2026

Sex differences in allergic asthma responses of mice prenatally exposed to wood smoke

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Introduction

Asthma is a chronic respiratory disease that affects more than 300 million people worldwide. It involves innate and adaptive immune cells and epithelial cell responses within lung tissue. The disease is characterized by bronchial hyperreactivity and airway remodeling. Allergic asthma is the most common form of asthma among children, which is identified by eosinophilic inflammation, an increase in Th2 cells and type 2 innate lymphoid cells (ILC2 cells) and their production of type 2 cytokines such as interleukin (IL)-4, IL-5, and IL-13[1-2]. Allergic asthma has become more prevalent over time. Recent research suggests that both genetic and environmental factors influence the development of asthma[3]. Furthermore, many studies have found that exposure to environmental factors, including wood or tobacco smoke, in early life or in utero can increase the likelihood of childhood asthma development.

The influence of sex on health-related issues has become a more prominent research topic in recent times. In order to provide better care for patients, it is necessary to understand the effects of sex on how an individual's body will react to both disease and treatment. Within the field of respiratory immunology, specifically regarding the research surrounding asthma and other allergies, many differences between the sexes have been discovered[4-6]. Sex differences have been increasingly reported in the incidence, prevalence, and severity of asthma as it has been found to play an essential role in most cases.

In a literature and clinical review by a research group from Vanderbilt University Medical Center, it was found that the prevalence of asthma between sexes changes throughout varying phases of life[6]. They discovered that following the onset of puberty/adolescence, an increase in allergic asthma is seen in the female population alongside a decrease in males. This has also been found to be true for most other allergic disorders, as well[7-9]. Based on these

findings, the group concluded that such information is vital for the progression of patient treatment; “deciphering how sex hormones regulate airway inflammation may also personalize treatment for asthma-based therapeutics, repurpose androgens as asthma therapeutics, and determine the percentage of women and men with different asthma phenotypes”[6]. Improvement of patient care can be achieved as the understanding of such differences and changes increases. Before such work can be done with human patients, these differences must first be tested in animals.

This study uses a mouse model of allergen-induced asthma to explore the potential sex differences in allergic asthma responses following prenatal wood smoke exposure. Based on the previous research surrounding the broader subject of allergic asthma processes[10-14] and the sex differences found within[4-6], it is hypothesized that the female mice samples will show a greater pulmonary inflammatory response to the HDM allergen following wood smoke exposure compared to the male samples. The samples used in these analyses are provided by Dr. Jaffar’s lab at the University of Montana, from their research determining the influence of prenatal wood smoke exposure on the exacerbation of allergic airway inflammation[14]. To further the understanding of these processes and potential factors that may influence them, experiments were completed comparing samples of the two sexes side-by-side.

Methods

A Model for Prenatal Wood Smoke Exposure

In order to monitor the effects of prenatal wood smoke exposure, we grouped 2 female and 1 male C57BL/6 mice into harems prior to the start of wood smoke inhalation. Following confirmation

of pregnancy, the pregnant mice were exposed to wood smoke or filtered air by a wood smoke exposure system in the inhalation and pulmonary physiology core facility of the Center for Environmental Health Sciences at the University of Montana. This exposure chamber is divided into 2 sections, one which receives filtered air and another which receives filtered air along with a predetermined concentration of wood smoke. Smoke exposure concentrations were determined based on levels that individuals who use wood stoves to heat their homes during winter months might experience. Specifically, groups of mice were placed in ventilated cages within the exposure chamber for 2 hours. The mice received wood smoke at a targeted level of 3 mg/m3 of 2.5 nanometer particulate matter ($PM_{2.5}$) over the course of the 2-hour exposure. Once the exposure was complete the mice were returned to their original cages and the process was repeated 24 hours later. The exposures took place on Monday through Friday for a total of 3 weeks. The female mice were monitored for pregnancy and removed with pups from the wood smoke once they had given birth to offspring. After birth the pups were kept with their mothers for 21 days then weaned. Male and female offspring mice were separated and housed in different cages. The mice described in these experiments were then left to "rest" for an additional 3 weeks before their responsiveness to inhaled house dust mite (HDM) allergen was evaluated (8 weeks old when challenged).

House Dust Mite (HDM) Allergen Challenge

Offspring mice were challenged with HDM allergen over a period of two weeks using a model of asthma previously described by the laboratory. Mice were lightly anesthetized with isofluorane to allow intranasal instillation of 30 µl solution of HDM allergen extract (Dermatophagoides pteronyssinus, Greer Laboratories) in sterile PBS, or administration of PBS alone (control) over a period of 2 weeks. Briefly, mice were first sensitized with HDM (100 µg) by intranasal instillation on Day 0 and then challenged with the allergen (50 µg) on Days 7 and 14. Forty-eight hours after

the last exposure (Day 16), the levels of airway inflammation were determined. Control groups were challenged with PBS (phosphate buffer solution).

Level of Airway Inflammation

Bronchoalveolar lavage was performed on the mice (using 3 × 0.5 ml PBS) to collect bronchoalveolar lavage fluid (BALF) for analysis of airway inflammation. BALF was cooled on ice and centrifuged at 4C for 10 min. The cell pellets were resuspended in PBS and the supernatants frozen (−80C) for analysis of cytokine production. Eosinophil peroxidase (EPO) levels in the bronchoalveolar lavage cells were determined by colorimetric analysis (as described below). Cells were centrifuged (Cytospin II, Shandon) onto glass slides and stained with hematoxylin and eosin (Hema 3, Fisher Scientific). The cells were counted by use of a hemocytometer. The four different cell types were counted based on percentage out of total cells per mouse.

The first analyses completed were cell differential counts to compare the levels of pulmonary inflammation between sexes. Under the direction of Dr. Zeina Jaffar, the samples were studied based on their macrophage, eosinophil, lymphocyte, and neutrophil levels. A total of 4 female and 4 male exposure groups were studied: (1) wood smoke exposure plus HDM allergen, (2) wood smoke exposure plus PBS, (3) air exposure plus HDM, and (4) air exposure plus PBS control.

The second analysis involved an eosinophil peroxidase assay (EPO). This data is used to supplement the results found in the cell differential counts. It is a procedure to measure the levels of eosinophil peroxidase within the samples to determine any changes in pulmonary inflammation. This measurement by colorimetric analysis was performed following the methods

Dr. Roberts and Dr. Jaffar have used in the past[15]. To begin, the OPD mixture was made which required 100 mL of 50mM Tris-HCL (pH 6.7), a crushed tablet of o-Phenylenediamine, 100 microliters of Triton X-100 (Fisher Scientific), and 20 microliters of 1mM hydrogen peroxide. Into each well of a 96-well ELISA plate was added 100 microliters of PBS, 100 microliters of cell sample (diluted down each row), and 100 microliters of the OPD mixture. Afterwards, the plate was left to sit for 5-10 minutes. After this period of rest, 50 microliters of stop solution (0.3M H_2SO_4) was added to each well. The plate was then analyzed with a SpectraMAX 190 machine with the SoftMax Pro program.

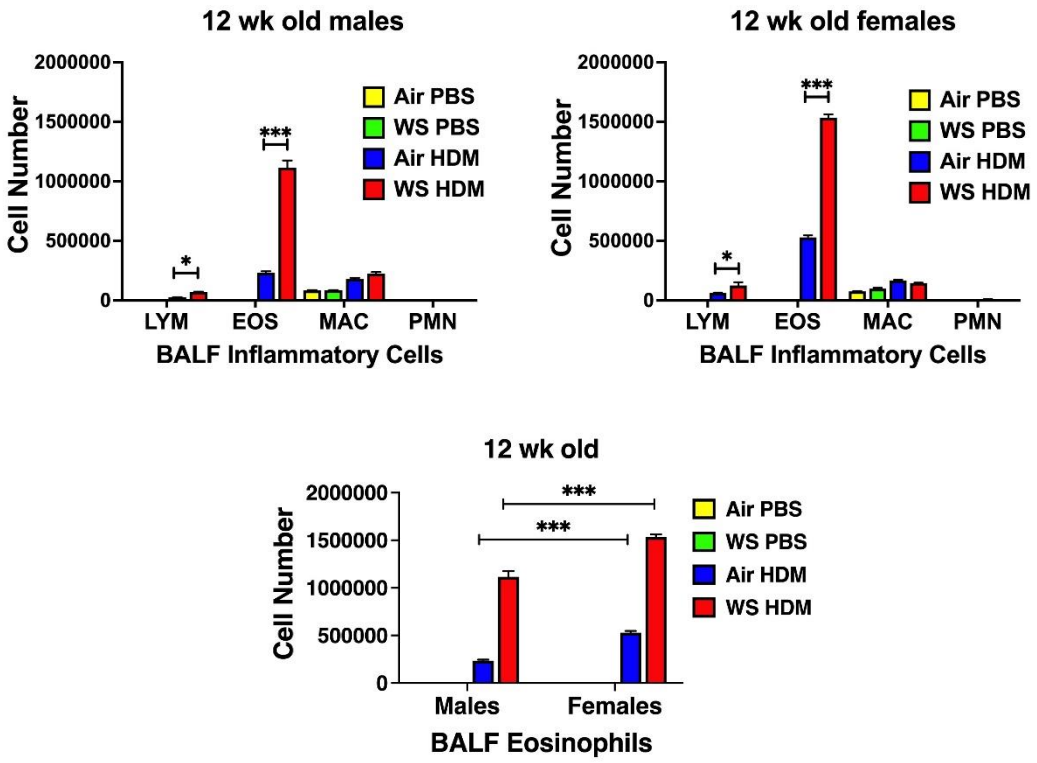
BALF Cytokine Levels

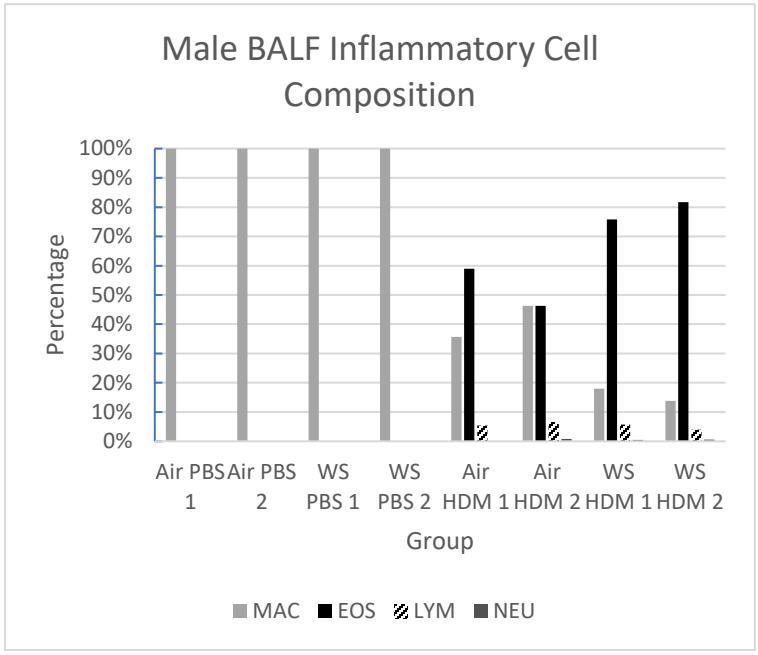
ELISA was used to determine the level of cytokines IL-5 and IL-4 in the BALF samples of both sexes. The analysis was conducted according to the manufacturer's protocol for both IL-5 and IL-4 cytokines. The kits used were the ELISA MAX Deluxe Set Mouse IL-5 (BioLegend, San Diego) and the ELISA MAX Deluxe Set Mouse IL-4 (BioLegend, San Diego).

Results

The adverse effects of prenatal exposure to wood smoke (WS) or filtered air on airway inflammation and cytokine production was assessed in both adult female mice and offspring mice (12 week old) after sensitization and challenge with intranasal HDM allergen over a period of two weeks using a model of allergic asthma. Control mice were not challenged with HDM allergen but treated with PBS instead. As seen in the graphs below (**Fig. 1**), the cell differential counts showed a significant increase in BALF inflammatory cells, especially eosinophils and lymphocytes, in the female and male offspring mice prenatally exposed to WS following HDM

allergen inhalation (WS HDM) compared to air exposed mice (Air HDM). In contrast, negligible levels of inflammatory cells were found in the BALF of control mice that inhaled PBS (WS PBS and Air PBS). However, the number of polymorphonuclear neutrophils (PMN) and macrophages did not significantly differ between the WS and air-exposed mice. Interestingly, a clear difference between the responses of each sex is also found. Female mice that were exposed to WS and inhaled allergen had a significantly elevated number of eosinophils in the BALF compared to male mice. The macrophage cell count was the only type of inflammatory cell found to be smaller in the female samples compared to the male samples. However, this may not be of great significance based on the potential sex difference in macrophage phenotypes found by previous studies[5]. Therefore, the higher levels of eosinophilia in the female samples compared to male samples (WS HDM groups, specifically) suggests that female mice exposed to WS in utero developed a more exacerbated allergic asthma in response to HDM inhalation than male mice, thus this can be used as evidence to prove the hypothesis proposed.





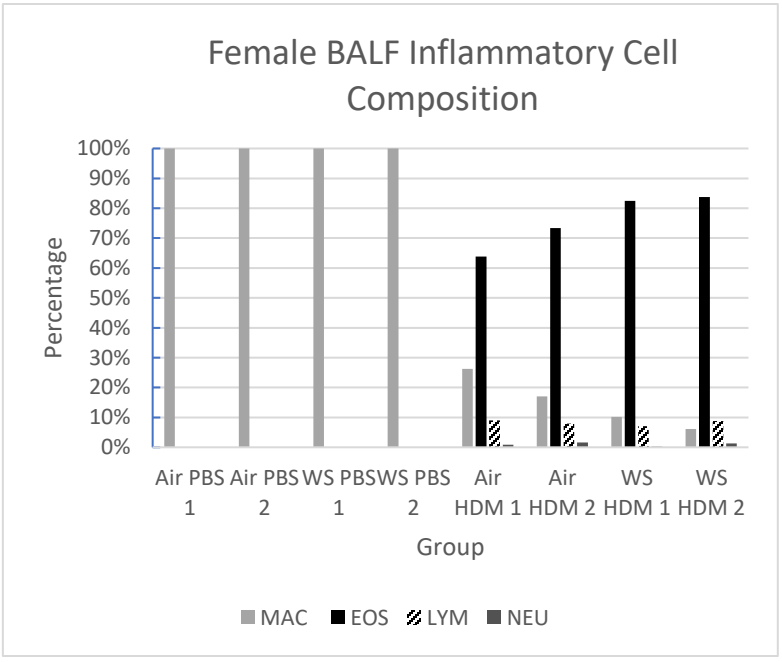


Fig. 1 Cell Differential Counts. Results are mean ±SEM (n = 6), ***p<0.001, *p<0.05. MAC = macrophages, EOS = eosinophils, LYM = lymphocytes, and NEU = neutrophils.

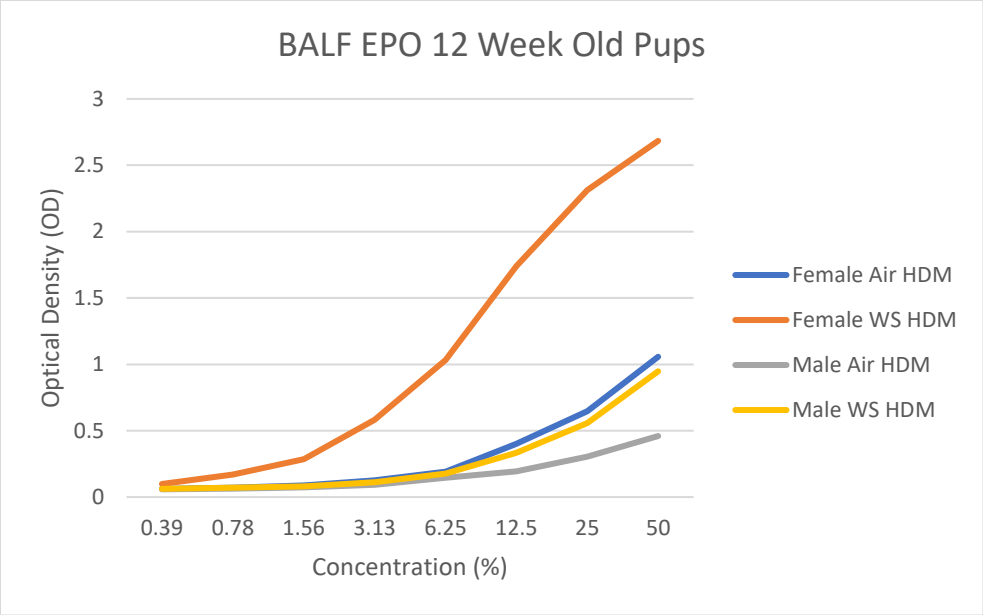
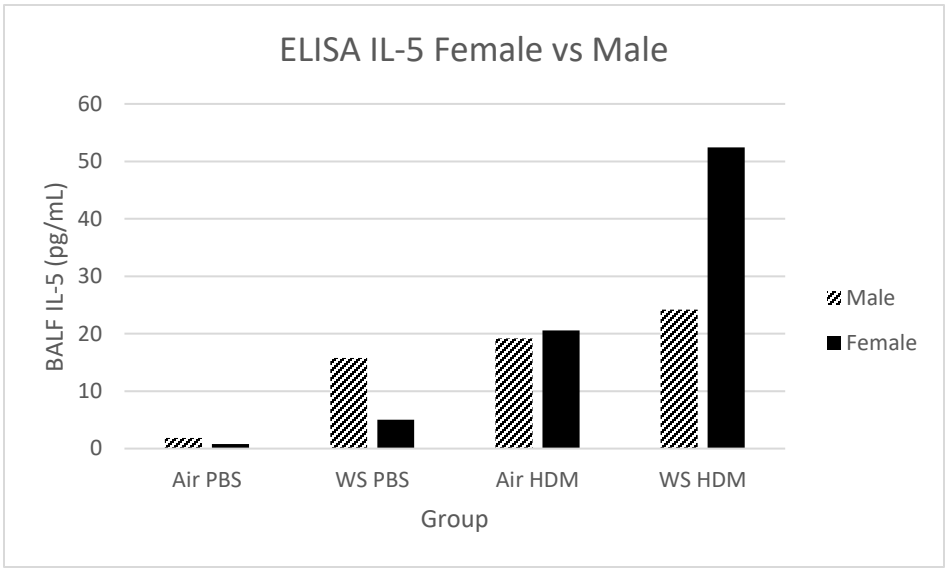


Fig. 2 BALF EPO Assay

In the graph above (**Fig. 2**), the results of the EPO Assay are shown. These results show that prenatal WS exposure caused an increase in the level of cell-associated EPO levels in the airways of both female and male following HDM inhalation. From these results, both female samples (Air HDM and WS HDM) had greater optical density of eosinophil peroxidase levels than that of the male samples (Air HDM and WS HDM). Additionally, the difference between the female Air HDM sample and female WS HDM sample is greater than that of the corresponding male samples. Therefore, the lungs of the female mice had a much greater influx of eosinophils during the inflammation response after exposure to wood smoke than the male mice. Such evidence suggests that the female mice experience a more exacerbated allergic asthma response than the male mice when prenatally exposed to wood smoke. This data agrees with the hypothesis of this study.



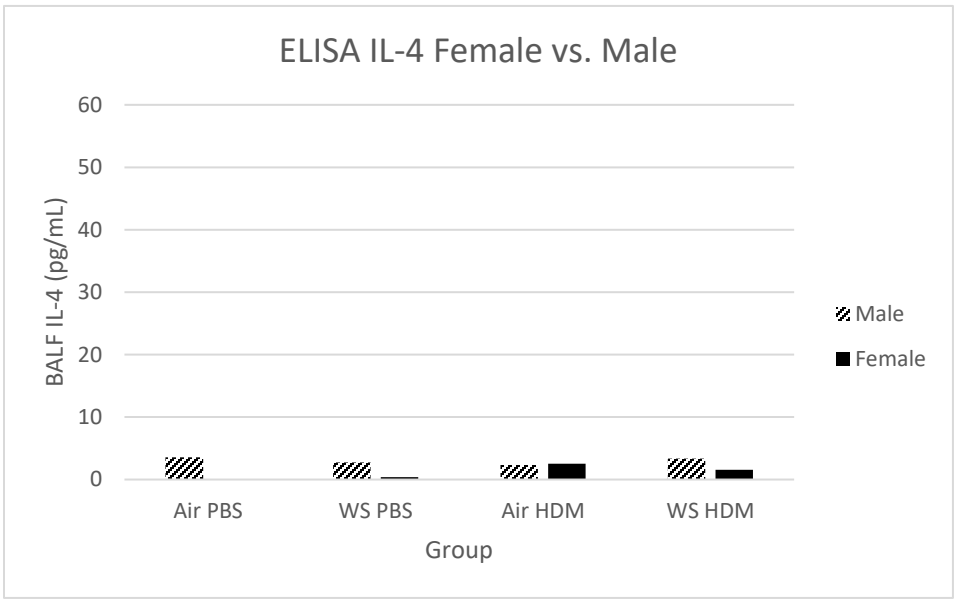


Fig. 3 BALF Cytokine ELISA

Asthma is characterized by type 2 immune responses and production of cytokines such as IL-4, IL-5 and IL-13 that drive the allergic disease. To examine events responsible for exacerbated allergic response following prenatal WS exposure, we assessed whether the exposure promotes generation of type 2 cytokine in the airways. The results of the IL-5 and IL-4 ELISAs are represented in the graphs above (**Fig. 3**). The data revealed that prenatal WS exposure caused

elevated HDM-induced levels of IL-5 in the BALF of both female and male mice compared to air-exposed mice (WS HDM compared to Air HDM). Little or no production of type IL-5 was observed in the airways of air-exposed control mice that did not inhale HDM allergen (controls). As can be seen, the BALF IL-5 levels of the WS HDM group are significantly higher in the female sample compared to the male sample. This is also true, in less magnitude, for the Air HDM samples. This cytokine plays an important role in the type 2 immune response of the asthma allergen and recruitment of eosinophils into the airways; if there is a large IL-5 cytokine production, then the allergic response will be greater. Therefore, the female mice experienced greater inflammation during their allergic asthma response than the male mice (WS HDM).

The IL-4 ELISA did not produce the same results. The levels of BALF IL-4 were very low in all the samples analyzed (all < 4pg/mL), suggesting that a more sensitive ELISA is needed to detect IL-4 levels in the BALF.

Discussion

The findings of this study provide evidence supporting the role of biological sex in allergic asthma. It is conclusive with similar research in the field of respiratory immunology, as well[4-9]. In fact, other investigators have found some specific mechanisms to explain these differences. For example, in a study by a group in Toulouse, France it was found that the sex differences in asthma of mice was influenced by the signaling of the male sex hormone, androgen[4]. The researchers claimed that the "ILC2 sex bias was exquisitely dependent on the male sexual hormones, androgens. Indeed, ovariectomy (removal of the ovaries) and ERα-deficiency* had no effect on ILC2 development and effector functions in females, ruling out any possible role for

estrogens"[4]. Due to this difference in sex hormones, it was discovered that, at least in the murine model, females were more susceptible to allergic asthma. Another study from the University of Montana found similar results when studying the inflammation of airway eosinophils in mice from nanoparticle exposure[5]. It was found that the female bias of the inflammatory allergic response due to MWCNTs (multiwalled carbon nanotube) was present even after considering body weight of the mice. They also determined that the alveolar macrophages played a major role in this response. The researchers claim the "identification of precise mechanisms for specific respiratory diseases and between sexes is essential for the development of effective therapeutics"[5]. Such a claim supports the need for exploration of sex differences in a variety allergic asthma responses. The present study sought to determine if this increased susceptibility for asthma in females would be seen in the allergic response of mice that have been prenatally exposed to wood smoke. This was found to be accurate.

Conclusion

This project found correlation between biological sex and severity of an allergic asthma response to prenatal wood smoke exposure in a murine model. Based on various samples and analyses, it was discovered that the female mice in this study presented higher respiratory exacerbation than the male mice. This is significant to public health as it provides a foundation for further study in humans. If other researchers find the same results in humans, then the therapy of asthma may change to reflect a more personalized treatment plan for patients based on sex. Future research directions may also involve studies comparing asthma therapy between male and female patients, as well.

Abbreviations

BALF: Bronchoalveolar lavage fluid; EPO: Eosinophil peroxidase; HDM: House Dust mite; WS: wood smoke; PBS: Phosphate buffer solution; IL: Interleukin; SEM: Standard error of mean; ELISA: Enzyme-linked immunosorbent assay; * ERα-deficiency: Estrogen receptor alpha deficiency

Acknowledgements

This project would not have been possible without the support and guidance of Dr. Zeina Jaffar and Dr. Kevan Roberts of the Department of Biomedical and Pharmaceutical Sciences at the University of Montana.

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