	0.80 ± 0.41	0.83 ± 0.35
Vitamin E, mg/L	16.0 ± 7.7	12.3 ± 5.0	14.4 ± 6.1
Fe, μmol/L	13.5 ± 8.3***	16.4 ± 7.2	19.2 ± 7.9
Ca, mmol/L	2.26 ± 0.16	2.24 ± 0.13	2.27 ± 0.13
Cu, μmol/L	21.9 ± 6.3***; +++	18.8 ± 4.5	15.6 ± 3.1
Zn, μmol/L	19.2 ± 4.7	19.9 ± 3.9*	17.8 ± 2.6
Zn/Cu	0.86 ± 0.34***; +++	1.13 ± 0.38	1.15 ± 0.40
Se, μg/L	31.3 ± 10.9***	43.1 ± 21.5*	58.9 ± 26.0
Albumin, g/L	41.4 (37.7–45.5)***; +++	45.7 (42.5–46.9)	47.2 (44.9–48.6)
Bilirubin, μmol/L	18.7 (11.0–64.1)***; +++	10.2 (7.2–14.2)	11.0 (8.0–15.3)
Uric acid, μmol/L	257 ± 108**; ++	324 ± 90	310 ± 76

Data are expressed as mean ± SD for parametric variables and as median and IQR (25th –75th percentile) for nonparametric variables;
PC or CP versus CON: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$; PC versus CP: $^{+++}P < 0.001$, $^{++}P < 0.01$, and $^{+}P < 0.05$ (one-way ANOVA with Newman-Keuls posttest).

TABLE 3. Discriminant Models for Classification of CP and PC

	F-Statistic (df)	Final Correct Classification	Percent
Model A			
ox-LDL/LDL	13.80 (1, 98)	CP	74.0
ox-LDL/LDL + CD/LDL	11.32 (2, 97)	PC	72.0
		Total	73.0
Model B			
PON1-L	31.77 (1, 98)	CP	68.0
PON1-L + SOD1	19.33 (2, 97)	PC	74.0
PON1-L + SOD1 + CAT	14.83 (3, 96)	Total	71.0
Model C			
PON1-L	31.77 (1, 98)	CP	80.0
PON1-L + ox-LDL/LDL	27.72 (2, 97)	PC	86.0
PON1-L + ox-LDL/LDL + CAT	21.32 (3, 96)	Total	83.0

Model A: Only oxidative stress and inflammatory markers included in the analysis (CD/LDL, Ox-LDL/LDL, and SAA). Model B: Only activities of antioxidant enzymes included in the analysis (SOD1, CAT, GPX1, GR, PON1-A, and PON1-L). Model C: combination of the model A and the model B.

df, degrees of freedom;

F statistic (so-called an approximate F statistic), transformation of Wilks λ.

and antioxidant variables giving the most discrimination power to separate CP and PC.

Multivariate discriminant analysis indicated that the activities of PON1-L and CAT, along with ox-LDL/LDL levels (in the order of PON1-L, ox-LDL/LDL, and CAT), are the independent factors discriminating the patients with PC and those with CP.

Antioxidant enzymes play an important role in the defense of cells against RONS and thus may protect the pancreas against development of CP, which is a risk factor of PC. The first scavenger of ROS is SOD, which converts superoxide radical to H_2O_2, which should be later removed by GPX1 and CAT. Our study found increased SOD1 activity in erythrocytes of the patients with PC in comparison with the patients with CP and the controls. Some experimental studies have described relationships between the expression of SOD, its activity, and PC cell growth in vitro, on the one hand, and tumor growth and survival in nude mice, on the other.[8] The insignificant differences in the erythrocyte activities of SOD1 in the patients with CP and controls found in our study were consistent with the study of Quillot et al.[18] On the other hand, decreased SOD1 activity in the patients with CP was found in the study of Girish et al.[15] Inconsistent results concerning serum SOD activities in hereditary and alcohol-related pancreatitis have been published. Some reports have described increased[23,33] serum SOD activity, and in some studies, no differences in serum SOD activities were found.[18,34] It could be supposed that discordance in elevated activity of SOD1 without an appropriate change in the GPX1 and/or CAT activities in the patients with PC resulted in the increased production of H_2O_2, which cannot be detoxified by the action of GPX1 and CAT. Accumulation of H_2O_2 can thus participate in a Haber-Weiss reaction and generate hydroxyl radicals.[35,36] Catalase and glutathione peroxidase are both able to detoxify H_2O_2. Under physiologic conditions, H_2O_2 is mainly removed by GPX1. The activity of CAT is involved in the degradation of H_2O_2 in severe oxidative stress connected with higher H_2O_2 concentrations.[37] It was previously shown that long-term exposure of CAT to H_2O_2 leads to the oxidation of the catalase-bound NADPH to $NADP^+$ and to a decrease in the activity of CAT to approximately 30% of the initial activity.[38] Because our patients with PC had the highest level of oxidative stress markers associated with decreased erythrocyte CAT activity, our results implicated that under stressed conditions, erythrocyte CAT is unable to detoxify H_2O_2. In contrast, no changes in serum CAT activity in the patients with PC were found.[39] The insignificant differences in CAT activities in erythrocytes of the patients with CP observed in our study were consistent with the results of Fukui et al,[39] dealing with serum CAT activities in patients with CP. In the contrast, other authors described increased serum CAT[23,40] or decreased serum and erythrocyte CAT[18] activities in patients with CP.

Glutathione peroxidases use GSH to metabolize H_2O_2 and lipid hydroperoxides to water/related alcohols.[1] We have found decreased activity of erythrocyte GPX1 in the patients with PC and those with CP compared with the controls. The decreased erythrocyte GPX1 in CP was also found in the study of Girish[15] but also no differences in erythrocyte GPX1 activity in patients with CP and the controls were observed.[18,33] Published results in serum and plasma GPX activities are inconsistent.[18,23,33,34,40] Decreased GPX1 activity may be explained by the lowered serum levels of selenium in both groups and/or decreased concentrations of reduced glutathione in erythrocytes found in the patients with PC. Selenium is bound as the selenocysteine at the active site of GPX1, and it is essential for its activity. Moreover, selenium deficiency leads to decreased GPX1 activities.[35]

Glutathione reductase is a NADPH-dependent enzyme that catalyzes the regeneration of GSH from oxidized glutathione (GSSG) and thus maintains a constant supply of GSH for GPX.[41] In the present study, a decrease in GR erythrocyte activity was observed in the patients with PC and those with CP and decreased erythrocyte levels of GSH in PC but not in the patients with CP in comparison with the controls. It is supposed that depletion in GSH concentration may be caused by accumulation of GSSG as a result of impaired GR (pentose-phosphate pathway may limit NADPH supply). Formed GSSG could react with the sulfhydryl group, via mixed disulphide reactions, or could be secreted out of the cell. It is supposed that the abovementioned processes may lead to GSH depletion.[42] In

contrast to our study, decreased levels of reduced glutathione were observed in the patients with CP.[43]

We found a decrease in serum activities of arylesterase as well as lactonase activity of PON1 in the patients with PC and those with CP. The lowest PON1 activities were observed in the patients with PC. At present, the decreased PON1 activity in patients with PC has been described only in one study,[44] and there is no study dealing with PON1 activities in CP. A number of studies have shown decreased serum arylesterase and/or paraoxonase activities of PON1 in different malignancies.[45]

Under conditions of systemic inflammation and/or oxidative stress, several mechanisms are implicated in a drop of PON1 activities. Among them, displacement of PON1 from its linkage to apolipoprotein A1 in HDL by SAA,[46] down-regulation of liver PON1 lipopolysaccharides and cytokines (tumor necrosis factor α and interleukin-1) via IL-6,[20] and inhibition of PON1 activity by oxidized phospholipids[47] are the most important. All the abovementioned mechanisms can be related to cancer-related decrease in PON1 activities. Using MDA, the PON1-L activity had the most discriminating power to differentiate PC from PC.

The finding of increased SAA levels in the patients with PC in our study is consistent with the results of other studies.[48,49] Serum amyloid A is implicated in carcinogenesis, and it was associated with tumor progression and its metastasizing.[50] Some authors considered SAA as a tumor marker for PC. However, SAA did not reach appropriate specificity and sensitivity as tumor marker for PC diagnostics.[48,49]

Human serum PON1 should contribute to the detoxification of organophosphorus compounds and carcinogenic lipid-soluble radicals from lipid peroxidation and, moreover, should impede oxidative modification of LDLs.[22] In this study, we found a negative correlation between PON1 activities and ox-LDL/LDL levels. The reduced PON1 activities in the patients with PC and those with CP could lead to the increase in ox-LDL/LDL levels. Oxidatively modified LDL represents heavily oxidized LDL characterized by oxidative altered both the lipid and the apolipoprotein B, moiety of particle. Low-density lipoprotein is supposed to be oxidize not only within the artery wall (by endothelial cells, smooth muscle cells, and monocyte/macrophages) but also at peripheral tissues altered by inflammation (by neutrophils and fibroblasts).[51]

In our study, increased concentrations of CD/LDL in the patients with PC and those with CP compared with the controls were found. Concentrations of CD/LDL are partly considered as a marker of systemic oxidative stress and partly reflect minimally modified LDL, in which only the lipid component is oxidatively modified.[29,52] Currently, serum concentration of CD in CP patients was described only in the study of Santini et al,[53] where the levels of CD and lipid hydroperoxides did not differ between patients with CP and controls. However, the patients with CP, in comparison with the controls, had increased levels of CD and lipid hydroperoxides in pancreatic juice after secretin stimulation. These results indicated local enhanced oxidative stress in pancreas without systemic oxidative stress response. An increase of lipid peroxidation connected with PC and CP were observed in many studies using products of lipid peroxidation (such as thiobarbiturate-reactive substances, malondialdehyde, 4-hydroxynonenal, lipid hydroperoxides).[1,3] The importance of ox-LDL/LDL and CD/LDL was pointed out in discriminating PC from CP using MDA.

In conclusion, our study demonstrates the persisting oxidative stress in patients with CP and those with PC, which is associated with the reduced capacity of the antioxidant defense system. The oxidative stress defense system seems to be more affected in patients with PC compared with those with CP. Multivariate discriminant analysis indicates the importance of PON1-L and CAT activities, along with ox-LDL/LDL levels, as the independent factors discriminating patients with PC and those with CP. Further studies concerning antioxidant defense systems and oxidative stress are warranted, especially with respect to potential diagnostic and therapeutic implications.

ACKNOWLEDGMENT

The authors thank Dr Vera Lanska, PhD, for her statistical assistance.

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Nutrition and Cancer, 64(7), 946–955

ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2012.716138

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Plasma Fatty Acid Composition in Patients with Pancreatic Cancer: Correlations to Clinical Parameters

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Pancreatic cancer (PC) ranks as the fourth cause of cancer-related deaths in the Czech Republic. Evidence exists that deregulation of fatty acid (FA) metabolism is connected with some malignancies; therefore, we decided to analyze FA profile in plasma lipid classes in patients with PC with relation to tumor staging, nutritional status, and survival. The study included 84 patients (47 males, 37 females) with PC and 68 controls (36 males, 32 females). FA patterns were analyzed in plasma lipid classes by gas-chromatography. We observed increased proportion of total monounsaturated FA (MUFA) in PC group in all plasma lipid classes. These changes were connected with increased Δ9-desaturase (SCD1) and Δ5-desaturase indices. Correlations of dihomo-γ-linolenic acid (DHGLA) with these variables were opposite. Longer survival of patients was connected with higher content of EPA, DHA, and with lower SCD1 index, respectively. Plasma phospholipid proportions of α-linolenic acid, DHGLA, EPA, and n-3 polyunsaturated fatty acids displayed negative trend with tumor staging. Plasma lipid FA pattern in PC patients resulted from decreased dietary fat intake and increased de novo synthesis of FA with transformation into MUFA. Changes in FA profile implicated some pathophysiological mechanisms responsible for disturbed FA metabolism in PC and importance of appropriate nutritional support.

Submitted 15 December 2011; accepted in final form 11 July 2012.
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INTRODUCTION

Pancreatic cancer (PC) is one of the most fatal human malignancies. It ranks as the fourth cause of cancer-related deaths in the United States (1) as well as in the Czech Republic (2). Its incidence varies worldwide with high rates in the United States, Canada, Australia, and Europe. In the Czech Republic, the incidence reached 18.9 per 100,000 inhabitants in men (18.6 in women) in 2009 (2).

Besides age, genetic risk factors, preexisting diseases (chronic pancreatitis, diabetes mellitus, obesity, and other insulin resistance states), several lifestyle and environmental factors have been reported to contribute to the development of PC (3–9). Cigarette smoking, the most well-established environmental risk factor, increases the risk of PC by 25%–30%; 9% is related to diabetes mellitus and 3% to heavy alcohol consumption (8). Dietary factors are supposed to contribute to the risk of PC by 20% (10–12).

There is growing evidence that the deregulation of fatty acid (FA) metabolism is connected with some malignancies similar to cardiovascular disease, metabolic and nutritional disease (such as obesity, diabetes mellitus, and other insulin resistance states) (13). FA composition in plasma phospholipids (PL) and cholesteryl esters (CE) reflects both dietary intake of FA over a 6-wk to 3-mo period as well as endogenous FA metabolism (synthesis of FA de novo, β-oxidation, enzymatic desaturation and elongation, conversion of polyunsaturated FA to eicosanoids, and lipoperoxidation) (14). The de novo biosynthesis of FA is induced in several types of malignant tumors by overexpression of FA synthase (FAS) and stearoyl-CoA desaturase (SCD1). FAS plays a role only in the liver and adipose tissue (15,16) in

healthy subjects, whereas tumor-induced expression and activities of FAS and SCD1 help to sustain the malignant phenotype, survival, and proliferation of cancer cells (16,17).

Several studies proved the association of risk for PC with total fat (18) and saturated FA (18–20) intake. When the saturated and monounsaturated FA were replaced with polyunsaturated FA (PUFA), the risk for PC decreased in obese individuals (21). High intake of n-6 PUFA, especially linoleic acid (LA; 18:2n-6), and the elevated ratio of n-6 PUFA to n-3 PUFA also increased the risk for alimentary tract tumors (colorectal carcinoma, pancreas) and breast and prostate cancer (22). Beneficial effects of n-3 PUFA (ratio n-6 PUFA/n-3 PUFA, respectively) in the risk and progression of several carcinoma were reported in epidemiological studies (22,23). In general, n-6 PUFA enhances tumor growth by supporting tumor proliferation, invasiveness, metastases formation, and apoptosis as well as the reaction of the organism (inflammation, immune responses, and angiogenesis) whereas n-3 PUFA opposes these effects (24).

The aim of the study was to analyze the profile of FA in the main plasma lipid classes: PL, CE, and triacylglycerols (TAG) in relation to tumor staging, nutritional status, and survival in the patients with PC.

MATERIALS AND METHODS

Subjects

The study included 84 patients (47 males/37 females) with PC and mean age of 64.7 ± 9.5 years (mean ± SD) and 68 control subjects (36 males/32 females) with a mean age of 59.3 ± 8.0. The study protocol was approved by the Joint Ethical Committee of the General University Hospital and the 1st Faculty of Medicine, Charles University in Prague. Written informed consent was obtained from each study participant.

The PC group was recruited from the consecutive patients hospitalized at the 4th Department of Internal Medicine between the years of 2008 and 2011. The control group was recruited from the medical staff of the institution and from outpatients with functional gastrointestinal disorders. Both groups of subjects were examined clinically, including an assessment of basic anthropometrical data using standard methods (25). The percentage of body fat was estimated according to the Durnin and Womersley method (26). The 7-day dietary intake was calculated from dietary record using NutriMaster SE software, as described earlier (27). Malnutrition was categorized into the mild, moderate, and severe form according to the Nutritional Risk Index (NRI) (28). The NRI was calculated according to formula: NRI = (1.519 * albumin + 0.417 * current body weight/usual body weight * 100) and the classification was as follows: normal nutrition: NRI > 100; mild malnutrition: NRI, 97.5–100; moderate malnutrition: NRI, 83.0–97.4; severe malnutrition: NRI < 83.0.

The homeostasis model assessment (HOMA) method was used as an index of insulin resistance (IR) (29). Diagnosis of PC was confirmed histologically in all of the patients (based on histological examination of pancreatic resection or endoscopic ultrasonography-guided aspiration cytology). PC staging was performed according to the TNM system and Union Internationale Contre le Cancer with the American Joint Committee on Cancer (UICC/AJCC 2003) (30). Blood samples were taken after 12 h of fasting. Routine biochemical and hematological analyses were performed immediately; samples for special analyses were stored at −80°C until use.

Laboratory Analyses

The routine biochemical parameters were analyzed by conventional methods on automatic analyzers according to standard procedures. The FA patterns in plasma PL, CE, and TAG were analyzed by gas chromatography (31). The molar percentages of FA were used for estimation of desaturase indices, which were calculated as the appropriate product/substrate ratio (see Tables 3 and 4 for details). These can serve as surrogate marker for the activities of the respective enzymes (32). Concentrations of conjugated dienes in precipitated LDL (CD-LDL) were determined spectrophotometrically (33).

Statistical Analysis

The data were processed with STATISTICA® statistical software for Windows. As the patients with PC were older than controls ($P < 0.001$, t-test), other variables were adjusted for age in the case of comparison between the PC vs. control group. The variables were log transformed where appropriate (non-Gaussian distribution of data). For the analyses within the PC group, we used nonparametric tests. $P < 0.05$ was considered to be statistically significant.

RESULTS

Demographic and clinical data of the patients are presented in Table 1. The patients with PC were older ($P < 0.001$, unpaired t-test with estimated variances) than controls and therefore further statistical analyses were performed after the adjustment for age when these 2 groups were compared. The PC group had a lower body mass index (BMI) and body weight. The decrease in weight is caused mainly by adipose tissue loss without changes in its centripetal distribution, as the waist circumference and waist-to-hip ratio remained similar. Nevertheless, the PC group had a higher ratio for subscapularis/triceps skinfold. Patients with PC had, in comparison with control group, the lower intake of total energy (CON vs. PC, 2240 ± 460 vs. 1560 ± 610, mean ± SD in Kcal/day, $P < 0.05$), lower relative intake of fat (CON vs. PC, 35 ± 8 vs. 25 ± 14, mean ± SD in % of total energy intake, NS), lower relative intake of protein (CON vs. PC, 20 ± 7 vs. 14 ± 8, mean ± SD in % of total energy intake, NS), and higher relative intake of saccharides (CON vs. PC, 45 ± 9 vs. 61 ± 18, mean ± SD in % of total energy intake, NS). Moreover, we found decrease in animal fat consumption, but differences were not statistically significant (CON vs. PC, 35 ± 17 vs. 22 ± 12, mean ± SD in g/day), probably because of high variance of variables. The patients with PC had also lower levels

TABLE 1
Demographic and clinical characteristics of studied groups

Characteristic	Control group	Pancreatic cancer	Stage II	Stage III	Stage IV	P (trend)[e]
Number of subjects (male/female)	36/32	47/37[b/ N.S.]	7/6	19/17	21/13[b/ N.S.]	
Age at diagnosis (yr)	59.3 ± 8.0[a]	64.7 ± 9.5[c/***]	66.6 (64.8–72.2)[f]	64.6 (56.8–71.8)	63.7 (59.1–70.1)	NS
Duration of symptoms (mo)	n.a.	5.3 ± 6.8	3.5 (2.3–7.5)	3.0 (1.0–6.0)	3.5 (2.0–6.0)	NS
Smoking status[g]						
Nonsmokers	38	24	9	5	10	
Exsmokers	11	28	1	16	11	
Smokers	19	30[b/**]	3	14	12[b/**]	
Diabetes mellitus[g]						
Absent	68	42	7	19	16	
Present <3 yr		23	1	7	15	
Present >3 yr		17	3	10	3[b/*]	
Body mass index (kg/m^2)	27.3 ± 5.4	24.7 ± 4.9**	25.3 (23.0–27.9)	25.1 (20.5–27.5)	23.6 (20.8–27.1)	NS
Nutritional risk index	113 ± 4	96 ± 11**	107 (95–110)	98 (92–103)	98 (86–101)	0.014↓
Fat mass (kg)	21.4 ± 11.5	16.3 ± 7.8*	18.7 (13.6–22.9)	17.0 (11.9–24.1)	13.4 (7.3–17.9)	0.007↓
Lean body mass (kg)	58.9 ± 14.7	53.9 ± 12.7	54.5 (45.7–59.9)	53.0 (45.1–60.7)	53.0 (43.3–61.7)	NS
Midarm circumference (cm)	30.2 ± 3.7	26.6 ± 3.6***	27.3 (26.1–28.4)	27.0 (24.0–29.5)	26.5 (24.5–28.5)	NS
Midarm muscle circumference (cm)	23.4 ± 3.6	22.6 ± 3.4	22.2 (20.6–25.0)	21.7 (20.2–24.8)	23.1 (20.0–24.3)	NS
Waist circumference (cm)	93.6 ± 13.5	92.5 ± 13.9	90 (85–105)	90 (82–101)	93 (85–100)	NS
Waist-to-hip circumference ratio	0.94 ± 0.08	0.95 ± 0.09	0.94 (0.89–1.02)	0.94 (0.88–1.00)	0.96 (0.91–1.01)	NS
Subscapularis to triceps skinfold ratio	1.34 ± 0.64	1.77 ± 0.95***	1.28 (1.01–2.18)	1.53 (1.18–1.97)	1.58 (1.25–2.00)	NS
Weight loss (kg/previous 3 mo)	−0.1 ± 1.1	11.5 ± 8.7***	6.3 (1.2–10.0)	9.4 (6.0–19.0)	14.5 (7.0–16.5)	0.022↑

Fat mass was calculated from % of fat mass (according to Durnin and Womersley). BMI, body mass index = weight(kg)/[(height(m)]^2; MAMC, midarm muscle circumference) = midarm circumference (cm) – 3.141 * triceps skinfold (cm), nutritional risk score = 1.519 x albumin (g/l) + 41.7 x (current weight / normal weight).

[a]Data are presented as mean ± SD.

[b]Chi-square test (with Yates' correction where appropriate).

[c]Unpaired t-test.

[d]Analysis of covariance with age as a covariate.

[e]Jonckheere-Terpstra test for ordered alternatives (↓ = decreasing, ↑ = increasing trend).

[f]The data are in the median (25th–75th percentile) format.

[g]The respective sums may not add to 84 because of missing data.

*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

TABLE 2
Biochemical characteristics of studied groups

Characteristic	Control group	Pancreatic cancer	stage II	stage III	stage IV	P(trend)[c]
Albumin (g/l)	46.7 ± 2.7[a]	40.4 ± 5.9[c***]	45.1 (38.6–46.8)[b]	41.6 (37.5–45.1)	40.3 (34.7–43.2)	0.016↓
Prealbumin (g/l)	0.25 ± 0.05	0.18 ± 0.08[c***]	0.21 (0.15–0.25)	0.19 (0.16–0.23)	0.14 (0.08–0.19)	0.001↓
CRP (mg/l)	5.5 ± 7.1	31.7 ± 38.2[c***]	12.7 (7.5–18.9)	8.1 (3.7–17.2)	36.7 (12.5–83.7)	0.005↑
Cholinesterase (μkat/l)	144 ± 30	104 ± 36[c***]	106 (91–153)	112 (88–140)	85 (65–113)	0.004↓
CA 19-9 (kU/l)	9 (6–14)[a]	273 (52–4514)[c***]	210 (29–662)	315 (51–2283)	256 (54–9178)	NS
CA 72-4 (kU/l)	1.5 (1.0–4.5)	3.1 (1.7–13.5)[c***]	2.2 (1.2–3.2)	2.1 (1.6–8.1)	9.1 (2.8–36.6)	0.001↑
CEA (μg/l)	0.6 (0.5–1.4)	3.8 (1.7–9.5)[c***]	2.3 (1.2–3.9)	2.4 (1.4–5.9)	6.5 (3.1–33.3)	0.001↑
Glucose (mmol/l)	5.18 ± 0.56	7.58 ± 3.17[c***]	6.10 (5.10–6.90)	7.40 (5.70–10.10)	6.44 (5.60–8.10)	NS
Insulin (mIU/l)	7.7 (5.4–13.3)	7.1 (4.6–11.1)	8.82 (7.41–12.34)	5.82 (4.44–11.96)	6.98 (4.44–10.70)	NS
HOMA-IR (ratio)	2.40 ± 1.74	3.49 ± 4.42	3.19 (2.15–4.36)	2.05 (1.28–4.96)	2.01 (1.14–3.14)	NS
LDL-CD (μmol/l)	48.7 ± 17.4	57.4 ± 26.9	43.8 (35.9–49.9)	53.0 (42.9–60.8)	55.6 (46.0–67.5)	0.043↑
Total cholesterol (mmol/l)	5.42 ± 1.01	5.43 ± 2.34	5.29 (4.23–6.62)	4.99 (4.11–5.98)	4.78 (3.70–5.65)	NS
LDL-cholesterol (mmol/l)	3.32 ± 0.81	3.59 ± 2.25	2.88 (2.29–5.28)	3.15 (2.44–3.83)	3.07 (2.13–3.99)	NS
HDL-cholesterol (mmol/l)	1.57 ± 0.38	0.94 ± 0.34 [c***]	1.06 (0.81–1.41)	0.97 (0.79–1.18)	0.90 (0.66–1.18)	NS
Triacylglycerols (mmol/l)	1.1 (0.8–1.4)	1.7 (1.2–2.1)[c***]	1.67 (1.33–2.56)	1.58 (1.12–2.18)	1.69 (1.38–1.97)	NS
FFA (mmol/l)	0.54 ± 0.24	0.75 ± 0.43[c**]	0.66 (0.51–0.85)	0.80 (0.49–0.89)	0.57 (0.40–0.98)	NS
apo A-I (g/l)	1.51 ± 0.29	0.93 ± 0.32[c***]	1.10 (0.90–1.48)	0.90 (0.73–1.10)	0.91 (0.64–1.09)	NS
apo B (g/l)	1.02 ± 0.25	1.27 ± 0.53[c**]	1.04 (0.88–1.66)	1.11 (0.92–1.45)	1.19 (0.91–1.37)	NS

CD, conjugated diene in precipitated LDL; CEA, carcinoembryonal antigen; CA, carbohydrate antigen; apo, apolipoprotein; FFA, free fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance = glucose (mmol/l) * insulin (mUI/l)/22.5.

[a]Values are expressed as mean ± SD or [b]median (25th–75th percentile).

[c]Analysis of covariance with age as a covariate; Jonckheere-Terpstra test for ordered alternatives (↓ = decreasing, ↑ = increasing trend).

[d]The data are in the median (25th–75th percentile) format.

$^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

of visceral proteins (albumin, prealbumin, cholinesterase) and increased concentrations of CRP and tumor markers: CA 19-9, CA 72-4, and carcinoembryonal antigen (Table 2).

In 40 patients from the PC group, DM2 was present in more than half of the cases (57%) lasting less than 3 yr. We found higher levels of plasma glucose in the PC group. HOMA-IR index and concentrations of insulin and C-peptide were similar in both groups. Analyses of parameters of lipid metabolism in the PC group revealed elevated TAG, free FA and apo B, as well as a lower concentration of apo A-I. No differences were noted in the values of TC, HDL-C, LDL-C and conjugated dienes in LDL particles (Table 2). There were no links of plasma lipid (lipoproteins, respectively) parameters to the stages of PC.

The plasma fatty acid profile in main lipid classes (PL, TAG, and CE) is shown in Table 3. The patients with PC had a decreased sum (Σ) of n-6 PUFA in PL and CE, which was accompanied with an increase in the sum of monoenoic fatty acids (MUFA) in PL, CE as well as TAG together with a decreased content of Σn-3 PUFA in PL and TAG. As for individual fatty acid composition in the PC group, we observed elevations of palmitoleic acid (POA; 16:1n-7) in both PL and CE, oleic (OA; 18:1n-9) in PL only, and CE and TAG and vaccenic (VA; 18:1n-7) acids in both PL and CE. On the contrary, lower contributions to the total FA sum were proved for LA in PL and CE, α-linolenic (ALA; 18:3n-3) in all lipid classes, whereas a drop in eicosapentaenoic acid (EPA; 20:5n-3) was seen in PC and TAG. The changes in the activities of delta desaturases (DD), estimated as the (product/substrate) ratio of respective fatty acids, are presented in Table 3. We proved a raised activity index of SCD1 for both stearic (18:0) and palmitic (16:0) acids, which was significant in PL and TAG (in CE only SCD1 for 18:0). The consistent result is the increase in activity index for delta 5 desaturase (D5D), demonstrable in all lipid classes, whereas a decrease of D6D activity index was seen only in TAG.

In Table 4, the trends between PC staging and composition of FA in plasma PL are presented. A negative trend was detected between the concentration of ALA, DHGLA, EPA, PUFA n-3 and PC staging (all $P < 0.05$). We did not observe any consistent changes in FA profiles between the subgroups of PC divided according to the presence/absence of DM (data not shown). In

TABLE 3
Fatty acid profiles in plasma main lipid classes

	Phospholipids		Triacylglycerols		Cholesteryl esters	
Fatty acid	CON	PC	CON	PC	CON	PC
16:0[a]	29.70 ± 1.20[b]	33.05 ± 2.93***	26.73 ± 3.30	26.80 ± 2.10	10.26 ± 2.00	10.24 ± 2.70
16:1n-7	0.49 ± 0.15	0.69 ± 0.35***	3.30 ± 1.01	3.42 ± 1.03	2.93 ± 0.99	3.69 ± 1.36**
18:0	13.58 ± 1.10	11.77 ± 1.61***	3.44 ± 0.83	2.78 ± 0.74***	0.64 ± 0.29	0.65 ± 0.33
18:1n-9	10.05 ± 1.13	12.15 ± 2.21***	40.35 ± 4.72	42.96 ± 2.89***	19.40 ± 2.60	21.46 ± 2.89***
18:1n-7	1.47 ± 0.22	1.92 ± 0.36***	2.47 ± 0.40	2.62 ± 0.35	1.19 ± 0.33	1.47 ± 0.32***
18:2n-6	23.09 ± 2.24	19.43 ± 3.07***	16.16 ± 4.95	15.02 ± 2.57	56.58 ± 4.32	51.24 ± 4.98***
18:3n-6	0.08 ± 0.03	0.07 ± 0.04*	0.29 ± 0.14	0.19 ± 0.10***	0.69 ± 0.31	0.68 ± 0.36
18:3n-3	0.20 ± 0.07	0.13 ± 0.04***	0.84 ± 0.34	0.57 ± 0.20***	0.52 ± 0.17	0.38 ± 0.12***
20:3n-6	3.07 ± 0.70	2.77 ± 0.87*	0.31 ± 0.17	0.28 ± 0.15	0.65 ± 0.17	0.69 ± 0.17
20:4n-6	11.37 ± 1.54	11.62 ± 2.81	1.51 ± 0.69	1.50 ± 0.52	5.42 ± 2.38	7.47 ± 3.18***
20:5n-3	1.07 ± 0.61	0.55 ± 0.26***	0.22 ± 0.19	0.12 ± 0.06***	0.28 ± 0.24	0.27 ± 0.18
22:6n-3	3.48 ± 0.86	3.59 ± 1.05	0.63 ± 0.61	0.51 ± 0.22	0.17 ± 0.12	0.30 ± 0.19***
ΣSFA	43.62 ± 0.98	45.10 ± 2.82**	32.11 ± 4.29	31.06 ± 2.41	11.65 ± 2.08	11.69 ± 2.61
ΣMUFA	12.25 ± 1.26	15.03 ± 2.71***	47.14 ± 4.96	49.95 ± 3.15***	23.92 ± 3.39	27.15 ± 3.68***
ΣPUFA n-6	38.46 ± 1.69	34.74 ± 3.84***	18.73 ± 5.48	17.46 ± 2.78	63.44 ± 4.91	60.18 ± 5.37**
ΣPUFA n-3	5.67 ± 1.35	5.12 ± 1.37*	2.02 ± 1.04	1.53 ± 0.44***	0.99 ± 0.46	0.99 ± 0.39
D9D-16[c]	0.017 ± 0.005	0.021 ± 0.009**	0.124 ± 0.037	0.128 ± 0.037	0.293 ± 0.098	0.390 ± 0.174***
D9D-18[d]	0.746 ± 0.120	1.065 ± 0.332***	12.52 ± 3.65	16.35 ± 4.14***	33.06 ± 8.54	39.58 ± 15.56**
D6D n-6[e]	0.004 ± 0.002	0.004 ± 0.002	0.018 ± 0.007	0.013 ± 0.008***	0.012 ± 0.006	0.014 ± 0.008
D6DE n-6[f]	0.135 ± 0.039	0.146 ± 0.052	0.020 ± 0.010	0.019 ± 0.010	0.012 ± 0.004	0.014 ± 0.004**
D5D n-6[g]	3.87 ± 0.96	4.55 ± 1.69**	5.02 ± 1.36	5.99 ± 2.16***	8.39 ± 3.60	11.49 ± 5.76***

Statistical analysis was performed with analysis of covariance (with age as covariate). Σ, sum; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA n-6, polyunsaturated fatty acids of n-6 family; PUFA n-3, polyunsaturated fatty acids of n-3 family. Only relevant fatty acids are presented.

[a]Shorthand notation of fatty acids—number of carbon atoms: number of double bonds, n = number of carbon atoms from methyl end to the nearest double bond.

[b]The data are presented as a mean ± SD (mol%).

[c]16:1n-7/16:0, Δ9 desaturase.

[d]18:1n-9/18:0, Δ9 desaturase.

[e]18:3n-6/18:2n-6, Δ6 desaturase.

[f]20:3n-6/18:2n-6, Δ6 desaturase+elongase.

[g]20:4n-6/20:3n-6, Δ5 desaturase.

*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

the PC group, proportion of MUFA and SCD1 index correlated negatively with cholinesterase (r = −0.551 and r = −0.483, both $P < 0.001$, Spearman rank order coefficients), albumin (r = −0.579 and r = −0.476, both $P < 0.001$), prealbumin (r = −0.333 and r = −0.223, both $P < 0.01$) and positively with CRP level (r = 0.197 and r = 0.225, both p < 0.05). Correlations of dihomo-γ-linolenic acid (DHGLA) with these variables were opposite (with cholinesterase r = 0.473, albumin r = 0.499, prealbumin r = 0.375, and with CRP r = −0.407, albumin r = 0.499, and with prealbumin 0.375, all $P < 0.001$).

The preliminary results indicate that better prognosis (survival >100 days at the time of the diagnosis) is in our patients with PC connected with higher content of EPA (0.49 ± 0.17 vs. 0.61 ± 0.32 mol%, $P = 0.05$; <100 days vs. >100 days, $n = 70$, unpaired t-test), DHA (3.30 ± 1.00 vs. 3.83 ± 1.13 mol%, $P = 0.04$) as well as lower SCD1 index (1.18 ± 0.41 vs.1.01 ± 0.28, $P = 0.05$) in plasma PL. These 2 subgroups did not differ in tumor staging and presence of severe/moderate malnutrition. Moreover, the SCD1 index in phospholipids is negatively correlated with time of the survival in PC patients ($n = 70$, Spearman rank coefficient = −0.356, $P < 0.01$).

DISCUSSION

It is known that PC belongs to the tumors with the highest incidence of malnutrition, which is induced by diminished food intake and higher resting energy expenditure (34). Significant depletion of adipose tissue represents a hallmark of metabolic

TABLE 4
Tumor stage and plasma phospholipids fatty acids

Fatty acid	Stage II	Stage III	Stage IV	*P* (trend)[b]
16:0	33.12 (31.64–33.66) [a]	32.75 (31.09–34.13)	33.04 (32.14–34.74)	NS
16:1n-7	0.61 (0.51–0.82)	0.60 (0.48–0.78)	0.56 (0.47–0.82)	NS
18:0	12.08 (11.14–12.48)	11.68 (10.59–13.04)	11.72 (10.92–12.88)	NS
18:1n-9	11.97 (10.36–13.23)	11.61 (10.88–13.52)	12.05 (10.61–13.00)	NS
18:1n-7	1.99 (1.68–2.18)	1.87 (1.65–2.15)	1.93 (1.70–2.05)	NS
18:2n-6	19.18 (16.67–21.94)	19.61 (18.28–21.89)	19.61 (18.14–20.83)	NS
18:3n-6	0.05 (0.05–0.06)	0.07 (0.05–0.09)	0.07 (0.04–0.10)	NS
18:3n-3	0.13 (0.11–0.16)	0.13 (0.10–0.15)	0.11 (0.09–0.14)	0.029↓
20:3n-6	2.72 (2.30–3.46)	2.95 (2.38–3.52)	2.40 (1.94–3.10)	0.033↓
20:4n-6	11.11 (9.19–14.51)	11.67 (9.92–12.70)	12.40 (10.07–13.76)	NS
20:5n-3	0.66 (0.44–0.76)	0.53 (0.40–0.70)	0.45 (0.37–0.62)	0.026↓
22:5n-3	0.92 (0.78–1.17)	0.92 (0.67–1.01)	0.78 (0.70–1.03)	NS
22:6n–3	3.80 (3.18–4.83)	3.48 (2.89–4.22)	3.49 (2.78–4.30)	NS
ΣSFA	44.83 (44.15–45.66)	44.56 (43.73–45.23)	45.16 (44.03–46.12)	NS
ΣMUFA	14.50 (13.22–16.20)	14.33 (13.38–16.27)	14.58 (12.78–15.93)	NS
ΣPUFA n-6	35.71 (32.90–36.80)	36.08 (33.47–37.09)	35.37 (33.30–36.53)	NS
ΣPUFA n-3	5.53 (4.88–6.38)	5.25 (4.22–5.92)	4.86 (3.97–5.78)	0.050↓
D9D-16[a]	0.018 (0.016–0.025)	0.019 (0.015–0.024)	0.017 (0.014–0.025)	NS
D9D-18[b]	0.970 (0.901–1.045)	0.976 (0.900–1.125)	1.034 (0.883–1.135)	NS
D6D n-6[c]	0.002 (0.002–0.003)	0.004 (0.002–0.005)	0.003 (0.002–0.005)	NS
D6DE n-6[d]	0.142 (0.109–0.199)	0.153 (0.119–0.177)	0.115 (0.098–0.165)	0.021↓
D5D n-6[e]	4.56 (3.26–5.55)	3.79 (3.08–4.95)	4.57 (3.68–6.95)	0.045↑

Σ, sum; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA n-6, polyunsaturated fatty acids of n-6 family; PUFA n-3, polyunsaturated fatty acids of n-3 family. Only relevant fatty acids are presented.

[a]The data are in the median (25th–75th percentile) format. 16:1n-7/16:0, Δ9 desaturase.

[b]Jonckheere-Terpstra test (↓ = decreasing, ↑ = increasing trend). 18:1n-9/18:0, Δ9 desaturase.

[c]18:3n-6/18:2n-6, Δ6 desaturase.

[d]20:3n-6/18:2n-6, Δ6 desaturase + elongase.

[e]20:4n-6/20:3n-6, Δ5 desaturase.

changes associated with cancer as well as with cancer cachexia. It was demonstrated that loss of body fat occurs before losing protein mass. Furthermore, it has been shown that adipose tissue depletion starts from the trunk followed by adipose tissue in lower and upper extremities (35). Anthropometric data found in our PC patients (decreased BMI without changes in lean body mass and midarm muscle circumference, decreased fat mass, and increased subscapularis to triceps skinfold ration) are consistent with these findings (35,36). The presence of cancer and/or cancer cachexia is associated with lipid metabolism changes that include reduction of fat mass, increased lipolysis and fatty acid oxidation connected with decreased lipogenesis. Observed changes of increased concentrations of TAG, FFA, apo B, accompanied with a decrease in HDL-C and apo A-I are consistent with cancer-induced dyslipidemia. Increased levels of hormone sensitive lipase mRNA and enzyme, which were detected in cancer patients led to increased hydrolysis of TAG and FFA turnover. Cancer-related dyslipidemia (hyperTAG, hyper apo B, hypo HDL-C, and hypo apo A-I) found in some cancers (colorectal, breast, endometrial, and pancreatic) (37–39) is similar to atherogenic dyslipidemia specific for metabolic syndrome (MS). Interestingly, both obese (40) and cancer cachectic (41) patients have increased intramyocellular lipid content, which may be related to the changes in energy balance. Fouladiun et al. found that body fat was lost more rapidly than lean tissue in progressive cancer cachexia, a phenomenon that was related highly to alterations in the levels of circulating hormones (insulin, leptin, ghrelin) and food intake (42).

We observed decreased levels of LA, ALA, and EPA in the PC group. With regard to the fact that the patients with PC did not exhibit increased concentrations of conjugated dienes in LDL particles, a marker of lipoperoxidation (43), it can be concluded that the observed decreased proportions of LA, ALA, and EPA are not caused by the systemic oxidative stress and/or lipoperoxidation but more likely by lowered dietary intake of these FA. The decreased LA, ALA, and EPA content can be also induced by malabsorption (44), which is highly probable in PC, and cigarette smoking as well as alcohol consumption (45).

Pratt et al. (46) reported in patients with advanced cancer a low content of total essential fatty acids, as well as decrease of ALA and LA in plasma PL regardless of total caloric or total fat intake. The effect of genetic and gender background on the fatty acid profile also cannot be excluded (47,48). Closer examination of the correlations between PUFA content and weight loss (mainly resulting from fat loss) revealed only a negative relationship with DHGLA. The lack of these correlations with essential fatty acids is not clear; the data about FA content in the fat tissue in cancer patients is scarce. In a study of patients with colorectal carcinoma published by Neoptolemos (49), it was found that in the healthy individuals, the adipose tissue LA content correlated well with the respective content in erythrocytes, whereas in the cancer group the correlation was missing. Another study described the negative relationship of dietary intake of animal fat with the PUFA/saturated fatty acids (SFA) ratio and C18 PUFA content in adipose tissue in endometrial cancer (50).

An important and consistent finding of this study is the increased composition of the $\sum$ of MUFA, because of elevated proportions of POA (16:1n-7), OA (18:1n-9), and VA (18:1n-7). The patients with PC were shown to have increased activities of desaturation of palmitate (16:0) and stearate (18:0), which implicate the increased activity of SCD1 (e.g., Δ9-desaturase activity) as well as D5D [e.g., fatty acid desaturase-1 (FADS1)]. The enzyme SCD1, which is, under physiological state, predominantly expressed in the liver, catalyzes the synthesis of monounsaturated long-chain FA from fatty acid acyl-CoA. The preferred substrates for SCD1 are stearoyl (18:0), and palmitoleyl (16:0) CoA, which are converted to oleolyl-CoA (18:1n-9) and palmitoleoyl-CoA (16:1n-7).

Increased activity of SCD1 is usually observed in various cancers. Aggressively growing tumors are characterized by an elevated synthesis of FA de novo and accelerated transformation of SFA to MFA, which is combined with synthetic (FAS) and desaturation (SCD1) activities. FAS is a multifunctional enzymatic complex that synthesizes palmitic acid (C16:0) from acetyl-CoA and malonyl-CoA.

SCD1, the isoform that is expressed in the liver, is an enzyme synthesizing MUFA: POA (16:1n-7) and OA (18:1n-9) from palmitic (C16:0) and stearic acids (18:0). POA and OA are key substrates for the formation of complex lipids (e.g., PL, CE, TG and waxes) (17). Increased content of POA was in several studies linked to higher level of lipogenesis (51). Activities of SCD1, which closely correlated with the OA/SA ratio, were found to be higher in several types of tumors (52). In our PC group, a higher OA/SA ratio was found that implicated a higher activity of SCD1 in these patients. Moreover, in all lipid classes analyzed, there was also higher content of vaccenic acid (18:1n-7), which is the known to be elongation product POA (16:1n-7). Our results are consistent with a study describing an increased ratio of 18:1 to SA (18:1n-9 + 18:1n-7/18:0) in patients with PC (53). FAS as well as SCD1 has elevated activities in cancer cells, where the de novo synthesis is important for cell membrane synthesis, membrane remodeling, and proliferation (15,17,54). In states with an absolute and relative lack of PUFA (n-6 and/or n-3), SCD1 is necessary for maintenance of cellular lipid homeostasis, because it keeps the synthesis of MUFA that is essential for complex lipid formation (17). Selective inhibition of SCD1 with cerulenin (natural mycotoxine) shortens the lifespan of human cancer cells (54). An experimental model of hepatocellular carcinoma in rats and mice has proven a higher expression of SCD1 (55). Women with a decreased ratio of MUFA in plasma phospholipids (as a surrogate marker of SCD1 activity) who were supposed to have a lower activity of SCD1, revealed decreased risk for breast cancer (56). In this study, we also analyzed the relationship of FA and tumor staging of PC; however, we did not find significant changes for the content of MUFA as well as SCD1 indices. Nevertheless, we observed trends for a decreased content of PUFA n-3, ALA, EPA, and DHGLA with an increased burden of disease. The lower content of plasma PUFA n-3 was found in some types of cancer (53) with its further loss pointing at a worse prognosis (57).

In the PC group, we proved higher SCD1 and D5D indices as well as both desaturation activity indices of palmitate (16:1-7/16:0) and stearate (18:1n-9/18:0). In an earlier study with metabolic syndrome patients, we found only higher desaturation index of palmitate (16:1-7/16:0), together with higher D6D and lower D5D indices (47). Because stearate is preferred to palmitate as a substrate of SCD1 this finding could be explained by dilution of products: POA (16:1-7) and OA (18:1n-9) by dietary fatty acids. Dietary lipids contain only small amounts of POA, whereas OA is the most abundant dietary FA. This finding could be explained by decreased intake of dietary lipids in the patients with PC. Therefore, the phenomenon of dilution of products (POA, OA) could not operate in the PC patients. As compared to MS, we did not find decreased activity of D6D. Increased activities of D6D have been ascribed to hyperinsulinemia and increased BMI and generally considered as a characteristic feature of IR. On the other hand, decreased D5D activities, an important feature of MS, was shown not to be dependent on BMI and on physical activity (32).

Our previous results indicated that in PC, the condition connected with high inflammatory response and low concentrations of the inhibitor of SCD1, leptin (58), the inhibition of SCD-1 concomitantly with anti-inflammatory intervention could be possible therapeutic strategy, as it was suggested for MS (59). Both the experimental animal studies and clinical data proved the protective effects of n-3 PUFA in prostate, breast, and colorectal cancer (60,61). We have observed the positive relationship of EPA and DHGLA (20:3n-6), on one hand, and the concentrations of visceral proteins and cholinesterase on the other, suggesting the beneficial effects of these FA on the protein metabolism.

In newly referred patients with nonsmall cell lung cancer, intervention with fish oil compared with standard of care led to increased or maintained muscle mass. Moreover, an increasing concentration of EPA positively correlated with

muscle gain (62). In patients with advanced PC, supplementation with n-3 PUFA led to weight gain, an increase in LBM, and changes in plasma EPA correlated positively with changes in body weight and LBM (63). EPA may support the anabolic potential of muscle acting against the insensitivity of skeletal muscle of cancer patients to insulin (64). Supplementation with EPA could attenuate muscle degradation by decreasing the expression of proteasome subunits, which are elevated in cancer cachexia (31), or by downregulating the acute-phase response.

High levels of CRP in the PC group point to the advanced stages of the disease with the invasions to the lymphatic nodes and peritoneum. The negative correlations of CRP with DPA (C22:5n-3) and DHGLA (C20:3n-6) implicate the therapeutic potential of these PUFA. The correlation of DHGLA exhibits a significant negative trend, which is dependent of the degree of malnutrition.

The limitations of the study include the estimation of the activities of desaturases with the help of substrate/product ratio, because the activities were not analyzed from the tissue biopsy because of ethical reasons. On the other hand, the literature data advocate the usage of the ratios in the PL lipid class. We also did not observe any patient at stage I. The number of the patients in the subgroups of PC was low, but sufficient for determining the effect according to the power analyses. Moreover, according to our knowledge, the study has included the highest number of the patients suffering from pancreatic ductal adenocarcinoma with determined FA profile in PL lipid class so far.

CONCLUSION

In conclusion, a specific plasma esters FA profile in patients with PC was described. In comparison with control subjects, patients with PC revealed increased concentrations of monounsaturated FA. These changes were associated with increased index of SCD1. Moreover, decreased concentrations of LA, ALA, and EPA were found in plasma lipid esters of PC patients, and these changes are probably caused by a lower intake of dietary fat. Positive correlations between levels of visceral proteins and concentrations of EPA and DHGLA were found. On the contrary, these FA negatively correlated with concentration of CRP. Index of SCD-1 in PL correlated negatively with survival time of the patients. Moreover, longer survival of the patients was connected with higher content of EPA, DHA, and with lower index of SCD-1 activity, respectively. Proportions of PUFA n-3 displayed a negative trend with tumor staging, whereas the positive trends of MUFA, SCD1, and on the degree of malnutrition as well as the negative trend of DHGLA content to the extent of malnutrition were found. The changes in FA profile implicate pathophysiological mechanisms responsible for disturbed FA metabolism in cancer patients and indicate the importance of appropriate nutritional support.

ACKNOWLEDGEMENTS

The study was supported by the grant IGA NS 9769-4, Ministry of Health, and Research project of Charles University in Prague, 1st Faculty of Medicine—PRVOUK—P25/LF1/2, Czech Republic.

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Klin. Biochem. Metab., 24 (45), 2016, No. 2, p. 88–93.

Changes in Paraoxonase 1 activity and concentration of conjugated dienes in connection with number of metabolic syndrome components

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SUMMARY

Objective: Paraoxonase 1 is an antioxidant enzyme with a variety of physiological roles – one of them is the inhibition of LDL (low-density lipoprotein) lipid peroxidation and inactivation of LDL-derived oxidized phospholipids. The aim of this study was to investigate the PON1 activity and levels of conjugated dienes in precipitated LDL (CD/LDL) in subjects, who fulfill different numbers of metabolic syndrome (MetS) criteria.

Design: Cross-sectional study

Settings: 4th Department of Internal Medicine, 1st Faculty of Medicine, Charles University and General University Hospital Prague

Material and methods: The population under study consisted of 354 Caucasian subjects (188 females/166 males) divided into 6 groups according to the number of presented components of metabolic syndrome. All groups were age matched. The activity of paraoxonase 1 and concentration of conjugated dienes in precipitated LDL (CD/LDL) were both assessed spectrophotometrically.

Results: The activity of PON1 was significantly decreased in subjects with all 5 components of MetS in comparison with those with 0 to 3 components of MetS ($p < 0.05$). The concentrations of CD/LDL were increased in subjects with 4 or 5 components of MetS compared to subjects with 0-3 components of MetS ($p < 0.001$).

Conclusion: As was shown in this study, the levels of PON1 are extensively affected by the concentration of HDL-C and ApoA1. PON1 activity is depressed and CD/LDL levels are increased mainly in subjects who fulfill all five criteria of MetS.

Keywords: Paraoxonase 1, conjugated dienes, metabolic syndrome components.

SOUHRN

Staňková B., Vávrová L., Rychlíková J., Žák A.: Changes in Paraoxonase 1 activity and concentration of conjugated dienes in connection with number of metabolic syndrome components

Cíl studie: Paraoxonasa 1 (PON1) je antioxidační enzym s širokým spektrem fyziologických úloh – jednou z důležitých funkcí PON1 je její schopnost inhibovat LDL-lipidovou peroxidaci a inaktivovat oxidované fosfolipidy. Cílem této studie bylo sledovat aktivitu PON1 a hladinu konjugovaných dienů v precipitovaných LDL částicích (CD/LDL) u subjektů s různým počtem splněných kritérií metabolického syndromu (MetS).

Typ studie: Observační.

Název a sídlo pracoviště: IV. Interní klinika 1. Lékařské fakulty Univerzity Karlovy a Všeobecné fakultní nemocnice v Praze.

Materiál a metody: Do studie bylo celkem zařazeno 254 subjektů (188 žen/166 mužů) rozdělených do šesti skupin, a to podle počtu splněných kritérií (podle počtu přítomných komponent) metabolického syndromu. Všechny skupiny byly spárovány na základě věku. Hlavními sledovanými parametry byla aktivita PON1 a koncentrace CD/LDL. Měření obou těchto parametrů bylo prováděno spektrofotometrickými metodami.

Výsledky: Pacienti, kteří splňovali všech pět kritérií MetS měli signifikantně snížené aktivity PON1 ve srovnání se skupinami pacientů s žádným až třemi parametry MetS ($p < 0.05$). Hladina CD/LDL byla naopak u pacientů, kteří splňovali čtyři nebo všech pět kritérií MetS signifikantně zvýšena ve srovnání s ostatními skupinami ($p < 0.001$).

Závěr: Naše studie ukázala, že aktivita PON1 je ve značné míře ovlivňována koncentrací HDL-C a apoA1 a dále, že aktivity PON1 jsou snížené a naopak hladiny CD/LDL zvýšené hlavně u pacientů, kteří splňují všech pět kritérií metabolického syndromu.

Klíčová slova: paraoxonasa 1, konjugované dieny, komponenty metabolického syndromu.

Introduction

Paraoxonase 1 (PON1; EC 3.1.8.1.) is synthesized in the liver and secreted into the blood, where associates with HDL (high-density lipoprotein) particles [1]. In anchoring of PON1 to HDL through Apo-A1, the hydrophobic N terminus of PON1 is thought to be involved [2].

A variety of physiological roles have been proposed for PONs. Serum PON1 catalyzes the hydrolysis and thereby the inactivation of oxons like paraoxon, is also able to hydrolyze the nerve agents sarin and soman. In addition, PON1 hydrolyzes arylesters and different aromatic and aliphatic lactones as well as cyclic carbonates [3-6]. It was hypothesizes that the physiological substrates could be some derivatives of fatty acid oxidation process such as 5-hydroxy - 6E, 8Z, 11Z, 14Z -eicosatetraenoic acid (5-HETE) lactone that resides in HDL or some lactones which are consumed as food ingredients or drug metabolites. Furthermore was shown that homocysteine thiolactone is naturally occurring substrate of PON1 [7].

In vitro assays demonstrated that PON1 can inhibit LDL (low-density lipoprotein) lipid peroxidation and inactivate LDL-derived oxidized phospholipids. This could potentially reduce the serum content of the oxidized lipids involved in the initiation of atherosclerosis [8,9]. Furthermore was shown, that PON1 have also peroxidase-like activity [10,11] – it is capable to hydrolyze hydrogen peroxide, reduce lipoprotein peroxides (by 19 %) and cholesteryl linoleate hydroperoxides (by 90 %).

One of the most sensitive indicators of lipid peroxidation is supposed to be the concentration of conjugated dienes in precipitated LDL (CD/LDL) [12].

The aim of this study was to investigate the PON1 activity and levels of CD/LDL in subjects, who fulfil different numbers of metabolic syndrome (MetS) criteria. The main components of MetS are accumulation of intraabdominal fat, impaired metabolism of glucose, atherogenic dyslipidemia (low HDL cholesterol, hypertriacylglyceridemia) and arterial hypertension.

Materials and methods

Settings and subjects

This cross-sectional study was carried out at the 4th Department of Internal Medicine of the General University Hospital in Prague from January 2012 to July 2015. The study protocol was approved by the institutional review board and the Ethics Committee of the General University Hospital in Prague. Informed consent was obtained from all participants.

The population under study consisted of 354 Caucasian subjects (188 females/166 males) divided into 6 groups according to the number of presented components of metabolic syndrome. All groups were age matched.

For the definition of metabolic syndrome components the criteria of the International Diabetes Federation [13] were used: central obesity (waist circumference ≥ 94 cm for men and ≥ 80 cm for women), raised TG level (≥ 1.7 mmol/l), reduced HDL-C (< 1.03 mmol/l in males and < 1.29 mmol/l in females), or specific treatment for these abnormalities, raised blood pressure (BP): systolic BP ≥ 130 (16.25kPa) or diastolic BP ≥ 85 mmHg (10.625kPa), or treatment of previously diagnosed hypertension, raised fasting plasma glucose (≥ 5.6 mmol/l), or previously diagnosed type 2 of diabetes mellitus.

Exclusion criteria for all groups were the following: current antioxidant therapy kidney disease (creatinine > 150 μmol/l), clinically manifest proteinuria (urinary protein > 500 mg/l), and liver cirrhosis, malignancies, chronic immunosuppressive and anti-inflammatory therapy, as well as chemotherapy. Further criteria for exclusion were: contraception, acute and chronic pancreatitis; heart insufficiency (NYHAIII/IV), unstable angina pectoris, stage within 1 year after acute myocardial infarction, respectively coronaro-aorto bypass grafting, or percutaneous coronary intervention, and stroke.

Blood samples

Blood samples were collected after a 12 hour overnight fast, puncturing a peripheral vein. All samples were marked with unique anonymized identification numbers, merging data only after assays had been completed. All parameters were assessed in serum. Serum was prepared following coagulation in vacutainer tubes; by centrifugation at 3500 rpm at 4 °C for 10 min. Samples were stored at -80 °C until the assay.

Methods

The arylesterase activity of PON1 was measured according to the method of Eckerson et al. (1983) [14] using phenylacetate as a substrate. The rate of phenol generation was monitored at 270 nm. Blank was run for each sample. Arylesterase activity of PON1 was calculated using the molar extinction coefficient of the produced phenol, 1310 M-1cm-1 and expressed as U/ml serum (U = μmol/min), as described earlier [15].

The concentration of CD in precipitated LDL was determined by the modified spectrophotometric method of Wieland at 234 nm [16,17]. All routine clinical tests were measured in Institute for Clinical Biochemistry and Laboratory Diagnostics of General University Hospital in Prague.

The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as HOMA-IR = [fasting serum glucose (mmol/l) * fasting serum insulin (μU/ml)]/22.5 [18].

Statistical analysis

Data are expressed as mean and standard deviation or median (25th-75th percentile) for data different from normal distribution. Normality of the distribution was tested by the Shapiro-Wilks W test. Comparisons between the groups were carried out by one-way ANOVA with Newman-Keuls post test. Kruskal-Wallis ANOVA was used for non-parametric comparisons. Spearman correlation coefficients were used for correlation analyses. All analyses were performed using version 12.0 of StatSoft Statistica software (2013, CZ). The p value < 0.05 was considered statistically significant.

Results

Table 1 summarizes basic clinical and biochemical data of the studied groups. As shown in Table 1 with increasing number of presented components of metabolic syndrome, the increase in levels of BMI, waist circumference, triacylglycerols, glucose, insulin and HOMA-IR and decrease in HDL-C and ApoA1 levels were observed.

The activity of PON1 was significantly decreased in subjects, who fulfill all 5 components of MetS in comparison with those, who fulfil 0 to 3 components of MetS. The concentrations of conjugated dienes in precipitated LDL were increased in subjects with 4 or 5 components of MetS compared to subjects with 0-3 components of MetS (Fig. 1).

Table 1. Basic characteristic of studied groups

MetS components	0	1	2	3	4	5
N (M/F)	22/31	21/45	44/54	48/36	20/16	11/6
Age (years)	54.5 ± 9.2	58.2 ± 11.2	57.7 ± 13.2	57.16 ± 11.7	60.0 ± 10.0	54.7 ± 8.6
Hypertension (N, %)	0 (0%)	19 (28.8%)	63 (64.3%)	66 (78.6%)	32 (88.9%)	17 (100%)
DM2/IFG (N)	0/0	1/1	7/6	16/29	14/17	9/8
Obese (N, %)	0 (0%)	44 (66.7%)	91 (92.9%)	83 (98.8%)	36 (100%)	17 (100%)
BMI (kg/m^2)	23.3 ± 2.8***,+++,000,bbb,x	25.6 ± 3.4***,+++,000,bbb	29.6 ± 5.6***,+	30.5 ± 5.0***	32.0 ± 4.3**	34.6 ± 5.5
Waist (cm)	78.9 ± 6.9***,+++,000,bbb,xx	87.2 ± 10.2***,+++,000,bbb	97.7 ± 12.5***,++,0	103.5 ± 11.5***	106.2 ± 13.6**	114.2 ± 13.8
TC (mmol/l)	5.18 (4.80-5.72)	5.33 (4.64-6.05)	5.12 (4.47-5.94)0	5.75 (5.05-6.82)*	5.04 (4.23-5.93)	4.55 (3.58-5.51)
TG (mmol/l)	0.91 (0.74-1.18)***,+++,000,bbb	0.9 (0.7-1.1)***,+++,000,bbb	1.2 (1.0-1.7)***,+++,000	2.0 (1.4-3.0)	2.2 (1.7-2.9)	2.4 (2.1-3.0)
HDL-C (mmol/l)	1.68 ± 0.32***,+++,000,bbb	1.77 ± 0.45***,+++,000,bbb	1.42 ± 0.31***,+++	1.35 ± 0.36***,++	1.12 ± 0.18**	0.90 ± 0.17
LDL-C (mmol/l)	3.23 ± 0.88**	3.17 ± 0.90**	3.26 ± 1.12**	3.39 ± 1.02***	2.99 ± 0.91**	2.38 ± 0.88
ApoA1 (g/l)	1.48 ± 0.22***,+++,x	1.63 ± 0.32***,+++,00,bbb	1.40 ± 0.27***,++	1.42 ± 0.33***,++	1.22 ± 0.23	1.11 ± 0.21
Glucose (mmol/l)	4.92 ± 0.40***,+++,00	4.94 ± 0.61***,+++,00	5.14 ± 0.86***,+++,0	5.8 ± 1.82***,++	6.52 ± 1.6***	7.64 ± 2.99
Insulin (U/l)	6.3 (4.5-8.7)***,+++,000,bb	7.4 (5.1-8.8)***,+++,000,b	9.5 (6.3-13.5)**,+,0	12.3 (8.5-18.4)	13.3 (10.2-17.3)	21.3 (11.4-23.8)
HOMA-IR	1.3 (1.0-2.0)***,+++,000,b	1.6 (1.1-1.9)***,+++,000	1.9 (1.3-3.1)**,+++,0	2.9 (1.8-4.1)	3.4 (2.8-5.4)	5.0 (3.2-7.2)

MetS: Metabolic syndrome, DM2: diabetes mellitus type 2, IFG: increased fasting glucose; BMI: body mass index, TC: total cholesterol, TG: triacylglycerols, HDL-C high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol; Data presented as mean ± standard deviation (S.D.) for parametric and median (IQR) for non-parametric variables; * 5 vs. 4,3,2,1,0 MetS-components; + 4 vs. 3,2,1,0 MetS-components; 0 3 vs. 2,1,0 MetS-components; b 2 vs. 1,0 MetS-components; x 1 vs. 0 MetS-components; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

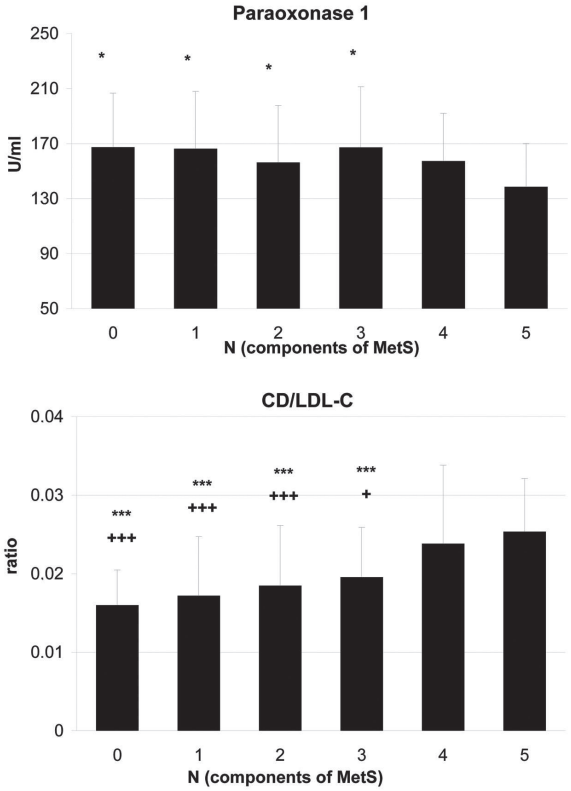

Fig. 1. Activity of Paraoxonase 1 and concentration of Conjugated dienes in precipitated LDL

MetS: Metabolic syndrome; CD: conjugated dienes, LDL-C: low density lipoprotein cholesterol; * 5 vs. 4, 3, 2, 1, 0 MetS-components; + 4 vs. 3, 2, 1, 0 MetS-components; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

We have also studied, if obesity or hypertension influences the levels of PON1 and CD/LDL; subjects with no component of MetS were compared with subjects with only one component (obesity or hypertension) of MetS. There was no difference between obese and non-obese subjects in activity of PON1 (163.4 ± 42.3 vs. 167.3 ± 39.2 U/ml; $p = 0.63$) or in concentration of CD/LDL (0.0177 ± 0.008 vs. 0.016 ± 0.004; $p = 0.22$). And also no difference in PON1 activity (167.2 ± 38.8 vs. 169.2 ± 40.0 U/ml; $p = 0.85$) or CD/LDL levels (0.0155 ± 0.0045 vs. 0.0162 ± 0.0045; $p = 0.55$) between subjects with and without hypertension was observed.

Strong correlation between PON1 activity and concentrations of HDL-C ($r = 0.331$; $p < 0.001$), ApoA1 ($r = 0.334$; $p < 0.001$) and TC ($r = 0.324$; $p < 0.001$) was observed. The levels of conjugated dienes correlated with concentrations of TC ($r = -0.388$; $p < 0.001$) TG ($r = 0.304$; $p < 0.001$) and HDL-C ($r = -0.303$; $p < 0.001$).

Studied subjects were also divided into 3 groups according to the levels of HDL-C and ApoA1. As shown in Fig. 2, the activity of PON1 was the lowest in subjects with depressed levels of both HDL-C and ApoA1 (H+A) and the highest in subjects with normal levels of both HDL-C and ApoA1 (N).

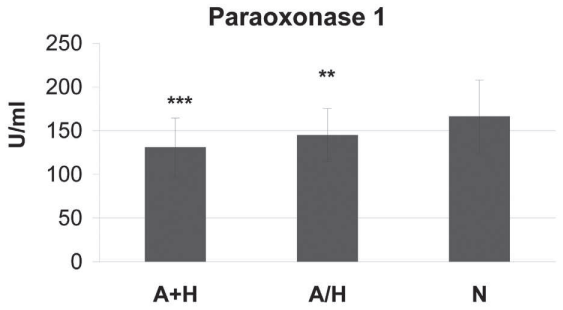

Fig. 2. Activity of Paraoxonase 1 according to the concentration of HDL-C and ApoA1

A+H: subjects with decreased levels of both HLC-C and ApoA1, (n = 21); A/H: subjects with decreased levels of only HLC-C or ApoA1, (n = 44); N: subjects with normal levels of both HDL-C and ApoA1 (n = 289); * N vs. A+H or A/H, * $p < 0.05$, *** $p < 0.001$

Discussion and Conclusion

The arylesterase activity of PON1 and levels of CD/LDL were measured in subjects with different numbers of presented components of MetS. Decreased activity of PON1 was found in subjects with 5 presented components of MetS compared to subjects with fewer components of MetS. The levels of CD/LDL were increased in subjects with 5 and 4 presented components of MetS in comparison with subjects with fewer components of MetS.

In our previous study we have investigated the activities of PON1 in subjects with metabolic syndrome compared with healthy controls [19] and found decreased PON1 activity and increased concentration of conjugated dienes in subjects with MetS compared to healthy controls. Also in other studies [20-23] decreased PON1 activity in MetS patients was observed. However in studies of Tabur et al. (2010) [24], Yilmaz et al. (2010) [25] and Lagos et al. (2009) [26] equivalent levels of PON1 in MetS patients and in CON were found. As was shown in this study PON1 activity is depressed mainly in subjects who fulfill all five criteria of MetS, in subjects with severe form of MetS. These findings could help to explain the inconsistent results in PON1 activity in MetS subjects.

Strong positive correlation between PON1 activity and HDL and ApoA1 concentrations were found in our studied subjects. When the subjects were divided into 3 groups according to the levels of HDL-C and ApoA1, it was shown, that subjects with decreased levels of both HDL-C a ApoA1 have the lowest PON1 activity, whereas subjects with normal levels of both HDL-C and ApoA1 the highest PON1 activity among the studied subjects. It could be hypothesize, that changes in composition of HDL influence the activity and function of PON1.

Several mechanisms are supposed to decrease PON1 activity, not only the changes in HDL composition. It was shown, that increased oxidative stress connected with elevated levels of oxidized LDL cause inactivation of PON1. Oxidized LDL appears to inactivate PON1 through interactions between the enzyme's free

sulfhydryl group and oxidized lipids, which are formed during LDL oxidation [26]. Also in our study levels of markers of lipid peroxidation (CD/LDL) were increased in subjects with decreased PON1 activity.

Other reason for the decrease in PON1 activity could be the glycation of the enzyme, which takes place as was shown in diabetes mellitus [27]. The acute phase response could also lead to the decreased activities of PON1 which are caused by the down-regulation of liver PON1 mRNA [28].

This study has shown that the levels of PON1 are extensively affected by the concentration of HDL-C and ApoA1. PON1 activity is depressed and CD/LDL levels are increased mainly in subjects who fulfill all five criteria of MetS.

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Acknowledgement: This study and preparation of this manuscript was supported by the research project NT13199 of Ministry of Health of the Czech Republic

Do redakce došlo: 1. 2. 2016

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Title

Polymorphisms *rs2167444* and *rs508384* in the *SCD1* gene are linked with high apoB-48 levels and adverse profile of cardiometabolic risk factors [1]

Keywords

Apolipoprotein B-48, cardiometabolic risk factors, metabolic syndrome components, small-dense LDL, oxidative stress, fatty acid desaturases, *SCD1* polymorphisms.

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[1] **Funding**

Financial support from the Ministry of Health of the Czech Republic (research project RVO-VFN64165/2012) and the Ministry of Education, Youth and Sports of the Czech Republic (research project PRVOUK25/LF1/2 – Charles University, 1st Faculty of Medicine) is gratefully acknowledged.

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[3] **Abbreviations**: ALT – alanine aminotransferase, apoB-48 – apoprotein B-48, BMI – body mass index, BP – blood pressure, CM – chylomicrons, CMR – chylomicron remnants, DM – diabetes mellitus, D6D – delta 6 desaturase, D9D – delta 9 desaturase, FA – fatty acid, GGT – gamma-glutamyl transferase, HDL – high density lipoproteins, HOMA-IR – homeostatic index of insulin resistance, IDL – intermediate density lipoprotein, LDL – low density lipoproteins, MetS – metabolic syndrome, NEFA – non-esterified fatty acids, ox-LDL – oxidized LDL, PAD – peripheral artery disease, SCD1 – stearoyl-CoA desaturase-1, sd-LDL – small dense LDL, SNP – single nucleotide polymorphism, TG – triglycerides, TRL – triglyceride rich lipoproteins, VLDL – very low density lipoproteins, WC – waist circumference

Abstract

Background: Elevated plasma concentration of apolipoprotein B-48 (apoB-48) is an independent risk factor of cardiovascular disease. Stearoyl-CoA desaturase-1 (SCD1) is a rate-limiting lipogenic enzyme and a key regulator of fuel metabolism.

The aim of this study was to analyze associations between clinical, biochemical, and genetic factors and different apoB-48 levels in subjects at increased cardiometabolic risk.

Methods and Findings: We examined 220 subjects exhibiting at least one metabolic syndrome (MetS) component. In conjunction with basic clinical, anthropometric and laboratory measurements, we analyzed various polymorphisms of stearoyl-CoA desaturase-1 (*SCD1*). Subjects were divided into two groups according to median apoB-48 level: (1) high apoB-48 (≥7.9 mg/L, n=112) and (2) low apoB-48 (<7.9 mg/L, n=108); Neither group differed significantly in anthropometric measures. High plasma apoB-48 levels were associated with increased systolic blood pressure (+3%; P<0.05), MetS prevalence (59.8 vs. 32.4%; P<0.001), small-dense LDL frequency (46.4 vs. 20.4%; P<0.001), triglycerides (+97%; P<0.001), non-HDL-cholesterol (+27%; P<0.001), and lower concentrations of HDL-cholesterol (-11%; P<0.01). This group was further characterized by a higher HOMA-IR index (+54%; P<0.001) and increased concentrations of conjugated dienes (+11%; P<0.001) and oxidatively modified LDL (+38%; P<0.05). Lower frequencies of *SCD1* minor genotypes (*rs2167444*, *rs508384*, P<0.05) were observed in subjects with elevated plasma concentrations of apoB-48.

Conclusions: Elevated plasma concentrations of apoB-48 are connected with an adverse lipid profile, higher systolic blood pressure, insulin resistance and oxidative stress. Lower proportions of minor *SCD1* genotypes (*rs2167444*, *rs508384*) implicate the role of genetic factors in the pathogenesis of elevated levels of apoB-48.

Introduction

Apolipoprotein B-48 (apoB-48) is the specific structural component of chylomicrons (CM) and their remnants (CMR). In humans, the apoB-48 protein is synthesized only in enterocytes and co-linear with 2152 aminoterminal residues of apoB-100. ApoB-48 mRNA is synthesized as a result of post-transcriptional enzymatic deamination, whereby cytidine in the CAA codon (for glutamine) is exchanged for uracil, creating a premature stop codon (UAA) in an apoB RNA editing. The apoB mRNA editing enzyme consists of several factors. Among the most important of these are catalytic deaminase APOBEC-1 and APOBEC-1 complementation factor (Fazio and Linton, 2015). CM particles and CMR always contain one molecule of apoB-48. Accordingly, plasma concentrations of apoB-48 provide information about the number of lipoprotein particles of intestinal origin (Sakai et al., 2003; Nakajima and Tanaka, 2018).

Triglyceride-rich lipoproteins (TRL), such as CM and CMR as well as VLDL and their remnants (intermediate density lipoprotein, IDL), containing apoB-100, are important, independent risk factors of atherosclerotic cardiovascular disease (Nakajima and Tanaka, 2018). Many experimental and clinical studies have proven that CMR directly or indirectly correlate with initiation and progression of atherosclerosis due to accumulation in the arterial wall, enhancement of systemic inflammation, platelet activation, and thrombus formation. Remnant lipoproteins can directly penetrate the arterial wall, infiltrate the sub-endothelial space, and accelerate foam cell formation. Adverse effects of CMR increase instability and progression of atherosclerotic plaque (Masuda and Yamashita, 2017). ApoB-48 concentration correlates with intima-media thickness of the common carotid artery endothelial dysfunction and with coronary heart disease. Fasting serum apoB-48 levels are reported to be an independent predictor of peripheral artery disease (PAD) in patients with type 2 diabetes. From the several risk factors, such as LDL-cholesterol, HDL-cholesterol, HBA1c, diabetes mellitus (DM) duration, smoking, and systolic blood pressure, only apoB-48 has been identified as an

independent predictor of PAD (Chan et al., 2012;Lapice et al., 2012; Mancera-Romero et al., 2013). High fasting serum apoB-48 concentration is understood to be a strong, independent predictor of both new onset and chronic coronary artery disease , irrespective of LDL-cholesterol level (Masuda et al., 2012; Mori et al., 2013). ApoB-48 has been shown to be a marker associated with coronary lesion progression in post-percutaneous coronary intervention patients undergoing LDL-lowering therapy (Mori et al., 2013). Other studies report higher fasting apoB-48 concentrations in men compared to women, and high apoB-48 levels in subjects with obesity, dyslipidemia, MetS, hypothyroidism, chronic kidney disease , and DM type 2 (DM2T) (reviewed in Nakajima and Tanaka, 2018; Masuda and Yamashita, 2017).

Stearoyl-coenzyme A desaturase 1 (SCD1) (EC:1.14.19.1) is a main regulator of fuel metabolism, body weight control, and central lipogenic enzyme activity (Uto, 2016). In humans and other species, SCD1 is a rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids from their saturated fatty acid precursors. Two isoforms in the human genome (1 and 5) are mapped to chromosome 10q24.31. The isoform SCD1 is highly expressed in lipogenic tissues, mainly in liver and adipose tissue, while isoform SCD5 is primarily expressed in the brain and pancreas (Uto, 2016). SCD1 and delta-9 desaturase (D9D) mainly catalyze the conversion of palmitic (C16:0) and stearic (C18:0) acids to palmitoleic (C16:1n-7) and oleic (C18:1n-9) acids. (Al-Johani et al., 2017; Tan et al., 2015). SCD1 gene expression is regulated by several intrinsic (genetic, hormonal) and extrinsic factors (diet, alcohol consumption, smoking, physical activity) (Poloni et al., 2015; Kamal et al., 2018). Experimental studies in mice (either with naturally occurring *scd1* or transgenic *scd1* deletions) show that *scd1* deficiency leads to reduced body weight adiposity, increased insulin sensitivity, and resistance to diet-induced obesity. These studies also report upregulation of several genes controlling lipid oxidation and downregulation of lipid synthesis genes (Paton and Ntambi, 2009). On the other hand, over-expression of *SCD1* is associated with numerous metabolic disorders, such as

obesity, DM2T, arterial hypertension, dyslipidemia, and non-alcoholic fatty liver disease (Koeberle et al., 2016; Kamal et al., 2018).

To measure SCD1 activity, human studies use a surrogate marker D9D (ratios of palmitoleic (16:1n-7) to palmitic (16:0) acid or oleic (18:1n-9) to stearic (18:0) acid) using various biological matrices, such as plasma lipids, erythrocyte membranes, and adipose tissue. Increased activity of D9D (representing SCD1) has been reported in obesity-related conditions, such as DM2T, MetS, arterial hypertension, malignancies, and depressive disorders (Warensjö et al., 2008; Mahendran et al. 2014; Assies et al., 2010; Vareka et al., 2012). Fatty acid (FA) metabolism and composition are subject to genetic regulation and can be modified by many factors including gender, age, ethnicity, health status, and diet (Saponaro et al., 2015).

Several studies have shown that some gene polymorphisms in the SCD1 gene are connected with various pathological states. British authors found a borderline association between a single nucleotide polymorphism (SNP) of *SCD1* (*rs41290540*) and the risk of DM2T (Liew et al., 2004). Swedish authors reported an association of four SNPs of *SCD1* (*rs10883463, rs7849, rs2167444,* and *rs508384*) with body mass index (BMI), waist circumference (WC), and insulin sensitivity. Their rare allele carriers had lower BMI and waist circumference as well as improved insulin sensitivity, with the rare allele *rs7849* exerting the strongest effect on WC and insulin sensitivity (Warensjö et al., 2007). A large population-based study in Costa Rica proved an association a *SCD1* polymorphism (*rs1502593*) and prevalence of MetS (Gong et al., 2011). Canadian authors found a positive association between CRP levels, concentrations of palmitic acid (16:0), and the SCD1 index for stearic acid (18:1/18:0) in European and Asian females. Of the nine *SCD1* polymorphisms analyzed, only SNP *rs2060792* was associated with concentrations of 16:0 and 18:0 in females of European descent. Additionally, the same SNP was associated with CRP levels in both ethnic groups of females (Stryjecki et al., 2012). In the EPIC-Potsdam Study, seven *SCD1* polymorphisms (*rs1502593,*

rs522951, rs11190480, rs3071, rs3793767, rs10883463, and *rs508384*) were investigated in relation to BMI, WC, fetuin-A, triglycerides (TG), glycated hemoglobin (HBA1c), high-sensitivity C-reactive protein (hs-CRP), gamma-glutamyltransferase (GGT), and alanine aminotransferase (ALT). Although *rs10883463* was initially weakly associated with TG, GGT, HBA1c, and *rs11190480*, these relationships became statistically non-significant after multiple testing ([Arregui et al., 2012).

To our knowledge, associations between *SCD1* polymorphisms and apoB-48 concentrationapoB48 concentrations have yet to be studied. The aim of this pilot study was to analyze basic clinical, and laboratory measurements in relation to selected SNPs of the *SCD1* gene in patients at elevated cardiometabolic risk with different plasma apoB-48 concentrations.

Materials and Methods

Study design and participants

This cross-sectional pilot study was carried out at the 4th Department of Medicine of the General University Hospital, Prague. The study protocol was approved by the Ethics Committee of the General University Hospital, Prague. Written informed consent was obtained from all participants prior to inclusion. The study was performed in accordance with the Declaration of Helsinki.

We examined a group of 220 Caucasian subjects at increased cardiometabolic risk from April 2014 to October 2015 at the Lipid and Diabetology Clinic of the 4th Department of Medicine. The group consisted of 104 men and 116 women, of whom 79 (68 %) were post-menopausal. Each subject had at least one MetS-related trait, with 164 persons (74.5 %) categorized as overweight or obese (BMI≥25.0 or 30 kg/m^2, respectively), with the same prevalence of abdominal fat distribution (waist circumference ≥94 cm and ≥80 cm in men and women, respectively). Increased blood pressure (BP) (or treatment of previously diagnosed arterial hypertension) was observed in 157 persons (71 %), and hyperuricemia (uric acid ≥420

μmol/L) in 26 persons (11.8 %). Impaired fasting glycemia was observed in 57 patients (57.9 %), hypertriglyceridemia (TG≥1.70 mmol/L) in 101 patients (46 %), and decreased HDL-C (<1.03 and <1.30 mmol/L for men and women, respectively) in 42 patients (19 %). All subjects had maintained a stable body weight in the preceding 3 months.

As our examination group did not contain healthy controls, we used the median apoB-48 concentration of all subjects examined as the cut-off level. The median value forapoB-48 was estimated at 7.9 mg/L (see Figure 1).

Fig. 1. Distribution of apoB-48 concentrations in the group studied

MetS was diagnosed according to the International Diabetes Federation criteria (IDF, 2005) (Alberti et al., 2006). The number of MetS components and related risk factors were determined based on current definitional criteria: waist circumference, concentrations of triglycerides (TG) and HDL-cholesterol (HDL-C), increased BP (or treatment of previously diagnosed arterial hypertension), impaired fasting glycemia (or previously diagnosed type 2 diabetes mellitus), hyperuricemia (>420 μmol/L), and predominance of small-dense LDL (pattern B of the LDL phenotype) (Das, 2010).

Basic clinical data and anthropometric parameters (body weight, height, waist circumference, thickness of four skin folds) were examined in all individuals using methods recommended by the Airlie Conference (Lohman et al., 1988). Body fat percentages were determined according to the Durnin and Womersley formula (Durnin and Womersley, 1974). Fat mass was calculated by multiplying body mass by the percentage ratio of fat mass.

Exclusion criteria were as follows: age <18 years, unstable clinical conditions (including body weight), current antioxidant therapy (e.g. vitamin C, vitamin E, allopurinol, N-acetylcysteine), immunosuppressive and/or anti-inflammatory therapy, supplementation with n-3 and/or n-6 polyunsaturated fatty acids, chronic kidney disease (creatinine >150 μmol/L; urinary protein >500 mg/L), liver cirrhosis, decompensated DM, endocrine disease, and

concomitant malignancies (or their treatment). Further criteria for exclusion included: acute pancreatitis or acute relapse of chronic pancreatitis, unstable angina pectoris, acute myocardial infarction occurring less than one year prior, coronary aortic bypass grafting, percutaneous coronary intervention, and stroke. Persons operated on in the gastrointestinal tract (within the previous year) and subjects having experienced systemic inflammation (within the previous 6 months) were also excluded as previously described (Zak et al., 2014; Zeman et al. 2017).

Laboratory measurements

Blood samples from all participants were obtained after overnight fasting (for at least 12 hours). Plasma was immediately cooled to 4-6 °C and separated within 30 min at the same temperature at 1000 g for 10 min. In line with routine biochemical and hematological analysis, samples were measured immediately according to standardized enzymatic-colorimetric methods, with samples requiring further analysis stored at -80 °C.

Concentrations of LDL-C were calculated according to Friedewald's equation based on recommended criteria. Given 35 patients had concentrations of TG above 2.5 mmol/L, LDL-C concentrations are not presented in Table 2.

Concentrations of insulin were measured using diagnostic sets and a modular analyzer (Roche) by electrochemiluminescence (ECLIA). Levels of hs-CRP were measured using the hs-CRP Human ELISA kit (BioVendor). Concentrations of apoB-48 were determined using a diagnostic set and the Human Apo B-48 ELISA Kit (Shibayagi Co., Ltd., Shibukawa, Gunma, Japan).

Subfractions of LDL were analyzed by high-performance discontinued gel electrophoresis using polyacrylamide gel tubes (Lipoprint® LDL System, Quantimetrix, USA). LDL particles were separated into 7 subfractions (LDL1 to LDL7). LDL1 and LDL2 subfractions represented large (buoyant) particles, while LDL3-7 represented small-dense LDL (sd-LDL). Concentrations of cholesterol in sd-LDL over 6 mg/dL or a peak LDL particle

diameter ≤ 26.8 nm denoted phenotype pattern B with a predominance of sd-LDL (Gazi et al., 2006).

Profiles of fatty acids (FA) were measured by chromatographic methods (Tvrzicka et al., 2002).

To measure conjugated diene concentrations in precipitated LDL, a modified spectrophotometric method was used (Ahotupa et al., 1996). Concentrations of circulating, oxidized LDL (ox-LDL) were measured by the ELISA method using reagent kits from Mercodia (Sweden). Concentrations of hs-CRP were measured using the hs-CRP Human ELISA reagent kit (BioVendor, CR).

Single nucleotide polymorphism selection and genotyping

Single nuclear polymorphisms (SNPs) of two genes – apoE (*APOE*) and stearoyl-CoA desaturase-1 (*SCD1*) – were analyzed in all individuals. We selected a total of eight *SCD1* SNPs for genotyping based on the following parameters in the case of a European Caucasian population: frequency [HapMap Rel 24-phaseII, NCBI B36 assembly, dbSNP b126; chr10:102089500-1022114800], known and implicated functional effect (Merino et al., 2010; Tosi et al., 2014; Warensjö et al., 2007; Gong et al., 2011; Stryjecki et al., 2012; Martin-Núñez et al., 2013), and positioning and spacing along the *SCD1* gene. Nearly all of the SNPs selected (except for *rs641996*) exhibited a minor allele frequency >0.05.

DNA samples were isolated from peripheral blood leukocytes according to standard desalting procedures (Miller et al., 1988). As essential parameters, DNA concentration and purity were assessed (Nanodrop ND 1000, Thermo Fisher Scientific, Wilmington, DE) in advance of subsequent methods.

Polymorphisms of *apoE* and *SCD1* were performed using the PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method. In cases where the

respective endonuclease or restrictive enzyme site was not found, direct DNA sequencing was employed (Sanger and Coulson, 1975; Sanger et al., 1977) using a genetic analyser (Applied Biosystems® 3500 Series Genetic Analyzer).

Isoforms of apolipoprotein E (*apoE*) were determined using the RFLP method based on a modified analytical procedure as previously described (Jachymova et al., 2001; Zak et al., 2007). Finally, fragments were detected by electrophoresis on 3% agarose gel with the addition of ethidium bromide, and visualized under a UV lamp. Each sample was verified independently by two team members. Reaction conditions for genetic analysis of the *SCD1* gene are given in Table 1.

Table 1: Analytic conditions for detection of polymorphisms in the *SCD1* gene

Polymorphism	**Forward primer 5´→3´** **Reverse primer 3´→5´**	**Annealing** **(°C)**	**Method:** **RFLP, ds**
rs2167444 T>A	gggagtttcttttggctgtg caagttgccagctggtgtta	59.8	AflII, SmlI
rs508384 C>A	gggagtttcttttggctgtg caagttgccagctggtgtta	59.8	HgaI, BmsFI
rs7849 T>C	agggtcactgaaccactgct ccagagagaggggactgaaa	59.5	ds
rs641996 T>C	ggtggccatgagttcaaagt gattagggtggcaggaaaca	59.9	ds
rs55710213 C>T	ggtggccatgagttcaaagt gattagggtggcaggaaaca	59.9	ds
rs56334587 C>T	ggtggccatgagttcaaagt gattagggtggcaggaaaca	59.9	ds
rs10883463 T>C	ggtggccatgagttcaaagt gattagggtggcaggaaaca	59.9	ds
CTTC deletion	ggtggccatgagttcaaagt tttggcagagaagatgacca	59.8	ds

Legend: Gene polymorphisms and allelic variants are denominated according to data obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Abbreviations: A – adenine; C – cytosine; G – guanine; T – thymine; SCD1 – stearoyl-CoA desaturase 1; ds – direct sequencing

The homeostasis model assessment method (HOMA-IR) was used as the index of insulin resistance (Matthews et al., 1985).

Desaturase activity was calculated as the product/substrate ratio in respect of two indices of delta-9 desaturase (DΔ9D), e.g. D9D16 as the ratio 16:1n-7/16:0 and DΔ9D18 as the ratio 18:1n-9/18:0. D6D activity was calculated as the ratio 18:3n-6/18:2n-6 and D5D as the ratio 20:4n-6/20:3n-6 (Zak et al., 2007; Tosi et al., 2014).

Statistical methods

STATISTICA® software for Windows was used for statistical evaluation of the results. Categorical values are given in absolute values and as percentages, continuous values are expressed as mean and standard deviations (median, 25^{th} and 75^{th} percentiles, respectively). Differences between continuous variables were analysed using Student´s t-test or Mann-Whitney U test. Distribution of the categorical values was assessed using the χ^2-test. Frequencies of all genotypes and alleles were calculated, and analysed according to the Hardy-Weinberg principle using the χ^2-test. Pearson's χ^2-test was used to analyze the frequency differences of individual genotypes and allelic variants (with Yates' correction for small numbers). Statistical significance was set at $P<0.05$ using the Benjamini-Hochberg procedure for multiple comparisons.

Results

Table 2 shows the clinical and biochemical characteristics of the patients divided into two groups, with plasma concentrations of apoB-48 above and below the median value (7.9 mg/L). Neither of the groups differed with regard to age, male/female ratio, proportion of postmenopausal women (33 vs. 45 %, NS), body mass index, fat mass, or concentrations of glucose, NEFA or hs-CRP. The two groups exhibited a similar prevalence of hypertension

(73.2 vs. 69.4 %; P=0.537), obesity/overweight (76.8 vs. 72.2 %; P=0.437), and atherosclerotic cardiovascular complications [coronary heart disease /stroke/peripheral vascular disease] (13.4 vs. 6.5 %; P=0.088). We found no differences in apoB-48 concentrations between men and women [9.34(4.70-22.80) vs. 7.10(4.14-12.60), median (25^{th} – 75^{th} percentile), NS] or between pre- and post-menopausal women [6.94(5.17-9.26) vs. 7.14(3.82-18.51), median (25^{th} – 75^{th} percentile), NS].

Table 2: Basic clinical and biochemical parameters of groups

	ApoB-48<7.9 (mg/L)	ApoB-48≥7.9 (mg/L)
Number of subjects	108	112
Males N (%)	44 (40.7)	60 (53.6) NS
Age (years)	49.9±14.7[a]	52.9±14.1
Probands with MSC [e]≥3 N (%)	35 (32.4)	67 (59.8)[c***]
LDL subclass phenotype B N (%)	22 (20.4)	52 (46.4)[c***]
BMI (kg/m^2)	28.6±5.7	29.4±5.8
Waist circumference >94/80 cm	78 (72%)	86 (77%)[c] NS
Waist circumference (cm)	94.6±15.2	98.9±14.8[b]NS†
Waist circumference men (cm)	100.0±12.5	103.4±13.8[b]NS†
Waist circumference women(cm)	90.5±16.0	93.7±14.5[b]NS†
SBP (mmHg)	131±15	135±18[b*]
DBP (mmHg)	87±10	86±11
Fat mass (kg)	32.5±10.9	33.2±10.8
TC (mmol/L)	5.08±1.04	5.78±1.35[b***]
TG (mmol/L)	0.99 [0.74–1.33]	1.95 [1.40–2.70][d***]
HDL-C (mmol/L)	1.59±0.47	1.36±0.36[b***]
Non-HDL-C (mmol/L)	3.49±0.96	4.42±1.22[b***]
ApoA1 (g/L)	1.52±0.33	1.40±0.28[b**]
ApoB-100 (g/L)	1.06±0.31	1.28±0.34[b***]
ApoB-48 (mg/L)	4.4 [2. 9–6.1]	18.3 [10.6–29.2][d***]
NEFA (mmol/L)	0.54 [0.35–0.71]	0.53 [0.39–0.71]

sd-LDL-C (mg/dL)	2.0 [0.0–5.0]	5.0 [2.0–17.0][d***]
Glucose (mmol/L)	5.08±0.64	5.20±0.66
HOMA-IR (index)	1.654 [1.249–2.824]	2.554 [1.692–3.830][d***]
Insulin (μU/mL)	7.48 [6.05–12.55]	10.55 [7.29–15.64][d**]
CD (μmol/L)	60.7±15.4	67. 8±16.8[b***]
ox-LDL (U/L)	39.3 [18.7–58.6]	54.4 [29.2–71.3][d**]
hs-CRP (mg/L)	2.17 [1.03–4.20]	2.32 [1.13–4.10]
Uric acid (μmol/L)	297±83	335±91[b***]

[a] Values are presented as means ± SD or medians [25th – 75th percentiles]; [b] Student's t-test; [c] χ^2 test; [d] Mann-Whitney U test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; † using multilinear regression with gender x group interaction

Abbreviations used:

[e] MSC – metabolic syndrome components: waist circumference >94/80 cm (M/F); TG>1.70 mmol/L; HDL-C<1.00/1.30 mmol/L (M/F); BP>130/85 mmHg (or specific antihypertensive treatment); glucose >5.60 mmol/L (or DM type 2 treatment); hyperuricemia >420 μmol/L; small-dense LDL (sdLDL-C >6 mg/dL);

Apo – apolipoprotein; BMI – body mass index; TC – total cholesterol; TG – triglyceride; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; hs-CRP – high-sensitivity C-reactive protein; sd-LDL-C – small-dense LDL cholesterol; NEFA – non-esterified fatty acids; ox-LDL – oxidized LDL; HOMA-IR – homeostatic index of insulin resistance; CD – conjugated dienes in LDL; MSC – metabolic syndrome components; BP – blood pressure; SBP – systolic BP; DBP – diastolic BP; M – male; F – female; N – number.

The group with apoB-48≥7.9 mg/L was characterized by increased systolic blood pressure, higher frequency of patients with three or more components of MetS (MSC≥3), higher proportions of smokers (36.6 vs. 22.2 %; P<0.05) and MetS patients, impaired fasting glucose (32.1 vs. 19.4 %; P<0.05), hyperuricemia (>420 μmol/L) (9.4 vs. 5.6 %, P<0.05), hypertriglyceridemia (67.9 vs. 23.1 %; P<0.001), and lower HDL-C (25 % vs. 13 %, P<0.05). Laboratory parameters for the group with apoB-48≥7.9 mg/L were characterized by higher concentrations of TC, TG, and non-HDL-C. We also observed significantly higher concentrations of apoB-100 and apoB-48 and lower concentrations of apoA1. In this group, hyperinsulinemia was connected with increased insulin resistance index values (HOMA-IR). Higher concentrations of apoB-48 were associated with increased concentrations of cholesterol in small-dense LDL (sd-LDL) and more frequent instances of LDL subclass phenotype B (46.4 vs. 20.4 %, P<0.001). Based on the surrogate parameters of oxidative stress analyzed, the group with higher concentrations of apoB-48 exhibited increased concentrations of conjugated dienes in LDL (CD-LDL) and oxidatively modified LDL particles (ox-LDL).

Concentrations of apoB-48 across the entire group showed significant correlations with TG (r=0.440, Spearman's rank correlation coefficient; P<0.001), sd-LDL (r=0.621; P<0.001), total cholesterol (r=0.466; P<0.001), non-HDL-C (r=0.419; P<0.001), and conjugated dienes in LDL (r=0.243; P<0.001). Moreover, apoB-48 levels correlated negatively with HDL-C (r=-0.176; P<0.01).

In the group with apoB-48≥7.9 mg/L (in comparison with those with low apoB-48), analysis of fatty acid profiles in plasma phosphatidylcholines revealed an increased D9D activity index for palmitic acid (0.0197±0.006 vs. 0.0178±0.0050; 16:1n-7/16:0 ratio; P<0.001), an increased D6D activity index (0.0051±0.0032 vs. 0.0043 ± 0.0017; 18:2n-6/18:3n-6 ratio; P<0.05), and a decreased D5D activity index (3.2610 ± 0.8857 vs. 3.6498 ± 1.0060; 20:4n-

6/20:3n-6 ratio; P<0.05). However, the D9D activity index for stearic acid was reduced (0.7198 ±0.1430 vs. 0.7871 ± 0.1280, 18:1n-9/18:0 ratio, P<0.001) (Table 3).

Table 3: Fatty acid composition of plasma phosphatidylcholines

Fatty acid[a]	ApoB-48<7.9 (mg/L)	ApoB-48≥7.9 (mg/L)
14:0	0.19±0.11	0.23±0.10[h*]
16:0	31.91±4.73	30.72±3.51[*]
16:1n-9	0.12±0.03	0.12±0.03
16:1n-7	0.57±0.20	0.61±0.20
18:0	14.13±1.66	14.89±1.45[***]
18:1n-9	10.99±1.43	10.65±2.00
18:1n-7	1.53±0.20	1.51±0.22
18:2n-6	23.60±3.03	23.30±3.56
18:3n-6	0.10±0.03	0.11±0.04[*]
18:3n-3	0.24±0.09	0.23±0.10
20:0	0.04±0.02	0.04±0.020
20:1n-9	0.12±0.04	0.12±0.04
20:2n-6	0.41±0.13	0.43±0.18
20:3n-6	2.76±0.79	3.06±0.75[**]
20:4n-6	9.58±2.41	9.64±2.34
20:5n-3	0.94±0.57	0.84±0.37
22:4n-6	0.20±0.10	0.24±0.11[**]
22:5n-6	0.14±0.07	0.16±0.08[*]
22:5n-3	0.50 [0.28–0.73]	0.67 [0.40–0.87][**]
22:6n-3	1.71 [1.01–2.85]	2.15 [1.28–3.30]
∑SFA	46.29±3.81	45.92±3.48
∑MUFA	13.35±1.55	13.00±2.19

Fatty acid[a]	ApoB-48<7.9 (mg/L)	ApoB-48≥7.9 (mg/L)
∑PUFAn-6	36.65±3.43	37.06±3.56
∑PUFAn-3	3.71±1.79	4.03±1.69
D5Dn6[c]	3.6498±1.0060	3.2610±0.8857**
D6Dn6[d]	0.0039 [0.0029–0.0051]	0.0045 [0.0035–0.0059]*
D9D16[e]	0.0178±0.0050	0.0197±0.0059**
D9D18[f]	0.7871±0.1280	0.7198±0.1430***

Legend and abbreviations: Only relevant fatty acids are shown (proportions of fatty acids 12:0 and 14:1n-5 are not given); ΣSFA – total content (sum) of saturated fatty acids; ΣMUFA – sum of monounsaturated fatty acids; ΣPUFAn-6 – sum of polyunsaturated fatty acids (n-6 family); ΣPUFAn-3 – sum of PUFA (n-3 family); [a] shorthand notation for fatty acids: number of carbon atoms/number of double bonds; n – number of carbon atoms from the methyl end to the nearest double bond; [b] values are means ± SD or medians [25 – 75th percentiles] (mol %); [c] D5D – delta 5 desaturase (20:4n-6/20:3n-6); [d] D6D – delta 6 desaturase (18:3n-6/18:2n-6); [e] D9D16 – delta 9 desaturase for palmitic acid (16:1n-7/16:0); [f] D9D18 – delta 9 desaturase for stearic acid (18:1n-9/18:0); [g] Mann-Whitney U test; [h] Student's t-test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

All genotypes were in Hardy-Weinberg equilibrium. Genotype analysis of *stearoyl-CoA desaturase-1* (*SCD1*) polymorphisms revealed significant differences in both allele and genotype frequencies for SCD1 in patients with different concentrations of apoB-48. The group with higher concentrations of apoB-48 exhibited lower prevalence of minor alleles and respective genotypes of the *rs2167444* and *rs508384* polymorphisms (Table 4). We found no associations between polymorphisms or alleles and *apoE (ε2, ε3, ε4*) in either group (data not presented).

Table 4: Genotype and allele distribution of *SCD1* polymorphisms in subjects with different apoB-48 concentrations

Polymorphisms ApoB-48 group		Genotype		Allele frequency		HWE	Genotype/allele analysis (χ^2 test)
rs2167444	TT	TA	AA	T	A		
<7.9 mg/L	65 (60.2)	38 (35.2)	5 (4.6)	168 (77.8)	48 (22.2)	χ^2=0.034	7.038 (P=0.030) genotype*
≥7.9 mg/L	85 (75.9)	27 (24.1)	-	197 (87.9)	27 (12.1)	χ^2=2.104	8.041 (P=0.005) allele
rs508384	CC	CA	AA	C	A		
<7.9 mg/L	64 (59.3)	39 (36.1)	5 (4.6)	167 (77.3)	49 (22.7)	χ^2=0.094	7.025 (P=0.030) genotype*
≥7.9 mg/L	84 (75.0)	28 (25.0)	-	196 (87.5)	28(12.5)	χ^2=2.286	7.901 (P=0.005) allele
rs7849	TT	TC	CC	T	C		
<7.9 mg/L	66 (61.1)	41 (38.0)	1 (0.9)	173 (80.1)	43 (19.9)	χ^2=3.919^x	3.906 (P=0.142) genotype*
≥7.9 mg/L	84 (75.0)	28 (25.0)	-	196 (87.5)	28 (12.5)	χ^2=2.286^x	4.459 (P=0.035) allele
rs55710213	CC	CT	TT	C	T		
<7.9 mg/L	45 (41.7)	48 (44.4)	15 (13.9)	138 (63.9)	78 (36.1)	χ^2=0.146	2.664 (P=0.264) genotype
≥7.9 mg/L	35 (31.2)	57 (50.9)	20 (17.9)	127 (56.7)	97 (43.3)	χ^2=0.149	2.375 (P=0.123) allele
rs56334587	CC	CT	TT	C	T		
<7.9 mg/L	45 (41.7)	48 (44.4)	15 (13.9)	138 (63.9)	78 (36.1)	χ^2=0.146	2.664 (P=0.264) genotype
≥7.9 mg/L	35 (31.2)	57 (50.9)	20 (17.9)	127 (56.7)	97 (43.3)	χ^2=0.149	2.375 (P=0.123) allele

Values represent the number of subjects with specific genotypes (alleles) in individual groups; numbers in parentheses represent the percentage of subjects with specific genotypes (alleles); ^{x} genotype distribution of *SCD1 rs7849* did not correspond with HWE (P>0.05); * Yates' correction

Abbreviations: HWE – Hardy-Weinberg equilibrium; A – adenine; C – cytosine; G – guanine; T – thymine.

Discussion

Increased concentrations of apoB-48, which reflect the concentrations of intestinally derived chylomicron lipoproteins (CM) and their remnant particles (CMR), are caused by increased production and secretion of CM and/or by their insufficient catabolism. Triglyceride-rich lipoproteins (TRL) originating in the liver, such as VLDL and their remnants (IDL), compete with lipoproteins of intestinal origin (CM and CMR) for lipoprotein lipase in skeletal muscle and adipose tissue, and for elimination by receptor-mediated uptake (LDLR and LRP) of IDL and CMR in the liver (Fazio and Linton, 2015; Nakajima and Tanaka, 2018).

Insulin resistance increases the expression of microsomal triglyceride transfer protein and Niemann-Pick C1-like 1 protein and reduces the activity of the ABCG5/G8 transporter. Consequently, the content of cholesterol and TG in CM along with the secretion of CM by enterocytes increase (Nakajima and Tanaka, 2018). It has been suggested that CMR particles, which are rich in cholesterol and other lipids, are also highly atherogenic. Increased concentrations of apoB-48 are understood to be associated with prolonged residence time of TRL and with increased concentrations of sd-LDL and ox-LDL. In vitro studies have proved the pro-atherogenic and pro-inflammatory properties of both CMR and ox-LDL (Nakajima et al., 2006). Increased concentrations of apoB-48 are recognized as a risk factor for atherothrombotic cardiovascular events (especially coronary heart disease and non-embolic ischemic stroke) and are understood to be independent of levels of total plasma, LDL- and HDL-cholesterol, and triglycerides in children, adults, diabetics and non-diabetics (Alipour et al., 2012; Mori et al., 2013; Wang et al., 2013). Elevated plasma concentrations of apoB-48 are recognized as risk factors for clinically manifest and subclinical atherosclerosis in both diabetics and non-diabetics (Nakajima and Tanaka, 2018; Mori et al., 2013; Valdivielso et al., 2010; Tanimura et al., 2008). Fasting apoB-48 has been independently associated with asymptomatic peripheral artery disease in patients with DM2T. Of the several risk factors

involved (LDL-cholesterol, HDL-cholesterol, HBA1c, DM duration, smoking, and systolic BP), only apoB-48 has been identified as an independent predictor of PAD (Lapice et al., 2012; Mancera-Romero et al., 2013).

High levels of apoB-48 have been reported in patients with diabetic nephropathy, chronic kidney disease, as well as end-stage renal disease (Hayashi et al., 2008), obesity and MetS (Kinoshita et al., 2009; Okubo et al., 2014; Masuda et al., 2014).

In our study, probands with increased concentrations of apoB-48 (≥7.9 mg/l) exhibited unfavorable lipid and lipoprotein profiles (increased TG, non-HDL-C and apoB-100; decreased HDL-C and apoA-I), elevated markers of oxidative stress (CD-LDL and ox-LDL), insulin resistance (higher insulinemia and HOMA-IR), and higher systolic blood pressure.

The most important result of our study was that probands with lower plasma concentrations of apoB-48 had higher proportions of minor alleles and genotypes of *rs2167444* and *rs508384 SCD1* polymorphisms. Our set of probands included persons with at least one component of MetS; however, no control group was used. As the normal range of apoB-48 concentrations could not be determined, we used the median for all 220 probands as the cut-off value. In the literature, data on the reference ranges of apoB-48 in healthy persons are scarce (Nakajima and Tanaka, 2018) and available only for Japanese normolipidemic population (Masuda et al., 2014).

The finding of a significant association between the *SCD1* genotype (allele) polymorphisms *rs2167444* and *rs508384* (*rs7849*) and apoB-48 concentrations indicates the higher proportion of minor, protective genotypes (alleles) in probands with lower concentrations of apoB-48. A Swedish study reported a significant association between minor alleles of four *SCD1* polymorphisms (*rs10883463*, *rs7849*, *rs2167444*, and *rs508384*) and lower BMI, lower waist circumference, and improved insulin sensitivity. The expected differences in D9D activities between minor and common allele carriers did not reach statistical

significance (Warensjö et al., 2007). In our study, we found no significant association between *SCD1* polymorphisms and D9D activity (data not shown). A Spanish study reported associations between several *SCD1* polymorphisms and obesity. However, although it revealed a lower risk of obesity among carriers of the minor alleles *rs7849* and *rs508384*, the opposite effect was observed for the minor allele *rs150259*. Moreover, the same study found a statistically significant association between D9D for palmitic and stearic acid and polymorphisms *rs508384*, *rs1267444*, and *rs7849* (Martín-Núñez et al., 2013). A Canadian study found an association between some cardiometabolic risk factors (TG, CRP, IL-6, and glycemia) and three polymorphisms of *SCD1* (*rs508384*, *rs3071*, and *rs3829160*), and a significant association between D9D activity and one *SCD1* polymorphism (*rs2234970*). The same study also noted a significant interaction between rs508384, dietary PUFA n-3 intake, and glycemic change (Rudkowska et al., 2014). The EPIC-Potsdam study, which employed univariate analysis, found a weak association between *SCD1*, *rs10883463*, TG, glycated hemoglobin, and liver function tests (GGT, ALT) and *rs11190480*. However, in multiple testing, this association was non-significant (Arregui et al., 2012). Warensjö et al. (2007) found associations between four *SCD1* polymorphisms (*rs10883463*, *rs7849*, *rs2167444*, and *rs508384*) and BMI, waist circumference, and insulin resistance. However, in our study, the group with higher concentrations (≥7.9 mg/L) of apoB-48 differed from the group with lower concentrations in respect of insulin resistance, but not for BMI or waist circumference.

Our results corresponded with the findings of higher apoB-48 concentrations in persons with dyslipidemia, obesity (overweight), MetS, and insulin resistance (Sakai et al., 2003; Alipour et al., 2012; Kinoshita et al., 2009; Masuda et al., 2014; Otokozawa et al., 2009). Higher apoB-48 concentrations have also been found in elderly men and post-menopausal women (Alipour et al., 2012; Kinoshita et al., 2009; Masuda et al., 2014). In our set of probands, the

group with concentrations of apoB-48≥7.9 mg/L did not significantly differ from the group with lower apoB-48 concentrations in respect of age, BMI, or fat mass.

Patients with higher concentrations of apoB-48 also exhibited higher concentrations of sd-LDL particles, indicating the predominance of LDL subclass phenotype B. Characterized by a preponderance of small-dense LDL particles, phenotype B is a dyslipidemia component of MetS. Liver lipase activity and TG concentrations are metabolic predictors of sd-LDL particle levels (Ng, 2013; Diffenderfer and Schaefer, 2014). Triglyceride-rich lipoprotein particles from the liver (VLDL and IDL) and gut (CM and CMR) compete for the same lipolytic system, and for receptors that bind IDL and CMR. As a result, triglyceride concentrations increase in tandem with prolonged TRL residence time. We found a positive correlation between concentrations of apoB-48 and concentrations of TG and sd-LDL particles and a negative correlation between concentrations of apoB-48 and HDL-C, as also previously reported by Sato et al. (2009).

Concentrations of both CD/LDL and ox-LDL reflect systemic oxidative stress. While CD/LDL is an indicator of minimally oxidized LDL (containing only oxidatively modified lipids), both lipid and protein components are oxidatively modified in ox-LDL particles (Ahotupa et al., 1996; Esterbauer et al., 1992). When evaluating the spectra of plasma phosphatidylcholine fatty acids, we observed higher activity of delta-9 desaturase for palmitic acid (D9D16) in the group with higher concentrations of apoB-48, higher D6D activity, and lower D5D activity. D9D activity for stearic acid (D9D18) was lower in the group with higher concentrations of apoB-48. The ratio of 16:1n-7/16:0 (D9D16) was a surrogate marker of SCD1 activity, the ratio of 20:4n-6/20:3n-6 (delta-5 desaturase, D5D) a marker of FADS1 activity, and the ratio of 18:3n-6/18:2n-6 (D6D) a marker of FADS2 activity.

The increase in D9D and D6D activity and the opposite changes in D5D activity have been described as a consequence of the characteristic alteration of fatty acid transformation in MetS (Zak et al., 2007; Tosi et al., 2014; Zak et al., 2014). SCD1 (or D9D) activity is influenced

by genetic and environmental factors and diseases (Merino et al., 2011; Mauvoisin and Mounier, 2011). Increased D9D activity, which is associated with dyslipidemia and ischemic heart disease, predicts hyperglycemia and DM2T manifestation (Warensjö et al., 2006; Mahendran et al. 2014). Elevated D9D activity is linked with tumor development (Byberg et al., 2014), obesity, adipose tissue distribution, insulin resistance (Warensjö et al., 2007), and MetS prevalence (Gong et al., 2011). Increased D6D activity is associated with hyperinsulinemia and higher BMI. However, low D5D activity specific to MetS is independent of BMI and physical activity (Warensjö et al., 2006), and is understood to be a predictor of development of abdominal obesity (Kawashima et al., 2009).

High D6D and low D5D activities are both characteristic of conditions associated with insulin resistance (Kröger and Schulze, 2012) and are known predictors for development of DM and MetS (Saito et al., 2013). One study reported a negative correlation in the case of both sexes between LDL-C and TG concentrations and D5D, and a positive correlation between LDL-C, TG, LDL-C, and non-HDL-C and D6D activity (Jacobs et al., 2015). A surprising finding in our study was the detection of higher D9D18 activity in the group with lower apoB-48 concentrations. Oleic acid (OA, 18:1n-9), unlike palmitoleic acid (POA, 16:1n-7), is abundantly supplied in the diet. Therefore, its concentration does not correspond with SCD1 activity. However, it does reflect OA intake. Unfortunately, we could not confirm decreased OA dietary intake because no dietary questionnaire was given to the probands in this study. Stearoyl-CoA desaturase-1 minor alleles are understood to be associated with lower D9D activity. Contrary to Martin-Núñez et al. (2013), neither we nor other authors (Warensjö et al., 2007; Stryjecki et al., 2012) have been able to detect differences in the 16:1n-7/16:0 (18:1n-9/18:0) indexes in minor allele homozygotes compared with common allele homozygotes and compound heterozygotes.

In agreement with Warensjö et al (2007), we found an association between two *SCD1* polymorphisms (*rs20167444* and *rs508384*) and insulin sensitivity, which might partly explain the increased concentration of apoB-48. However, other factors can also modify apoB-48 levels, such as the amount of dietary cholesterol, diverse composition of dietary fatty acids, and factors influencing cholesterol homeostasis (such as activities of Niemann-Pick C1-like 1 protein and of the ABCG5/G8 transporter), CM synthesis, and catabolism.

The limitations of the study are: (1) the small number of subjects, (2) the cross-sectional study design, (3) the lack of a dietary questionnaire, and (4) the absence of a healthy control group.

The major strength of the study is its well-defined group of high-risk individuals, none of whom were treated with lipid-lowering drugs or n-3/n-6 polyunsaturated FA supplements. Additionally, at least according to our knowledge, this is the first study to describe associations between different apoB-48 concentrations and *SCD1* polymorphisms. Our study supports the role of the *SCD1* variant in the etiology and pathogenesis of increased apoB-48 levels. However, further studies are undoubtedly warranted, especially in relation to factors influencing homeostasis of chylomicrons and their remnants.

Conclusions

(1) We found an association between increased apoB-48 concentrations and an unfavorable lipid profile characterized by higher concentrations of TC, non-HDL-C, TG, and apoB-100, lower HDL-C, higher systolic blood pressure, insulin resistance, and oxidative stress. (2) These changes were independent of age, sex, BMI, adipose tissue mass, and adipose tissue distribution. (3) The lower prevalence of minor alleles and genotypes for two *SCD1* polymorphisms, *rs2167444* and *rs508384*, in the subgroup with higher apoB-48 concentrations implicates the contribution of genetic factors in the pathogenesis of elevated apoB-48 concentrations, a trend connected with higher cardiometabolic risk.

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Prohloubení oxidačního stresu u karcinomu pankreatu vlivem malnutrice

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SOUHRN

Cíl studie: Posoudit vliv malnutrice u pacientů s karcinomem pankreatu (PC) na míru oxidačního stresu a na antioxidační systém organismu.

Typ studie: Observační, strukturálně vyvážená studie případů a kontrol.

Název a sídlo pracoviště: IV. Interní klinika, 1. LF UK a VFN v Praze, U Nemocnice 2, 128 01 Praha 2, Česká republika

Materiál a metody: Do studie bylo zařazeno 68 pacientů (M/F = 36/32) s PC, kteří byli podle indexu NRI (nutrition risk index) rozděleni do dvou skupin na pacienty se středně těžkou až těžkou malnutricí (PC-MAL) a pacienty s lehkou malnutricí či bez malnutrice (PC-NOR). Každá skupina čítala 34 osob (M/F = 18/16) a mezi skupinami nebyl signifikantní rozdíl ve věku. Dále byla do studie zařazena na základě věku a pohlaví spárovaná kontrolní skupina (CON). Sledovaným subjektům byly odebírány vzorky po celonočním lačnění a kromě základních klinických a biochemických parametrů byly stanovovány markery oxidačního stresu (konjugované dieny v precipitovaných LDL; CD/LDL a oxidované LDL; ox-LDL/LDL), aktivity antioxidačních enzymů a koncentrace redukovaného glutathionu (GSH). Ke statistickému zpracování výsledků byl použit program STATISTICA (Stat Soft, CZ).

Výsledky: Výsledky naší studie potvrzují zvýšený oxidační stres u pacientů s PC a to zvýšenými hladinami ox-LDL/LDL a CD/LDL v porovnání s CON ($p < 0,01$). Signifikantně vyšší hladiny těchto markerů měli pacienti s malnutricí než bez malnutrice. Pozorovány byly rovněž výrazné změny v antioxidačním systému u pacientů s PC; kteří oproti CON skupině měli sníženou aktivitu katalázy (CAT, $p < 0,01$) glutationperoxidázy ($p < 0,01$), arylesterázovou (PON-A) i laktonázovou aktivitu (PON-L) paraoxonázy ($p < 0,01$) a koncentraci redukovaného glutathionu ($p < 0,001$) a zvýšené hladiny sérového amyloidu A (SAA, $p < 0,001$). Ovlivnění aktivit CAT, PON-A a PON-L a hladiny SAA bylo signifikantně větší u pacientů s podvýživou oproti PC pacientům bez příznaků malnutrice.

Závěr: V této studii bylo prokázáno prohloubení oxidačního stresu a výraznější ovlivnění funkce antioxidačního systému organismu pacientů vlivem malnutrice.

Klíčová slova: karcinom pankreatu, malnutrice, oxidační stres, antioxidační enzymy, paraoxonáza.

SUMMARY

Vávrová L., Staňková B., Rychlíková J., Žák A.: Deepening oxidative stress in pancreatic cancer due to malnutrition

Objective: To assess the influence of malnutrition in patients with pancreatic carcinoma (PC) on the oxidative stress and antioxidant system.

Design: Observation, matched case-control study.

Settings: This study was conducted at the 4th Department of Internal Medicine of General University Hospital in Prague, U Nemocnice 2, 128 01 Prague 2, Czech Republic

Material and methods: In to our study 68 patients (M/F = 36/32) with PC were included and divided according to the nutrition risk index into two groups – patients with moderate or severe malnutrition (PC-MAL) and mild or no malnutrition (PC-NOR). In both groups there were 34 patients (M/F = 18/16) with no difference in age between both groups. Furthermore, group of 34 sex- and age-matched healthy controls (CON) were enrolled into the study. The samplings were taken after overnight fast and apart from basic clinical and biochemical parameters markers of oxidative stress (level of conjugated dienes in precipitated LDL, CD/LDL and oxidized LDL, ox-LDL/LDL), activities of antioxidant enzymes and concentration of reduced glutathione (GSH) were assessed. For all statistical analysis the statistical program STATISTICA (Stat Soft, CZ) was used.

Results: In our study we confirmed increased oxidative stress in PC, with higher levels of ox-LDL/LDL and CD/LDL compared to CON ($p < 0.01$). Significantly higher levels of these markers were in patients with malnutrition then without malnutrition. We observed also changes in antioxidant system of PC patients – these patients had decreased activity of catalase (CAT, $p < 0.01$), glutathione peroxidase ($p < 0.01$), arylesterase (PON-A) and also lactonase activity (PON-L) of paraoxonase ($p < 0.01$) and concentration of GSH ($p < 0.001$) and higher levels of serum amyloid A (SAA, $p < 0.001$). The changes in CAT, PON-A, PON-L and SAA levels were significantly higher in PC patients with malnutrition then without.

Conclusion: In this study we proved the deepening of oxidative stress and the strongly impaired function of antioxidant system in PC patients due to malnutrition.

Keywords: pancreatic cancer, malnutrition, oxidative stress, antioxidant enzymes, paraoxonase.

Úvod

Předpokládá se, že v patogenezi karcinomu pankreatu (PC) se uplatňuje oxidační stres, podmíněný zvýšenou tvorbou reaktivních forem kyslíku a dusíku (Reactive oxygen and nitrogen species, RONS). K rizikovým faktorům pro vznik PC se řadí nadměrná konzumace alkoholu, kouření či chronická pankreatitida, kde všechny tyto faktory jsou samy o sobě spojeny se zvýšeným oxidačním stresem [1].

Oxidační stres je charakterizován jako nerovnováha mezi tvorbou a odbouráváním RONS. Udržení oxidační rovnováhy organismů zajišťuje antioxidační systém, tvořený antioxidačními enzymy – superoxiddismutáza (SOD), kataláza (CAT), glutationperoxidáza (GPx), glutationreduktáza GR) a paraoxonáza (PON) – a neenzymovými antioxidanty, kde nejdůležitějším je redukovaný glutation (GSH) [2].

U většiny pacientů s PC je v době diagnostiky tohoto onemocnění již pozorován výrazný úbytek na váze, který se velmi často rozvine ve vážnou formu kachexie. Kachexie je potom jednou z hlavních příčin snížení kvality života pacienta a je spojena se zvýšenou jak morbiditou, tak mortalitou pacientů [3, 4]. Stabilizace a udržení stálé váhy a kompozice organismu je potom spojeno s lepší prognózou pro pacienta [5, 6]. K predikci nutričního rizika je velmi často využíván nutriční rizikový index (Nutritional risk index, NRI).

Cílem této studie bylo posoudit vliv malnutrice u pacientů s karcinomem pankreatu na míru oxidačního stresu a na antioxidační systém organismu.

Materiál a metody

Z celkového počtu 93 pacientů s PC hospitalizovaných na IV. Interní klinice bylo do této observační studie zařazeno 68 pacientů (M/F = 36/32), u kterých byly dostupné potřebné údaje pro výpočet NRI indexu. Podle hodnot NRI indexu byli pacienti rozděleni do dvou skupin na pacienty se středně těžkou až těžkou malnutricí (PC-MAL; NRI < 97,5) a pacienty s lehkou malnutricí či bez malnutrice (PC-NOR). Každá skupina čítala 34 osob (M/F = 18/16) a mezi skupinami nebyl signifikantní rozdíl ve věku. Dále byla do studie zařazena na základě věku a pohlaví spárovaná kontrolní skupina (CON). Diagnóza PC byla u všech pacientů potvrzena na základě histologického vyšetření po pankreatické resekci nebo aspirační cytologií vedenou endoskopickou ultrasonografií. Určení stádia pokročilosti nádoru pankreatu bylo provedeno na základě systému TNM a Union Internationale Contrele Cancer with American Joint Committee on Cancer (UICC/AJCC 2003) [7].

Pro všechny osoby platila stejná vylučovací kritéria: zavedená terapie antioxidanty (farmakologické dávky vitaminu C a E, allopurinol, N-acetylcystein), jiná nádorová onemocnění, chronická dialýza, onemocnění ledvin (kreatinin > 500 μmol/l), imunosuprese, protizánětlivá terapie, chemoterapie, dekompenzovaný diabetes mellitus, cirhóza jater, akutní pankreatitida.

Studie byla schválena Etickou komisí VFN Praha. Všechny osoby zařazené do studie podepsaly informovaný souhlas. U všech osob zařazených do studie byly prováděny odběry krevních vzorků po celonočním lačnění (min. 10 hodin). Odebrané krevní vzorky byly zpracovány do 1 hodiny od náběru a materiál pro další analýzy byl uchováván při -80°C. Vzorkům byly přiřazeny unikátní anonymizační kódy, které byly dekódovány až po skončení pokusu.

U pacientů byly sledovány základní klinické, antropometrické a biochemické parametry, dále pak byly stanovovány aktivity antioxidačních enzymů CAT, GPx1, GR, SOD v erytrocytech a arylesterázové a laktonázové aktivity PON1 v séru, koncentrace GSH a koncentrace sérového amyloidu A (SAA). Jako marker oxidačního stresu byla měřena koncentrace konjugovaných dienů v precipitovaných LDL (CD/LDL) a hladina oxidovaných LDL (ox-LDL/LDL) v séru. Speciální vyšetření (hladiny antioxidantů, markery oxidačního stresu) byla prováděna v laboratořích IV. Interní kliniky, rutinní biochemické parametry spolu s hladinami Cu a Zn byly stanoveny v Ústavu lékařské biochemie a laboratorní diagnostiky VFN Praha. Metody ke stanovení aktivity antioxidačních enzymů a koncentrací GSH a CD/LDL byly podrobně popsány v publikaci Kodydkova et al. [8], ke stanovení ox-LDL byl využit komerčně dodávaný ELISA kit od firmy Mercodia, ke stanovení koncentrace SAA byl použit komerčně dodávaný ELISA kit od firmy Invitrogen.

Hodnoty počítané celkové antioxidační kapacity organismu (calculatedtotalperoxylradicaltrapping, cTRAP) byly počítány podle vzorce: cTRAP = [0,63 (albumin) + 1,02 (kyselina močová) + 1,50 (bilirubin)] [9]. Index (Nutritional Risk Index, NRI) byl počítán podle vzorce: NRI = 1,519* (albumin, g/l) + 41,7 * (současná váha/obvyklá váha) [10]. Mezní hodnota NRI pro rozdělení na středně těžkou až těžkou a lehkou malnutrici byla 97,5.

Výsledky jsou vyjádřeny jako průměr ± S.D. pro parametrické veličiny a jako medián (0,25 - 0,75 percentil) pro neparametrické veličiny. Normalita byla testována prostřednictvím Shapiro-Wilkova W testu. Rozdíly mezi jednotlivými skupinami byly zkoumány pomocí jedno-faktorové ANOVY s Neuman-Keulsovým post-testem. Pro neparametrickou analýzu byla použita Kruskal-Wallisova ANOVA. Pro korelační analýzu byl použit Spearmanův korelační koeficient. Pro všechny statistické analýzy byl používán program STATISTICA 12.0 (Stat Soft, CZ). Za statisticky signifikantní byly považovány výsledky s $p < 0,05$.

Výsledky

Základní klinické a biochemické charakteristiky jednotlivých skupin jsou shrnuty v Tabulce 1. Jak je zde ukázáno, pacienti s PC mají signifikantně zvýšené hodnoty nádorových markerů (CA19-9, CA72-4 a CEA), koncentrace triacylglycerolů, glukózy a C-reaktivního proteinu a snížené koncentrace HDL-C oproti CON.

V rámci této studie byl sledován vliv malnutrice u PC pacientů jednak na markery oxidačního stresu, jednak

Table 1. Basic clinical and biochemical characteristics of the studied groups

	PC-MAL	PC-NOR	CON
N (M/F)	34 (18/16)	34 (18/16)	34 (18/16)
Age (years)	67 ± 9	64 ± 9	65 ± 8
Stage - T2 T3 T4	4 17 13	8 14 12a	-
DM (N, %)	12 (35.3%)	15 (44.1%)	0 (0%)
Waist (cm)	91.1 ± 14.3	95.2 ± 14.4	91.7 ± 8.6
BMI	23.9 ± 5.6	26.4 ± 4.7	25.3 ± 2.9
NRI	86.4 ± 7.9^{xxxx}	105.3 ± 5.3	-
CEA (µg/L)	4.6 (2.4 – 7.5)****	2.2 (1.5 – 4.7)*	1.3 (0.5 – 2.0)
CA 19-9 (kU/L)	343.6 (52.5 – 3876.7)****	117.1 (34.1 – 1060.4)****	8.2 (5.8 – 10.5)
CA 72-4 (kU/L)	2.5 (1.6 – 5.1)*	7.2 (1.8 – 25.4)****	1.2 (0.9 – 2.5)
CRP (mg/L)	24.1 (10.6 – 71.0)****	8.6 (4.8 – 27.1)****	1.4 (1.0 – 4.2)
TC (mmol/L)	4.8 (3.7 – 6.0)	5.3 (4.2 – 6.6)	5.3 (4.5 – 6.0)
TAG (mmol/L)	1.70 (1.37 – 2.56)***	1.65 (1.38 – 1.98)***	0.97 (0.70 – 1.24)
HDL-C (mmol/L)	0.8 (0.5 – 1.0)^{***,xxx}	1.0 (0.9 – 1.3)***	1.7 (1.5 – 1.8)
LDL-C(mmol/L)	3.1 (2.1 – 4.0)	3.3 (2.5 – 4.3)	3.2 (2.6 – 3.7)
Glucose (mmol/lL)	7.6 ± 3.6***	7.8 ± 3.5***	5.1 ± 0.5

N: number of subjects, M: male, F: female, PC: pancreatic carcinoma, MAL: malnutrition, NOR: normal nutrition, CON: healthy controls, DM: diabetes mellitus, BMI: body mass index, NRI: nutrition risk index, CRP: C-reactive protein, CEA: carcinoembryonic cancer antigen, CA 19-9 carbohydrate cancer antigen, CA 72-4: carbohydrate cancer antigen CA 72-4. TC: total cholesterol, TAG: triacylglycerols, HDL-C: highdensity lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol. Data presented as mean ± S.D. for parametric and median (25^{th}-75^{th} percentile) for nonparametric variables; one-way ANOVA with Newman-Keuls post-test for parametric data and Kruskal-Wallis ANOVA for non-parametric data; * PC vs CON, ** p < 0.01; *** p < 0.001; x MAL vs NOR, xx p < 0.01; xxx p < 0.001;a χ^2 = 1.664; p = 0.44

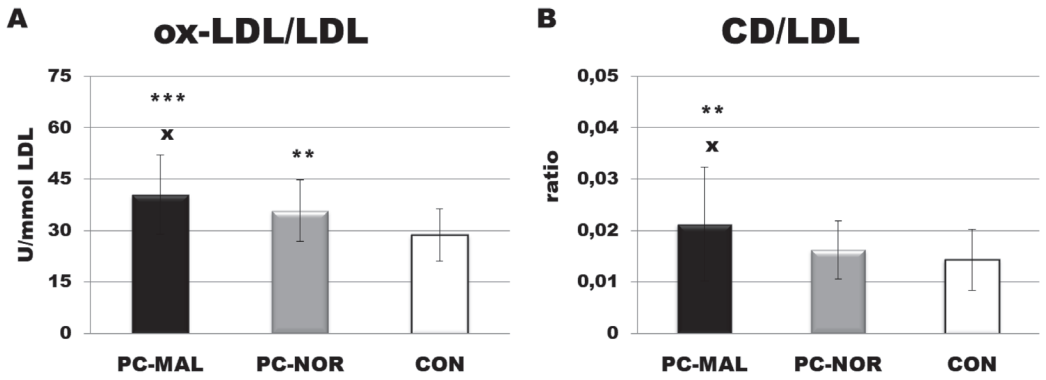

Fig. 1: Markers of oxidative stress

PC: pancreatic carcinoma, MAL: malnutrition, NOR: normal nutrition, CON: healthy controls; one-way ANOVA with Newman-Keuls post-test; * PC vs. CON, ** p < 0.01; x MAL vs. NOR, x p < 0.05.

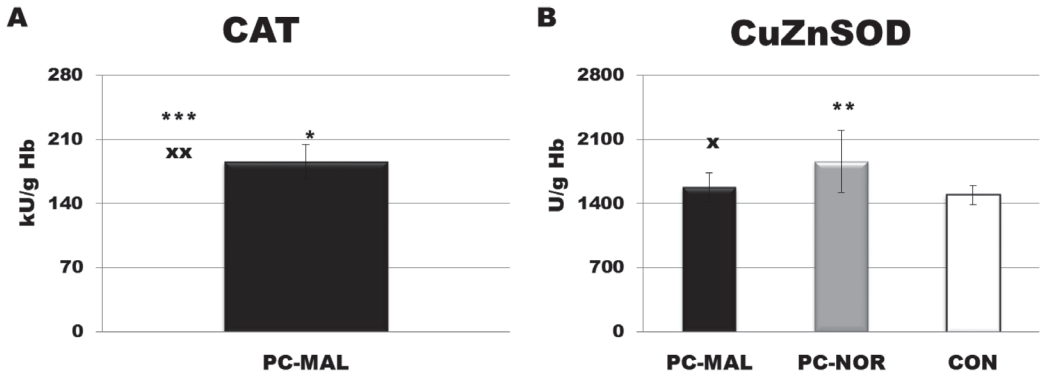

Fig. 2: Antioxidant enzymes

PC: pancreatic carcinoma, MAL: malnutrition, NOR: normal nutrition, CON: healthy controls; one-way ANOVA with Newman-Keuls post-test; *PC vs. CON, **p < 0.01; ***p < 0.001; x MAL vs. NOR, x p < 0.05; xx p < 0.01.

Table 2. Antioxidants and their cofactors and other parameters of antioxidant capacity

	PC-MAL	PC-NOR	CON
GPX1 (U/g Hb)	45.9 ± 12.9**	43.8 ± 10.5***	55.8 ± 14.5
GR (U/g Hb)	7.76 ± 1.75	7.63 ± 1.68	8.04 ± 1.57
GSH (mg/g Hb)	1.9 (0.11 – 4.0)**	1.0 (0.09 – 3.70)**	6.4 (1.5 – 8.9)
Ca(mmol/l)	2.20 ± 0.15^{**,xxx}	2.37 ± 0.09**	2.27 ± 0.11
Fe (μmol/l)	11.1 ± 6.9***	14.2 ± 6.8**	18.7 ± 6.8
Cu (μmol/l)	23.0 ± 3.9^{***,x}	24.9 ± 4.8***	16.9 ± 3.1
Zn (μmol/l)	18.3 ± 3.2x	20.2 ± 4.4**	17.2 ± 2.3
Albumin (g/l)	35.2 ± 5.0^{***,xxx}	44.6 ± 3.2	45.7 ± 3.1
Bilirubin (μmol/l)	46.9 (12.0 – 117.0)^{***,xxx}	19.6 (11.0 –38.4)	9.9 (8.0 – 12.4)
Uric acid (μmol/l)	220.5 (141.5 – 327.0)*	246.0 (209.0 – 323.0)	297.0 (260.0 – 338.0)
cTRAP (μmol/l)	685.8 (574.5 – 808.1)	743.9 (694.9 – 811.9)	767.0 (715.5 – 811.0)

PC: pancreatic carcinoma, MAL: malnutrition, NOR: normal nutrition, CON: healthy controls, GPX1: glutathione peroxidase 1, GR: glutathione reductase, GSH: reduced glutathione, cTRAP: calculated total peroxyl radical trapping - calculation: [0.63 (albumin) + 1.02 (uric acid) + 1.50 (bilirubin)]; Data presented as mean ± S.D. for parametric and median (25th-75th percentile) for nonparametric variables; one-way ANOVA with Newman-Keuls post-test for parametric data and Kruskal-Wallis ANOVA for non-parametric data; * PC vs. CON, * $p < 0.05$;** $p < 0.01$; *** $p < 0.001$; x MAL vs. NOR, x $p < 0.05$, xx $p < 0.01$; xxx$p < 0.001$.

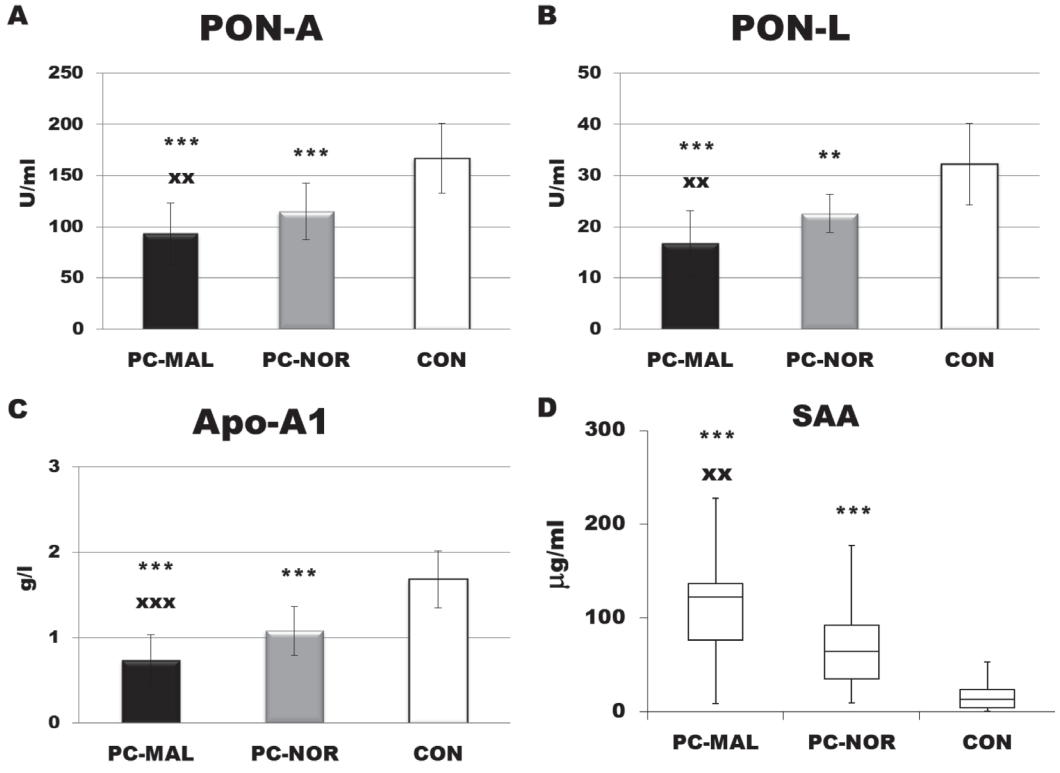

Fig. 3: Paraoxonase and SAA

PON: paraoxonase, A- arylesterase activity, L-lactonase activity, Apo: apolipoprotein, SAA: serum amyloid A, PC: pancreatic carcinoma, MAL: malnutrition, NOR: normal nutrition, CON: healthy controls; one-way ANOVA with Newman-Keuls post-test for parametric data and Kruskal-Wallis ANOVA for non-parametric data; * PC vs. CON, ** $p < 0.01$; *** $p < 0.001$; x MAL vs. NOR, xx $p < 0.01$; xxx$p < 0.001$.

na antioxidační systém. Naše výsledky ukazují, že PC pacienti s malnutricí mají vyšší hladiny jak ox-LDL/LDL tak CD/LDL než PC pacienti bez malnutrice a než CON (obr. 1).

Sledujeme-li parametry antioxidačního systému, pozorujeme výrazné rozdíly mezi subjekty s karcinomem pankreatu a zdravými kontrolami. U aktivity SOD bylo pozorováno její zvýšení u PC pacientů bez malnutrice oproti CON i PC s malnutricí (obr. 2B). Pacienti s PC mají snížené aktivity CAT (obr. 2A), GPx1 (Tabulka 2), PON-A i PON-L (obr. 3A, 3B) a koncentrace GSH (Tabulka 2) a zvýšené koncentrace SAA (obr. 3D) ve srovnání s CON. U některých parametrů byl zjištěn následně i rozdíl mezi PC pacienty s a bez malnutrice. Aktivity CAT (obr. 2A), PON-A i PON-L (obr. 3A, 3B) byly signifikantně sníženy a koncentrace SAA (obr. 3D) zvýšeny u PC pacientů s malnutricí oproti PC pacientům bez malnutrice.

Dále byly sledovány koncentrace některých dalších neenzymatických antioxidantů a kofaktorů antioxidačních enzymů (Tabulka 2). Zjištěny byly snížené koncentrace Fe a zvýšené koncentrace Cu u PC pa-

cientů v porovnání s CON. PC pacienti s malnutricí měli snížené hladiny albuminu, Ca, Zn, Cu a zvýšené koncentrace bilirubinu oproti pacientům bez malnutrice.

Mezi aktivitou CAT a koncentrací železa byla pozorována pozitivní korelace ($r = 0,304$; $p < 0,05$), negativní korelace byla potom pozorována mezi aktivitou CAT a koncentrací ox-LDL/LDL ($r = - 0,332$; $p < 0,05$) a CD/LDL ($r = - 0,322$; $p < 0,05$). Dále byla pozorována silná pozitivní korelace mezi aktivitami PON-A a PON-L navzájem ($r = 0,841$; $p < 0,0001$) a mezi aktivitami PON-A a PON-L a koncentracemi HDL-C ($r = 0,567$; $p < 0,0001$ resp. $r = 0,559$; $p < 0,0001$) a ApoA1 ($r = 0,623$; $p < 0,0001$ resp. $r = 0,573$; $p < 0,0001$) a negativní korelace PON-A s SAA ($r = - 0,426$; $p < 0,001$), CD/LDL ($r = - 0,324$; $p < 0,05$) a ox-LDL/LDL ($r = - 0,374$; $p < 0,01$).

Diskuse

V naší studii jsme se zaměřili na sledování jednotlivých komponent antioxidačního systému a měření markerů peroxidace u pacientů s karcinomem pankreatu v závislosti na stavu malnutrice a jejich srovnání se zdravou kontrolou.

Jako markery oxidačního stresu byly sledovány koncentrace ox-LDL/LDL a CD/LDL, kdy nejvyšší hodnoty byly nalezeny u PC pacientů s malnutricí. Hladina ox-LDL/LDL byla zvýšena i u PC pacientů bez malnutrice oproti CON. Naše výsledky tedy ukazují na zvýšený oxidační stres u PC a jeho prohloubení při malnutrici u PC pacientů.

Oxidační stres je podmíněn zvýšenou tvrobou RONS a nebo jejich nedostatečným odbouráváním. Za odbourávání RONS je zodpovědný antioxidační systém. K degradaci superoxidového radikálu v organismu slouží SOD, která ho degraduje na peroxid vodíku. V naší studii jsme pozorovali zvýšenou aktivitu SOD u PC bez malnutrice, kdežto u pacientů s malnutricí byla aktivita SOD na stejné úrovni jako u CON. U pacientů s PC bez malnutrice byly též pozorovány zvýšené hodnoty koncentrací jejích kofaktorů – zinku a mědi jak oproti CON tak oproti PC s malnutricí, i když analýza neukázala signifikantní korelaci. V dříve publikované studii byla nalezena zvýšená aktivita SOD mezi PC a CON [11].

Peroxid vodíku může být v organismu odbouráván buďto enzymaticky prostřednictvím CAT a GPx nebo působením peroxiredoxinů. To, který děj se uplatní, závisí jednak na koncentraci peroxidu vodíku, jednak na místě působení. Při nízkých koncentracích je peroxid vodíku degradován převážně GPx, naopak při vysokých koncentracích působí hlavně CAT [12]. Některé studie ukazují, že genová exprese CAT, a tím i zvýšené hladiny CAT mohou být stimulovány prostřednictvím peroxidu vodíku či oxidovaných lipidů [13]. Na druhou stranu bylo dokázáno, že je-li CAT dlouhodobě vystavena působení vysokých hladin peroxidu vodíku, dochází k oxidaci NADPH vázaného na CAT a následně k poklesu její aktivity až na třetinu původní hodnoty [14]. Naše studie ukazuje, že se zvyšujícím se oxidačním stresem aktivita CAT klesá. U pacientů s PC byla v dřívějších studiích pozorována jak snížená [11], tak nezměněná [15] aktivita CAT ve srovnání s CON. Kofaktorem CAT je železo, jehož koncentrace byla nejnižší u PC pacientů s malnutricí a snížená u PC pacientů bez malnutrice oproti CON, což je ve shodě s aktivitami CAT, oba parametry spolu pozitivně korelují.

Druhým enzymem schopným degradovat peroxid vodíku je GPx, která ale může katalyzovat i degradaci lipoperoxidů; pro své působení potřebuje však ještě druhý substrát GSH [16]. V naší studii jsme pozorovali snížené aktivity GPx i koncentrace GSH u obou skupin pacientů s PC ve srovnání s CON. Snížená koncentrace GSH může být jednou z příčin snížené aktivity GPx. Mezi oběma skupinami pacientů s PC nebyl pozorován žádný rozdíl ani u GPx, ani u GSH.

Při reakci peroxidů s GSH dochází k jeho oxidaci, za jeho zpětnou redukci je potom zodpovědná GR, která tak zajišťuje stálý přísun GSH pro GPx [17]. V naší studii jsme pozorovali trend ke sníženým hodnotám GR u PC pacientů, který však nedosáhl hranice významnosti.

Mezi antioxidační enzymy se řadí i PON, která vykazuje tři různé druhy aktivit paraoxonázovou, arylesterázovou a laktonázovou a přispívá k detoxikaci organofosfátových sloučenin a karcinogenních radikálů vzniklých při lipidové peroxidaci a dále zabraňuje oxidační modifikaci LDL [18]. V této studii byly měřeny PON-A a PON-L a obě aktivity byly sníženy u PC pacientů ve srovnání s CON, výraznější snížení pak bylo pozorováno u PC pacientů s malnutricí. Snížené aktivity PON u PC byly již pozorovány v dřívějších studiích [11, 19]. V publikovaných studiích, které sledovaly aktivitu PON, bylo prokázáno, že aktivita PON koresponduje se závažností oxidačního stresu [20-22], což potvrzuje i naše studie.

Pokles aktivity PON je v případě systémového zánětu nebo oxidačního stresu spojen s jejím vytěsněním z vazby na HDL přes apo-A1 prostřednictvím SAA [23]. V naší studii jsme ukázali, že koncentrace SAA rostla s klesající aktivitou PON. Nejvyšší koncentrace SAA byla u PC pacientů s malnutricí v porovnání s PC pa-cienty bez malnutrice a CON. Zvýšené koncentrace SAA u PC pacientů pozorované v naší studii jsou ve shodě s již dříve publikovanými výsledky [11, 24, 25].

Zvýšené koncetrace oxidovaných LDL a konjugovaných dienů ukazují na zvýšený oxidační stres u PC a jeho prohloubení u pacientů s malnutricí. Zvýšený oxidační stres je též doprovázen změnou ve fungování některých složek antioxidačního systému. Pozorována byla zvýšená aktivita SOD, snížená aktivita CAT, GPx1 a koncentrace GSH u pacientů s PC. Největší změny bylo možno pozorovat v arylesterázové a laktonázové aktivitě PON a dále pak v koncentraci SAA, u kterých opět došlo k prohloubení změn vlivem malnutrice.

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Studie byla podpořena výzkumným záměrem RVO-VFN64165/2012, PROGRES Q25

Autoři nejsou ve střetu zájmů.

Do redakce došlo 14. 7. 2017

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Obes Facts 2013;6:39–47
DOI: 10.1159/000348569
Received: October 12, 2011
Accepted: April 8, 2012
Published online: February 21, 2013

1662–4033/13/0061–0039$38.00/0
www.karger.com/ofa

Original Article

Altered Activities of Antioxidant Enzymes in Patients with Metabolic Syndrome

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Key Words
Metabolic syndrome · Antioxidant enzymes · Reduced glutathione · Conjugated dienes

Abstract
Objective: In the pathogenesis of the metabolic syndrome (MetS), an increase of oxidative stress could play an important role which is closely linked with insulin resistance, endothelial dysfunction, and chronic inflammation. The aim of our study was to assess several parameters of the antioxidant status in MetS. ***Methods:*** 40 subjects with MetS and 40 age- and sex-matched volunteers without MetS were examined for activities of superoxide dismutase (CuZnSOD), catalase (CAT), glutathione peroxidase 1 (GPX1), glutathione reductase (GR), paraoxonase1 (PON1), concentrations of reduced glutathione (GSH), and conjugated dienes in low-density lipoprotein (CD-LDL). ***Results:*** Subjects with MetS had higher activities of CuZnSOD ($p < 0.05$) and GR ($p < 0.001$), higher concentrations of CD-LDL ($p < 0.001$), lower activities of CAT ($p < 0.05$) and PON1 ($p < 0.05$), and lower concentrations of GSH ($p < 0.05$), as compared with controls. Activity of GPX1 was not significantly changed. ***Conclusions:*** Our results implicated an increased oxidative stress in MetS and a decreased antioxidative defense that correlated with some laboratory (triglycerides, high-density lipoprotein cholesterol (HDL-C)) and clinical (waist circumference, blood pressure) components of MetS.

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Obes Facts 2013;6:39–47
DOI: 10.1159/000348569

Introduction

Currently, the prevailing notion of the metabolic syndrome (MetS) is that it is characterized by a cluster of risk factors for atherosclerosis and type 2 diabetes mellitus and can be regarded as a physiological and clinical entity [1]. The main components of MetS are accumulation of intra-abdominal fat, impaired metabolism of glucose, atherogenic dyslipidemia (low high-density lipoprotein cholesterol (HDL-C), hypertriglyceridemia), and arterial hypertension. In pathogenesis, several mechanisms were shown to take part, namely insulin resistance, chronic low-grade inflammation, endothelial dysfunction, and oxidative stress; their interactions have not been fully elucidated at present. Elevated levels of oxidative stress in subjects with MetS were demonstrated in many experimental and clinical studies [2].

Oxidative stress is defined as an imbalance between the production of reactive oxygen and nitrogen species (RONS) and their insufficient decomposition by the antioxidant system which results in macromolecular damage and disruption of redox signaling and control [3]. Free radicals and non-radical oxidants belong to RONS. Free radicals could induce DNA mutations, structural disorders in proteins, and peroxidative damage of cell membrane and plasma lipids [4]. RONS play an important role in the pathogenesis of many cardiovascular and neurodegenerative diseases as well as in type 2 diabetes mellitus and its complications [5].

The defense mechanisms of the human body against oxidative stress are complex and involve cellular and extracellular antioxidant systems which are regulated at multiple levels [6]. Various enzymes, e.g. superoxide dismutase (CuZnSOD), glutathione peroxidase 1 (GPX1), catalase (CAT), paraoxonase 1 (PON1), glutathione reductase (GR), as well as nonenzymatic antioxidant compounds (e.g. metal chelators, low-molecular-weight antioxidants) take part in the antioxidant defense.

In the first step of the defense mechanism against superoxide anions (O_2^-), the enzyme CuZnSOD catalyzes their dismutation into oxygen and H_2O_2. In the second step, CAT and GPX1 independently convert H_2O_2 to water. Any increase in the CuZnSOD catalytic activity produces an excess of H_2O_2 that must be efficiently neutralized by either CAT or GPX1; otherwise, H_2O_2 reacts with O_2^- producing in a two-step reaction (the Haber-Weiss reaction) hydroxyl radicals OH which are even more dangerous [5]. Cytosolic GPX1 detoxifies H_2O_2 in the presence of reduced glutathione (GSH), which is oxidized to oxidized glutathione (GSSG) and subsequently recycled by GR. GPX1 with the aid of GSH protects lipids against peroxidation. The pool of GSH has to be replenished by de novo synthesis that is catalyzed by the enzyme glutamate-cystein ligase. The PON1 enzyme as HDL-associated enzyme is implicated in the anti-inflammatory and antioxidant activities of HDL and impedes oxidative modification of low-density lipoprotein (LDL) thus protecting cell membranes from the damage caused by products of lipoperoxidation [7].

This study is focused on the state of the antioxidant defense system in patients with MetS. We intend to investigate the wide variety of known antioxidants in association with MetS. The activities of several antioxidant enzymes as well as the concentration of GSH were determined in the erythrocytes. It has been noted that these cells maintain fairly constant concentrations of enzymes throughout the life span which had been synthesized during the maturation of erythroid precursors [8]. Furthermore, levels of albumin, bilirubin, and calculated total peroxyl radical trapping (cTRAP) were assessed in serum. As a global marker of systemic oxidative stress, conjugated dienes in precipitated low-density lipoproteins (CD-LDL) were determined.

Obes Facts 2013;6:39–47
DOI: 10.1159/000348569

Participants and Methods

Participants

40 Caucasian subjects with MetS (20 male / 20 female) were recruited from outpatients who had been subsequently examined (from January 2008 until August 2010) at the Lipid Clinic of the 4th Department of Medicine, First Faculty of Medicine, Charles University in Prague. This study group was compared with a control group constituted from 40 volunteers without MetS matched for sex and age (20 male / 20 female), all Caucasian.

MetS was diagnosed according to the International Diabetes Federation criteria [9]. To be included, patients had to have central obesity (waist circumference ≥ 94 cm for men and ≥ 80 cm for women) and fulfill any two of the following four criteria: i) raised TG level (≥1.7 mmol/l), ii) reduced HDL-C (<1.03 mmol/l in males and <1.29 mmol/l in females) or specific treatment for these abnormalities, iii) raised blood pressure (BP) with systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension, and iv) raised fasting plasma glucose (≥5.6 mmol/l) or previously diagnosed type 2 diabetes mellitus. All samples were marked with unique anonymized identification numbers, and the data was merged only after the assays had been completed.

In the MetS group, 21 patients (52.5%) had three, 13 patients (32.5%) four, and 6 patients (15.0%) had all five of the above mentioned components of MetS. In the control group, only three subjects (7.5%) met two components of MetS, 15 (37.5%) controls met one, and the 22 (55.0%) volunteers showed no components of MetS. In the MetS group, 35 patients suffered from hypertension, and of these patients, 21 were under antihypertensive treatment. Among them, 12 were treated with an angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor type 1 blockers, and the 9 remaining subjects were on a combination of ACE inhibitor with calcium channel blockers.

Exclusion criteria for both groups were the following: current antioxidant therapy, excessive alcohol consumption (>30 g/day), hormonal replacement therapy, supplementation with polyunsaturated fatty acids, manifestation of cardiovascular and/or cerebrovascular diseases, type 1 diabetes mellitus, liver (with exception of nonalcoholic fatty liver disease) and kidney diseases (creatinine >130 μmol/l), microalbuminuria (urinary albumine 30–300 mg/day), hypothyroidism as well as recent infections and malignancies.

Informed consent was obtained from all participants. The study protocol was approved by the Ethical Committee of the First Faculty of Medicine, Charles University in Prague.

Blood Samples

Blood samples were collected after a 12-hour overnight fast. Activities of antioxidant enzymes (with exception of PON1) and concentrations of GSH were measured in hemolysed erythrocytes which had been separated from the EDTA plasma and washed three times with saline. Serum was used for all other parameters. Samples were stored at –80 °C until the assay.

Methods

Activities of antioxidant enzymes were measured spectrophotometrically using kinetic methods previously described [10]. Briefly, the activity of GPX1 was measured using tert-butyl hydroperoxide as a substrate, and the rate of NADPH degradation was monitored. The molar extinction coefficient of NADPH (6,220 mol/l/cm) was used for calculation of activity which was then expressed as U/g hemoglobin. The activity of GR was measured by monitoring the rate of NADPH degradation. Activity was calculated using the molar extinction coefficient of NADPH and expressed as U/g hemoglobin. The CAT activity was calculated using the molar extinction coefficient of H_2O_2 (43.6 mol/l/cm), whose degradation rate was monitored at 240 nm. Activity is expressed as kU/g hemoglobin. The method of CuZnSOD activity assessment is based on the monitoring of the rate of NBT-formazan generation. Superoxide dismutase activity was calculated by means of a calibrating curve; superoxide dismutase standard (Cat. No. S9636-1kU) was purchased from Sigma Aldrich (St. Louis, MO, USA). Activity was expressed as U/g hemoglobin. The arylesterase activity of PON1 was measured using phenylacetate as a substrate. Arylesterase activity of PON1 was calculated using the molar extinction coefficient of the produced phenol (1,310 mol/l/cm) and expressed as U/ml serum.

GSH was assessed by the modified spectrophotometric method according to Griffith [11]; this method is based on the determination of the relatively stable product of the reduction of 5,5′ dithiobis-2-nitrobenzoic acid (DTNB). The concentration of CD-LDL was assessed by the modified method of Wieland and Seidel at 234 nm [12]; both methods have been fully described in the previously mentioned paper [10].

Obes Facts 2013;6:39–47
DOI: 10.1159/000348569

All routine clinical tests were performed at the Institute for Clinical Biochemistry and Laboratory Diagnostics of General University Hospital in Prague: C-reactive protein (CRP) was determined by an immunoturbidimetric method using a K-ASSAY CRP kit (Kamiya Biomedical Company, Seattle, WA, USA; cv = max. 7.6%) on a Hitachi Modular analyzer (Tokyo, Japan). Copper and zinc were measured using atomic absorption spectrometry, uric acid by an enzymatic colorimetric method with the uricase-peroxidase system, and bilirubin by the 2,5-dichlorophenyldiazonium method with a Hitachi Modular analyzer. Plasma albumin was assessed by a colorimetric method using bromocresol green. Plasma concentrations of total cholesterol (TC) and triglycerides (TG) were measured by enzymatic-colorimetric methods (Boehringer, Mannheim, Germany). HDL-C was determined in the supernatant after precipitation of lipoproteins B by PTA/Mg^{2+}, using the kit from the same manufacturer; LDL-C was calculated according to Friedewald's formula. Concentrations of apolipoproteins apo B and apo A1 were measured by the Laurell rocket electroimmunoassay using standard and specific antibodies (Behringwerke, Marburg, Germany). The concentrations of insulin and C-peptide were determined with an electrochemiluminescence immunoassay (Roche, Basel, Switzerland). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as HOMA-IR = (fasting serum glucose (mmol/l) × fasting serum insulin (μU/ml)) / 22.5 [13]. The TRAP was calculated according to the formula: (0.63 (albumin) + 1.02 (uric acid) + 1.50 (bilirubin)) [14].

Statistical Analysis

Data was expressed as mean and standard deviation or median (25th–75th percentile) for data different from normal distribution. Normality of the distribution was tested by the Shapiro-Wilks W test. Comparisons between the groups were carried out by the independent t-test. Mann-Whitney U test was used for nonparametric comparisons and Spearman correlation coefficients for correlation analyses. All analyses were performed using version 8.0 of StatSoft Statistica software (2007, Czech version). The p value < 0.05 was considered statistically significant.

Results

Clinical and biochemical characteristics of the group of subjects with MetS and that of healthy controls are shown in table 1. The groups did not differ in age. In both groups there were no subjects with either type 1 or type 2 diabetes mellitus . The subjects included in the MetS group suffer from insulin resistance when the metabolism of glucose was impaired.

As expected, subjects with MetS had significantly higher values of body mass index and waist circumference. They also had higher values of systolic BP and diastolic BP, glucose, TC, TG, apolipoprotein B (apo B), and uric acid as well as a higher level of insulin and insulin resistance, as assessed by the homeostatic model HOMA-IR. Decreased values were observed for plasma concentrations of HDL-C and apo A1. The difference in CRP did not reach statistical significance. As expected, men have decreased levels of HDL-C and Cu and increased values of waist circumference compared to women.

Activities of antioxidant enzymes and concentrations of GSH and CD-LDL together with levels of cTRAP are presented in table 2. In the group of subjects with MetS, activities of CuZnSOD and GR as well as concentrations of CD-LDL and levels of cTRAP were significantly elevated. On the other hand, activities of CAT and PON1 as well as concentrations of GSH were found to be decreased.

Spearmen correlations (after Bonferroni adjustment) between selected variables are shown in table 3. All risk factors of MetS correlated significantly with the number of components of MetS, namely abnormal levels of glucose, waist circumference, TG, HDL-C, and SBP. Concentrations of CD-LDL significantly correlated with concentrations of TG and HDL-C.

Activities of CuZnSOD correlated positively with those of GR ($r = 0.341$, $p < 0.01$) and GPX1 ($r = 0.260$, $p < 0.05$), and with concentrations of Zn ($r = 0.363$, $p < 0.01$) as well as negatively with the ratio Cu/Zn ($r = -0.278$, $p < 0.05$). Activities of PON1 correlated positively with

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Table 1. Clinical and biochemical characteristics of subjects with the metabolic syndrome and of healthy controls[a]

	Metabolic syndrome			Controls		
	all	M	F	all	M	F
N	40	20	20	40	20	20
Age, years	58.4 (53.4–62.2)	57.0 (50.1–63.1)	58.7 (55.9–61.6)	58.5 (52.6–64.5)	57.8 (50.1–64.0)	59.5 (54.0–65.0)
Waist, cm	101.4 ± 9.1***	104.4 ± 6.4***+	98.3 ± 10.5***	82.5 ± 11.0	86.9 ± 13.2+	78.3 ± 6.6
BMI, kg/m^2	29.4 (27.4–31.7)***	29.0 (27.7–30.3)**	30.6 (25.9–32.4)***	23.9 (21.9–25.5)	25.0 (21.8–26.3)	23.8 (21.9–25.4)
Smoking, N (%)	10 (25.0)	6 (30.0)	4 (20.0)	4 (10.0)	0 (0.0)	4 (20.0)
Hypertension, N (%)	35 (87.5)	18 (90.0)	17 (85.0)	8 (20.0)	5 (25.0)	3 (15.0)
Systolic BP, mm Hg	140 (130–143)**	140 (130–140)***	140 (130–145)	130 (120–130)	128 (120–130)	130 (120–140)
Diastolic BP, mm Hg	90 (88–95)***	90 (90–95)***	90 (83–95)***	80 (80–80)	80 (80–85)	80 (80–85)
Glucose, mmol/l	5.4 (4.8–6.1)***	5.0 (4.7–5.8)	5.6 (5.1–6.3)**	4.7 (4.5–5.1)	4.9 (4.5–5.4)	4.7 (4.3–5.0)
Insulin, mU/l	11.4 (8.7–14.8)***	11.4 (8.6–15.1)*	11.3 (9.4–14.3)*	7.8 (4.6–9.5)	8.5 (4.8–10.1)	7.6 (4.6–9.1)
C-peptid, nmol/l	0.97 (0.84–1.19)***	0.99 (0.86–1.14)***	0.93 (0.81–1.28)***	0.64 (0.51–0.75)	0.59 (0.46–0.75)	0.68 (0.55–0.73)
HOMA-IR	3.0 (1.9–3.8)***	3.0 (1.8–3.7)*	3.0 (2.1–4.5)**	1.6 (1.0–2.1)	1.8 (1.0–2.2)	1.6 (1.0–1.9)
TC, mmol/l	6.3 (5.2–7.3)*	6.2 (5.2–7.1)*	6.5 (5.2–7.4)	5.7 (5.0–6.2)	5.7 (4.8–6.1)	5.8 (5.0–6.6)
TG, mmol/l	2.6 (1.9–3.7)***	2.3 (1.9–3.6)**	2.7 (1.7–3.9)***	1.1 (0.9–1.4)	1.1 (0.8–1.4)	1.0 (0.9–1.3)
HDL-C, mmol/l	1.2 (1.1–1.3)***	1.1 (1.0–1.2)***+	1.2 (1.1–1.3)***	1.6 (1.3–1.8)	1.5 (1.3–1.8)	1.6 (1.5–1.9)
LDL-C, mmol/l	3.6 (3.1–4.3)	3.5 (3.2–4.2)	3.6 (3.0–4.3)	3.5 (2.8–4.3)	3.4 (2.9–3.8)	3.6 (2.8–4.3)
Apo A1, g/l	1.26 ± 0.25**	1.24 ± 0.25	1.28 ± 0.26**	1.43 ± 0.21	1.36 ± 0.20+	1.50 ± 0.20
Apo B, g/l	1.34 ± 0.32***	1.39 ± 0.26***	1.28 ± 0.37	1.09 ± 0.25	1.06 ± 0.22	1.13 ± 0.28
NEFA, mmol/l	0.50 (0.39–0.72)	0.43 (0.35–0.68)	0.51 (0.43–0.75)	0.55 (0.43–0.71)	0.59 (0.435–0.83)	0.55 (0.40–0.61)
CRP, mg/l	2.7 (2.0–6.3)	2.8 (2.0–4.3)	2.7 (2.0–7.4)	2.3 (2.0–6.5)	2.0 (2.0–4.3)	4.6 (2.1–7.3)
Cu, µmol/l	17.7 (16.0–20.5)	17.0 (15.5–18.4)	19.6 (16.3–21.7)	18.5 (16.3–21.5)	16.3 (14.3–18.6)++	19.9 (18.5–23.4)
Zn, µmol/l	16.0 (13.4–17.7)	15.8 (13.4–17.8)	16.3 (13.8–17.1)	15.4 (14.6–19.9)	16.0 (14.5–18.3)	15.2 (14.7–20.8)
Bilirubin, µmol/l	9.1 (6.8–12.9)	10.9 (7.9–13.8)+	7.2 (6.1–9.6)	10.6 (8.0–15.2)	13.9 (9.8–18.2)++	9.0 (7.5–12.3)
Uric acid, µmol/l	346 (290–390)**	355 (312–420)*	329 (275–352)**	293 (236–346)	320 (291–370)+++	251 (195–293)

BP = Blood pressure; TC = total cholesterol; TG = triglycerides; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; Apo = apolipoprotein; HOMA-IR = homeostasis model assessment of insulin resistance; QUICKI = quantitative insulin sensitivity check index; NEFA = non-esterified fatty acids; CRP = C-reactive protein; Met = metabolic syndrome.

[a]Data presented as mean ± standard deviation (SD) for parametric and median (IQR) for non-parametric variables; MetS versus controls: *p < 0.05, ** p < 0.01, ***p < 0.001. Female versus male: +p < 0.05, ++p < 0.01, +++p < 0.001.

apo A1 (r = 0.479, p < 0.001). Concentrations of CD-LDL correlated positively with TC (r = 0.565, p < 0.001), apo B (r = 0.597, p < 0.001), and LDL-C (r = 0.384, p < 0.001), and negatively with CAT (r = –0.233, p < 0.05).

Discussion

In this study, comparing MetS patients with an age- and sex-matched control group, increased activities of CuZnSOD (+15%, p < 0.05) and GR (+19%; p < 0.001) and increased levels of CD-LDL (+14.4%; p < 0.001) and cTRAP (+6.5%; p < 0.01) were found in MetS

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Table 2. Parameters of oxidative stress of studied groups

	Metabolic syndrome			Controls		
	all	M	F	all	M	F
GPX1, U/g Hb	59.4 ± 15.8	57.6 ± 18.1	61.1 ± 13.4	59.1 ± 17.7	55.4 ± 19.2	62.8 ± 15.7
GR, U/g Hb	8.19 ± 1.54***	8.74 ± 1.21***$^{+}$	7.63 ± 1.67	6.88 ± 1.66	6.92 ± 1.76	6.83 ± 1.60
GSH, mg/g Hb	0.57 (0.38–2.73)*	0.56 (0.40–0.70)	1.51 (0.38–5.01)	1.46 (0.41–5.22)	1.22 (0.43–5.40)	1.70 (0.40–5.05)
CAT, kU/g Hb	189.6 ± 31.8*	192.5 ± 27.8	186.7 ± 35.8	204.6 ± 33.0	206.1 ± 32.5	203.1 ± 34.4
CuZnSOD, kU/g Hb	2.3 (1.9–2.5)**	2.3 (2.2–2.5)*	2.0 (1.7–2.5)$^{+}$	2.0 (1.2–2.5)	2.2 (1.1–2.6)	2.0 (1.3–2.4)
PON1, kU/l	158.9 ± 41.9*	152.0 ± 47.4	165.7 ± 35.4	179.9 ± 42.3	170.2 ± 36.1	189.5 ± 46.5
CD, mmol/l	61.9 (54.1–84.3)***	57.3 (53.4–68.7)*	63.8 (55.3–94.2)*	54.1 (41.3–63.6)	53.3 (33.8–63.6)	57.5 (42.9–68.1)
cTRAP, µmol/l	823 (766–877)**	875 (816–909)$^{+++}$	785 (732–835)**	773 (691–820)	809 (768–865)$^{+++}$	701 (655–776)

GPX1 = glutathione peroxidase 1; GR = glutathione reductase; GSH = reduced glutathione; CAT = catalase; CuZnSOD = CuZn-superoxide dismutase; PON1 = paraoxonase1 – arylesterase activity; CD = conjugated dienes in precipitated LDL; cTRAP = calculated total peroxyl radical trapping – calculation: [0.63 (albumin) + 1.02 (uric acid) + 1.50 (bilirubin)]; Met = metabolic syndrome; Data presented as mean ± standard deviation (S.D.) for parametric and median (IQR) for non-parametric variables.

MetS versus controls: *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Female versus male: $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$.

Table 3. Spearman correlation coefficients for components of the metabolic syndrome and parameters of oxidative stress in the combined group (metabolic syndrome plus controls) (N = 80)

	SBP	TG	HDL-C	Glucose	HOMA-IR	MetSC	CD	PON1	GR	GPX1	CAT	CuZnSOD
Waist	0.313	0.533^{+++}	−0.602^{+++}	0.402^{++}	0.570^{+++}	0.717^{+++}	0.336	−0.103	0.377^{+}	−0.160	−0.115	0.049
SBP	–	0.270	−0.147	0.141	0.103	0.405^{++}	0.338	−0.039	0.129	0.076	−0.108	−0.097
TG	-	–	−0.631^{+++}	0.396^{+}	0.453^{++}	0.736^{+++}	0.571^{+++}	−0.170	0.219	−0.067	−0.182	0.017
HDL-C	-	-	–	−0.357^{+}	−0.405^{+}	−0.681^{+++}	−0.374^{+}	0.321	−0.148	0.086	0.133	−0.015
Glucose	–	-	-	–	0.555^{+++}	0.540^{+++}	0.019	−0.103	0.127	−0.286	−0.081	−0.118
HOMA-IR	–	-	-	-	–	0.493^{+++}	0.099	−0.088	0.216	0.025	−0.066	−0.073
MetSC	-	-	-	-	–	–	0.442^{++}	−0.193	0.261	−0.097	−0.249	−0.115

SBP = Systolic blood pressure; TG = triglycerides; HDL-C = high density lipoprotein; HOMA-IR = homeostasis model assessment of insulin resistance; Met = metabolic syndrome; MetSC = number of components of the MetS (N = 1–5; waist circumference, glucose, triglycerides, HDL-C, SBP); GPX1 = glutathione peroxidase 1; GR = glutathione reductase; CAT = catalase; CuZnSOD = CuZn-superoxide dismutase; PON1 = paraoxonase-1-arylesterase activity; CD = conjugated dienes in precipitated LDL. $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$; after Bonferroni adjustment.

patients. In contrast, activities of CAT (–7.3%; $p < 0.05$) and PON1 (–11.7%; $p < 0.05$) as well as serum concentration of GSH (–61%; $p < 0.05$) were significantly decreased. The HOMA-IR demonstrated evidence of a significantly increased insulin resistance in subjects with MetS.

Under resting physiological conditions, biologic systems generate only small amounts of the superoxide anion. Its overproduction can result from mitochondrial electron leakage in hyperglycemia [15]. Other causes of superoxide overproduction are increased activities of NAD(P)H oxidases [16], xanthine oxidase, lipoxygenase, and cyclooxygenase as well as an imbalance in the thioredoxin system [17]. Large amounts of superoxide and other RONS arise in the accumulated fat, mainly due to increased activities of NAD(P)H oxidases and a decreased expression of antioxidant enzymes [18]. Adipose tissue is an important generator of oxidative stress and inflammation, contributing to the production of pro-inflammatory cytokines

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(TNFα, IL-1, IL-6 etc.). Oxidative stress is supposed to worsen the inflammatory state in MetS via activation of redox-sensitive transcription factors (particularly NFκB) by RONS, inducing the expression of TNFα and IL-6. These cytokines increased CRP synthesis. However, in our study, we did not find a statistically significant difference in CRP levels between MetS patients and controls. This could be caused by the method used for CRP measurement. The method used in our study lacks the sensitivity to differ between low-grade inflammation in MetS (CRP between 1.0 and 3 mg/l) and subjects without MetS (CRP < 1.0 mg/l).

The raised CuZnSOD activities in the erythrocytes of patients with MetS found in our study may be compared with the results of Mitrijevic-Sreckovic et al. [19], who described slightly increased CuZnSOD activities in children with MetS in comparison with obese children without MetS. Studies on serum CuZnSOD activities did not show consistent results [20, 21]. Increased CuZnSOD activity results in raised amounts of H_2O_2 which becomes toxic when activity of CAT is normal or decreased. Induction of one enzyme (CAT or CuZnSOD) does not necessarily lead to the induction of the other one [22]. Another source of H_2O_2 is its passage through the erythrocyte membrane [23]. The elevated production of ROS in the endothelium could thus lead to increased levels of ROS also in erythrocytes.

In our study, we have found a significantly decreased activity of CAT. Because of the increased activity of CuZnSOD in our study, elevated levels of H_2O_2 have to be expected. According to study of Kirkman et al. [24], during lengthy exposure of CAT to H_2O_2, the CAT-bound NADPH became oxidized to $NADP^+$ and activity of CAT fell to about one third of the initial activity. Consequently, the cause of the decrease of CAT activity could be the damage of erythrocyte CAT by H_2O_2. Contrary to our study, Cardona et al. [20, 21] found increased activities of CAT in patients with hypertriglyceridemia (concentration of TG > 1.7 mmol/l) apart from the presence of MetS, and these activities were further increased after fat overload. Decreased activities of CAT were described in patients bearing only individual components of MetS – obesity [25], hypertension [26], or insulin resistance [27]. Decreased activity of CAT implies stressed condition of erythrocytes when complete removal of H_2O_2 is not possible [28]. Low activities of CAT were associated with an increased risk of diabetes mellitus and its complications [5, 29].

The GPX1 activity in our study was not altered in MetS patients. This result is in accordance with the study of Mitrijevic-Sreckovic [19]. On the contrary, Cardona et al. [20, 21] found lower activities of GPX1 in a group of subjects with hypertriglyceridemia, a part of MetS presence, and the drop of its activity was almost to 75% of that of the control group. Bougoulia et al. [30] showed decreased activity of GPX1 in obese subjects as well as an increase after weight reduction.

As expected, concentrations of GSH were significantly decreased and activities of GR increased in our group of subjects with MetS. Decreased concentrations of GSH with opposite changes in GSSG levels were also found in MetS subjects in the study of Cardona et al. [20]. On the other hand, Cardona et al. [21] registered a significant drop in GR activity in MetS subjects. Increased activity of GR could be attributed to a compensatory protective mechanism of the cells against ROS. Furthermore, our expected increase in the GSSG/GSH ratio due to lower levels of GSH may stimulate compensatory increase in GR activity in blood to reduce higher levels of GSSG into GSH [31].

The finding of decreased arylesterase activities of PON1 in our subjects with MetS is in accordance with other studies [32, 33]. Because it was shown [34] that there is a strong positive correlation between arylesterase and paraoxonase activity of PON1, we could therefore discuss arylesterase and paraoxonase activity of PON1 together. Low activities of PON1 have been shown to be associated with oxidative stress, hypercholesterolemia, diabetes mellitus, cardiovascular diseases, and sepsis [34, 35].

In the present study, we found significantly higher concentrations of CD-LDL in subjects with MetS. This test was shown to be the most sensitive indicator of lipid peroxidation and can be regarded as a global marker of systemic oxidative stress [36]. In this study, several

anthropometric and biochemical characteristics of MetS correlated significantly with increased concentrations of CD-LDL, which reflect oxidation of the lipid component of LDL. This finding is in agreement with the results of our studies concerning the severity of MetS, oxidative stress, hypertriglyceridemia, and fatty acid metabolism [37, 38]. The important role of lipid peroxidation in the pathogenesis of MetS has been proven in many experimental and clinical studies [39].

Conclusion

In the present study, we estimated a wide variety of antioxidant enzymes, and activities of several enzymes were changed in subjects with MetS. Enzyme activities were assessed in the erythrocytes where the concentration of enzymes remain stable throughout the life span and reflect adaptive changes in their expression in erythroid precursors. According to our results, alterations of antioxidant enzymes related to MetS are not uniform. While activities of CuZnSOD and GR were higher in the MetS group than in healthy subjects, a decrease in CAT and PON1 as well as the absence of the expected increase in GPX1 indicate a disorder in antioxidant defense mechanisms. Our results could be interpreted that the erythrocytes and their GSH levels and activities of GR and GPX1 protect against oxidative stress in MetS. The severity of MetS, as assessed by the number of its components, significantly correlated with the concentrations of CD-LDL.

Acknowledgments

This work was supported by by the Research Project of Charles University in Prague, First Faculty of Medicine – PRVOUK-P25/LF1/2, and by the grant IGA NS9769-4 of the Ministry of Health of the Czech Republic.

Disclosure Statement

We hereby state that there is no conflict of interest.

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METABOLIC SYNDROME AND RELATED DISORDERS
Volume 15, Number 7, 2017

Pp. 371–378
DOI: 10.1089/met.2017.0040

Fatty Acid Composition of Plasma Phosphatidylcholine Determines Body Fat Parameters in Subjects with Metabolic Syndrome-Related Traits

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Abstract

Background: This study examines the associations of fatty acids (FAs) in plasma phosphatidylcholine (PC) with the anthropometrical and biochemical characteristic of patients with metabolic syndrome (MetS)-related traits.
Methods: We analyzed the FA profiles of PC in 300 persons with MetS-related traits (152 M/148F, mean age 46.9 ± 9.0 years) and in 70 healthy controls of the same age using a balanced men/women ratio and gas–liquid chromatography. Multivariate linear regression analysis was performed to determine the coefficients of determination (R^2) using FA proportions of the mentioned proband characteristics.
Results: The FA composition of PC in patients with MetS traits was only associated with waist circumference ($R^2 = 0.27$), waist-to-hip ratio (WHR; $R^2 = 0.41$), body fat percentage ($R^2 = 0.62$), and fat mass ($R^2 = 0.29$). Positive associations were found for dihomo-γ-linolenic (DGLA), palmitic, stearic (SA), α-linolenic (ALA), and eicosapentaenoic acids, whereas negative associations were found for linoleic (LA), oleic, and docosapentaenoic acids. Palmitoleic acid (POA) was positively associated with waist circumference but negatively with fat percentage. In controls, significant associations were found for waist circumference ($R^2 = 0.51$), WHR ($R^2 = 0.53$), body fat percentage ($R^2 = 0.60$), and fat mass ($R^2 = 0.34$). DGLA and saturated FA (SFA) were positively associated, whereas docosahexaenoic, adrenic, and *cis*-vaccenic acids were negatively associated. The study group differed from controls as follows: lower concentrations of LA and total n-6 FA, higher indices of delta-9-desaturase and delta-6 desaturase activity and higher proportions of POA, SA, ALA, DGLA, and SFA.
Conclusions: We found significant associations ($R^2 > 0.25$) of FA in plasma PC with adiposity in middle-aged persons with MetS-related traits, but not with metabolic indices.

Keywords: fatty acids, phosphatidylcholine, *de novo* lipogenesis, multivariate linear regression analysis, anthropometric variables

Introduction

Fatty acids (FAs) are among the basic structural parts of lipid molecules, which, together with carbohydrates and proteins, represent the main components of living organisms. The most important metabolic function of FAs is as an energy source. Furthermore, some FAs (as essential components of cell membranes) are known for their physicochemical properties and membrane functions, for example, interactions of receptors, characteristics of ion channels, and other membrane-bound protein structures, whereas other FAs modulate gene expression. Individual FA molecules released from membranes by various stimuli are precursors of prostaglandins, leukotrienes, thromboxanes, and other compounds (e.g., docosanoids) with miscellaneous biological functions.[1]

In humans, FAs originate on the one hand from their nutritional intake and on the other from endogenous synthesis.[2] FA profiles are further modulated by desaturases, elongases, and degradation mechanisms stemming from enzymatic as well as nonenzymatic actions.[3–5] In addition, resulting FA content in different compartments is affected in every individual by racial, genetic, and pathophysiological factors.[2,6] Therefore, the metabolic conversion of individual

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FA can differ between various populations,[7] leading to pathological states such as metabolic syndrome (MetS),[8,9] type 2 diabetes mellitus (T2DM)[10] as well as cardiovascular,[11] neuropsychiatric,[12] and malignant[13,14] diseases.

Incidence of MetS, which is characterized by significant insulin resistance, is mostly connected with increased proportions of serum-saturated FA (SFA), palmitoleic acid (POA), and dihomo-γ-linolenic (DGLA) acid, whereas proportions of linoleic acid (LA) and total n-6 FAs are decreased.[8,9] Moreover, increased estimated activities are usually found for the enzymes delta-9 desaturase (D9D) and delta-6 desaturase (D6D), which catalyze the synthesis of POA from palmitic acid (PA), oleic acid (OA) from stearic acid (SA), and γ-linolenic (GLA) acid from LA.[8,9] In contrast, the activity of D5D, which catalyzes the synthesis of arachidonic acid (AA, 20:4n-6) from DGLA 20:3n-6, is decreased. Similar FA alterations correlate with both total and cardiovascular mortality as well as T2DM risk.[10,11]

Interestingly, some of these FA alterations, such as increased D9D activity, increased SFA, and decreased n-6 FA, have also been described in some malignancies and depressive disorders[12–14]; diseases in which insulin resistance but also obesity and fat accumulation are supposed to play a role. The associations between dysregulated FA metabolism and body fat size and distribution are not yet fully understood. Altered profiles of FAs for various lipid classes (PL, triacylglycerols [TAGs], and CE) may also potentially serve as biomarkers or treatment targets for the already mentioned pathological conditions.

The aim of this pilot study was to elucidate the connections between individual FAs in plasma phosphatidylcholine (PC) and the basic anthropometric, clinical, and biochemical parameters in patients with MetS-related traits. All patients were referred to an outpatient lipid clinic.

Materials and Methods

Study design and participants

This cross-sectional study was carried out at the 4th Department of Internal Medicine at General University Hospital, Prague, from January 2012 to March 2015. The study protocol was approved by the Joint Ethical Committee of the General University Hospital and the 1st Medical Faculty, Charles University, Prague. Written informed consent was obtained from all participants. A total of 300 patients were examined at the Lipid Clinic of the 4th Department of Medicine, the 1st Faculty of Medicine, Charles University, and the General University Hospital, Prague.

The study population comprised 158 men and 142 women, consecutively examined as outpatients at the 4th Department of Medicine between the years 2012 and 2015. All probands were mostly in the central obesity category as defined by the International Diabetes Federation[15] and exhibited at least one component of MetS.

Eighty-three percent had increased waist circumference (≥94/80 cm for men and women, respectively), whereas high blood pressure (or specific treatment for hypertension) was present in 44% of probands. Other components of MetS, such as impaired fasting glycemia (≥5.60 mmol/L), hypertriacylglycerolemia (>1.70 mmol/L), and low concentrations of high-density lipoprotein-cholesterol (HDL-C) (<1.00 mmol/L for men, 1.30 mmol/L for women) were present in 22%, 55%, and 29% of cases, respectively. A total of 32% from the high cardiovascular risk group of patients were cigarette smokers.

We also examined a smaller group of 70 healthy controls (CON) of the same age (balanced men/women ratio), 14% of whom were smokers. The following exclusion criteria were applied to the study: ongoing antioxidant, lipid-lowering or antidiabetic medication, excessive alcohol consumption (>30 g/day), hormonal replacement therapy, supplementation with polyunsaturated fatty acids (PUFAs; n-3 and n-6 families), manifestation of cardiovascular disease (CVD) and/or cerebrovascular disease, diabetes mellitus, liver disease (except for nonalcoholic fatty liver disease), kidney disease (creatinine >130 μmol/L), hypothyroidism, and recent infections and malignancies.

Laboratory measurements

Basic clinical and anthropometric data, including the assessment of body fat, were analyzed using standard methods as described previously.[16] Plasma concentrations of biochemical parameters were measured using routine methods, whereas concentrations of nonesterified fatty acids (NEFAs) were determined using an enzymatic colorimetric method (Randox Laboratories, United Kingdom). The homeostasis model assessment method (HOMA-IR) was used as the index of insulin resistance.[17]

FA patterns in plasma PC were examined using analytical procedures (as described previously) and expressed as molar percentages. Method variability, presented as a relative standard deviation, ranged from 1.07% for PA (16:0) to 8.60% for 16:1n-9.[18] We analyzed the complete range of FA profiles in plasma PC. However, minor FAs (<0.1 mol%) were not included, such as 12:0, 14:1n-5, and 20:0 FA. Minor FAs exhibited considerable analytical variance, thus increasing the demands on group size and/or detectable differences. Desaturase activities were estimated using FA product/precursor ratios.[9]

Statistical methods

Comparisons between the study and control groups were carried out using the nonparametric two-sample Wilcoxon rank-sum test for numeric variables and the chi-squared test for categorical variables. Reported p-values were adjusted using Benjamini–Hochberg correction for multiple comparisons to control the false discovery rate, that is, the expected proportion of false discoveries among the rejected hypotheses.[19] Stepwise multivariate linear regression analysis was performed to determine the coefficients of determination (R^2) using FA concentrations from both the anthropometric and biochemical characteristics of the probands.

As this is a cross-sectional exploratory study that uses multiple biological data sources, we selected a value of $R^2 > 0.25$ as a moderate effect size index.[20] All data were processed and statistical analysis was performed using the R statistical environment, version 3.3.2.[21] Data were expressed as mean ± standard deviation or as medians (interquartile range) for data with non-normal distribution. Values of $p < 0.05$ were considered statistically significant.

Results

The basic demographic, anthropometric, and biochemical parameters of the study and control groups are shown in

Table 1. Basic Anthropometric and Biochemical Data of the Analyzed Groups

	Study group	*Control group*	P
Number	300	70	—
Age (years)	47 (40–55)	43 (33.2–54.8)	NS
M/F	152/148	36/34	NS[a]
No. of smokers	97/300	10/70	NS
No. of MetS components	2 (2–3)	0 (0–0)	<0.001
Systolic blood pressure (mmHg)	130 (120–140)	120 (110–125)	<0.001
Diastolic blood pressure (mmHg)	85 (80–90)	78.5 (70–80)	<0.001
Body weight (kg)	83.8 (73–96)	68.2 (60.5–75.2)	<0.001
BMI (kg/m^2)	27.9 (25.4–30.9)	23.2 (21.3–25.1)	<0.001
Waist circumference (cm)	96 (88–105)	78 (75–86.5)	<0.001
WHR (1)	0.9 (0.9–1)	0.8 (0.8–0.9)	<0.001
Body fat (% body weight)[b]	34.2 (26.5–39.6)	26.1 (20.2–31.5)	<0.001
Fat mass (kg)	28.1 (20.9–33.4)	18.1 (13–21.7)	<0.001
CRP (mg/L)	3 (1.5–5.2)	2 (1–4.2)	<0.05
Uric acid (μmol/L)	318 (256–377.2)	263.5 (218.5–309.8)	<0.001
TC (mmol/L)	6 (5.1–7.1)	5.2 (4.7–5.9)	<0.001
TAG	1.9 (1.2–3.1)	1 (0.8–1.1)	<0.001
HDL-C (mmol/L)	1.3 (1.1–1.6)	1.6 (1.3–1.8)	<0.001
LDL-C (mmol/L)	3.3 (2.7–4.2)	3 (2.5–3.7)	NS
Non-HDL-C (mmol/L)	4.5 (3.7–5.6)	3.6 (3–4.4)	<0.001
Apo A1 (g/L)	1.4 (1.2–1.6)	1.4 (1.3–1.6)	NS
Apo B (g/L)	1.2 (1–1.5)	0.9 (0.8–1.1)	<0.001
Glucose (mmol/L)	4.9 (4.6–5.5)	4.8 (4.4–5)	<0.001
HOMA-IR (index)[c]	2 (1.4–3.2)	1.2 (0.9–1.8)	<0.001
Insulin (mU/L)	9.2 (6.6–13.5)	5.8 (4.2–8.6)	<0.001
C-peptide (ng/L)	0.8 (0.7–1.1)	0.6 (0.5–0.7)	<0.001
NEFAs (mmol/L)	0.6 (0.4–0.8)	0.4 (0.3–0.6)	<0.01

Numeric values are medians (interquartile range).
[a]Chi-square test.
[b]According to Durnin–Womersley equations.[16]
[c]HOMA-IR, fasting glucose (mmol/L)×insulin (mU/L)/0.22.5.
Apo, apolipoprotein; CD, conjugated dienes in LDL particles; CRP, C reactive protein; HDL-C, cholesterol in high-density lipoproteins; HOMA-IR, homeostasis model assessment method; LDL-C, cholesterol in low-density lipoproteins; MetS, metabolic syndrome; NEFAs, nonesterified fatty acids; TAGs, triacylglycerols; TC, total plasma cholesterol; NS, nonsignificant; WHR, waist-to-hip ratio.

Table 1. As expected, the study group differed significantly from controls for both anthropometric and biochemical variables. The study group registered higher values for systolic and diastolic blood pressures, weight, body mass index (BMI), waist circumference, waist-to-hip ratio (WHR), body fat percentage, and fat mass. Thirty-two percent of the study group participants were smokers, compared with only 10% of the control group. Furthermore, the study group had significantly higher concentrations of total plasma cholesterol (TC), TAGs, non-HDL-C, apolipoprotein B, uric acid, glucose, insulin, C-peptide, and insulin resistance index (HOMA-IR), but lower HDL-C. The study group also differed from controls with higher concentrations of plasma C reactive protein (CRP) and NEFAs.

Differences in the FA composition of plasma PC between the study and control groups are shown in Table 2. The study group had a significantly higher molar percentage of SFAs, especially SA (18:0). In comparison with the control group, the study group exhibited significantly higher percentages of POA (16:1n-7), DGLA (20:3n-6), and GLA (18:3n-6), but significantly lower percentages of total n-6 PUFAs, especially LA (18:2n-6) and AA (20:4n-6). In comparison with controls, significantly higher activities of D9D for POA (D9D16) and D6D were found in the group of patients with MetS-related traits.

Table 3 shows the results of multivariate linear model analysis for associations between the basic anthropometric and biochemical parameters of patients with MetS-related traits, namely plasma PC, FA composition, age, and gender. The models obtained by linear discriminative analysis in the study group of patients showed that the most important links between parameters were visceral fat accumulation and FAs in plasma PC. We found significant positive associations with indices of fat accumulation, primarily for DGLA and SFA [(PA, 16:0) and SA (18:0)]. Fat percentage was positively associated with α-linolenic acid (ALA, 18:3n-3). Similarly, fat mass was positively associated with both ALA and eicosapentaenoic acid (EPA, 20:5n-3). Unexpectedly, POA was positively associated with waist circumference, but negatively with fat percentage.

Furthermore, negative associations for parameters correlating with visceral fat were found for LA (18:2n-6), OA (18:1n-9), and docosapentaenoic acid (DPA, 22:5n-3). Fat percentage was also negatively associated with gondoic acid (20:1n-9). In the study group, male gender and age contributed to higher values of waist circumference and WHR. In contrast, body fat and body fat percentage were negatively associated with male gender. Age was positively associated with WHR and fat percentage in the study group only.

In the study group, other MetS traits did not show strong associations with FA profiles in plasma PC such as mean arterial pressure ($R_{adj}^2 = 0.069$, $F = 6.51$), CRP (R_{adj}^2 0.101, $F = 6.57$), conjugated dienes in LDL (R_{adj}^2 0.137, $F = 8.52$), and uric acid (R_{adj}^2 0.210, $F = 19.64$), nor did lipid

Table 2. Fatty Acid Profiles of Plasma Phosphatidylcholine

	Study group	*Control group*	P
14:0	0.26 (0.2–0.32)	0.26 (0.19–0.34)	NS
16:0	29.98 (28.73–31.97)	29.7 (28.53–30.85)	NS
16:1n-9	0.12 (0.09–0.14)	0.11 (0.09–0.13)	NS
16:1n-7	0.56 (0.46–0.72)	0.47 (0.39–0.6)	<0.001
18:0	14.2 (13.3–15.27)	13.4 (12.72–14.3)	<0.001
18:1n-9	10.15 (9.19–11.53)	10.33 (9.17–11.74)	NS
18:1n-7	1.53 (1.34–1.7)	1.58 (1.4–1.86)	NS
18:2n-6	23.03 (20.93–24.98)	24.37 (22.6–25.98)	<0.01
18:3n-6	0.09 (0.07–0.12)	0.07 (0.05–0.1)	<0.01
18:3n-3	0.21 (0.16–0.27)	0.2 (0.16–0.28)	NS
20:1n-9	0.12 (0.1–0.15)	0.13 (0.11–0.17)	NS
20:2n-6	0.38 (0.31–0.46)	0.4 (0.32–0.44)	NS
20:3n-6	3.02 (2.54–3.46)	2.77 (2.27–3.22)	<0.05
20:4n-6	10.39 (8.48–11.82)	10.2 (8.5–11.74)	NS
20:5n-3	0.89 (0.69–1.18)	0.78 (0.64–1)	NS
22:4n-6	0.28 (0.21–0.34)	0.28 (0.2–0.34)	NS
22:5n-6	0.17 (0.13–0.21)	0.18 (0.14–0.24)	NS
22:5n-3	0.81 (0.6–0.96)	0.8 (0.63–0.96)	NS
22:6n-3	2.85 (1.98–3.63)	2.88 (2.2–3.86)	NS
ΣSFA	44.68 (43.34–46.45)	43.29 (42.4–44.46)	<0.001
ΣMFA	12.54 (11.36–14.19)	12.8 (11.31–14.32)	NS
ΣPUFAn-6	37.17 (34.73–39.34)	38.54 (36.58–40.35)	<0.05
ΣPUFAn-3	4.75 (3.78–5.85)	4.74 (3.94–5.97)	NS
D9D16: 16:1n-7/16:0	0.0186 (0.0155–0.0227)	0.0158 (0.0132–0.0198)	<0.001
D9D18: 18:1n-9/18:0	0.7148 (0.6446–0.8099)	0.7679 (0.6721–0.8363)	NS
D5D: 20:4n-6/20:3n-6	3.4120 (2.8099–4.0478)	3.4436 (3.0575–4.2888)	NS
D6D: 18:3n-6/18:2n-6	0.0038 (0.0028–0.0054)	0.0029 (0.0021–0.0042)	<0.001

Numeric values are medians (interquartile range). Data are in molar%, only relevant fatty acids are shown; shorthand notation for fatty acids: number of carbon atoms: number of double bonds; n—number of carbon atoms from the methyl end to the nearest double bond.
SFAs, saturated fatty acids; MFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids, Σ, sum.

parameters such as TC (R_{adj}^2 0.069, F = 12.14), TAG (R_{adj}^2 0.109, F = 10.12), HDL-C (R_{adj}^2 0.242, F = 16.92), and LDL-C (R_{adj}^2 0.111, F = 8.26). Neither glucose (R_{adj}^2 0.100, F = 7.67), insulin (R_{adj}^2 0.150, F = 13.19), nor HOMA-IR (R_{adj}^2 0.142, F = 12.38) showed a close relationship with FA profiles of plasma PC.

Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/met) shows results obtained in the small group of healthy persons of the same age. In this set of probands, we found positive associations between body fat indices and DGLA, 14:0, 18:0, and AA, as well as, surprisingly in the case of body fat percentage, LA. Negative associations with visceral fat were noted for adrenic (22:4n-6), docosahexaenoic (DHA, 22:6n-3), and vaccenic (18:1n-7) acids. Male gender was positively associated with waist circumference and WHR, but negatively with fat mass and body fat percentage. In the control group, age was not associated with indices of body fat accumulation except for fat percentage, in which case a positive association was found.

Discussion

In this study, we found significant associations between the FA composition of plasma PC and anthropometric parameters (waist circumference, WHR, fat percentage, and body fat mass). Waist circumference and WHR are considered markers of visceral fat accumulation, which is the central component of MetS.[15,22] In the study group, male gender and age contributed to higher values of waist circumference and WHR, which is in correspondence with the data in the literature on the influence of gender on body fat distribution.[23] We demonstrated that aging is connected with increasing adiposity both in men and in women. In comparison with women, we found that men have characteristically higher visceral fat mass but lower subcutaneous fat.[23]

The links between individual FAs and different clinical and metabolic parameters have been observed in both experimental and clinical studies.[24] Proportions of 16:0 and 18:0 in plasma and erythrocytes are associated with insulin resistance and altered glucose metabolism in nondiabetic individuals,[25] higher risk of T2DM in both genders,[26] worsening of glycemia in men,[27] and coronary heart disease (CHD) risk in postmenopausal women.[28]

The higher proportion of POA in plasma lipid classes is an independent marker of high TAG and abdominal adiposity in men,[29] whereas the content of this type of POA in adipose tissue has been shown to positively correlate with the extent of obesity.[30] POA in plasma CE has been found to be positively associated with plasma glucose concentrations[31] as well as incidence of T2DM,[24] BMI, TC, and HDL-C.[32]

In erythrocyte membranes, POA content has been shown to be associated with inflammation and higher risk of MetS,[33] worsening of glycemia,[27] and to positively correlate with CHD in men.[34] In contrast, some authors indicate that circulating POA may favorably influence insulin sensitivity in the liver and skeletal muscle by acting as a ''lipokine''.[35] The relationship between POA circulating in NEFAs and insulin sensitivity has been investigated by several other studies with equivocal results.[36–38]

In our group of individuals with MetS-related traits, we observed an opposite (positive) association between POA

Table 3. Multivariate Linear Model Analysis for the Group of Patients at High Cardiovascular Risk ($N = 300$)

	F	*Regression coefficient β*	*Standard error*	*t*	*Contribution (%)*	P
			Dependent variable: **Waist,** F = 17.094: adjusted $\mathbf{R^2 = 0.27}$			
Intercept		+109.807	12.037	9.122		<0.0001
Gender/male	37.91	+7.158	1.293	5.536	10.99	<0.0001
20:3n6[a]	17.94	+2.899	0.900	3.221	4.66	0.0014
22:5n3	0.23	−12.211	2.610	−4.679	*2.47*	<0.0001
18:2n6	19.65	−0.949	0.267	−3.551	*3.41*	0.0004
18:1n9	4.42	−2.236	0.477	−4.684	*3.16*	<0.0001
18:0	12.79	+1.618	0.474	3.412	1.52	0.0007
16:1n7	1.24	+9.467	3.953	2.395	1.17	0.0173
			Dependent variable: **WHR,** F = 43.26784: adjusted $\mathbf{R^2 = 0.41}$			
Intercept		+0.424	0.075	5.669		<0.0001
Gender/male	158.38	+0.099	0.008	13.040	34.48	<0.0001
18:0	9.99	+0.013	0.003	4.678	1.73	<0.0001
16:0	2.29	+0.006	0.001	4.605	2.64	<0.0001
Age	1.99	+0.001	0.000	3.059	1.54	0.0024
20:3n6	2.80	+0.012	0.005	2.477	1.02	0.0138
			Dependent variable: **Fat percentage,** F = 50.24893: adjusted $\mathbf{R^2 = 0.62}$			
Intercept		+5.641	8.258	0.683		0.4951
Gender/male	243.90	−10.103	0.668	−15.119	*44.82*	<0.0001
22:5n3	65.21	−8.697	1.581	−5.499	*10.30*	<0.0001
Age	38.14	+0.172	0.036	4.789	2.13	<0.0001
20:3n6	0.13	+2.104	0.447	4.703	1.67	<0.0001
20:1n9	9.63	−16.525	7.752	−2.132	*0.73*	0.0339
20:5n3	14.20	+1.404	0.620	2.265	0.60	0.0243
18:0	0.30	+0.755	0.248	3.044	0.28	0.0026
16:0	11.30	+0.505	0.145	3.475	0.71	0.0006
16:1n7	1.94	−3.677	1.471	−2.499	*0.36*	0.0130
18:3n3	4.85	+8.792	3.646	2.412	0.63	0.0165
			Dependent variable: **Fat mass,** F = 16.27317: adjusted $\mathbf{R^2 = 0.29}$			
Intercept		−8.459	11.812	−0.716		0.474463
22:5n3	49.22	−12.771	2.414	−5.291	*13.89*	<0.0001
20:3n6	3.49	+3.771	0.698	5.402	5.96	<0.0001
Gender/male	20.29	−5.016	1.005	−4.992	*3.57*	<0.0001
20:5n3	3.51	+2.323	0.981	2.369	1.38	0.0185
18:0	3.57	+1.373	3.412	3.412	1.70	0.0007
16:0	11.89	+0.742	0.202	3.679	0.79	0.0003
18:1n9	1.71	−0.913	0.309	−2.950	*1.12*	0.0034
18:3n3	1.51	+11.424	6.152	1.857	0.60	0.0643

Determination by plasma phosphatidylcholine fatty acid composition, age, and gender. *Italics* are used to indicate negative contributions to the variance of the dependent variable. Variable and its R2 are emphasized in *bold*.

[a]Shorthand notation of fatty acids: number of carbon atoms: number of double bonds.

n—number of carbon atoms from the methyl end to the nearest double bond; R^2—proportion of variance explained by the model (adjusted by the number of variables used).

and waist circumference on the one hand and a negative association with body fat percentage on the other. It is acknowledged that waist circumference is a preferential indicator of abdominal/visceral fat and is associated with cardiovascular risk factors. Furthermore, total body fat includes gluteofemoral fat, which is known to be a major source of circulating lipokine POA from adipose tissue.[38] Insulin resistance has a significant negative correlation with the proportion of POA in the plasma NEFA pool.

In a population-based sample of 1150 Chinese individuals, Wu et al.[39] observed that ''trunk fat'' approximately corresponds to visceral fat. In contrast, ''large leg fat,'' which represents gluteofemoral fat mass, was associated with unfavorable adipokine profiles, such as lower adiponectin levels and higher PAI-1, as well as inflammatory parameters (higher CRP and IL-6). Similar to our findings, Warensjö et al.[40] found significant positive correlations between markers of obesity and proportions of DGLA, PA, SA, and POA and an inverse correlation with LA in serum cholesteryl esters. DGLA, an important determinant of fat accumulation in our study, is associated with many clinical conditions.

Increased levels of DGLA in both plasma and erythrocyte lipids are connected with insulin resistance,[41] MetS, BMI, plasma TAGs,[42] chronic pancreatitis,[43] and markers of inflammation.[44] DGLA has been shown to predict worsening of hyperglycemia[45] and T2DM[24,46] and to correlate with CHD risk[28] and obesity.[47] In our group of patients with MetS-related traits, we observed beneficial associations for OA, gondoic acid (20:1 n-9), and PUFAs, especially LA and DPA.

Substituting SFAs for monounsaturated fatty acids (MFAs) (mainly OA) is associated with improved insulin sensitivity and glycemic control in patients with T2DM.[48] In the large population-based study of Finnish men, METabolic syndrome in men (METSIM), MFAs were biomarkers

for increased risk of developing hyperglycemia and T2DM relative to total FAs.[49] *Cis*-vaccenic acid (18:1 n-7), which was negatively associated with fat mass in our model, was linked to lower risk of T2DM in the Cardiovascular Health Study.[26] Moreover, the erythrocyte membrane *cis*-vaccenic acid was inversely related to CHD for participants in the Physicians' Health Study.[34]

The metabolic effects of *cis*-vaccenic acid have not been sufficiently studied. In one experimental work, *cis*-vaccenic acid 18:1 n-7 acted as a mediator of FA elongase-5 (Elovl5), suppressing hepatic gluconeogenic gene expression. Decreased hepatic abundance of 18:1 n-7 is associated with increased expression of enzymes involved in gluconeogenesis and glucose intolerance.[50]

In our study, LA (18:2n-6) in plasma PC was negatively associated with waist circumference. Similarly, in a study of overweight adolescents carried out by Steffen et al.,[51] levels of LA in serum PL and CE negatively correlated with BMI, WHR, and concentrations of TAG, whereas DGLA was found to have positive associations with BMI, waist circumference, and TAG. In a prospective study of Finnish men, LA in plasma lipid fractions predicted a decrease in glycemia, whereas POA and DGLA predicted an increase.[27] A recently published prospective study on nonobese women revealed a positive association between PUFA n-6 content in erythrocyte membranes and weight gain, but a negative association with PUFA n-3 for a 10-year period.[52]

In our study group, DPA (22:5n-3) was significantly and negatively associated with most body fat accumulation indices. This FA is an elongated metabolite of EPA and, as an intermediary product between EPA and DHA, exerts different health effects, including anti-inflammatory actions, platelet aggregation inhibition, lipid profile improvement,[53] and boosting of insulin secretion.[54] In one study, higher serum total n-6 PUFA, LA, and AA (20:4n-6) concentrations as well as estimated Δ5 desaturase activity were associated with lower risk of incident T2DM, whereas higher GLA (18:3n-6) and DGLA (20:3n-6) concentrations as well as estimated Δ6 desaturase activity were associated with higher risk of T2DM.[55]

The potential mechanisms involved in the negative associations between adrenic acid (22:4n-6) and indices of fat accumulation are unclear, because there is a scarcity of literature on the metabolic effects of adrenic acid. Adrenic acid, abundant in the vasculature, is produced by the elongation of AA. Like AA, adrenic acid can be converted into oxygenated metabolites that have important physiological roles.[56]

Adrenic acid may be of great importance for the pathophysiological processes of MetS, with one study finding higher levels of adrenic acid in plasma-free fatty acids (FFAs) of persons with MetS in comparison with healthy controls.[57] In an experimental metabolomics study of rats, adrenic acid was increased in FFAs and lysophosphatidylcholine fractions in skeletal muscle after glucose challenge tests and oral glucose tolerance tests.[58] In the DIOGENES project,[59] weight reduction because of a low-calorie diet was associated with increased percentages of adrenic acid and other PUFA n-6 (LA, DGLA, and AA) in TAG of adipose tissue.

Contrary to our assumptions, we found positive associations between ALA (18:3n-3) and EPA (20:5n-3) on the one hand and fat percentage and fat mass on the other in the study group of persons with MetS-related traits. This was not proved in the healthy control group. Studies dealing with PUFA n-3 concentrations for individual plasma lipid classes or erythrocyte membranes in obese persons and patients with MetS and T2DM have produced equivocal results. Similarly, results from studies that examine associations between individual PUFA n-3 and obesity, MetS and T2DM have not shown consistency either.[9,27,41,60,61]

Ebbesson et al.[62] studied the associations between specific FA and CVD risk factors in 758 men and women. With regard to PUFA n-3, they found that ALA was associated with increased TAG, BMI, and 2-hr insulin, which suggests that this FA may have some detrimental effects. EPA and DHA in erythrocytes were negatively associated with TAG, CRP, diastolic blood pressure, and 2-hr insulin, but positively associated with LDL-C. It has been shown that concentrations of PUFA n-3 among different lipid classes and their associations with obesity, MetS, T2DM, and CVD may be significantly modified by dietary FA composition[63] and genetic factors.[64]

Elevated estimated activity of D6D and D9D in our group of patients with MetS-related traits is a usual finding in persons suffering from MetS[9] and is connected with increased both total and cardiovascular mortality.[11] We have observed higher estimated activity of D6D in patients with depression[12] and increased D9D activity in cholesteryl esters of patients suffering from pancreatic cancer.[13] These conditions are supposed to be related to adiposity and insulin resistance.[12,14] Increased D6D and D9D activities were found in other pathological conditions, for example, chronic pancreatitis.[43]

The limitations of our study are that the persons investigated were not given a dietary questionnaire and trans-FAs were not measured. In contrast, the strengths of our study lie in its relatively large set of mostly overweight patients using a balanced ratio of men and women. Furthermore, the probands in this study were not treated with lipid-lowering drugs, antioxidants, or dietary supplements containing n-3 or n-6 PUFA.

Conclusions

In our pilot study of a middle-aged population comprising patients bearing at least one component of MetS, we found with the help of multivariate regression analysis that the composition of FAs in plasma PC significantly determines anthropometric parameters (waist circumference, body fat percentage, and fat mass). The variances of other studied parameters, for example, lipids and insulin resistance indices, were not influenced by FAs in plasma PC for this group. Our results support the hypothesis that body fat deregulation is associated with altered FA profiles. Nevertheless, it must be acknowledged that this study was carried out on mostly overweight probands. These results need to be studied further and applied to specific individual male and female populations, such as healthy individuals and patients with MetS or T2DM.

Acknowledgments

The work was supported by grants PROGRES Q25/LF1 UK (MŠMT ČR) and RVO VFN64165/2012 (MZ ČR) and by the project ''LQ1602 IT4 Innovations Excellence in Science.''

Author Disclosure Statement

No competing financial interests exist.

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Prostaglandins, Leukotrienes and Essential Fatty Acids 108 (2016) 38–44

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Chronic pancreatitis and the composition of plasma phosphatidylcholine fatty acids

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ARTICLE INFO

Article history:
Received 28 January 2016
Received in revised form
16 March 2016
Accepted 22 March 2016

Keywords:
Chronic pancreatitis
Fatty acid profile
Discriminant analysis
Diabetes mellitus
Malnutrition

ABSTRACT

Chronic pancreatitis (CP) is an irreversible inflammatory disorder characterized by the destruction of both exocrine and endocrine tissue. There is growing evidence that dysregulation of fatty acid (FA) metabolism is connected with many diseases; however, there are few data concerning FA composition in CP. Therefore, we analyzed FA profiles in plasma phosphatidylcholines in 96 patients with CP and in 108 control subjects (CON).

The patients with CP had, in comparison with CON, increased sum of monounsaturated FA (ΣMUFA) and decreased content of polyunsaturated FA (PUFA) in both n-6 and n-3 families. Moreover, CP patients had increased indexes for delta-9, delta-6 desaturases, and fall in activity of delta-5 desaturase. Increased ratio of 16:1n-7/18:2n-6 (marker of essential n-6 FA deficiency), was more prevalent among CP patients.

These changes implicated decreased fat intake, including n-3 as well as n-6 PUFA, and intrinsic changes in FA metabolism due to the alteration of delta desaturase activities.

1. Introduction

Chronic pancreatitis (CP) is a condition characterized by progressive and irreversible damage to both the exocrine and endocrine parts of the gland. In addition to many complications, CP patients are at increased risk of developing pancreatic cancer. The pathological findings of CP are characterized by progressive pancreatic inflammation, fibrosis, acinar atrophy and distorted and blocked ducts. The basic etiopathogenetic mechanism responsible for the development of CP is inflammation-led fibrosis. Pancreatic fibrosis in CP is caused by recurrent episodes of acute pancreatitis, which causes interstitial acinar and fatty tissue necrosis and consequently leads to acinar fibrosis and distorted and/or blocked pancreatic ducts. Fibrotic destruction of the pancreatic gland is irreversible, and morphological and structural changes lead to the functional impairment of both exocrine and endocrine functions, eventually leading to malnutrition and/or diabetes [1,2].

Annual incidence of CP worldwide ranges between 1.8 and 14.0/100,000 subjects. CP prevalence varies at an average of about 50/100,000 persons. Approximately 70% of CP cases are caused by alcohol abuse, and the remaining cases are associated with genetic disorders, pancreatic duct obstruction, recurrent acute pancreatitis, autoimmune pancreatitis and unknown mechanisms [1].

There is growing evidence that dysregulation of fatty acid (FA) metabolism is connected with chronic diseases such as metabolic syndrome (MS) [3], obesity [4], diabetes mellitus type 2 (DM2T) [5], cardiovascular diseases [6], neuro-psychiatric disorders [7], various cancers [8], inflammation [6], allergies and autoimmune diseases [9].

In addition to the abovementioned diseases, the role of fat (and FA) has been extensively studied in many gastrointestinal diseases, such as non-alcoholic fatty liver (NAFLD)/non-alcoholic steatohepatitis (NASH) [10] and acute pancreatitis (AP), as well as in connection with AP severity and AP outcomes (reviewed in [11]).

Abbreviations: AP, acute pancreatitis; BMI, body mass index; CF, cystic fibrosis; CON, healthy controls; CP, chronic pancreatitis; CRP, C-reactive protein; CT, computer tomography; DD, delta desaturases; DGLA, dihomo-γ-linolenic acid (20:3n-6); DHA, docosahexaenoic acid (22:6n-3); DM, diabetes mellitus; DM2T, diabetes mellitus type 2; EFAD, essential fatty acid deficiency; EPA, eicosapentaenoic acid (20:5n-3); ERCP, endoscopic retrograde cholangiopancreatography; EUS, endoscopic ultrasonography; FA, fatty acid; FELA, fecal elastase activity; HOMA-IR, homeostasis model assessment method; LDL-C, low density lipoprotein cholesterol; MRCP, magnetic resonance cholnagiopancreatography; MS, metabolic syndrome; MUFA, monounsaturated fatty acids; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NEFA, non-esterified fatty acids; PUFA, polyunsaturated fatty acids; SCD-1, steraoyl-CoA desaturase, synonym for delta 9 desaturase (D9D); SFA, saturated fatty acids; TG, triacylglycerols.

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http://dx.doi.org/10.1016/j.plefa.2016.03.012
0952-3278/

Although there is growing evidence that dysregulation of FA metabolism is connected with a number of diseases, there are few data concerning FA composition in pancreatitis. The composition of FA in plasma lipids and the RBC membrane has been evaluated both in acute [12] and chronic pancreatitis [13–16], as well as in CP and with and without diabetes mellitus (DM) [14,15].

FA composition in plasma phospholipids and cholesteryl esters reflects both dietary intake of FA over a six-week to three-month period as well as endogenous FA metabolism (synthesis of FA *de novo*, β-oxidation, enzymatic desaturation and elongation, conversion of polyunsaturated FA to eicosanoids and lipoperoxidation) [17].

The aim of the study was to evaluate changes in the composition of plasma phosphatidylcholines FA in patients with (i) CP in comparison with healthy controls (CON) and in relation to (ii) concomitant diabetes mellitus and (iii) malnutrition. Furthermore, multivariate linear discriminant analysis was used to evaluate the discriminative power of different FAs as independent variables (iv) capable of differentiating CP from healthy controls.

2. Patients and methods

2.1. Study design and participants

This study was carried out at the 4th Department of Medicine of the General University Hospital from January 2010 to September 2013. The study group consisted of 108 (55M/53F) controls (CON) and 96 consecutively diagnosed patients (70M/26F) with chronic pancreatitis (CP). All samples were marked with unique anonymized identification numbers, and the data was merged only after the assays had been completed. The study protocol was approved by the Joint Ethics Committee of the General University Hospital and the 1st Medical Faculty of Charles University in Prague (decision no. 3311/2011) and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Clinical diagnosis of CP was based on clinical features (abdominal pain, nausea and/or vomiting, anorexia and/or malnutrition and steatorrhea) confirmed by two or more imaging methods (abdominal – USG, contrast-enhanced computed tomography – CT), endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography (MRCP) and endoscopic ultrasonography (EUS). Only patients with definite CP were included. The grade of CP (mild – moderate – severe) was assessed according to M-ANNHEIM pancreatic imaging criteria. All patients were assessed using a combination of EUS and other imaging methods (CT, or USG, or MRCP), since EUS does not differentiate between moderate and severe grades, and other methods (CT, USG or MRCP) cannot differentiate between mild and moderate changes [18].

The exclusion criteria for both groups were: ongoing antioxidant therapy (e.g. vitamin C, vitamin E, allopurinol, N-acetylcysteine), lipid-lowering treatment (fibrates, statins), supplementation with n-3 polyunsaturated fatty acids, kidney disease (creatinine $>$ 150 μmol/l), clinically manifest proteinuria (urinary protein $>$ 500 mg/l), liver cirrhosis, decompensated diabetes mellitus, concomitant malignancies, chronic immunosuppressive and anti-inflammatory therapy as well as chemotherapy. Further criteria for exclusion were: endocrine disease, acute pancreatitis or acute relapse of CP, unstable angina pectoris, acute myocardial infarction $<$ 1 year prior to enrollment, either coronary artery bypass grafting or percutaneous coronary intervention, and stroke. Persons who were operated on in the upper gastrointestinal tract (in the previous 12 months) and subjects who exhibited systemic inflammation in the previous 6 months were also excluded. Patients with CP enrolled in the study were re-examined after 2 years in order to exclude the development of pancreatic cancer and thus to avoid enrolling patients with the initial stages of pancreatic cancer in the study.

The diagnosis of T2DM was based on recommended guidelines [19]. In the CP group, there were 34 DM2T patients (30M/4F). Among all of the 34 DM2T patients, 12 (11M/1F) were treated with insulin therapy and 22 (19M/3F) were placed under peroral antidiabetic therapy or on a diet.

Alcoholic CP was diagnosed in 72 patients, obstructive CP in 9 subjects; 15 patients experienced idiopathic CP. Among the 96 patients with CP, 58 patients had severe grade, 22 had moderate and 16 had mild grade changes in pancreatic morphology according to the imaging methods used. Severe exocrine dysfunction [FELA (fecal elastase activity) $<$ 200 ng/g] was found in 61 patients with CP.

Complications (ascites, bleeding, obstruction and/or *ductus choledochus* stricture, pancreatic fistula, duodenal stenosis, splenic and/or portal vein thrombosis, segmental portal hypertension) were found in 43 patients with CP. According to the M-ANNHEIM severity index of CP, 26 patients were categorized as being at a minor level, 27 subjects at an increased level, 30 cases at an advanced level and 13 at a marked level of CP [18]. A total of 70 patients in the CP group were on pancreatic enzyme replacement therapy.

Among the CP group, 9 subjects were underweight (BMI $<$ 18.5 kg/m^2), 5 patients had plasma albumin lower than 35 g/l and 19 subjects had CRP higher than 10 mg/l. All CP patients were screened for nutritional risk. Among all of the CP patients, 15 patients were nutritionally at-risk (NRS score $\geq$ 3) [20]. The patients were asked to maintain their regular diet without using any supplements that might affect the intake of FA and they were recommended: (1) to avoid alcohol consumption and smoking; (2) not to skip meals; (3) to take small (low volume) and frequent meals; (4) to minimize high-sugar (high-glycemic, resp.) index food or fluids. Recommended energy intake was 30–35 Kcal/kg/day, with a protein 1–1.5 g/kg/day, and approximately 30% of calories may (should) be given as fat.

2.2. Blood sampling and anthropometry

Blood samples were taken after 12 h of fasting. Routine biochemical and hematological analyses were performed immediately and samples for special analyses were stored at –80 °C until use. Basic clinical and anthropometrical data, including assessment of body fat, were examined using standard methods, as described previously [3].

2.3. Laboratory measurements

Plasma concentrations of total cholesterol and triglycerides were measured using enzymatic-colorimetric methods (Boehringer, Mannheim, Germany). HDL-C was determined in supernatant after precipitation of B lipoproteins by PTA/Mg^{2+}, using a kit from the same manufacturer. Low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald's formula. Concentration of apolipoprotein (apo) *B* was measured by Laurell rocket electroimmunoassay using standard and specific antibodies (Behringwerke, Marburg, Germany). Immunoreactive insulin was determined using the RIA method and double monoclonal antibodies (Insulin IRMA, Immunotech Prague, Czech Republic). The concentrations of non-esterified fatty acids (NEFA) were determined using an enzymatic-colorimetric method (NEFA, Randox Laboratories, UK).

Fatty acid patterns in the main plasma lipid classes were examined using analytical procedures described previously and

expressed as molar percentages. Method variability presented as relative standard deviation ranged from 1.07% for palmitic acid (16:0) to 8.60% for 16:1n-9. Relevant variability data were 1.25% for dihomo-γ-linolenic acid; 0.76% for stearic acid; 8.06% for myristic acid; 2.38% for docosahexaenoic acid; 1.91% for docosapentaenoic acid; and 0.725% for linoleic acid [21].

All routine clinical tests were measured at the Institute for Clinical Biochemistry and Laboratory Diagnostics of the General University Hospital in Prague. The concentrations of C-reactive protein (CRP), prealbumin, bilirubin, C-peptide, glucose, lipid parameters, liver enzymes and tumor markers were measured in serum. C-reactive protein (CRP) was determined using an immunoturbidimetric method with the K-ASSAY CRP kit (Kamiya Biomedical Company, USA; CV = max. 7.6%) on a Hitachi Modular analyzer (Japan). Bilirubin was measured with 2,5-dichlorophenyldiazonium on a Hitachi Modular analyzer. Plasma albumin was assessed used a colorimetric method with bromocresol green. Levels of glucose, ALT, AST and GGT were estimated using photometric methods on the same Modular analyzer. Activities of ALP and AMS were measured using an IFCC enzymatic method on a Cobas Integra analyzer. C-peptide concentrations were determined by electrochemiluminescence immunoassay (Roche Diagnostics, USA). Tumor markers CEA and CA 19-9 were measured by chemiluminescence immunoassay – CLIA Architect (Abbott, USA).

The homeostasis model assessment method (HOMA-IR) was used as an index of insulin resistance [22]. Desaturase activities were estimated using FA product/precursor ratios [3]. The ratio of palmitoleic to linoleic acid (16:1n-7/18:2n-6) above 0.086 was used as a surrogate marker of n-6 essential fatty acid deficiency (EFAD) [23].

2.4. Statistical analyses

Prior to the analyses, all data were cleaned and preprocessed: extreme values were examined and double-checked, and rows with missing values were omitted. After that, scaling was performed to achieve centering and constant variance. Numerical data are expressed as median and inter-quartile ranges (IQR, 25–75th percentile), and categorical data are summarized with the absolute number of occurrence and relative frequency (in %). Comparisons between the groups were carried out using the Wilcoxon test (or the chi-squared test) for numerical (or categorical) data. *P*-values were adjusted for multiple comparisons using Benjamini–Hochberg correction.

The process of linear discriminant analysis was carried out in a stepwise manner utilizing, at a minimum, Wilks's λ (within-groups sum of squares: total sum of squares ratio) as a measure of group discrimination. At each step in this process, the variable containing the most discriminating power is identified and its coefficient determined. The relative importance of each variable is indicated by what is known as the approximate *F* statistic. This is a transformation of Wilks's λ, which can be compared with *F* distribution. The process is stopped when the remaining variables are considered as lacking significant discriminating power ($p > 0.05$). We used discriminant models for classification of CP and CON into groups. Only variables with an appropriate final *F* statistic ($p < 0.05$) were included in our discriminant functions. The individual fatty acids in plasma phosphatidylcholines (22 fatty acids were analyzed initially) as independent variables were subjected to the discriminant function.

All statistical analyses were performed using R software version 3.2.2 [24].

3. Results

3.1. Clinical and biochemical characteristics of the studied groups

Basic clinical and biochemical parameters of the studied groups are shown in Table 1. There were no differences in BMI, fat mass or percent of fat between both groups studied. CP subjects had increased abdominal fat distribution (waist circumference and subscapular/triceps skinfold ratio) in comparison with CON. Moreover, only CP subjects had increased systolic BP. Both groups differed in sex ratio ($\chi 2 = 12.067$, d.f. = 1, $p < 0.001$) and in smoking prevalence ($\chi 2 = 27.928$, d.f. = 1, $p < 0.001$).

Fasting plasma glucose levels were significantly different in the CP group, but fasting plasma insulin and C-peptide levels did not statistically differ. Insulin resistance, calculated from HOMA analysis, was not statistically different between both groups.

In a set of functional liver tests, only increased activities of ALP

Table 1
Basic clinical and biochemical parameters of the studied groups.

	Chronic pancreatitis	Controls
Number of subjects	96	108
Sex ratio (M/F)	70/26[b,***]	53/55
Age (years)	56.2 [49.0–64.3][a]	55.6 [50.2–63.4]
Diabetes mellitus *N* (M/F)	43 (39/4)	–
Smokers *N* (%)	73 (76.4)[b,***]	33 (30.4)
Body weight (kg)	72.0 [62.00–85.8]	68.2 [61.9–74.1]
BMI (kg m^{-2})	24.7 [21.3–27.0]	23.6 [22.0–25.4]
Waist circumference (cm)	92 [82–101][c,***]	83 [77–89]
Fat mass (kg)	21.1 [17.9–29.2]	22.49 [5.00–7.03]
Percentage of fat mass	31.2 [24.5–36.5]	31.2 [25.2–34.5]
WHCR (ratio)	1.00 [0.95–1.03][***]	0.89 [0.83–0.94]
SCI/TRI (ratio)	1.00 [1.00–1.83][***]	0.98 [0.75–1.00]
SBP (mm Hg)	130 [120–140][**]	120 [115–130]
DBP (mm Hg)	80 [75–85]	80 [72–80]
Glucose (mmol/l)	5.60 [5.00–7.03][***]	4.90 [4.60–5.20]
Insulin (mIU/l)	6.65 [3.85–10.72]	5.76 [4.18–7.97]
HOMA-IR (ratio)	1.53 [0.93–3.22]	1.27 [0.90–1.67]
C-peptide (nmol/l)	0.59 [0.47–0.78]	0.59 [0.49–0.68]
NEFA (mmol/l)	0.54 [0.38–0.78]	0.50 [0.33–0.62]
TC (mmol/l)	4.77 [4.09–5.65][**]	5.12 [4.73–5.73]
TG (mmol/l)	1.30 [5.00–7.03][***]	0.95 [0.78–1.22]
HDL-C (mmol/l)	1.37 [1.13–1.59][**]	1.56 [1.29–1.82]
LDL-C (mmol/l)	2.70 [2.24–3.20][**]	3.01 [2.61–3.53]
Apo *B* (g/l)	0.98 [0.82–1.13]	0.94 [0.83–1.15]
Bilirubin (μmol/l)	9.8 [6.9–14.2]	10.8 [8.0–14.8]
ALT (μkat/l)	0.41 [0.31–0.62]	0.37 [0.31–0.57]
AST (μkat/l)	0.45 [0.35–0.58]	0.43 [0.37–0.48]
GGT (μkat/l)	0.70 [0.40–1.76][***]	0.33 [0.23–0.52]
ALP (μkat/l)	1.23 [1.02–1.79][**]	0.99 [0.85–1.20]
CHES (μkat/l)	124 [103–142][*]	142 [119–152]
Albumin (g/l)	45.7 [43.4–47.2]	46.4 [44.4–48.3]
Prealbumin (g/l)	0.26 [0.21–0.29]	0.26 [0.23–0.28]
CRP (mg/l)	3.3 [1.20– 7.2]	3.9 [2.0–5.9]
CEA (μU/l)	2.22 [1.13–3.35][***]	0.50 [0.50–1.43]
CA 19-9 (μU/l)	11.40 [6.30–21.80][*]	8.05 [5.75–9.88]

Apo: apolipoprotein; BMI: body mass index; CRP: C-reactive protein; HOMA-IR: homeostasis model assessment of insulin resistance; TC: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density cholesterol; CEA: carcinoembryonal antigen; CA 19-9: carbohydrate antigen 19-9; SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR (homeostatic index of insulin resistance) = glucose (mmol/l)*insulin (mUI/l)/22.5; CHES: cholinesterase; TG: triglyceride; NEFA: non-esterified fatty acids; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; WHCR: waist-to-hip circumference; SCI/TRI: subscapular-to-triceps skinfold.

[a] median [25–75th percentile].
[b] χ^2 test: *** $p < 0.001$.
[c] Wilcoxon test; *p* – values were adjusted for multiple comparisons using Benjamini–Hochberg corrections.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

(+36%, $p < 0.01$) and GTP (+79%, $p < 0.001$) were found in the CP group; however, the changes were not clinically relevant.

Fasting levels of albumin, prealbumin and CRP were not different between the groups. Activity of cholinesterase (CHES), as a surrogate marker of proteosynthesis, was significantly decreased in CP patients (−13%, $p < 0.05$). Concentrations of total cholesterol (TC), LDL-C and HDL-C were significantly decreased in the CP group (all $p < 0.01$). Opposite changes in levels of triglycerides (TG) were found ($p < 0.001$). Patients with CP had significantly increased levels of CEA ($p < 0.001$) and CA 19-9 ($p < 0.05$) in comparison with CON subjects, even though these changes were not clinically relevant.

3.2. Fatty acid composition of plasma phosphatidylcholines

The FA profile and corresponding derived parameters (delta desaturases, DD) in plasma phosphatidylcholines of CP patients and CON subjects are shown in Table 2. There were no statistically significant differences in FA profiles between men and women either in the CP patients or in the CON group. Therefore, subsequent statistical analysis concerning the FA composition between CP patients and CON subjects comprised both men and women.

The patients with CP had a decreased sum (Σ) of both n-3 polyunsaturated fatty acids (PUFA) and n-6 PUFA, which was accompanied with an increase in Σ monounsaturated fatty acids. There was neither significant difference in the Σ of saturated fatty acids (ΣSFA) nor in the individual saturated fatty acids [myristic (14:0), palmitic (16:0) and stearic (18:0) acids]. The increase in the ΣMUFA was caused by the elevation of *cis*-7-hexadecenoic (16:1n-9), palmitoleic (16:1n-7), oleic (18:1n-9), vaccenic (18:1n-7) and gondoic (20:1n-9) acids. The drop in the Σn-6 PUFA was accompanied by decreased levels of linoleic (18:2n-6) acid with concomitant increased content of γ-linolenic (18:3n-6), dihomo-γ-linolenic (20:3n-6), adrenic (22:4n-6), *cis,cis*-11,14-eicosadienoic (20:2n-6) and all *cis*-4,7,10,13,16-docosapentaeoic (22:5n-6) acids. There was no change in the content of arachidonic acid (20:4n-6) between the studied groups. Analysis of n-3 PUFA did not show statistically significant changes in the content of both α-linolenic (18:3n-3) and eicosapentaenoic (20:5n-3) acids, which was associated with decreased level of docosahexaenoic acid (22:6n-3) and opposite changes of docosapentaenoic (22:5n-3) acids.

The changes in the activities of delta desaturases (DD), estimated as the (product/substrate) ratio of respective fatty acids, showed statistically significant changes in CP patients. We proved raised activity of D9D18 for both stearic (18:0) and palmitic (16:0) acids (D9D16), which was accompanied by a decrease in the activity of D5D and opposite changes in D6D activity.

As gender, malnutrition and presence of diabetes belong to the factors that might influence the FA profiles in CP groups, we tried to elucidate the effect of diabetes and malnutrition on the FA profiles. We did not find any statistically significant differences in FA profiles between men and women with CP, or between CP patients with and without DM. The CP patients with diabetes mellitus (subgroup CP-DM+) had, in comparison with CP patients without DM (subgroup CP-DM-), increased plasma glucose levels [7.60 (6.17–10.60) vs. 5.20 (4.80–5.85) mmol/l; median (25–75th percentile), $P < 0.001$], HOMA-IR [3.23 (1.67–4.93) vs. 1.00 (0.65–1.39) ratio, $P < 0.001$] and insulin [9.62 (4.98–13.90) vs. 3.96 (2.91–6.16) mIU/l, $P < 0.01$]. Moreover, there were no significant differences in the FA composition of CP subjects with DM treated with insulin and CP diabetics on peroral antidiabetic drugs and a diet. Smoking had only a little effect on FA profile in CP patients, we found only proportion of 18:1n-9 to be lower in non-smoker subgroup of CP [12.4(11.0–15.1) vs. 10.9(10.3–11.7), mol%, $P=0.02$, Wilcoxon test].

Table 2
Plasma phosphatidylcholines fatty acid composition of the studied groups.

Fatty acid[a]	Chronic pancreatitis	Controls
14:0	0.27 [0.20–0.33][b]	0.26 [0.21–0.33]
16:0	30.00 [28.56–32.00]	29.55 [28.69–30.41]
18:0	13.57 [12.76–14.58]	13.36 [12.89–14.02]
ΣSFA	43.52 [42.76–44.82]	43.21 [42.44–44.09]
16:1n-9	0.11 [0.09–0.14][g,*]	0.10 [0.09–0.12]
16:1n-7	0.68 [0.52–0.98][***]	0.46 [0.39 –0.53]
18:1n-9	11.67 [10.29–14.22][***]	9.84 [8.90–10.82]
18:1n-7	1.75 [1.57–1.98][***]	1.48 [1.34–1.69]
20:1n-9	0.16 [0.13–0.19][**]	0.14 [0.12–0.19]
ΣMUFA	14.48 [12.68–17.49][***]	12.06 [10.90–13.07]
18:2n-6	20.07 [17.60–21.76][***]	24.71 [22.76–26.26]
18:3n-6	0.09 [0.07–0.19][**]	0.07 [0.05–0.08]
20:2n-6	0.37 [0.31–0.55][**]	0.34 [0.29–0.42]
20:3n-6	3.25 [2.74–3.85][**]	2.86 [2.48–3.22]
20:4n-6	11.02 [8.97–13.35]	11.09 [9.53–12.27]
22:4n-6	0.37 [0.31–0.42][***]	0.31 [0.28–0.34]
22:5n-6	0.25 [0.21–0.30][***]	0.20 [0.16–0.22]
ΣPUFA n-6	36.75 [33.55–38.57][***]	38.90 [37.97–40.51]
18:3n-3	0.18 [0.13–0.23]	0.19 [0.16–0.24]
20:5n-3	0.77 [0.58–1.03]	0.84 [0.69–1.00]
22:5n-3	0.94 [0.78–1.08]	0.88 [0.80–0.99]
22:6n-3	2.60 [2.20–3.34][***]	3.33 [2.79–3.99]
ΣPUFA n-3	4.66 [3.99–4.99][**]	5.31 [4.55–6.04]
D9D16[c]	0.023 [0.019–0.031][***]	0.017 [0.010–0.023]
D9D18[d]	0.85 [0.73–1.05][***]	0.74 [0.66–0.81]
D6Dn6[e]	0.0046 [0.0032–0.0065][**]	0.0030 [0.0022–0.0040]
D5Dn6[f]	3.26 [2.71–3.91][***]	3.82 [3.29–4.54]
n-6 EFAD index (≥ 0.086/ < 0.086)	14/82[***]	none/108

ΣSFA – total content (the sum) of saturated fatty acids; ΣMUFA – the sum of monounsaturated fatty acids; ΣPUFAn-6 – the sum of polyunsaturated fatty acids (n-6 family); ΣPUFAn-3 – the sum of PUFA (n-3 family); n-6 EFAD index – n-6 essential fatty acid deficiency index 16:1n-7/ 18:2n-6 ≥ 0.086.

[a] Shorthand notation of fatty acids: number of carbon atoms; number of double bonds; n – number of carbon atoms from the methyl end to the nearest double bond. Only relevant fatty acids are presented (content of 12:0, 14:1n-5, and 20:0 is not presented).

[b] median [25–75th percentile].

[c] D9D16 – delta 9 desaturase for palmitic acid (16:1n-7/16:0).

[d] D9D18 – delta 9 desaturase for stearic acid (18:1n-9/18:0).

[e] D6D – delta 6 desaturase (18:3n-6/18:2n-6).

[f] D5D – delta 5 desaturase (20:4n-6/20:3n-6).

[g] Statistical analysis: Wilcoxon test; p – values were adjusted for multiple comparisons using Benjamini–Hochberg corrections.

[*] $p < 0.05$.

[**] $p < 0.01$.

[***] $p < 0.001$.

Moreover, we found no significant difference in the FA profile of CP patients with malnutrition (subgroup CP-MN+, $n=27$) in comparison with patients without malnutrition (subgroup CP-MN-, $n=67$). The subgroup of CP-MN+ consisted of 9 patients with underweight (BMI < 18.5 kg/m^2) and 18 subjects with decreased plasma albumin (< 35 g/l), and/or elevation of CRP (> 10 mg/l).

There were no statistical differences in the FA composition between CP patients nutritionally at-risk (NRS score ≥ 3, $n=15$) in comparison with the rest of the CP patients (NRS score ≤ 2; $n=76$; the group of CP patients categorized according to the NRS score comprised only 91 subjects because there were no available data for 5 patients).

The increased ratio of 16:1n-7/18:2n-6 (> 0.086) as a surrogate marker of n-6 essential fatty acid deficiency (EFAD) was found in

Table 3
Discriminant models for classification of chronic pancreatitis and healthy controls.

	F-statistic (d.f.)	Coefficients	Final correct classification (%)
18:2n-6	105.42 (1, 193)	1.045	Chronic pancreatitis 89.3
20:3n-6	72.16 (2, 192)	−0.563	Controls 91.2
22:6n-3	66.63 (3, 191)	0.831	All cases 90.3
20:1n-9	57.92 (4, 190)	−0.350	
16:0	49.08 (5, 189)	0.326	
18:1n-7	42.15 (6, 188)	−0.311	
16:1n-9	37.06 (7, 187)	0.200	
22:5n-3	32.83 (8, 186)	−0.189	

Negative linear discriminant analysis function (or variable coefficient) represents classification as CP; positive linear discriminant analysis function represents classification as control. Only 22 primarily analyzed individual FAs are included in the analysis.
d.f.: degrees of freedom; F statistic (an approximate "F statistic"): transformation of Wilks's λ; 18:2n-6 – linoleic acid; 20:3n-6 – dihomo-γ-linolenic acid; 22:6n-3 – docosahexaenoic acid; 20:1n-9 – gondoic acid; 16:0 – palmitic acid; 18:1n-7 – vaccenic acid; 16:1n-9 – *cis*-7-hexadecenoic acid; 22:5n-3 – all *cis*, 7,10,13,16,19-docosapentaenoic acids.

14 CP patients and in no subject of the CON group ($\chi 2 = 14.71$, $p < 0.001$). The CP patients with underweight as compared with CP patients with normal body weight exhibited increased prevalence for the increased 16:1n-7/18:2n-6 ratio ($\chi 2 = 5.04$, $p < 0.05$). Statistical analysis of other CP subgroups, which comprised patients with malnutrition (CP-MN+), diabetes mellitus (CP-DM+) as well as patients nutritionally at-risk revealed no statistically significant differences in the prevalence of the 16:1n-7/18:2n-6 ratio in comparison with CP subjects without these markers.

A discriminant model for the classification of CON and CP into groups is shown in Table 3. When 22 analyzed FAs were initially entered into linear discriminant analysis, the concentrations of 18:2n-6, 20:3n-6, 22:6n-3, 20:1n-9, 16:0, 18:1n-7, 16:1n-9 and 22:5n-3 (in that order) were the best discriminators for the differentiation of CP and CON with 90.3% final correct classification.

We also correlated the proportion of fatty acids with anthropometric and laboratory measurements within the CP group (for results, see Supplementary Table 1).

4. Discussion

The present study demonstrated altered FA composition of plasma phosphatidylcholines in patients suffering from chronic pancreatitis (CP) as compared with healthy controls. Among the patients with CP in comparison with the control group (CON), we found a significant increase of total monounsaturated FA concurrent with a significant decrease of both Σn-6 and Σn-3 polyunsaturated FA. This pattern of FA changes in our CP patients resembles, with the exception of the unchanged total saturated FA, the FA composition seen in patients with chronic gastrointestinal disorders, which are known to have high prevalence (31%) of essential FA deficiency (EFAD). In this type of EFAD, manifested in patients with terminal ileitis (Crohn's disease), ulcerative colitis, celiac sprue and short-bowel syndrome, elevation of total SFA and total MUFA (both n-7 and n-9 families) along with a decrease in PUFA of both n-3 and n-6 families was found [23]. Other states of pancreatic insufficiency, such as pancreatic cancer and cystic fibrosis, also exhibit the lower content of total n-3 and n-6 PUFA (especially LA, EPA and DHA) along with increased activities of D9D, D6D and D5D [25,29].

The content of saturated FA (SFA) in CP was not altered in our study. The literature data on this family of FA in CP are contradictory: some authors described no changes [13], other reported increased content of palmitic (16:0) and stearic acids (18:0) and total SFA in cholesteryl esters in CP [16], and a French study demonstrated a decreased concentration of stearic and very long-chain FA (20:0, 22:0, 24:0) in CP [14].

In agreement with published studies dealing with CP, we found increased concentrations of monounsaturated FA (MUFA), both the n-7 and n-9 family, in CP. The finding of increased proportions of POA (16:1n-7), *cis*-7 hexadecenoic acid (16:1n-9), oleic (18:1n-9) and vaccenic acids (18:1n-7) in our CP patients is considered a marker of *de novo lipogenesis*, the features of which have been described in many pathological states, such as metabolic syndrome [3], diabetes mellitus type 2 (DM2T), and some cancers [25].

Furthermore, our CP patients displayed an elevated proportion of gondoic acid (20:1n-9), which arises from oleic acid by elongation. Gondoic acid and vaccenic (18:1n-7) acid discriminated patients into the CP group in our model. In contrast, *cis*-7 hexadecenoic acid (16:1n-9), which discriminates subjects into the CON group, arises by β-oxidation of oleic acid and can play other metabolic roles in the organism [26].

Our patients with CP have been shown to display increased activities of the enzyme stearoyl-CoA desaturase-1 (SCD1), a synonym for delta 9-desaturase (D9D). The surrogate markers of this activity are the ratios of 16:1n-7/16:0 (D9D16) and 18:1n-9/18:0 (D9D18). The preferred substrates for SCD1 are stearoyl-CoA and palmitoyl-CoA, which are converted to oleoyl-CoA (18:1n-9) and palmitoleoyl-CoA (16:1n-7), respectively. In contrast to other authors [14] who have described only increased activities of D9D for palmitic acid (D9D16), we found increased activity of D9D both for palmitic (D9D16) and stearic (D9D18) acids. Because the human diet contains only very small amounts of palmitoleic acid (whereas oleic acid is usually present in high abundance), increased activity of D9D (D9D18) for stearic acid in the CP group implicated low dietary intake of vegetable fat, rich in oleic acid.

Increased D9D activities have been positively associated with BMI, waist circumference, body fat mass and triglyceride levels. Moreover, increased SCD-1 activity has been described as being connected with other diseases, such as dyslipidemia, atherosclerosis, some cancers, bone fractures and non-alcoholic fatty liver disease. Activity of SCD-1 depends on genetic background [27] and other factors, especially dietary, hormonal and environmental ones. SCD-1 expression increases with a high-carbohydrate fat-free diet, glucose, fructose, cholesterol, insulin and some drugs (fibrates, retinoic acid). In contrast, PUFA, especially the n-3 and n-6 families, ethanol, some proinflammatory cytokines (TNF-α, interleukin-11), thyroxin and cAMP (including drugs that increase its intracellular level) inhibit SCD-1 activity [28]. Increased D9D activity has been described in connection with lung and breast cancers, as well as with pancreatic cancer [25] or cystic fibrosis [29].

In contrast to other studies, in the CP group we found decreased content of total PUFA n-3, which was caused by a drop in the content of docosahexaenoic acid (22:6n-3). Higher contents of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids in CP patients were attributed in a Japanese study to the relatively high intake of fish oil in combination with the relatively low intake of dietary fat [13], whereas both French and Hungarian studies found unchanged total content of PUFA n-3 [14,16]. Lower proportion of DHA was previously observed only in the subsets of CP patients with DM [14], possibly due to the deficit of selenium in the diet, or in the cystic fibrosis patients with symptomatic related liver disease [31].

The decreased docosahexaenoic acid (22:6n-3) was the only changed PUFA n-3 in CP patients in our study. There are several potential explanations for this phenomenon: (i) Eicosapentaenoic acid (20:5n-3) could be shunted to pathways other than DHA production (e.g. EPA is a substrate for synthesis of 3-series prostaglandins and 5-series leukotrienes); (ii) a further explanation is the increased retroconversion of DHA to EPA through β-oxidation

in peroxisomes; (iii) and yet another hypothesis is the increased conversion of DHA to docosanoids (e.g. resolvins, protectins and maresins). Our results implicate the block in the downstream conversion of DPA n-3 (22:5n-3) to 24:5n-3 (or 24:6n-3) and DHA (22:6n-3), since docosapentaenoic acid of the n-3 family (22:5n-3) was the only PUFA n-3, of which the elevated proportion in our model assigned probands to the CP group. This phenomenon in CP subjects may be connected, at least partly, with the disturbed conversion of EPA to the downstream metabolites [35].

The observed decrease in concentration of linoleic acid (18:2n-6) seen in our CP group was previously described in plasma phosphatidylcholines [13,14] and cholesteryl esters [16]. The lower content of linoleic acid in our CP patients and higher concentrations of γ-linolenic (18:3n-6) and dihomo-γ-linolenic (20:3n-6) acids could reflect elevated activity of D6D and have not yet been described in CP patients. Contrary to other studies [13,16] we did not find changes in arachidonic acid level in CP. Additionally, decreased D5D activity in CP, found in our study, is the result of elevated dihomo-γ-linolenic acid and unchanged arachidonic acid (20:4n-6) concentration.

The decreased linoleic acid content in plasma lipid classes in CP can also be explained by several other mechanisms, among which, low dietary intake of PUFA n-6, fat malabsorption [32], increased oxidative stress [33] together with enhanced conversion of arachidonic acid to prostaglandins *via* cyclooxygenase-2 [34] are more plausible.

In different studies, increased levels of DGLA in both plasma and erythrocyte lipids have been connected with insulin resistance, metabolic syndrome [3], BMI and plasma triglycerides [36], low-grade inflammation [37], predicted worsening of hyperglycemia [38] and the onset of DM2T [39]. Patients with cystic fibrosis and pancreatic insufficiency have displayed lower concentrations of linoleic and arachidonic acid and higher concentrations of DGLA in plasma and erythrocyte membranes in comparison with pancreatic-sufficient cystic fibrosis subjects and healthy controls [30].

In comparison to controls, our CP group displayed increased levels of adrenic (22:4n-6) and docosapentaenoic n-6 (22:5n-6) acids. These FAs are converted from arachidonic acid under the catalytic action of elongase (El) 2 and El5, D6D and β-oxidation [29]. These changes in FA profiles have not yet been described in CP patients.

This study demonstrated significantly increased activities of D6D and D9D, associated with decreased D5D activity, in CP patients in comparison with control persons. To our knowledge, this is the first study to describe the estimated activity of D6D and D5D in patients with CP. The changes in estimated activities of these enzymes have been described in other pathological states. Increased D6D activity has been found in metabolic syndrome [3], correlated with the risk of DM2T [5,38], with the risk of cardiovascular disease [4], and has also been described in persons with depression [7]. On the other hand, D5D is lowered in metabolic syndrome [3] and correlates negatively with cardiovascular disease risk [4] and risk of DM2T [5]. Decreased concentrations of PUFA n-3, as were found in our CP group, are known to be associated with the upregulation of D6D activity [40].

Using multivariate discriminant analysis, we established a model with eight FA which correctly classified 90% of probands in the CP or CON groups, respectively. In this model, increased proportions of 20:3n-6, 20:1n-9, 18:1n-7 and 22:5n-3 assigned the probands to the CP group and decreased others to the control group. In contrast to these FA, decreased proportions of 18:2n-6, 22:6n-3, 16:0 a 16:1n-9 assigned probands to the CP group.

Besides the alterations of plasma PC FA composition, in patients with CP we found some clinical and laboratory features, potentially denoting insulin resistance (higher waist circumference, HOMA-IR, higher TG and lower HDL-C). Recently, the issue of pancreatic fat accumulation, known as pancreatic lipomatosis, was discussed in relation to CP and DM [41]. It was not known before then whether fat accumulation in the pancreas led to exocrine secretion insufficiency. This condition reveals some similarities to non-alcoholic fatty liver disease, considered as a component of metabolic syndrome.

The limitations of our study are the unequal number of men and women in the CP and control groups (however, we did not find any statistically significant differences in FA profiles between men and women both in the CP and control groups); the absence of a dietary questionnaire in the studied groups; and the absence of simultaneously measured oxidative stress and low-grade inflammation. Furthermore, the estimation of delta desaturase activities was indirect, calculated as product/precursor ratios, reflecting both the FA metabolisms and their intake. Also, laboratory analysis of the FA profile did not involve the measurement of Mead acid (20:3n-9).

It is known that dietary assessment methods have many limitations. Among them, the most important are deficient data (e.g. finite food list, no quantification or imprecise estimation of portion size, absence of dietary details, underreporting of data) and measurement error. It is supposed that the accuracy for individual dietary components reaches maximally 70% [42]. However, all patients of the CP group were screened for nutritional risk according to the ESPEN Guidelines [20]. We did not find statistically significant differences in the FA pattern between CP patients nutritionally at-risk (NRS score ≥ 3), in CP subjects with underweight (or malnutrition) or in the CP patients without these markers. Although we did not simultaneously measure parameters of low-grade inflammation and oxidative stress in this set of patients, in an earlier study of CP subjects we described increased low-grade inflammation as well as increased oxidative stress levels [33].

Although we did not measure Mead acid (the Mead/arachidonic acid ratio) or the surrogate markers of essential FA deficiency due to the time consuming analyses of the FA profile (including Mead acid), we included another parameter derived from the FA profile concerning n-6 EFA deficiency (EFAD index), which possesses similar sensitivity to essential FA deficiency [23].

The strengths of our study include assembling the largest set of patients in which FA have yet been analyzed, the studied CP group was well defined (using clinical, anthropometric, biochemical and imaging methods) and the FA profile was analyzed in relation to disease complications as well as to CP severity index. Furthermore, subjects with CP were not given dietary supplements containing n-3 PUFA/n-6 PUFA or antioxidants. Moreover, to our best knowledge, this is the first study to deal with D6D and D5D in patients with CP.

5. Conclusions

In conclusion, this study shows that plasma phosphatidylcholines FA composition was significantly and markedly altered in CP patients compared with healthy persons. FA status in CP was characterized especially by an increased proportion of MUFA indicating *de novo* lipogenesis, decreased levels of LA and DHA, an altered spectrum of PUFA, both of n-3 and n-6 families, accompanied by increased estimated activities of D9D and D6D, whilst D5D was decreased. There were no significant differences in the FA composition in CP patients with DM and malnutrition, compared with CP subjects without these conditions. The whole group of CP patients is at risk of n-6 EFAD, and among CP patients those at highest risk of n-6 EFAD were CP patients with underweight.

The abovementioned changes of FA patterns in CP implicated decreased fat intake, including n-3 as well as n-6 PUFA, increased *de novo* lipogenesis, intrinsic changes in FA metabolism due to

alteration of delta desaturases and β-oxidation activities. Other mechanisms, such as the higher degree of oxidative stress (or lipoperoxidation), increased synthesis of proinflammatory eicosanoids, and other yet unknown mechanisms could also have been involved.

The results of this study warrant further research concerning dietary FA intake and the genetic and environmental factors that influence FA metabolism. It can be hypothesized that changes in FA profiles could improve our understanding of the pathophysiology, prevention and treatment of this disease and its complications.

Funding

Financial support from the Ministry of Health of the Czech Republic (project RVO-VFN64165/2012, and the grant NT/13199, IGA), the Ministry of Education, Youth and Sports of the Czech Republic (research project PRVOUK-P25/LF1/2 of Charles University in Prague, the 1st Faculty of Medicine) and the IT41I XS project number LQ1602 are gratefully acknowledged.

Competing interests

The authors declare no competing interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2016.03.012.

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Physiol. Res. 63 (Suppl. 3): S375-S385, 2014

Fatty Acid Composition Indicates Two Types of Metabolic Syndrome Independent of Clinical and Laboratory Parameters

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Received July 10, 2014
Accepted July 23, 2014

Summary

Dietary composition and metabolism of fatty acids (FA) influence insulin resistance, atherogenic dyslipidemia and other components of the metabolic syndrome (MS). It is known that patients with MS exhibit a heterogeneous phenotype; however, the relationships of individual FA to MS components have not yet been consistently studied. We examined the plasma phosphatidylcholine FA composition of 166 individuals (68F/98M) with MS and of 188 (87F/101M) controls. Cluster analysis of FA divided the groups into two clusters. In cluster 1, there were 65.7 % of MS patients and 37.8 % of controls, cluster 2 contained 34.3 % of patients and 62.2 % of controls ($P<0.001$). Those with MS within cluster 1 (MS1) differed from individuals with MS in cluster 2 (MS2) by concentrations of glucose ($P<0.05$), NEFA ($P<0.001$), HOMA-IR ($P<0.05$), and levels of conjugated dienes in LDL ($P<0.05$). The FA composition in MS1 group differed from MS2 by higher contents of palmitoleic (+30 %), γ-linolenic (+22 %), dihomo-γ-linolenic (+9 %) acids and by a lower content of linoleic acid (–25 %) (all $P<0.01$). These FA patterns are supposed to be connected with the progression and/or impaired biochemical measures of MS (lipolysis, oxidative stress, dysglycidemia, and insulin resistance).

Key words

Metabolic syndrome • Fatty acids • Delta desaturase activities • Cluster analysis

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Introduction

Metabolic syndrome (MS) is one of the most important health issues in developed countries over recent decades. Its increasing prevalence ranges between 20-30 % in the middle aged (Bruce and Byrne 2009, Ervin 2009, Sethom *et al.* 2011). MS significantly increases the risk for cardiovascular diseases, type 2 diabetes mellitus and other diseases (neuropsychiatric disorders and some cancers) (Bruce and Byrne 2009).

MS represents a cluster of cardiovascular risk factors connected with insulin resistance, visceral obesity, disturbed glucose metabolism, atherogenic dyslipidemia and arterial hypertension. These components of MS are complemented with chronic low-grade inflammation, coagulopathy, endothelial dysfunction and oxidative stress. It is supposed that the link between visceral obesity and metabolic disturbances, such as insulin resistance, impaired secretion of insulin or dyslipidemia, is caused by dysregulation of FA metabolism and/or chronic low-grade inflammation of the adipose tissue (Kalupahana *et al.* 2012).

MS develops in consequence of an increased energy intake and physical inactivity resulting in overweight and obesity; other important factors include composition of the diet, aging of the population and genetic background (Bruce and Byrne 2009, Lottenberg *et al.* 2012, Murphy *et al.* 2013, Walsh *et al.* 2014).

Epidemiological data indicate that increased fat intake is connected with a higher prevalence of overweight and obesity. The individual components of MS are variably influenced by saturated fatty acids

(SFA), monounsaturated fatty acids (MFA) and polyunsaturated fatty acids (PUFA) of n-3 as well as n-6 series, probably *via* their effects on plasma lipids (lipoproteins), blood pressure, insulin secretion and its action on target tissues and low-grade inflammation (Králová Lesná *et al.* 2013). Also, recent findings attribute an important influence on MS components to *trans* isomers of FA (Lottenberg *et al.* 2012).

Fatty acid profiles of individual lipid classes, especially those of cholesteryl esters and phosphatidylcholine, reflect dietary FA intake over several-weeks (i), FA metabolism (SFA synthesis, desaturation and elongation processes) (ii), as well as both enzymatic (β-oxidation) and nonenzymatic (lipoperoxidation) degradation (iii). The resulting profiles of FA are also influenced by racial, ethnic, geographic, genetic factors and concomitant diseases (Hodson *et al.* 2008).

The FA profiles in MS are characterized by an increased content of SFA [especially palmitic acid (16:0)], palmitoleic (16:1n-7), γ-linolenic (18:3n-6) and dihomo-γ-linolenic (20:3n-6) acids, accompanied by a lower concentration of linoleic acid (18:2n-6). Moreover, there is enhanced activity of delta 9 desaturase (D9D, synonym for steraoyl-CoA desaturase 1, SCD-1) and delta 6 desaturase (D6D) together with lower activity of delta 5 desaturase (D5D) (Warensjö *et al.* 2005, Žák *et al.* 2007, Paillard *et al.* 2008, Kawashima *et al.* 2009, Mayneris-Perxachs *et al.* 2014).

Lower content of linoleic acid, higher ratios of both γ-linolenic and dihomo-γ-linolenic acids reflecting increased activities of D6D, as well as a decreased activity of D5D were described in obese children with other components of MS, but not in simple obesity. Analogous changes in FA profiles resembling insulin resistance were described in patients with recent myocardial infarction (Marangoni *et al.* 2014) and depressive disorder (Vařeka *et al.* 2012). FA profiles in adolescents with MS correlate not only with insulin resistance, but also with systemic markers of inflammation (Decsi *et al.* 2000, Klein-Platat *et al.* 2005). It must be stated that none of the cited papers have presented all these changes simultaneously.

Since MS is a heterogeneous group of diseases, with a different genetic background, as well as absence of a uniting definition based on various pathophysiological mechanisms (e.g. from interaction of genetic and environmental factors), the serum FA profile is not consistent. The aim of this study was to analyze the FA composition in the MS and control groups with the help of cluster analysis. We tried to characterize the patients with MS by cluster analysis of FA profile independently of clinical and laboratory parameters.

Methods

Study design

This study was carried out at the 4th Department of Internal Medicine of General University Hospital from January 2009 to September 2013. The study protocol was approved by the Joint Ethical Committee of the General University Hospital and the 1st Medical Faculty, Charles University in Prague. Written informed consent was obtained from all participants. In this period of time, a total of 354 persons were examined at the Lipid Clinic of the 4th Department of Medicine, the 1st Faculty of Medicine, Charles University and the General University Hospital in Prague.

Participants

The study group consisted of 188 (101 men and 87 women) controls (CON) and 166 patients (98 men and 68 women) with MS. MS were diagnosed according to the International Diabetes Federation criteria (Alberti *et al.* 2006). Combinations of individual MS components and their prevalence are shown in Table 1. All samples were marked with unique anonymized identification numbers, and the data was merged only after the assays had been completed.

The control group consisted of 42 healthy subjects (22 men and 20 women) that were recruited from employees of the General University Hospital, and 146 probands (79 men and 67 women) with at least one component of MS who failed to fulfil the diagnostic criteria for MS (see Table 1). None of control subjects was treated for dyslipidemia, hypertension or diabetes mellitus (prediabetic state, respectively).

Exclusion criteria for both groups were the following: current antioxidant, lipid-lowering and antidiabetic medication, excessive alcohol consumption (>30 g/day), hormonal replacement therapy, supplementation with polyunsaturated fatty acids (both of n-3 and n-6 families), manifestation of cardiovascular and/or cerebrovascular diseases, type 1 diabetes mellitus, liver (with exception of nonalcoholic fatty liver disease) and kidney diseases (creatinine >130 μmol/l), microalbuminuria (urinary albumin 30-300 mg/day),

hypothyroidism as well as recent infections and malignancies.

Table 1. Components of the metabolic syndrome in studied groups.

Control group (n=188)	
without any component [a]	42 (22.3) [b]
W	44 (23.4)
TG	11 (5.8)
HDL-C	5 (2.7)
fs-G	4 (2.1)
W + TG	42 (22.3)
W + BP	22 (11.7)
W + fs-G	7 (3.7)
W + HDL-C	6 (3.2)
BP + fs-G	5 (2.6)
Metabolic syndrome (n=166)	
W + TG + HDL-C	44 (26.5)
W + TG + BP	29 (17.5)
W + TG + BP + fs-G	19 (11.4)
W + TG + HDL-C + fs-G	18 (10.8)
W + TG + fs-G	17 (10.2)
W + BP + fs-G	13 (7.8)
W + TG + HDL-C + BP	12 (7.2)
W + TG + HDL-C + BP + fs-G	11 (6.6)
W + HDL-C + BP	2 (1.2)
W + HDL-C + fs-G	1 (0.6)

[a] – presence (absence, respectively) of MS component(-s); [b] – number of cases (%); fs-G – fasting serum glucose; BP – blood pressure; W – waist circumference; TG – triglycerides; HDL-C – cholesterol in HDL. Metabolic syndrome: waist circumference >94 (88, respectively) cm for men (women, respectively), and further at least 2 factors of following: triglycerides >1.70 mmol/l; HDL-C<1.04 (1.29, respectively) mmol/l for men (women, respectively); fasting serum glucose ≥5.60 mmol/l (or presence of diabetes mellitus type 2); elevated BP >130/85 mm Hg (or antihypertensive therapy).

Blood sampling

Blood samples were taken after 12 h of fasting. Routine biochemical and hematological analyses were performed immediately; samples for special analyses were stored at –70 °C until use.

Dietary habits

The nutritional intake of the main dietary components, based on a regular 7-day dietary questionnaire, was assessed in all study subjects. The nutritional data were analyzed by Nutrimaster SE software, version 1.0.

Anthropometry

Basal clinical and anthropometrical data, including assessment of body fat, were examined using standard methods, as described previously (Žák *et al.* 2007).

Laboratory measurements

Plasma concentrations of total cholesterol and triglycerides were measured by enzymatic-colorimetric methods (Boehringer, Mannheim, Germany). HDL-C was determined in the supernatant after precipitation of lipoproteins B by PTA/Mg^{2+}, using the kit from the same manufacturer. Concentration of apolipoprotein (apo) B was measured by a Laurell rocket electroimmunoassay using standard and specific antibodies (Behringwerke, Marburg, Germany). Immunoreactive insulin was determined by a RIA method using double monoclonal antibodies (Insulin IRMA, Imunotech Prague, Czech Republic). Concentrations of conjugated dienes in precipitated LDL (CD-LDL) were determined spectrophotometrically (Ahotupa *et al.* 1996). The concentrations of non-esterified fatty acids (NEFA) were determined by enzymatic-colorimetric method (NEFA, Randox Laboratories, U.K.).

Fatty acid patterns in main plasma lipid classes were examined by analytical procedures described previously (Tvrzická *et al.* 2002). The method variability presented as relative standard deviation (RSD) ranged from 1.07 % for palmitic acid (16:0) to 8.60 % for 16:1n-9. The relevant variability data (RSD) were for dihomo-γ-linolenic 1.25 %; stearic 0.76 %, myristic 8.06 %; docosahexaenoic 2.38 %; docosapentaenoic 1.91 %, and linoleic acids 0.725 %.

Calculated parameters

The Homeostasis model assessment method, HOMA-IR, was used as an index of insulin resistance (Matthews *et al.* 1985). Desaturase activities were estimated using FA product/precursor ratios (Žák *et al.* 2007).

Statistical analyses

Prior analyses, all data were cleaned and pre-processed: extreme values were examined and double-checked. After that, a power-transformation was

performed to achieve symmetry and constant variance. Data are expressed as mean and standard deviation or median and inter-quartile range (IQR, 25^{th}-75^{th} percentile) in cases of non-Gaussian distribution of data. Normality of the distribution was tested by the Shapiro-Wilks W test. Comparisons between the groups were carried out by the independent t-test, and the Wilcoxon test, respectively. P-value was adjusted for multiple comparisons using Benjamini-Hochberg correction.

Cluster analysis

Cluster analysis was performed in two steps. In the first step, the number of individual fatty acids in plasma phosphatidylcholine was reduced, and in the second step the grouping of subjects into the clusters was carried out.

To reduce number of individual fatty acids, a linear discriminant analysis was performed with stepwise forward variable selection, using the Wilk's lambda criterion: an initial model was created from the variable that mostly separated the groups. This model was then iteratively extended by including further variables depending on the Wilk's lambda criterion. Incorporation of additional variables stopped when the newly added variable did not show a statistically significant improvement (P>0.05). We used 22 initially analyzed FAs of all the probands to separate CON and MS groups using linear discriminant analysis. This resulted in overall 69.8 % correct classification. Final correct classification was 74.5 % for CON, 64.5 % for MS, respectively. Variables subjected into the linear discriminant analysis were dihomo-γ-linolenic (20:3n-6; F=30.41), stearic (18:0; F=24.2), myristic (14:0; F=21.55), docosahexaenoic (22:6n-3; F=17.66), docosapentaenoic (22:5n-3; F=14.92), and linoleic (18:2n-6, F=13.19) acids. These six individual fatty acids were included into the cluster analysis. On the selected six fatty acids, a hierarchical clustering was applied using the Ward's method with Euclidean measure (Ward 1963).

All the statistical analyses were performed using the R software version 3.1.0 (The R Development Core Team 2014).

Results

Basic clinical and biochemical parameters of the subjects with MS and in the CON group are shown in Table 2. As expected, subjects with MS differed from controls in nearly all parameters examined. There was no difference in sex ratio between MS and CON. Table 3 shows the composition of FA in plasma phosphatidylcholine and the corresponding derived parameters. In MS patients, significantly increased concentrations of palmitoleic (16:1n-7), stearic (18:0), dihomo-γ-linolenic (20:3n-6) acids, and the sum of SFA (ΣSFA) were found. In contrast, patients with the MS had a decreased content of linoleic acid (18:2n-6), and the sum of PUFA n-6 (ΣPUFAn-6).

Table 2. Clinical and biochemical characteristics of the studied groups.

	Metabolic syndrome	**Controls**
Number of persons	166	188
Gender (M/F)	98/68	101/87
Age (years)	55.2 ± 10.6 [a]	54.5 ± 11.9
Weight (kg)	88.4 ± 15.6 [a] ***	77.4 ± 14.4
BMI (kg.m^{-2})	29.8 ± 4.0 [a] ***	26.5 ± 4.1
Waist circumference (cm)	103 ± 10 [a] ***	91 ± 12
Systolic BP (mm Hg)	141 ± 17 [a] ***	129 ± 14
Diastolic BP (mm Hg)	88 ± 10 [a] ***	80 ± 9
Relative fat mass (%)	33.9 ± 6.9 [a] ***	30.0 ± 7.6
Fat mass (kg)	29.8 ± 7.6 [a] ***	23.3 ± 7.9
Glucose (mmol/l)	5.96 ± 1.92 [a] ***	4.99 ± 1.27
Insulin (mU/l)	10.70/7.24[b]***	7.70/5.56
HOMA-IR (ratio)	2.59/2.14 [b]***	1.62/1.20
TC (mmol/l)	6.42 ± 1.43 [a]*	6.05 ± 1.26
TG (mmol/l)	2.69/2.09 [b]***	1.40/0.83
HDL-C (mmol/l)	1.27 ± 0.35 [a]***	1.53 ± 0.37
NEFA (mmol/l)	0.60/0.36 [b]***	0.53/0.36
Apo B (g/l)	1.36 ± 0.34 [a]***	1.21 ± 0.37
CD-LDL (μmol/l)	66.7/23.7[b]***	56.4/22.9

[a] – mean ± SD; [b] – median/IQR. Statistical analysis: Student's t-test, or Wilcoxon test (in cases of non-Gaussian distribution of data); p-values were adjusted for multiple comparisons using Benjamini-Hochberg corrections: * P<0.05, ** P<0.01, *** P<0.001. M – males; F – females; BMI – body mass index; BP – blood pressure; NEFA – nonesterified fatty acids; CD-LDL – conjugated dienes in LDL; TC – total cholesterol; TG – triglycerides; LDL – low density lipoproteins; HDL – high density lipoproteins; Apo – apolipoprotein; HOMA-IR – homeostasis model assessment for insulin resistance (f-insulin (μU/ml) x f-glucose (mmol/l)/22.5); IQR – interquartile range.

A significant increase in activities of delta 9 desaturase for palmitoleic (D9D16) and delta 6 desaturase (D6D), as well as a decreased activity of delta 5 desaturase (D5D), were found in MS.

Table 3. Plasma phosphatidylcholine fatty acid composition of the studied groups.

Fatty acid [c]	Metabolic syndrome	Controls
Number of persons	166	188
14:0	0.28 ± 0.08 [a]	0.28 ± 0.09
16:0	29.68 ± 1.73 [a]	29.4 ± 1.60
16:1n-9	0.11 ± 0.03 [a]	0.11 ± 0.03
16:1n-7	0.59/24 [b** g]	0.52/0.20
18:0	14.44 ± 1.28 [a ***]	13.84 ± 1.14
18:1n-9	10.06 ± 1.62 [a]	10.03 ± 1.73
18:1n-7	1.56 ± 0.36 [a]	1.61 ± 0.37
18:2n-6	21.94 ± 3.16 [a ***]	23.54 ± 3.00
18:3n-6	0.08/0.05 [b]	0.08/0.05
18:3n-3	0.19/0.08 [b]	0.21/0.10
20:1n-9	0.14 ± 0.04 [a]	0.14 ± 0.04
20:2n-6	0.41 ± 0.12	0.40 ± 0.11
20:3n-6	3.35 ± 0.70 [a ***]	2.98 ± 0.56
20:4n-6	10.99 ± 2.05 [a]	10.91 ± 1.83
20:5n-3	0.94/0.50 [b]	0.92/0.48
22:4n-6	0.31 ± 0.08 [a]	0.32 ± 0.07
22:5n-6	0.20 ± 0.06 [a]	0.20 ± 0.05
22:5n-3	0.90 ± 0.18 [a]	0.91 ± 0.17
22:6n-3	3.56 ± 1.11 [a]	3.36 ± 0.95
ΣSFA	44.47 ± 1.68 [a ***]	43.59 ± 1.43
ΣMFA	12.48 ± 2.04 [a]	12.45 ± 2.12
ΣPUFA n-6	37.22 ± 3.20 [a **]	38.38 ± 2.77
ΣPUFA n-3	5.79 ± 1.77 [a]	5.55 ± 1.53
D9D16[d]	0.020/0.008 [b**]	0.018/0.007
D9D18[e]	0.700 ± 0.120 [a]	0.730 ± 0.150
D6Dn6[e]	0.004/0.003 [b**]	0.003/0.002
D5Dn6[f]	3.45 ± 1.08 [a **]	3.79 ± 0.98

[a] – mean ± SD (mol%); [b] – median/IQR; [c] – shorthand notation of fatty acids: number of carbon atoms: number of double bonds n – number of carbon atom from the methyl end to the nearest double bond. Only relevant fatty acids are presented; [d] – D9D16, delta 9 desaturase for palmitoleic acid (16:1n-7/16:0); [e] – D9D18, delta 9 desaturase for oleic (18:1n-9/18:0); [f] – D6D, delta 6 desaturase (18:3n-6/18:2n-6); [g] – D5D, delta 5 desaturase (20:4n-6/20:3n-6); statistical analysis: Wilcoxon test; p – values were adjusted for multiple comparisons using Benjamini-Hochberg corrections: * P<0.05, ** P<0.01, *** P<0.001. IQR – interquartile range; ΣSFA – total content (the sum) of saturated fatty acids; ΣMFA – the sum of monounsaturated fatty acids; Σn-6 PUFA – the sum of polyunsaturated fatty acids n-6 family; Σn-3 PUFA – the sum of polyunsaturated fatty acids n-3 family.

Based on the cluster analysis that includes six selected FA obtained from linear discriminant analysis, two clusters were found both in the CON and MS groups. In the MS group, 109 patients (65.7 %) were classified as cluster 1 (MS1) and 57 patients (34.3 %) were classified as cluster 2 (MS2). Conversely, in the control group, 71 probands (37.8 %) were classified as cluster 1 (CON1) and 117 subjects (62.2 %) were classified as cluster 2 (CON2). These results indicated a non-random distribution of subjects in both groups into cluster 1 and cluster 2 (χ^2=26.35; P<0.001). This means that MS was present mainly in cluster 1, while the CON probands presented mainly in cluster 2.

Table 4. Clinical and biochemical characteristics of metabolic syndrome according to cluster analysis.

	Cluster 1	Cluster 2
Number of persons	109	57
Gender (M/F)	67/42	31/26
Age (years)	54.6 ± 11.1 [a]	56.3 ± 9.5
Weight (kg)	89.9 ± 15.7 [a]	85.5 ± 15.0
BMI (kg.m^{-2})	30.2 ± 4.1 [a]	29.1 ± 3.8
Waist circumference (cm)	104 ± 11 [a*]	101 ± 9
Systolic BP (mm Hg)	141 ± 15 [a]	142 ± 15
Diastolic BP (mm Hg)	88 ± 9 [a]	89 ± 10
Relative fat mass (%)	33.3 ± 6.8 [a]	35.3 ± 6.9
Fat mass (kg)	29.7 ± 7.7 [a]	29.9 ± 7.4
Phenotypes of metabolic syndrome		
MS_{gly}	6 (5.5) [c, d *]	7 (12.3)
MS_{glylip}	50 (45.9)	16 (28.1)
MS_{lip}	53 (48.6)	34 (59.6)
Glucose (mmol/l)	6.24 ± 2.22 [a**]	5.44 ± 0.96
Insulin (mU/l)	11.75/7.17 [b]	9.40/5.83
HOMA-IR (ratio)	3.03/2.30 [b*]	2.07/1.94
TC (mmol/l)	6.51 ± 1.53	6.26 ± 1.21
TG (mmol/l)	2.86/3.09 [b]	2.43/1.60
HDL-C (mmol/l)	1.25 ± 0.34	1.30 ± 0.36
NEFA (mmol/l)	0.69/0.73 [b***]	0.44/0.40
Apo B (g/l)	1.36 ± 0.38	1.36 ± 0.26
CD-LDL (μmol/l)	70.9/34.9 [b*]	60.9/19.7

[a] – mean ± SD; [b] – median/IQR; [c] – number of subjects (%) in individual phenotypes of MS. Statistical analysis: Student's t-test, or Wilcoxon test (in cases of non-Gaussian distribution of data); p – values were adjusted for multiple comparisons using Benjamini-Hochberg corrections: * P<0.05, ** P<0.01, *** P<0.001. [d] – Fisher's exact test: * P<0.05. Abbreviations: see Table 2. Characterization of metabolic syndrome phenotypes according to combination of metabolic syndrome components: MS_{gly} – increased waist, elevated BP (or hypertension), increased fasting serum glycemia; MS_{lip} – increased waist, elevated TG and/or decreased HDL-C, elevated BP (or hypertension) (alternatively); MS_{glylip} – increased waist, elevated TG and/or decreased HDL-C, increased fasting serum glycemia, elevated BP (alternatively).

The basic clinical and biochemical characteristics of patients with MS according to cluster analysis are shown in Table 4. Patients in cluster 1 had a more detrimental metabolic profile in comparison with patients in cluster 2. There were no statistically significant differences between both clusters in sex ratio, age, BMI, fat mass, systolic and diastolic blood pressure, total cholesterol, HDL-C, triglycerides, and apoB concentrations. Between both clusters of MS, there was a non-random distribution of patients with different phenotypes of MS. In cluster 1, in comparison with cluster 2, the phenotype of MS with fasting hyperglycemia and dyslipidemia ($MS_{glyclip}$) was more prevalent ($P<0.05$). The patients with MS in cluster 1 (MS1) were characterized by increased waist circumference and HOMA-IR (both $P<0.05$). Moreover, they had increased glucose ($P<0.01$), NEFA ($P<0.001$), and CD-LDL concentrations ($P<0.05$). There were no statistically significant differences in the concentration of hs-CRP (data not shown).

The plasma FA patterns, including derived parameters, of MS patients categorized according to cluster analysis are shown in Table 5. Apart from FAs used in the cluster analysis (see Table 5), the levels of palmitic (16:0), palmitoleic (16:1n-7), oleic (18:1n-9), vaccenic (18:1n-7), γ-linolenic (18:3n-6), eicosapentaenoic (20:5n-3), and arachidonic (20:4n-6) acids were increased in cluster 1. In addition, patients in cluster 1 were characterized by higher activities of D9D for palmitoleic acid (D9D16), D9D for oleic acid (D9D18), and D6D. Contrary to the comparison of the whole MS with CON, there was no significant difference between cluster 1 and cluster 2 in the D5D activity.

In comparison of controls in cluster 1 (CON1) with those in cluster 2 (CON2) significantly increased BMI (27.5±4.5 vs 25.9±3.7 kg/m^2, $P<0.01$), and waist circumference (95.5±12.2 vs 88.9±10.6 cm, $P<0.01$) were found. After ANCOVA adjustment (with BMI and waist circumference as covariates), probands in CON1 had, in comparison with CON2, increased concentrations of palmitoleic (16:1n-7) (0.60/0.29 vs 0.49/0.18, median/IQR, mol%, $P<0.01$), oleic (18:1n-9) (10.14±2.30 vs 9.57±1.91, $P<0.01$), and arachidonic (20:4n-6) (11.66±1.91 vs 10.46±1.62, $P<0.01$) acids. In addition, higher contents of ΣSFA (44.48±1.68 vs 43.10±1.43, $P<0.001$), ΣMFA (12.59±2.68 vs 11.74±2.20, $P<0.001$), ΣPUFA n-3 (5.76±1.50 vs 4.88±1.42, $P<0.01$), as well as a lower content of ΣPUFA n-6 (36.69±3.36 vs 39.64±2.89, $P<0.001$) were found. Similarly, probands in

Table 5. Plasma phosphatidylcholine fatty acid composition of metabolic syndrome according to cluster analysis.

Fatty acid [c]	Cluster 1	Cluster 2
Number of persons	109	57
14:0 [h]	0.28 ± 0.08 [a]	0.28 ± 0.08
16:0	29.89 ± 1.87 [a **]	29.29 ± 1.34
16:1n-9	0.11 ± 0.03 [a]	0.10 ± 0.02
16:1n-7	0.63/0.28 [b***]	0.48/0.18
18:0 [h]	14.59 ± 1.34 [a]	14.16 ± 1.12
18:1n-9	10.50 ± 1.56 [a]	9.20 ± 1.38
18:1n-7	1.62 ± 0.38 [a ***]	1.45 ± 0.30
18:2n-6 [h]	20.17 ± 2.07 [a]	25.31 ± 1.88
18:3n-6	0.09/05 [b*]	0.07/0.04
18:3n-3	0.20/0.08 [b]	0.19/0.08
20:1n-9	0.14 ± 0.05 [a]	0.13 ± 0.03
20:2n-6	0.42 ± 0.13 [a]	0.40 ± 0.10
20:3n-6 [h]	3.45 ± 0.70 [a]	3.16 ± 0.68
20:4n-6	11.34 ± 2.03 [a **]	10.34 ± 1.95
20:5n-3	1.09/0.49 [b***]	0.80/0.28
22:4n-6	0.32 ± 0.08 [a]	0.29 ± 0.06
22:5n-6	0.21 ± 0.06 [a]	0.18 ± 0.05
22:5n-3 [h]	0.94 ± 0.19 [a]	0.82 ± 0.13
22:6n-3 [h]	3.76 ± 1.18 [a]	3.20 ± 0.85
ΣSFA	44.85 ± 1.84 [a***]	43.79 ± 1.04
ΣMFA	13.08 ± 1.96 [a***]	11.40 ± 1.74
ΣPUFA n-6	35.84 ± 2.79 [a***]	39.70 ± 2.28
ΣPUFA n-3	6.19 ± 1.95 [a***]	5.07 ± 1.06
D9D16 [d]	0.021/0.01 [b***]	0.016/0.005
D9D18 [e]	0.726 ± 0.120 [a**]	0.655 ± 0.118
D6Dn6 [f]	0.005/0.003 [b***]	0.003/0.002
D5Dn6 [g]	3.45 ± 1.06 [a]	3.46 ± 1.13

[a] – mean ± SD (mol%); [b] – median/IQR; [c] – shorthand notation of fatty acids: number of carbon atoms: number of double bonds n – number of carbon atom from the methyl end to the nearest double bond. Only relevant fatty acids are presented. [d] – D9D16, delta 9 desaturase for palmitoleic acid (16:1n-7/16:0); [e] – D9D18, delta 9 desaturase for oleic (18:1n-9/18:0); [f] – D6D, delta 6 desaturase (18:3n-6/18:2n-6); [g] – D5D, delta 5 desaturase (20:4n-6/20:3n-6); statistical analysis: Wilcoxon test; p – values were adjusted for multiple comparisons using Benjamini-Hochberg corrections: * $P<0.05$, ** $P<0.01$, *** $P<0.001$. [h] – Fatty acids used in cluster analysis separating MS patients into cluster 1 and cluster 2: dihomo-γ-linolenic (20:3n-6), stearic (18:0), myristic (14:0), docosahexaenoic (22:6n-3), docosapentaenoic (22:5n-3), and linoleic (18:2n-6) acids. Therefore, the respective P values for these FAs are not indicated. IQR – interquartile range; ΣSFA – total content (the sum) of saturated fatty acids; ΣMFA – the sum of monounsaturated fatty acids; Σn-6 PUFA – the sum of polyunsaturated fatty acids n-6 family; Σn-3 PUFA – the sum of polyunsaturated fatty acids n-3 family.

CON1 presented increased activities of D9D for palmitoleic, (0.020/0.010 vs 0.017/0.06, P<0.01), and D9D for oleic (0.729/0.164 vs 0.708/0.141, P<0.05) acids, along with increased activity of D6D (0.004/0.003 vs 0.003/0.002, P<0.001). The differences in FA profiles between cluster 1 and cluster 2 in CON group were similar to those observed for MS 1 and MS 2 cluster.

The control persons did not differ from the patients with MS in the daily energy intake, the energy content of proteins, fats as well as carbohydrates. No differences were observed in the intakes of dietary FA – saturated FA, monounsaturated FA and PUFA (as sum of both n-3 and n-6 PUFA). Similarly, there were not statistically significant differences of macronutrients consumed in diets between the probands in cluster 1 and those in cluster 2 of MS (data not shown).

Discussion

The metabolic syndrome is a heterogeneous clustering of metabolic and non-metabolic abnormalities that are associated with various degrees of cardiovascular risk. Most clinical studies use either the diagnostic criteria according to ATP-III (NCEP Expert Panel, 2001), or those according to IDF (Alberti *et al.* 2006), which require the presence of central (abdominal) obesity and minimally two of four further risk factors (elevated glucose, triglycerides and arterial blood pressure, low HDL-C levels). Obesity is a key factor in the development of MS; increased body fat percentage unfavorably influences insulin resistance, oxidative stress and chronic systemic inflammation, as well as prothrombotic and proatherogenic metabolic traits (Phillips *et al.* 2013). In comparison with BMI, the values of waist circumference correlate more tightly with hemodynamic (blood pressure, endothelial dysfunction), hemostatic (coagulation, fibrinolysis), and metabolic parameters (plasma lipids, glucose, uric acid, insulin sensitivity) involved in MS (Savva *et al.* 2013). Visceral fat displays a high degree of lipolysis; it is almost exclusively perfused by the portal vein which delivers triglycerides, fatty acids and adipokines directly into the liver; this fact significantly influences hepatic metabolic processes (Carey 1998, Miranda *et al.* 2005).

Both definitions of MS, according to ATP-III and IDF, allow considerable inter-individual differences concerning the presence of the constitutive components (risk factors). In the German study PROCAM, for example, 72.9 % of participants with MS had elevated blood pressure, 32 % dyslipidemia with dysglycidemia, and 20 % dyslipidemia without dysglycidemia (Assmann and Seedorf 2009). In our study, dyslipidemia (dyslipidemia with and without dysglycidemia) was almost twice more frequent in comparison with the PROCAM study. On the other hand, only 51.8 % of patients in our MS group had elevated blood pressure (hypertension, respectively).

The MS_{glylip} phenotype (MS with dyslipidemia and dysglycidemia) is associated with high plasma concentrations of NEFA that point to insulin resistance of the adipose tissue. One Finnish prospective study has shown that increased NEFA levels predict the development of hyperglycemia and type 2 diabetes mellitus (Mahendran *et al.* 2013). Furthermore, elevated NEFA can contribute to low-grade systemic inflammation. In this study, we observed higher concentrations of CD-LDL in the MS group. The levels of CD-LDL partially reflect systemic oxidative stress and/or the presence of minimally modified LDL particles (Ahotupa *et al.* 1996). In our previous study, subjects with MS had low values of the antioxidative potential, mainly due to decreased activities of paraoxonase 1 (Vávrová *et al.* 2013).

To extend upon the investigations of visceral fat and NEFA concentrations, several recent clinical studies have concentrated on plasma fatty acid patterns. The spectrum of plasma FAs in MS depends both on the quality of dietary fat and on the metabolic processes (e.g. endogenous synthesis, activity of desaturases) that are partly influenced by the genotype (Hodson *et al.* 2008).

In this study, we performed a cluster analysis of FA profiles in plasma phosphatidylcholine for the participants of the MS and CON groups. Cluster analysis is a method for statistical data exploration that groups a set of probands in such a way that probands in the same group (called a cluster) are more similar to each other than to those in other groups (clusters). In our MS group, this analysis rendered two clusters independent of clinical and biochemical parameters, that were characterized by six FAs: dihomo-γ-linolenic (20:3n-6), stearic (18:0), myristic (14:0), docosahexaenoic (22:6n-3), docosapentaenoic (22:5n-3), and linoleic (18:2n-6) acids. In comparison with cluster 2, cluster 1 displayed a higher consistency and a more adverse risk profile (waist circumference, HOMA insulin resistance index, concentrations of NEFA, glucose and CD-LDL). To our knowledge, this is the first phenotyping of MS based on cluster analysis of plasma FA.

The FA profiles in cluster 1 differed from those in cluster 2 in higher proportions of ΣSFA, ΣMFA and ΣPUFA n-3, while ΣPUFA n-6 were decreased. Enhanced proportion of ΣSFA was particularly due to palmitic (16:0) and stearic (18:0) acids, that of ΣMFA to palmitoleic (16:1n-7), oleic (18:1n-9) and vaccenic (18:1n-7) acids; lower ΣPUFA n-6 were caused by the drop in concentrations of linoleic acid (18:2n-6). The patients in cluster 1 had higher activities of D9D for palmitoleic acid, D9D for oleic acid and D6D. Elevated activities of D6D were connected with higher concentrations of γ-linolenic (18:3n-6) and dihomo-γ-linolenic (20:3n-6) acids. Our previous study has shown significant positive correlations between the number of MS components and the concentrations of palmitic, palmitoleic, stearic and dihomo-γ-linolenic acids, as well as negative correlations for linoleic acid. ΣSFA and activities of both D9D for palmitoleic acid and D6D correlated positively, ΣPUFA n-6 and activities of D5D negatively with the number of MS components (Žák *et al.* 2007). In several other studies, SFA, MFA, D9D and D6D correlated positively with cardiovascular risk factors (BMI, waist circumference, HOMA-IR, glycemia, triglycerides, blood pressure), while negative correlations were proved with both PUFA n-3 and n-6 series (Lee *et al.* 2008, Sethom *et al.* 2011, Mayneris-Perxachs *et al.* 2014). In a long-term prospective study, serum FA composition and activities of desaturases predicted the development of MS in middle-aged men (Warensjö *et al.* 2005).

The proportions of MFA, especially palmitoleic, oleic and vaccenic acids are indicators of *de novo* synthesis of FAs; higher concentrations of MFA were shown to predict an elevated risk of sudden cardiac arrest (Wu 2011). Increased level of palmitoleic acid was associated with enhanced lipogenesis induced by carbohydrates (Aarsland *et al.* 1997), as well as with hyperglycemia in Indian women in Peru (Lindgärde *et al.* 2006). Middle-aged Chinese with higher concentrations of palmitoleic acid in erythrocyte phospholipids had higher levels of several MS components and decreased concentrations of adiponectin (Zong *et al.* 2012).

We did not prove decreased concentrations of n-3 PUFA, which are – according to several authors – associated with the progression of MS (Warensjö *et al.* 2006, Lee *et al.* 2008, Chien *et al.* 2011, Mahendran *et al.* 2013). On the contrary, a prospective Finnish study failed to prove any association between the incidence of MS and n-3 PUFA, while relative proportions of n-6 PUFA correlated negatively (Vanhala *et al*. 2012). In our study, elevated ΣPUFA n-3 in cluster 1 can be explained by a more pronounced decrease in ΣPUFA n-6, expressed in relative concentrations. A similar phenomenon was described for FA composition in patients with anorexia nervosa (Žák *et al.* 2005).

Decreased plasma concentrations of n-6 PUFA (mainly due to low concentrations of linoleic acid) as a hallmark of MS were observed in obese adults (Vessby 2003), children (Decsi *et al.* 2000), and postinfarction middle-aged men (Leskinen *et al.* 2005). Low concentrations of linoleic acid were found to be associated with the progression of MS (Laaksonen *et al.* 2002, Warensjö *et al.* 2006). The decrease in linoleic acid content can be explained by a higher degree of oxidative stress, lipoperoxidation, and synthesis of proinflammatory eicosanoids (Hardwick *et al.* 2013).

Patients with MS were shown to have increased activities of the enzyme stearoyl-CoA desaturase-1 (SCD1), synonym delta 9-desaturase (D9D). The preferred substrates for this enzyme are stearoyl-CoA and palmitoyl-CoA, which are converted to oleoyl-CoA (18:1n-9) and palmitoleoyl-CoA (16:1n-7), respectively. The human diet contains only very small amounts of palmitoleic acid, whereas oleic acid is usually present in abundance. In our study, activities of D9D for palmitoleic acid (D9D16) and D9Dfor oleic acid (D9D18) were significantly higher in cluster 1 than in cluster 2; high activities of D9D18 suggest a low dietary intake of fats rich in oleic acid. Increased activities of D6D are associated with hyperinsulinemia and higher BMI (Decsi *et al.* 2000, Vessby 2003), while decreased activities of D5D, specific for MS, are independent of BMI and physical activity (Warensjö *et al.* 2005). In a group of Japanese men, low D5D activities predicted the development of abdominal obesity (Kawashima *et al.* 2009).

We did not prove statistically significant differences in dietary habits between MS and CON subjects, as well as between MS patients in cluster 1 and in cluster 2. It is known that dietary assessment methods have many limitations. Among them, the most important are deficient data (e.g. finite food list, no quantification or imprecise estimation of portion size, absence of dietary details, underreporting of data), and the measurement error. The accuracy for individual dietary component reaches maximally 70 to 80 % (Thompson and Subar 2013).

The limitations of our study are a relatively

small number of participants and the cross sectional design. Estimated activities of desaturases are calculated as product/precursor ratio, reflecting both the metabolism of FAs and their dietary intake. For technical reasons, we did not estimate *trans*-FAs that are supposed to play an important role in the etiopathology of MS (Lottenberg *et al.* 2012). Among the strengths of our study is the fact that the participants with MS were not treated with lipid-lowering drugs, dietary supplements containing n-3 PUFA/n-6 PUFA, or antioxidants. The implementation of the cluster analysis of plasma FAs seems to be a promising approach to identify distinct phenotypes of MS.

Conclusions

By means of the cluster analysis of plasma FAs, MS can be categorized into two clusters, independent of clinical and biochemical parameters. These clusters differ in the biochemical abnormalities associated with insulin resistance, lipolysis, and oxidative stress, as well as in the degree of the risk for type 2 diabetes mellitus and cardiovascular disease. The results of this study warrant further research concerning dietary FA intake and genetic factors that influence FA metabolism. Improved understanding of fatty acid patterns in the pathogenesis of metabolic syndrome could lead to novel approaches in its prevention and treatment.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This research was supported by the grant NT/13199, IGA, Ministry of Health, the Czech Republic, RVO VFN64165/2012, PRVOUK P25/LF1/2, and by the European Regional Development Fund in the project of IT4 Innovations Centre of Excellence (CZ.1.05/1.1.00/02.0070, VP6).

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