



BASIC SCIENCE ARTICLE

Dlk1 expression relates to visceral fat expansion and insulin resistance in male and female rats with postnatal catch-up growth

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BACKGROUND: Although prenatal and postnatal programming of metabolic diseases in adulthood is well established, the mechanisms underpinning metabolic programming are not. *Dlk1*, a key regulator of fetal development, inhibits adipocyte differentiation and restricts fetal growth.

METHODS: Assess *Dlk1* expression in a *Wistar* rat model of catch-up growth following intrauterine restriction. Dams fed *ad libitum* delivered control pups (C) and dams on a 50% calorie-restricted diet delivered pups with low birth weight (R). Restricted offspring fed a standard rat chow showed catch-up growth (R/C) but those kept on a calorie-restricted diet did not (R/R).

RESULTS: Decreased *Dlk1* expression was observed in adipose tissue and skeletal muscle of R/C pups along with excessive visceral fat accumulation, decreased circulating adiponectin, increased triglycerides and HOMA-IR (from $p < 0.05$ to $p < 0.0001$). Moreover, in R/C pups the reduced *Dlk1* expression in adipose tissue and skeletal muscle correlated with visceral fat ($r = -0.820$, $p < 0.0001$) and HOMA-IR ($r = -0.745$, $p = 0.002$).

CONCLUSIONS: Decreased *Dlk1* expression relates to visceral fat expansion and insulin resistance in a rat model of catch-up growth following prenatal growth restriction. Modulation of *Dlk1* expression could be among the targets for the early prevention of fetal programming of adult metabolic disorders.

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INTRODUCTION

Fetal programming is a process by which the fetus' adaptations to the adverse conditions during gestation and early postnatal life have long-term effects on the newborn.¹ Clinical studies, and subsequent work in animal models, have demonstrated that adverse conditions in these critical developmental windows can program physiological processes and cause metabolic alterations leading to future adult diseases, such as obesity, type 2 diabetes, metabolic syndrome and cardiovascular disease.^{1–3}

Fetal development is sensitive to the nutritional environment. Adaptations to adverse conditions serve to optimize and improve the chances of fetal survival. However, when there is a mismatch between fetal and postnatal nutrition, this adapted thrifty phenotype is inappropriate, becomes detrimental and contributes to metabolic disorders in adulthood.^{3,4} Consequently, the major risk of developing these disorders later in life has been associated with postnatal catch-up growth following low birth weight, a condition occurring in ~8% of pregnancies worldwide.^{1,2,5}

The impact of early life insults on childhood and adult health is certainly apparent; however, the mechanisms underlying the alterations in metabolic programming remain largely unknown. The study of genes that regulate fat storage, whose alterations lead to insulin resistance, are of central importance for understanding the pathophysiology of catch-up growth.

Delta-like protein 1 (*Dlk1*) gene is a key regulator of fetal development and it is involved in energy and nutrient metabolism. In adults, its expression is limited to endocrine tissues: *Dlk1* inhibits adipocyte differentiation in adipose tissue and is involved in regeneration processes in skeletal muscle.^{6–8} Our group has previously linked prenatal and postnatal growth with the placental imprinted *DLK1-DIO3* domain in human studies;⁹ others have described that impairments in fetally derived *DLK1* cause fetal growth restriction.¹⁰ Studies, however, linking *Dlk1* gene expression and catch-up growth following prenatal restriction are lacking.

The aim of the study was to assess whether changes in *Dlk1* gene expression relate to metabolic risk markers in an animal model of postnatal catch-up growth following fetal weight restriction.

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METHODS

Rat model of catch-up growth following prenatal growth restriction

A *Wistar* rat model of intrauterine growth restriction induced by calorie restriction throughout gestation was used. Studies were approved by the Animal Research Ethic Committee (C.E.E.A., #DAAM 6911) of the University of Barcelona and were in accordance with the Catalan Government guideline 214/97. First-time-pregnant *Wistar* rats (Janvier, Le Genest Saint Isle, France) were housed in a facility with constant temperature ($21 \pm 1^\circ\text{C}$) and humidity (50–60%) and a controlled 12:12-h light-dark cycle. The first day of gestation was determined by observation of spermatozoa in vaginal smears. From day 1 of pregnancy to term, control dams (C) were provided an *ad libitum* diet consisting of a standard laboratory chow (Teklad Global Rodent Maintenance Diet, Harlan Laboratories Inc.; 14% protein, 4% fat, 2900 kcal/kg) and restricted dams (R) were fed a 50% less of diet used in control dams (Supplementary Figure S1). Rat dams gave birth normally and pups were limited to ten per litter at day 1 after birth to normalize pups feeding. Throughout the 21-day lactation period, different control or restricted diets were provided in order to create 3 experimental groups: control dams were fed an *ad libitum* diet (C/C) and restricted dams were fed either an *ad libitum* diet (R/C) or a restricted diet (R/R) to create catch-up or non-catch-up growth groups, respectively (Supplementary Figure S1). At 22-days of age, after the lactation period, offspring in all groups were weaned to *ad libitum* feed. All pups were weighed twice a week from postnatal day 1 (PD1) until PD42. At PD42 all pups ($N = 14$ per group; 7 females and 7 males) fasted overnight were anesthetized with 3% isoflurane and euthanized by cardiac exsanguination. Blood was collected via cardiac puncture in heparinized syringes and centrifuged at 3500 rpm for 15 min at 4°C to obtain plasma samples which were stored at -80°C . Adipose tissue from different locations in the abdomen and skeletal gastrocnemius muscle samples were immediately frozen in liquid nitrogen. Additionally, an approximation to body composition was obtained by calculating visceral fat tissue mass (sum of weights of perigonadal, retroperitoneal and mesenteric adipose tissues). For gene and protein expression studies, rat retroperitoneal adipose tissue was selected as being equivalent to human omental adipose tissue.

Plasma metabolic profile

Plasma adiponectin and insulin concentrations were determined by ELISA using a commercial kit (*Rat Adiponectin ELISA Kit*, and *Ultrasensitive Rat Insulin ELISA kit*; Crystal Chem Inc.). For adiponectin, the sensitivity of the kit was 0.25 mg/l and intra-assay CVs were $<10\%$ and for insulin, the sensitivity was 0.1 ng/ml and intra-assay CVs were less than 10%. Plasma glucose, total cholesterol, HDL-cholesterol and triglycerides were determined by dry chemistry technique (*Spotchem II*, *Spotchem EZ SP-4410*, Menarini Diagnostics-Akray, Japan). Homeostatic model assessment of insulin resistance (HOMA-IR) was assessed using the following formula: $\text{HOMA-IR index} = \text{glucose} \times \text{insulin} / 2430$.¹¹

Gene and protein expression

For gene expression, RNA (*RNAeasy mini kit*, Qiagen) was extracted and 2 μg of total RNA was retro-transcribed to cDNA (High-capacity cDNA Reverse Transcription Kit, Applied Biosystems, Thermo Fisher Scientific Inc.). cDNA samples were used to quantify the relative expression by real-time PCR (*Taqman Assays 20* \times [*Dlk1*: Rn00587011_m1 and endogenous control *GAPDH*: Rn01775763_g1], Applied Biosystems, Thermo Fisher Scientific Inc.). For protein quantification, total protein was extracted from tissue (*DC protein assay*, Bio-Rad Laboratories Inc.). Total protein concentration was quantified by the Lowry method and *Dlk1* protein concentrations by an ELISA commercial kit (*Rat Delta Like 1*

Homolog Dlk1 ELISA kit, MyBioSource, Inc.). The sensitivity of the kit was 0.27 ng/ml and intra-assay CVs were $<9\%$.

Tissue morphology

For morphometric studies, retroperitoneal adipose tissue from the abdominal cavity and skeletal gastrocnemius muscle from a set of 12 animals ($N = 4$ animals per each treatment, 2 males and 2 females) were used. Tissues were paraffin-embedded, tissue sections were deparaffinized by the xylene-ethanol sequence and processed using the hematoxylin-eosin dye method. Images were taken at $\times 200$ (Olympus BX51, XC50 CellVe). One histological section of each animal was randomly selected from the histological slides. Twelve randomly chosen fields per section were considered for the measurement of both adipocyte area and density (>250 adipocytes were analyzed per group) and skeletal muscle cross-sectional myofiber density and cross-sectional myofiber area. The measurements were analyzed in a blinded manner and both manual and automatic tools of the Image J software (NIH, public domain) were used.

Statistical analysis

All statistics were performed with SPSS 22.0 (SPSS software, IBM, Inc.) and graphs were done with Prism 6.0 (GraphPad Software, Inc.). Differences between experimental groups were compared using ANOVA test and least significance difference (LSD) *post hoc* tests. Spearman correlation was used to analyze associations between variables. Combined data for males and females are shown, since no sex differences were evident. Data are expressed as mean \pm standard deviation (SD) and *p* value was set at 0.05.

RESULTS

Catch-up growth *Wistar* rats following prenatal growth restriction exhibited increased fat mass and higher HOMA-IR

At birth, R/R and R/C pups showed less body weight ($p < 0.0001$, Table 1) relative to C/C pups: both R/R and R/C pups showed a 22% lower value in body weight at birth in relation to C/C pups. At weaning (PD22), R/C pups showed higher body weight ($p < 0.0001$; Table 1) and at PD42 these pups experienced further postnatal weight gain ($p < 0.0001$; Table 1), together with higher retroperitoneal adipose tissue weight and higher visceral fat (from $p < 0.05$ to $p < 0.0001$; Table 1), as compared to other pup groups. At PD42, R/C pups showed also lower total adiponectin, higher plasma triglycerides (both $p < 0.01$; Table 1) and higher insulin concentration and HOMA-IR index (both $p < 0.0001$; Table 1), as compared to R/R pups.

Decreased *Dlk1* expression in adipose tissue and skeletal muscle relates to visceral fat and HOMA-IR in catch-up growth *Wistar* rats following prenatal growth restriction

Relative *Dlk1* expression in R/C pups was decreased in both retroperitoneal adipose tissue and skeletal muscle compared to R/R pups ($p < 0.0001$; Table 1). Likewise, *Dlk1* protein concentrations in retroperitoneal adipose tissue were lower in R/C pups ($p < 0.001$; Table 1) compared to other pup groups. In adipose tissue, negative correlations between *Dlk1* relative expression and body weight, postnatal body weight gain and visceral fat at PD42 were observed (all between $r = -0.820$ and $r = -0.895$, $p < 0.0001$; Fig. 1 and Supplementary Table S1). In muscle, negative correlations between *Dlk1* relative expression and plasma insulin and HOMA-IR index were observed ($r = -0.701$, $p = 0.005$ and $r = -0.745$, $p = 0.002$; respectively; Fig. 1 and Supplementary Table S1).

Catch-up growth *Wistar* rats following prenatal growth restriction showed hypertrophied adipocytes and enlarged skeletal muscle myofibers

The histological studies showed higher adipocyte area ($4933 \mu\text{m}^2 \pm 1336$; $3277 \mu\text{m}^2 \pm 900$; $2144 \mu\text{m}^2 \pm 533$; $p < 0.0001$) and lower

Table 1. Somatometric, metabolic and gene expression characteristics of the studied groups according to pre and postnatal growth: restricted pups (R/R), control pups (C/C) and catch-up growth pups (R/C)

	R/R N = 14	C/C N = 14	R/C N = 14	p	p for trend
Somatometric parameters					
Body weight PD1 g	5.10 ± 0.23 *	6.47 ± 0.29	5.08 ± 0.28 *	<0.0001	0.912
Body weight PD22 g	17.81 ± 1.13	43.08 ± 3.56 #	51.32 ± 3.94 # *	<0.0001	<0.0001
Body weight PD42 g	79.43 ± 11.64	170.62 ± 20.37 #	179.42 ± 20.43 #	<0.0001	<0.0001
Postnatal body weight gain g	74.33 ± 11.69	164.15 ± 20.28 #	173.62 ± 20.66 #	<0.0001	<0.0001
Postnatal body weight gain %	14.62 ± 2.54	25.36 ± 3.04 #	33.87 ± 4.17 # *	<0.0001	<0.0001
Retroperitoneal AT g	0.22 ± 0.06	0.75 ± 0.26 #	1.02 ± 0.33 # *	<0.0001	<0.0001
Mesenteric AT g	0.17 ± 0.04	0.35 ± 0.07 #	0.41 ± 0.09 #	<0.0001	<0.0001
Visceral fat g	0.67 ± 0.16	2.12 ± 0.60 #	2.71 ± 0.87 # *	<0.0001	<0.0001
Metabolic parameters					
	N = 14	N = 14	N = 14	p	p for trend
Total adiponectin mg/g	19.81 ± 9.33	12.41 ± 4.71 #	10.42 ± 5.65 #	0.002	0.001
Glucose mmol/L	8.28 ± 0.74	10.15 ± 0.90 #	9.04 ± 0.56 # *	<0.0001	0.011
Insulin pmol/L	159.1 ± 73.6	267.2 ± 24.6 #	360.1 ± 26.8 # *	<0.0001	<0.0001
HOMA-IR	1.62 ± 0.77	3.53 ± 0.67 #	3.97 ± 1.34 #	<0.0001	<0.0001
Total cholesterol mmol/L	2.47 ± 0.36	2.31 ± 0.16	2.34 ± 0.42	0.448	0.318
HDL cholesterol mmol/L	0.91 ± 0.19	0.80 ± 0.09	0.89 ± 0.17	0.211	0.787
Triglycerides mmol/L	0.82 ± 0.27	1.41 ± 0.40 #	1.38 ± 0.58 #	0.001	0.002
Gene expression					
	N = 14	N = 14	N = 14	p	p for trend
Retroperitoneal adipose tissue					
<i>Dlk1</i> relative expression 2- <i>ACT</i>	0.93 ± 0.93	0.02 ± 0.02 #	0.01 ± 0.01 #	<0.0001	<0.0001
<i>Dlk1</i> protein ng/g	392.3 ± 169.5	209.3 ± 148.2 #	82.3 ± 46.1 * #	<0.0001	<0.0001
Skeletal muscle					
<i>Dlk1</i> relative expression 2- <i>ACT</i>	1.87 ± 1.55	0.39 ± 0.24 #	0.30 ± 0.28 #	<0.0001	<0.0001

Values are expressed in mean ± deviation score. R/R restricted, C/C control, R/C catch-up growth, PD postnatal day; AT adipose tissue, HOMA-IR homeostatic model assessment of insulin resistance, HDL high-density lipoprotein. Postnatal body weight gain percentage in relation to birth body gain, specific adipose tissue weights percentage in relation to total body weight and total adiponectin was corrected for body weight. Anova *p* and *p* for trend values are shown (LSD post-hoc tests are shown with **p* < 0.05 compared to C/C and #*p* < 0.05 compared to R/R). Significant *p* values are marked in bold style

adipocyte density (242N/mm² ± 85; 340N/mm² ± 83; 531N/mm² ± 153; *p* < 0.0001) in retroperitoneal adipose tissue and lower density or number of myofibers per area (491N/mm² ± 90; 474N/mm² ± 45; 726N/mm² ± 95; *p* = 0.003) in skeletal muscle of R/C pups, as compared to C/C and R/R groups, respectively (Fig. 2).

DISCUSSION

In the present model of catch-up growth following fetal growth restriction, we observed decreased *Dlk1* in both visceral fat and skeletal muscle in pups experiencing catch-up growth. Moreover, in these pups, reduced *Dlk1* gene expression in retroperitoneal adipose tissue was related to visceral fat mass accumulation, and reduced *Dlk1* gene expression in skeletal muscle was associated with insulin resistance.

As expected, in our experimental model of catch-up growth following fetal weight restriction, R/C pups successfully presented catch-up growth, as judged by higher body weight gain and visceral adiposity at P42. Previous studies have shown excessive visceral fat accumulation during catch-up growth.¹² It has also been suggested that permanent changes in the expression of regulatory proteins in liver, muscle and adipose during catch-up growth relate to insulin resistance and metabolic disorders later in life.⁴ In our study, R/C pups showed not only increased visceral fat but also decreased circulating adiponectin and increased plasma triglycerides. It is well known that circulating adiponectin concentrations decrease along with visceral fat accumulation.¹³ Consequently, the observed visceral fat mass accumulation, low adiponectin levels and high triglycerides during catch-up growth

may reflect dysregulation of adipose tissue function, which may plausibly contribute to the development of insulin resistance in these animals. This is substantiated by previous studies relating catch-up growth and insulin resistance in *Wistar* rats¹⁴ and by our results showing higher HOMA-IR values in R/C pups. Catch-up growth in these pups caused both visceral fat accumulation and insulin resistance as early as at postnatal period PD42. On the contrary, R/R pups kept postnatally under restricted conditions, in order to prevent the mismatch between fetal and postnatal nutrition, exhibited rather insulin sensitivity. Metabolic programming has been described to present both altered fat expansion and disrupted insulin signaling pathway leading to insulin resistance, although the molecular mechanisms implicated in these processes are not fully understood.

Dlk1 is a regulator of adipocyte differentiation and adipogenesis.^{6,15} The decrease in *Dlk1* relative gene expression and *Dlk1* protein concentrations in retroperitoneal adipose tissue at P42 together with the negative correlations between *Dlk1* expression and body weight, body weight gain and visceral fat mass in R/C pups point to a relevant role of *Dlk1* in the control of adipocyte growth and differentiation during catch-up growth. It was shown that mice with *Dlk1* deficiency presented higher amounts of visceral fat, circulating lipids and adipocyte cell size at weaning and developed obesity and fatty liver disease.^{6,8,16} Histologically, hypertrophied adipocytes have been consistently found in adipose tissue of individuals with obesity,¹⁷ as well as in restricted pups at weaning.^{12,18,19} This observation has been related to a lower number of progenitor adipocytes capable of differentiating into healthy adipocytes.²⁰ Accordingly, our results

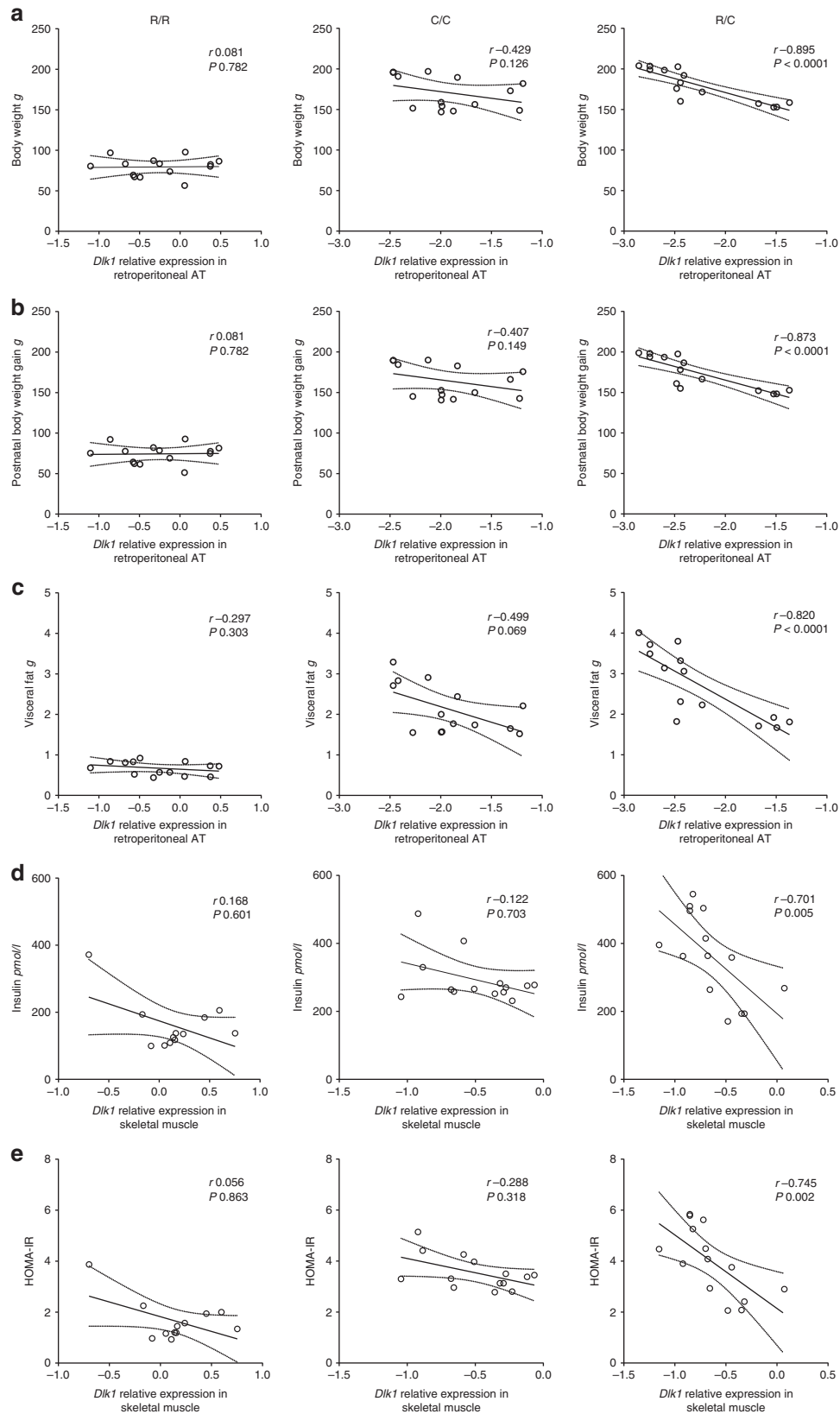


Fig. 1 Correlation graphs between *Dlk1* expression in adipose and skeletal muscle tissues and studied parameters in a *Wistar* rat catch-up growth model. Correlation graphs of different R/R, C/C and R/C groups between *Dlk1* relative expression in retroperitoneal adipose tissue (AT) and **a** body weight at PD42, **b** postnatal body weight gain and **c** visceral fat; as well as *Dlk1* relative expression in skeletal muscle and **d** insulin and **e** HOMA-IR values. Spearman r and p values are shown, P value was set at $p < 0.05$ and significant correlations are marked in bold style. Gene expression variables have been logarithmically transformed (\log_{10}). R/R: restricted, C/C: control, R/C: catch-up growth

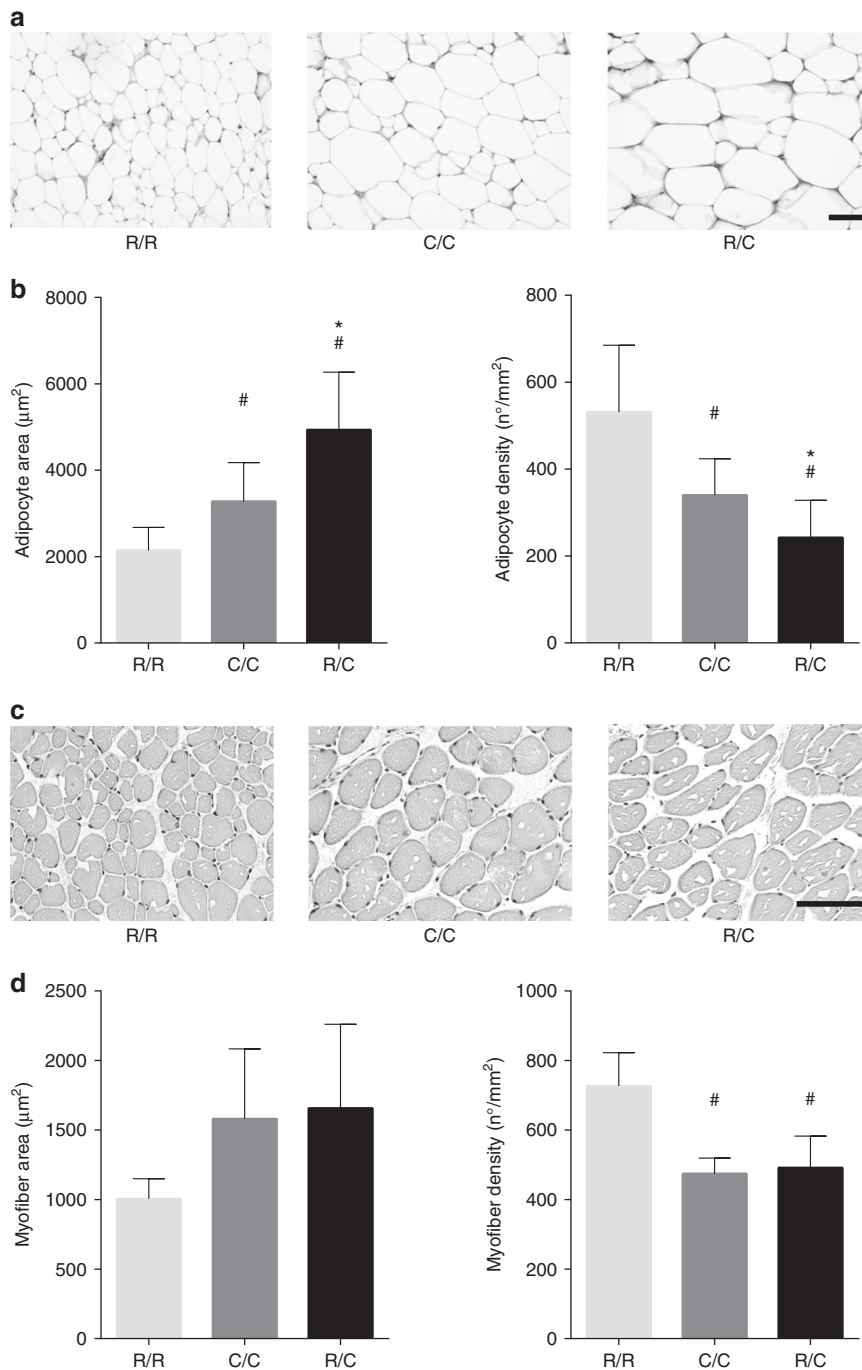


Fig. 2 Adipose tissue and skeletal muscle morphometric parameters in a *Wistar* rat catch-up growth model. Representative images of **a** retroperitoneal adipose tissue and **c** skeletal muscle at PD42 are shown (scale bar: 50 μm). Bar graphs showing **b** adipocyte area and adipocyte density (>250 adipocytes per group were measured), **d** myofibers area and myofiber density (>20 fields per group were measured) are shown (* $p < 0.05$ compared to C/C and [#] $p < 0.05$ compared to R/R)

showed hypertrophy of the retroperitoneal adipose tissue in R/C pups at P42, which was characterized by higher adipocyte area and lower adipocyte density. The low *Dlk1* expression observed in R/C pups may thus predispose these pups to postnatal adipose tissue expansion, especially of visceral fat, with a low number of preadipocytes being capable of differentiating into mature adipocytes, in turn contributing to adipocyte dysfunction. Indeed, low *Dlk1* gene expression in adipose tissue is likely to cause excessive adipocyte lipid storage, inflammation and altered adipokine secretion, all known to be involved in the development of insulin resistance and obesity.¹⁸ As it has been described that

cell size and/or lipid droplet locularity influence cortical actin structures that mediate GLUT4 translocation,²¹ we hypothesize that low *Dlk1* and related adipocyte hypertrophy could directly influence the development of insulin resistance.

Dlk1 is known to be a crucial regulator of the myogenic program in skeletal muscle, and it has been shown that *Dlk1* has a dual biological function in this tissue, both as an enhancer of muscle development and as an inhibitor of adult muscle regeneration.²² The observed low *Dlk1* expression in skeletal muscle could be involved in restraining of muscle development as we observed histologically lower number of myofibers per area

(myofiber density) in skeletal muscle of R/C pups at P42. In this sense, catch-up growth towards fat mass accumulation in detriment of muscular mass has been described in humans.²³ Low birth weight following free access to food during the growing-finishing period in pigs also showed enlarged cross-sectional myofiber areas due to enhanced fat deposition.²⁴ Moreover, fat mass accumulation in detriment of muscular mass has been related to insulin resistance and overexpression of insulin receptor in skeletal muscle in experimental animals.²⁵ In our model we observed decreased *Dlk1* expression in skeletal muscle in R/C pups that was related to increased HOMA-IR values. We therefore suggest that *Dlk1* expression in skeletal muscle is contributing to the development of insulin resistance during catch-up growth. It is tempting to speculate that lower *Dlk1* gene expression could enhance C/EBP α , a known downstream *Dlk1* target molecule, which in turn would regulate GLUT4 and affect glucose homeostasis.²⁶ Then, *Dlk1* dysregulation would be among the mechanisms explaining why skeletal muscle presents lower insulin-stimulated glucose utilization and develops insulin resistance during catch-up growth.

We acknowledge some limitations in our study. Major differences were observed between R/C and R/R pups, with fewer differences being evident between R/C and C/C pups. It should be highlighted, however, that there were strong linear differences between the 3 groups of pups in most of the examined parameters, which give relevance to our results. In future studies, our goal will be to examine more severe fat mass accumulation or obesity phenotypes during catch-up growth as well as to extend the growth period since P42 may have been rather a short juvenile time-point to examine the association between *Dlk1* and metabolic risk markers. Interestingly though, our findings indicate that these metabolic abnormalities take place at an early age following catch-up growth. Future studies assessing these parameters for a longer period should take into account the age-related sex dimorphism in insulin sensitivity that is evident starting at PD90 (3 months), and which has not been described in early stages, such as at PD26 or PD40, when the juvenile rats are still not sexually mature.^{27–29} Finally, we could not analyze the direction of causality of the molecular changes observed at PD42 in pups experiencing catch-up growth since the *Wistar* rat model of intrauterine growth restriction induced by calorie restriction throughout gestation used in the present study did not include tissues at birth: a few pups were removed to equal litter sizes within groups but they were insufficient in number to provide tissue samples at birth. Despite these limitations, we have observed novel molecular changes in *Dlk1* gene expression during catch-up growth.

In conclusion, decreased gene expression of *Dlk1* in adipose tissue and skeletal muscle may contribute to visceral fat expansion and development of insulin resistance during catch-up growth following fetal growth restriction. It is tempting to speculate that future intervention strategies based on *Dlk1* activation may improve the outcome of catch-up growth following prenatal growth restriction.

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AUTHOR CONTRIBUTIONS

All authors read and approved the final manuscript. G.C.-B., X.R., J.B. and A.L.-B. designed research; G.C.-B. and A.L.-B. wrote the first draft of the manuscript; G.C.-B., X.R. and A.P.-P. conducted research; G.C.-B., S.X.-T. and E.L.-M. analyzed data; and F.d.Z. and L.I. contributed to review the manuscript.

ADDITIONAL INFORMATION

The online version of this article (<https://doi.org/10.1038/s41390-019-0428-2>) contains supplementary material, which is available to authorized users.

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