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RESEARCH ARTICLE

Omics of Sex Differences

Lung proinflammatory microRNA and cytokine expression in a mouse model of allergic inflammation: role of sex chromosome complement and gonadal hormones

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Abstract

Epigenetic alterations such as dysregulation of miRNAs have been reported to play important roles in interactions between genetic and environmental factors. In this study, we tested the hypothesis that induction of lung inflammation by inhaled allergens triggers a sex-specific miRNA regulation that is dependent on chromosome complement and hormonal milieu. We challenged the four core genotypes (FCGs) model through intranasal sensitization with a house dust mite (HDM) solution (or PBS as a control) for 5 wk. The FCG model allows four combinations of gonads and sex chromosomes: *1*) XX mice with ovaries (XXF), *2*) XY mice with testes (XYM), *3*) XX mice with testes (XXM), and *4*) XY mice with ovaries (XYF). Following the challenge (n = 5–7/group), we assessed the expression of 84 inflammatory miRNAs in lung tissue using a PCR array and cytokine levels in bronchoalveolar lavage fluid (BAL) by a multiplex protein assay (n = 4–7 animals/group). Our results showed higher levels of the chemokine KC (an Il-8 homolog) and IL-7 in BAL from XYF mice challenged with HDM. In addition, IL-17A was significantly higher in BAL from both XXF and XYF mice. A three-way interaction among treatment, gonads, and sex chromosome revealed 60 of 64 miRNAs that differed in expression depending on genotype; XXF, XXM, XYF, and XYM mice had 45, 32, 4, and 52 differentially expressed miRNAs, respectively. Regulatory networks of miRNAs identified in this study were implicated in pathways associated with asthma. Female gonadal hormonal effects may alter miRNA expression and contribute to the higher susceptibility of females to asthma.

NEW & NOTEWORTHY miRNAs play important roles in regulating gene and environmental interactions. However, their role in mediating sex differences in allergic responses and lung diseases has not been elucidated. Our study used a targeted omics approach to characterize the contributions of gonadal hormones and chromosomal components to lung responses to an allergen challenge. Our results point to the influence of sex hormones in miRNA expression and proinflammatory markers in allergic airway inflammation.

allergens; asthma; four core genotypes model; miRNA; sex differences

INTRODUCTION

Epigenetic alterations such as dysregulation of miRNAs have been reported to play important roles in interactions between genetic and environmental factors, as well as in the initiation and development of a variety of lung diseases (1). miRNAs, a class of small noncoding RNAs with ≤25 nucleotides, play a vital regulatory role in a wide range of cellular and biological processes including immune regulation, cell differentiation, developmental processes, apoptosis, and inflammatory responses (2). These posttranscriptional regulators fine-tune gene expression by direct translational inhibition and/or induction of target mRNA degradation (3).

Abnormal miRNA expression has been associated with both the development and exacerbation of pulmonary pathophysiology in clinical and animal models (4). Dysregulation of miRNAs has been linked with a variety of respiratory diseases including asthma, chronic obstructive pulmonary disease, and lung cancer (2) among young and older populations (5, 6). Yet, despite the known sex disparities in the incidence and severity of these diseases, very few studies have investigated the role of miRNAs in mediating such sex disparities

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(7). Moreover, the mechanisms by which the male and female lungs respond to environmental stimuli, and the exact role of miRNAs remain understudied.

To date, few studies have addressed the mechanisms by which sex hormones contribute to the overall pathogenesis of the most common chronic respiratory disease, namely, asthma (8). The current study expands this knowledge by investigating epigenetic mechanisms (specifically, miRNA expression) by which gonadal sex hormones control inflammation in the lung. Understanding the inflammatory miRNA networks and hormonal influences in response to allergen challenges will help in the future development of sex-specific treatments and prevention strategies for inflammatory lung disease.

Thus, we aimed to investigate changes in miRNA expression triggered by a house dust mite (HDM)-exposure model that reflects sex differences in the propensity to develop airway inflammation in asthma. Prior studies from our research group using mouse models have reported sex differences and influences of the estrous cycle and sex hormones in the inflammatory response to environmental exposures (9–11). In addition, a number of key studies have also examined sex-related differences in house dust mite-challenged mice, including in various mouse strains (12–15). Based on these findings, we hypothesized that sex-specific miRNA expression mediates immune responses to HDM challenge via modulation of lung inflammatory gene expression.

MATERIALS AND METHODS

Animals

Adult male and female mice (8–10 wk of age) from the C57BL/6 background were purchased from JAX laboratories (Bar Harbor, ME) and housed, bred, and maintained in a 12:12-h light-dark cycle with food and water available ad libitum. Specifically, we used the four core genotypes (FCGs) model, in which the *Sry* gene is deleted from the Y chromosome and transplanted as a transgene onto an autosome (16). The results are four combinations of gonads and sex chromosomes: XX mice with ovaries (XXM), XY mice with testes (XYM), XX mice with testes (XXM), and XY mice with ovaries (XYF).

PBS and HDM Exposure

The treatment group was challenged by administering 50 μL of HDM solution consisting of 25 μg of HDM extract from two species, *Dermatophagoides pteronyssinus* and *Dermatophagoides farina* (Citeq Biologics, Groningen, the Netherlands) in PBS five times a week for 5 wk. The control group received 50 μL of PBS solution following the same schedule and route. The 50 μL HDM or PBS was intranasally administered to the mice using 20–200 μL pipette tips after a light anesthetic of 5% isoflurane using the SomnoSuite device (Kent Scientific). Then, 48 h after the last exposure, animals were anesthetized, lungs were lavaged, and whole lung tissues were collected to subsequently assess miRNA expression, as previously described by us (9, 10). Results were examined 48 h after the last HDM challenge because allergic inflammation typically peaks approximately 2 days after the final exposure (17). The Indiana University Bloomington Institutional Animal Care and Use Committee approved all procedures (Protocol No. 21-012). The institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Lung Inflammation Profiling

Approximately 100 μL of bronchoalveolar lavage fluid (BAL) from a subset of the mice used in this study were sent to Eve Technologies Corp (Calgary, ON, Canada) to assess cytokine and chemokine expression using the Mouse High Sensitivity 18-Plex Discovery Assay. The multiplexing analysis was performed using the Luminex 200 system (Luminex, Austin, TX) by Eve Technologies (Calgary, ON, Alberta). Eighteen markers were simultaneously measured in the samples using Eve Technologies' Mouse High Sensitivity 18-Plex Discovery Assay (MilliporeSigma, Burlington, MA) according to the manufacturer's protocol. The 18-plex consisted of GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p70), IL-13, IL-17A, KC/CXCL1, LIX, MCP-1, MIP-2, and TNFα. Assay sensitivities of these markers ranged from 0.06 to 9.06 pg/mL for the 18-plex, and samples were run in duplicates.

miRNA Profiling

Total RNA was extracted from pulverized whole lung tissue using TRIzol and the Direct-Zol RNA extraction kit (Zymo Research, Irvine, CA), and the manufacturer's instructions were followed without deviation. Small RNAs were retrotranscribed from 20 ng of total RNA using the miRCURY LNA RT Kit (Vendor Item No. 339340, Qiagen, Hilden, Germany). The expression of 84 mouse miRNAs predicted to regulate inflammatory genes was then assayed with the Mouse Inflammatory Response & Autoimmunity Focus V2, miRCURY LNA miRNA Focus PCR Panel (Vendor Item No. YAMM-205YE-2, Qiagen), following the manufacturer's instructions.

Data Analysis

For the lung inflammation profile data, we used three-way ANOVAs to compare PBS- versus HDM-treated mice with XX or XY chromosomes and male and female gonads. Tukey's honest significant difference test was conducted on the main effects of the treatment (HDM vs. PBS). In addition, the following parameters were considered: *1*) if there were missing concentration values due to the fluorescence being out of range, markers were excluded; and *2*) ANOVA was performed only if one or more PBS-treatment categories had two values and if there was one or more HDM-treatment category.

We also imputed missing values with the minimum detectable value of the marker. A second ANOVA was conducted after imputation. Most of the treatment categories had six data points, although PBS-treated XYF had $n = 5$ and HDM-treated XYF had $n = 7$. Values were log-transformed for the ANOVA. Shapiro–Wilk normality tests were conducted on the data with ANOVA results. Tukey's honest significant difference tests were conducted to assess the effects of treatment, gonads, and sex chromosomes. We present raw data and summarize data with boxplots. P values of ≤ 0.1 were considered statistically significant.

For the miRNA array data, data were processed as have been previously described in studies with similar samples (10, 18). In brief, PCR results were analyzed using QuantStudio 12 K Flex software, and threshold cycle (C_t) values were exported to an MS Excel spreadsheet. Data were analyzed using C_t values for each sample as follows. First, 64 of 86 miRNAs were detected in at least 80% of samples, and subsequent analysis focused on this subset of the array data. Second, the arithmetic mean of the four reference genes for each sample was calculated. Then, the ΔC_t for each sample was determined by subtracting the reference gene mean for each gene within each sample. C_t values are inverse to gene expression thus, values were subtracted from the max number of cycles (40), $40 - \Delta C_t$. Statistical analyses were performed with *R*. Hierarchical clustering and principal components analysis (PCA) were performed. Finally, three-way ANOVA was used to detect differences among treatments (HDM vs. PBS). We used the *R* package emmeans to obtain estimates of marginal means for the three-way ANOVAs. *P* values of ≤ 0.15 were considered statistically significant.

Ingenuity Pathway Analysis

Networks of predicted miRNA-regulated genes were constructed using miRNAs that have significant interaction at the HDM, gonadal, and chromosomal level for each genotype to visualize the potential targets of individual miRNAs and their associations with canonical pathways implicated in asthma-associated lung inflammation. We used the ingenuity pathway analysis (IPA) miRNA analysis function to understand both experimentally demonstrated and in silico-predicted miRNA associations with various disease processes.

RESULTS

Assessment of Lung Inflammation in HDM-Challenged FCG Mice

We measured cytokine and chemokine expression in BAL from mice treated with HDM or PBS for 5 wk. We found differences across the FCG treated with HDM. Specifically, when we compared measured concentrations, mice with ovaries, regardless of chromosomal component (XXF and XYF), showed higher KC expression (Il-8 homolog) (Tables 1 and 2, and Fig. 1) compared with genotypes with male gonads (XXM and XYM). After imputation, Il-17A also displayed higher levels in XXF and XYF HDM-challenged mice compared with their PBS controls (Tables 1 and 2, and Fig. 2), whereas Il-7 had higher levels in XYF HDM-challenged mice compared with its PBS control (Tables 1 and 2).

miRNA Expression in HDM-Challenged FCG Mice

The Qiagen array used for this study had 84 miRNAs associated with inflammation and autoimmunity. Twenty miRNAs were excluded from the analysis because they were not detected in the majority (80%+) of the samples. Thus, 64 miRNAs used in subsequent analyses were detected in at least 83% of all samples. To assess the quality of the 24 samples used in this study, the hierarchical distances of the ΔC_t values were calculated, and dendrograms were created and plotted as a heatmap. In addition, PCA plots were created to assess sample relatedness.

Clustering of the Lung miRNA HDM Levels

The results of hierarchical clustering with a heatmap of the expression of the 64 miRNAs for each sample type are shown in Fig. 3. There appeared to be no clustering by treatment or even sex chromosome. The only notable fact was that there appeared to be two clusters split in the middle of the plot: underexpressed miRNAs (blue) and overexpressed miRNAs (orange). Notably, all the HDM XXF were underexpressed and were located in the blue cluster.

Differences in FCG Lung miRNA Expression After HDM Challenge

There were 10 miRNAs that were differentially downregulated in HDM-challenged mice compared with their

Table 1. *Summary statistics [mean ± standard deviation (SD)] of cytokines by genotype, gonad, and chromosome*

	PBS-Treated Mice				HDM-Treated Mice			
Cytokine	XX F (*n* = 5)	XX M (*n* = 5)	XY F (*n* = 4)	XY M (*n* = 5)	XX F (*n* = 6)	XX M (*n* = 5)	XY F (*n* = 7)	XY M (*n* = 6)
GM-CSF	NaN ± NA	NaN ± NA	NaN ± NA	NaN ± NA	NaN ± NA	NaN ± NA	NaN ± NA	2.05 ± 2.31
IFNγ	0.09 ± NA	0.07 ± 0.08	0.17 ± NA	0.08 ± 0.07	0.25 ± NA	0.02 ± NA	0.63 ± NA	0.52 ± NA
IL-10	1.63 ± 0.27	0.86 ± 0.27	0.55 ± 0.54	1.31 ± 0.83	0.70 ± 0.23	0.88 ± 0.19	1.16 ± 1.01	1.01 ± 0.27
IL-12p70	20.08 ± 17.12	24.98 ± 22.67	17.31 ± 18.67	19.40 ± 20.10	14.95 ± 14.76	15.90 ± 14.74	17.74 ± 16.99	17.05 ± 17.14
IL-13	6.02 ± 1.29	6.40 ± 0.47	5.70 ± 0.67	5.97 ± 0.78	5.40 ± 0.58	5.94 ± 0.63	6.33 ± 1.97	5.90 ± 2.13
IL-17A	NaN ± NA	1.89 ± 3.24	0.22 ± NA	0.02 ± 0.01	3.00 ± 3.96	NaN ± NA	2.21 ± 2.39	0.25 ± NA
IL-1α	19.49 ± 10.39	16.50 ± 4.94	31.66 ± 33.19	27.86 ± 15.95	18.79 ± 8.39	20.23 ± 6.85	28.56 ± 43.25	32.48 ± 37.17
IL-1β	0.42 ± 0.27	0.86 ± 0.35	0.30 ± 0.37	0.40 ± 0	0.59 ± 0.24	0.32 ± 0.13	0.53 ± 0.26	0.80 ± 0.36
IL-2	3.47 ± 1.15	2.98 ± 1.10	4.05 ± 2.45	3.74 ± 1.75	2.34 ± 1.09	2.36 ± 1.60	6.88 ± 11.47	8.19 ± 12.01
IL-4	0.07 ± 0.03	1.81 ± 3.01	0.08 ± 0.01	0.08 ± 0.03	0.11 ± 0.08	0.03 ± 0.02	1.58 ± 2.76	0.06 ± 0.06
IL-5	0.71 ± 0.52	0.61 ± 0.21	0.79 ± 0.59	0.55 ± 0.46	0.77 ± 0.55	0.56 ± 0.45	1.10 ± 0.58	0.88 ± 0.80
IL-6	0.65 ± 0.01	0.48 ± 0.04	0.88 ± 0.19	0.55 ± 0.21	0.41 ± 0.36	0.36 ± 0.26	0.78 ± 0.42	1.04 ± 0.51
IL-7	0.92 ± 0.46	0.84 ± 0.42	0.60 ± 0.31	0.72 ± 0.34	0.62 ± 0.28	0.53 ± 0.31	0.79 ± 0.47	0.66 ± 0.77
KC	3.55 ± 1.66	9.51 ± 11.40	3.23 ± 2.83	3.36 ± 1.00	16.97 ± 11.11	3.85 ± 2.06	20.48 ± 17.09	2.90 ± 2.52
LIX	74.13 ± 0	118.78 ± 52.90	91.82 ± 0	74.98 ± 13.66	59.51 ± 30.95	74.13 ± 11.63	64.74 ± 49.09	64.84 ± 8.04
MCP-1	7.99 ± 4.66	9.20 ± 1.88	3.96 ± NA	13.36 ± 0.65	7.64 ± 3.49	10.14 ± 2.10	10.51 ± 0.80	12.07 ± 2.82
MIP-2	42.97 ± 10.65	45.87 ± 12.05	53.58 ± 30.02	52.21 ± 25.58	52.42 ± 10.50	41.26 ± 20.34	53.29 ± 55.01	66.26 ± 63.40
TNFα	1.91 ± 1.24	2.82 ± 1.77	1.46 ± 1.49	2.73 ± 0.39	1.30 ± 1.22	1.82 ± 1.38	1.59 ± 1.37	1.63 ± 1.25

NaN implies missing values due to fluorescence being out of range for the multiplex assay (mean values) and NA means SD not applicable.

Table 2. *P value of the effects on cytokines after conducting a three-way analysis of variance (ANOVA) of house dust mite exposure (HDM vs. PBS control), gonadal (ovaries vs. testes), and chromosomal (XX vs. XY)*

Cytokine	Exposure	Gonadal	Chromosomal	Exposure × Gonadal	Exposure × Chromosomal	Gonadal × Chromosomal	Exposure × Gonadal × Chromosomal
GM-CSF	0.380	0.307	0.358	0.329	0.380	0.307	0.364
IFNγ	0.713	0.897	0.441	0.743	0.992	0.712	0.765
IL-1α	0.869	0.427	0.690	0.810	0.643	0.095	0.830
IL-1β	0.448	0.800	0.791	0.881	0.632	0.726	0.629
IL-2	0.702	0.849	0.235	0.877	0.768	0.343	0.924
IL-4	0.839	0.305	0.948	0.103	0.264	0.484	0.754
IL-5	0.827	0.508	0.441	0.709	0.915	0.452	0.869
IL-6	0.696	0.639	0.452	0.514	0.948	0.713	0.680
IL-7	**0.008**	0.238	0.164	0.227	0.512	0.171	0.121
IL-10	0.934	0.530	0.838	0.803	0.715	0.703	0.433
IL-12p70	0.665	0.987	0.699	0.870	0.823	0.712	0.844
IL-13	0.540	0.687	0.976	0.659	0.424	0.358	0.566
IL-17A	**0.0002**	**0.00003**	0.858	**0.00002**	0.810	0.847	**0.077**
KC	**0.014**	**0.003**	0.475	**0.00007**	0.359	0.368	0.743
LIX	0.578	0.660	0.855	0.303	0.939	0.814	0.597
MCP-1	0.594	0.670	0.688	0.676	0.905	0.189	0.455
MIP-2	0.978	0.643	0.900	0.696	0.702	0.586	0.884
TNFα	0.831	0.868	0.835	0.961	0.742	0.468	0.669

Bolded *P* values have significance levels of ≤0.10.

respective PBS controls, whereas seven and two miRNAs were differentially expressed depending on gonads (male vs. female) and chromosome (XX vs. XY) (Table 3). Three-way interaction among treatment, gonads, and chromosomes revealed that 61 of 64 miRNAs differed in expression by the levels of gonadal and chromosomal and depending on treatment (Table 4). A look at the marginal means comparing HDM versus PBS revealed that the combination of treatment, gonads, and chromosomes can alter the expression of miRNAs in the FCGs ($P \leq 0.15$). Specifically, XXF, XXM, XYF, and XYM had 45 (underexpressed), 32 (overexpressed), 4 (3 overexpressed and 1 underexpressed), and 52 (underexpressed) miRNAs, respectively, with some of these miRNAs overlapping.

Pathway Analysis of Differentially Expressed miRNAs

IPA of the miRNAs that were significantly differentially expressed in HDM-treated XXF mice compared with PBS

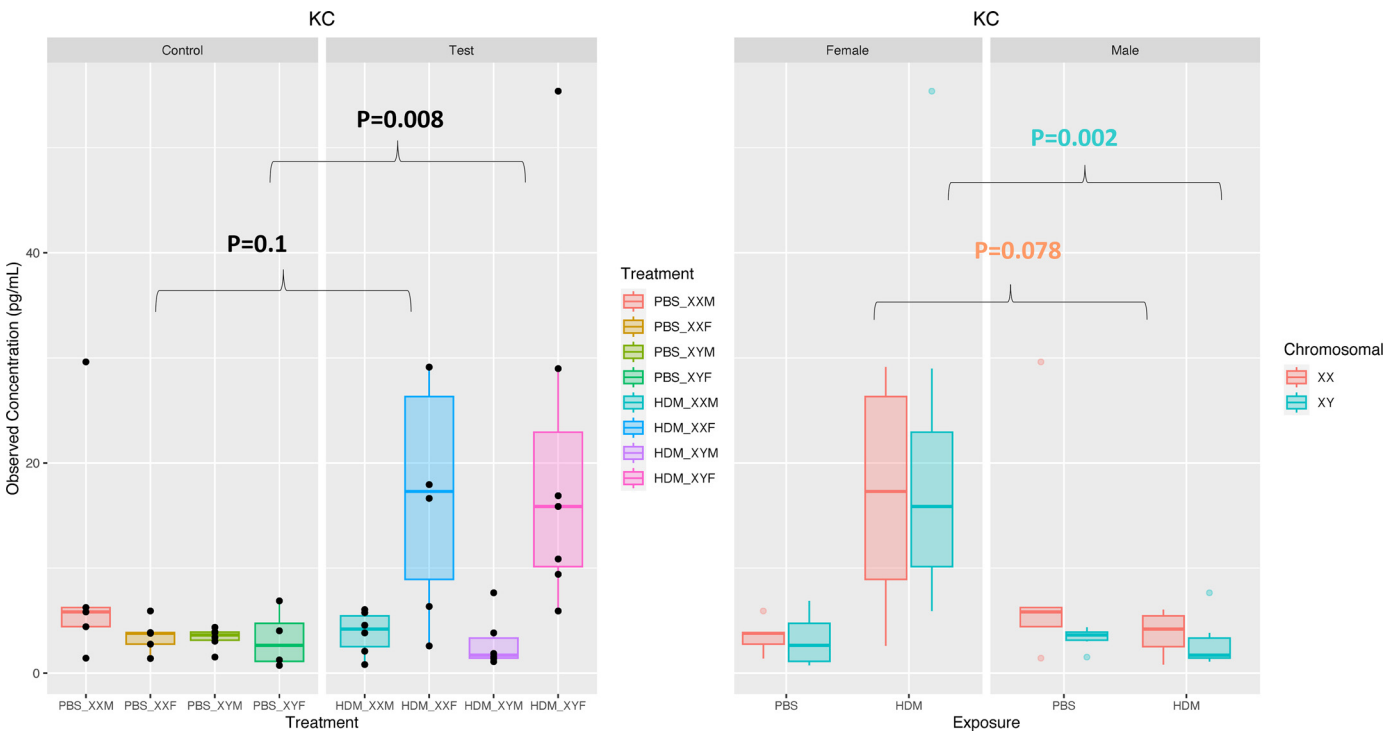

Figure 1. Differences in bronchoalveolar lavage (BAL) fluid levels of the proinflammatory chemokine KC/CXCL1 in the four core genotypes after 5 wk of house dust mite (HDM) challenge compared with those exposed to PBS. *Left*: actual concentrations by genotype; *right*: actual concentrations by male and female ($n = 5$ or 6 for all genotypes except XYF, where HDM = 7 and PBS = 4). *P* values were obtained by three-way ANOVA followed by Tukey's test.

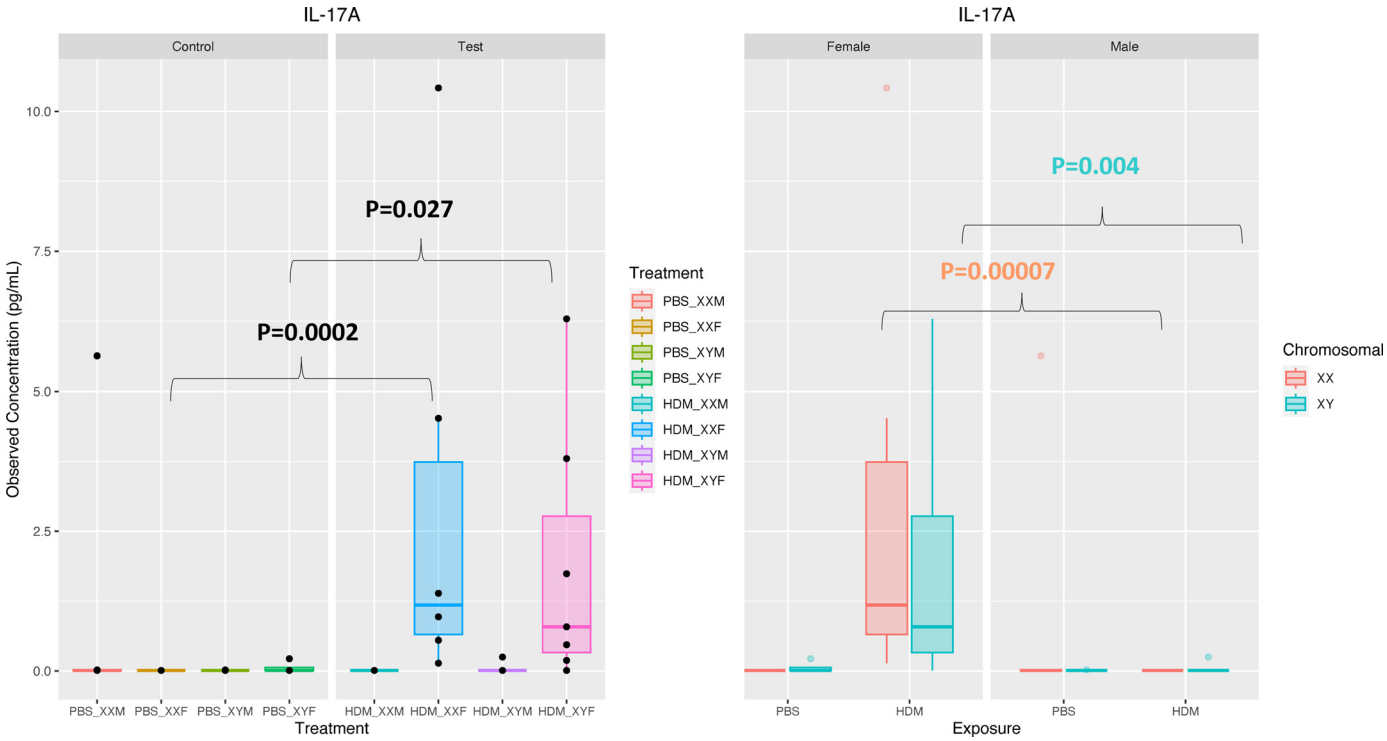

Figure 2. Differences in bronchoalveolar lavage (BAL) fluid levels of the proinflammatory cytokine IL-17A in the four core genotypes and by male and female after 5 wk of house dust mite (HDM) challenge compared with those exposed to PBS (n = 5 or 6 for all genotypes except XYF, where HDM = 7 and PBS = 4). P values were obtained by three-way ANOVA followed by Tukey's test.

revealed the top disease and biological functions (P value range) involving neurological diseases ($4.53e^{-2}$ – $1.59e^{-20}$), organismal injury and abnormalities ($4.95e^{-2}$ – $1.65e^{-22}$), and reproductive system disease ($4.77e^{-2}$ – $1.65e^{-22}$). Top cellular functions included cellular movement, cellular development, cellular growth and proliferation, cell death and survival, and cell cycle (Table 5). The top network functions found were neurological disease, organismal injury and abnormalities, and psychological disorders. The top gene targets of these differentially expressed miRNAs were *CG*, *CREBL2*, and *estrogen receptor* (Supplemental Fig. S1). Interestingly, canonical pathways associated with some of the target genes of these miRNAs included IL-17A signaling in airway cells and IL-8 signaling (Supplemental Fig. S1).

For XXM challenged with HDM, organismal injury and abnormalities ($4.63e^{-2}$ – $1.52e^{-17}$) and reproductive system disease ($3.89e^{-2}$ – $1.52e^{-17}$) were the top diseases and disorders (Table 5). In addition, cellular movement, cellular development, cell cycle, and cellular growth and development were the top molecular and cellular functions. The top physiological system development and functions included embryonic development and connective tissue development and function. The top gene targets of miR-29b included *BIRC5*, *CG*, *CLDN12*, *Gnasas1*, *Gulo*, *HNF1A-AS1*, and *HOTTIP* (Supplemental Fig. S2). Of interest, the neutrophil extracellular trap signaling pathway, which can be activated due to inflammatory stimuli, was a canonical pathway associated with some of the target genes of this miRNA (e.g., *COL15A1*, *COL4A5*, *COL5A2*, and *COL5A3*) (Supplemental Fig. S2).

For XYF-challenged mice, the top diseases and disorders found were cancer ($4.85e^{-2}$ – $2.69e^{-8}$), organismal injury and abnormalities ($4.85e^{-2}$ – $2.69e^{-8}$), and reproductive system disease ($4.85e^{-2}$ – $4.12e^{-8}$) (Table 5). On the other hand, cell cycle, cellular assembly and organization, cell-to-cell signaling, and interaction were associated with molecular and cellular functions. For the top physiological system development and function, nervous system development and function, tissue morphology, hematological system development and function, hematopoiesis, and humoral immune response came up. The top network functions were cellular movement, cancer, and organismal injury and abnormalities. The top gene targets were *ACSL6*, *ALOX5AP*, *CCL18*, *CD209*, *CLDN1*, *ERBB2*, *FCER2*, *FGF7*, *HTR1A*, *JARID2*, and *MSI2* (Supplemental Fig. S3). Canonical pathways associated with the target genes of this miRNA include IL-17 signaling.

Finally, for XYM-challenged mice, cancer ($4.83e^{-2}$ – $9.18e^{-25}$), organismal injury and abnormalities ($4.83e^{-2}$ – $9.18e^{-25}$), and reproductive system disease ($4.83e^{-2}$ – $9.18e^{-25}$) were some of the associated disorders (Table 5). Cellular movement, cellular development, and cellular growth and proliferation were some molecular functions associated with the miRNAs that were significant in the three-way interaction term. The associated canonical pathways included IL-17, IL-8, and IL-7 signaling and the neutrophil extracellular trap signaling pathway. Important gene targets included DLK1, estrogen receptor, FSH, Gulo, HOTTIP, and interferon-α (Table 5 and Supplemental Fig. S4).

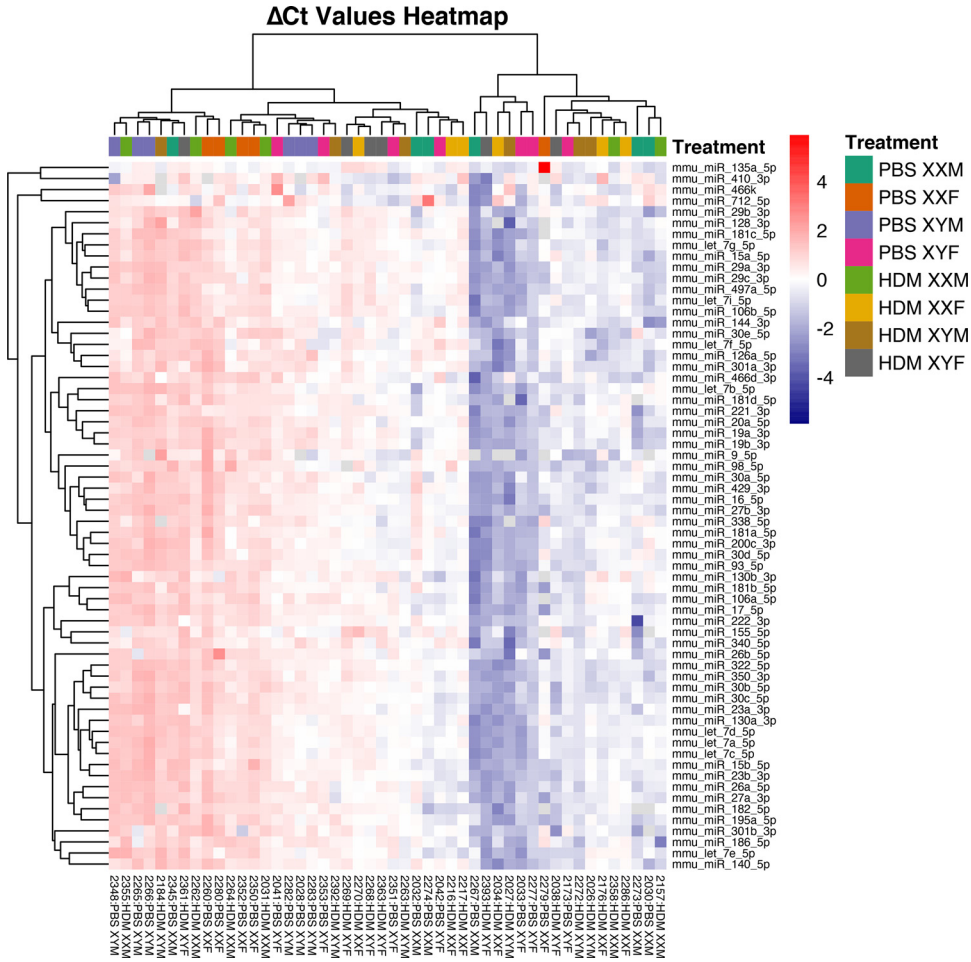

Figure 3. Hierarchical cluster analysis of 64 miRNAs in lung extracts from the four core genotype mice exposed challenged with house dust mite (HDM) or PBS for 5 wk. Two main clusters exist (orange and blue, with all XXF HDM-treated mice located in the blue cluster). NA refers to out of range below the standard curve. HDM and PBS refer to house dust mite and phosphate-buffered saline, respectively.

When we analyzed data for all four genotypes, the miRNAs that were significant at the three-way interaction term for treatment, gonad, and sex chromosome were associated with asthma, including severe asthma in the IPA.

DISCUSSION

In this study, we combined an HDM allergen challenge model with the FCG murine model to demonstrate an association of chromosome components and gonadal hormones with an expression of proinflammatory mediators in BAL and inflammatory miRNAs in lung tissue. Confirming our hypothesis, the observed differential expression of proinflammatory mediators in the BAL in the FCGs indicate that the previously reported sex differences in airway inflammation are mediated by gonadal female hormones rather than by sex chromosomes. We report higher levels of the chemokine KC, an IL-8 homolog, in both XX and XY mice with ovaries in response to HDM challenge. In addition, there were increased levels of IL-7 (in XY mice with ovaries) and IL-17A (in both XX and XY mice with ovaries).

Recently, Mostafa et al. (12) reported sexual dimorphism in HDM-mediated airway inflammation in BALB/c mice. The authors observed a T helper (Th)17-biased response, resulting in higher IL-17 levels in female mice compared with male mice. Although there are strain differences, our study results also pointed to higher IL-17 levels in mice with ovaries and implicate female sex hormones. From these findings, we infer that hormones from the female gonads, including but not limited to estrogens, have proinflammatory effects in response to the allergen challenge and may help explain some of the sex disparities observed in adult females with allergic asthma. Although prior studies from us and others have indicated proinflammatory effects of estrogens in the lung (9, 10, 19, 20), the roles of other female gonadal hormones have not been studied in detail and therefore cannot be excluded.

Allergic asthma comprises a range of disease phenotypes (21) and there are many animal models of allergic airway disease that depict pulmonary allergen sensitization, inflammation, and airway hyperresponsiveness (22). Given that allergic asthma is heterogeneous and cannot be represented by a single animal model, the current study findings are generalizable only in regard to the type of allergen intranasally instilled into the animal over a 5-wk exposure period. As such any sex-specific differences observed in the current study are for this chronic exposure model. In a recent study (23), the authors observed pulmonary Th17 > Th2 > Th1 response with lung eosinophilia and some neutrophil infiltration associated with airway hyperresponsiveness after challenging mice intranasally with HDM, 5 days/week for 3 wk. Although the authors did

Table 3. *P value of the effects on miRNA after conducting a three-way analysis of variance (ANOVA) of house dust mite exposure (HDM vs. PBS control), gonadal (ovaries vs. testes), and chromosomal (XX vs. XY)*

MiRNA	Exposure	Gonadal	Chromosomal	Exposure × Gonadal	Exposure × Chromosomal	Gonadal × Chromosomal	Exposure × Gonadal × Chromosomal
mmu_let_7a_5p	0.494	0.273	0.809	0.781	0.651	0.138	**0.005**
mmu_let_7b_5p	0.882	0.222	0.792	0.395	0.548	0.123	**0.006**
mmu_let_7c_5p	0.620	0.258	0.671	0.612	0.683	0.191	**0.005**
mmu_let_7d_5p	0.571	0.177	0.725	0.594	0.644	0.256	**0.007**
mmu_let_7e_5p	0.671	0.114	0.647	0.747	0.657	0.398	**0.025**
mmu_let_7f_5p	**0.100**	0.521	0.926	0.917	0.873	0.961	**0.022**
mmu_let_7g_5p	0.609	0.576	0.676	0.493	0.663	0.320	**0.003**
mmu_let_7i_5p	0.404	0.821	0.744	0.934	0.481	0.313	**0.002**
mmu_miR_106a_5p	0.760	0.634	0.959	0.466	0.165	0.041	**0.001**
mmu_miR_106b_5p	0.961	0.607	0.655	0.880	0.490	0.165	**0.002**
mmu_miR_126a_5p	**0.012**	0.756	0.903	0.976	0.973	0.590	**0.002**
mmu_miR_128_3p	0.655	0.884	0.976	0.992	0.501	0.195	**0.006**
mmu_miR_130a_3p	0.696	0.411	0.799	0.602	0.987	0.234	**0.006**
mmu_miR_130b_3p	0.385	0.640	0.450	0.763	0.424	0.332	**0.014**
mmu_miR_135a_5p	0.833	**0.042**	0.285	0.675	0.253	0.160	**0.037**
mmu_miR_140_5p	0.502	0.295	0.705	0.861	0.927	0.287	**0.026**
mmu_miR_144_3p	0.281	0.609	0.191	0.566	0.666	0.262	**0.081**
mmu_miR_155_5p	0.494	**0.087**	0.613	0.062	0.851	0.172	**0.004**
mmu_miR_15a_5p	0.806	0.832	0.309	0.770	0.951	0.386	**0.005**
mmu_miR_15b_5p	0.556	0.150	0.934	0.850	0.576	0.254	**0.003**
mmu_miR_16_5p	0.222	0.380	0.604	0.987	0.479	0.532	**0.006**
mmu_miR_17_5p	0.766	0.398	0.719	0.988	0.194	0.380	**0.008**
mmu_miR_181a_5p	0.329	0.371	0.828	0.807	0.816	0.228	**0.013**
mmu_miR_181b_5p	0.696	0.180	0.411	0.860	0.346	0.476	**0.042**
mmu_miR_181c_5p	0.602	0.358	0.574	0.700	0.788	0.395	**0.010**
mmu_miR_181d_5p	0.463	0.164	0.909	0.606	0.906	0.240	**0.003**
mmu_miR_182_5p	0.115	0.163	0.881	0.499	0.713	0.741	**0.0002**
mmu_miR_186_5p	0.212	0.971	0.353	0.899	0.919	0.249	**0.017**
mmu_miR_195a_5p	0.224	0.171	0.931	0.500	0.643	0.315	**0.009**
mmu_miR_19a_3p	0.545	0.840	0.586	0.443	0.114	**0.033**	**0.001**
mmu_miR_19b_3p	0.511	0.876	0.500	0.418	0.318	**0.069**	**0.001**
mmu_miR_200c_3p	0.309	0.149	0.411	0.841	0.795	0.199	**0.011**
mmu_miR_20a_5p	0.652	0.686	0.774	0.834	0.109	0.173	**0.004**
mmu_miR_221_3p	0.269	1.000	0.349	0.654	**0.096**	0.175	**0.007**
mmu_miR_222_3p	0.718	0.740	0.718	0.458	0.135	**0.093**	**0.003**
mmu_miR_23a_3p	**0.075**	**0.075**	0.490	0.232	0.291	**0.068**	**0.004**
mmu_miR_23b_3p	0.213	**0.070**	0.721	0.485	0.489	0.245	**0.008**
mmu_miR_26a_5p	**0.097**	0.340	0.728	0.504	0.309	0.518	**0.001**
mmu_miR_26b_5p	0.371	0.990	0.825	0.287	0.379	0.658	**0.031**
mmu_miR_27a_3p	0.145	0.370	0.820	0.887	0.177	0.468	**0.006**
mmu_miR_27b_3p	0.145	0.238	0.586	0.945	0.483	0.275	**0.010**
mmu_miR_29a_3p	0.904	0.535	0.884	0.984	0.359	0.239	**0.020**
mmu_miR_29b_3p	0.942	0.584	0.177	0.678	0.893	0.227	**0.002**
mmu_miR_29c_3p	0.961	0.342	0.454	0.746	0.470	0.342	**0.018**
mmu_miR_301a_3p	0.127	0.521	0.892	0.629	0.636	0.213	**0.007**
mmu_miR_301b_3p	0.705	0.721	0.870	0.256	0.369	0.180	**0.045**
mmu_miR_30a_5p	**0.032**	0.338	0.552	0.668	0.332	0.257	**0.004**
mmu_miR_30b_5p	**0.061**	0.322	0.652	0.707	0.707	0.357	**0.003**
mmu_miR_30c_5p	**0.065**	**0.065**	0.928	0.555	0.695	0.246	**0.001**
mmu_miR_30d_5p	0.114	0.112	0.304	0.914	0.651	0.244	**0.013**
mmu_miR_30e_5p	**0.016**	0.441	0.646	0.683	0.503	0.922	**0.002**
mmu_miR_322_5p	0.163	0.537	0.983	0.764	0.682	0.366	**0.004**
mmu_miR_338_5p	**0.079**	**0.065**	0.214	0.774	0.697	**0.061**	**0.014**
mmu_miR_340_5p	0.861	0.325	0.618	0.577	0.258	0.294	**0.005**
mmu_miR_350_3p	0.445	0.364	0.995	0.918	0.532	0.288	**0.002**
mmu_miR_410_3p	0.467	0.786	0.298	0.716	0.430	0.817	0.933
mmu_miR_429_3p	**0.056**	0.542	0.290	0.613	0.510	0.249	**0.005**
mmu_miR_466d_3p	0.293	0.752	0.368	**0.054**	0.320	0.126	**0.009**
mmu_miR_466k	0.385	0.127	0.782	**0.099**	0.200	0.298	0.769
mmu_miR_497a_5p	0.621	0.495	0.633	0.780	0.245	0.364	**0.017**
mmu_miR_712_5p	0.138	**0.045**	0.217	0.471	0.677	**0.080**	0.687
mmu_miR_9_5p	0.996	0.485	**0.013**	0.870	0.563	0.232	0.156
mmu_miR_93_5p	0.407	0.176	0.371	0.971	0.417	0.182	**0.008**
mmu_miR_98_5p	0.223	0.153	**0.069**	0.749	0.505	0.487	**0.027**

Bolded *P* values have significance levels of ≤0.10. miRNAs in bold are associated with HDM exposure ($P \leq 0.10$).

Table 4. *Estimated marginal means for microRNAs by genotype with significant three-way ANOVA interaction terms (exposure × gonadal × chromosomal)*

miRNA	Contrast	Gonadal	Chromosomal	Estimate	Standard Error	Degrees of Freedom	*t* ratio	*P* Value
mmu-let-7a-5p	HDM - PBS	Female	XX	−1.88	1.02	40	−1.85	**0.072**
		Male	XX	1.45	0.97	40	1.49	**0.144**
		Female	XY	0.60	0.93	40	0.64	0.527
		Male	XY	−1.85	0.97	40	−1.91	**0.063**
mmu-let-7b-5p	HDM - PBS	Female	XX	−1.66	0.94	40	−1.75	**0.087**
		Male	XX	1.88	0.90	40	2.09	**0.043**
		Female	XY	0.44	0.87	40	0.51	0.616
		Male	XY	−1.25	0.90	40	−1.39	0.172
mmu-let-7c-5p	HDM - PBS	Female	XX	−1.72	0.91	40	−1.89	**0.066**
		Male	XX	1.46	0.87	40	1.68	**0.100**
		Female	XY	0.54	0.84	40	0.65	0.522
		Male	XY	−1.46	0.87	40	−1.69	**0.099**
mmu-let-7d-5p	HDM - PBS	Female	XX	−1.79	0.98	40	−1.84	**0.074**
		Male	XX	1.51	0.93	40	1.63	**0.111**
		Female	XY	0.46	0.90	40	0.52	0.607
		Male	XY	−1.56	0.93	40	−1.68	**0.101**
mmu-let-7f-5p	HDM - PBS	Female	XX	−3.08	1.41	40	−2.19	**0.035**
		Male	XX	0.43	1.34	40	0.32	0.752
		Female	XY	0.38	1.29	40	0.29	0.773
		Male	XY	−2.55	1.34	40	−1.90	**0.065**
mmu-let-7g-5p	HDM - PBS	Female	XX	−2.20	1.07	40	−2.05	**0.046**
		Male	XX	1.95	1.02	40	1.91	**0.064**
		Female	XY	0.66	0.98	40	0.67	0.509
		Male	XY	−1.75	1.02	40	−1.71	**0.094**
mmu-let-7i-5p	HDM - PBS	Female	XX	−0.95	0.92	40	−1.03	0.309
		Male	XX	2.16	0.88	40	2.47	**0.018**
		Female	XY	1.36	0.84	40	1.60	**0.116**
		Male	XY	−1.34	0.88	40	−1.53	**0.135**
mmu-miR-106a-5p	HDM - PBS	Female	XX	−1.91	1.04	39	−1.84	**0.073**
		Male	XX	2.42	0.93	39	2.60	**0.013**
		Female	XY	0.30	0.90	39	0.33	0.740
		Male	XY	−2.03	0.93	39	−2.18	**0.035**
mmu-miR-106b-5p	HDM - PBS	Female	XX	−1.60	1.04	40	−1.53	**0.134**
		Male	XX	2.06	1.00	40	2.06	**0.046**
		Female	XY	1.07	0.96	40	1.11	0.272
		Male	XY	−1.94	1.00	40	−1.94	**0.059**
mmu-miR-126a-5p	HDM - PBS	Female	XX	−4.10	1.34	40	−3.07	**0.004**
		Male	XX	0.45	1.28	40	0.35	0.726
		Female	XY	0.28	1.23	40	0.23	0.823
		Male	XY	−3.77	1.28	40	−2.95	**0.005**
mmu-miR-128-3p	HDM - PBS	Female	XX	−1.38	1.19	38	−1.16	0.253
		Male	XX	2.03	1.02	38	1.99	**0.054**
		Female	XY	1.22	0.99	38	1.24	0.223
		Male	XY	−1.56	1.02	38	−1.52	**0.137**
mmu-miR-130a-3p	HDM - PBS	Female	XX	−1.75	0.88	40	−2.00	**0.053**
		Male	XX	1.25	0.83	40	1.50	**0.141**
		Female	XY	0.68	0.80	40	0.85	0.402
		Male	XY	−1.15	0.83	40	−1.38	0.175
mmu-miR-130b-3p	HDM - PBS	Female	XX	−0.54	0.98	39	−0.55	0.583
		Male	XX	1.69	0.88	39	1.93	**0.061**
		Female	XY	1.18	0.84	39	1.40	0.170
		Male	XY	−1.21	0.88	39	−1.38	0.176
mmu-miR-135a-5p	HDM - PBS	Female	XX	−4.57	2.67	40	−1.71	**0.095**
		Male	XX	0.38	2.55	40	0.15	0.882
		Female	XY	3.97	2.45	40	1.62	**0.113**
		Male	XY	−2.11	2.55	40	−0.83	0.412
mmu-miR-140-5p	HDM - PBS	Female	XX	−1.58	1.02	40	−1.55	**0.129**
		Male	XX	0.65	0.97	40	0.67	0.509
		Female	XY	0.79	0.94	40	0.84	0.405
		Male	XY	−1.50	0.97	40	−1.54	**0.132**
mmu-miR-144-3p	HDM - PBS	Female	XX	−1.89	1.23	40	−1.53	**0.133**
		Male	XX	1.03	1.17	40	0.88	0.386
		Female	XY	−0.26	1.13	40	−0.23	0.817
		Male	XY	−1.57	1.17	40	−1.34	0.189
mmu-miR-155-5p	HDM - PBS	Female	XX	−0.55	1.16	38	−0.48	0.635
		Male	XX	1.14	1.09	38	1.05	0.301

Continued

Table 4.— *Continued*

miRNA	Contrast	Gonadal	Chromosomal	Estimate	Standard Error	Degrees of Freedom	*t* ratio	*P* Value
		Female	XY	2.61	1.00	38	2.62	**0.013**
		Male	XY	−2.26	1.03	38	−2.18	**0.035**
mmu-miR-15a-5p	HDM -	Female	XX	−1.98	1.11	40	−1.79	**0.081**
	PBS	Male	XX	1.63	1.05	40	1.55	**0.130**
		Female	XY	1.12	1.02	40	1.10	0.279
		Male	XY	−1.54	1.05	40	−1.46	**0.153**
mmu-miR-15b-5p	HDM -	Female	XX	−1.84	1.05	40	−1.76	**0.086**
	PBS	Male	XX	1.63	1.00	40	1.63	**0.111**
		Female	XY	0.75	0.96	40	0.77	0.444
		Male	XY	−2.03	1.00	40	−2.03	**0.049**
mmu-miR-16-5p	HDM -	Female	XX	−1.87	1.05	40	−1.78	**0.083**
	PBS	Male	XX	1.18	1.00	40	1.17	0.248
		Female	XY	0.38	0.97	40	0.39	0.700
		Male	XY	−2.46	1.00	40	−2.45	**0.019**
mmu-miR-17-5p	HDM -	Female	XX	−1.07	1.13	40	−0.94	0.353
	PBS	Male	XX	2.05	1.08	40	1.90	**0.065**
		Female	XY	0.56	1.04	40	0.53	0.597
		Male	XY	−2.38	1.08	40	−2.20	**0.034**
mmu-miR-181a-5p	HDM -	Female	XX	−1.76	0.93	40	−1.89	**0.066**
	PBS	Male	XX	0.92	0.89	40	1.04	0.306
		Female	XY	0.38	0.86	40	0.45	0.658
		Male	XY	−1.59	0.89	40	−1.79	**0.081**
mmu-miR-181b-5p	HDM -	Female	XX	−0.72	0.96	40	−0.75	0.456
	PBS	Male	XX	1.12	0.92	40	1.22	0.230
		Female	XY	0.35	0.88	40	0.39	0.696
		Male	XY	−1.67	0.92	40	−1.82	**0.076**
mmu-miR-181c-5p	HDM -	Female	XX	−1.67	1.16	39	−1.44	0.158
	PBS	Male	XX	1.05	1.04	39	1.01	0.318
		Female	XY	1.06	1.00	39	1.06	0.294
		Male	XY	−1.94	1.04	39	−1.87	**0.069**
mmu-miR-181d-5p	HDM -	Female	XX	−2.42	1.04	38	−2.33	**0.025**
	PBS	Male	XX	1.56	1.04	38	1.50	**0.142**
		Female	XY	0.79	0.95	38	0.83	0.414
		Male	XY	−1.65	1.04	38	−1.59	**0.120**
mmu-miR-182-5p	HDM -	Female	XX	−3.32	1.09	36	−3.05	**0.004**
	PBS	Male	XX	2.11	1.09	36	1.94	**0.060**
		Female	XY	0.40	0.94	36	0.42	0.675
		Male	XY	−2.69	1.02	36	−2.64	**0.012**
mmu-miR-186-5p	HDM -	Female	XX	−2.18	1.04	40	−2.10	**0.042**
	PBS	Male	XX	0.60	0.99	40	0.60	0.551
		Female	XY	0.44	0.96	40	0.46	0.649
		Male	XY	−1.77	0.99	40	−1.78	**0.082**
mmu-miR-195a-5p	HDM -	Female	XX	−2.42	1.12	40	−2.16	**0.037**
	PBS	Male	XX	1.41	1.07	40	1.32	0.194
		Female	XY	0.06	1.03	40	0.06	0.953
		Male	XY	−2.01	1.07	40	−1.88	**0.067**
mmu-miR-19a-3p	HDM -	Female	XX	−1.82	0.98	40	−1.86	**0.070**
	PBS	Male	XX	2.58	0.93	40	2.76	**0.009**
		Female	XY	0.20	0.90	40	0.22	0.824
		Male	XY	−2.40	0.93	40	−2.57	**0.014**
mmu-miR-19b-3p	HDM -	Female	XX	−2.28	1.05	40	−2.16	**0.037**
	PBS	Male	XX	2.42	1.00	40	2.41	**0.020**
		Female	XY	0.43	0.97	40	0.44	0.660
		Male	XY	−2.24	1.00	40	−2.23	**0.031**
mmu-miR-200c-3p	HDM -	Female	XX	−1.75	0.91	40	−1.92	**0.063**
	PBS	Male	XX	0.89	0.87	40	1.02	**0.314**
		Female	XY	0.37	0.84	40	0.44	0.661
		Male	XY	−1.64	0.87	40	−1.88	**0.067**
mmu-miR-20a-5p	HDM -	Female	XX	−1.21	1.09	40	−1.12	0.271
	PBS	Male	XX	2.28	1.04	40	2.20	**0.034**
		Female	XY	0.26	1.00	40	0.27	0.792
		Male	XY	−2.54	1.04	40	−2.45	**0.019**
mmu-miR-221-3p	HDM -	Female	XX	−0.33	1.02	40	−0.32	0.747
	PBS	Male	XX	2.98	0.97	40	3.07	**0.004**
		Female	XY	0.81	0.94	40	0.86	0.394
		Male	XY	−1.42	0.97	40	−1.46	**0.152**

Continued

Table 4.— *Continued*

miRNA	Contrast	Gonadal	Chromosomal	Estimate	Standard Error	Degrees of Freedom	*t* ratio	*P* Value
mmu-miR-222-3p	HDM - PBS	Female	XX	−1.62	1.12	40	−1.45	**0.154**
		Male	XX	2.66	1.07	40	2.50	**0.017**
		Female	XY	0.12	1.03	40	0.11	0.911
		Male	XY	−2.27	1.07	40	−2.13	**0.040**
mmu-miR-23a-3p	HDM - PBS	Female	XX	−2.37	0.91	40	−2.61	**0.013**
		Male	XX	1.52	0.86	40	1.76	**0.087**
		Female	XY	−0.58	0.83	40	−0.69	0.494
		Male	XY	−2.07	0.86	40	−2.40	**0.021**
mmu-miR-23b-3p	HDM - PBS	Female	XX	−2.08	0.99	40	−2.10	**0.042**
		Male	XX	1.37	0.94	40	1.45	**0.153**
		Female	XY	−0.06	0.91	40	−0.07	0.948
		Male	XY	−1.91	0.94	40	−2.03	**0.049**
mmu-miR-26a-5p	HDM - PBS	Female	XX	−2.72	1.08	40	−2.53	**0.016**
		Male	XX	1.84	1.03	40	1.80	**0.080**
		Female	XY	−0.02	0.99	40	−0.02	0.984
		Male	XY	−2.91	1.03	40	−2.84	**0.007**
mmu-miR-26b-5p	HDM - PBS	Female	XX	−2.36	1.40	40	−1.68	**0.100**
		Male	XX	2.20	1.34	40	1.64	**0.108**
		Female	XY	−0.52	1.29	40	−0.40	0.689
		Male	XY	−1.98	1.34	40	−1.48	**0.147**
mmu-miR-27a-3p	HDM - PBS	Female	XX	−1.71	1.06	40	−1.61	**0.116**
		Male	XX	1.47	1.01	40	1.45	0.156
		Female	XY	−0.15	0.98	40	−0.15	0.880
		Male	XY	−2.84	1.01	40	−2.80	**0.008**
mmu-miR-27b-3p	HDM - PBS	Female	XX	−1.90	1.06	40	−1.79	**0.081**
		Male	XX	0.93	1.01	40	0.92	0.364
		Female	XY	0.17	0.98	40	0.18	0.860
		Male	XY	−2.53	1.01	40	−2.50	**0.017**
mmu-miR-29a-3p	HDM - PBS	Female	XX	−0.77	0.87	40	−0.88	0.384
		Male	XX	1.33	0.83	40	1.60	**0.118**
		Female	XY	0.50	0.80	40	0.62	0.538
		Male	XY	−1.44	0.83	40	−1.73	**0.091**
mmu-miR-29b-3p	HDM - PBS	Female	XX	−2.11	1.08	40	−1.96	**0.058**
		Male	XX	2.00	1.03	40	1.95	**0.059**
		Female	XY	1.27	0.99	40	1.28	0.207
		Male	XY	−1.59	1.03	40	−1.55	**0.130**
mmu-miR-29c-3p	HDM - PBS	Female	XX	−0.73	0.92	40	−0.80	0.428
		Male	XX	1.24	0.87	40	1.41	0.165
		Female	XY	0.80	0.84	40	0.95	0.346
		Male	XY	−1.54	0.87	40	−1.76	**0.086**
mmu-miR-301a-3p	HDM - PBS	Female	XX	−2.74	1.21	40	−2.27	**0.029**
		Male	XX	1.28	1.15	40	1.11	0.274
		Female	XY	0.02	1.11	40	0.02	0.985
		Male	XY	−2.52	1.15	40	−2.19	**0.035**
mmu-miR-301b-3p	HDM - PBS	Female	XX	−1.75	1.27	39	−1.37	0.178
		Male	XX	2.48	1.27	39	1.95	**0.058**
		Female	XY	−0.38	1.17	39	−0.33	0.746
		Male	XY	−1.27	1.21	39	−1.05	0.301
mmu-miR-30a-5p	HDM - PBS	Female	XX	−2.20	0.92	40	−2.40	**0.021**
		Male	XX	0.95	0.87	40	1.09	0.281
		Female	XY	−0.38	0.84	40	−0.45	0.657
		Male	XY	−2.54	0.87	40	−2.90	**0.006**
mmu-miR-30b-5p	HDM - PBS	Female	XX	−2.85	1.10	40	−2.58	**0.014**
		Male	XX	1.01	1.05	40	0.96	0.343
		Female	XY	0.08	1.01	40	0.08	0.940
		Male	XY	−2.66	1.05	40	−2.53	**0.016**
mmu-miR-30c-5p	HDM - PBS	Female	XX	−2.85	1.00	40	−2.87	**0.007**
		Male	XX	1.22	0.95	40	1.28	0.206
		Female	XY	0.14	0.91	40	0.15	0.883
		Male	XY	−2.46	0.95	40	−2.59	**0.013**
mmu-miR-30d-5p	HDM - PBS	Female	XX	−1.81	0.89	40	−2.03	**0.050**
		Male	XX	0.64	0.85	40	0.75	0.458
		Female	XY	0.06	0.82	40	0.07	0.944
		Male	XY	−1.96	0.85	40	−2.30	**0.027**
mmu-miR-30e-5p	HDM - PBS	Female	XX	−4.26	1.39	40	−3.07	**0.004**
		Male	XX	−0.26	1.32	40	−0.20	0.846

Continued

Table 4.— *Continued*

miRNA	Contrast	Gonadal	Chromosomal	Estimate	Standard Error	Degrees of Freedom	*t* ratio	*P* Value
		Female	XY	0.99	1.27	40	0.77	0.443
		Male	XY	−3.65	1.32	40	−2.76	**0.009**
mmu-miR-322-5p	HDM - PBS	Female	XX	−2.49	1.11	40	−2.24	**0.031**
		Male	XX	1.23	1.06	40	1.17	0.251
		Female	XY	0.34	1.02	40	0.34	0.738
		Male	XY	−2.42	1.06	40	−2.28	**0.028**
mmu-miR-338-5p	HDM - PBS	Female	XX	−2.21	0.98	38	−2.26	**0.029**
		Male	XX	0.64	0.93	38	0.69	0.496
		Female	XY	−0.13	0.90	38	−0.14	0.890
		Male	XY	−2.22	1.04	38	−2.13	**0.040**
mmu-miR-340-5p	HDM - PBS	Female	XX	−1.02	1.26	40	−0.81	0.424
		Male	XX	2.03	1.20	40	1.69	**0.098**
		Female	XY	1.21	1.16	40	1.05	0.302
		Male	XY	−2.90	1.20	40	−2.41	**0.021**
mmu-miR-350-3p	HDM - PBS	Female	XX	−1.64	0.94	40	−1.74	**0.089**
		Male	XX	1.35	0.90	40	1.50	**0.141**
		Female	XY	0.76	0.87	40	0.88	0.386
		Male	XY	−2.14	0.90	40	−2.38	0.022
mmu-miR-429-3p	HDM - PBS	Female	XX	−2.07	1.05	40	−1.97	**0.056**
		Male	XX	0.54	1.00	40	0.54	0.591
		Female	XY	0.27	0.97	40	0.27	0.785
		Male	XY	−3.08	1.00	40	−3.07	**0.004**
mmu-miR-466d-3p	HDM - PBS	Female	XX	−2.50	1.04	40	−2.41	**0.020**
		Male	XX	2.31	0.99	40	2.34	**0.024**
		Female	XY	−0.76	0.95	40	−0.80	0.430
		Male	XY	−1.37	0.99	40	−1.38	0.174
mmu-miR-466k	HDM - PBS	Female	XX	−0.65	0.90	39	−0.73	0.473
		Male	XX	1.06	0.86	39	1.24	0.222
		Female	XY	−1.50	0.83	39	−1.82	**0.076**
		Male	XY	−0.30	0.90	39	−0.34	0.738
mmu-miR-497a-5p	HDM - PBS	Female	XX	−0.64	1.03	40	−0.61	0.542
		Male	XX	2.20	0.99	40	2.23	**0.032**
		Female	XY	0.68	0.95	40	0.72	0.478
		Male	XY	−1.41	0.99	40	−1.43	0.161
mmu-miR-712-5p	HDM - PBS	Female	XX	−0.41	0.86	40	−0.47	0.640
		Male	XX	−0.61	0.82	40	−0.74	0.464
		Female	XY	−0.41	0.79	40	−0.51	0.612
		Male	XY	−1.28	0.82	40	−1.55	**0.129**
mmu-miR-93-5p	HDM - PBS	Female	XX	−1.43	0.93	40	−1.53	**0.133**
		Male	XX	1.23	0.89	40	1.38	0.175
		Female	XY	0.36	0.86	40	0.42	0.673
		Male	XY	−1.98	0.89	40	−2.22	**0.032**
mmu-miR-98-5p	HDM - PBS	Female	XX	−2.02	1.18	39	−1.71	**0.094**
		Male	XX	1.05	1.12	39	0.93	0.356
		Female	XY	−0.10	1.14	39	−0.09	0.929
		Male	XY	−2.27	1.12	39	−2.02	**0.050**

Bolded *P* values have significance levels of ≤0.15. miRNAs in bold are associated with HDM exposure ($P \leq 0.10$).

not stratify results by males and females, they used mice with the same C57BL/6 background as FCG in our study. With higher KC and IL-17A in HDM-challenged mice with ovaries, our results indicate that this model resembles that of allergic airway inflammation (24) and has implications for males and females with asthma as well as patients receiving hormonal treatments.

We also found that IL-7 was increased in XYF with ovaries and this finding was not unusual since another study observed elevated levels of IL-7 in the lung lavage fluid of humans sensitized to fungi (25). Furthermore, the IL-7 levels were negatively correlated with lung function measures (25). Further studies are needed to understand the role of this cytokine in sex-specific asthma phenotypes. Conversely, androgens are associated with anti-inflammatory effects, whereas estrogens produce proinflammatory effects (26–29). In our study, XY mice with testes did not demonstrate increased lung inflammation, even after the HDM challenge. Although the XX mice with testes showed some trends suggesting increased airway inflammation, these results were not statistically significant or consistent across markers. These findings are plausible given that current evidence supports the fact that *1*) adult men are less susceptible to developing asthma compared with women and *2*) adult women exhibit more severe asthma than adult men (30). Given that allergic asthma often persists (31) and lung cytokines correlate with circulating levels of estradiol in females (32), it is important to gain a deeper understanding of the underlying mechanisms in sex-specific allergic asthma to help guide effective treatment, such as targeting allergic triggers and the key players in the Th2 pathway.

Table 5. *IPA summary of the miRNAs with statistical significance in three-way ANOVA interaction models for the four core genotypes and their associations with various molecular and cellular functions*

Genotype	Key miRNAs in Pathway (Downregulated↓; Upregulated↑)	Top Genes Targeted by Differentially Expressed miRNA	Diseases and Disorders	*P* Value Range	Molecular and Cellular Functions	*P* Value Range	Physiological System Development and Function	*P* Value Range
XXF	miR-135a-5p↓ miR-30c-5p↓ miR-126a-5p↓ miR-182-5p↓ let-7a-5p↓ miR-130a-3p↓ miR-26a-5p↓ miR-297a-3p↓ miR-16-5p↓ miR-181a-5p↓	ADAMTS14, ADAMTS15, ADAMTS8, Akt, calcifediol, CG, CREBL2, estrogen receptor	Cancer	4.87E-02–1.65E-22	Cellular Movement	4.53E-02–8.84E-15	Digestive System Development and Function	3.06E-09–3.06E-09
			Organismal Injury and Abnormalities	4.95E-02–1.65E-22	Cellular Development	Cellular Development 4.99E-02–1.83E-09	Hepatic System Development and Function	3.06E-09–3.06E-09
			Reproductive System Disease	4.77E-02–1.65E-22	Cellular Growth and Proliferation	4.99E-02–1.83E-09	Organ Development	2.76E-02–3.06E-09
			Neurological Disease	4.53E-02–1.59E-20	Cell Death and Survival	4.53E-02–1.59E-07	Organismal Development	4.87E-02–3.19E-07
			Psychological Disorders	9.57E-03–1.59E-20	Cell Cycle	4.99E-02–1.83E-09	Embryonic Development	3.10E-02–8.76E-07
XXM	miR-221-3p↓ miR-19b-3p↓ miR-130a-3p↓ miR-17-5p↓ miR-297a-3p↓ miR-26a-5p↓ miR-16-5p↓ let-7a-5p↓ miR-182-5p↓ miR-128-3p↓	ADAMTS14, ADAMTS15, BIRC5, CG, CLDN12, Gnasas1, Gulo, HNF1A-AS1, HOTTIP	Organismal Injury and Abnormalities	4.63E-02–1.52E-17	Cellular Movement	4.90E-02–1.04E-09	Embryonic Development	4.62E-02–2.41E-07
			Reproductive System Disease	3.89E-02–1.52E-17	Cellular Development	3.66E-02–2.02E-08	Connective Tissue Development and Function	4.34E-02–2.05E-06
			Cancer	4.63E-02–4.66E-17	Cellular Growth and Proliferation	3.66E-02–2.02E-08	Digestive System Development and Function	3.55E-02–7.71E-06
			Neurological Disease	4.30E-02–2.44E-16	Cell Cycle	3.72E-02–2.41E-07	Hepatic System Development and Function	4.34E-02–7.71E-06
			Psychological Disorders	2.19E-02–2.44E-16	Cell Death and Survival	4.06E-02–9.09E-06	Organ Development	4.62E-02–7.71E-06
XYF	miR-135a-5p↑ miR-155-5p↑ let-7a-5p↑ miR-466d-5p↓	ACSL6, ALOX5AP, CCL18, CD209, CLDN1, ERBB2, FCER2, FGF7, HTR1A, JARID2, MSI2	Cancer	4.85E-02–2.69E-08	Cellular Movement	2.94E-02–2.38E-05	Nervous System Development and Function	4.74E-03–3.74E-04
			Organismal Injury and Abnormalities	4.85E-02–2.69E-08	Cell Cycle	5.48E-03–1.27E-04	Tissue Morphology	2.51E-02–3.74E-04
			Reproductive System Disease	4.85E-02–4.12E-08	Cellular Development	3.77E-02–2.50E-04	Hematological System Development and Function	2.51E-02–7.49E-04
			Neurological Disease	4.70E-02–1.48E-05	Cellular Assembly and Organization	5.85E-03–3.74E-04	Organ Morphology	7.49E-04–7.49E-04
			Inflammatory Disease	4.70E-02–1.96E-05	Gene Expression	6.24E-04–6.24E-04	Respiratory System Development and Function	7.49E-04–7.49E-04
XYM	miR-126a-5p↓ miR-182-5p↓ miR-27a-3p↓ miR-340-5p↓ miR-350↓ miR-30c-5p↓ miR-19b-3p↓ miR-130a-3p↓ miR-155-5p↓ miR-338-5p↓	ADAMTS14, ADAMTS15, Akt, CG, DLK1, estrogen receptor, FSH, Gulo, HOTTIP, Insulin, Interferon alpha	Cancer	4.83E-02–9.18E-25	Cellular Movement	4.48E-02–3.55E-14	Organismal Development	4.39E-02–3.70E-07
			Organismal Injury and Abnormalities	4.83E-02–9.18E-25	Cellular Development	4.74E-02–2.00E-11	Digestive System Development and Function	4.12E-07–4.12E-07
			Reproductive System Disease	4.83E-02–9.18E-25	Cellular Growth and Proliferation	4.74E-02–2.00E-11	Hepatic System Development and Function	4.12E-07–4.12E-07
			Neurological Disease	4.74E-02–2.05E-22	Cell Death and Survival	4.74E-02–2.81E-08	Organ Development	4.39E-02–4.12E-07
			Psychological Disorders	1.00E-02–2.05E-22	Cell Cycle	3.86E-02–1.01E-06	Embryonic Development	4.39E-02–1.01E-06

miRNA levels in biological fluids are often altered compared with healthy controls and can therefore be used as noninvasive biomarkers for lung diseases (33). In this study, we observed differential expression of miRNAs associated with HDM challenge compared with PBS controls. Specifically, we observed that let_7f_5p, miR_126a_5p, miR_23a_3p, miR_26a_5p, miR_30a_5p, miR_30b_5p, miR_30c_5p, miR_30e_5p, mmu_miR_429_3p, and miR_338_5p were associated with HDM exposure and were differentially expressed depending on gonads and sex chromosomes.

Although testing of these microRNA signatures in other datasets and studies is warranted, these results present additional evidence that hormonal effects may alter miRNA expression, which in turn contribute to the mechanisms responsible for the higher susceptibility of adult females to asthma.

miRNAs, whether they are encoded on the X chromosome or elsewhere in the genome, can contribute to biological sex differences in lung disease (34). We looked up the locations of each of the mouse miRNAs identified in the present study (using https://rnacentral.org/), and only one (miR-19b) was found on chromosome 14 as well as the X chromosome. The rest were all found on autosomes (chromosomes 1, 2, 4, 6, 8, 9, 10, 11, 13, 14, 15, and 16). These non-X chromosome locations suggest that other factors, for instance, hormones, may be the drivers of the observed differential miRNA expression in these genotypes.

In addition, IPA results showed a few canonical pathways associated with some of the gene targets of the significantly expressed miRNAs in our study. Agarwal et al. (35) highlighted the importance of canonical sites for miRNA binding and pointed to the fact that noncanonical sites may not mediate the repression of mRNAs despite the binding of the miRNAs. Detailed understanding of miRNA-mRNA targeting, and interactions can aid in the development of novel molecular interventions to treat and prevent sex-specific adverse lung health effects. Taken together, the regulatory networks containing the miRNAs identified in this study have gene targets implicated in pathways associated with asthma and even severe asthma.

The functions of most of the miRNAs differently expressed in HDM-challenged mice in the current study have been studied in other contexts, particularly as they relate to asthma diagnosis and severity assessment (36). For instance, miRNA-135a has been implicated in mediating airway inflammatory response in allergic airway disease models by targeting the JAK/STAT signaling pathway (37). miRNA-155 has been implicated in allergic asthma (38). miRNA-155 has also been discovered to be overexpressed in the skin of patients with atopic dermatitis, and its expression is positively correlated with the disease severity, the number of Th17 cells, IL-17 mRNA expression, and IL-17 plasma concentrations (39). Mechanistic studies have shown that miR-29b regulates inflammatory cytokine expression by directly targeting specific genes such as *T-bet* or via the Sp1-NF-κB-HDAC-miR-29b regulatory network (40). Inhibition of miR-29b reduces inflammation and pulmonary fibrosis in vivo and in vitro (41). miRNA-26b influences the NF-κB pathway in alveolar macrophages by regulating PTEN (42). When PTEN is silenced, there is an increased expression of several key cytokines, including IL-8 (42). As such, miR-26b participates in the inflammatory response in the airways. Most of the miRNAs that had reduced expression in the HDM-challenged XX mice with ovaries are discussed in a recent review on miRNA research in asthma (43) with several of these miRNAs playing key roles in both adult and childhood asthma.

Our study is not without limitations. First, we were limited by a small sample size, which may have affected the statistical significance of our data. However, to our knowledge, this is the first time that miRNA and protein expression have been studied in HDM-challenged FCG models and our results are hypothesis-generating. Particularly, the role of miRNA in mediating sex differences in allergic responses and lung diseases is understudied and we present novel results using the FCG model. We expect to include larger sample sizes in future studies for each genotype and continue to test our hypothesis. We also studied airway inflammation using a novel mouse model (the FCG), which was developed in the C57BL/6 background, a strain that is less sensitive to HDM challenge than other strains. However, our study results pointed to airway inflammation characteristics typical of the responses observed in asthma mouse models (or "allergic airway disease" models) in wild-type mice (also 5 wk of HDM challenge) (44). Future studies will focus on confirming the target gene expression of the identified miRNAs through transcriptomic approaches as well as through mechanistic assessment by in vitro models. However, although these steps are beyond the scope of this current project, our study will pave the way for future work in our understanding of sex differences in asthma using targeted omics approaches. Finally, this study was designed to assess whether differences in asthma in males and females are mediated by hormonal or chromosomal effects. We provide sex-specific data for both cytokine and miRNA data; results are analyzed to assess the influence of sex on study results, and we incorporate sex-based analysis in our discussion and limitations. The methods and results are provided according to Animal Research: Reporting of In Vivo Experiments (ARRIVE; https://arriveguidelines.org/) guidelines.

Perspectives and Significance

Our study used a targeted omics approach to characterize the contributions of gonadal hormones and chromosomal components to lung responses to an allergen challenge. Our results point to the influence of sex hormones in miRNA expression and proinflammatory markers in allergic airway inflammation. These results have implications for our understanding of the mechanisms mediating asthma development and progression in males and females as well as in patients receiving hormonal treatments. Additional studies are needed to address sex disparities in asthma and provide knowledge about the mechanisms by which gonadal sex hormones regulate inflammation in the lung, particularly in the presence of allergens.

DATA AVAILABILITY

All source data can be found here: https://github.com/exposurelabiu/microRNA-and-protein-data.git.

SUPPLEMENTAL DATA

Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.23277056.v2.
Supplemental Fig. S2: https://doi.org/10.6084/m9.figshare.23277053.v2.
Supplemental Fig. S3: https://doi.org/10.6084/m9.figshare.23277050.v2.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.S. conceived and designed research; S.C., C.D.E., D.R., R.A., M.B., S.S., and P.S. performed experiments; S.C., D.R., A.B., D.B.R., and P.S. analyzed data; S.C., A.B., D.B.R., and P.S. interpreted results of experiments; S.C., A.B., and D.B.R. prepared figures; S.C. and P.S. drafted manuscript; S.C., C.D.E., D.R., R.A., M.B., S.S., A.B., D.B.R., and P.S. edited and revised manuscript; S.C., C.D.E., D.R., R.A., M.B., S.S., A.B., D.B.R., and P.S. approved final version of manuscript.

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