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*Article*

## Liver proteome profile of growth restricted and appropriately grown newborn Wistar rats associated with maternal undernutrition

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**Abstract:** (1) Background: Fetal Growth Restriction (FGR) has been associated with

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adverse perinatal outcomes and epigenetic modifications that impact gene expression leading to permanent changes of fetal metabolic pathways and thereby influence development of disease in childhood and adult life. Both clinical and experimental studies showed that maternal nutrition during pregnancy is critical since malnutrition adversely affects fetal growth and physiology. In this study, we investigated the result of maternal food restriction on liver protein expression in Wistar male newborn pups. (2) Materials & methods: Pups born to food restricted mothers were subdivided to FGR and non-FGR groups. Livers of control, FGR and non-FGR groups were analyzed using quantitative proteomics. (3) Results: In total 6665 proteins were profiled. Of these, 451 and 751 were differentially expressed in FGR and non-FGR vs. control respectively, whereas 229 were common between the two groups. Bioinformatics analysis of the differentially expressed proteins (DEPs) in FGR vs. control revealed: induction of the super-pathway of cholesterol biosynthesis and inhibition of thyroid hormone metabolism, fatty acid beta oxidation and apelin liver signaling pathway. In the DEPs of non-FGR vs. control groups there was inhibition of thyroid hormone metabolism, fatty acid beta oxidation and apelin liver signaling pathway as well. (4) Conclusion: This study demonstrates the impact of prenatal food restriction on the proteomic liver profile of FGR and non-FGR offspring underlying the importance of both prenatal adversities and birth weight on liver dependent postnatal disease.

**Keywords:** FGR; fetal programming; food restriction; metabolic disorders; liver proteomics

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## 1. Introduction

Fetal Growth Restriction (FGR) refers to a fetus that has failed to achieve its biological growth potential due to pathological conditions such as maternal / fetal disease and placental dysfunction. Fetal growth impairment is associated with perinatal morbidity and mortality, a 5 to 10-fold risk of *in utero* demise [14] and adverse neonatal outcomes [28]. Furthermore, according to Barker's hypothesis, an unfavorable intrauterine environment may have negative long-term effects in adult life [1]. According to the thrifty phenotype hypothesis [2], FGR impairs the growth of organs such as the liver in order to maintain homeostasis of other crucial for survival organs and systems. These metabolic adaptations, enable fetuses to survive in a malnourished intrauterine environment. However, the cost of these adaptations is permanent physiological and epigenetic phenotypical alterations that are responsible for development of disease later in life such as obesity, diabetes and cardiovascular disease.

Nutrition is one of the environmental variables with the widest range of effects on both physical growth and metabolism [3,33]. An expanding body of epidemiological evidence suggests that the nutritional environment experienced in fetal life increases the risk of chronic non-communicable diseases associated with human ageing. Maternal undernutrition constitutes a serious public health problem exhibiting large regional and within-country variations across the globe. Proper nutrition from preconception to delivery is critical for avoiding poor pregnancy and long-term outcomes for both the mother and child [51]. To date, many experimental approaches have been designed to study the impact of FGR intervening either in maternal nutrition, placental blood flow or fetal wellness. Restricting maternal food intake is advantageous since it leads to an altered intrauterine nutritional milieu and growth impairment avoiding surgical intervention. Moreover, this type of animal model is closer to pregnancy malnutrition effects observed in humans [35,45]. Although a large number of animal models of FGR have investigated the impact of intrauterine environment on fetal epigenetic programming, there is little knowledge about the effects of

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maternal undernutrition on liver growth and physiology of appropriately grown (non-FGR) offspring of undernourished pregnancies.

Liver plays a major role in nutrients' absorption and metabolism. During pregnancy, fetal growth restriction not only affects adversely liver's growth but also its physiological function [43]. Metabolic disorders namely, reduced oxidative phosphorylation, impaired mitochondrial function, antioxidant capacity and altered nutrient metabolism are commonly found in FGR livers [42,34,22]. It has been demonstrated that liver of FGR offspring seems to have an abnormally increased rate of glycogenesis contributing to insulin resistance and hyperglycemia [24,39]. Nevertheless, the exact mechanisms which are responsible for alterations in development, growth and liver function leading to hepatic diseases are not adequately described.

Our aim was to investigate the impact of maternal food deprivation on liver proteomic profile in three groups of newborn male Wistar rats: a) offspring of mothers that received standard laboratory diet (control group) , b) offspring of food restricted mothers with low birth weight (FGR group) and c) appropriately grown offspring of food restricted mothers (non – FGR group).

Furthermore, the aim of this study was to examine whether prenatal food restriction during late gestation affects offspring liver proteome irrespective of birth weight and propose possible underlying pathophysiological mechanisms of liver fetal programming

## 2. Results

### Experimental model

There was no statistical difference in post-delivery maternal bodyweight in both diet groups (control:  $265 \pm 25$  gr, starved  $270 \pm 20$  gr  $p=0.769$ ). Control group mothers (ad libitum food access) gave birth to control pups with mean body weight of 6.419 gr (SD: 0.436). The mean birthweight of the food restricted group was 5.423 gr, significantly different compared to controls ( $5.423 \pm 0.610$  gr vs.  $6.419 \pm 0.436$  gr;  $p<0.001$ ). Male neonates were heavier compared to females in the control group (6.659g vs 6.2g,  $p<0.001$ ) but there was no statistically significant difference between them in the starved group ( $p=0.666$ ).

Newborn pups delivered by starved mothers, were further divided to Fetal Growth Restricted (FGR) group when birthweight was  $< - 2$  SDs of the mean BW of the control offspring and non-FGR group when birthweight was  $> - 2$  SDs of the mean BW of the control. The cut-off between FGR and non-FGR neonates was set at 5.547gr according to the aforementioned definition. Furthermore, there was statistically significant birthweight difference between FGR ( $4.796$  gr  $\pm 0.479$  gr) and non-FGR ( $5.914$  gr  $\pm 0.479$  gr) groups ( $p<0.001$ ).

Although liver weight of the non-FGR group was statistically significant higher compared to FGR pups ( $0.211 \pm 0.047$  vs.  $0.280 \pm 0.073$ ,  $p<0.0014$ ), there was no difference in the liver weight to body weight ratio between groups ( $0.04274 \pm 0.00743$  vs.  $0.04721 \pm 0.01220$ ,  $p=0.10337$ ) (Table 1)

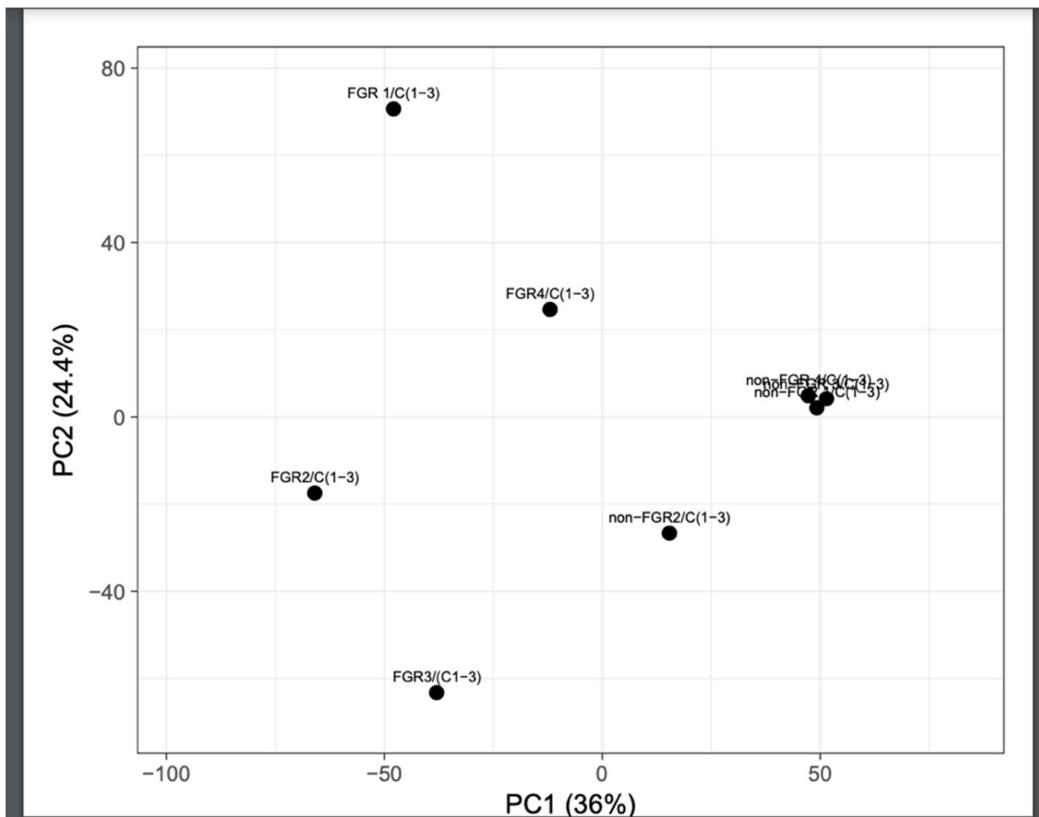
**Table 1.** Mean values for all experimental outcomes and comparisons between study groups

	Group	Mean	SD	p-value
<b>Length of gestation (days)</b>	Starved control	21.22 20.73	0.47 0.06	0.081
<b>Litter size (pups)</b>	Starved control	10.83 11.50	1.72 1.29	0.530
<b>Post delivery maternal weight(g)</b>	Starved Control	268.83 264.50	26.75 10.47	0.304
<b>Birth weight (g)</b>	Starved control	5.423 6.419	0.610 0.436	< 0.001
<b>Liver weight (g)</b>	Starved	0.245	0.070	0.117
	Control	0.266	0.057	
	Fgr non-Fgr	0.211 0.280	0.047 0.073	<0.001
<b>Liver to body weight (g)</b>	Starved Control	0.04498 0.41753	0.01026 0.00946	0.112
	Fgr non-Fgr	0.04274 0.04721	0.00743 0.01220	0.103
	Starved Control	0.150 0.180	0.045 0.044	<0.001
	Fgr non-Fgr	0.151 0.148	0.058 0.043	0.783
<b>Brain weight (g)</b>	Starved Control	0.02826 0.02806	0.00968 0.00598	0.905
	Fgr non-Fgr	0.03157 0.02496	0.01093 0.00698	0.009

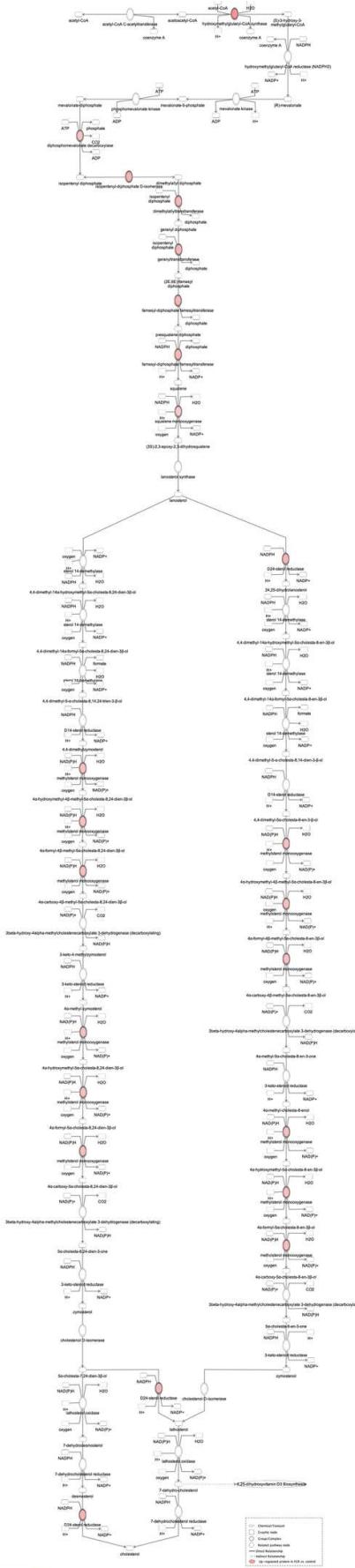
## Proteomic Analysis

Proteomic analysis of male offspring livers ended up in the profiling of 6,665 proteins (peptide level  $q<0.05$ ) (Supplementary Table 1). Among the quantified proteins, 451 proteins were differentially expressed in FGR vs. control (Supplementary Table 2) and 782 in non-FGR vs. control group (Supplementary Table 3). Of these, 76 were commonly up-regulated and 153 commonly down-regulated in both FGR and non-FGR compared to control (Supplementary Table 3).

Principal component analysis (PCA) of all quantified proteins showed a distinct proteomic liver profile of FGR compared to non-FGR rats (figure 1). Bioinformatics analysis of differentially expressed proteins (DEPs) in FGR compared to control groups using Ingenuity Pathway Analysis (IPA) showed: a. induction of the super pathway of cholesterol biosynthesis ( $z=2.2$ ;  $p=1.5e-4$ ) (figure 2), and b. inhibition of thyroid hormone metabolism (figure 3) ( $z=-2.0$ ;  $p=4.6e-3$ ), fatty acid beta oxidation ( $z=-2.0$ ;  $p=2.7e-3$ ) (figure 4) and apelin liver signaling pathway (figure 5) ( $z=-2.2$ ;  $p=8.5e-5$ ). Enrichment analysis of the DEPs in non-FGR vs. control groups using IPA showed: a. induction of immune cell adhesion ( $z=2.9$ ;  $p= 1.1e-7$ ) and b. inhibition of thyroid hormone metabolism ( $z=-2.0$ ;  $p=2.5e-2$ ), fatty acid beta oxidation ( $z= -2.0$ ;  $p=1.6e-2$ ) and apelin liver signaling pathway ( $z=-2.0$ ;  $p=6.7e-3$ ) (figure 6).



**Figure 1.** Principal component analysis (PCA) of all quantified proteins revealed that liver of fetal growth restricted pups had a heterogeneous proteomic profile compared to non-FGR ones.



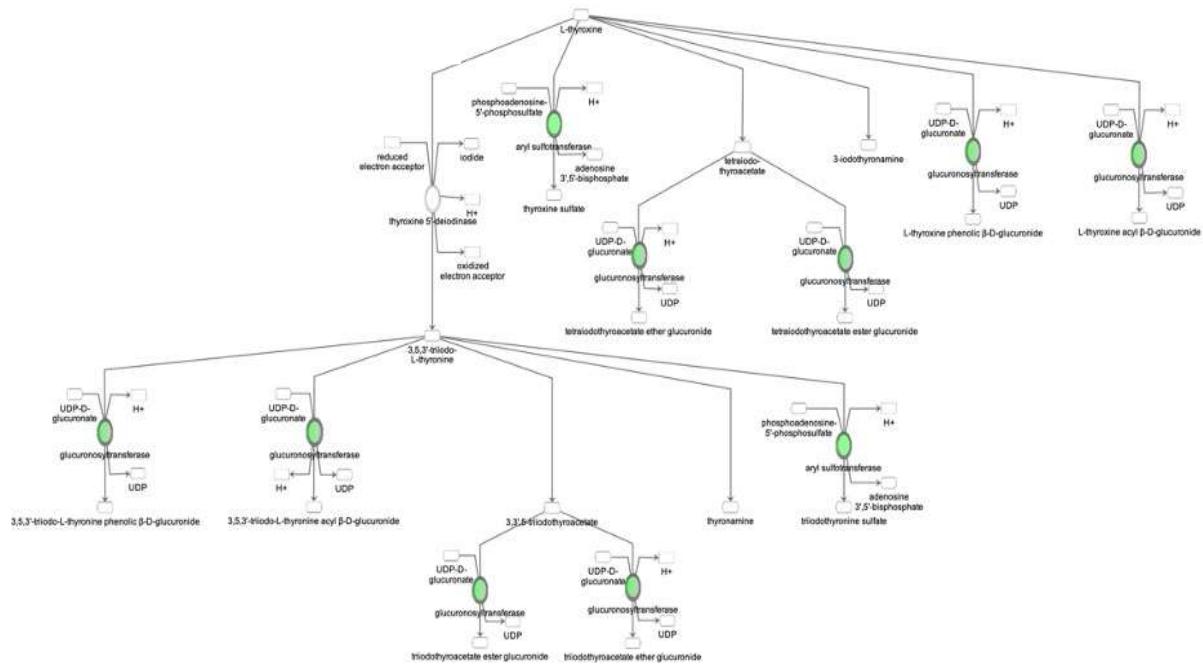
**Figure 2. Ingenuity Pathway Analysis of DEPs between FGR vs. Control group.**

*Induction of the super pathway of cholesterol biosynthesis.*

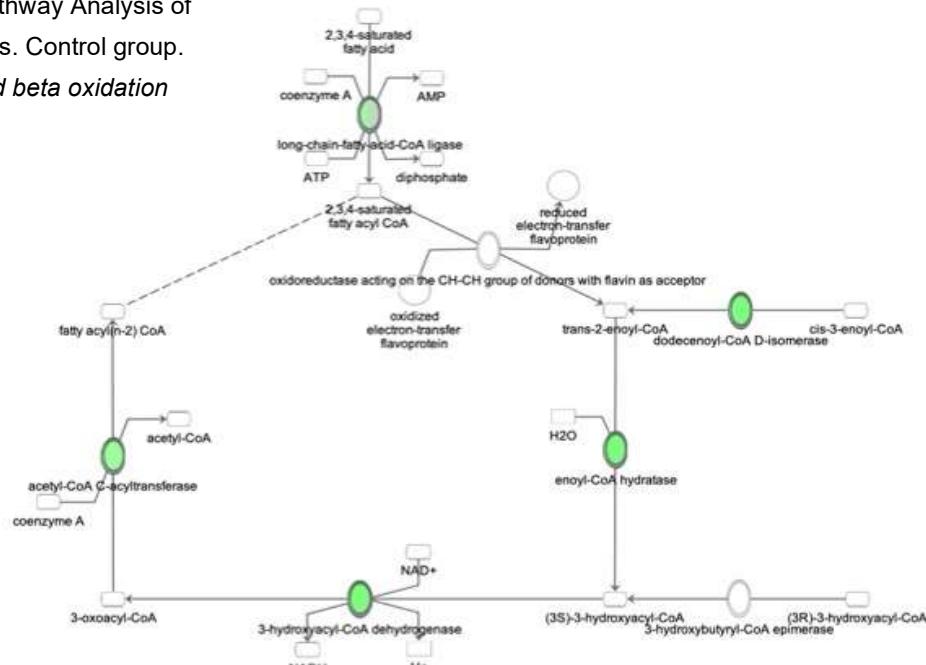
$z=2.2$   $p=1.5e-4$

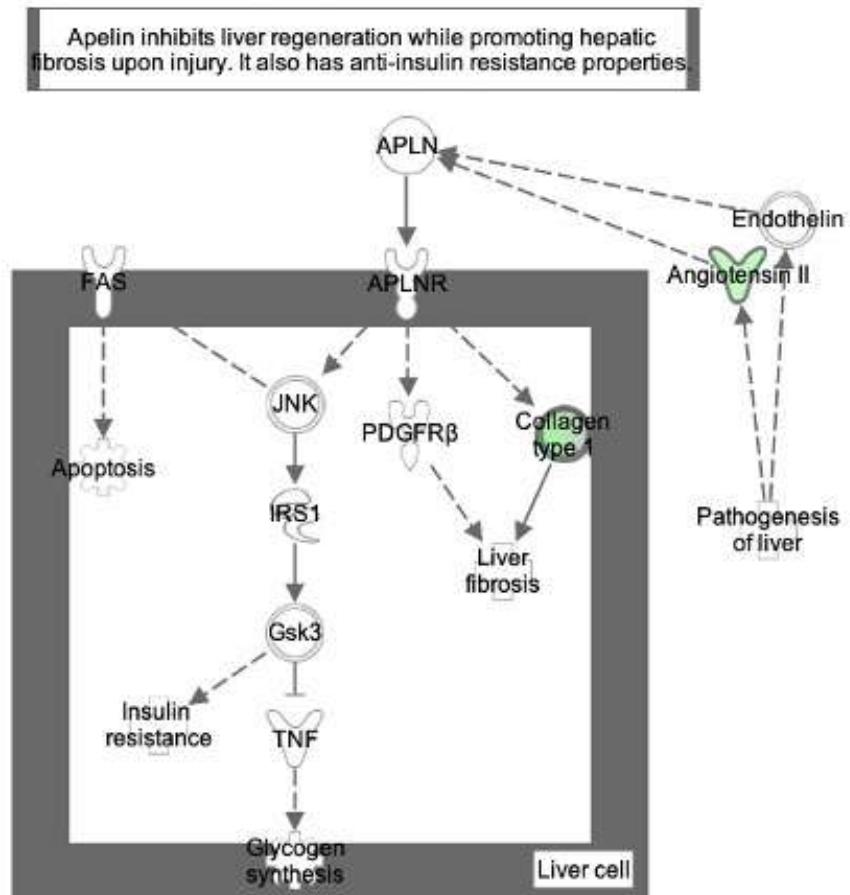
**Figure 3. Ingenuity Pathway Analysis of**

DEPs between FGR vs. Control group.

*Inhibition of thyroid hormone metabolism* **$z = -2.0 \ p = 4.6e-3$** **Figure 4. Ingenuity Pathway Analysis of**

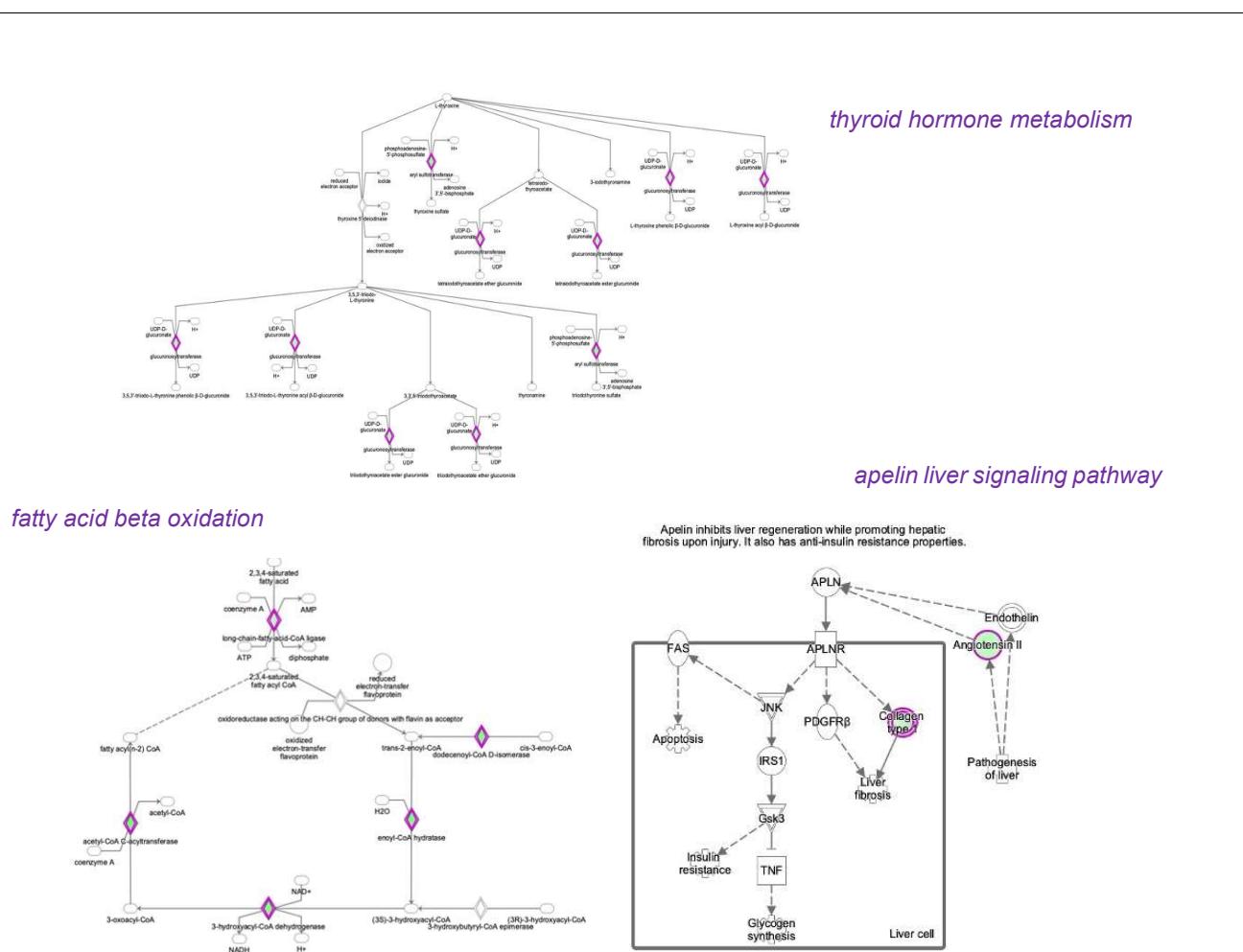
DEPs between FGR vs. Control group.

*Inhibition of fatty acid beta oxidation* **$z = -2.0 \ p = 2.7e-3$** 



**Figure 5.** Ingenuity Pathway Analysis of DEPs between FGR vs. Control group. *Inhibition of apelin liver signaling pathway*

$z = -2.2$   $p = 8.5 \times 10^{-5}$



**Figure 6.** Ingenuity Pathway Analysis of differentially expressed proteins in non-FGR compared to control group showed inhibition of thyroid hormone metabolism ( $z = -2.0$   $p = 2.5e-2$ ), fatty acid beta oxidation ( $z = -2.0$   $p = 1.6e-2$ ) and apelin liver signaling pathway ( $z = -2.0$   $p = 6.7e-3$ )

### 3. Discussion

Numerous studies have shown the impact of adverse early-life environment on disease during infancy, childhood and adult life [5]. FGR is associated with significant perinatal and subsequent long-term morbidity and mortality. FGR neonates and infants demonstrate a variety of complications involving multiple organs and systems such as pulmonary, gastrointestinal, immune and central nervous system. Regarding the endocrine system, FGR is associated with altered glucose metabolism, transiently low thyroxin levels and cortisol deficiency.

Furthermore, FGR programs both childhood and adult disease, associated with increased risk of obesity, insulin resistance and cardiovascular disease.

Using a well-defined FGR rat model, this study shows that maternal food restriction plays a crucial role, impairing liver intrauterine growth and altering its proteomic expression. In our study liver weight was reduced in proportion to body weight in FGR compared to non-FGR pups. On the contrary, brain weight did not differ significantly between the abovementioned two groups (Table 1) indicating a late-onset FGR model resembling to the commonest FGR phenotype in human population [23]. This study aimed to a better understanding of the proteomic mechanisms of liver developmental dysfunction induced by prenatal food restriction investigating possible differentiations in liver proteomic expression in both growth restricted (FGR) and appropriately grown (non-FGR) offspring born to starved mothers.

Bioinformatics analysis of DEPs in the FGR group vs. control showed induction of cholesterol biosynthesis. Regarding cholesterol biosynthesis, metabolomic studies have shown that FGR fetuses have higher concentrations of cholesterol such as VLDL and LDL, lipoproteins and triglycerides [29]. Lipids are vital molecules for life, providing energy for metabolic processes. Furthermore, cholesterol is a key element for brain neurodevelopment and a precursor of many hormones like sex steroids [6,20]. Fetal liver is the main source of circulating lipoproteins, as in adults. Alterations of VLDL concentrations, which is mostly synthesized in fetal liver, implies an altered hepatic synthesis of lipoproteins caused by FGR. Remarkably, the lipid profile of FGR fetuses resembles to adults presenting with atherosclerosis and dyslipidemia [48].

The apelin signaling pathway, thyroid metabolism and fatty acid beta oxidation were inbibited in both FGR and non-FGR neonate rats, indicating these might be a result of maternal undernutrion regardless the fetus' growth.

Apelin is a regulatory peptide and in conjunction with its receptor, are both expressed in a wide range of tissues such as central nervous system, heart and

liver. Apelin is also produced by adipocytes and latest studies proposed its crucial role in energy metabolism and enhancement of insulin sensitivity [27]. Our study in accordance with previous ones, have shown inhibition of apelin signaling and reduced plasma concentrations as a potential response to undernutrition [7]. Recent studies have highlighted the paramount importance of apelin and its receptor, since they have been proposed as a valuable new treatment target in type 2 diabetes [10,30].

Our study showed that in both FGR and non-FGR offspring of calorie restricted mothers, liver thyroid hormones' metabolism is inhibited. Thyroid hormones play a key role to thermoregulation, specifically in norepinephrine (NE) controlled thermogenesis [44]. Brown adipose tissue thermogenic activity which is triggered by NE is under triiodothyronine ( $T_3$ ) control [32]. Low  $T_3$  plasma levels are associated with impaired thermogenesis and predisposition to diet-induced obesity in neonatal and adult life despite later normalization of  $T_3$  plasma concentrations [9,37]. Hypothermia and transiently low thyroxine levels are common neonatal complications of FGR however no information is available in appropriately grown neonates born to undernourished mothers [36].

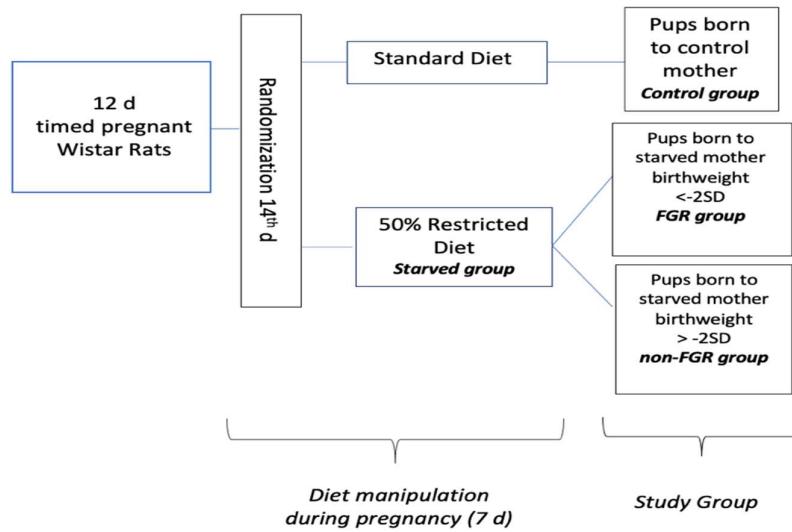
Our model suggests inhibition of fatty acid metabolism not only in FGR liver but in non-FGR liver as well. Liver is the central organ of fatty acid metabolism. Both obesity and insulin resistance are close related with disrupted fatty acid metabolism [40]. Inhibition of this metabolic process leads to non-alcoholic fatty liver, liver steatosis and subsequent insulin resistance deterioration. In a previous study of our team where NEFA (Non Esterified Fatty Acids) concentrations were compared between FGR and non-FGR rats at one year of age there was no statistical difference between groups. It seems that food restriction produces the same adipose tissue response in both the FGR and non-FGR groups, suggesting that it is the adverse prenatal event that determines certain metabolic profiles rather than birthweight [12].

## 4. Materials and Methods

### Animal model

Ten (n=10) timed pregnant Wistar rats, on their 12<sup>th</sup> day of gestation (Janvier Labs – Rodent research models & associated services, France), were hosted individually in 36 × 20 × 14 cm breeding boxes at the Laboratory of Experimental Surgery of the Second Department of General Surgery at Aretaieion Hospital, National and Kapodistrian University of Athens, Athens, Greece. Animals were housed under standard conditions (temperature between 22° and 23° C, humidity 55-65% and 12-hour light/dark cycles). All animals were fed with standard formula diet containing 18.5% protein (Mucedola S.r.l., Settimo Milanese, Italy) with ad libitum access to food and water as well, until day 14. Following randomization, pregnant dams were assigned to starved group (n=6, diet restricted by 50%) and control group (n=4 ad libitum access to food). Both groups had free access to fresh water. Control group's food intake was measured on a daily basis. During the experimental period (from day 15th onwards), rats of the starved group, were given half the amount of food that was on average consumed by the control group, based on measurements taken place the day before. Food restriction of the starved group lasted from 15<sup>th</sup> gestational day to delivery. All rats delivered spontaneously on the 21<sup>st</sup> gestational day and neonates were immediately weighted (figure 7).

**Figure 7.** Experimental design of the study.



Starved group's offspring were categorized according to their birth weight as FGR (birth weight < mean birth weight of control group's offspring –  $2 \times$  Standard Deviation) and non FGR (birth weight > mean birth weight of control group's offspring –  $2 \times$  Standard Deviation) as previously described [11,38,50,52].

Immediately after delivery, offspring were separated from their mothers and weighted. Neonates were anesthetized using inhaled sevoflurane, and euthanized. Liver tissues were rapidly removed. The time interval between rat's sacrifice and specimens' storage at  $-80^{\circ}\text{C}$  did not exceed 15 minutes.

All liver tissues were cleaned from blood with PBS (phosphate buffered saline). Specimens were stored at  $-80^{\circ}\text{C}$  and sent packed in dry ice to the Centre for Proteomic Research, Institute for Life Sciences, University Southampton for proteomic analysis.

Growth characteristics of mothers, gestation duration, litter size, birth weight of the pups and organ weight were compared using the independent-samples t-test (IBM SPSS Statistics 22.0). Statistical significance was considered at  $p < 0.05$ .

All experimental procedures were approved by the Greek Directorate of Veterinary Services (1211/19-03-2018) and the Ethical Committee of Areteiaeion Hospital

(011/21-11-2017) and were in accordance with both European Union and National legislation.

#### Quantitative proteomics:

Each liver tissue was dissolved in 200 µL of 0.5 M triethylammonium bicarbonate, 0.05% sodium dodecyl sulfate and homogenised using the FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). Lysates were subjected to pulsed probe sonication (Misonix, Farmingdale, NY, USA) and centrifuged (16,000 g, 10 min, 4°C). The supernatant had been measured for its protein content using the Direct Detect™ system (Merck Millipore, Darmstadt, Germany). From each lysate, 100 µg of protein subjected to reduction, alkylation, trypsin proteolysis and 11-plex TMT labelling according to manufacturer's instructions.

The resulting TMT peptides were initially fractionated with alkaline C<sub>4</sub> reversed phase (RP) liquid chromatography. Each peptide fraction further separated with on-line nano-capillary C<sub>18</sub> reverse phase liquid chromatography under acidic conditions, subjected to nanospray ionization and measured with ultra-high resolution mass spectrometry using the hybrid ion-trap / FT-Orbitrap Elite platform.

The unprocessed raw data files were submitted to Proteome Discoverer 1.4 for target decoy searching with SequestHT against the TREMBL Uniprot database for *rattus norvegicus* (release date: January 2018). Reporter ion ratios derived from unique peptides only were used for the relative quantitation of each respective protein. Quantification ratios were median-normalized and log<sub>2</sub> transformed. The threshold of percent co-isolation excluding peptides from quantification was set at 50. A one-sample T-Test was performed to identify proteins that were differentially expressed in the tissue from FGR and non-FGR compared to control rats. The two-stage step-up method of Benjamini, Krieger and Yekutieli was used for multiple hypothesis correction. A *q-value* ≤ 0.05 was considered significant. Proteomics

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data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository.

## 5. Conclusions

According to fetal programming theory, fetal malnutrition induces adaptive processes that permanently change growth, physiology and metabolism of the offspring. Maternal undernutrition alters the proteomic profile of the neonatal liver which is a key organ of many metabolic processes supporting homeostasis. In our study, FGR (representing a model of human neonates with growth restriction) and non-FGR pups (representing a model of human infants having experienced adverse intrauterine conditions but born with normal body weight) have developed both common and different metabolic phenotypes. Thus suggesting that both intrauterine adversities and birthweight determine the metabolic profiles of the offspring. This study contributes to a better understanding of the proteomic mechanisms of liver developmental dysfunction induced by prenatal food restriction and helps to explain the intrauterine origin of adult metabolic disease.

**Author contributions:** Conceptualization, Makarios Eleftheriades; Formal analysis, Polyxeni - Maria Sarli, Antigoni Manousopoulou, Anastasios Potiris, Andreas Zouridis and Elias Efthymiou; Funding acquisition, Polyxeni- Maria Sarli and Makarios Eleftheriades; Investigation, Polyxeni-Maria Sarli, Antigoni Manousopoulou, Anastasios Potiris, Andreas Zouridis and Makarios Eleftheriades; Methodology, Polyxeni-Maria Sarli, Antigoni Manousopoulou, Spiros Garbis and Makarios Eleftheriades; Project administration, Makarios Eleftheriades; Resources, Makarios Eleftheriades and Spiros Garbis; Supervision, Konstantinos Panoulis, Panagiota Pervanidou, Nikolaos Vlahos, Spiros Garbis and Makarios Eleftheriades; Visualization, Polyxeni-Maria Sarli and Antigoni Manousopoulou; Writing –original draft, Polyxeni-Maria Sarli and Antigoni Manousopoulou; Writing

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– review & editing, Antigoni Manousopoulou, Konstantinos Panoulis, Efthymios Deligeoroglou, Nikolaos Vlahos Panagiota Pervanidou, Spiros Garbis and Makarios Eleftheriades.

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**Institutional Review Board Statement:** All animal procedures were approved by the Directorate of Veterinary Services (protocol number 1211/19-03-2018) and the Ethical Committee of Areteiaeion Hospital (protocol number 011/21-11-2017) and were in accordance with both EU and National legislation.

**Informed Consent Statement:** Not applicable

**Data Availability Statement:** The data presented in this study are available within the article. All proteomics data are uploaded at the ProteomeXchange Consortium via the PRIDE partner repository (dataset identifier PXD011407)

**Conflicts of Interest:** Spiros D. Garbis, PhD is Founder, President and CEO of Proteas Bioanalytics Inc., BioLabs at the Lundquist Institute, 1124 West Carson Street, MRL Building, 3rd Floor, Torrance, CA 90502.

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**Appendix A.** Supplementary tables.

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