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ALTERATIONS IN THE LIVER OF INTRAUTERINE GROWTH RETARDED PIGLETS MAY PREDISPOSE TO DEVELOPMENT OF INSULIN RESISTANCE AND OBESITY IN LATER LIFE

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Intrauterine growth retardation (IUGR) leads to increased predisposition to metabolic syndrome in adult life but the mechanisms remain obscure. Considering a significant number of functional similarities, IUGR piglets appear to be a good model to study the development of this syndrome in humans. The aim of the present study was to investigate the ultrastructure and proteomic profile of the liver in IUGR pig neonates to discover early markers of predisposition to obesity and insulin resistance. In our study intestine and liver tissue samples were investigated in 7 day old IUGR and normal body weight (NBW) littermate piglets using histometry, mass spectrometry, in-tissue cytometry analysis and confocal microscopy. Compared to NBW, the liver in IUGR neonates was characterized by a significantly enhanced ratio of Kupffer cells to hepatocytes and insulin receptor abundance as well as higher percentages of cells expressing receptors for adipokines (resistin and adiponectin), increased expression of TNF-α (as marker of inflammation), and increased expression of insulin receptor and uncoupling protein 3 (UCP3). Moreover, NBW and IUGR differed in proteomic profile, including protein metabolism (proteasomes, cathepsin D, phermitin, phosphoglucomutase), carbohydrate metabolism (hexokinase 1, phosphoglucokinase, galactokinase, aldolase B, glucose-6-phosphate isomerase), oxidative stress and chromatin organization and DNA uptake (histones, lamin a/c). Reduction of hepatocyte numbers concomitant with significant modifications of expression of key hormones and enzymes for protein and carbohydrate metabolism in IUGR neonates may predispose to insulin resistance and obesity in adult life.

Key words: *inflammation, intrauterine growth retardation, liver receptors, adipokines, Kupffer cells, insulin resistance, obesity*

INTRODUCTION

Intrauterine growth retarded (IUGR) individuals show a high prevalence for developing obesity and type 2 diabetes in adult age (1, 2). The precise mechanisms underlying this relationship remain obscure, however, studies suggest that the major reasons are associated with the 'thrifty phenotype' metabolism enabling foetuses to survive despite intrauterine nutritional restrictions and epigenetic changes in the phenotype enabling adaptation to the extrauterine environment (3, 4). These life-saving modifications taking place in the second half of the foetal period and soon after birth cause problems resulting from aberrant digestive function (5, 6) and modifications of energy metabolism. For instance, in pig neonates the supply of energy *via* colostrum and glycogen mobilization in the liver are of major importance until about 33 hours after the onset of parturition. At least 200 g of colostrum per piglet is required to maintain life during the neonatal phase (7-9). However, Amdi and co-workers (10) demonstrated that only normal, but not IUGR piglets, ingested the correct amounts. In IUGR neonates, the deficit in colostrum intake resulted in decreased plasma glucose levels and lower residual glycogen depots in the liver (10). Vuguin *et al*. showed that basal hepatic glucose production in IUGR neonates rats was increased, and insulin suppression of hepatic glucose production as well as insulin receptor substrate 2 and Akt-2 phosphorylation were reduced compared to non-IUGR rats (11). In IUGR rats, they also observed increased levels of PEPCK, a key enzyme of gluconeogenesis. Earlier studies showed that in IUGR rat neonates, despite low insulin concentrations, glucose uptake and hepatic GLUT-1 protein and mRNA levels were high compared to non-IUGR neonates, suggesting abnormal glucose homeostasis (12-14). These studies suggest that a primary defect in gene expression and aberrant hepatic metabolism in IUGRs may predispose to development of metabolic diseases in later life.

Bouchard (15) reviewed a version of the human obesity gene map and demonstrated that there were 127 genes with at least one positive association with phenotypes relevant to obesity. Among them, 22 are supported by at least five positive studies. The latter genes are *ACE*, *ADIPOQ* (adiponectin), *ADRB2*, *ADRB3*, *DRD2*, *GNB3*, *HTR2C*, *IL6* (interleukin 6), *INS* (insulin receptor), *LDLR*, *LEP* (leptin), *LEPR* (leptin receptor), *LIPE*, *MC4R*, *NR3C1*,

PPARG, *RETN* (resistin), *TNF-α* (tumor necrosis factor alpha), *UCP1*, *UCP2*, *UCP3* and *VDR* (15).

The aim of the present study was to investigate microscopic structure as well as protein profiles of the liver as a crucial organ for carbohydrate and lipid metabolism. Using the pig as a model both for itself and for humans (5, 16, 17), liver samples from 7 day old IUGR piglets and normal body weight littermates were analysed. We wanted to ascertain which markers are changed on the transcript level in order to verify which type of obesity genotype or epigenetic changes may predominantly influence the predisposition to obesity and diabetes type 2 development in IUGR syndrome. Recently, we have found several modifications in the expression of enterocyte proteins responsible for nutrients, especially sugars, digestion and absorption in IUGR neonatal pigs (6).

MATERIALS AND METHODS

Animals, tissue collection, histologic analyses

The study was conducted in compliance with the European Union regulations concerning the protection of experimental animals. The study protocol was approved by the Local Ethical Committee, Warsaw University of Life Sciences, Warsaw, Poland. Briefly, 8 pairs (4 male and 4 female pairs) of neonatal piglets (*Sus scrofa domesticus*, Landrace × Pietrain) of both sexes, each pair selected from a different litter, were used in the study. The selection of the pairs was as follows: one piglet was of normal birth body weight (NBW), i.e., representing the average weight of all littermates (range between 1.3 and 1.6 kg), and the other one was of low birth weight, recognized as asymmetric IUGR with spontaneous background, and with birth body weight between 0.6 and 0.9 kg.

Sows were kept on a standard diet during pregnancy (dry matter (DM) 87.6%, metabolizable energy (ME) 11.35 MJ/kg, crude protein (CP) 13.1%), and lactation (DM 87.3%, ME 12.93 MJ/kg, CP 15.4%). Fresh diet and water were provided daily *ad libitum*. Piglets were delivered at term and were clinically healthy. On the postnatal day 3, all piglets were injected intramuscularly with 100 mg iron dextran (FeDex, Ferran100, 10% solution, Vet-Agro, Lublin, Poland). NBW and IUGR piglets were kept together with their litters and fed by the sow until postnatal day 7. On that day, the NBW and IUGR piglets were killed by barbiturate overdose, exsanguinated, the livers were gently removed and measured for weight and size.

In each piglet, a 1 cm^3 block from the right hepatic lobe was isolated, snap frozen and stored at –80°C. Frozen tissues were subsequently ground and aliquoted, snap frozen and stored at –80°C until further analysis. Another block of tissue was fixed in 4% buffered formaldehyde and stored in ethanol; subsequently, these liver samples were embedded in paraffin. Samples were sliced into 5 µm sections and mounted on microscopy glass. Deparaffinisation of slides included 2 washes in xylene for 15 min and rehydratation in decreasing concentrations of ethanol (from 100% to 70%). Serial histological 5 µm sections were stained with hematoxylin and eosin for morphometric analysis under a light microscope. Five slides from each tissue sample were prepared and 30 measurements were performed using an optical binocular microscope (Olympus BX60; Olympus, Warszawa, Poland) coupled *via* a digital camera to a personal computer equipped with cell^P (Olympus) software.

Analysis of proteomic profiling

Frozen samples of liver tissues were homogenized and pooled into one IUGR and one NBW sample, and the homogenates were incubated with trypsin after reduction and alkylation of protein disulfide bonds. Separation of peptide mixtures was performed by liquid chromatography (LC) and molecular mass was measured using the mass spectrometry assay (LC-MS-MS/MS) - Orbitrap spectrometer (ThermoScientific). Searching of acquired spectra, protein identification and formatting were done using NCBI, UniProt database and the Mascot program (matrixscience.com). The fold-change was calculated using score data. Mass spectrometry studies were carried out at the Laboratory of Mass Spectrometry (Institute of Biochemistry and Biophysics Polish Academy of Sciences).

Confocal microscopy/tissue cytometry

Tissues from 7 day old IUGR piglets were fixed in 4% formaldehyde solution for 48 hours and then stored in ethanol. Subsequently, samples were embedded in paraffin in a tissue processor (STP 120-2, Microm GmbH) for 26 hours. Samples were sliced into 5 µm sections. Deparaffinisation of samples included 2 washes in xylene for 15 min and rehydration in decreasing concentrations of alcohol (from 100% to 70%). Antigen retrievals were performed by boiling in citrate buffer (pH = 6.5) twice for 3 min. Non-specific binding was blocked with 1% BSA (Sigma) in PBS at room temperature for 1 hours. Samples were labeled with a specific set of antibodies (insulin receptor sc-559; Cap receptor- sc-134637, adiponectin receptor 2- sc-99184, ucp2-sc6526, ucp3-sc31387, leptin receptor-sc-1832, GLUT2-sc-7580, TNF-α-sc-1348, Il-6-sc 28343, IL1-sc-12742; Santa Cruz Biotechnology). Cell nuclei were stained with Hoechst 33342 (Sigma-Aldrich, 14533) at a concentration of 15 µg/ml for 10 s at room temperature. For primary visualization of secondary antibodies conjugated with Alexa Fluor 568 (Life Technology) were used. Lack of tissue auto-fluorescence and nonspecific secondary antibody fluorescence were checked. After each incubation, slides were washed 3 times each for 5 min in PBS buffer. Slides were mounted in Fluoromount Aqueous Mounting Medium (Sigma). Slides for comparative analysis between groups were prepared during one series of staining. Until analysis, slides were stored at +4°C in the dark. For tissue cytometry, whole cross-sections were scanned and analysed with the SCAN^R scanning cytometry system (Olympus Polska) with the cell nucleus as a reference point (18, 19). Each liver sample from one pig had from 30 to 45 thousand cells for measurements. Two main parameters were examined: mean intensity of fluorescence per cell (AB) and percentage of cells with high expression of markers (%). Visualization of receptors was performed using a confocal microscope (Olympus FV500) with Fluoroview v5 software.

Statistical analysis

The results obtained were subjected to a two-stage statistical analysis. In the first step, data were checked for uniformity of the SD and the Kolmogorov-Smirnov-A test of normal distribution. Depending on the outcome, the data were then analyzed with an unpaired t-test or a Welch (normal distribution) or Mann-Whitney test-A (in its absence). All statistical analyses were done using Graph-Pad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA); $P < 0.05$ was considered as significant.

RESULTS

The liver weights of IUGR 7 day old piglets (38.0 ± 11.2 g) were significantly smaller compared to their NBW littermates (85.6 ± 14.7 g) ($P < 0.002$), while the histological structure remained intact. Noteworthy, the liver weight in relation to body weight in IUGR and NBW was 4.5% and 5.1% ($P > 0.05$),

respectively, whereas in relation to brain weight (known for major life-saving effect in IUGRs, (3-6)) it was 140% and 284% ($P > 0.001$), respectively. Low liver weight in IUGR (and thereby the reduction in total number of hepatocytes, *Fig. 1*) compared to NBW piglets was associated with a 50% increase in the number of Kupffer cells per field of vision (0.098 ± 0.009 in NBW versus 0.148 ± 0.0068 in IUGR; $P < 0.05$).

The expression of major cytokines produced by Kupffer cells was significantly higher in liver from IUGR compared to NBW piglets (*Table 1*). In-tissue cytometry analysis showed abundant expression of leptin receptor (Ob-R) as well as GLUT-2 and UCP2 in both the NBW and IUGR hepatic samples. However, expression of the insulin receptor (IR) per cell in the IUGR liver was significantly greater compared to that in NBW. Also, the percentage of cells expressing resistin receptor (CAP) and adipokine receptor (AdipoR2) was increased. The most dramatic difference was a more than 5-fold increase in the number of cells expressing UCP3, and more than a 2-fold increase in mean fluorescence intensity per cell (*Table 2*). In addition, the increased number of cells expressing IR, AdipoR2 and UCP3 was associated with an uniform increase in the intensity of expression in the cells (*Fig. 2*) but abundance of the resistin receptor was strictly limited to Kupffer cells (*Fig. 3*). Proteomic profiles showed differences in liver homogenates between IUGR neonates and their NBW littermates in expression of several proteins involved in oxidative stress, carbohydrate metabolism and protein metabolism, as well as gene uptake and chromatin organization (*Table 3*).

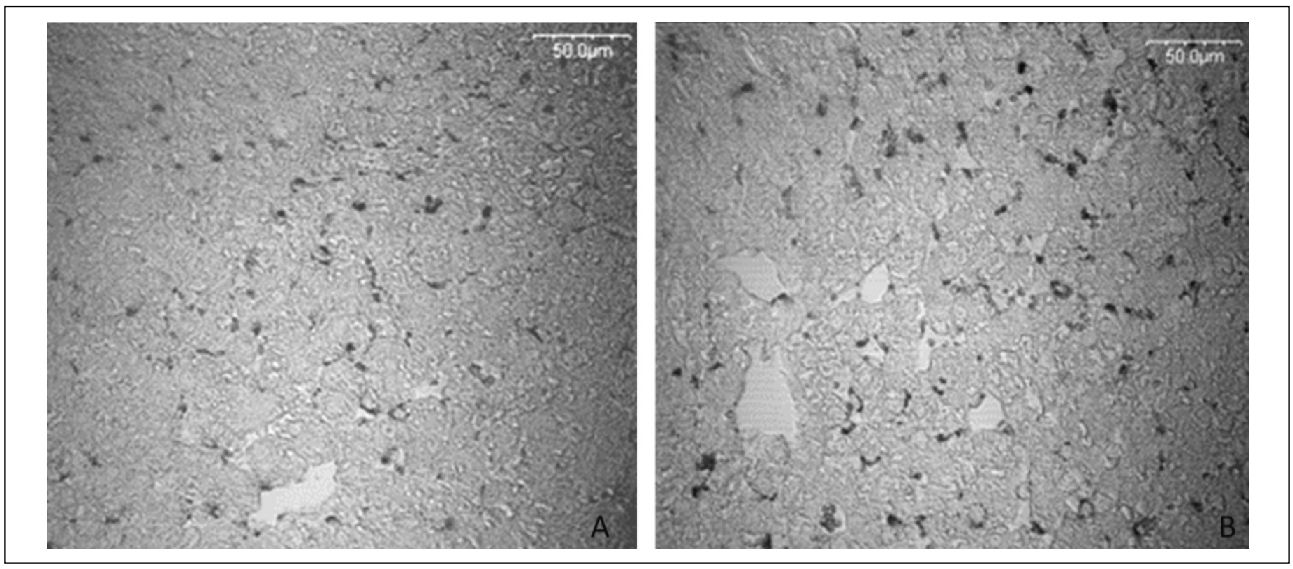

Fig. 1. Abundance of Kupffer cells in the liver of 7 day old normal body weight (NBW - left) and intrauterine growth retarded (IUGR - right) piglets. The Kupffer cells stain darker than hepatocytes because of a higher density of cytoplasm. Magnification × 20.

Table 1. Expression of cytokines by Kupffer cells in 7 day old intrauterine growth retarded (IUGR) piglets compared to their normal body weight (NBW) littermates. Mean fluorescence intensity per cell is given ± SD; arbitrary units.

	NBW	**IUGR**	**P**
TNF-α	45.7 ± 5.7	71.4 ± 9.6	< 0.001
IL-1	52.8 ± 6.5	61.5 ± 13.5	NS
IL-2	56.0 ± 17.3	69.8 ± 10.8	NS

Table 2. Changes in the expression of liver receptors in 7 day old normal body weight (NBW) and intrauterine growth retarded (IUGR) piglets analyzed by in-tissue cytometry. Left column (%) shows percentage of cells expressing markers, and right column (AB) depicts mean intensity of fluorescence per cell (mean ± SD, arbitrary units, unpaired Student's t test).

Receptor	**NBW**		**IURG**		**P for %**	**P for AB**
	%	AB	%	AB		
IR	65.8 ± 16.3	41.3 ± 7.6	72 ± 17.4	60.8 ± 8.6	NS	< 0.05
GLUT-2	72.3 ± 16.6	43.5 ± 16	83.2 ± 10.8	68 ± 27.9	NS	NS
Cap	16.6 ± 3.9	23.9 ± 2.2	32.3 ± 5.3	25.8 ± 3.8	< 0.05	NS
AdipoR2	41.1 ± 3.8	34.3 ± 3.7	70.9 ± 2.3	37.5 ± 5.0	< 0.0001	NS
Ob-R	74.3 ± 17.6	26.9 ± 6.2	84.3 ± 6.0	29.4 ± 4.8	NS	NS
UPC2	59.2 ± 13.9	81.4 ± 27.4	67.3 ± 12.1	100 ± 36.8	NS	NS
UPC3	3.7 ± 3.4	22.5 ± 5.8	25 ± 5.0	48.3 ± 5.7	< 0.0001	< 0.0002

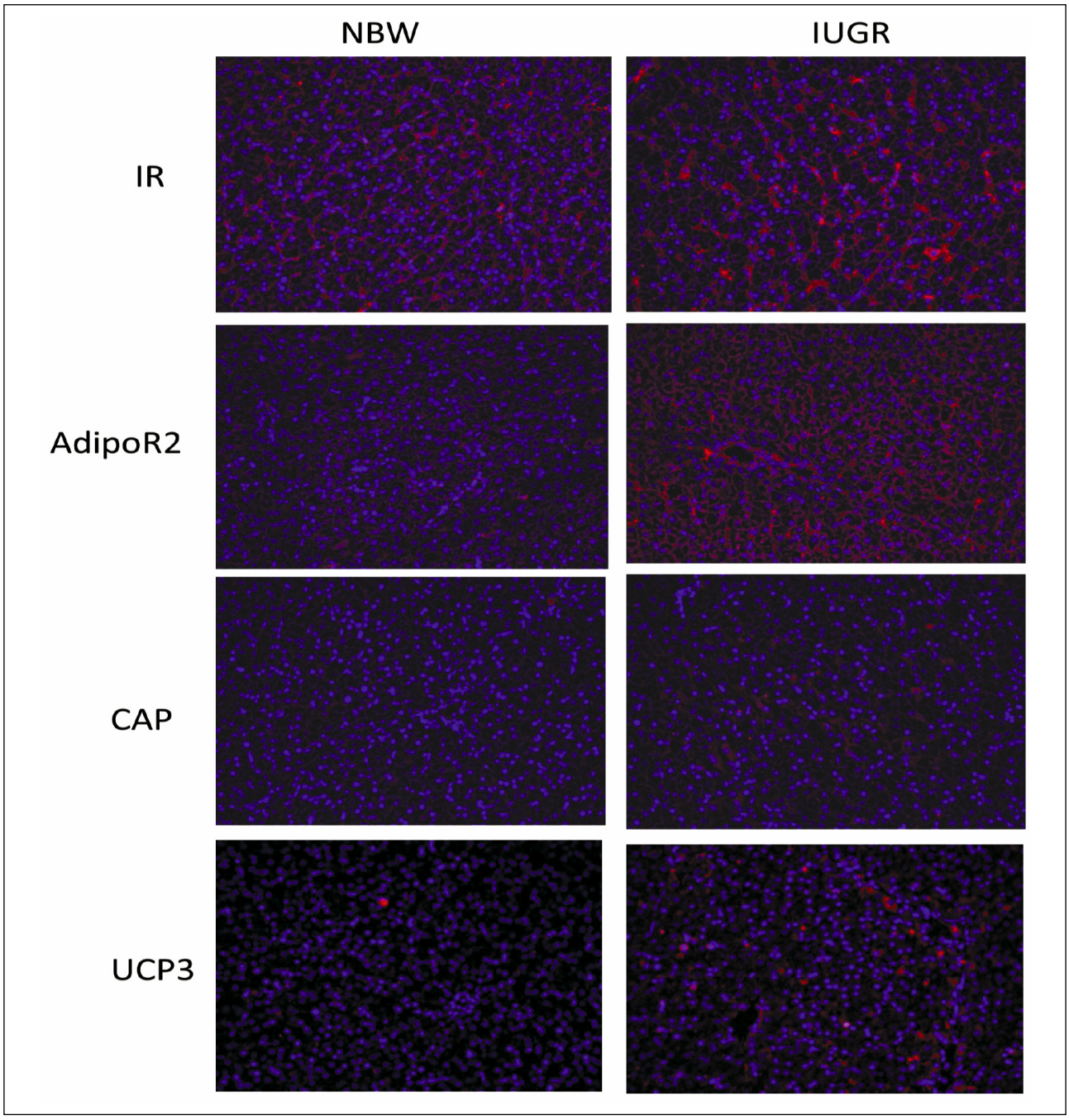

Fig. 2. Distribution of insulin receptor (IR), adiponectin receptor 2 (AdipoR2), resistin receptor (CAP) and uncoupling protein 3 (UCP3) expression in the liver in normal body weight (NBW, left panel) and intrauterine growth retarded (IUGR, right panel) 7 day old piglets. Compared to NBW, in IUGR piglets the IR expression per cell is increased, AdipoR2 and CAP receptors are expressed in higher numbers of cells, and UCP3 expression is more intense as well as the percentage of cells with marker being increased. Red fluorescence (Alexa Fluor 568) - receptors, blue fluorescence (Hoechst 33342) - cell nuclei. Magnification × 20.

DISCUSSION

In our study we searched for early markers of obesity and diabetes type 2 development in the liver - a crucial organ for carbohydrate and lipid metabolism. Our data demonstrated a number of changes in the liver of IUGR neonates, both at the morphological and molecular levels, that may directly influence metabolism. These changes might be a result of genetic predisposition (10) and/or restriction of nutrients during pregnancy leading to creation of the 'thrifty phenotype' (3, 4). Creation of this phenotype may have epigenetic background and could be a consequence of biological plasticity to environmental adaptation (20). Independent of the background, the observed changes may influence predisposition to obesity and diabetes type 2 development and may have long term consequences. Alternatively, we should consider the poor nutritional status of IUGR piglets due to their reduced access to colostrum and low milk intake and utilization (15).

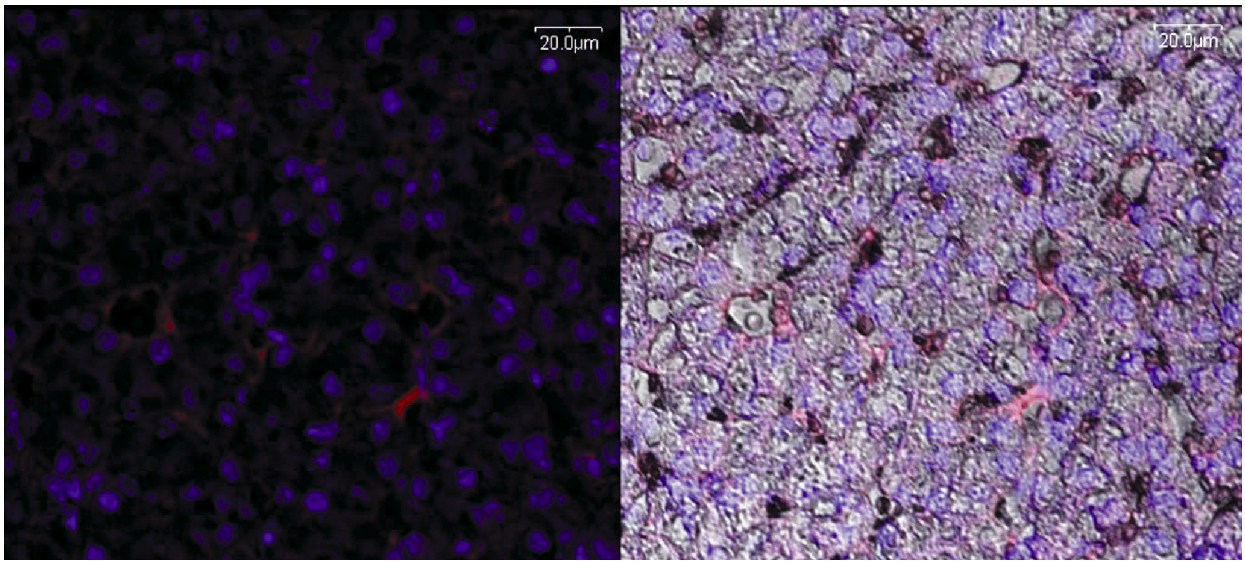

Fig. 3. Abundance of Kupffer cells in the liver of 7 day old intrauterine growth retarded (IUGR) piglets. Right panel - red fluorescence (Alexa Fluor 568) resistin receptor expression, blue fluorescence (Hoechst 33342) - cell nuclei. Left panel - additional channel with transparent unstained view of liver tissue. Expression of resistin receptor is mainly localized on Kupffer cells. The Kupffer cells are stained darker than hepatocytes because of a higher density of cytoplasm. Magnification × 40.

Table 3. Changes in proteomic profile in 7 day old intrauterine growth restricted (IUGR) piglets; fold change shown as plus or in minus compared to normal body weight littermates.

Role in	Full name of protein	Short name	Fold change
Oxidative stress	Secretory carrier-associated membrane protein 1	Scamp1	–1.99
	Glutaredoxin-1	Glrx	+1.85
Protein metabolism	Vesicle-fusing ATPase	Nsf	–1.54
	Proteasome subunit β type-6	Psmb6	–5.81
	Protein phosphatase 1 regulatory subunit 1B	Poo1r1b	–2.41
	Cathepsin D	Ctsd	–1.94
	Fermitin family homolog 2	Fermt2	+2.09
Carbohydrate metabolism	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	Dlat	–1.67*
	Hexokinase-1	Hk1	–1.42*
	cAMP-dependent protein kinase type II-α regulatory subunit	Prkar2a	+2.08
	Galactokinase	Galk1	+1.81*
	Glucose-6-phosphate isomerase	Gpi	+1.46*
	Fructose-bisphosphate aldolase B	Aldob	+1.41*
DNA uptake	Histone H2B	Hist1h2bh	–4.02
	Histone H4	Hist2h4	–2.89
	Histone H2A	hist 1h2af	–2.64
Chromatin organization	Prelamin-A/C	Lmna	–2.2

*Involvement in the glycolysis pathway.

Kupffer cells and cytokines

Our novel and most striking finding was an increased ratio of Kupffer cells to hepatocytes. We believe that this might be the consequence of decreased number of hepatocytes (21, 22) and/or increased number of monocytes infiltrating into the liver of IUGR neonates. Following Boutsikou *et al*. lower birth weight, reflecting reduced fat mass in the former might indicate more intense inflammation in IUGR neonates in the other organs as well (23). Accordingly, we demonstrated significantly enhanced

expression of TNF-α, one of the inflammatory cytokines produced by the Kupffer cells (24, 25). Our findings support the hypothesis that chronic local low-grade inflammation in the IUGR liver stimulates the development of insulin resistance (26, 27). Previous studies on IUGR liver also showed increased levels of mRNA and proteins involved in inflammation such as heat shock proteins, IL-6 and TNF-α (28-30).

Liver receptors

In obesity, levels of adipokines and their receptors changed significantly (31). Accordingly, levels of insulin and adipokines, such as leptin, adiponectin and resistin in the blood of IUGR neonates changed too (13, 14, 30, 32, 33), but the expression of their receptors on the tissue level was not described until now. Using in-tissue cytometry, two main parameters can be investigated, namely, changes in percentages of cells expressing receptors, as well as average expression per cell. These key data cannot be accessed with commonly used methods, such as Western blot.

Increased insulin receptor expression per cell in the liver suggests that in IUGR neonates there may already exist an early response to decreased levels of insulin in the circulating blood by increased insulin release (13, 14). On the other hand, as Thorn *et al*. suggested, it may result from blocking of proximal insulin signaling and upregulation of the insulin receptor as a compensatory mechanism (35).

In contrast to the insulin receptor, the percentage of cells expressing adiponectin and resistin receptors, but not average expression per cell, in IUGR neonates was higher than in normal body weight piglets. This makes more IUGR hepatic cells sensitive to adipokine stimulation. Therefore, the increased adiponectin receptor expression shown in the present study may serve to compensate for low levels of adiponectin (32, 36), and may lead to greater insulin sensitization through adiponectin signaling mechanisms. Another reason may result from increased levels of TNF-α which modulates expression of adiponectin receptor level (37). Increased numbers of cells expressing the resistin receptor CAP1 in IUGR liver may support the development of insulin-resistance by a resistin-mediated proinflammatory activity of monocytes (38). This hypothesis is further supported by increased expression of the resistin receptor, mainly in the Kupffer cells, in our IUGR neonates.

Increased expression of UCP3 in the liver of IUGR neonates may be explained by lower energy intake compared to NBW piglets due to a metabolic adaptation to fasting (10, 39). Also, it has been shown that overexpression of UCP3 can protect against fat-induced defects in insulin signaling and its action in the liver and in skeletal muscles (40). Another idea is that the up-regulation of UCP3 is a result of increased levels of fatty acid delivery to the mitochondria which exceeds their oxidative capacity (41).

Protein profile

Previous results, in fetal IUGR piglets at late gestation (110 – 114 days of pregnancy), showed important proteomic profile changes in the liver (42, 43), but our results are not in agreement with those reports. The reason for this discrepancy may be difference in age (fetuses versus 7 day old neonates) and a possible influence of environmental factors during the early postnatal period, such as colostrum and milk feeding. Differences in proteome analysis could also contribute. In an IUGR rat model, similar changes in gene expression for some proteins were found by Cianfarani and co-workers representing changes in levels of hexokinase 2, glucokinase, fructose-1,6-biphosphatase 1 and GLUT1 (44). However, the data concerning gene expression levels are opposite to our proteomic results. This divergence may be the result of the fact that the level of mRNA does not necessarily correspond to the level of protein. This phenomenon comes from cellular processes such as mRNA level regulation, posttranslational protein modifications or its degradation (45). On the other hand, dynamics of changes linked with glucose transport proteins in the neonatal period may be flexible (46). On the other hand, we have previously shown several changes in IUGR intestinal mucosa, which are also present in the liver, such as decreased levels of lamin-A/C, histones and hexokinase 1 (6).

Nonetheless, our protein results fit within the whole context of possible changes in IUGR syndrome. In IUGR neonates we found a decreased level of lamin A/C which is involved in chromatin organization during mitosis. This result may also explain the decreased number of hepatocytes in IUGR liver and their reduced capacity for regeneration which predisposes IUGR neonates to non-alcoholic fatty liver disease (NAFLD) in later life (47).

Also, decreased level of histones may negatively affect the organization and packing of chromatin and lead to altered gene expression. Significantly decreased level of histones in the protein profile may also be the result of a lack of matches during mass spectrometry analysis due to hypomethylation and hyperacetylation of these proteins as was shown in the IUGR rat model. In addition, it was demonstrated that both of these types of changes are linked to development of diabetes in later life (42, 48).

Our results indicate that the main changes in the protein profile in IUGR liver occur in carbohydrate metabolism, especially in the glycolysis pathway. Our data are supported by another studies in a gestational undernourishment rat model in which gene expression was also altered for both glycolysis and the gluconeogenesis pathway. The authors postulated that IUGR individuals may shift to a preferential use of fatty acids as an energy source rather than carbohydrates (44, 49). In other studies using IUGR rat and pig models, increased levels of PEPCK were also observed. PEPCK is a key enzyme in the regulation of gluconeogenesis (10, 11). Up to now, dis-regulation of the glycolysis pathway in proteomic studies in IUGRs was described only in small intestine (6, 28) but not in the liver (42, 43).

According to our results in IUGR neonates, changes in protein profiles are primarily linked to a decreased ability to degrade protein as decreased levels of cathepsin D may influence metabolic degradation of intracellular proteins, ability to degrade polypeptide hormones and growth factors, activation of enzymatic precursors, and processing of enzyme activators and inhibitors (50-53). In addition to decreased levels of proteasomes, which play a key role in the maintenance of protein homeostasis by utilizing misfolded, damaged or unnecessary proteins (54, 55), these changes may decrease the pool of free amino acids, which serve as building blocks for proteins in neonatal IUGRs.

Conclusions

In summary, we have shown for the first time morphological and proteomic changes in IUGR liver during the neonatal period which may directly predispose to obesity and insulin resistance in later life. There is evidence that in IUGRs perturbed chromatin organization due to a decreased number of histones may lead to changes in gene expression. Hepatic mitosis may be decreased due to altered levels of prelamin a/c. In IUGR piglets we also observed low-grade inflammation, which may directly lead to insulin resistance, caused by an increased ratio of Kupffer

cells to hepatocytes, increased levels of cytokine-TNF-α and increased expression of the resistin receptor on macrophages. Changes in genetic markers of obesity on the protein level seen as increased expression of the insulin receptor, increased numbers of cells expressing resistin and adiponectin, increased UCP3 expression and number of cells expressing UCP3 are direct evidence of early predisposition to obesity in IUGR neonates. Changes in the proteome involved in carbohydrate metabolism, especially in the glycolysis pathway, may be evidence of a shift in IUGR individuals to a preferential utilization of fatty acids as an energy source, and also may be a crucial factor in development of diabetes type 2 in later life.

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Conflict of interests: None declared.

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