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Plasma proteome alterations in prediabetic, obese Zucker rats – possible cardiovascular risk implications

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Abstract. Hyperphagia and obesity, which underlie metabolic syndrome, have been linked to multiple health complications and increased mortality. Here, we investigate the differences in plasma proteome between obese and lean Zucker rats in order to identify circulating proteins involved in obesity-related conditions. Plasma samples of male Zucker fatty (obese) rats carrying fatty fa/fa mutation (–/–) and their lean controls were enriched using ProteoMiner technology and labeled with isobaric tags (iTRAQ) for mass spectrometry-based quantitation. We found elevation in levels of coagulation factors whereas levels of serine protease inhibitors were decreased. Levels of acute phase proteins were also altered, as well as complement components. We also noticed differences in the abundance of apolipoproteins. In summary, quantitative proteomic assessment of plasma protein composition in obese Zucker rats revealed a profound landscape of changes, reflecting altered hemostasis, disturbed metabolic processes involving insulin resistance and lipid metabolism and ongoing low-grade inflammation.

Key words: Obesity — Metabolic syndrome — Thrombophilia — Inflammation — Proteomics

Introduction

Obesity – hallmark of metabolic syndrome (metS) – is associated with chronic inflammation (Berg and Scherer 2005) and insulin resistance which may progress to the diabetes (Fujimoto 2000) and have been linked to the cardiovascular mortality (Coutinho et al. 2011). It was proved that obesity-related metabolic changes, including altered triglycerides profiles and hyperinsulinemia are associated with hypercoagulant and hypofibrynolytic states and may contribute to increased thrombotic risk (Alessi and Juhan-Vague 2008), however the exact mechanistic links are complex and not yet completely comprehended. Most of the obese subjects show elevated plasma concentration of inhibitor of fibrinolysis-plasminogen activator inhibitor-1 (PAI-1) (Tschoner et al. 2012) and increased levels of tissue factor (TF), the key initiator of coagulation (Diamant et al. 2002). Moreover, it has been shown that plasma from subjects with metS formed denser clots than that from healthy people (Carter et al. 2007). Importantly, weight loss has been demonstrated to attenuate hypercoagulability (Kopp et al. 2003; Ay et al. 2010).

It is widely recognized, that comprehensive plasma proteome profiling represents valuable approach in studies of metabolic diseases. It contributed to discovery of novel diagnostic markers as well as provided better understanding of mechanisms of disease and pointed to novel therapeutic targets (Anderson 2010; Geyer et al. 2017). Thus, it may well be that plasma analysis by the methods of differential proteomics may shed a new light on the mechanisms of obesity-related thrombophilia (Stein et al. 2005).

The Zucker fatty rats, carrying the mutation in the leptin receptor gene, are used in studies pertaining to pathomecha-

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nisms of metS (Bouvet Céline et al. 2007; Raza et al. 2013; Tomassoni et al. 2020). These animals, characterized by hyperphagia and decreased energy expenditure, typically develop severe obesity, insulin resistance and dyslipidemia as well as marked hypercoagulability state, evidenced by shortened prothrombin (PT) and partial thromboplastin time (aPTT), accelerated thrombin generation and thrombus formation, and decreased fibrinolysis (Vera et al. 2013; Shang et al. 2014). In this work we applied isobaric tag for absolute and relative quantitation (iTRAQ) labeling to investigate the differences in plasma proteome between obese and lean animals.

Materials and Methods

Animals

Research meets European Union (EU) standards for the protection and use of animals for scientific purposes and feed legislation. All experimental procedures were carried out according to the Jagiellonian University Ethical Committee on Animal Experiments (No. 172/2012).

Male Zucker fatty (obese) rats and lean controls of the same strain were purchased from Harlan (Udine, Italy). Animals were maintained on 12-h light/dark cycle and allowed *ad libitum* access to water and standard diet. After acclimatization, both lean ($n = 4$) and obese ($n = 4$) rats aged 33 weeks were fasted overnight and sacrificed by decapitation. The trunk blood was collected in cooled tubes containing EDTA as anticoagulant (to ensure compatibility with the subsequent enrichment protocol) and centrifuged immediately at 4°C (1000 × *g*, 10 min) to separate plasma, which was then stored in aliquots at –80°C until analyzed. As we showed previously, obese Zucker rats exhibited a profound difference in body mass, circulating lipids, showing a marked increase in total cholesterol, LDL- and HDL-cholesterol fractions, as well as triglycerides (Krskova et al. 2020).

Mass spectrometry 2D-LC-nESI-MS/MS

ProteoMiner (Combinatorial Peptide Ligand Library beads, CPLL) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The enrichment procedure was optimized with the reference to previously published protocol (Lam et al. 2007; Fasoli et al. 2010). 1 ml of rat serum diluted with deionized water (1:3 ratio) to reduce the physiological salt concentration below 50 mM was acidified to pH = 3 to enlarge the number of proteins harvested after enrichment. Samples were centrifuged (10,000 × *g*, 10 min) to eliminate possible particles in suspension. Each serum sample was intimately mixed with 100 µl of pre-equilibrated ProteoMiner in wash buffer (25 mM sodium chloride buffered with 25 mM acetate, pH 4.0). The suspensions were gently shaken overnight at 4°C. After supernatant separation by centrifugation (1000 × *g*, 1 min) the beads were washed twice with the wash buffer to remove the unbound protein excess. The captured proteins were eluted from the peptide libraries by three consecutive injections of 200 µl boiling 3% SDS, 3% DTT solution (95°C, 5 min, vigorously mixed on termomixer). After elution samples were precipitated overnight with ice-cold acetone (Sigma, St. Louis, MO, USA) (1:6 v:v). The protein concentration was determined in the harvested supernatant using the Bradford method (Bradford 1976). 100 µg of measured protein content of each sample was reduced, and alkylated, as recommended by iTRAQ protocol (ABSciex, Framingham, MA, USA). Proteins were digested with modified trypsin (Thermo Scientific, Waltham, MA, USA) overnight, with 1:50 (w:w) ratio at 37°C. Samples were labeled with iTRAQ reagents as recommended by producer and ordered as follows: controls – 113, 114, 115, 116 and obese rats – 117, 118, 119 and 121. Labeled samples were combined and dried in vacuum concentrator (Eppendorf, Hamburg, Germany). Next, trypsin-digested peptides were dissolved in 5% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) and purified with C18 MacroSpin Columns (Harvard Apparatus, Holliston, MA, USA). Eluate was dried in a vacuum concentrator, reconstituted in 5% ACN, 0.1% formic acid (FA), and subjected to strong cation exchange (SCX) fractionation. Samples were loaded onto previously conditioned SCX Macrospin columns (Harvard Apparatus), after which flow-through fraction and 11 consecutive injections of the eluent buffer, which consisted of 5, 10, 20, 40, 60, 80, 100, 150, 200, 300, 500 mM ammonium acetate in 5% ACN, 0.1% FA, respectively, were collected by centrifugation (2000 × *g*, 1 min). Thus, the labeled peptides from each sample were distributed across 12 SCX fractions.

Labeled peptides were injected on PepMap100 RP C18 75 µm i.d. ×15 cm column (Thermo Scientific, Waltham, MA, USA) *via* trap column PepMap100 RP C18 75 µm i.d. ×2 cm column (Thermo Scientific, Waltham, MA, USA). Each peptide fraction was separated in 65 min 7–45% B phase linear gradient (A phase – 2% ACN and 0.1% FA; B phase – 80% ACN and 0.1% FA) with a flow rate of 300 nl/min by UltiMate 3000 HPLC system (Thermo Scientific, Waltham, MA, USA) and applied on-line to a Velos Pro (Thermo Scientific, Waltham, MA, USA) dual-pressure ion-trap mass spectrometer. The main working nano-electrospray ion source (Nanospray Flex, Thermo Scientific, Waltham, MA, USA) parameters were as follows: ion spray voltage 1.9 kV and capillary temperature 250°C. Spectra were collected in full scan mode (400–1500 Da), followed by five higher energy collisional dissociation (HCD) MS/MS scans of the five most intense ions from the preceding survey full scan under dynamic exclusion criteria.

Collected data were analyzed by the X!Tandem (The GPM Organization) (Craig and Beavis 2004) and Comet (Eng et al. 2013) search engines, statistically validated with PeptideProphet and integrated with iProphet (Shteynberg et al. 2011) under trans-proteomic pipeline (TPP) suite of software (Institute for Systems Biology, Seattle, WA, USA) (Deutsch et al. 2010). Search parameters were set as follows: taxonomy: rat (UniProtKB/Swiss-Prot), enzyme: trypsin, missed cleavage sites allowed: 2, fixed modification: methylthio (C), iTRAQ8plex (K), iTRAQ8plex (N-term), variable modifications: methionine oxidation (M), iTRAQ8plex (Y); parent mass terror: –1.5 to +3.0 Da and peptide fragment mass tolerance: 0.7 Da. Quantitative information of peptides abundances (iTRAQ reorted ion intensities) were extracted from the HCD MS2 scans by Libra software (TPP). Peptide false discovery rate (FDR) was estimated by Mayu (Reiter et al. 2009) (TPP) and peptide identifications with FDR below 1% were considered as correct matches. DanteR software (Taverner et al. 2012) was used for statistical analysis of iTRAQ-labeled peptides. In brief, replicate peptides across iTRAQ channels were aggregated to unique peptides, while the corresponding reporter ion intensities were summed up. The dataset was normalized using linear regression and ANOVA was performed at the peptide level using a linear model with minimum 2 and maximum 100 peptides. Finally, the Benjamini and Hochberg FDR correction was used to adjust *p*-values. Protein fold change was reports as median value of corresponding unique peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaíno et al. 2014) *via* the PRIDE partner repository with the data set identifier PXD024178.

Results

In total, the identification of 1535 unique peptides enabled for the quantitation of 136 plasma proteins by iTRAQ-2D-LC-MS/MS analysis, while the plasma concentrations of 48 of these proteins were significantly different between lean and obese animals (Table 1). Altered proteins were engaged in several biological processes, including inflammation, metabolism, plasma lipid turnover, hemostasis and coagulation, as well as plasma protease activity regulation. Figure 1 presents volcano plot of obtained quantitative data. Normalized protein abundances for each animal included in the experiment for the selected subset of key study findings are collected on Figure 2.

Discussion

Accumulating evidence indicate association between obesity and increased cardiovascular risk. It may well be that either fat or other obesity-affected tissues could be a source of circulating proteins responsible for increased risk of cardiovascular events. In this study we analyzed plasma proteomic profiles of obese, prediabetic Zucker fatty rats and their lean littermates in order to identify proteins which may be linked to potential cardiovascular hazard. In our setting among

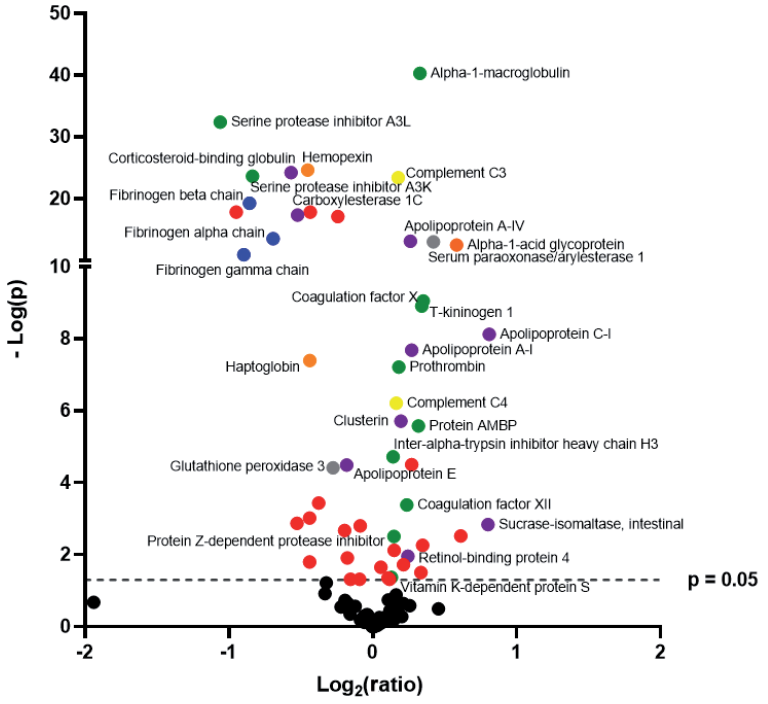

Figure 1. Differentially regulated plasma proteins in obese Zucker rats. The volcano plot combines the quantitative estimate of protein plasma concentration with the statistical confidence of its significant differential abundance between the experimental groups. The x-axis presents the ratio of measured protein MS signal intensities for obese and lean animals. Statistically significant regulated protein results were first highlighted in red, and then a subset of key results was colour-coded by protein function.

Table 1. Differentially regulated proteins in obese Zucker rat plasma

UniProt Accession	UniProt protein name	Total peptides	Unique peptides	Fold change	Adj. *p*-value
P19939	Apolipoprotein C-I	34	4	1.75	7,6E−09
P23739	Sucrase-isomaltase, intestinal	7	2	1.74	1,5E−03
Q99PS8	Histidine-rich glycoprotein	22	4	1.53	3,0E−03
P02764	Alpha-1-acid glycoprotein	1007	17	1.50	3,4E−13
P55159	Serum paraoxonase/arylesterase 1	483	18	1.34	1,0E−13
Q63207	Coagulation factor X	157	13	1.28	9,0E−10
P18424	Phosphatidylcholine-sterol acyltransferase	14	2	1.27	5,5E−03
P01048	T-kininogen 1	171	18	1.27	1,2E−09
Q63041	Alpha-1-macroglobulin	1637	100	1.25	5,2E−41
Q64240	Protein AMBP	302	14	1.25	2,7E−06
P04639	Apolipoprotein A-I	677	30	1.21	2,1E−08
P55314	Complement component C8 beta chain	82	14	1.21	3,1E−05
P02651	Apolipoprotein A-IV	408	31	1.20	7,9E−14
P04916	Retinol-binding protein 4	51	6	1.18	1,1E−02
D3ZTE0	Coagulation factor XII	46	9	1.18	4,1E−04
P19999	Mannose-binding protein A	32	5	1.16	1,9E−02
P05371	Clusterin	313	19	1.14	2,0E−06
P18292	Prothrombin	700	41	1.13	6,2E−08
P01026	Complement C3	2472	116	1.13	4,2E−24
P08649	Complement C4	399	47	1.12	6,2E−07
P04937	Fibronectin	72	19	1.11	7,6E−03
Q62975	Protein Z-dependent protease inhibitor	152	15	1.11	3,1E−03
Q63416	Inter-α-trypsin inhibitor heavy chain H3	895	39	1.10	1,9E−05
P53813	Vitamin K-dependent protein S	20	8	1.09	4,3E−02
P35859	IGFBP complex acid labile subunit	249	15	1.08	4,8E−02
P08934	Kininogen-1	344	19	1.08	4,4E−02
P12346	Serotransferrin	634	41	1.04	2,2E−02
P13635	Ceruloplasmin	816	53	−1.06	1,6E−03
Q03626	Murinoglobulin-1	460	24	−1.07	4,8E−02
P36953	Afamin	54	13	−1.11	4,8E−02
Q68FP1	Gelsolin	58	14	−1.13	1,2E−02
P02650	Apolipoprotein E	644	26	−1.13	3,2E−05
P24090	Fetuin A	177	7	−1.15	2,1E−03
P02770	Serum albumin	1580	66	−1.18	8,0E−18
P23764	Glutathione peroxidase 3	189	10	−1.21	3,9E−05
P26051	CD44 antigen	17	4	−1.30	3,7E−04
P17475	Alpha-1-antitrypsin	459	22	−1.35	1,6E−18
P06866	Haptoglobin	446	26	−1.35	4,1E−08
P51886	Lumican	30	5	−1.36	9,6E−04
P20059	Hemopexin	613	29	−1.37	2,5E−25
P10959	Carboxylesterase 1C	187	17	−1.44	4,8E−18
P20761	Ig gamma-2B chain C region	20	5	−1.44	1,3E−03
P31211	Corticosteroid-binding globulin	311	21	−1.48	6,2E−25
P06399	Fibrinogen alpha chain	174	24	−1.62	3,1E−14
P05545	Serine protease inhibitor A3K	424	15	−1.78	2,3E−24
P14480	Fibrinogen beta chain	172	23	−1.81	5,9E−20
P02680	Fibrinogen gamma chain	97	17	−1.86	1,3E−11
P05544	Serine protease inhibitor A3L	471	26	−2.08	4,1E−33

IGFBP, insulin-like growth factor-binding protein.

the most significantly up- or down-regulated proteins are elements of coagulation and complement cascades, acute phase proteins, lipoprotein metabolism-related proteins as well as proteins reflecting systemic low-grade inflammation and insulin resistance.

Strikingly, the obese Zucker rats were characterized by plasma protein changes collectively pointing to increased blood coagulability. We showed elevation in plasma levels of two coagulation factors: XII (fXII) and X (fX), an initiator of intrinsic coagulation pathway after contact activation (Cheng et al. 2010), and a convergence point for extrinsic and intrinsic pathways of coagulation cascade, respectively; the level of circulating prothrombin, was also elevated in obese animals. What is more, rat-specific T-kininogen, which takes part in enhancing prekallikrein activation by fXIIa and fibronectin, was also up-regulated in obese Zucker rats.

Interestingly, in plasma of obese animals we have also detected decreased levels of serine protease inhibitors (serpins A3K and A3L), and alpha-1-antitrypsin (serpine A1, AT) – proteins, which are known to inhibit of activated protein C (APC), which is able to inactivate factors Va and VIIIa. Given the fact, that in our setting some other anticoagulant proteins (APC co-factor protein Z-dependent protease inhibitor and vitamin K-dependent protein S) are also elevated, it is tempting to speculate, that these changes could represent a part of counter-regulatory mechanism, however this remains to be tested. On the other hand, it is known, that obesity and insulin resistance are linked to hypofibrynolytic state. Interestingly, the levels of two plasmin inhibitors: alpha-1-macroglobulin and alpha-1-microglobulin (AMBP) (Schaller and Gerber 2011) were significantly up-regulated in plasma of obese Zucker rats. Both the mechanistic explana-

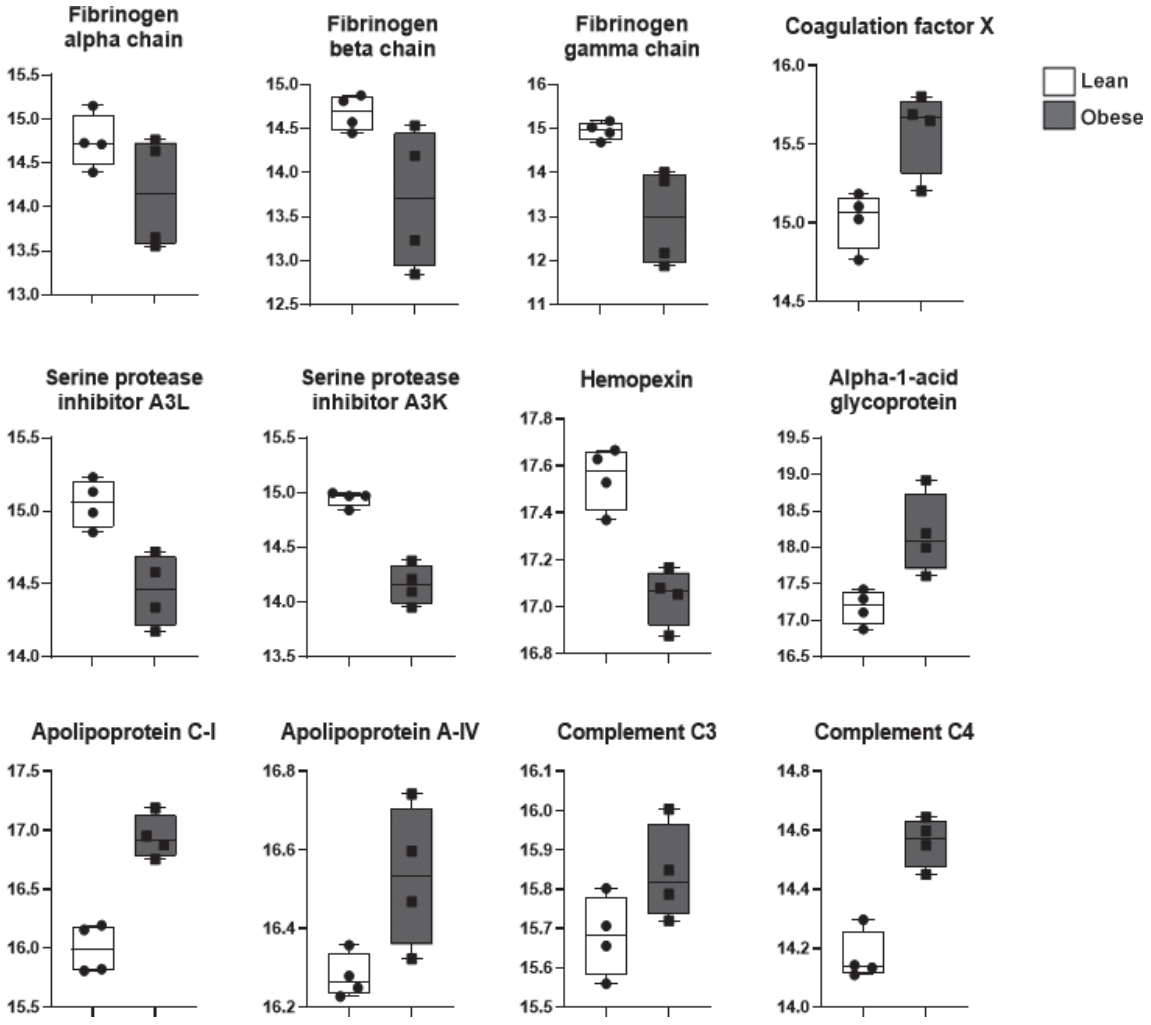

Figure 2. Mass spectrometry-based quantification of rat plasma proteins. The box-and-whisker plots present specific values for each animal in the experimental groups, as well as a median normalized protein abundance (y-axis) for selected subset of differentially regulated proteins.

tion and the relevance of this finding to human pathology require further investigation.

In the context of literature data showing positive correlation of plasma fibrinogen levels with body fat content (Stensel et al. 2001), the down-regulation of all three chains of fibrinogen in plasma of obese animals may seem surprising, however taking into account parallel decrease of serum albumin levels, it could be probably at least partially explained by altered hepatic protein synthesis due to hepatic steatosis in obese Zucker rats (Toblli et al. 2008). It has been shown that this condition could result in impaired protein synthesis manifested as hypofibrinogenemia (de Maat et al. 1995). Noteworthy, abundance of fibrinogen is not the necessary condition to develop thrombosis, as a proportion of patients with decreased plasma levels of fibrinogen suffer from thrombotic disease (Morris et al. 2009; Lisman and Ariëns 2016; Nagler et al. 2016). In keeping with this fact, it was shown that abundant thrombi formed in $Fg^{-/-}$ mice in ferric chloride-injured arterioles (Ni et al. 2000). Clearly, both the background and the biological meaning of changes in plasma fibrinogen in obese Zucker rats remain unclear and require further studies.

The alpha-1-acid glycoprotein (orosomucoid, ORM) was highly up-regulated in obese Zucker rats. ORM is acute phase protein (APP), released into blood from the liver under conditions such as tissue injury, infection, and inflammation (Lee et al. 2010). ORM levels were shown to be markedly elevated in obesity (Benedek et al. 1984; Rødgaard et al. 2013; Sun et al. 2016) and diabetes (Muhammad et al. 2016). Interestingly, ORM similar to leptin was proposed to be also a negative regulator of food intake (Sun et al. 2016). Whether ORM increase could represent regulatory mechanism trying to compensate defective leptin signaling in obese Zucker rats, such attractive hypothesis remains to be tested. Interestingly, the levels of two other APP in obese animals – haptoglobin and hemopexin – were decreased, what in turn could be linked to the increased risk of thrombotic events (Insenser et al. 2014).

A growing amount of evidence points to the interplay between coagulation cascade and complement system. It has been shown that the coagulation factors FXa, plasmin, thrombin, FIXa, FXIa may cleave C3 and generate active C3a (Amara et al. 2008). This communication is bidirectional as activated complement cascade may trigger inflammation (TNF-α, IL-6) what potentiates coagulation (Esmon 2004) but also directly influence hemostasis by altering the tissue factor expression and platelet activation (Markiewski et al. 2007). In our study, we have shown elevated levels of complement components C3 and C4 in plasma of obese Zucker rats. Our results are in keeping with data from literature showing that increased levels of C3 and C4, both in the liver and adipocytes, could be associated with obesity and insulin resistance up-regulation of some elements of complement system may predict future cardiovascular events (Gabrielsson et al. 2003; Hernández-Mijares et al. 2007).

In keeping with the majority of reports about obesity, and our previous reports showing dyslipidemia in obese Zucker rats (Krskova et al. 2020), we have found many, multifaceted alterations in plasma proteome of fatty rats regarding factors involved in lipids metabolism (Witztum and Schonfeld 1979). The higher levels of ApoC-I could be linked to increased total plasma cholesterol, very low density lipoprotein (VLDL) and hepatic triglyceride (TG) content (Muurling et al. 2004). In turn, increase in ApoA-IV levels could partially depend on the Zucker rats genotype, as ApoA-IV synthesis in small intestine has been shown to be inhibited by leptin signaling (Tso and Liu 2004). On the other hand, elevated apoA-IV level was also found in hypertriglyceridemic (Vergès et al. 1994) and obese patients (Vergès et al. 2001), and its decrease was reported in obese patients undergoing weight reduction (Lingenhel et al. 2004). Interestingly, the administration of exogenous apoA-IV has been shown to improve glucose tolerance and restore insulin secretion, so its elevation could be considered as compensatory in Zucker rat model of obesity and insulin resistance (Kohan et al. 2015).

Other changes in apoproteins – the slight down-regulation of plasma apoE and increase of clusterin (ApoJ), could contribute to the increased level of LDL cholesterol and decreased HDL in obese animals. Interestingly, in HDL-C particles, clusterin forms complexes with ApoA-I and paraoxonase 1, which both were increased in the present study.

The paraoxonase 1 (PON1) is a liver enzyme associated with high-density lipoprotein (HDL), having an important role in the protection of LDL, HDL and macrophages against oxidative stress and stimulation of HDL-mediated cholesterol efflux from the cells (Efrat and Aviram 2010). In our study plasma PON1 levels were increased in obese animals. Interestingly, plasma level of other antioxidant protein – glutathione peroxidase 3 (GPx3), which reduces systemic oxidative stress by scavenging H_2O_2 and peroxidized organic molecules in plasma, was markedly down-regulated in obese rats. Increase in PON1 seems to be contradictory to several reports, showing negative correlation between PON1 activity and BMI (Agirbasli et al. 2014). Yet, the matter of changes in plasma PON1 levels in obesity remains controversial. In obese adults PON1 levels was reported to remain unchanged (Tabur et al. 2010), whereas high PON1 values were detected in children with obesity and also in a group with metS (Eren et al. 2014). Clearly, the mechanism of increase in plasma PON1 levels in obese Zucker rats requires further investigation.

The changes in plasma levels of several proteins in obese Zucker rats seem to be in keeping with previous reports. The elevation of plasma intestinal sucrase-isomaltase (SI) was reported in diabetic state, and normalized by insulin action

(Takenoshita et al. 1998). Decreased levels of corticosteroid-binding globulin (CBG), resulting in elevated free cortisol, were proved to be associated with insulin resistance and low-grade inflammation (Fernandez-Real et al. 2002). Also, down-regulation of carboxylesterase 1 (CES1) has been associated with metabolic disturbances. The CES1-deficient mice developed insulin resistance, hyperinsulinemia, hyperlipidemia and presented decreased energy expenditure and obesity with hepatic steatosis as a consequence (Quiroga et al. 2012a). It was also shown that overexpression of CES1 lowered hepatic TG and plasma glucose levels in both wild-type and diabetic mice, in contrast to CES1 knockdown. Moreover pharmacological activation of farnesoid X receptors, known therapeutic target for treatment of nonalcoholic fatty liver disease (NAFLD), induced expression of CES1 mRNA (Xu et al. 2014a). Interestingly, CES1 was proved to regulate hepatic chylomicrons assembly and secretion (Quiroga et al. 2012b) and postprandial glucose metabolism, which dysregulation is significant manifestation of diabetes mellitus type 2 (Xu et al. 2014b). On the other hand, the level of retinol-binding protein 4 (RBP4) in plasma of obese rats was significantly elevated. The expression of RBP4 was reported to be increased in adipose tissue of mice and humans with type 2 diabetes and obesity and normalized in by insulin sensitizing drug – rosiglitazone. Moreover, overexpression of RBP4 in mice resulted in augmentation of insulin release. Since circulating RBP4 induces expression of gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) which regulates hepatic glucose production and impairs insulin sensitivity in muscle (Graham et al. 2006) our observations raise the possibility that elevated serum RBP4 level might contribute to altered insulin signaling in obese Zucker rats. This however, as well as confirmation of CES1 involvement in disturbances in lipid and glucose metabolism requires further investigation.

Of note, the limitation of our study is that it was performed on male animals only, which should be taken into account when interpreting the data, as sex-specific differences in circulating proteome between healthy males and females are known (Silliman et al. 2012), as well as diverse secretory response to obesity-induced inflammation (Ter Horst et al. 2020).

In conclusion, the results of this study suggest that plasma proteome alterations in obese Zucker rats are reflecting altered hemostasis, disturbed metabolic processes involving insulin resistance and lipid metabolism, as well as ongoing low-grade inflammation. Both, the mechanisms responsible for these changes, and whether they may play a role in increased susceptibility to thrombosis in obese subjects, or implicate other functional consequences require further studies.

Conflict of interest. The authors declare no conflict of interest.

Author contributions. JTZ – analysis and interpretation of data, manuscript writing. MS – analysis and interpretation of data, manuscript revision. BBG, MS – proteomic analyses. LB, KK – experiments on animals, acquisition of data. SZ, RO – study design, acquisition of funding conceptualization, manuscript revision.

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Availability of data and material. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaíno et al. 2014) *via* the PRIDE partner repository with the data set identifier PXD024178.

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