Puberty is an important developmental period for the establishment of adipose tissue mass and metabolic homeostasis

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ABSTRACT
Over the past 2 decades, the incidence of childhood obesity has risen dramatically. This recent rise in childhood obesity is particularly concerning as adults who were obese during childhood develop type II diabetes that is intractable to current forms of treatment compared with individuals who develop obesity in adulthood. While the mechanisms responsible for the exacerbated diabetic phenotype associated with childhood obesity is not clear, it is well known that childhood is an important time period for the establishment of normal white adipose tissue in humans. This association suggests that exposure to obesogenic stimuli during adipose development may have detrimental effects on adipose function and metabolic homeostasis. In this study, we identify the period of development associated with puberty, postnatal days 18–34, as critical for the establishment of normal adipose mass in mice. Exposure of mice to high fat diet only during this time period results in metabolic dysfunction, increased leptin expression, and increased adipocyte size in adulthood in the absence of sustained increased fat mass or body weight. These findings indicate that exposure to obesogenic stimuli during critical developmental periods have prolonged effects on adipose tissue function that may contribute to the exacerbated metabolic dysfunctions associated with childhood obesity.

KEYWORDS
adipocyte precursor; adipogenesis; adipose development; adipose stem cell; adipose tissue biology; childhood obesity; metabolism; obesity; puberty

Introduction
Obesity, which is defined as the excess accumulation of white adipose tissue, is associated with increased risk for developing several metabolic complications, including cardiovascular disease (CVD), type II diabetes (T2D), and certain forms of cancer.1-3 While adult obesity is linked to severe health problems, evidence suggests that being obese during childhood has an even greater impact on health. Compared to people who become obese as adults, people who are overweight or obese in childhood or adolescence have increased risk for developing CVD independent of whether they remain obese as adults4-6 and have an increased severity of diabetes that is more resistant to current methods of intervention.7-9 These findings suggest that exposure to obesogenic stimuli is particularly harmful early in life. The poor health outcomes associated with childhood obesity are particularly concerning given that there has been a recent and rapid increase in the incidence of childhood obesity and T2D, with more than one-third of US children currently being obese or overweight as of year 2000.10-12 The recent rise in childhood obesity rates foreshadows increased numbers of adults with intractable T2D, which will eventually lead to an even greater burden on the healthcare system. However, the basis of the exacerbated metabolic outcomes associated with childhood obesity is not well understood.

It is well established that early childhood and puberty are important periods for the development of normal adipose tissue mass. Early studies have shown that there are 2 intervals of cellular proliferation in human adipose tissue, one before the age of 2 and the other during puberty.13-15 Concurrent to the cellular proliferation is an increase in adipocyte size, which is augmented in obese individuals. After puberty, adipocyte number and size become static in lean individuals.15,16 The development of...
adipose tissue in rodents follows a similar pattern to that observed in humans. In both rats and mice, adipocyte cell size and number increase steadily during the postnatal period and puberty under standard diet conditions.\textsuperscript{17,18} Thus, normal adipose tissue is established during precise developmental periods.

In development and adulthood, increases in adipose mass in obesity can result from both increased adipocyte size and increased adipocyte number.\textsuperscript{19–22} In adults, high fat diet (HFD) feeding transiently induces adipocyte precursors to proliferate, which leads to the increased formation of adipocytes in diet-induced obesity.\textsuperscript{19,20} We recently showed that the molecular mechanisms that drive adipocyte hyperplasia in adult obesity are distinct from the mechanisms that regulate the establishment of normal adipocyte number during development.\textsuperscript{20} This finding suggests that simultaneous exposure to different adipogenic signals may affect adipocyte formation and function, thereby influencing metabolic outcomes resulting from childhood obesity. Indeed, studies have shown that HFD feeding from early development through adulthood alters adipose tissue biology and metabolism. Mice given high fat diet from weaning into adulthood display an immediate increase in cell size followed by an increase in adipocyte number compared with standard diet-fed controls.\textsuperscript{17} In another study, rats given HFD starting at weaning develop liver steatosis, have increased visceral fat growth rate, and have increased cholesterol levels compared with mice fed standard diet.\textsuperscript{23} However, since animals in these studies were fed HFD continuously through adulthood, the effect of HFD feeding during the critical periods for adipose tissue development remain unclear.

Here we define the precise timeframe for the establishment of white adipose tissue (WAT) during puberty and assess the effects of restricting HFD feeding to this critical developmental window. Using BrdU pulse-chase and flow cytometry, we quantify cellular proliferation and adipogenesis in vivo. Our data show that over half of visceral adipocytes and over 20% of subcutaneous adipocytes in adult mice are formed from differentiation of proliferative adipocyte precursors in early puberty. We show that even transient HFD during puberty leads to increased adipocyte size, aberrant adipokine regulation, and metabolic complications in adulthood in the absence of sustained increases in fat mass.

**Results and discussion**

**Contribution of adipocyte precursor activation to early postnatal WAT development**

To determine the important time periods for the establishment of body mass, we measured body weight daily from postnatal day 0 (P0) to 6 weeks of age (P42). Analysis of the daily percentage increase in body weight during this time frame reveal 2 distinct periods of rapid body weight gain (Fig. 1A). This includes a period directly after birth in which mice exhibit an increase of approximately 10–20% of body weight per day from P0 to P16 (termed perinatal/pre-puberty). We also observe a second period of 16 days, with an increase of approximately 10% per day, observed from P18 to P34 (Fig. 1A). As this time period is associated with hallmarks of puberty in mice, we termed this time period 'puberty.'\textsuperscript{24} After P33, weight gain returns to a basal level of about 1–2% per day. These data highlight that, similar to humans, mice display rapid growth both early in development and during puberty, and identify the period of P18-P34 as an important period for growth.

We next analyzed adipose tissue formation over this developmental time course. The formation of mature adipocytes in subcutaneous WAT (SWAT) occurs directly after birth, and adipocytes in the visceral depot (gonadal) WAT (GWAT) are first observed at postnatal day 7 (P7).\textsuperscript{25,26} However, the period in which adipocyte precursor cells (APCs) are proliferative during development has not been established. Thus, we characterized APC activation by assessing cellular proliferation via bromodeoxyuridine (BrdU) incorporation throughout pre-puberty and puberty in SWAT. Similar to humans, APC proliferation is highly elevated before birth (e17.5) and remains high immediately after birth, with proliferation decreasing throughout the first 2 postnatal weeks in SWAT to a rate similar to that seen in adult mice by P16 (Fig. 1B, Fig. S1C-D).\textsuperscript{15} This is followed by a second increase in proliferation during the growth spurt associated with puberty from P21-P34 (Fig. 1B). Although the size of the GWAT depot is not adequate for analysis in the early postnatal period,\textsuperscript{25} analysis of GWAT APC BrdU incorporation from P19-P34 indicates that GWAT APCs also display increased proliferation during this time period in both males and females, with the increased BrdU incorporation spanning P21-P33 (Fig. 1C). Interestingly, there is an increase in APC proliferation in GWAT of male mice at p24 that is not observed in female mice (Fig. 1C) which may be due to differences in sex hormones between males and females.\textsuperscript{27} These data establish that APCs have increased proliferation during puberty in both SWAT and GWAT in males and females.

The activation of APCs during puberty is consistent with previous work that has demonstrated that a considerable amount of adipogenesis occurs after P20 in GWAT.\textsuperscript{19} To determine if the activation of APCs during puberty results in increased formation of adipocytes, we pulsed mice with low-dose BrdU from P21-P35, then
analyzed the incorporation of BrdU into mature adipocytes after a 7-week chase in the absence of BrdU, as described previously (Fig. 1D).28 Immunofluorescence staining of adipose tissue sections after BrdU pulse labeling during puberty and a 7-week chase revealed significant adipogenesis from proliferative APCs during puberty in both SWAT and GWAT. 49.8 ± 3.3% of adipocytes in the GWAT and 19.5 ± 1.3% of adipocytes in SWAT are derived from mitotically activated APCs during puberty, compared with only 11.9 ± 2.7% and 0.6 ± 0.5%, respectively, from post-puberty adipogenesis (Fig. 1E). Furthermore, this establishment of adipose tissue coincides with a rapid increase in body weight, fat mass, and lean mass in both males and females (Fig. S1A, B). Taken together, these data suggest that, similar to humans,15 puberty is an important period for

Figure 1. Adipocyte Precursor Activation During Embryonic and Postnatal Development. (A) The daily percentage change in body weight for male (red) and female (green) C57BL/6J mice during postnatal development leading to 2 16-day periods of perinatal (p1–p16) and puberty (p18–34) development (male n = 23–59, female n = 8–46). (B) SWAT adipocyte precursor proliferation, assessed by BrdU incorporation and flow cytometry from embryonic day 17.5 (e17.5) to postnatal day 34 (p34). Male and female mice used (n = 5–32). Asterisks indicate significance over p19. (C) GWAT adipocyte precursor proliferation for male (red) and female (green) during puberty (p19–34). Asterisks indicate significance over p19. (D) Representative image of male SWAT (BrdU+ adipocyte nuclei – green arrow, BrdU+ non-adipocyte nuclei – purple arrow, BrdU-adipocyte nuclei – white arrow). Data are represented as mean ± SEM. ‘(p < 0.05), **(p < 0.01), ****(p < 0.001).
adipose tissue formation and the establishment of adipocyte number. Furthermore, this work establishes the time period of P18-P34 as critical for the formation of normal adipose mass in mice.

**Transient puberty HFD**

Due to the importance of adipogenesis during puberty in establishing normal WAT mass, we focused on the effect of high-fat diet (HFD) feeding during this time period. As the increased period of adipogenesis in mice during puberty occurs well after the switch from ingesting milk to eating solid food, we were able to determine the direct effect of feeding a high fat diet (HFD) during puberty. HFD feeding is well known to increase body weight and fat mass in C57Bl6/J mice, and we have previously shown that HFD feeding of adult male mice transiently induces adipogenesis in GWAT. Transient HFD feeding only during puberty (P18-P34), with a switch back to SD at P35 (Fig. S2A), results in altered body composition, despite no overall significant change in body weight, due to an increase in fat mass and a reduction in lean mass (Fig. 2A-C, Fig. S2B). At the end of the pulse, both SWAT and GWAT have increased mass over standard diet controls (Figure S2C). The increase in fat mass is transient and persists for approximately 6 weeks until 11 weeks of age at which point body composition is equivalent to SD controls (Fig. 2C; Fig. S2B).

We next determined if HFD treatment during puberty affects adipogenesis. Analysis of APC proliferation during the entire P18-P34 puberty window shows that even though levels of BrdU incorporation in SD-fed mice are high, HFD feeding from P18-P34 still significantly increases APC proliferation in both SWAT and GWAT (Fig. 2D). To determine if the increase in APC proliferation leads to increased adipocyte formation, we pulsed with low dose BrdU concurrent with HFD feeding from P18-P34 and then switched back to SD and chased in the absence of BrdU for 7 weeks. IHC analysis of BrdU incorporation into adipocyte nuclei after the 7-week chase shows that, despite the increase in APC proliferation in response to HFD feeding during puberty, there is not a significant increase in adipocyte formation (Fig. 2E). After 7 weeks of switching back to SD, at 12 weeks of age, the SWAT and GWAT mass is equalized between HFD puberty pulsed mice and SD controls (Fig. 2F-G). Intriguingly, at this time point, mice given HFD during puberty have significantly larger adipocytes in both SWAT and GWAT (Fig. 2H-I, Fig. S2F-G) despite a lack of difference in total mass in either depot, suggesting that HFD feeding during puberty may actually decrease adipocyte formation. These data indicate that exposure to an obesogenic stimulus during puberty can alter adipose morphology even in the absence of a sustained effect on fat mass.

**Transient adult HFD**

For comparison to the effect of HFD feeding during puberty, we also determined the effect of transient HFD treatment in adulthood. We fed mice a HFD for 16 d starting at 13 weeks of age (P90–106), then returned the mice to SD for an additional 12 weeks (Figure S2A). Transient adult HFD significantly increases body weight and fat mass compared with puberty HFD pulsed mice and SD controls (Fig. 2B-C). Furthermore, upon switch back to SD, the mice lose weight and fat mass compared with the HFD feeding, but maintain increased body weight and fat mass compared with SD controls until the terminal end point at 6 months of age (Fig. 2B-C). At 6 months of age, adult HFD pulsed mice exhibit significantly elevated SWAT and GWAT mass, compared with SD and puberty HFD pulsed mice (Fig. 2F-G). This shows that unlike in mice fed HFD during puberty, even short periods of HFD during adulthood have long-lasting impacts on total body fat mass and body weight.

**Transient HFD and energy homeostasis**

Transient adult HFD results in reduced metabolic rate demonstrated by decreased energy expenditure, carbon dioxide production, and RER, compared with SD controls at 16 weeks of age (Figure S3A-D). Activity and food intake are unchanged in adult HFD pulsed mice, compared with SD controls (Fig. S3E, F). Transient HFD during puberty does not alter metabolic rate, activity or food intake but does increase RER, compared with SD controls (Fig. S3A-F).

**Transient HFD and glucose homeostasis**

Being obese during childhood leads to increased severity of T2D and increased risk for developing the metabolic syndrome in humans, therefore, we assessed glucose homeostasis and adipose function in our transient HFD models. Directly after feeding HFD during puberty, blood glucose was increased compared with controls, but plasma insulin levels were not significantly changed (Fig. 3A-B). Adipokine levels are altered by HFD feeding during puberty in a manner similar to that observed in adult HFD, with a reduction in plasma adiponectin and an increase in plasma leptin (Fig. 3C-D). When metabolic parameters were assessed after a return to SD, we observe that glucose tolerance is significantly altered by transient HFD in both puberty and adults after 18 weeks.
Figure 2. Transient High-Fat Diet Activates Adipocyte Precursors During Puberty. (A) Body weight throughout puberty HFD in male mice (SD n = 10, HFD n = 14). (B, C) Body weight (B) and fat mass (C) for male mice pulsed transiently with HFD only during puberty (p18–34) or adulthood (p90–106) and then switched back to SD until 6 months of age (SD n = 6–11, puberty HFD n = 8–14, adult HFD n = 7–10). (D) Adipocyte precursor proliferation, assessed by BrdU incorporation and flow cytometry with low-dose BrdU from p18–34 in male GWAT and SWAT in response to SD and HFD (n = 5). (E) Amount of BrdU+ adipocytes in male mice after puberty HFD with 7-week chase relative to SD controls shown in Figure 1D (n = 3). (F, G) SWAT (F) and GWAT (G) fat pad mass in male mice after puberty (p18–34) or adulthood (p90–106) HFD and 7-week chase on SD (SD n = 5–16, puberty HFD n = 4–17, adult HFD n = 4–14). (H, I). Adipocyte diameter after 7-week chase in male mice given HFD or SD during puberty (p21–35) in SWAT (H) and GWAT (I) (SWAT SD n = 5, puberty HFD n = 3; GWAT SD n = 5, puberty HFD = 4). Data are represented as mean ± SEM. *(p < 0.05), ***(p < 0.01), ****(p < 0.001).
of SD. However, by 26 weeks of age, the animals transiently fed HFD in adulthood maintained impaired glucose tolerance while the puberty HFD group had recovered to SD levels. (Fig. 3E-F, Fig. S4A-B). Plasma insulin levels are significantly elevated over SD controls at 18 weeks of age for both transient HFD treatments (Fig. 3G); however, at 26 weeks of age, insulin levels are still elevated in animals that received adult HFD, while animals that received HFD during puberty have intermediate insulin levels that are not significantly different from either SD controls of adult transient HFD (Fig. 3G). Interestingly, plasma leptin levels are significantly higher at 26 weeks of age in animals that received transient HFD in adulthood, which is consistent with their increased fat mass (Fig. 3H). Similar to plasma insulin levels, the plasma leptin levels in mice fed HFD only during puberty are intermediate to the SD controls and transient adult HFD fed animals (Fig. H). Analysis of leptin expression in WAT depots from the puberty HFD mice reveal that leptin expression is higher at 26 weeks in GWAT (Fig. 3I), but that SWAT leptin expression is not changed in SWAT in these animals (Fig. S4D). There is no difference in expression of adiponectin or TNFa in either depot (Fig. S4C-D).
Taken together, these data indicate that HFD during puberty (p18–34) leads to immediate changes in important metabolic parameters. Importantly, at 18 weeks of age, a full 13 weeks post-HFD, these mice still display altered metabolic parameters, similar to those seen in mice with twice as much fat mass, in the absence of any increase in body weight, total fat mass, or lean mass. Specifically, there is sustained adipocyte hypertrophy after HFD during puberty as compared with HFD during adulthood, which mainly leads to adipocyte hyperplasia. This could be because adipocyte hyperplasia is near its maximum levels during adolescence, even in the absence of HFD. Therefore, the protective mechanism of adult HFD-induced adipogenesis is not available, which leads to sustained increases in adipocyte size, potentially contributing to WAT dysfunction and ultimately leading to altered glucose homeostasis. Additionally, previous studies have shown that HFD during adulthood, as well as maternal HFD during gestation, leads to long-lasting effects on the epigenome. Therefore, the sustained metabolic changes in mice pulsed with HFD during puberty could be attributed to epigenetic changes. The sustained effect on glucose tolerance and leptin expression that result from exposure to an obesogenic stimulus during puberty supports these ideas and suggests that similar mechanisms may be responsible for the exacerbated metabolic dysfunction that results from childhood obesity in humans.

The data presented in this study highlight the importance of childhood nutrition in establishing normal metabolic homeostasis, and indicate that poor nutrition during childhood can lead to long-lasting effects on adipocyte size, altered adipocyte function and impaired metabolism, even in the absence of sustained fat mass. Our data provide the framework to study the detrimental effects of poor nutrition during puberty. But to fully understand the long-term consequences, more extensive studies need to be done to assess if poor diet during puberty leads to altered cardiac function, increased susceptibility to obesity in adulthood, or increased WAT inflammation and fibrosis. In 2008, it was estimated that obesity adds an extra $147 billion in cost to the US healthcare system, and that number is poised to increase exponentially if the rates of obesity continue to rise. Therefore more focus on childhood nutrition is needed to increase the long-term health of our population and ultimately decrease healthcare costs in the United States.

Materials and methods

Animals

The Institutional Animal Care and Use Committee at Yale University approved all animal studies. Mice (male C57BL/6J) were group housed (3–5 mice per cage) and food and water were provided ad libitum. All mice were weaned at p18 except for the BrdU pulse chase experiment (Figs. 1D-E, 2E, Fig. S2D-E), in which mice were weaned at p21. Male and female mice were housed separately post-weaning. Control SD is from Harlan Laboratories (2018S; 3.1kcal/g, 18% kcal fat derived from soybean oil, 24% kcal protein, 58% kcal carbohydrate). High-fat diets were purchased from Open Source Diets Research Diets (D12492).

Body composition

Analysis of body composition was performed by NMR using the Echo MRI whole body composition analyzer (Echo Medical System, Houston, TX). Mice were marked with permanent marker and tracked during postnatal development. Fat depots were carefully dissected and weighed on a scale to 0.01 g. GWAT refers to the epidymal depot, SWAT refers to the inguinal depot.

Flow cytometry analysis of In Vivo adipocyte precursor cell proliferation

In experiments assessing daily proliferation levels, BrdU was administered at 0.8 mg/ml in the drinking water along with an injection of BrdU (50 mg/kg) in PBS (10 mg/ml) in the morning and evening. For BrdU experiments lasting longer than 24 hours, low dose BrdU (0.4 mg/ml) was administered in the drinking water. BrdU water was changed every other day. For prenatal BrdU timepoints, pregnant mothers were given BrdU injections and 0.8 mg/ml BrdU water as described above and in previous studies. For BrdU analysis, cells were stained as described previously with the following antibodies: CD45 APC-eFluor 780 (eBioscience; 47–0451–80), CD31 PE-Cy7 (eBioscience, 25–0311–82), CD29 Alexa Fluor 700 (BioLegend, 102218) and Sca-1 Pacific Blue (BD Biosciences, 560653) or Sca-1 V500 (BD Horizon, 561228). Cells were washed and then fixed and permeabilized using Phosflow lyse/fix and Perm Buffer III (BD Biosciences) according to the manufacturer’s recommendations. Cells were then treated with DNsase (deoxyribonuclease I; Worthington; x 6000 units/ml) in dPBS (Sigma; with calcium chloride and magnesium chloride) for 2 hrs at 37°C and then washed in HBSS with 3% BSA. Cells were then stained with anti-BrdU antibody (Alexa Fluor 488; Phoenix Flow Systems; AX488 or Alexa Fluor 647; Phoenix Flow Systems; AX647) in HBSS with 3% BSA overnight in the dark at 4°C. Cells were then washed in HBSS with 3% BSA and incubated with CD34 Alexa Fluor 647 (BioLegend, 119314) or CD34 Brilliant Violet 421 (BioLegend...
and CD24 PerCP-Cyanine 5.5 (eBioscience, 45–0242–80). Following antibody incubation, samples were washed and analyzed on a BD LSRII analyzer. Data analysis was performed using BD FACS Diva software (BD Biosciences).

BrdU sections immunofluorescence
Adipose tissue was prepared as described for paraffin-embedded tissue.32 5 μm sections were deparaffinized and rehydrated, followed by antigen retrieval in 10mM sodium citrate, pH 6.0, under pressure in a 2100 Retriever (PickCell Laboratories). Blocking and staining was performed in 2%BSA in PBS. Sections were incubated in primary antibodies including rat anti-BrdU (Abcam #ab6326) and rabbit anti-Caveolin-1 (Cell Signaling #3238) overnight at 4°C. Secondary antibodies including goat anti-rat-A488 and goat anti-rabbit rhodamine-X-red were purchased from Jackson Immunoresearch, and incubated with tissue for 1–2 hours at room temperature. Slides were mounted with DAPI Fluoromount-G mounting media (Southern Biotech) and imaged by confocal microscopy. Several confocal images of each tissue section were acquired, and analyzed for the presence of BrdU and adipocyte nuclei. Adipocyte nuclei were identified by their location inside adipocyte membranes as described.28

Metabolic measurements
Mice were acclimated in individual metabolic chambers (TSE Systems, Germany; CMED Metabolic Phenotyping Center, Yale University) for 3 d before the start of the recordings. Mice were continuously recorded for 7 d with the following measurements being taken every 30 minutes: water intake, food intake, ambulatory activity (in X and Y axes), and gas exchange (O2 and CO2) (using the TSE LabMaster system, Germany). VO2, VCO2 and energy expenditure were calculated according to the manufacturer’s guidelines (PhenoMaster Software, TSE Systems). The respiratory exchange rate (RER) was estimated by calculating the ratio of VCO2/VO2. Values were adjusted by body weight and lean mass where mentioned.

Glucose levels were measured with Contour Glucometer blood glucose meter (Bayer Corp). Mice were fasted for 6hrs in the light period and plasma collected in lithium heperate microvette capillary tubes (Sarsted) and leptin, insulin and adiponectin levels were measured using a mouse metabolic kit (MSD, Mesoscale Discovery) and a SECTOR Imager 2400 (Mesoscale Discovery) according to the manufacturer’s instructions. Intraperitoneal glucose tolerance tests were performed by measuring glucose at 0 (6-hr light period fast) 10, 20, 30, 60 and 120 minutes after intraperitoneal glucose injection (2 g/kg of body weight) using a Contour Glucometer (Bayer Corp).

Statistical analysis
Longitudinal body weight and fat mass were analyzed using 2-way analysis of variance with Bonferroni’s post-test for multiple comparisons. All other statistical analyses were performed with a Student’s t-test (for 2 groups) or One-way ANOVA (for > 2 groups; followed by Bonferroni’s post-test for multiple comparisons) for independent samples using GraphPad Prism version 6.00 for Mac, (Prism: GraphPad Software, Inc.). Area under the curve for glucose tolerance test were calculated using GraphPad Prism (GraphPad Software, Inc.). Comparisons are indicated in figure legends. Data are expressed as mean ± SEM, and P < 0.05 was considered as statistically significant.

Disclosure of potential conflicts of interest
The authors report no conflict of interest.

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Author contributions
BH, CC, RB, NM and MSR conceived experiments and analyzed data. BH, CC, RB, LC, EJ and NM and performed experiments. BH, CC, EJ, and MSR were involved in writing the paper and had final approval of the submitted and published versions.

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