

Reduced levels of maternal progesterone during pregnancy increase the risk for allergic airway diseases in females only

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Abstract

Observational as well as experimental studies support that prenatal challenges seemed to be associated with an increased risk for allergic airway diseases in the offspring. However, insights into biomarkers involved in mediating this risk are largely elusive. We here aimed to test the association between endogenous and exogenous factors documented in pregnant women, including psychosocial, endocrine, and life style parameters, and the risk for allergic airway diseases in the children later in life. We further pursued to functionally test identified factors in a mouse model of an allergic airway response. In a prospectively designed pregnancy cohort ($n=409$ families), women were recruited between the 4th and 12th week of pregnancy. To investigate an association between exposures during pregnancy and the incidence of allergic

airway disease in children between 3 and 5 years of age, multiple logistic regression analyses were applied. Further, in prenatally stressed adult offspring of BALB/c-mated BALB/c female mice, asthma was experimentally induced by ovalbumin (OVA) sensitization. In addition to the prenatal stress challenge, some pregnant females were treated with the progesterone derivative dihydrodydrogesterone (DHD). In humans, we observed that high levels of maternal progesterone in early human pregnancies were associated with a decreased risk for an allergic airway disease (asthma or allergic rhinitis) in daughters (adjusted OR 0.92; 95 % confidence interval [CI] 0.84 to 1.00) but not sons (aOR 1.02, 95 % CI 0.94–1.10). In mice, prenatal DHD supplementation of stress-challenged dams attenuated prenatal stress-induced airway hyperresponsiveness exclusively in female offspring. Reduced levels of maternal

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progesterone during pregnancy—which can result from high stress perception—increase the risk for allergic airway diseases in females but not in males.

Key messages

- Lower maternal progesterone during pregnancy increases the risk for allergic airway disease only in female offspring.
- Prenatal progesterone supplementation ameliorates airway hyperreactivity in prenatally stressed murine offspring.

Keywords Allergic airway disease · Asthma · Allergic rhinitis · Progesterone · Pregnancy · Pregnancy cohort · Mice

Introduction

The incidence of allergic airway diseases such as asthma and rhinitis has been increasing in young children in most Western societies [1]. A hereditary component is well known to increase the risk for allergic airway diseases in children. However, the rapid rise in disease prevalence observed over the last decades suggests that environmental and lifestyle changes contribute to the increased incidence of childhood allergies. In this context, an emerging field of research investigating the developmental origin of health and disease has drawn attention to the lasting influence of the prenatal period in determining the risk for allergic diseases [2–5]. To date, a number of chronic diseases have been linked to prenatal challenges, such as coronary heart disease [6], obesity, metabolic syndrome, and type I diabetes [7]. In the context of an increased risk for allergic airway diseases, prenatal tobacco smoke exposure [8, 9], urban living [10], maternal diet during pregnancy [11] and prenatal stress challenges [12–15] were identified as risk factors.

Prenatal stress is known to critically impair maternal endocrine adaptation to pregnancy [16]. In this context, a decrease of maternal progesterone in response to stress appears to be a phenomenon highly conserved between species, as it could be confirmed in mice [17], elks [18], and humans [16, 19]. Progesterone among other endocrine factors, such as estradiol, is important in promoting and sustaining pregnancy and therefore fetal development [20].

In this context, we previously unveiled that reduced levels of progesterone during early pregnancy are associated with an increased risk for female children to develop atopic dermatitis at an age of 3 years [21]. An association with other pregnancy hormones, such as estradiol, could not be shown. Here, we now present data from a prospectively designed birth cohort supporting that lower levels of maternal progesterone during pregnancy increase the risk for allergic airway disease up to an age of 5 years in a gender-dependent way. In a translational, bedside-to-bench approach, we provide evidence that maternal supplementation with progesterone during pregnancy in

mice ameliorates the enhanced airway response in prenatally stressed female adult offspring.

Materials and methods

Prospective birth cohort

Study design and setting

Pregnant women were recruited during the first trimester (between 4 and 12 weeks of gestation) in private obstetrical practices in Berlin, Germany, from February 2002 to August 2003. Pregnancy was confirmed by a positive urine test or via ultrasound. Medical, obstetric, and gynecological history was documented, and standardized questionnaires were used to examine maternal quality of life (QoL-SF12) [22], perceived stress (PSQ) [23], and social support (F-SOZU) [24, 25]. Further, blood was taken at recruitment by venous puncture from all women and delivered to our laboratory within 1 to 3 h by courier, and serum was harvested from all blood samples after centrifugation (2,000 rpm/20 min) and stored at -80°C until hormone levels were determined.

The present study resulted from the continuation of a study aiming to identify early markers for subsequent pregnancy complications [19, 26]. Originally, 1,089 pregnant women had been recruited. After birth, women were asked whether they would be willing to participate in the continuation of the study. Of the women, 473 agreed and were subsequently contacted on an annual basis. The research protocol was approved by the ethics committee of the Charité University Medicine Berlin, and all participants gave written informed consent.

Outcome assessments

The main outcome was the cumulative incidence of allergic airway diseases (asthma and allergic rhinitis) in the children between the ages of 3 and 5 years. This was assessed with an annual questionnaire filled out by the parents, employing previously published study designs [1]. Asthma between ages 3 and 5 was defined by a positive response to the questions “ever having suffered from a wheeze” and additionally “taking asthma medication” or “having been diagnosed with asthma by a doctor.” Allergic rhinitis was specified as “suffering from sneezing attacks or runny, blocked, or itchy nose in the absence of a common cold.” If the child suffered once from asthma, allergic rhinitis, or both between the ages of 3 and 5, he or she was assumed to suffer from an allergic airway disease. We here assessed allergic airway disease via parental questionnaires, since obtaining a physician’s diagnosis was not feasible in the present setting. A published evidence supports the validity of such approach, as the specificity of the items used for self-reported allergic

rhinitis as well as asthma assessment is high, allowing for an accurate identification. Noteworthy, the sensitivity of such approach is still acceptable but lower compared to a physician's diagnosis [27–29].

Exposure of interest

Maternal serum progesterone and estradiol levels were measured during the first trimester (between 4 and 12 weeks of gestation) using commercially available ELISA kits (DRG Instruments GmbH, Marburg, Germany). Serum samples were thawed, and—if required by the kit's protocol—steroids were extracted and assayed according to the manual provided by the manufacturer. Maternal progesterone and estradiol levels were used as continuous predictors of allergic airway disease in the statistical models.

Potential confounders

Maternal characteristics such as age at birth, body mass index, quality of life, gestational age at time of recruitment, and smoking during pregnancy documented at recruitment were considered potential confounders to increase the risk for allergic airway diseases in the children [8, 9, 30]. Further, perinatal parameters including duration of pregnancy, maternal body mass index (BMI) in the first trimester, fetal birth weight, and duration of breast-feeding; parental factors, such as parental allergy and education; and environmental factors like postnatal parental smoking, the presence of older siblings, day care attendance, and cat ownership were documented after birth and included in the analyses. Educational background was defined as more than or less than 12 years of maternal education, which is equivalent to a German secondary school diploma. Postnatal tobacco smoke exposure was defined as “never” if she reported not smoking at all three postnatal follow-up assessments, “irregular” when she reported smoking at one of the assessments, and “regular” when the mother reported smoking at two or more follow-up assessments.

Experimental setting of allergic airway inflammation in mice

Mouse model

Adult male and female BALB/c mice were obtained from Charles River and maintained in an animal facility with a 12 h light/dark cycle and provided with water and chow ad libitum. Animal care and experimental procedures were followed in accordance with guidelines of the Canadian Council for Animal Care.

Maternal stress exposure and progesterone administration

Six- to eight-week old BALB/c females were allowed to acclimatize to the animal facility 1 week before being mated with BALB/c male mice. Successful mating was assumed upon the appearance of a vaginal plug. Pregnant females were segregated and divided into three different subgroups. One group served as a control and was left undisturbed during pregnancy. The second and the third groups were exposed to sound stress for 24 h on days 12.5 and 14.5 of gestation according to our previously established prenatal sound stress model (Fig. 1) [31]. The times of gestational stress exposure in mice were chosen to pinpoint effects on the fetal immune development, since this was of primary interest. Critical phases of thymic development take place in mid-gestation in mice and are comparable to those occurring between early and mid-gestation in humans [32]. Additionally, pregnant mice are rather resistant to fetal loss in response to sound stress challenge at mid-gestation, whereas challenges early in gestation have been linked to a high frequency of fetal loss, which would have resulted in fewer offspring or forced us to generate more litter [33]. In this model, pregnant dams have previously been shown to have lower progesterone levels in response to the stressor ([34] and Solano and Arck, unpublished observation). The sound stress was emitted by a rodent repellent device (Conrad Electronic) with 70 dB at a frequency of 300 Hz in intervals of 15 s. To prevent the mice from escaping sound exposure, the stress device was placed into the mouse cage.

In addition to exposure to the sound stressor, the third group of pregnant females was subcutaneously injected with single doses of 1.25 µg of dihydroprogesterone (DHD), dissolved in a 200-µl solution of sterile castor oil (80 %) and benzyl benzoate (20 %) on gestational day 11.5, 13.5, and 15.5. Pregnant mice from the control group and stress group received a sham injection of 200 µl sterile castor oil/benzyl benzoate solution on the same gestational days. This DHD supplementation protocol prior to sound stress challenge induces supra-physiological progesterone levels, which was our intention in order to provide the mice with a progesterone deposit. Progesterone has no known toxic effect during pregnancy; hence, the temporary increase of progesterone above physiological levels is assumed to be negligible with regard to the outcome analysis in the offspring. At delivery, gestational age and litter size were documented. Offspring were weaned 21 days after delivery. Female and male offspring were used for subsequent induction of experimental allergic airway inflammation. We considered to include a group of pregnant mice that receives DHD, but no prenatal stress challenge. However, we dismissed this idea as it was our aim to supplement the stress-challenged dams with progesterone rather than inducing supra-physiological progesterone levels by injecting DHD in control dams.

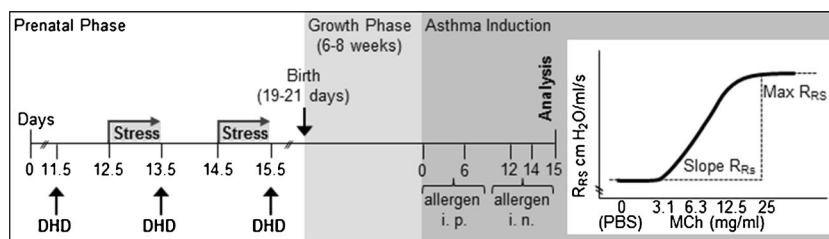


Fig. 1 The experimental set up is shown: In timed pregnancies, syngenerically mated BALB/c female mice were stressed on gestation days (gd) 12.5 and 14.5. One group of pregnant mice received DHD at the same time as the stress exposure on days 11.5, 13.5, and 15.5. At an age between 6 and 8 weeks (early adolescence), the offspring were immunized twice with OVA i.p. This treatment was repeated 6 days later and

followed by two intranasal (i.n.) OVA applications at days 12 and 14 after the first OVA treatment. Airway responsiveness was measured in response to increasing doses of methacholine (MCh). Two components of airway responsiveness were assessed: airway reactivity, which is the slope of increase in total respiratory system resistance (R_{RS}) for an increase in MCh dose, and maximum inducible bronchoconstriction (maximum R_{RS})

Experimental allergic airway inflammation in the offspring

Since asthma-like symptoms do not occur spontaneously in mice [35], this study used an ovalbumin (OVA)-sensitized mouse model of allergic airway inflammation. Briefly, between the ages of 6 and 8 weeks, male and female offspring were sensitized to OVA by two intraperitoneal injections of 20 μ g OVA (grade VI; Sigma Chemie) adsorbed with 500 μ g mg $Al(OH)_3$ (alum) in saline on experimental days 0 and 6. On days 12 and 14, mice were challenged intranasally with 5 μ g OVA per mouse [36]. Twenty-four hours after the last challenge (day 15), airway responsiveness was measured in these mice as described below. Alum-injected, saline-challenged offspring of the respective groups served as control [37]. Theoretically, it may be advantageous to test the severity of experimentally induced asthma in response to prenatal challenges in pre-pubescent rather than adult offspring to mirror effects seen in children in human studies. However, we were restricted to assess allergic airway inflammation in adult offspring, to act in accordance with the guidelines of the Canadian Council of Animal Care. Similarly, published evidences from other groups on experimentally induced asthma also generally arise from adult or pubescent mice [35, 37, 38], which facilitates direct comparison of effects.

Airway responsiveness

Airway responsiveness was determined as airway reactivity and maximum inducible bronchoconstriction. Both parameters were based on respiratory system resistance (R_{RS}) in response to saline and increasing doses of methacholine (MCh) using the flow interrupter technique. The experimental setup was followed as described previously [37], modified by a maximum MCh challenge of 25 mg/ml. Airway reactivity was assessed as the slope of the dose response, calculated by linear regression between the measured R_{RS} and the $\log_{10}$ -transformed MCh dose at 3.1, 6.3, 12.5, and

25 mg/ml, as described previously [37, 39]. Maximum inducible bronchoconstriction was assessed as the maximum R_{RS} measured in centimeter H_2O per milliliter per second.

Bronchoalveolar lavage (BAL)

Two aliquots of 250 μ l PBS were injected and withdrawn through a tracheal cannula. Airway inflammation was assessed by inflammatory cell counts in BAL fluids. Cells were removed from BAL fluid by centrifugation at $200\times g$ for 15 min. Cells were resuspended in PBS (1 ml). BAL cells were stained with trypan blue, and viable cells were counted using a hemocytometer. Smears of BAL cells were prepared with Cytospin (Thermo Shandon, Pittsburgh, PA, USA) and stained with HEMA 3 reagent (Biomedical Sciences, Swedsboro, NJ, USA) for differential cell counts (see Fig. 2a). An independent observer blinded to the experimental conditions performed all cell counts [37].

Cytokine determination by CBA

Cytokines were analyzed in BAL samples using CBA. Samples were stored at $-80^\circ C$ until cytokines were determined. IL-4, IL-5, and IL-13 were detected simultaneously using the mouse cytokine CBA-enhanced sensitivity kit (BD Biosciences). Briefly, 50 μ l of each sample was mixed with 20 μ l of mixed capture beads and 50 μ l of mouse inflammation PE detection reagent. Samples were incubated twice for 2 h at room temperature. After incubation, samples were washed once and resuspended with 300 μ l of washing buffer before acquisition on a LSRFortessa cytometer (BD Biosciences). Standard curves were generated for each cytokine using a mixed cytokine standard from the kit. Data were analyzed using FCAP Array v.3.0.1 (BD, Softflow). The concentration of each cytokine was determined by interpolation to the corresponding standard curve.

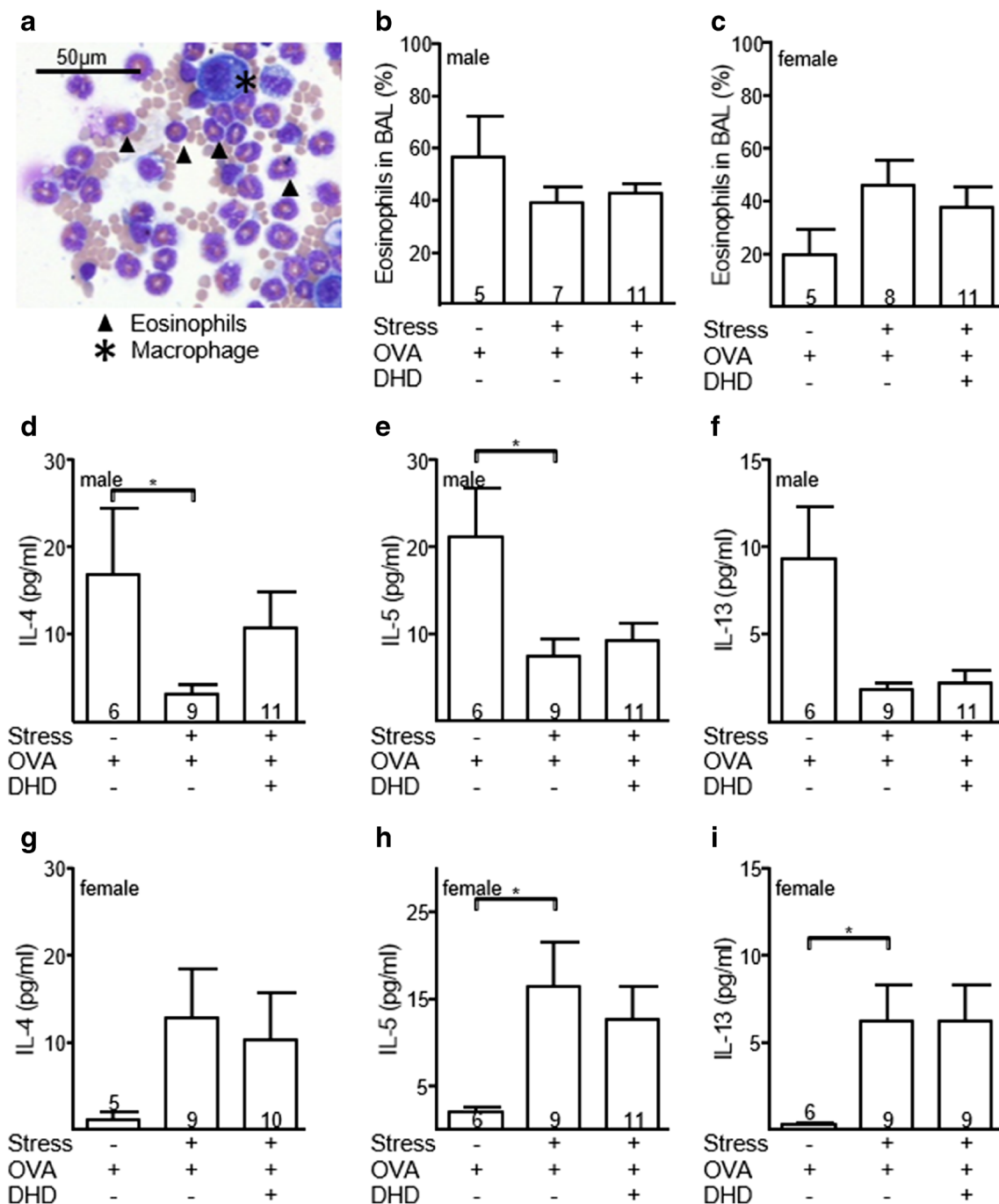


Fig. 2 Representative photomicrograph of a Cytospin prepared from BAL samples for cell differentiation; eosinophils are marked with arrow heads (a). Frequencies of eosinophils in BAL for male (b) and female (c) offspring. Levels of IL-4 (d), IL-5 (e), and IL-13 (f) in BAL supernatants obtained from male offspring from the respective groups of prenatal challenges and

DHD treatment. Levels of IL-4 (g), IL-5 (h), and IL-13 (i) in female offspring from the respective groups of prenatal challenges and DHD treatment. Each column represents the mean (SEM). Data were not normally distributed, so non-parametric tests were used to assess statistically significant differences between groups. **p* value <0.05; ***p* value <0.01

Statistical analyses

Animal experiments

Experimental results are expressed as the means and the standard errors of the means (SEM). Since data was normally

distributed, analyses of variance (ANOVA) were used to compare levels of airway reactivity (the slope of the R_{RS} –MCh dose–response curve) and maximum bronchoconstriction (Max R_{RS}) between treatment groups. A *p* value of less than 0.05 was considered statistically significant. Post hoc analyses were conducted using Tukey’s honestly significant difference

(HSD) tests, given a statistically significant omnibus ANOVA *F* test. Data that was not normally distributed were analyzed using Kruskal–Wallis and Mann–Whitney *U* tests.

Birth cohort analyses

For descriptive analyses, we calculated means and standard errors of the mean (SEM) for continuous variables. Differences between study participants who were included in the analysis and those who were lost to follow-up were tested by the χ^2 test (for categorical variables) and the Mann–Whitney *U* test (for continuous variables). For all children combined and stratified for boy and girls, we estimated the association between progesterone and allergic airway disease by calculating crude odds ratios (OR) and 95 % confidence intervals (CI) to indicate uncertainty. In multivariable logistic regression analyses, we calculated ORs and 95 % CIs adjusting for potential confounders based on the literature.

The present analyses were based on a total of 409 observations. The adjusted model included gestational age when progesterone was assessed (in weeks), first trimester estradiol levels, maternal BMI during first trimester, maternal smoking during pregnancy, parental allergy status (2 and 1 vs. non-allergic parents), parental educational attainment, postnatal parental smoking, presence of older siblings, and cat ownership. A *p* value of less than 0.05 was considered significant, though the *p* values cannot be interpreted as error probabilities in a confirmatory sense.

Results

Incidence of allergic airway diseases in human study population

Among the 473 pregnant women enrolled in the continuation of the birth cohort study, 409 women and their children continued to participate until the children reached the age of 5 years. Subjects who dropped out of the study were similar to those who participated until the children reached 5 years of age with regard to demographic, medical, psychosocial, and biological parameters (Table 1). Among the children included in the analyses, the incidence of allergic airway diseases during the first 5 years was 37.1 % (41.9 % in boys and 30.3 % in girls). Furthermore, 19.7 % of those children with allergic airway disease suffered exclusively from asthma, 61.3 % suffered only from allergic rhinitis, and 18.9 % suffered from both.

We performed univariable logistic regression analyses and observed that maternal, prenatal endocrine, or perinatal factors did not significantly affect the children's odds of developing an allergic airway disease. Parental allergy significantly

increased children's odds as well as irregular postnatal parental smoking (see Supplement Table 1).

Inverse association between progesterone and allergic airway disease in girls

Next, we stratified the sample by sex and calculated crude odds ratios for boys and girls separately. Stratification revealed that increasing levels of first trimester maternal progesterone by 1 ng/ml reduced the odds to develop allergic airway inflammation in the first 5 years of life exclusively in girls (crude OR 0.96, 95 % CI 0.91–1.01, *p* value=0.09). No other factors were significantly associated with the development of allergic airway disease during the first 5 years in girls (Supplement Table 2). In boys, one allergic parent and perceived stress significantly increased the odds of developing allergic airway disease (Supplement Table 2).

We then further examined the relationship between maternal progesterone levels during early pregnancy and allergic airway disease by controlling for confounding variables in a multivariable logistic regression. In boys, no association could be observed between maternal progesterone levels during pregnancy and the development of allergic airway disease until the age of 5 (adjusted OR 1.02, 0.94–1.10). In girls, however, higher maternal progesterone levels during pregnancy significantly reduced the odds to develop an allergic airway disease (adjusted OR 0.92, 0.84–1.00; *p* value <0.05). Moreover, parental allergy significantly heightened the risk toward the development of allergic airway disease in boys only, whereas all other factors documented in this study revealed no statistically significant associations in the adjusted analyses (Table 2). Progesterone is produced by the corpus luteum until completion of the luteal–placental shift at approximately 6–12 weeks following last menstruation. Hence, we explored whether a failure or delay of the luteal–placental shift and associated lower levels of progesterone during the first trimester differed in daughters with or without allergic airway disease. We identified that progesterone levels significantly increased with gestational week in mothers of daughters who did not develop allergic airway disease (Supplement Figure 1A). Conversely, in mothers whose daughters developed allergic airway disease, progesterone levels did not significantly increase with gestational age but very slightly decreased (Supplement Figure 1B). In mothers, who were pregnant with a boy, progesterone increased regardless of later disease development (data not shown). Further, we excluded that maternal progesterone levels during pregnancy were affected by maternal atopy. When comparing progesterone levels, no significant differences could be observed between atopic (mean=16.53 ng/ml, SEM=0.51) and non-atopic mothers (mean=17.81 ng/ml, SEM=0.76; *t*(378)=1.428, *p*=0.15).

Table 1 Comparison of demographic, anamnestic, and psychosocial parameters at time of recruitment between continuously participating families compared to discontinued participation

Characteristics ^a	Continuous participation	Discontinued participation	<i>p</i> value
Prenatal maternal factors			
Maternal age, years (available data) ^b	29.8±0.2 (407)	29.0±0.7 (56)	0.28
Maternal BMI, kg/m ² (available data) ^c	23.4±0.2 (399)	23.1±0.7 (52)	0.42
Gestational week at recruitment (available data) ^c	8.8±0.2 (407)	8.8±0.3 (56)	0.82
Quality of life (available data) ^c	49.0±0.4 (345)	50.2±1.0 (57)	0.23
Social support, score from 1 to 5 (available data) ^c	4.5±0.0 (372)	4.6±0.1 (51)	0.24
Perceived stress (available data) ^{c,d}	32.58±0.8 (372)	34.04±2.2 (52)	0.54
Mother's smoking during pregnancy, % (available data) ^d	15.6 (358)	21.7 (60)	0.26
Endocrine factors			
Progesterone, ng/ml (available data)	18.2±0.7 (387)	16.8±0.9 (50)	0.68
Estradiol, ng/ml (available data)	658.2±29.4 (347)	714.1±103.3 (50)	0.81
Perinatal factors			
Week of delivery (available data)	39.6±0.09 (403)	39.3±0.3 (50)	0.25
Fetal birth weight, grams (available data)	3,424.4±26.5 (409)	3,375.1±80.4 (51)	0.60
Breast-feeding duration, months (available data)	3.7±0.2 (332)	3.5±0.4 (52)	0.54
Parental factors			
Parental allergy, % (available data)	15.7 (363)	13.3 (60)	0.70
Education ≥12 years of schooling, % (available data)	53.7 (402)	47.3 (55)	0.30
Mothers who are employed, % (available data)	98.0 (402)	83.3 (54)	0.53
Environmental factors			
Children with postnatal parental smoke exposure, % (available data)	25.0 (362)	18.8 (60)	0.29
Study children with older siblings, % (available data)	36.7 (406)	28.6 (56)	0.23

^a If not stated differently, characteristics are described as mean±SEM; available data indicates *n*

^b At birth of child

^c Assessed at recruitment during first trimester

^d Assessed with the perceived stress questionnaire

Airway response in prenatally stressed offspring in mice

The ANOVA revealed a significant difference between treatment groups in female offspring (airway reactivity $F(4,43)=4.584$, $p=0.004$; maximum bronchoconstriction $F(4,42)=5.062$, $p=0.002$). Post hoc tests revealed prenatally stressed female offspring had significantly increased airway responsiveness in OVA-sensitized female offspring (see Fig. 3b, d), which confirmed our previous observations. In OVA-sensitized male offspring, exposure to prenatal sound stress or pre-treatment with DHD did not alter airway responsiveness compared to control offspring (see Fig. 3a, c). Strikingly, the prenatal stress-induced enhanced airway responsiveness observed in female offspring was ameliorated if dams had been treated with DHD during pregnancy (see Fig. 3 b, d; Supplement Figure 2B). Noteworthy, the response to OVA sensitization was more profound in male offspring compared to females (see Fig. 3a, c; Supplement Figure 2A). The percentage of eosinophils in BAL shows a similar pattern to the airway responsiveness, which does not reach levels of significance (Fig. 2). There is no difference between stressed male

offspring and offspring that was injected with DHD (see Fig. 2b). In female offspring, eosinophils increase in the prenatal stress group. This effect is decreased in the dams, who also received DHD (Fig. 2c). Similarly, prenatal stress significantly increases levels of IL-5 and IL-13 in female offspring (Fig. 2h, i). DHD supplementation decreased IL-4, IL-5, and IL-13 in female offspring. However, this decrease did not reach levels of significance (Fig. 2h, i). In male offspring, prenatal stress decreased the production of these cytokines (Fig. 2d–f). Moreover, prenatal exposure to sound stress challenge or DHD supplementation did not affect gestational length or the number of offspring (data not shown). The sex ratio within the respective litters was also not affected (data not shown).

OVA-specific immunoglobulin E (IgE) did not differ between prenatally stressed, DHD treated, and non-stressed female offspring (data not shown), confirming that the differences of clinical asthma features among the various groups of female mice were not due to a variation in the effectiveness of sensitization. DHD does not affect subsequent sensitization of offspring with OVA.

Table 2 Odds for a child to develop an allergic airway disease between 3 and 5 years, stratified by gender, based on multivariable logistic regression analysis^a

Independent factors	Boys (<i>n</i> =202)			Girls (<i>n</i> =176)		
	Adjusted OR ^a	95 % CI	<i>p</i> value	Adjusted OR ^a	95 % CI	<i>p</i> value
Prenatal maternal factors						
Maternal BMI (kg/m ²) ^b						
<18.5	NE	NE	NE	0.58	0.07–5.03	0.62
18.5–25.0	1	–	–	1	–	–
25.0–30.0	1.68	0.31–9.18	0.55	0.48	0.11–2.04	0.32
>30.0	1.42	0.31–6.57	0.66	0.45	0.02–9.12	0.60
Smoking during pregnancy						
Yes	0.35	0.07–1.73	0.20	0.56	0.11–2.83	0.48
No	1	–	–	1	–	–
Endocrine factors						
Progesterone (ng/ml) ^{b,c}	1.02	0.94–1.10	0.65	0.92	0.84–1.00	0.048
Estradiol (ng/ml) ^{b,c}	1.00	1.00–1.00	0.76	1.00	1.00–1.00	0.13
Parental factors						
Parental allergy						
2 allergic parents	7.16	0.71–72.13	0.01	NE	NE	NE
1 allergic parent	5.62	1.64–19.22	0.10	0.94	0.18–4.96	0.94
No allergic parent	1	–	–	1	–	–
Parents' educational attainment						
≥12 years	0.61	0.24–1.60	0.32	0.60	0.20–1.87	0.38
<12 years	1	–	–	1	–	–
Environmental factors						
Older siblings						
Yes	0.62	0.25–1.55	0.30	0.88	0.31–2.52	0.81
No	1	–	–	1	–	–
Cat ownership ^c						
Yes	0.80	0.26–2.47	0.70	0.88	0.31–2.52	0.81
No	1	–	–	1	–	–
Postnatal parental smoking						
Regular	4.37	0.80–23.91	0.09	0.23	0.03–1.68	0.15
Irregular	1.38	0.18–10.70	0.76	0.13	0.01–1.32	0.09
Never	1	–	–	1	–	–

NE not estimable, OR odds ratio

^a Adjusted for all variables listed here and gestational week at progesterone assessment^b At recruitment during first trimester^c Per unit increase^c Cat lived in household during first 5 years

Discussion

Previous studies have provided convincing epidemiological evidence for prenatal modulation of the risk for allergic diseases later in the child's life. However, mediators through which this risk may be altered are largely elusive. We here provide evidence that reduced levels of maternal progesterone during pregnancy may be involved

in increasing the risk for allergic airway diseases in children in a sex-dependent fashion. In a mouse model, which mirrors the findings in human pregnancies, we show that progesterone substitution ameliorates this effect. These recent observations are in line with the identification of an inverse association between maternal progesterone levels during pregnancy and the risk for atopic dermatitis in daughters at an age of 3 years [21].

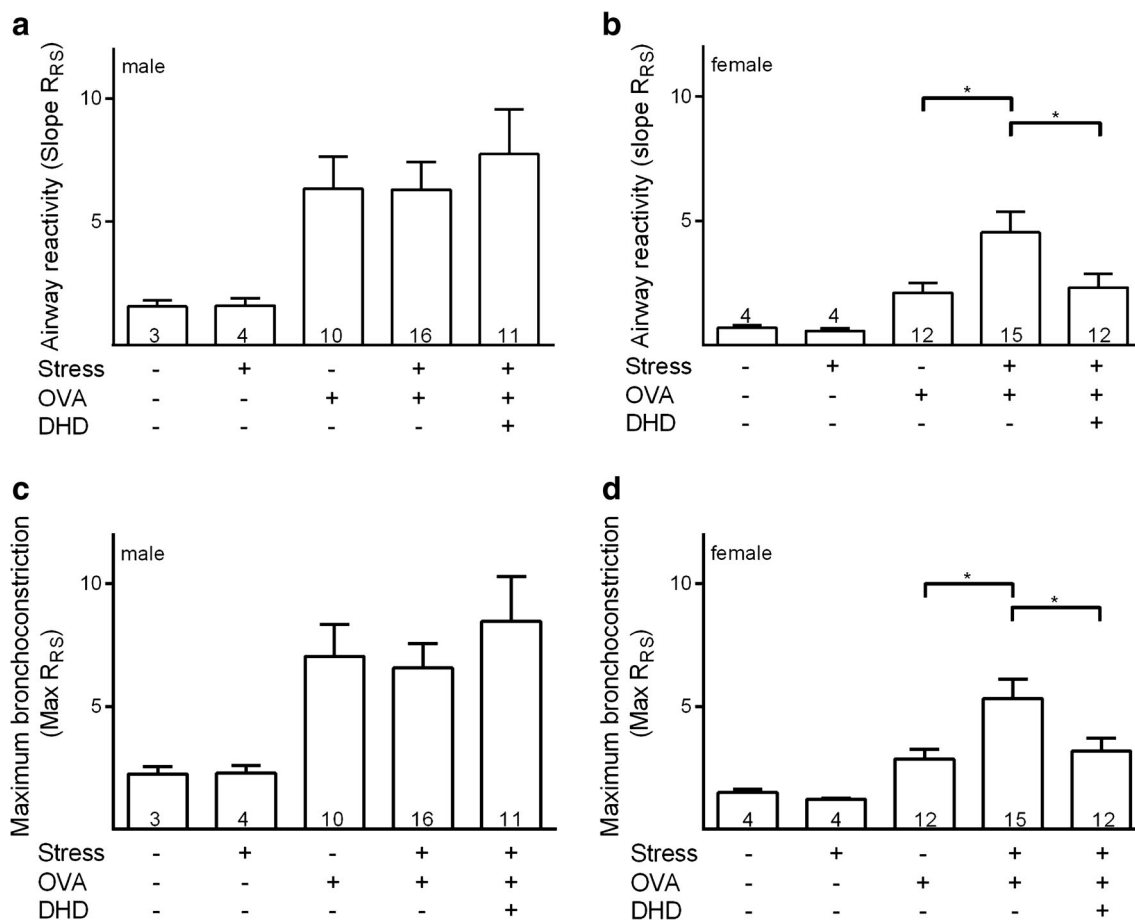


Fig. 3 The effect of treatment with prenatal stress and DHD in male (**a**) and female (**b**) offspring on airway reactivity (slope R_{RS}) and maximum MCh-inducible bronchoconstriction (Max R_{RS} , **c** for male and **d** for female offspring) in OVA-sensitized mice 24 h after challenge with

intranasal OVA or saline. ANOVA results: airway reactivity $F(4,43)=4.584$, $p=0.004$; maximum bronchoconstriction $F(4,42)=5.062$, $p=0.002$. Each column represents the mean (SEM). * p value <0.05 and ** p value <0.01 from post hoc Tukey's tests

However, one must consider that the effect we observed in the cohort study of first trimester progesterone on girls is rather weak and that our findings are limited by the questionnaire-based diagnosis of allergic airway disease. An independent confirmation of these findings is needed, and we hope that our present findings encourage researcher to address the role of prenatal progesterone levels in modulating children's risk for allergies later in life. In the present study, a clinical validation of this diagnosis by a physician would have been more desirable compared to parentally reported disease incidences in the children, especially since assessments via parental questionnaires have been shown previously to under diagnose asthma and allergic rhinitis [27, 29, 40]. It remains to be elucidated if a clear-cut clinical diagnosis would have led to an even more robust effect of prenatal progesterone levels decreasing the risk for allergic airway disease. Additionally, results from skin prick tests or specific IgE levels would have been useful to characterize whether the children suffered from allergic or non-allergic airway disease.

The timeframe between gestational week 4 and 12 during which progesterone was measured encompasses different stages

of fetal development, i.e., the embryonic and pseudoglandular stages of lung development, maturation of the immune system, as well as a shift in the production of progesterone from the corpus luteum to the placenta [41–43]. Our results indicate that a delay of the luteal–placental shift and associated lower levels of progesterone may account for the increased risk for allergic airway diseases. Future studies in which levels of progesterone are repeatedly measured in narrowly defined assessment periods are urgently required to assess whether a subclinical delay of the luteal–placental shift is indeed involved in increasing the risk for allergic airway diseases in girls.

The verification that prenatal stress challenge indeed reduces maternal progesterone level is pivotal. We have previously demonstrated that stress challenges during early gestation in mice and humans are associated with low levels of progesterone and an increased risk for fetal loss [17, 19, 34]. Moreover, stress challenge at mid-gestation in mice—as also applied in the present study—also resulted in significantly reduced levels of maternal progesterone, as shown in urine or plasma samples obtained throughout mid-gestation or close to term (Solano et al., unpublished data). Since the repeated handling required to obtain

insights on progesterone levels in dams, e.g., by daily collection of urine, causes an additional stress challenge, we refrained from including such analyses in the present study. In the cohort study, we were unable to detect an effect of prenatal maternal perceived stress on progesterone. This may relate to the questionnaire used to assess stress perception, which did not include the assessment of chronic stressors, state anxiety, and negative life events in addition to perceived stress. Further, since measures of stress and, therefore, the facets of stress they capture vary considerably, a multidimensional approach may be useful, which includes not only perceived stress but also environmental stressors as well as maternal coping strategies. Moreover, environmental pollution, such as pesticide exposure [44], as well as prenatal smoking [30, 45], diet [46], age, and parity [30] have all been shown to decrease progesterone levels. Interestingly, prenatal smoking has been linked to an increased risk for asthma in the offspring [8, 9]. Hence, lower levels of maternal progesterone may serve as a common denominator through which an increased allergy risk is communicated in response to prenatal stress perception or prenatal smoking. Such in depth evaluation should be included in future pregnancy cohorts in order to determine factors causally involved in reducing levels of maternal progesterone.

Surprisingly, assessments of maternal progesterone levels during pregnancy are not routinely pursued. Thus, routine screenings for reduced levels of progesterone are impossible at this time and limit the identification of a threshold below which maternal progesterone levels increase the risk for allergic airway diseases in daughters.

One issue that requires discussion in the light of the present findings is the putative pathway through which maternal progesterone during pregnancy affects the risk for allergic airway disease in offspring. Progesterone is well known to promote decidualization, placentation [20], and uterine relaxation [47, 48]. Further, progesterone has been shown to act as an immunosteroid, ensuring immune tolerance toward semiallogeneic antigens expressed on fetal tissue [34, 49]. Through these mechanisms, maternal progesterone may promote placental function and fetal development. Indeed, a positive correlation has been observed between maternal progesterone levels during pregnancy and birth weight of female babies [50]. Yet, in the present cohort study, we included birth weight as a confounder and could exclude a mediation effect of progesterone levels on subsequent airway diseases via low birth weight. Besides an indirect effect of the placenta, maternal progesterone may also directly affect the fetus. In rodents, maternal plasma progesterone levels during pregnancy are strongly associated with levels in their fetuses, suggesting that maternal progesterone is indeed the predominant endogenous ligand for progesterone receptors in developing fetal tissues and binds to a fetal progesterone receptor [51]. Considering the association between maternal progesterone and allergic airway disease in the offspring, a progesterone-dependent programming of the fetal lung or immune system can be hypothesized.

Our results highlight a sex-specific effect of progesterone. This may be due to sex differences in progesterone receptor expression, which has been shown in other tissue, such as rat fetal and neonatal brain tissue [51]. Although no insights are available on the expression of progesterone receptors in fetal lungs, it is tempting to speculate that—if progesterone receptor expression is also higher in lung tissue of male fetuses compared to females—modulation of maternal progesterone may have a more profound effect if receptor expression is reduced, hereby causing the increased risk for allergic airway diseases seen in prenatally challenged daughters/female offspring.

We here also observe an enhanced airway response in control male mice compared to control female mice, which suggests a sex-specific sensitization effect. Indeed, comparative analyses of male and female mice sensitized with OVA revealed that male mice show a greater sensitization response [52]. Hence, it is conceivable that the failure to identify differences between groups in male mice in the present setting is due to an overshooting response to the OVA sensitization. Careful dose response analyses using decreasing levels of OVA may be helpful to identify a threshold dose that would allow to test a potential sensitivity of male mice toward an enhance airway response upon prenatal stress. However, since such an approach would require a large number of offspring and the evidence from our human study does not support an interaction between prenatal stress and subsequent risk for allergic airway diseases in boys, we refrained from performing such experiments. Further, the more severe asthma-like phenotype in male offspring might be associated with a stronger atopic phenotype observed in boys, as boys have higher IgE concentrations at the age of 6 months [53] which persist and steadily increase up to the age of 10 years [54]. The disease pattern observed in allergic airway disease, such as an increased expression of inflammatory cytokines, i.e., IL-13, IL-5, IL-4, and IL-2 as shown previously [35], and a greater frequency of eosinophils into the lungs in response to prenatal stress in female offspring, could be confirmed in the present study. Yet, whether this effect may be fully ameliorated by DHD substitution remains unclear, since levels of significance were not reached.

Besides the decrease of progesterone, prenatal stress challenges may also affect a wealth of parameters from the nervous, endocrine, or immune system [55–57]. In this cascade of downstream mediators involved in the stress response, it is likely the greatest challenge to identify which markers may be redundant, as adaptational processes may compensate for their modulation. Considering that progesterone supplementation abrogated the effect of prenatal stress on airway response severity in the offspring, a decrease of maternal progesterone may be a potent upstream mediator in the hierarchy of systemic stress response elements.

One aspect of the translational approach of the studies was the challenge to harmonize the timing of events such as the prenatal challenges or offspring's age at time of assessment. Since fetal immune development takes place later in mice

compared to humans [32], we adjusted the timing of the intervention during pregnancy in the mouse model accordingly and exposed dams to sound stress at mid-gestation. Further, allergic airway inflammation and subsequent airway responsiveness could only be assessed in adult mice, which does not match the assessment time in the human study. This is a common limitation of translational studies and requires cautious extrapolation of findings. Further, prenatally stressed offspring mice do not show a spontaneous onset of asthma-like symptoms without prior sensitization with the OVA allergen [35]. However, the risk for allergic airway diseases in female children in response to low levels of prenatal progesterone appeared to have occurred spontaneously. Considering that most of these children are likely suffering from allergic airway diseases, future studies are needed to identify when and how a sensitization with an allergen has occurred. In the present study, we provide translational evidence that reduced levels of maternal progesterone during early pregnancy are a significant risk factor for allergic airway diseases in girls. In our rodent model, we demonstrate that the prenatal supplementation of pregnant dams with a progesterone derivative ameliorates the adverse effects of prenatal challenges on increased severity of allergic airway responses in female offspring. Our data raise the intriguing possibility of a defined, sex-dependent window of development during early prenatal life in which fetal programming of allergic diseases is operative and may be therapeutically influenced. Clearly, future studies are now urgently required to unveil pathways through which maternal progesterone during pregnancy affects the risk for allergic airway disease in offspring. Since progesterone is pivotal to maintain maternal immune adaptation during pregnancy, i.e., via the generation of CD4⁺ Treg and CD8⁺ Treg or suppressor cells (reviewed in [58, 59]) or the epigenetic silencing of leukocyte recruitment to the feto-maternal interface in mice [60], an inhibition of progesterone synthesis during pregnancy may impair fetal development by the subsequent failure to maintain immune tolerance toward fetal tissue such as the placenta.

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