

## ARTICLE

# Identification of Critical Windows of Metabolic Programming of Metabolism and Lung Function in Male Offspring of Obese Dams

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Perinatal nutritional determinants known as metabolic programming could be either detrimental or protective. Maternal obesity in the perinatal period determines susceptibility for diseases, such as obesity, metabolic disorders, and lung disease. Although this adverse metabolic programming is well-recognized, the critical developmental window for susceptibility risk remains elusive. Thus, we aimed to define the vulnerable window for impaired lung function after maternal obesity; and to test if dietary intervention protects. First, we studied the impact of high-fat diet (HFD)-induced maternal obesity during intrauterine (HFD<sup>iu</sup>), postnatal (HFD<sup>post</sup>), or perinatal (i.e., intrauterine and postnatal (HFD<sup>peri</sup>)) phase on body weight, white adipose tissue (WAT), glucose tolerance, and airway resistance. Although HFD<sup>iu</sup>, HFD<sup>post</sup>, and HFD<sup>peri</sup> induced overweight in the offspring, only HFD<sup>peri</sup> and HFD<sup>iu</sup> led to increased WAT in the offspring early in life. This early-onset adiposity was linked to impaired glucose tolerance in HFD<sup>peri</sup>-offspring. Interestingly, these metabolic findings in HFD<sup>peri</sup>-offspring, but not in HFD<sup>iu</sup>-offspring and HFD<sup>post</sup>-offspring, were linked to persistent adiposity and increased airway resistance later in life. Second, we tested if the withdrawal of a HFD immediately after conception protects from early-onset metabolic changes by maternal obesity. Indeed, we found a protection from early-onset overweight, but not from impaired glucose tolerance and increased airway resistance. Our study identified critical windows for metabolic programming of susceptibility to impaired lung function, highlighting thereby windows of opportunity for prevention.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Maternal and perinatal obesity determine metabolism of the offspring. Obesity and adverse metabolic conditions are related to impaired lung development and reduced lung function. The condition, in which adverse perinatal metabolic influences have lifelong impact on health, has been coined as “perinatal metabolic programming.”

### WHAT QUESTION DID THIS STUDY ADDRESS?

☑ The study aimed to identify which window during development is critical for determining the metabolism and lung function later in life.

### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ The present study does not only highlight the importance of perinatal metabolic influences on long-term body

composition and metabolism in male offspring, but also its impact on lung function. Moreover, we identified critical developmental windows for metabolic programming of glucose metabolism and susceptibility to impaired lung function, highlighting thereby windows of opportunity for prevention.

### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Perinatal nutrition, including postnatal feeding of newborns, is a highly relevant pediatric topic. Our study highlights that early nutritive interventions could beneficially affect the offspring's health in later life.

The incidence of chronic lung diseases, such as bronchial asthma and chronic obstructive pulmonary disease, has increased over the last decades in adults and children.<sup>1,2</sup> The pathogenesis of lung diseases is multifactorial, not only including environmental factors and genetic predisposition, but also obesity.<sup>3</sup> Obesity and childhood obesity

have become a worldwide epidemic and were recognized to contribute to impaired lung function.<sup>4</sup> Recent studies have shown that metabolic dysregulation, including cytokines secreted by the adipose tissue, occurs in patients with chronic lung diseases and could thereby be pathomechanistically important for chronic obstructive

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Received: February 20, 2020; accepted: April 14, 2020. doi:10.1111/cts.12811

pulmonary disease, bronchopulmonary dysplasia, and asthma.<sup>5–8</sup>

Children of obese women are not only at high risk to become overweight or obese,<sup>9</sup> but also to evolve wheezing illnesses, asthma, and atopic diseases.<sup>10</sup> The condition, in which perinatal metabolic influences have lifelong impact on health, has been coined as “perinatal metabolic programming.”<sup>11,12</sup> Although some studies have shown positive effects on metabolic programming,<sup>13</sup> experimental animal studies demonstrate that exposure of a maternal high-fat diet (HFD) impairs fetal lung development and increases risk for bronchiolitis.<sup>14,15</sup> Moreover, our group showed that catch-up growth after intrauterine growth restriction (IUGR) or postnatal maternal HFD increase respiratory airway resistance. These findings were related to increased production of adipocytokines in white adipose tissue (WAT),<sup>16,17</sup> a condition that has been previously linked with the pathogenesis of pulmonary diseases, such as bronchial asthma.<sup>18,19</sup>

Although there is strong evidence that maternal obesity or maternal exposure to HFD adversely affect the developing lung, the critical window for metabolic programming of metabolism, obesity, and increased airway resistance remains uncertain. The aim of the present study was to define the critical window for programming of obesity, metabolism, and lung function in the offspring using a mouse model of maternal obesity.

## METHODS

Please see the online supplementary information for a detailed description of the materials and methods.

### Animal studies

All animal studies were approved by the local government authorities (LANUV, NRW, Germany; AZ84-02.04.2012. A424). C57BL/6N mice were housed in humidity-controlled and temperature-controlled rooms with a 12-hour dark/light cycle and were allowed food and water ad libitum. Obesity was induced in female mice feeding HFD (C1057; Altromin, Lage, Germany) 7 weeks prior to conception. The control group received a standard diet (SD; R/M-H V1534; Ssniff, Soest, Germany). Prior to mating, dams received an intraperitoneal glucose tolerance test (ipGTT), as described previously.<sup>17,20</sup> Five study groups were defined: (1) control group (Co): dams were fed a SD prior to mating, during gestation, and lactation; (2) HFD<sup>pre</sup>: to determine the effect of dietary intervention, dams were fed HFD prior to conception, followed by an SD during gestation and lactation; (3) HFD<sup>iu</sup>: to define the impact of exposure to obese dams during the intrauterine (iu) period offspring of HFD-dams were nursed

by SD-dams from postnatal day (P)1 to P21; (4) HFD<sup>post</sup>: to study the effect of postnatal exposure to obese dams, offspring of SD-dams were nursed by HFD<sup>peri</sup>-dams from P1 to P21; and (5) HFD<sup>peri</sup>: dams received HFD prior to conception, during gestation, and lactation. The experimental design is illustrated in **Figure 1a**. Details about number of dams, litters, as well as offspring analyzed are mentioned in the respective figure legends. To avoid sex-related effects on the outcome, only male offspring were analyzed; each group comprised different litters.

### Physiological data of animals after litter size reduction

Body weight in grams (g) of dams and of offspring was obtained weekly prior to mating as well as at P21 and P70, respectively. At P21 and P70, the offspring was euthanized and epigonadal white adipose tissue (WAT) was excised, weighed, and preserved.

### Intraperitoneal glucose tolerance test (ipGTT)

Dams and offspring underwent an ipGTT, as previously described.<sup>17,20</sup> In brief, after fasting overnight (12 hours), blood glucose levels were determined (0 minutes), followed by i.p. injection of 20% glucose (0.1 mL/10 g body weight). Blood glucose levels were measured using a glucose meter (GlucoMen LX; A. Menarini Diagnostics) after 15, 30, 60, and 120 minutes.

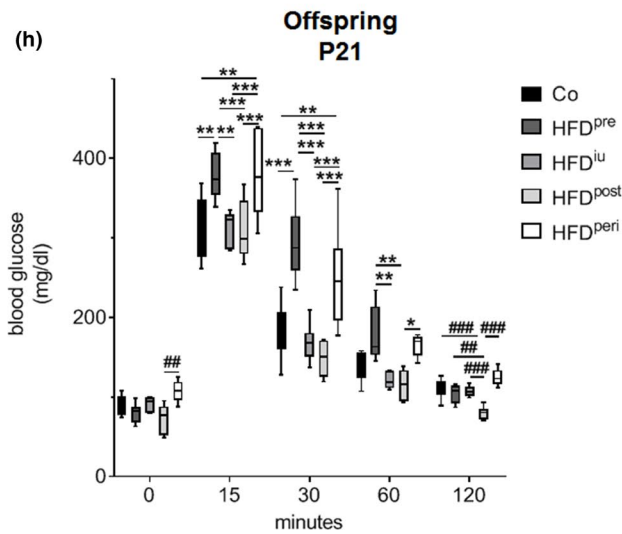
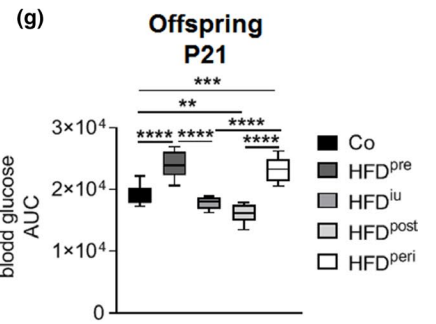
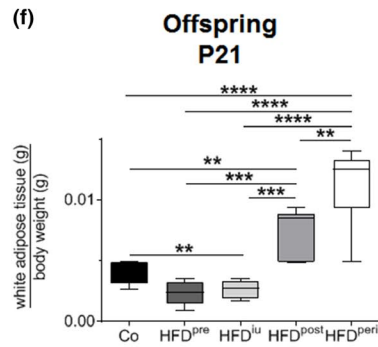
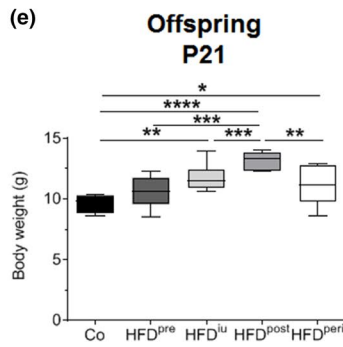
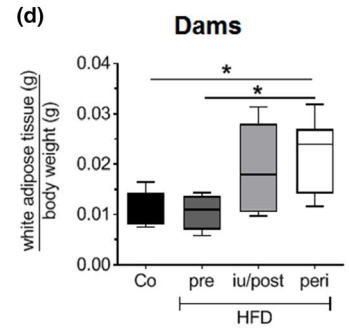
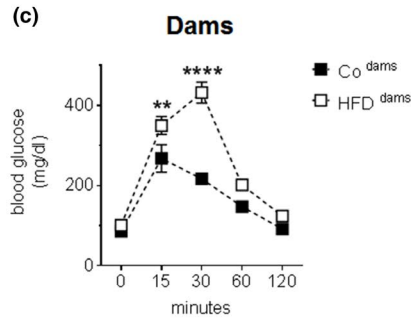
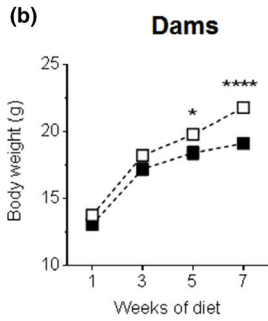
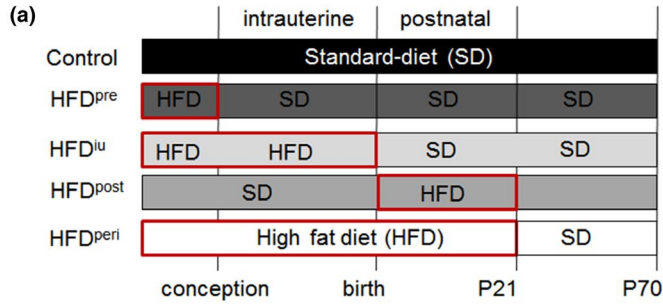
### Measurement of airway resistance

At P70, respiratory system resistance and airway responsiveness were assessed using direct plethysmography for mice (FinePointeRC; Buxco, Wellington, NC) as described previously.<sup>16</sup> Resistance was measured after exposure to phosphate buffered saline, and after stimulation with increasing concentrations of methacholine (6.25 and 12.5 mg/mL), a bronchoconstrictor.

### Analysis of data

Values are shown as means  $\pm$  SEM or as whiskers plots (minimum to maximum). One-way or two-way analysis of variance followed by Bonferroni post-test or Student *t*-test were used to test significant differences. For glucose tolerance test, area under the curve (AUC) was calculated using the trapezoidal estimation method. The trapezoidal rule is a numerical integration method to be used to approximate the integral or the area under a curve. A *P* value < 0.05 was considered as significant. The procedures were carried out using the Graph Pad Prism software (GraphPad Software Version 7.0, San Diego, CA).

**Figure 1** Perinatal windows of metabolic programming of body weight, body composition, and glucose tolerance early after birth (postnatal day 21, P21). **(a)** Experimental design: standard diet (SD), prenatal high-fat diet (HFD; HFD<sup>pre</sup>), intrauterine HFD (HFD<sup>iu</sup>), postnatal HFD (HFD<sup>post</sup>), and perinatal HFD (HFD<sup>peri</sup>). **(b, c, d)** Measurement of body weight **(b)**, and intraperitoneal glucose tolerance of dams prior to mating (SD-fed dams, Co<sup>dams</sup>; HFD-fed dams, HFD<sup>dams</sup>) **(c)**; retroperitoneal white adipose tissue (WAT) of dams relative to body weight in dams after weaning **(d)**. There were 4–6 dams per group analyzed. **(e, f)** Early-onset effects of maternal obesity at postnatal day 21 (P21). Body weight **(e)**, epigonadal WAT relative to body weight **(f)**. **(g, h)** Intraperitoneal glucose tolerance test in the offspring at postnatal day 21 (P21). Blood glucose was measured before intraperitoneal injection (0.1 mL 20% glucose/10 g body weight) at 0 minutes; as well as 15 minutes, 30 minutes, 60 minutes, and 120 minutes after injection. Area under the curve **(g)** and glucose measurements at single time points **(h)**. Co; prenatal HFD (HFD<sup>pre</sup>), intrauterine HFD (HFD<sup>iu</sup>), postnatal HFD (HFD<sup>post</sup>), and perinatal HFD (HFD<sup>peri</sup>). Four to 6 litters per group were analyzed, per offspring group 4–6 animals were analyzed. \*One-way analysis of variance (ANOVA) followed by Bonferroni post-test **(a, b)**. \*Two-way ANOVA followed by Bonferroni post-test or #Student *t*-test **(c)**; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001; #*P* < 0.05.



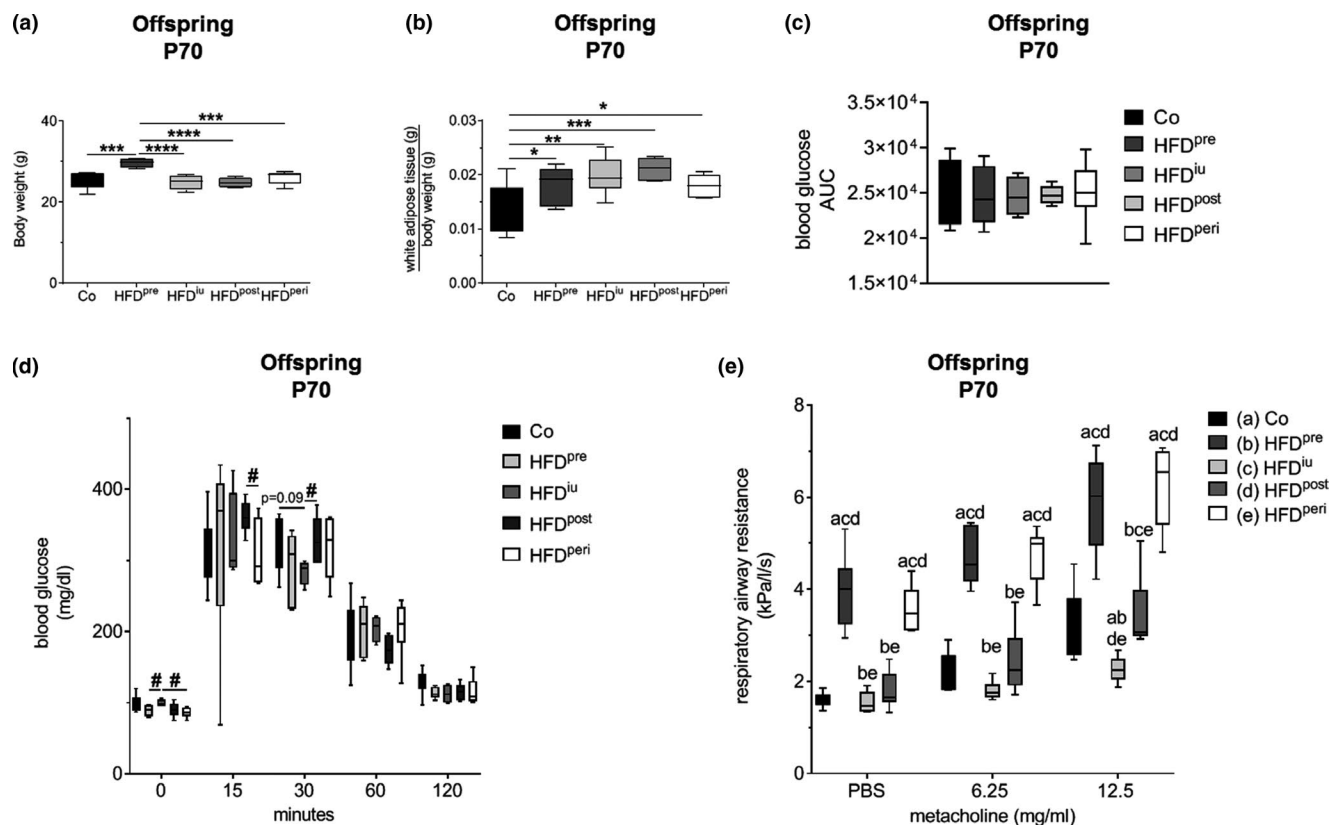
**RESULTS**

**HFD induces obesity and impaired glucose tolerance in dams prior to mating; dietary intervention to SD prior to mating reverses increased white adipose tissue during gestation**

Female mice fed HFD exhibited an increased body weight when compared to SD-fed control mice (Figure 1b). The ipGTT showed significantly higher serum glucose 15 and 30 minutes (Figure 1c) in the HFD-dams than in the Co-dams, indicating impaired glucose tolerance (Figure 1c). After 7 weeks of HFD or SD, female mice were mated to lean male mice. When mated some of the HFD-fed female mice underwent a dietary intervention by switching from HFD to SD during gestation and lactation (P1–P21; HFD<sup>pre</sup>); the other mice remained on HFD or continued on their SD, defining HFD<sup>peri</sup> (including dams of the HFD<sup>iu</sup> and HFD<sup>post</sup> groups) and Co, respectively. For better illustration, please see Figure 1a. At P21, the offspring were weaned and the dams were euthanized. Assessment of WAT showed a significant increased amount relative to body weight in HFD<sup>peri</sup>-dams when compared to Co-dams; in contrast, relative WAT in the HFD<sup>pre</sup>-dams was similar to the Co-group (Figure 1d).

**The interaction of intrauterine and postnatal window is most critical for evolving adiposity and impaired glucose tolerance in the offspring; Dietary intervention (HFD<sup>pre</sup>) is protective**

To determine which window of development is critical for metabolic programming by maternal obesity, we measured body weight as well as relative WAT and assessed glucose tolerance using ipGTT in the offspring at P21. We found that the HFD<sup>post</sup>-offspring exhibited markedly higher body weight than the Co-offspring, HFD<sup>peri</sup>-offspring, HFD<sup>iu</sup>-offspring, and HFD<sup>pre</sup>-offspring (Figure 1e). Moreover, the HFD<sup>peri</sup>- and HFD<sup>iu</sup>-offspring were heavier than the control group, whereas the HFD<sup>pre</sup>-offspring was protected at P21 (Figure 1e). We next related WAT to body weight and determined that the HFD<sup>peri</sup>-offspring and HFD<sup>post</sup>-offspring showed significantly greater WAT relative to body weight than Co-offspring; the increase of this relative WAT in HFD<sup>post</sup>-offspring, however, was less than in the HFD<sup>peri</sup>-offspring. Interestingly, HFD<sup>iu</sup>-offspring and dietary intervention (HFD<sup>pre</sup>-offspring) protected offspring from increased relative WAT (Figure 1f). Finally, we studied glucose tolerance of the offspring at P21. HFD<sup>pre</sup>-offspring, and HFD<sup>post</sup>-offspring



**Figure 2** Long-term effects of maternal obesity at postnatal day 70 (P70). (a, b): Measurement of body weight (grams) (a), and epigonadal white adipose tissue (WAT) relative to body weight (b). (c, d) Glucose tolerance test after intraperitoneal injection of 20% glucose (0.1 mL/10 g body weight). Serum glucose was measured before injection (0 minutes) as well as 15 minutes, 30 minutes, 60 minutes, and 120 minutes after injection. Area under the curve (c) and glucose measurements at single time points (d). a–d: \*One-way ANOVA followed by Bonferroni post-test (a, b) or #Student *t*-test (c); \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001; #*P* < 0.05. (e) Assessment of respiratory airway resistance. Standard-diet (SD, Co), high-fat diet (HFD), prenatal HFD (HFD<sup>pre</sup>), intrauterine HFD (HFD<sup>iu</sup>), postnatal HFD (HFD<sup>post</sup>), and perinatal HFD (HFD<sup>peri</sup>). Two-way ANOVA followed by Bonferroni post-test; significant difference as indicated: <sup>a</sup>vs. Co, <sup>b</sup>vs. HFD<sup>peri</sup>, <sup>c</sup>vs. HFD<sup>iu</sup>, <sup>d</sup>vs. HFD<sup>iu</sup>, and <sup>e</sup>vs. HFD<sup>peri</sup>. Standard-diet (SD, Co), high-fat diet (HFD), prenatal HFD (HFD<sup>pre</sup>), intrauterine HFD (HFD<sup>iu</sup>), postnatal HFD (HFD<sup>post</sup>), and perinatal HFD (HFD<sup>peri</sup>). Four to 6 litters per group were analyzed, per offspring group 4–6 animals were analyzed. AUC, area under the curve; PBS, phosphate buffered saline.

exhibited lower fasting blood glucose levels when compared to Co-offspring (**Figure 1h**). Blood glucose was higher in the HFD<sup>pre</sup>-offspring and HFD<sup>peri</sup>-offspring than in Co at 15 and 30 minutes after i.p. injection of glucose. In contrast, both HFD<sup>iu</sup>-offspring and HFD<sup>post</sup>-offspring showed significantly lower blood glucose than the HFD<sup>pre</sup>-offspring and HFD<sup>peri</sup>-offspring at 15, 30, and 60 minutes. The HFD<sup>iu</sup>-offspring and HFD<sup>post</sup>-offspring were protected from changes in glucose tolerance. Similar findings were found when we calculated the AUC (**Figure 1g,h**).

### **Maternal obesity in any window of development induces an adipose body composition in the offspring; whereas the postnatal period is more critical for long-term glucose metabolism**

To determine the long-term impact of maternal obesity, offspring of all groups were fed an SD after P21, and euthanized at P70. Body weight of HFD<sup>iu</sup>-offspring, HFD<sup>post</sup>-offspring, and HFD<sup>peri</sup>-offspring was similar to the Co-offspring; in contrast, the HFD<sup>pre</sup>-offspring were significantly heavier than Co, HFD<sup>iu</sup>-offspring, HFD<sup>post</sup>-offspring, and HFD<sup>peri</sup>-offspring (**Figure 2a**). Interestingly, WAT related to body weight was higher in HFD<sup>pre</sup>-offspring, HFD<sup>iu</sup>-offspring, HFD<sup>post</sup>-offspring, and HFD<sup>peri</sup>-offspring when compared to the Co (**Figure 2b**), not only showing that each perinatal developmental window is critical for body composition beyond infancy, but also demonstrating that dietary intervention (HFD<sup>pre</sup>) has no long-term protective effect. We next studied the glucose tolerance and found significantly reduced fasting blood glucose at single time points in HFD<sup>pre</sup>-offspring and HFD<sup>peri</sup>-offspring when compared with HFD<sup>iu</sup>-offspring. The HFD<sup>post</sup>-offspring exhibited higher blood glucose than HFD<sup>peri</sup>-offspring and HFD<sup>iu</sup>-offspring at 15 and 30 minutes, respectively. These mild changes in glucose tolerance at P70 were not detectable when we calculated the AUC (**Figure 2c,d**).

### **Maternal obesity during the intrauterine window is critical for metabolic programming of lung function**

Finally, we assessed lung function at P70 and found that exposure to obese dams during the intrauterine (HFD<sup>iu</sup>-offspring) and postnatal window (HFD<sup>post</sup>-offspring) did not adversely impact airway. In contrast, interaction of intrauterine and postnatal period (HFD<sup>peri</sup>-offspring) induced a marked increase of airway resistance at baseline, but no hyper-responsiveness to methacholine in comparison to the control group. The dietary intervention did not protect from impaired respiratory function; airway resistance in the HFD<sup>pre</sup>-offspring was similar to the HFD<sup>peri</sup>-offspring (**Figure 2e**).

## **DISCUSSION**

Our present study defines critical windows for metabolic programming of glucose tolerance, body composition, and lung function in male offspring of obese dams. The postnatal window seems to be most critical for early postnatal overweight (P21), and impaired glucose tolerance in adulthood (P70). In contrast, the intrauterine window strongly determines the persistence of early postnatal obese phenotype and early postnatal impaired glucose tolerance at P21, which is related

to increased airway resistance. Dietary intervention of obese dams only protected from early-onset adiposity at P21, but not from early postnatal impaired glucose tolerance and increased airway resistance in adulthood (P70). Prior studies have shown that maternal HFD induces increased concentrations of insulin in the offspring; in contrast, dietary intervention with an SD postnatally attenuated this effect.<sup>21</sup> The present study, however, primarily focused on glucose tolerance and did not investigate insulin resistance. Because insulin-mediated pathways have been implicated in airway resistance<sup>22</sup> further, the impact of critical windows of metabolic programming of insulin function should be addressed in future studies.

Both maternal and gestational weight gain are closely related to pulmonary function and bronchial asthma in children.<sup>10</sup> Previous studies have shown that obesity in general<sup>19,23-25</sup> as well as perinatal obesity or HFD<sup>17,21</sup> are associated with an increased level of adipokines linked to chronic airway disease. HFD<sup>peri</sup> does not only induce early-onset overweight and impaired glucose tolerance, but also long-term obese body composition and increased airway resistance. Although the HFD<sup>post</sup>-offspring showed the highest content of WAT at P21, the WAT:body weight ratio was higher in the HFD<sup>peri</sup>-offspring, indicating an altered body composition between the groups. Interestingly, although postnatal exposure to maternal obesity had a strong impact on obese body composition in adulthood, HFD<sup>post</sup>-offspring did not exhibit impaired lung function. These findings are in contrast to our previous studies, in which offspring of dams fed with HFD during lactation exhibited an asthma-like phenotype.<sup>17</sup> Differences in animal model as well as body composition and metabolism in dams could account for differences in lung function in both studies.<sup>17</sup>

In the present study, we cross-fostered offspring of HFD-dams and SD-dams to guarantee an isolated exposure to maternal obesity during the intrauterine phase (HFD<sup>iu</sup>). HFD<sup>iu</sup> induced an obese phenotype, but exhibited reduced fasting blood glucose and partially improved glucose uptake. The interaction of intrauterine and postnatal exposure to maternal obesity, however, had a strong effect on early-onset adiposity and impaired glucose metabolism at P21 as well as impaired lung function at P70, suggesting that the transition from the intrauterine to the postnatal period is of utmost importance for metabolic programming of airway resistance. In line with these findings, our group reported that prevention of postnatal hyperalimentation with early-onset adiposity protects from increased airway resistance after IUGR.<sup>26</sup> Supportive clinical studies show that children of obese mothers and with accelerated postnatal weight gain are prone to become obese and evolve asthma later in life.<sup>1</sup>

Perinatal dietary intervention is important for metabolism of the infants and for lung function.<sup>21</sup> For example, breastfeeding instead of infant formula reduces the risk for bronchopulmonary dysplasia,<sup>27</sup> highlighting the perinatal window of opportunity to preserve lung development. We show that HFD<sup>pre</sup> is protected from early-onset overweight but not from impaired glucose tolerance. At P70, however, the HFD<sup>pre</sup>-offspring exhibited increased body weight, WAT, and airway resistance when compared to the Co. The switch in nutrition from HFD to SD after conception induces loss of WAT. This metabolic stress could affect placental

function and thereby the intrauterine nutritive supply of the offspring and resemble features seen in IUGR.<sup>28</sup>

Finally, the impaired lung function with increased airway resistance in the HFD<sup>peri</sup>-offspring and HFD<sup>pre</sup>-offspring does not show a bronchial hyperreagibility, but rather increased airway resistance at baseline. Obese asthmatic children show a decreased functional residual capacity and FEV1/FVC ratios and a poor response to bronchodilator.<sup>29,30</sup> These human data coupled with our findings suggest that structural changes might occur in obese asthmatic children.

The present study has a few limitations that need to be taken in account for the interpretation of the data. First, we did not study insulin resistance, but glucose tolerance. Although both terms are intimately linked, they should not be used interchangeably. Whereas prior studies indicate that perinatal HFD increases insulin concentrations in the offspring, future studies should determine the critical window of metabolic programming of insulin signaling. Second, the present study design does not allow to distinguish between the impact of maternal overweight, hyperglycemia and direct dietary impact. This must be taken into account when interpreting the data. Third, cross-fostering represents an additional stress factor that could affect metabolic response and programming in the offspring.

To conclude, our data do not only highlight the importance of perinatal metabolic influences on long-term body composition and metabolism in the offspring, but also its impact on lung function. Both perinatal maternal obesity and intrauterine stress, such as dietary intervention, induce metabolic programming of lung function. Further studies investigating the sex effect and in-depth mechanistic studies in this model are necessary. Elucidating the molecular mechanism is fundamental to define preventative strategies for a worldwide epidemic.

**Supporting Information.** Supplementary information accompanies this paper on the *Clinical and Translational Science* website ([www.cts-journal.com](http://www.cts-journal.com)).

**Funding.** This study was funded by Marga und Walter Boll Stiftung (210-03-15, 210-02-16); Köln Fortune, University of Cologne, Faculty of Medicine and University Hospital Cologne (283/2017); and Deutsche Forschungsgemeinschaft (DFG; 1632/2-1).

**Conflicts of Interest.** All authors declared no competing interests for this work.

**Author Contributions.** K.D. and M.A.A.A. wrote the manuscript. K.D. and M.A.A.A. designed the research. K.D. performed the research. K.D., S.v.K.R., J.D., and M.A.A.A. analyzed the data.

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