

## ORIGINAL ARTICLE

## Catch-up growth following intra-uterine growth-restriction programmes an insulin-resistant phenotype in adipose tissue

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**BACKGROUND:** It is now widely accepted that the early-life nutritional environment is important in determining susceptibility to metabolic diseases. In particular, intra-uterine growth restriction followed by accelerated postnatal growth is associated with an increased risk of obesity, type-2 diabetes and other features of the metabolic syndrome. The mechanisms underlying these observations are not fully understood.

**AIM:** Using a well-established maternal protein-restriction rodent model, our aim was to determine if exposure to mismatched nutrition in early-life programmes adipose tissue structure and function, and expression of key components of the insulin-signalling pathway.

**METHODS:** Offspring of dams fed a low-protein (8%) diet during pregnancy were suckled by control (20%)-fed dams to drive catch-up growth. This 'recuperated' group was compared with offspring of dams fed a 20% protein diet during pregnancy and lactation (control group). Epididymal adipose tissue from 22-day and 3-month-old control and recuperated male rats was studied using histological analysis. Expression and phosphorylation of insulin-signalling proteins and gene expression were assessed by western blotting and reverse-transcriptase PCR, respectively.

**RESULTS:** Recuperated offspring at both ages had larger adipocytes ( $P < 0.001$ ). Fasting serum glucose, insulin and leptin levels were comparable between groups but increased with age. Recuperated offspring had reduced expression of IRS-1 ( $P < 0.01$ ) and PI3K p110 $\beta$  ( $P < 0.001$ ) in adipose tissue. In adult recuperated rats, Akt phosphorylation ( $P < 0.01$ ) and protein levels of Akt-2 ( $P < 0.01$ ) were also reduced. Messenger RNA expression levels of these proteins were not different, indicating a post-transcriptional effect.

**CONCLUSION:** Early-life nutrition programmes alterations in adipocyte cell size and impairs the protein expression of several insulin-signalling proteins through post-transcriptional mechanisms. These indices may represent early markers of insulin resistance and metabolic disease risk.

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**Keywords:** developmental programming; maternal under-nutrition; catch-up growth; adipose tissue; insulin resistance; type-2 diabetes

## INTRODUCTION

The prevalence of non-communicable diseases, such as obesity and type-2 diabetes (T2D), has reached epidemic proportions globally. Although traditionally viewed as being related to adult-lifestyle behaviours, in recent years, the role of the early-life environment in determining later disease risk has been the focus of numerous human studies worldwide.

Low birth weight (LBW) is associated with detrimental long-term metabolic consequences in humans.<sup>1–3</sup> The concept of developmental programming suggests that environmental insults during critical periods of development can trigger maladaptive changes in organ structure and function thus increasing susceptibility to obesity,<sup>4</sup> T2D,<sup>1</sup> cardiovascular disease<sup>5,6</sup> and metabolic syndrome in later life.<sup>2,7–9</sup> Accelerated postnatal growth, or 'catch-up growth', following intra-uterine growth restriction (IUGR) has also been shown to be important in the programming of later metabolic disease risk<sup>10,11</sup> as well as being an independent risk factor for overweight that can manifest as early as childhood.<sup>12</sup>

With the consistency of findings over the last 20 years, it is now essential to establish a better understanding of the underlying

mechanisms by which disease risk is programmed. Using a well-established rodent model of maternal protein restriction, we previously demonstrated that a mild nutritional insult during critical periods of development can give rise to offspring with aberrant early-life growth trajectories,<sup>13,14</sup> altered longevity<sup>15,16</sup> and changes in metabolism. An insulin-resistant phenotype has been observed in both male and female adult rats born to dams fed a low-protein diet throughout pregnancy and lactation.<sup>17,18</sup> This was associated with impaired expression of key insulin-signalling proteins.<sup>19–21</sup> However, it is not known if these effects are a result of exposure to maternal protein restriction during pregnancy alone and the mechanisms mediating such effects are unknown.

Postnatal exposure to a higher nutritional plane following IUGR in rodents—either through cross-fostering pups or changing the maternal diet—as well as reducing litter sizes drives accelerated postnatal growth. This is associated with increased adiposity and metabolic dysfunction.<sup>22–24</sup> Accelerated early postnatal growth in humans is also associated with increased risk of obesity later in life.<sup>25</sup> In addition, randomised intervention studies with different formula milk compositions have suggested that nutritionally

induced differences in neonatal growth are associated with long-term changes in adiposity.<sup>26,27</sup>

In the current study, we aimed to identify mechanisms of adipose tissue dysregulation arising as a consequence of LBW followed by accelerated growth using a rat model. We determined if adipose tissue of male 'recuperated' animals is susceptible to metabolic programming with a focus on canonical components of the insulin-signalling pathway. We then established if programmed changes occurred through transcriptional or post-transcriptional mechanisms. As the early postnatal period is important with regards to the programming of obesity risk in both humans and rodents,<sup>22,28,29</sup> we chose to study offspring just before weaning, and as young adults, enabling us to determine whether (mal)-adaptive changes take place early on as a consequence of the accelerated postnatal growth.

## MATERIALS AND METHODS

### Animals

All procedures involving animals were conducted in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act, 1986. Virgin female Wistar rats (240–260 g) were housed individually on a 12-h light/dark cycle (lights on between 0700–1900 hours) at 22 °C. After mating, pregnancy was confirmed by the presence and expulsion of a vaginal plug. Throughout gestation, pregnant dams were given *ad lib* access to either a control (20% protein, w/v) or an isocaloric low-protein diet (8% protein, w/v), the compositions of which have been published previously.<sup>14</sup> This dietary manipulation did not affect litter size and is in agreement with observations from previous cohorts of both mice<sup>30</sup> and rats<sup>13</sup> generated using this model. Control offspring were born to and suckled by control-diet-fed dams, and litter size was standardised to eight pups per dam on postnatal day 3 (PND3). To generate the 'recuperated' group, randomly selected male offspring of low-protein-fed dams were cross-fostered on PND3 to control-fed dams. Litter size was reduced to four pups per dam to maximise nutrition availability thus driving catch-up growth. Body weight of pups was recorded on the day of cross-fostering and then throughout the lactation period (PND7, PND14 and PND21). Pups were weaned on PND21 and fasted overnight. On the experimental day, 22-day-old males were killed by exposure to a rising concentration of CO<sub>2</sub>. Blood serum was collected via cardiac puncture. Blood glucose measurements from tail blood were recorded using a blood glucose analyser (Hemocue, Angelholm, Sweden). Epididymal fat pads were dissected, weighed, snap-frozen and stored at –80 °C. A portion of each epididymal fat pad was fixed in formalin.

One male from each of the control and recuperated litters was weaned on PND21 onto a standard laboratory chow diet (19.6 g protein, 58.3 g carbohydrate and 3.0 g fat, all per 100 g dry weight) (LAD1, SDS, Witham, Essex, UK). Body weight and food intake were recorded weekly thereafter. At 3 months ( $\pm$  3 days) of age, fasted males were killed and fat pads were collected as described above.

Blood glucose measurements were taken as described above. Serum concentrations of leptin and insulin were measured by enzyme-linked immunosorbent assay (CrystalChem, Downers Grove, IL, USA and Merckodia, Uppsala, Sweden, respectively). Serum lipid and cholesterol measurements were determined using a Dade Behring Dimension RXL analyser (Mouse Phenotyping Facility, Department of Clinical Biochemistry, Cambridge University Hospitals NHS Foundation Trust).

### Light microscopy: adipocyte morphology analysis

Epididymal adipose tissue from 22-day and 3-month-old control and recuperated rats was post-fixed, sectioned (5  $\mu$ m) and stained with haematoxylin and eosin. Images were captured on an inverted light microscope (Olympus BX41, Olympus UK Ltd, Southend-on-Sea, UK) and then cell area (whole cells only in field of view) was measured using CellAD software (Olympus). Analysis was carried out using either one field per section (for 22-day time point, sections:  $n=10$  control,  $n=11$  recuperated) or four fields per section (for 3-month time point, sections:  $n=4$  control,  $n=4$  recuperated).

### Protein expression analysis: western blotting

Epididymal tissue was homogenised in cold TK lysis buffer.<sup>14</sup> Total protein concentration in the lysates was determined using a Sigma copper/

bicinchoninic assay with bovine serum albumin (Sigma, Gillingham, UK) standards. The final protein concentration of samples was standardised to 0.5 mg ml<sup>-1</sup>. Ten micrograms protein was loaded onto a 10% polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA, USA) and the membrane blocked (5% Marvel, 1% TBS and 0.1% Tween-20) for 1 h at room temperature. Membranes were probed overnight (4 °C) with primary antibodies (diluted 1:1000 in 1  $\times$  TBS, 0.1% Tween-20 and 5% bovine serum albumin, unless otherwise specified): phospho-Akt Ser 473 (p-Akt, #4585), Akt-1 (#2967), Akt-2 (#2962, 1:5000), PI3-K catalytic subunit p110 $\beta$  (#30115) (all from Cell Signalling, New England Biolabs, Hitchin, UK); PI3-K regulatory subunit p85 $\alpha$  (06–195) and insulin receptor substrate-1 (IRS-1, #32682) (Upstate, Millipore); and insulin receptor- $\beta$  (IR- $\beta$ , C19 sc-711, 1:200 in phosphate buffered saline) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was then placed in anti-rabbit or anti-mouse secondary (1:10 000, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature (1  $\times$  TBS, 0.1% Tween-20 and 5% Marvel) and detected onto hyperfilm (GE Healthcare, Amersham, UK) using a chemiluminescent enhancer substrate (West Pico SuperSignal, Thermo Scientific, Rockford, IL, USA). Optical densities of the immunoreactive protein bands were quantified by spot-densitometry (AlphaEase 4.0, Alpha Innotech/Protein Simple, Santa Clara, CA, USA). Twenty-four samples ( $n=6$  for each of the four groups) were run on a single gel alongside molecular weight markers. In addition, 10  $\mu$ g and 5  $\mu$ g of pooled sample were loaded onto each gel to confirm the linearity of the signal.

### Gene expression analysis: quantitative reverse-transcriptase PCR

Gene expression analysis was measured in epididymal adipose tissue from 3-month-old male rats. RNA was isolated from 50 mg of tissue using a mirVana miRNA isolation kit (Life Technologies/Ambion, Austin, TX, USA). RNA was then quantified on a NanoDrop spectrophotometer (NanoDrop Technologies/Thermo Scientific, Wilmington, DE, USA). Complementary DNA was synthesised using a High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, Warrington, UK) with incubation steps of 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C, on a thermal cycler (MJ Research PTC-200/Bio-Rad, Hemel Hempstead, UK). Primers (Figure 4a) (Sigma Custom Products, Haverhill, UK) were designed using Universal Probe Library Assay Design Centre (Roche, Welwyn Garden City, UK) and their specificity for the target was tested using Primer-blast (NCBI). Quantitative reverse-transcriptase PCR was carried out using Sybr green PCR master mix (Applied Biosystems) on the StepOnePlus PCR machine (Applied Biosystems, Paisley, UK). Dissociation curves revealed a single product. Messenger RNA (mRNA) expression was normalised to that of the housekeeping gene *cyclophilin-A*, the expression of which did not differ between experimental groups.

### Statistical analysis

Prism (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. A standard two-tailed Student's *t*-test was used for analysis of phenotypic characteristics between control and recuperated groups within each age group and for pre-weaning growth trajectory analyses. Serum measures and protein expression data were analysed by two-way analysis of variance with maternal diet and age as independent variables. Bonferroni *post-hoc* tests were applied where appropriate. mRNA expression data were analysed using standard two-tailed student's *t*-test and are expressed relative to that of the housekeeping gene *cyclophilin-A*. Repeated measures analysis of variance was used to analyse food intake and body weight beyond weaning. Insulin measurements were log-transformed before testing as data were not normally distributed, and are presented as geometric means (95% confidence intervals). Non-parametric Mann-Whitney *t*-test was used to analyse differences in adipocyte area between control and recuperated groups as data were not normally distributed, and data are presented as median values (with interquartile range). All other data are presented as mean  $\pm$  s.e.m. In all cases, *P*-values < 0.05 were accepted as significant and *n* refers to the number of litters (no more than one animal per litter was studied for each parameter).

## RESULTS

### Offspring body weight, fat mass and serum characteristics

Table 1 shows the postnatal body weight of control and recuperated rats throughout the lactation period. Recuperated rat offspring were significantly lighter than controls on PND3

**Table 1.** Offspring postnatal growth trajectory

Postnatal day	Body weight (g)	
	Control	Recuperated
3	7.42 ± 0.16	6.16 ± 0.12***
7	15.85 ± 0.75	13.36 ± 0.61*
14	31.99 ± 1.12	32.47 ± 1.36
21	52.17 ± 1.36	55.06 ± 1.78

Body weight of control ( $n = 13$ ) and recuperated ( $n = 10$ ) male rats during the lactation period. Data are presented as mean ± s.e.m. Student's *t*-test; \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with same age controls.

( $P < 0.0001$ ) and remained lighter at PND7 ( $P = 0.022$ ). However, they then underwent catch-up growth and by PND14 had attained body weights similar to control offspring. By PND21, recuperated offspring were heavier than controls; however, this difference was not statistically significant.

The growth trajectory of control and recuperated rats beyond weaning can be seen in Figure 1a. Body weight gain from postnatal day 28–70 tended to be higher in recuperated offspring than in controls ( $P = 0.062$ ). Body weight at the time of postmortem was higher in recuperated animals compared with controls ( $P < 0.05$ ). Mean food intake and mean cumulative food intake between weaning and 3 months of age was similar in both groups (Figure 1b and Table 2).

As shown in Table 2, recuperated rats have a significantly higher absolute epididymal fat mass compared with control offspring at 22 days of age ( $P = 0.039$ ); however, the increased fat mass was not significantly different between groups at 3 months of age.

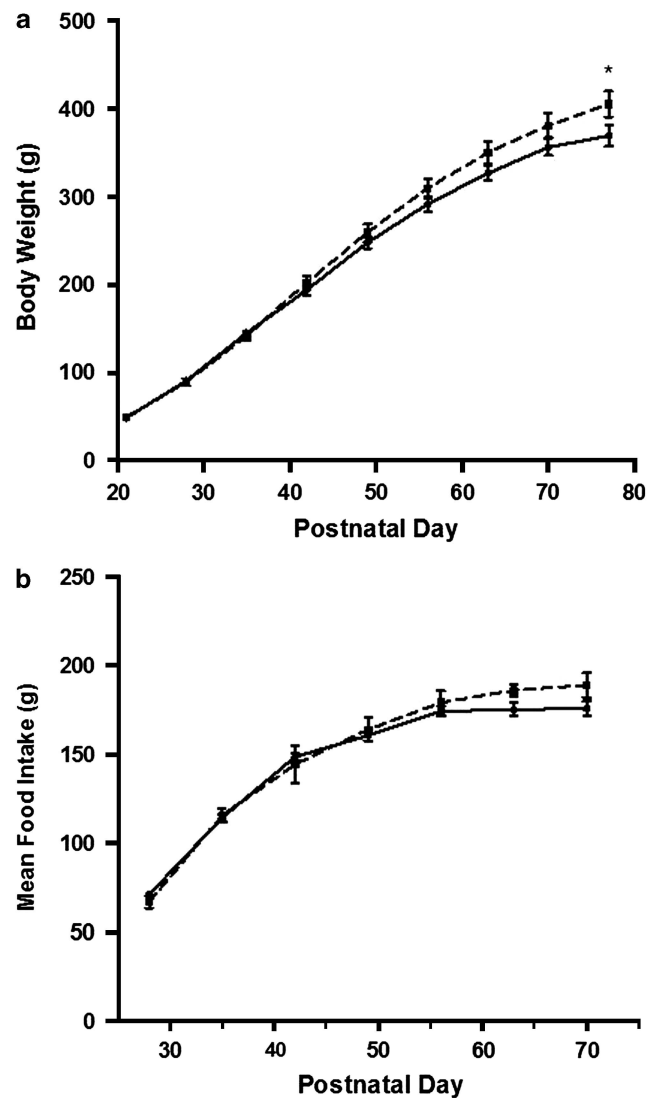
As shown in Table 2, fasting plasma glucose was similar between experimental groups and did not change with age. There was an overall effect of age on fasting serum levels of insulin ( $P < 0.001$ ), leptin ( $P < 0.001$ ), triglycerides ( $P < 0.01$ ) and cholesterol ( $P < 0.001$ ). For insulin, leptin and triglycerides, levels increased with age but levels of cholesterol decreased with age. There was also an overall effect of maternal diet on serum cholesterol ( $P < 0.05$ ) with recuperated animals showing elevated levels compared with controls.

#### Epididymal adipose tissue morphology

At 22 days of age, epididymal adipose tissue from recuperated animals contained a lower percentage of small adipocytes compared with controls ( $P < 0.001$ ) (Figure 2 and Table 2). At 3 months of age, recuperated epididymal adipose tissue still contained a lower percentage of small adipocytes ( $P < 0.001$ ) (Figure 2 and Table 2). For representative images see Figure 2a.

#### Epididymal adipose tissue levels of insulin-signalling proteins

As shown in Figure 3, protein levels of IR- $\beta$  were not different between experimental groups at either age. There was an overall effect of both maternal diet and age on IRS-1. Recuperated animals at both ages had significantly reduced levels of IRS-1 compared with controls ( $P < 0.001$ ). Three-month-old animals had higher protein levels of IRS-1 compared with 22-day-old rats ( $P < 0.05$ ). Levels of the regulatory PI3-kinase subunit, p85 $\alpha$ , were not different between experimental groups at either age. In contrast, there was an overall effect of both maternal diet and age on the p110 $\beta$  catalytic subunit of PI3-kinase. Recuperated animals had significantly reduced levels of p110 $\beta$  compared with controls ( $P < 0.001$ ). Three-month-old animals had higher protein levels of p110 $\beta$  compared with 22-day-old rats ( $P < 0.001$ ). There was an interaction between maternal diet and age on the expression of



**Figure 1.** (a) Post-weaning growth trajectory and (b) mean food intake (per rat per week) of control (solid line) and recuperated (dashed line) rat offspring. Data are presented as mean ± s.e.m. and were examined using repeated measures analysis of variance, \* $P < 0.05$ . Control  $n = 11$ , recuperated  $n = 10$ .

Akt-1 ( $P < 0.05$ ) and Akt-2 ( $P < 0.01$ ) such that levels of these proteins were lower in recuperated animals compared with controls at 3 months of age but not at 22 days of age. There was also an interaction between maternal diet and age on phosphorylated levels of Akt (Ser473) ( $P < 0.05$ ) with pAkt being undetectable in 3-month-old recuperated offspring. Furthermore, Akt phosphorylation levels decreased between weaning and 3 months ( $P < 0.01$ ). Adipose tissue GLUT-4 protein levels were higher in 3-month-old rats compared with 22-day olds ( $P < 0.001$ ), but were not affected by maternal diet.

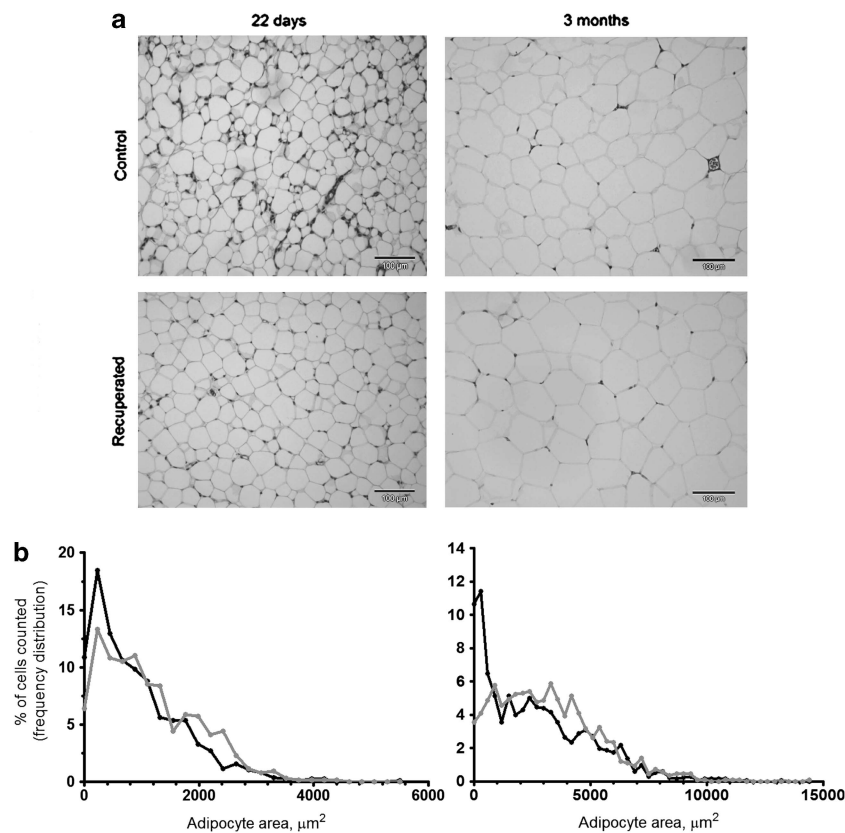
#### Epididymal adipose tissue mRNA expression of insulin-signalling genes

The mRNA expression of IRS-1, p110 $\beta$  and Akt-2 were comparable between control and recuperated animals at 3 months of age (Figure 4b). mRNA expression levels were normalised to that of cyclophilin-A, which did not differ between groups (control  $371.7 \pm 65.8$  vs recuperated  $408.5 \pm 105.5$ ).

**Table 2.** Offspring characteristics

	22d Control	22d Recuperated	3m Control	3m Recuperated
	(n = 13)	(n = 10)	(n = 11)	(n = 10)
Body weight (g)	50.1 ± 1.5	51.6 ± 1.8	369.5 ± 12.1	405.2 ± 14.7
Mean cumulative food intake, PND 28–70 (g)	—	—	1019 ± 14	1044 ± 31
Epididymal mass (g)	0.12 ± 0.01	0.19 ± 0.03 <sup>^</sup>	5.3 ± 0.3	5.9 ± 0.4
Adipocyte area (μm <sup>2</sup> )	707 (268, 1337)	957*** (439, 1700)	2202 (570, 4068)	2928*** (1448, 4496)
Plasma glucose (mmol l <sup>-1</sup> )	4.4 ± 0.3	5.0 ± 0.3	4.3 ± 0.2	4.6 ± 0.2
Insulin (pmol l <sup>-1</sup> )	20 (13–30)	10 (5–22)	131 (81–212)	135 (85–213) <sup>†</sup>
Leptin (ng ml <sup>-1</sup> )	1.2 ± 0.1	1.6 ± 0.15	5.5 ± 0.6	5.7 ± 0.04 <sup>†</sup>
Cholesterol (mmol l <sup>-1</sup> )	2.7 ± 0.2	3.1 ± 0.2	1.2 ± 0.06	1.5 ± 0.1 <sup>†</sup> ,#
Triglycerides (mmol l <sup>-1</sup> )	1.3 ± 0.1	1.1 ± 0.1	1.8 ± 0.2	1.5 ± 0.2 <sup>†</sup>
Free fatty acids (μmol l <sup>-1</sup> )	687 ± 35	762 ± 33	850 ± 71	818 ± 77

Abbreviation: PND, postnatal day. Control and recuperated rat offspring characteristics at 22 days (22d) and 3 months (3m) of age. Insulin data are presented as geometric means (95% confidence intervals) and were logged before statistical analysis. Adipocyte area is presented as median values (interquartile range). All other data are presented as mean ± s.e.m. Plasma and serum measures were taken in the fasted state. Student's *t*-test; <sup>^</sup>*P* < 0.05 vs same age control. Two-way analysis of variance; <sup>#</sup>*P* < 0.05 for effect of maternal diet, <sup>†</sup>*P* < 0.01 for effect of age. Mann–Whitney *t*-test, \*\*\**P* < 0.001 vs same aged controls.



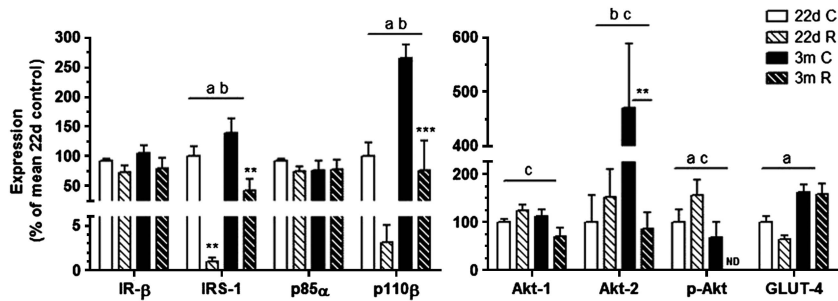
**Figure 2.** Epididymal adipose tissue morphology at 22 days and 3 months of age in control and recuperated rats. **(a)** Representative images of epididymal adipose tissue sections from control (top) and recuperated (bottom) male rats at 22 days (left) and 3 months (right) of age. **(b)** Adipocyte area frequency distribution of control (black line, *n* = 10) and recuperated (grey line, *n* = 11), 22-day-(left) and 3-month-(right) old rats. Median adipocyte area in recuperated epididymal adipose tissue differs from that of control at both ages (*P* < 0.001, Mann–Whitney test). Sections stained with haematoxylin and eosin. Scale bar, 100 μm.

## DISCUSSION

It is well established that LBW individuals are at increased risk of developing insulin resistance and T2D,<sup>31</sup> and that accelerated postnatal growth is also an important determinant of metabolic disease risk. Using a well-established rodent model of IUGR and catch-up growth, here we show that recuperated rats have reduced

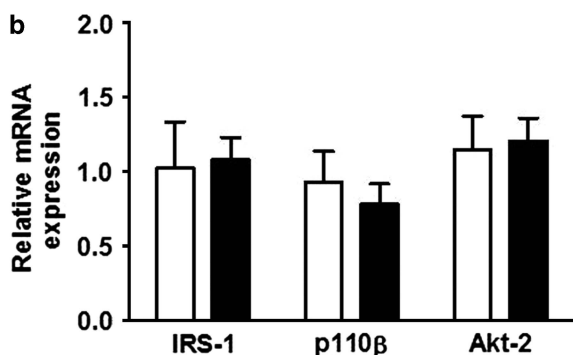
levels of key components of the insulin-signalling pathway in epididymal adipose tissue. Many of these signalling impairments are apparent at 3 weeks of age indicating that metabolic adaptations occur early in life, in the absence of differences in circulating glucose or insulin levels. They may therefore contribute to the increased risk of metabolic disease and are not a consequence of it.





**Figure 3.** Insulin-signalling protein phosphorylation and expression in epididymal adipose tissue from 22-day- (22d) and 3-month-old (3m) control (C) and recuperated (R) male rats. Two-way analysis of variance revealed an overall effect of age (a), maternal diet (b) and/or an interaction between age and maternal diet (c); \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with same age controls according to Bonferroni's *post hoc* test. Data are expressed as a percentage of mean 22-day controls  $\pm$  s.e.m.,  $n = 4-6$  per group. ND, not detected.

Target	Primer Sequence
IRS-1	F CCATGCGGGCCATGAGCGAT
	R CCGAGTCAGCCCCACTTGCC
p110-β	F ACGCGTACCTGATTTTACGG
	R TTTGACTGATGTGAGCTCGG
Akt-2	F GGAGGCCCGGTACTTCCTT
	R CGGGGGCCTCAGGTCTCTCT
Cyc-A	F TTTGCAGACGCCGCTGTCTCTT
	R ACCCAAGGGCTCGCCATCAGC



**Figure 4.** mRNA expression of insulin-signalling components in epididymal adipose tissue from 3-month-old control and recuperated male rats. (a) Forward (F) and reverse (R) primer sequences. (b) mRNA expression determined by quantitative reverse-transcriptase PCR and normalised to mRNA expression of housekeeping gene *cyclophilin-A* (*Cyc-A*). Data presented as mean  $\pm$  s.e.m.;  $n = 6$  per group.

Adipocytes from individuals with a known heritable predisposition for diabetes (those with at least two first-degree relatives with T2D) show low levels of IRS-1 protein, reduced serine phosphorylation of Akt, impaired cellular insulin-stimulated PI3-kinase activity and impaired glucose transport. Such defects are comparable to those seen in adipocytes from individuals with frank diabetes<sup>32</sup> and are similar to those observed in this study of nutritionally programmed animals. In adult recuperated rats, adipose tissue protein expression of IRS-1, p110β and Akt-2 as well as phosphorylation levels of Akt (Ser473) were all significantly reduced compared with controls. It is therefore possible that genetic and programmed predisposition operate through similar pathways.

One of the earliest, as well as most pronounced, defects in insulin-signalling protein expression was that of IRS-1. It is therefore likely that IRS-1 in adipose tissue may be an early target of nutritional programming. In rats, short-term *in vivo* inhibition of IRS-1 expression has been shown to lead to a reduction in insulin-stimulated phosphorylation of Akt and whole-body insulin resistance.<sup>33</sup> Furthermore, IRS-1-targeted gene deletion in mice results in impaired glucose tolerance, hyperinsulinaemia, defective beta cell function and impaired insulin signalling.<sup>34,35</sup>

Significant effects of maternal diet were also observed in relation to p110β protein expression with lower levels seen in adipose tissue of recuperated offspring. It is well established that PI3K is important in the metabolic response to insulin in adipocytes.<sup>36</sup> PI3K-p110β is an important component of the signalling cascade and has a key role in glucose transport and inhibition of lipolysis in response to insulin stimulation.<sup>37</sup> We demonstrated previously that p110β is reduced in adipose tissue from rats born to dams fed a low-protein diet throughout both pregnancy and lactation.<sup>21</sup> The current findings suggest that suboptimal nutrition during fetal life alone is sufficient to lead to a programmed reduction in p110β protein expression. This is consistent with our observation in young LBW men in whom we demonstrated a reduction in p110β protein in adipose tissue.<sup>38</sup> We have also demonstrated reduced p110β and IRS-1 in muscle and liver, respectively, from female offspring of obese mouse dams.<sup>39,40</sup> Such observations indicate that these proteins forming key nodes of the canonical insulin-signalling pathway<sup>36</sup> are particularly vulnerable to the adverse effects of early-life nutritional programming.

There are a number of mechanisms by which programmed changes in protein expression of p110β, IRS-1 and Akt-2 could arise. There has been much recent focus in the programming field on the potential role of epigenetic changes, such as altered DNA methylation or histone modifications.<sup>41,42</sup> Here, we showed that mRNA expression of these signalling components were not affected, suggesting that the programmed changes were occurring at the post-transcriptional level. This is again consistent with our observation in adipose tissue of LBW men.<sup>38</sup> One

potential mechanism by which post-transcriptional gene regulation could occur is through microRNAs (small non-protein-coding RNA species). The deregulation of numerous microRNAs has been implicated in obesity and T2D pathophysiology,<sup>43</sup> and we have also previously shown that microRNAs in adipose tissue are susceptible to programming.<sup>44</sup>

In our rat model, recuperated offspring typically do not become overweight compared with controls when fed a chow diet.<sup>16,45</sup> However, adipocyte hypertrophy was observed in recuperated rats at 22 days and 3 months of age. At 22 days of age, this was accompanied by an increase in absolute fat mass. Increased adiposity and adipocyte hypertrophy have previously been observed in 38-week-old offspring of both protein- and food-restricted rats that then underwent catch-up growth induced by postnatal overfeeding.<sup>22</sup> Using *in vitro* experiments, Bol *et al.*<sup>46</sup> demonstrated an altered mitotic rate of preadipocytes from rats exposed to IUGR and catch-up growth suggesting that increased adiposity could also be related to an increase in cell number.

At weaning and as young adults, recuperated rats did not have altered concentrations of glucose or insulin. Previous studies suggest that the development of insulin resistance following suboptimal nutrition *in utero* occurs much later in life.<sup>47</sup> Indeed, the development of insulin resistance is associated with ageing. Proximal components of the insulin-signalling pathway, including expression of IRS-1, have been shown to be reduced in epididymal adipocytes from aged (~2 years old) compared with adult (3-month old) rats.<sup>48</sup> That we observe similar changes in our adult recuperated rats to these non-IUGR aged rats suggests a state of accelerated aging in recuperated animals owing to their prior exposure to an early-life nutritional insult. These findings are in agreement with the accelerated-aging phenotype we see in aorta, kidney and pancreas of recuperated rat offspring,<sup>16,45,49</sup> and the reduced longevity observed in both recuperated rats and mice.<sup>15,16</sup>

Only male offspring were studied here. Reports in the literature indicate that early-life programming outcomes are gender specific, as well as being dependent on the timing and nature of the insult.<sup>22,50</sup> Based on previously published findings, female recuperated offspring develop increased adiposity<sup>50</sup> and hyperglycemia.<sup>51</sup> We have previously shown that female offspring of dams fed a low-protein diet throughout both pregnancy and lactation develop hyperinsulinemia but at a later age to males.<sup>17</sup> In addition, we have shown that although female rats live longer than male rats, recuperated females demonstrate the same reduction in life span as males. Based on these observations, we would expect female recuperated animals to also develop a metabolic phenotype, albeit at a later age.

In summary, our results show that LBW followed by accelerated postnatal growth programmes adipose tissue dysfunction and provide a possible mechanism leading to insulin resistance, increased T2D risk and accelerated ageing. In particular, programming of the key insulin-signalling proteins IRS-1 and p110 $\beta$  observed here is similar to defects seen in individuals predisposed to diabetes as well as LBW individuals at risk of diabetes. These therefore represent identifiable early disease risk markers. Further deciphering the underlying mechanisms leading to programmed changes in insulin-signalling protein expression and their causal role in linking early-life nutrition and metabolic disease could help to identify at-risk individuals and facilitate interventions to curb age-associated metabolic decline.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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