Adipose tissue plasticity during catch-up fat driven by thrifty metabolism:
Relevance for muscle-adipose glucose redistribution during catch-up growth

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Short running title: Adipose tissue plasticity during catch-up growth

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Objective: Catch-up growth – a risk factor for later type 2 diabetes – is characterized by hyperinsulinemia, accelerated body fat recovery (catch-up fat) and enhanced glucose utilization in adipose tissue. Our objective was to characterize the determinants of enhanced glucose utilization in adipose tissue during catch-up fat.

*Research Design and Methodology***:** White adipose tissue morphometry, lipogenic capacity, fatty acid composition, insulin signalling, *in-vivo* glucose homeostasis, and insulinemic response to glucose were assessed in a rat model of semistarvation-refeeding. This model is characterized by glucose redistribution from skeletal muscle to adipose tissue during catch-up fat that results solely from suppressed thermogenesis (i.e. without hyperphagia).
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Results: Adipose tissue recovery during the dynamic phase of catch-up fat is accompanied by increased adipocyte number with smaller diameter, increased expression of genes for adipogenesis and *de-novo* lipogenesis, increased fatty acid synthase activity, increased proportion of saturated fatty acids in triglyceride (storage) fraction but not in phospholipid (membrane) fraction, and no impairment in insulin signalling. Furthermore, it is shown that hyperinsulinemia and enhanced adipose tissue *de-novo* lipogenesis occur concomitantly and are very early events in catch-up fat.

Conclusions: These findings suggest that increased adipose tissue insulin stimulation and consequential increase in intracellular glucose flux play an important role in initiating catch-up fat. Once activated, the machinery for lipogenesis and adipogenesis contribute to sustain an increased insulin-stimulated glucose flux towards fat storage. Such adipose tissue plasticity could play an active role in the thrifty metabolism that underlies glucose redistribution from skeletal muscle to adipose tissue.

The pattern of growth early in life is now recognized to be an important predictor of chronic metabolic diseases. In particular, people who had low birth weight or whose growth faltered during infancy and childhood, but who subsequently showed catch-up growth, had higher propensity for the development of abdominal obesity, type 2 diabetes and cardiovascular diseases later in life (1-8). The mechanistic basis of the link between catch-up growth and risks for these chronic diseases is poorly understood. There is however compelling evidence that mammalian catch-up growth is characterized by a disproportionately higher rate of body fat than lean tissue gain (9), and that an early feature of such 'preferential catch-up fat' is concomitant hyperinsulinemia (10).

Using a rat model of semistarvation-refeeding (11), in which catch-up fat is studied in the absence of hyperphagia, we previously showed that the hyperinsulinemic state of catch-up fat preceded the development of excess adiposity and could be linked to suppressed thermogenesis *per se* in the absence of hyperphagia (12). Subsequent studies of hyperinsulinemic-euglycemic clamps during catch-up fat showed that *in vivo* insulin-mediated glucose utilization was diminished in skeletal muscle but enhanced in white adipose tissue (WAT), suggesting that preferential catch-up fat is characterized by glucose redistribution from skeletal muscle to WAT (13). Consistent with this hypothesis are the demonstrations, in this rat model of catch-up fat, of diminished mitochondrial mass and lower IRS1-associated phosphatidylinositol-3-kinase (PI3K) activity in the skeletal muscle (14, 15). Furthermore, *ex-vivo* studies in WAT have previously shown that glucose uptake and utilization are enhanced during refeeding after fasting or caloric restriction (16, 17).

Elucidating the mechanisms that underlie such enhancement in glucose uptake and glucose flux towards lipid synthesis in WAT is therefore of central importance in understanding the mechanisms of glucose redistribution during catch-up fat. In addressing this topic here, we have characterized our rat model of catch-up fat for changes in adipose tissue morphometry (adipocyte size and number) and fatty acid composition given their importance as determinants of WAT responsiveness to the action of insulin on glucose utilization. Indeed, it is established that small adipocytes have a greater capacity for insulin-mediated glucose uptake and *de-novo* lipogenesis than larger ones (18-22), while alterations in adipocyte membrane phospholipid composition in favor of a high ratio of polyunsaturated to saturated fatty acids correlates with increased rate of insulin-stimulated glucose transport and glucose flux towards *de-novo* lipogenesis in WAT (23-24). We have therefore investigated here the extent to which differences in adipocyte number and diameter, key gene markers for adipocyte proliferation as well as the fatty acid composition of phospholipid and triglyceride lipid fractions of WAT might be involved in the enhanced glucose flux towards lipogenesis. Furthermore, given the importance of insulin signalling in adipocyte growth (25) and in controlling glucose flux towards lipogenesis (26, 27), we have also evaluated the *in-vivo* insulinemic response to glucose and investigated proximal insulin signalling in WAT under basal and *in-vivo* insulin-stimulated conditions during catch-up fat.

RESEARCH DESIGN AND METHODS

Animals and Diet. Male Sprague Dawley rats (Elevage Janvier, France), caged singly in a temperature-controlled room (22 ±1°C) with a 12-h light/dark cycle, were

maintained on a commercial chow diet (Kliba, Cossonay, Switzerland) consisting, by energy, of 24% protein, 66% carbohydrates, and 10% fat, and had free access to tap water. Animals were maintained in accordance with our institute's regulations and guide for the care and use of laboratory animals.

Design of study . The experiments were performed in rats within an age-range characterized by high rate of growth for controls and by catch-up growth in the refed group during ad libitum access to food (supplementary file available at http://diabetes.diabetesjournals.org). The experimental design is similar to that previously described in establishing a rat model for studying changes in energy expenditure that occur during accelerated fat deposition (i.e. Catch-up fat) upon refeeding after growth arrest (11, 12), i.e. an approach that allows suppressed thermogenesis specific for accelerated fat recovery (catch-up fat) to be studied in the absence of confounding variables, such food intake and differential rates of protein gain, on energy expenditure. In brief, groups of 7 wk old rats (mean body weight of 225g) are food-restricted at 50% of their spontaneous food intake for 2 wks. After this period of growth arrest, they are refed the same chow diet at a level equal in metabolizable energy content to the spontaneous food intake of control rats matched for weight at the onset of refeeding. Under these conditions, the refed animals show similar gain in lean mass, but about 2-fold increase in body fat gain than controls for a period of 2 wks, due to 10-13% lower energy expenditure resulting from suppressed thermogenesis (11, 12).

Body composition. Body composition was determined at the end of semistarvation (corresponding to day 0 of refeeding), and subsequently on day 5, 10, 15 and 20 of refeeding in refed and control groups, as indicated in Fig. 1. After sacrifice, the whole carcasses were dried to a constant weight in an oven maintained at 70 °C and subsequently homogenized for analysis of fat content by the Soxhlet extraction method as previously described (11); the dry lean body mass (a proxy of protein mass) was determined by subtracting total body fat and body water content from body weight.

Glucose tolerance test. Intraperitoneal glucose tolerance test (GTT) was performed as previously described (12). Plasma glucose was determined using a Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA) while plasma insulin was assessed using rat insulin ELISA kit (Crystal Chem Inc, IL, USA).

Adipocyte number and size. Fixation with osmium tetroxide and isolation of adipocytes for cell counting/sizing were performed according to the method of Hirsch and Gallian (28), and suspensions of adipocytes were analyzed using Multisizer™ 3 Coulter Counter®. For determination of cell size (adipocyte diameter distribution), similar amounts of cells (approximately 10'000) were aspirated by the machine and classified according to their diameter and frequency.

Molecular measurements and lipid biochemistry. After harvesting, the adipose tissue was snap-frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated from 50–150 mg of powdered adipose tissue using the method of Chomczynski and Sacchi (29). After phase separation, RNA was precipitated with isopropanol, cDNA was synthesized from 250ng of total RNA and RT-PCR was performed. Cyclophilin was used as an invariant control since we found that its mRNA levels in white adipose tissue are not significantly altered in response to semistarvation or to refeeding in our rat model. The relative quantification for a given gene was thus corrected for the cyclophilin mRNA values.

Fatty acid synthase (FAS) activity was measured according to the protocol described by Pénicaud et al. (30). For fatty acid

profiling, equal amounts of tissue samples were suspended in (1:2:0.8, v/v/v), vortexed and incubated for 10 min at room temperature. Lipids were extracted using the method of Bligh and Dyer (31) and the fatty acid methyl esters prepared as described by Morrison and Smith (32) were analyzed by gas chromatography/mass spectrometry as detailed previously (33).

For PI3K assay, 200-500 mg of protein extract was immunoprecipitated with IRS-1 (Upstate) polyclonal antibody. The kinase reaction, thin-layer chromatography separation and signal detection were performed as previously described (15, 34). Akt (Ser 473) and ERK (p44/42 MAPK) were evaluated by immunoblot analysis. 40 μg of protein extract were separated by SDS-PAGE and blotted on PVDF membranes that were analyzed with polyclonal antibodies (Cell Signaling) raised against the indicated phosphorylation sites. Bands within the linear range were quantified densitometrically. Total protein amount of Akt and ERK were measured in the same way by using corresponding antibodies (Cell Signaling).

***In vivo* bolus administration of insulin.** The rats were fasted from 7:00 a.m, and 4-7 h later subgroups were anesthetized by injection of ketamine/xylazine (39/5 mg/kg b.w) and surgically prepared for a bolus injection (through the jugular vein) of insulin (10 U/kg b.w) (Actrapid®, Novo Nordisk) or an equal volume of saline vehicle. Insulin or saline was injected 3 min before sacrifice and tissue harvest. Immediately after sacrifice, the epididymal adipose tissue as well as skeletal muscles were rapidly dissected, frozen in liquid nitrogen, and stored at –80 °C until analysis. The timing for tissue harvesting after hormonal administration, and the dose of insulin utilized, correspond to maximal activation of PI3K assessed in preliminary studies, and are consistent with those obtained by others (35).

Data analysis and statistics All data are presented as means ± SEM. Direct comparisons between the two groups were performed using unpaired t-tests. Between-group differences in distribution curves for adipocyte diameter were analysed by the Kolmogorov-Smirnov test - which takes into account the ordering (i.e. cell diameter) of the categories and is therefore a suitable test for analysis of curve shifts. Between-group differences across time were analysed by regression analysis incorporating between-group comparison of slopes. For the study comparing refed and control animals in response to hormone or saline, the data were analyzed by two-factor ANOVA for the main effects of groups (refed *vs*. control), treatment (hormone *vs*. saline) and group x treatment interactions. All statistical treatment of data was performed using the computer software *STATISTIX 8* (Analytical Software, Tallahassee, FL, USA).

RESULTS

Body composition and glucose tolerance test. In this rat model of catch-up fat, the end of semistarvation (or onset of refeeding) is characterized by the fact that the semistarved and control animals display similar body weight (**Fig. 1A**) and dry lean body mass (**Fig. 1C**), but compared to control rats, the semistarved animals have 50% less fat mass (8 *vs* 15.7 g, p<0.001) (**Fig. 1D**) and higher body water content (155 *vs* 148g, p<0.01). Between day 0-15 of refeeding on an isocaloric food intake relative to the controls (**Fig. 1B**), the refed rats gained fat at a rate which is about 2 times greater than that of controls (**Fig. 1D**), whereas during day 15-20, both groups display similar rate of fat gain. This enhanced efficiency of catch-up fat during day 0-15 contrasts with no differences in lean body mass gain (**Fig. 1C**). GTT conducted on days 8-9 of refeeding show no between-group differences in glucose homeostasis (**Fig. 1E**), but reveal plasma

insulin concentrations after the glucose load which are clearly higher in refed than in control animals (p<0.01 by ANOVA test) (**Fig. 1F**).

Adipocyte number and size. The data on adipocyte cell size distribution (**Fig. 2A**, left-hand panel), mean adipocyte diameter (**Fig. 2D**) and adipocyte cell number (**Fig. 2C**) from epididymal white adipose tissue (EWAT) are presented for refed and control animals at different time-points during the phase of catch-up fat. After 2 wks of semistarvation (corresponding to day 0 of refeeding), the weight of EWAT in semistarved animals is found to be significantly lower than in controls (**Fig. 2B**) and is associated with a significant shift-to-the-left of adipocyte diameter distribution (p<0.001) and hence reduction in average adipocyte diameter, without a significant reduction in adipocyte cell number (**Fig. 2C**). During the first 15 days of isocaloric refeeding, EWAT weight increases faster in refed than in control groups (**Fig. 2B**), and the rate at which adipocyte cell number increases is also found to be significantly greater in refed than in control groups, as indicated by statistical comparisons of their slopes of regression (**Fig. 2C**). Adipocyte diameter remains significantly lower in EWAT from refed animals than in controls on day 5 and on day 10 (**Fig. 2A** and **Fig. 2D**), and only exceeded those of controls at day 15. Thus, the recovery of adipose tissue, even in the absence of hyperphagia, is accompanied by an increase in adipocyte number, while the adipocyte diameter remains lower in refed than in control animals during the dynamic phase of catch-up fat, lasting for at least 10 days in this study.

Adipose tissue FAS activity. The data on FAS activity in EWAT on day 0, 5, 10 and 15 of refeeding are shown in **Fig. 3**. At the end of semistarvation (day 0), the activity of FAS is found to be lower than in controls by about 30% (p<0.05), but overshoots above

control upon refeeding. It is significantly higher in refed animals than in controls on day 5 (+60%, p<0.01) and on day 10 (+25%, p<0.05), whereas at day 15, there are no longer significant differences between refed and controls.

Adipose fatty acid composition. The data on the fatty acid profile of the triglyceride and phospholipid fractions obtained from EWAT of animals on day 8-9 of refeeding are presented in **Fig. 4**. In the triglyceride fraction (fig. 4A), the proportion of saturated fatty acids (SFA) is significantly higher (P<0.01) in refed than in controls, while the proportion of polyunsaturated fatty acids (PUFA), as well as the PUFA/SFA ratio are significantly lower (P<0.001) in refed than in controls. By contrast, the proportions of SFA, monounsaturated fatty acids (MUFA), total PUFA and the PUFA/SFA ratio in the phospholipid fraction (fig. 4B) are not different in EWAT from refed and control groups.

Adipose insulin signalling. To assess proximal insulin signalling in adipose tissue, we measured IRS1-associated PI3K activity in EWAT from refed and control animals after *in vivo* administration of insulin or saline control on day 8-9 of refeeding. The results, presented in **Figure 5**A show that PI3K activity is marginally higher in refed than in control animals (overall group effect **by** 2-factor **ANOVA**, i.e. RF *vs*. C: p =0.045); with post-hoc pair-wise comparisons by unpaired t-test indicating that between-group difference in insulin-stimulated PI3K activity (+12%, p=0.09) or in basal PI3K activity (+31%, p=0.07) failing to reach statistical significance; similar lack of statistical significance is also observed with other pairwise comparison tests (e.g. Scheffe's, Tukey). Furthermore, measurement of downstream signalling (Akt phosphorylation) (Fig. 5B) shows no statistically significant group effect by ANOVA. Similarly, there are no differences between refed and controls in

ERK phosphorylation (data not shown). Taken together, these data on day 8-9 of catch-up fat suggest that, at the dose of insulin tested, there is no significance difference in the sensitivity of the insulin receptor IRS1-PI3K-Akt signalling pathway.

Analysis of early catch-up fat. We also investigated *in-vivo* insulinemic response to glucose, adipose tissue insulin signalling and gene-expression reprogramming within the first day of catch-up fat (20-23 hours after the start of the semistarvation-refeeding transition). This time-point was chosen to be as close as possible to the initial process of catch-up fat while minimizing the gorging effects that typically occurs at the onset of refeeding. This was achieved by providing the first day's refeeding diet in three equal parts at 15:00, 19:00 and 23:00 during this transition, with all food removed at 7:00 the next day and GTT measurements starting 4 hours later. As shown in **Figure 6** (panels A and B), refed and control rats display similar glucose tolerance, but the hyperinsulinemic response to the glucose load is already present within day 1 of refeeding. Furthermore, we found that on day 1 of refeeding, FAS activity was higher in refed than in controls two hours after the glucose load, but not in saline controls (Fig. 6C). FAS activity 2h after the glucose load was also found to correlate positively with both basal and post-glucose insulin levels, with the strongest correlations observed with the 30 min (peak) insulin values ($r^2 = 0.5$, p< 0.02). To investigate post-receptor events at this early stage of catch-up fat, we measured Akt phosphorylation, ERK phosphorylation and GLUT4 protein expression in EWAT harvested from refed and control rats after injection of insulin or saline. The results (Figure 6D) did not reveal any difference in these post-receptor events.

Induction of genes implicated in lipogenesis and adipogenesis. We performed a detailed time course of the expression of genes involved in lipogenesis (GLUT4, FAS, SREBP1c) and in adipogenesis (PPARγ and CEBPα). The data in **Figure 7** show that the expression of these genes are all rapidly induced within day 1 of refeeding relative to semistarvation (day 0), with FAS expression significantly exceeding fed control values on this first day of refeeding. By day 3 of refeeding, all these genes for lipogenesis and adipogenesis were upregulated relative to fed controls. These inductions were still present after 9 days of catch-up fat (p <0.01). By contrast, whereas the gene expression of steoryl CoA desaturase 1 (SCD1) was rapidly induced upon refeeding (relative to semistarvation), SCD1 mRNA levels were similar in refed and controls during catch-up fat.

DISCUSSION

Using a rat model of semistarvation-refeeding in which, from a perspective of energy balance, catch-up fat results solely from lower energy expenditure (and not from hyperphagia), we previously reported that during catch-up fat, the *in-vivo* glucose utilization, under insulin stimulation, is diminished in skeletal muscle but enhanced in white adipose tissue (WAT) (13). The studies reported here suggest that this enhanced capacity for glucose utilization in WAT during catch-up fat is associated with *(i)* adipose tissue hyperplasia and controlled hypertrophic growth, with adipocyte size remaining significantly smaller than controls, *(ii)* an early and sustained enhancement in the capacity for lipogenesis, in parallel to glucose-induced hyperinsulinemia and preserved proximal insulin signalling, and *(iii)* an increase in the proportion of saturated fatty acids specifically in the triglyceride (storage) fraction of WAT, but not in the phospholipids (membrane) component. Taken together, these findings during catch-up fat underscore adipose tissue plasticity in favour of an enhanced glucose flux towards fat

storage in WAT, and suggests that hyperinsulinemia might play a major role in the intitiation and promotion of catch-up fat.

Adipose tissue morphometry. An inverse relation between adipocyte size and insulin-stimulated glucose utilization is well established (18-22), and is directly supported by *in vitro* studies comparing large and small adipocytes isolated from animals varying in size (20, 21), or by comparing adipocytes varying in size from the same animal (36). While the mechanisms of enhanced insulin sensitivity pertaining to glucose uptake in small adipocytes are poorly understood, they have often been linked to an increase in the number of adipocytes and to enhanced adipogenesis (37). Consequently, the question arose whether the enhanced *in vivo* insulin-mediated glucose utilization in WAT found in our rat model of catch-up fat (13) could also be related to the progressive recovery of adipocyte size accompanied by an increase in the number of adipocytes. The results presented here show that adipocyte size, which is lower at the end of semistarvation, remains smaller in refed animals than in controls for at least 10 days of refeeding (fig. 2A). This lower adipocyte size during catch-up fat can in part be explained by enhanced adipogenesis as evidenced from the faster increase in adipocyte number, together with the upregulation of PPARγ and CEBPα gene expression. While these observations are consistent with several studies describing WAT hyperplasia during refeeding following caloric restriction (38-41), our studies here, however, demonstrate for the first time that hyperphagia is not a requirement for the induction of hyperplasia in response to refeeding after growth arrest.

Adipose tissue *de-novo* lipogenesis and fatty acid profiling. The present studies showing that the adipose tissue of refed animals display marked increases in FAS activity and in the expression of key genes implicated in *de-novo* lipogenesis (GLUT4, SREBP1c, FAS) are also consistent with our previous demonstration of catch-up fat as a state of increased insulin-mediated glucose utilization in WAT (13). In contrast to the marked upregulation of *de-novo* lipogenic genes in WAT, however, the expression of SCD1, the enzyme that catalyses desaturation of saturated fatty acids to monounsaturates, was not found to differ from that of controls during catch-up fat. The enhancement of *de-novo* lipogenesis (relative to fed controls) without a parallel increase in SCD1-induced desaturation could explain the increase in the proportion of saturated fatty acid in the triglyceride lipid fraction of adipose tissue during catch-up fat.

Adipose tissue *de-novo* lipogenesis vs insulin signalling. To test whether the increased lipogenesis and glucose uptake in WAT observed during catch up-fat depend on increased insulin sensitivity, we measured proximal-insulin signalling following *in-vivo* administration of an insulin bolus on day 8-9 of refeeding. Measurements of IRS1-associated PI3K activity, AKT and ERK phosphorylation revealed a small statistically significant induction of IRS1-associated PI3K activity during catch-up fat, which, however, was not reflected in an increased AKT phosphorylation nor in ERK activation. Although these results show only a marginal increase in insulin signalling, it is important to emphasize that insulin signalling is clearly not impaired, which is in sharp contrast to the diminished PI3K activity in skeletal muscle obtained from the same animals (15). Furthermore, unlike in skeletal muscle where the insulin resistant state can also be associated with a decreased ratio of PUFA:SFA in the phospholipid fraction (33), the data presented here on adipose tissue show no such alterations in phospholipids composition. These findings of differential regulation of PI3K signaling and phospholipids composition in skeletal muscle and adipose tissue are in line with the

previously reported studies of hyperinsulinemic-euglycemic clamps (13) showing that glucose uptake is reduced in skeletal muscle and enhanced in white adipose tissue during catch-up fat.

Whether adipose tissue reprogramming precedes or is consequential to skeletal muscle insulin resistance is not known. However, a state of whole-body insulin resistance is already evident on day 1 of refeeding as judged by a higher insulin response curve but normal glucose tolerance in refed animals relative to controls following administration of a glucose load (fig. 6). Whether this hyperinsulinemia can be explained entirely by peripheral muscle insulin resistance or whether it may also be contributed by pancreatic β-cell hyperresponsiveness to glucose is not known, and is an interesting avenue for future investigations. Nonetheless, the data obtained here on day 1 of catch-up fat showing rapid induction in the expression of genes controlling *de-novo* lipogenesis in WAT concomitant to a state of whole-body insulin resistance suggest that hyperinsulinemia is an early event and could therefore be a causal factor in the induction of *de-novo* lipogenesis in adipose tissue at the onset of refeeding. As this hyperinsulemia persists during the course of catch-up fat, it may also be important in maintaining the high rate of *de-novo* lipogenesis, as reflected by the marked upregulation of lipogenic genes and/or high FAS activity between days 3 to 10 of refeeding. However, while increased insulin is undeniably a major factor in initiating and sustaining enhancing glucose uptake and *de novo* lipogenesis, the results of our previous hyperinsulinemic-euglycemic clamp studies (13) showing enhanced glucose utilisation in adipose tissue on day 7 of catch-up fat implies increased insulin-dependent glucose utilization by adipocytes for the same insulin stimulation. Consequently, it can be speculated that the marked increases in *de-*

novo lipogenic capacity observed here between day 3-10 of catch up fat could also be contributing to the increased glucose uptake in adipose tissue. This concept, which embodies interactions between insulin-stimulated glucose uptake and *de-novo* lipogenesis in adipose tissue (depicted in **Figure 8),** is consistent with studies showing that pharmacological inhibitors of *de-novo* lipogenesis decrease the ability of insulin to stimulate both the pentose shunt glucose oxidation and overall glucose utilization, but not Krebs cycle or glyceride-glycerol synthesis (18, 20). In other words, the increased demand in acetyl-CoA and NADPH for lipogenesis would lead to enhanced glucose metabolism through glycolysis and the pentose phosphate pathway, thereby driving glucose influx. These studies led to the proposal that the enzymatic capacity for fatty acid synthesis is an important factor in determining insulin-stimulated glucose utilization in white adipose tissue (18, 20).

CONCLUSIONS

We show here that the process of catch-up fat involves an early and sustained induction of hyperinsulinemia, increased glucose flux towards lipogenesis, increased saturated fatty acid specifically channelled to the triglycerides stores and not to membrane phospholipids, and that the recovery of adipocyte size is accompanied by enhanced adipogenesis, thereby limiting adipocyte hypertrophy. Such adipose tissue plasticity suggest that white adipose tissue plays an active role in glucose redistribution towards catch-up fat during catch-up growth. Impairment in adipose tissue plasticity may underlie some of the pathophysiological consequences of catch-up growth. Indeed, enhanced lipogenesis in the absence of hyperplasia was recently reported in a murine model of postnatal catch-up growth characterized by hypertrophic adipocytes and glucose intolerance (42). However, as

underscored by apparently conflicting findings that postnatal catch-up growth after protein malnutrition programs proliferation of preadipocytes in rats (43), it is likely that genetics and epigenetics-environment interactions play a critical role in defining adipose tissue plasticity during catch-up growth (44).

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FIGURE LEGENDS
Figure 1:
Hyperinsulinemia in our rat model of semistarvation-refeeding in which the accelerated fat recovery (catch-up fat) results only from diminished energy expenditure (suppressed thermogenesis) and not from hyperphagia. In this particular investigation here, body weight and body composition (panels A,C,D) were determined at day 0, 5, 10, 15 and 20 of refeeding with both groups consuming isocaloric amount of chow diet (panel B). The acceleration of fat mass but not that of lean mass (i.e. preferential catch-up fat) lasts for about 2 weeks. Panel E and F shows the plasma glucose and insulin concentrations before and for 2h after an intraperitoneal injection of glucose (2g/kg body weight); the test of glucose tolerance was performed on day 8-9 of refeeding. All values are means ±SE (n = 6); ** $p<0.01$; *** $p<0.001$.

Figure 2:
Epididymal white adipose tissue (EWAT) morphometry showing in panel A: the frequency distribution curves for adipocyte cell diameter, in panel B: the EWAT pad weight, in panel C: the total adipocyte cell number per EWAT pad, and in panel D: the mean adipocyte diameter, in refed (RF) or control (C) animals at the end of semistarvation (Day 0) and day 5, 10 and 15 of isocaloric refeeding. Values are means ±SE (n=6). Statistical analysis for differences in refed and controls for adipocyte diameter distribution curves were performed by Kolmogorov-Smirnov tests, while between-group differences across time in EWAT pad weight, adipocyte number, and mean adipocyte diameter were determined by regression analysis and comparison of slopes. The regression coefficients (r^2) of the regression lines in panels B, C and D are as follows: in panel B, $r^2 = 0.64$ and 0.93 for control and refed, respectively; in panel C: $r^2 = 0.52$ and 0.90 for control and refed, respectively; in panel D: $r^2 = 0.47$ and 0.71 for control and refed, respectively. In each panel B, C, D, the above-mentioned r2 values are statistically significant ($p<0.01$).

Figure 3:
Time-course analysis of fatty acid synthase (FAS) activity in epididymal white adipose tissue in refed (RF) or control (C) animals at the end of semistarvation (day 0) and on days 5, 10, and 15 of isocaloric refeeding. Values are means ± SE (n=6); statistical significance of differences is indicated as follows: * $p<0.05$; ** $p<0.01$, as assessed by unpaired t-test.

Figure 4:
Fatty acids composition of triglyceride fraction (panel A) and phospholipid fraction (panel B) extracted from epididymal WAT pads of control (C) and refed (RF) animals on day 8-9 of refeeding. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values are means ± SE (n=6) ** = $p<0.01$; *** = $p<0.001$, as assessed by unpaired t-test.

Figure 5:
PI3K activity and Akt phosphorylation in epididymal fat pads in response to either an acute bolus of intravenous administration of 10 U/kg of insulin (black or grey bars) or a similar volume of saline (white bars) in refed (RF) and control (C) groups on days 8-9 of refeeding. Values are means ± SE (n=6); statistical significance of differences is indicated as follows: @, effect of group (C *vs*. RF); #, effect of treatment (hormone *vs*. saline), as assessed by ANOVA.; Triple

symbols, p<0.001. Post-hoc between-group comparison treated with either saline or insulin was performed by unpaired t-test.

Figure 6:
Analysis of early catch-up fat (day 1 of refeeding). Panel A and B shows the plasma glucose and insulin concentrations before and for 2h after an intraperitoneal injection of glucose (2g/kg body weight) during glucose tolerance test (GTT). Panel C indicates the activity of fatty acid synthase (FAS) in EWAT obtained at the end of the GTT test (i.e. 2 h post-glucose load) or after saline injection. Panel D shows the results of a representative western blot pertaining to proximal insulin signalling in response to bolus administration of insulin or saline; for ERK, the upper and lower bands correspond to P44 and P42, respectively. C = Control; RF= Refed. All values are means ±SE (n = 6-8); ** p<0.01 by unpaired t-test.

Figure 7:
Time-course gene expression analysis of key genes implicated in adipogenesis and in glucose flux towards lipogenesis during the course of catch-up fat, namely at the end of semistarvation (day 0), and on day 1, 3 and 9 of refeeding. The data in EWAT from semistarved (day 0) or refed animals (day 1, 3, 9) are expressed as a percentage of that of the respective fed controls (represented as 100% - the dotted line). Values are means ± SE, (n=6-8); for each time-point, statistical significance of differences are relative to fed controls, and indicated as follows: * p<0.05, ** p<0.01, as assessed by unpaired t-test.

Figure 8:
A model integrating of our previous (13) and current findings based on the interrelationships between hyperinsulinemia, insulin-dependent glucose uptake and *de-novo* lipogenesis (*DNL*). In this model, hyperinsulinemia on day one of refeeding plays an important role in initiating and sustaining catch-up fat through the stimulation of glucose uptake and *DNL* in adipocytes. Once the machinery for *DNL* is fully activated, the increased glucose metabolism to *DNL* will contribute to the enhanced insulin-dependent glucose uptake observed in adipose tissue during catch-up fat. According to this model the enzymatic capacity for fatty acid synthesis is hence an important factor in determining insulin-stimulated glucose utilization in white adipose tissue. The newly synthesized lipids will lead to a moderate adipocytes hypertrophic growth since hyperplastic growth will channel part of the lipid pool in newly formed adipocytes. The net effect is a delayed adipocyte size restoration, which could possibly play a role in maintaining the high lipogenic activity and insulin sensitivity observed in adipose tissue during catch-up fat.
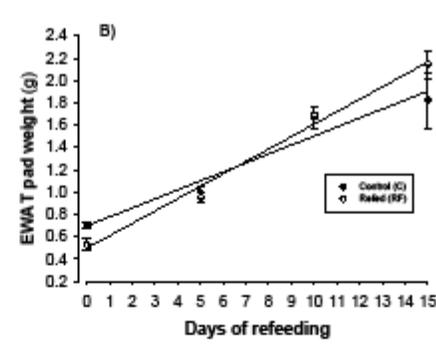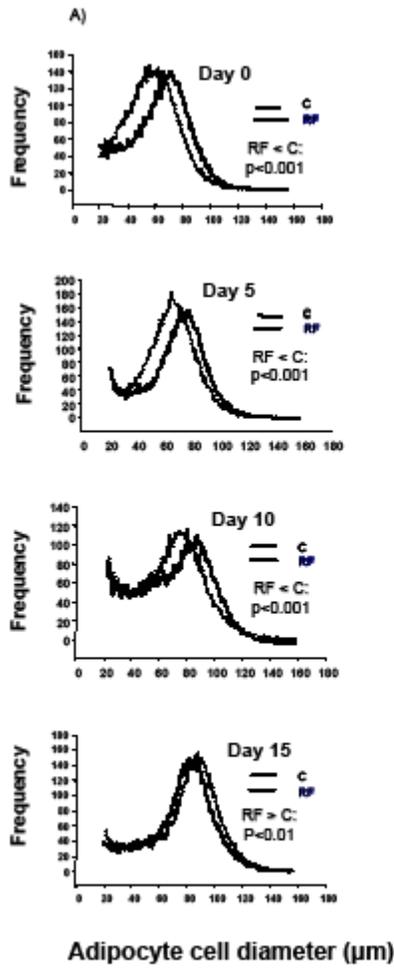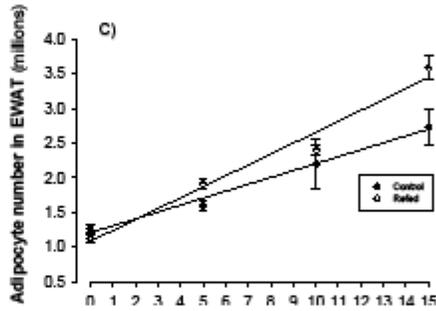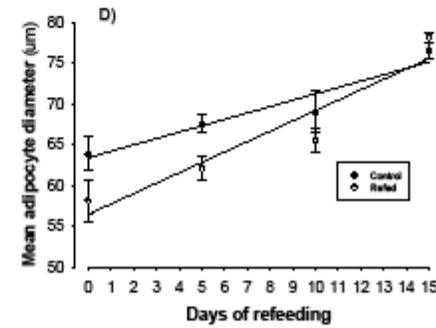
Figure 1
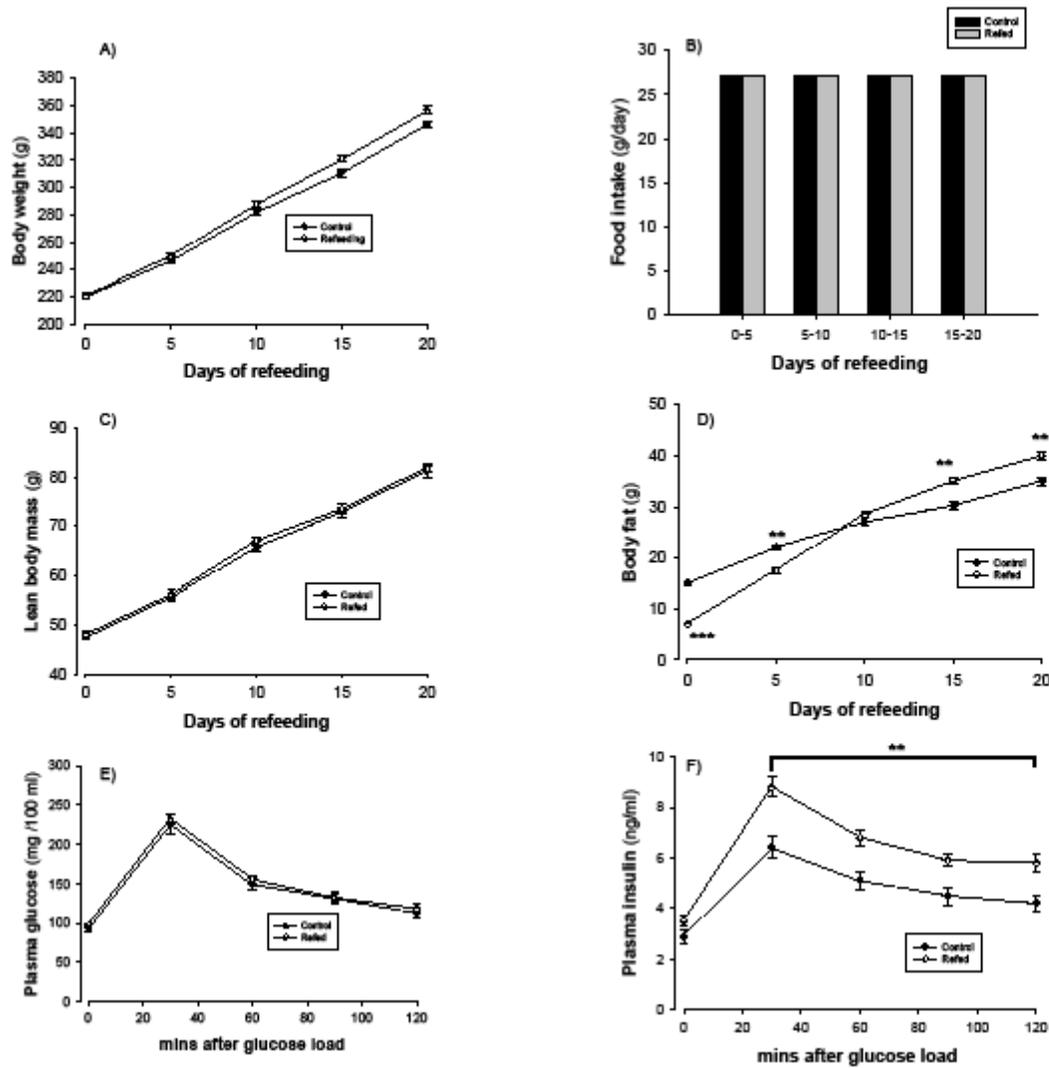
A)

Day 0

Frequency

C
RF

RF < C:
p<0.001

Day 5

Frequency

C
RF

RF < C:
p<0.001

Day 10

Frequency

C
RF

RF < C:
p<0.001

Day 15

Frequency

C
RF

RF > C:
P<0.01

Adipocyte cell diameter (μm)

Figure 2

B)

EWAT pad weight (g)

Control (C)
Refed (RF)

Days of refeeding

Comparison of slopes
RF > C: p<0.05

C)

Adipocyte number in EWAT (millions)

Control
Refed

Comparison of slopes
RF > C: p<0.05

D)

Mean adipocyte diameter (μm)

Control
Refed

Days of refeeding

Comparison of slopes
RF > C: p<0.05

Figure 3

Figure 4

A) **Triglycerides**

B) **Phospholipids**

Figure 5

Figure 6

A)

B)

D)

C)

Figure 7

Figure 8