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Addressing Sex as a Biological Variable in Preclinical Models of Lung Disease

An Official American Thoracic Society Research Statement

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Abstract

Background: Pulmonary diseases have sex-specific predilections across the lifespan. The rigor of preclinical research is paramount to ensure the reproducibility and applicability of findings to clinical studies. The overarching goal was to identify current research gaps and the need for consideration of sex as a biological variable (SABV) in preclinical pulmonary research. The objective was to provide a roadmap and the best standards to incorporate and investigate the role of biological sex in preclinical models of lung diseases.

Methods: A multidisciplinary working group of 17 international investigators from the American Thoracic Society Assembly on Allergy, Immunology, and Inflammation, external content experts, and researchers engaged in lung basic and translational research. They reviewed the literature, identified critical knowledge gaps, and provided recommendations.

Results: The research statement provides an updated summary of the currently available evidence on the standards of SABV research in preclinical models and then offers specific research recommendations focused on the needs of researchers in the pulmonary field. The statement identifies knowledge gaps and develops guidance for experimental design and key considerations for incorporating SABV in two major topic areas: *1*) *in vivo*; and *2*) *in vitro* models. Furthermore, the group developed a checklist to guide researchers in including SABV in preclinical studies.

Conclusions: This statement provides a roadmap for the investigation of SABV in preclinical models. This will increase the applicability of findings to both sexes, uncover sex-biased mechanisms in lung diseases, and identify novel therapeutic targets.

Keywords: sex; sex-specific differences; sex as a biological variable; preclinical models; cellular sex

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Overview

Many lung diseases have a different incidence or progression in female and male individuals, suggesting that each sex has different inherent biological factors that protect from or exacerbate the disease. However, an objective inclusion of biological sex in preclinical studies is needed to increase the applicability of findings to both sexes and to tailor the right therapy for the right patient. These efforts will enable precision medicine approaches to understand the complex molecular mechanisms that drive the pathophysiology of chronic lung diseases and devise novel therapies in the future. Outcomes of preclinical research, if done rigorously to include sex as a biological variable (SABV), will reveal important sex-dependent therapeutic targets and eventually guide precision medicine efforts in this field. The proposed research statement was formulated in collaboration with researchers specialized in basic and translational lung research. The research statement includes an updated summary of the currently available evidence on the standards of SABV research in preclinical models. It provides specific research recommendations focused on the needs of researchers in the pulmonary field. Furthermore, we aspired to achieve a consensus on a working document and a checklist to guide researchers in including SABV in their ongoing and future preclinical studies. The key recommendations of this research statement are summarized below.

Key Recommendations

***In vivo* models:**

- Consider the stage of the estrous cycle, the timing of experimental interventions, circadian rhythms, endpoint assessments, dietary factors, use of tamoxifen, strain differences, and environmental factors that can lead to or confound sex-based differences in endpoints and phenotypes.
- Consider the organizational and activational effects of gonadal hormones. Consider local production of sex steroids in the lung as a possible modulator of outcomes.
- Consider the role of sex chromosomes in contributing to sex-biased findings in lung diseases.
- Consider the differential sex-biased effects of puberty, menopause, and aging on animal models of lung disease.
- Consider the appropriate experimental design and power to accurately and transparently report findings based on biological sex.

***In vitro* models:**

- Verify and document the cellular sex of commercially sourced cell lines and primary cells before conducting experiments. If cells are derived from patient samples, report the donor's sex, ethnic background, age, menopausal status, hormone therapy use, and pregnancy history. These data help researchers contextualize findings from female donors and should be collected in biorepositories.
- Document culture conditions and avoid media components that can activate sex steroid receptor signaling. Use phenol red–free and charcoal-stripped media if experimental conditions allow.
- Consider the potential autocrine or paracrine effects of sex steroids. Catalog sex hormone and receptor expression in cells. Culture media should be formulated to control hormonal and growth factors, as some media components can affect steroidal pathways and growth factor interactions. Replicate sex-specific physiologic conditions.
- Consider how donor sex can introduce variability in the reprogramming, pluripotency, and differentiation in models using induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs).
- Consider the sex of cells when building complex three-dimensional (3D) models, recognizing the increased potential for sex-based variability.

Introduction

Pulmonary diseases have sex-specific manifestations across the life course. SABV plays a crucial role not only in disease pathophysiology but also in responses to therapy (1–3). SABV is not limited to comparing outcomes between males and females; it also includes considering sex-specific factors, such as the effects of the estrous cycle and menopause. The rigor of preclinical research is paramount to ensure the reproducibility and applicability of findings to clinical studies. Consideration of SABV is a prerequisite for all basic and translational research categories for federal funding. Despite these efforts in studies using animal models, the sex of the biological replicates is often not specified, or a scientific justification is not provided for using one biological sex. Table 1 summarizes the sex differences in selected lung diseases in humans, the commonly used preclinical models used for research, and their limitations. Operationally, three types of sex differences have been described: *1*) sexual

Table 1. Sex Differences in Human Lung Diseases and Preclinical Animal Models

Disease	Sex Differences (Human)	Sex-Specific Differences (Animal Models)	Limitations of Animal Models
COPD (194–202)	Women are less likely to be diagnosed, possibly more susceptible for similar tobacco exposure, exhibit different symptom profile, have more exacerbations, and show less emphysema, more small airway disease, and lower mortality than men. The growing global burden is in women.	Mouse models of COPD have revealed significant sex differences in disease progression and manifestation. Female mice exposed to chronic cigarette smoke tend to develop more small airway disease, inflammation, airflow obstruction, and airway remodeling, whereas male mice are more prone to developing emphysema. Gonadectomized females display a male-like phenotype, suggesting a role of female sex hormones in sex-specific COPD mechanisms.	Most animal models of COPD cannot be directly extrapolated to human phenotypes. COPD mouse studies typically use males to avoid potential female hormone effects.
PAH (82, 99, 132, 203–208)	Women are more susceptible to disease development. Female patients exhibit better RV adaptation and survival. Sexual dimorphisms exist in response to pulmonary vasodilators.	Some animal models demonstrate female susceptibility, whereas others exhibit male susceptibility; female RV resilience usually recapitulated in animal models; conflicting results on whether estrogens promote or protect against pulmonary vascular remodeling; estrogens consistently protective in promoting RV adaptation; certain estrogen metabolites (e.g., 16a-OHE1) drive pulmonary vascular remodeling.	Not all animal models recapitulate human PAH phenotypes, especially mouse models (e.g., female disease susceptibility seen in humans is not consistently seen in animal models).
IPF (209–214)	IPF is more prevalent in men than in women.	Young male mice developed more severe fibrotic lung disease than control females with bleomycin. Increased sensitivity and development of progressive nonresolving disease in aged males compared with aged females, who resolve fibrotic disease. Female mice have less fibrosis after exposure to nitrogen mustard and hydrochloric acid.	Single-dose bleomycin does not fully mimic human IPF histopathologic features, and aged females do not develop progressive fibrotic disease.
ARDS/ALI (215–221)	Women demonstrate higher rates of alveolar fluid clearance than men. In some studies, men demonstrate an increased risk of ALI; however, after adjustment for baseline differences between the sexes, these differences are no longer significant.	A meta-analysis of preclinical studies demonstrates male mice exhibiting a higher risk of ALI severity than female mice. Changes in lung vascular barrier regulation may be explained by estrogen-mediated upregulation of angiotensin via the Mas receptor. Lowering of gonadal hormone levels increases the risk of indirect ALI (for example, brain death–induced lung injury or trauma-induced hemorrhagic shock).	Most studies demonstrate variable levels of bias and are also primarily in mouse models, rats, or hamsters.
Pulmonary infections (218, 220, 222–228)	Men have a higher risk for COVID-19 infection, hospitalization, disease severity, ICU admission and death. Men also have a higher risk of tuberculosis.	Male mice demonstrate worse bacterial pneumonia severity than female mice. Gonadal hormones affect survival and severity of infection in response to bacterial pneumonia. Male hamsters are at a higher risk of direct ALI from infection (SARS-CoV-2).	Preclinical studies on sex differences are likely to be influenced by other factors, such as obesity and baseline levels of mediators (e.g. SP-A) that need to be accounted for.
ILD/RA-ILD (229–240)	Males have a higher incidence and mortality rate than females in occupational ILD (silicosis). RA-ILD: RA is more common in women, but men with RA are more likely to develop ILD.	No sex differences are observed in mice in the development of silicotic nodules. In the SKG model of RA-ILD, female mice develop more arthritis with a nonspecific interstitial pneumonia pattern of fibrosis in ~30% compared with males, whereas males have increased expression of proinflammatory cytokines and fibrotic markers in lung tissue. Aged males develop a progressive fibrotic ILD.	In the SKG model, only 30% of female mice develop disease, requiring larger cohorts for study if pulmonary screening by microCT is not available.

(*Continued*)

Table 1. (*Continued*)

Disease	Sex Differences (Human)	Sex-Specific Differences (Animal Models)	Limitations of Animal Models
Allergy/asthma (241–246)	A higher incidence of asthma and allergy is observed in boys vs. girls, before puberty. After puberty, there is a higher incidence of asthma in women than men. Women have more severe asthma and higher rates of exacerbations, hospitalizations, and mortality than men. Women tend to mount stronger immune responses, contributing to their higher rates of allergies and autoimmune diseases.	Female mice typically have higher levels of inflammation and IgE production. Neutrophil and eosinophil counts display sex differences that vary with challenge and strain. AHR tends to be higher in females with ovalbumin challenge and males with house dust mite challenge.	Allergen-induced inflammation, AHR, and T-cell response phenotypes vary depending on strain, duration, and type of allergen challenge.
LAM (173, 247, 248)	LAM disease is a pulmonary disease primarily of women of childbearing age, but patients may also have multisystemic pathologies, including kidney angiomyolipomas, lymphatic involvement, and chylous effusions. There is increasing appreciation for the role of hormonal fluxes in the pathogenesis of this orphan disease, including variations in symptoms during the menstrual cycle and exacerbations associated with pregnancy, exogenous estrogen use, and childbirth. Patients most often present between menarche and menopause with lung collapse or with dyspnea later in the disease course.	Mouse LAM model with targeted TSC2-dependent mTORC1 hyperactivation in lung mesenchyme develops female-specific lung function and structure decline exacerbated by pregnancies. Inactivation of *Tsc2* gene in the mouse uterus resulted in myometrial tumors exhibiting LAM features, and approximately 50% of animals developed metastatic myometrial lung tumors.	Animal models of LAM cannot be directly extrapolated to human LAM. However, experimental mouse models of LAM recapitulating some features of disease provide useful tools to study cellular and molecular mechanisms and use for preclinical testing of novel therapeutic approaches.
CF-related bronchiectasis (249–253)	Females demonstrate earlier mortality, increase in pulmonary exacerbations, and earlier acquisition of *Pseudomonas aeruginosa*.	Female mice with CF show higher levels of inflammatory markers and serine proteases, including IL-8, IL-6, TNF-α, neutrophil elastase, and MPO.	Lung disease in mouse models may not always mimic human disease, and thus findings should ideally be replicated in pig or ferret models.
Neonatal lung diseases (83–85, 254–257)	Increased incidence of RDS, TTN, and BPD. Lower lung function in males born preterm.	Male mice have greater alveolar simplification. Sex-specific differences in pulmonary microvascular endothelial cells and macrophages.	Sex-specific differences have not been validated in all mouse strains; different disease models and long-term effects have not been studied.

Definition of abbreviations: AHR = airway hyperresponsiveness; ALI = acute lung injury; ARDS = acute respiratory distress syndrome; BPD = bronchopulmonary dysplasia; CF = cystic fibrosis; COPD = chronic obstructive pulmonary disease; COVID-19 = coronavirus disease; ILD = interstitial lung disease; IPF = idiopathic pulmonary fibrosis; LAM = lymphangioleiomyomatosis; microCT = micro-computed tomography; PAH = pulmonary arterial hypertension; RA = rheumatoid arthritis; RDS = respiratory distress syndrome; RV = right ventricle; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; SKG = specific strain of mice developed as a genetic model for rheumatoid arthritis and other autoimmune diseases; TTN = transient tachypnea of the newborn.

dimorphism: the outcome is found either exclusively or predominantly in one sex and not in the other; *2*) sex differences: when the outcome exists on a continuum and is quantitatively different between the sexes; and *3*) sex convergence and divergence: the outcome is the same in males and females, but the pathophysiological mechanisms are different. Alternatively, there may be no differences at baseline or in the unperturbed state, but a sex difference may appear in response to a challenge such as injury or stress (4).

Similarly, in cell-based models, the biological sex of the primary cells and cell lines is often not specified, even though cellular sex can profoundly influence cellular phenotype, function, and response to experimental stimuli/conditions. Sex differences can arise from the effects of gonadal hormones or sex chromosomes. Mammalian cells show intrinsic sex-specific differences and respond differently to various intrinsic and extrinsic stressors. Genes on the

X and Y chromosomes can be differentially expressed between male and female cells because of X chromosome inactivation, gene dosage, or genomic imprinting.

Assessing SABV is of critical importance for designing and interpreting preclinical studies. However, clear guidelines on how to accurately do this are lacking. This research statement brings together researchers engaged in basic and translational pulmonary research and aims to provide clear standards for including and assessing SABV in preclinical research and fill knowledge gaps in accounting for SABV in lung disease research. The overarching goal is to identify current research gaps and the need for the consideration of SABV in preclinical pulmonary research. "Preclinical research" refers to research before testing in human volunteers, including animal studies and cell-based model systems (animal- and human-derived). Our objective is to provide a roadmap and the best-practice standards to incorporate and investigate the role of biological sex in preclinical models of lung diseases. This research statement provides an updated summary of the currently available evidence on the standards of SABV research in preclinical models and specific research recommendations focused on the needs of researchers in the pulmonary field. The statement is organized in two sections, focusing on *1*) animal models; and *2*) *in vitro* models. In addition, we provide a checklist to guide researchers in including SABV in their ongoing and future preclinical studies.

Methods

A multidisciplinary working group comprising 17 international investigators from the American Thoracic Society (ATS) Assembly on Allergy, Immunology, and Inflammation, together with external content experts and researchers specialized in basic and translational lung research, collaborated on this effort. The investigators (Ph.D., M.D.) were selected from all facets of pulmonary medicine (adult and pediatric medicine) based on their track record of publications on SABV in pulmonary diseases. The following steps were adhered to for the generation of the research statement recommendations:

1. Articulate the scope of the research statement and the specific objectives
2. Review existing literature and data: Conduct a comprehensive review of relevant published literature and existing data to gather insights and best practices for studying SABV in preclinical models.
3. Identify key issues and challenges: Determine the critical issues, challenges, or gaps in the current state of knowledge or practice in the lung research field.
4. Prioritize recommendations and provide a rationale: Prioritize the solutions based on their relevance, potential impact, and alignment with the research objectives. Identify the most important recommendations. Clearly explain the rationale behind each recommendation. Why is it necessary? How will it address the identified issue or challenge?
5. Support with evidence: Back the recommendations with evidence from the existing literature review, stakeholder input, or relevant research findings to increase credibility.

Before beginning work on the project, all participants had their conflict-of-interest disclosures vetted by the ATS. In addition, the co-chairs reviewed for potential conflicts of interest from other authors.

Section 1: Integrating and Investigating SABV in Preclinical Animal Models

The goals of this section include:

- Identifying rigorous experimental approaches and study designs to integrate and investigate the role of sex (chromosomal and gonadal) in preclinical animal models.
- Create a checklist for researchers as a guide and for journals and reviewers to assess rigor in scientific work and reproducibility for the consideration of SABV.

Experimental Design and Key Considerations for Incorporating SABV in Preclinical Models

The following section includes considerations and recommendations for appropriately considering SABV and avoiding potential confounders in preclinical animal models.

Estrous cycle and synchronization of cycles. To investigate potential sex differences in lung disease, gonad-intact, age-matched, wild-type males and females should first be compared. The investigator must keep in mind that the levels of hormones (especially estrogens) in female rodents vary during the menstrual cycle (known as the estrous cycle) and, therefore, could induce variabilities if all female rodents are not used at the same stage (5, 6). The estrous cycle is important because it is known to affect several major indicators of lung physiology (e.g., diffusing capacity and vasoreactivity). The estrous cycle in rodents lasts ∼4–5 days and is divided into four stages: proestrus (lasting ∼14 h), estrus (24–48 h), metestrus (6–8 h), and diestrus (48–72 h) (7). The stage of the estrous cycle is determined by vaginal smear cytology based on the abundance of the three main cell types: nucleated epithelial cells (mainly in proestrus), cornified epithelial cells (mainly in estrus), a mix of leukocytes and a few nucleated epithelial and/or cornified epithelial cells (metestrus), and leukocytes (diestrus). During the estrous cycle there are changes in plasma hormone levels, especially estrogen (E2), with an estrogen peak at proestrus hours before estrus; hence, the estrous stage reflects the effects of the high E2 surge at proestrus (8). On the other hand, diestrus presents a low estrogenic condition. The stage of the female estrous cycle can be synchronized easily by group housing 4–5 females together for 10–14 days and then exposing them to soiled bedding from a male's cage (male urine contains pheromones that can trigger estrus) (9).

In studies investigating sex differences in rodents, the estrous cycle stage should be known at the time of experimental interventions and endpoint assessment. Although obtaining vaginal smears for cytology is the gold standard for assessing the stage of the estrous cycle, this can be stressful for animals and is not always feasible. A less invasive but still accurate method to determine the stage of the estrous cycle in rodents is assessing the appearance of the vagina (7). Assays for measuring plasma E2 levels may not be sensitive or specific enough to differentiate the various stages of the estrous cycle accurately. Methods such as assessing the histological appearance of reproductive organs, measuring vaginal wall impedance, and determining urine protein and lipid levels, although generally considered accurate, are not widely

established (7). To date, there is little investigation on sex hormone receptor expression and other downstream pathways that may be regulated during the menstrual cycle in nonreproductive organs. These studies are critical for the understanding of menstrual cycle effects in the lung.

Circadian factors. Effects of circadian rhythm should be considered when investigating sex differences (10). Hypothalamic suprachiasmatic nuclei are the principal pacemakers that gather data on light or darkness, socialization, and mealtime to frame sleep or wakefulness, hormonal, and metabolic rhythms. Therefore, to control for the effects of circadian rhythm, investigators should synchronize factors such as feeding time, duration of light exposure or darkness, and number of rodents in cages (same socialization) for both females and males (11). Next, the investigators should perform their interventions and assessments at the same time of the day, because estrogens can modulate the expression of circadian clock genes (12). Estrogens regulate circadian behavior during the estrous cycle, and there is an interplay between estrogens and the circadian system. Thus, synchrony in the estrous cycle and experimental times may be needed (13). Apart from sex hormones, sex chromosomes can also influence circadian rhythms. For example, in four core genotype mouse models, XX animals have longer activity durations than XY animals, regardless of their gonadal phenotype (14).

Diet requirement. As some diets have been linked to altering sex hormone levels (in particular, estrogen levels), investigators should attempt to lessen the effect of dietary factors while exploring the impact of sex differences in pulmonary diseases (15, 16). Diet composition is the first factor that should be considered. In rodents, a high-calorie diet has been shown to increase levels of sex hormones such as testosterone and estradiol in female rats compared with rats with a standard diet (17). The sex differences in high-fat diets have also been reported in rodents; a high-fat diet increases body weight to a greater extent in male mice than female mice because females are more resistant to weight gain (18). Phytoestrogens in soy products may have estrogen-like effects, influencing hormone levels (19). Therefore, when investigating sex differences in rodents, diets free of or reduced in phytoestrogen should be used (20).

Use of tamoxifen. Tamoxifen is a selective estrogen receptor modulator that can act as an agonist or antagonist on estrogen receptors. This response is influenced by the distribution of estrogen receptors, their ligand-binding specificity, and interactions with other coactivators or corepressors. Tamoxifen has been used in preclinical research models to achieve temporally controlled recombination of floxed target genes achieved by the CreERT2/loxP system and to tag specific lung cells, creating inducible mouse models. This is extremely useful in understanding the cell-specific role of a gene or biological pathway in lung disease pathophysiology. It is also important to investigate any sex-specific effects of tamoxifen administration in preclinical models of lung disease. Several studies have reported the direct effects of tamoxifen and differing effects of tamoxifen treatment by biological sex (21–23). Using appropriate controls that did not receive tamoxifen is important to identify effects attributable to tamoxifen administration in the disease model being studied (24). Focused experiments on the effects of tamoxifen on the specific lung cells (in wild-type mice without the floxed gene) being studied can provide reassurance that tamoxifen can be used as a Cre-recombinase inducer without confounding the experimental results (25).

Classes of factors causing sex differences. The sex chromosomes cause the development of different gonads in the two sexes, which secrete different gonadal hormones that act throughout the body (26). Many actions of gonadal hormones are reversible, but some are long-lasting. For example, testicular hormones act on the brain prenatally to cause permanent changes (27). Possible long-lasting effects on the lungs have not been studied to date. Sex-steroid signaling has profound effects in the pathophysiology of lung diseases (28). The sex chromosomes can also act directly on nongonadal tissues, including the lung, to cause sex differences (29–31). One goal is to differentiate the effects of gonadal hormones and sex chromosomes in driving sex differences in lung disease, with the aim of identifying molecular pathways that could serve as therapeutic targets.

Gonadectomy. The investigator wishing to determine the factors causing sex differences in disease phenotypes in mice should start by comparing wild-type males and females to document the direction, size, and parameters of the sex differences when all sex-biasing factors operate. If a sex difference is detected at baseline or after the disease, the first logical step might be to search for hormonal effects. Manipulating hormones is often easier than manipulating sex chromosomes and requires no special genetic models (*see below*). The first experiment might be to remove gonads to see if that reduces or abolishes the sex difference. If it does, then replacing the gonadal hormone(s) will test which are responsible for the hormone effect. Treating gonadectomized animals of both sexes tests if the hormones have the same effect in the two sexes.

First, gonadectomy (GDX) should be performed by removing the ovaries in females to suppress E2 and progesterone secretion and orchidectomy in males to suppress testosterone secretion. Because GDX surgery affects stress hormones, a sham procedure should be performed in control animals. Off-target effects of GDX (e.g., metabolic impairments in females) need to be considered as a potential bias (32). In females, apoptosis of oocytes can be induced with 4-vinylcyclohexene diepoxide, making this a potentially more physiologically relevant model of menopause than GDX (33–35). If gonadectomy eliminates or reduces the sex differences, hormone replacement therapy in GDX animals should be performed next to assess which sex-specific hormones are mediating the gonadal hormone effect. Special attention should be given to the dose and duration of the sex hormone treatments to ensure that the physiological concentration of the sex hormones is achieved similarly to the intact male or female animals (using accurate and validated assays for sex hormone measurement) (8, 36, 37). Subcutaneous osmotic pressure minipumps (36) or continuous-release pellet (37) implants have an advantage over injection as they assure constant release of exogenous sex hormones. However, it should be noted that a continuous release of sex hormones does not mimic the cyclical and pulsatile levels of sex steroids that occur in intact animals. For example, if ovareictomy (OVX) eliminates a sex difference, to examine if E2 treatment is sufficient to restore the sex differences observed in females versus males, at least three groups of animals should be used: *1*) intact females; *2*) OVX females treated with vehicle; and *3*) OVX females treated with E2. Similarly, if removing the testes reduces sex differences, then the investigator might treat GDX mice with testosterone or a placebo to

determine if testosterone mimics the effect of testes. Once the effect of the specific sex hormone (e.g., E2) is identified, the subsequent experiment can be designed to examine the role of the main estrogen receptors (e.g., ERα, ERβ, and GPER) using selective gain- or loss-of-function strategies for each receptor. Sex hormone receptor agonists and antagonists are widely available but are not always completely specific for the targeted receptor (37, 38). Gene-targeting approaches may, therefore, be preferable if the experimental question allows. Levels of relevant sex steroid receptors in the target system and sex steroid levels should be measured after sex steroid receptor manipulation interventions. Comprehensive strategies for the design of preclinical studies focused on sex differences are reviewed in detail in References (39) and (40).

If gonadectomy does not eliminate the sex difference of interest, the investigator concludes that the sex difference was either caused by gonadal hormones acting at time points before the gonadectomy was performed or by sex chromosome effects outside of the gonad. The investigator can consider manipulating hormone levels at earlier ages to study the long-lasting effects of gonadal hormones.

Investigating Effects of Gonadal Hormones and Sex Chromosomes

The four core genotypes (FCG) mouse model (41) offers advantages for determining if the XX versus XY sex chromosome complement has a differential effect on the phenotype, partly because the FCG model also tests if gonadal hormones contribute to the sex difference. In the FCG model, the *Sry* gene responsible for testis development has been relocated from the Y chromosome to an autosome. It is the most widely used method for distinguishing the effects of sex chromosome complement from gonadal influences. By comparing mice with the same gonadal type but different sex chromosomes, the model highlights the impact of sex chromosomes. Conversely, comparing mice with identical sex chromosomes but different gonads will uncover the effects of gonadal hormones. Investigators should be aware of the caveats when using the FCG mice pertaining to the genetic background, differences in *Sry* expression between the transgene and the wild-type gene, and differences in hormonal status between mice with the same gonads but different chromosomal sex. A detailed discussion of these factors has been reported in prior publications (42). Panten and colleagues reported the translocation of a 3.2-MB region of the X chromosome to the Y Sry-chromosome in the FCG mouse strain (43). A new FCG strain (Strain number: 039108) does not carry the translocation.

The FCG model also detects interactions of gonadal hormones and sex chromosomes—for example, if the hormones have different effects in XX and XY mice. Many sex differences in phenotypes are found to be influenced by sex chromosomes, which are also affected by gonadal hormones. If gonadal hormones contribute to the sex difference, the investigator can better understand the hormonal regulation by determining the receptors that mediate the effect (e.g., estrogen receptors, androgen receptors), the cellular sites of action of the hormone(s), and the downstream molecular pathways that are regulated by the hormones that cause the sex difference in phenotype. Estrogen-mediated effects due to generation in the lung versus ovary should be considered. For example, cell-specific aromatase expression may be considered in the study design. Conversion of androgens to estrogens by the enzyme aromatase (CYP19A1), expressed in lung tissue, may play a role in lung diseases (44–46). Local estrogen synthesis may be modulated by either gonadal hormones or sex chromosomes (47–49).

If a sex chromosome effect is detected using the FCG model, the investigator can use the XY* mouse model to determine if the effect is caused by X or Y genes. XY* mice possess an abnormal pseudoautosomal region on the Y chromosome, leading to atypical recombination with the X chromosome (50). When XY* males are mated with XX females, they produce offspring comparable to XX and XO females with ovaries and XY and XXY males with testes. In the XY* model, the presence of a Y chromosome results in gonadal males. The XY* model compares mice with one versus two X chromosomes (XO vs. XX, XY vs. XXY) and mice with zero versus one Y chromosome (XO vs. XY, XX vs. XXY). The investigator may then manipulate the expression of specific X or Y candidate genes that mediate the sex chromosome effect discovered with the FCG model (51).

Downstream molecular pathways regulated by the X- or Y-linked genes can then be elucidated. Multiple sex chromosome factors may influence the phenotype, such that the XX versus XY comparison reveals the balanced effects of multiple factors. If both gonadal hormones and sex chromosome factors modify the phenotype, further studies manipulating both factors at once can uncover the molecular mechanisms of their interaction. Figure 1 outlines the logic tree for an investigator who is setting out to delineate the mechanisms behind sex differences in an experimental phenotype of lung disease.

Sex Differences in Lung Diseases Across the Lifespan

Sex and gender play a crucial role in the susceptibility, pathogenesis, and outcomes of lung diseases. It is important to match the sex of the animal used in the preclinical model to the sex predilection of the human disease. The sections below summarize key concepts for researchers investigating lung diseases during specific ages or experiments investigating the long-term impacts of early-life exposures.

Study of disease phenotypes across puberty. Differential lung disease manifestations can occur before and after puberty (52). When choosing an animal model, it is essential to consider the differential timing of pubertal onset between sexes and across species. In rodents, early markers of puberty are vaginal opening, first vaginal cornification, and the onset of cyclicity in females, whereas balanopreputial separation is used as a marker in males (53). In C57BL/6 females, vaginal opening occurs at 25 days of age on average, whereas in males of the same strain, balanopreputial separation occurs between 27 and 28 days (54). The peripubertal period in mice is generally considered 28–40 days for females and 30–40 days for males (55).

When designing experiments across puberty, researchers should consider that rapid changes in body mass can account for differences in lung-related phenotypes (56). These differences are important for dosing while administering drugs, treatments, or anesthesia (57). Similarly, equipment needs may vary depending on the age of the animal. For example, lung function measures using a rodent ventilator will require adjusting ventilation parameters according to the mouse's age and size. Similarly, treatments and interventions such as gonadectomy should consider the timing relative to pubertal development and account for potential changes in body composition that may indirectly affect lung function and

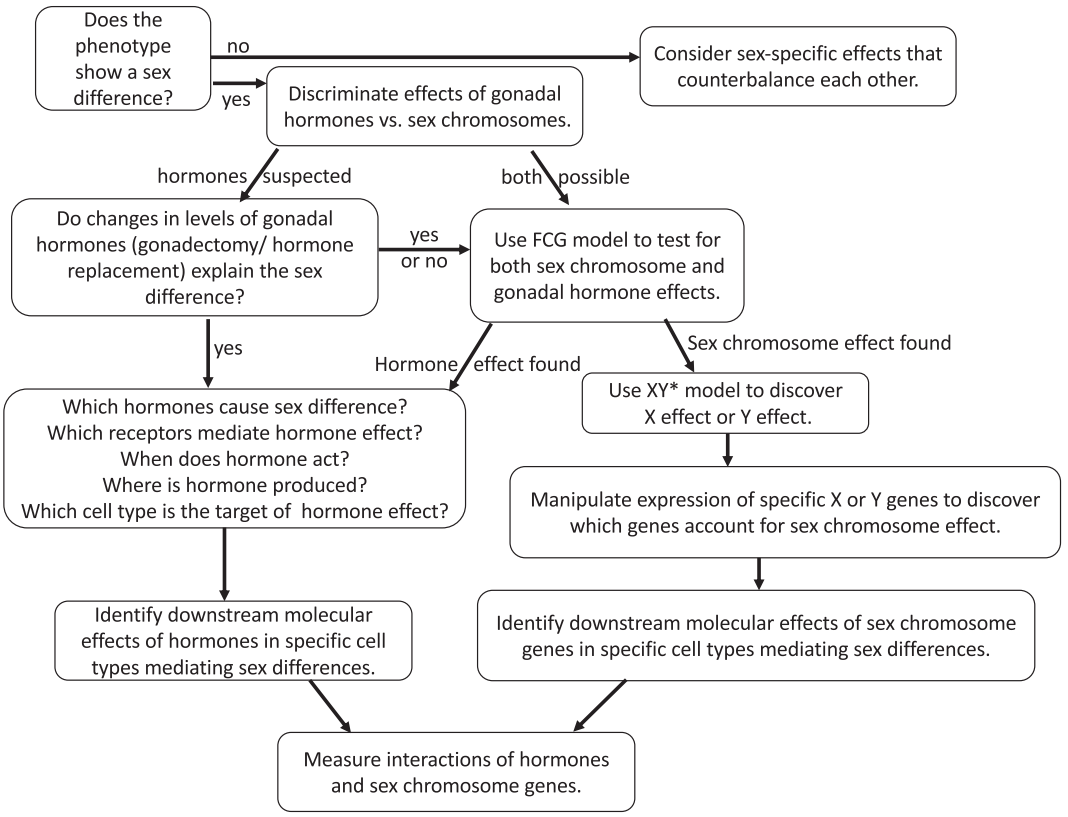

Figure 1. The logic tree for an investigator who is setting out to delineate the mechanisms behind sex differences in an experimental phenotype of lung disease. FCG = four core genotypes.

other measurements. Although gonadectomy is more commonly performed after puberty, some researchers have conducted the surgery by 3–4 weeks of age. It is important to note that gonadectomy before and after puberty produces different phenotypes in males and females, including body weight and composition changes, which could impact lung phenotypes (58).

Study of sex differences in lung diseases influenced by aging. Aging is a complex biological process marked by distinct hallmark features that accumulate over a person's lifespan, influencing potential disease risks. The aging process varies significantly between individuals, with notable differences between men and women. In both human and preclinical studies, evidence for sex differences in the biology of aging may result from differences in gene expression (59, 60), the immune system (61), mitochondrial biology (62–64), proteostasis (65), telomeres (66, 67), and senescence (68). Growth factors can stimulate hormone receptors independent of the hormone in the lung, leading to pathological changes (69).

Lung aging is linked to molecular and physiological changes that lead to reduced lung function, impaired pulmonary remodeling and regeneration, and heightened vulnerability to acute and chronic lung diseases. Diseases impacted by aging include asthma, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis (IPF), and pulmonary arterial hypertension (70). Interestingly, mechanisms such as mitochondrial dysfunction, increased oxidative stress, and telomere shortening related to pulmonary pathophysiology associated with aging are different in the male and female sex (71).

Menopause in women is associated with many biological changes, significant hormonal changes, and secondary changes in physiological and cellular functions. Lung function (particularly FVC) declines more rapidly among postmenopausal women (72). Sex-specific and age-related differences in immunophenotypes, hormonal landscapes, and molecular determinants may contribute to pulmonary pathophysiology (73). In summary, designing experiments with *a priori* attention to SABV is crucial for investigating aging-related lung diseases. Animal models and experimental paradigms outlined in previous sections should also be considered by researchers to address the role of SABV.

Study of the impact of early-life exposures modified by sex across the lifespan. Recognizing SABV is crucial in studies of early-life exposure. Research involving animal models examining the effects of early-life adversity on lung disease often highlights significant differences between males and females. This growing body of evidence suggests that males and females may respond differently to early-life challenges, including lung development and responses to environmental exposures (74, 75). To account for this, researchers must assess both sexes in the design and statistical testing designed to accurately identify sex-specific effects of early exposures on organ development and subsequent health outcomes and to test the statistical interaction between sex and early-life environments explicitly.

The timing of early-life exposures may also impact sex differences in lung outcomes (52, 76). Study designs may, therefore, examine if males and females are differentially affected by exposures occurring at specific developmental time points or if these effects on lung development emerge at different times in males versus females. Similarly, the persistence of these effects into adulthood may differ between males and females (77).

Researchers examining the potential mechanisms underlying sex differences in early-life exposure effects should also consider hormonal, chromosomal, and epigenetic influences. Sex hormones such as estrogen and testosterone can differentially affect lung development and responses to early-life stressors (78–81). On the other hand, sex chromosome genes may contribute to sex differences in susceptibility to lung disease triggered by early-life exposures (30, 82–91). Finally, early-life exposures may induce sex-specific epigenetic modifications affecting lung development and health outcomes across the lifespan (92). Recognizing how environmental exposures affect each sex differently is essential for evaluating environmental risks, shaping treatment guidelines, and uncovering new biological insights. Sex hormones and sex-specific occupational exposures may modify susceptibility and pathophysiology of lung diseases (93–95).

Key takeaways for consideration of SABV in *in vivo* models are summarized in Figure 2.

Research Recommendations for *In Vivo* Models

- Consider the stage of the estrous cycle, the timing of experimental interventions, circadian rhythms, endpoint assessments, dietary factors, use of tamoxifen, strain differences, and environmental factors that can lead to or confound sex-based differences in endpoints and phenotypes.
- Consider the organizational and activational effects of gonadal hormones. Consider local production of sex steroids in the lung as a possible modulator of outcomes.
- Consider the role of sex chromosomes in contributing to sex-biased findings in lung diseases.
- Consider the differential sex-biased effects of puberty, menopause, and aging on animal models of lung disease.
- Consider the appropriate experimental design and power to accurately and transparently report findings based on biological sex.

Section 2: Integrating and Investigating SABV in *In Vitro* Lung Models

The goals of this section include:

- Highlighting the importance of ascertaining cellular sex and the effect of cellular sex on phenotype and function. Emphasize the culture-related variables

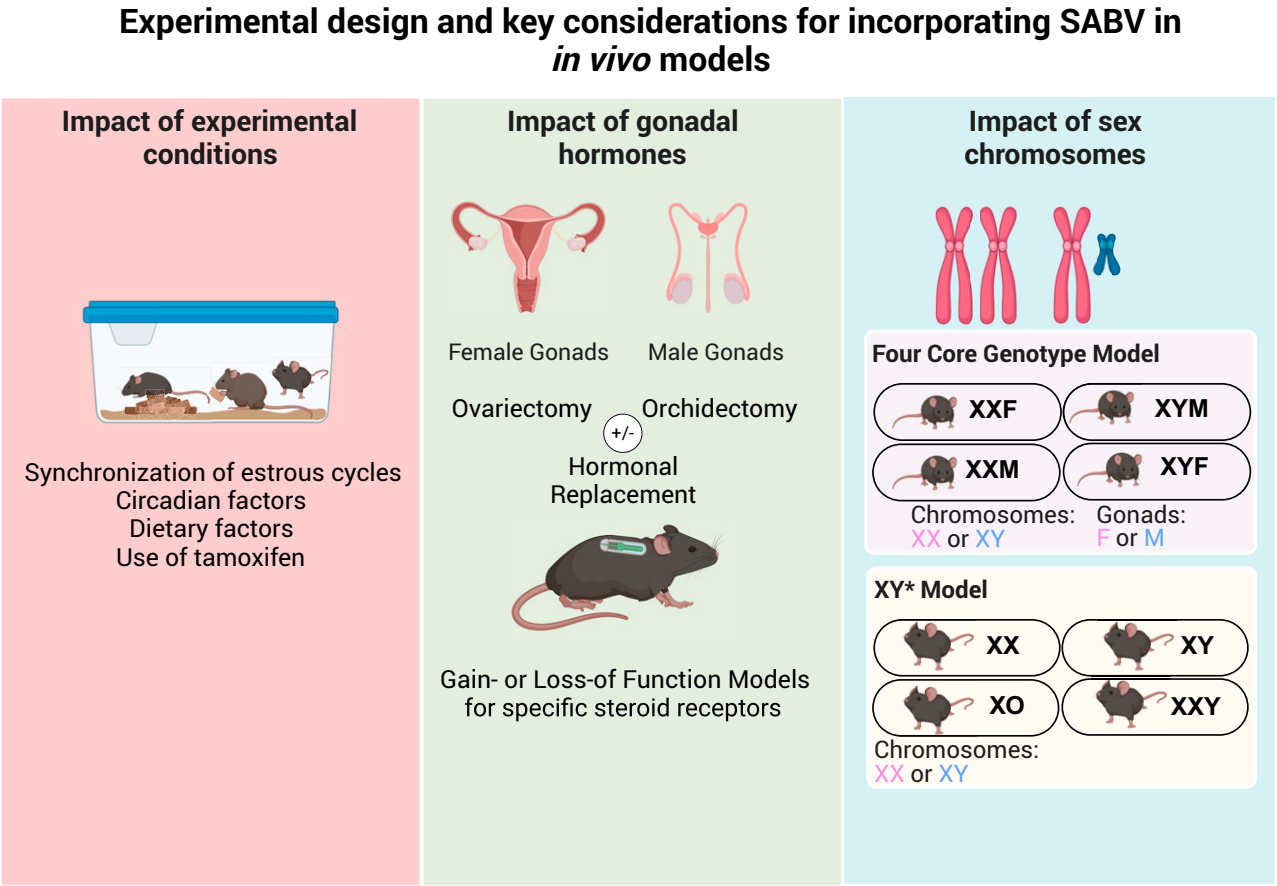

Figure 2. Experimental design and key considerations for incorporating SABV in *in vivo* models. SABV = sex as a biological variable. Created in BioRender. Lingappan K. (2025) https://BioRender.com/o51u227.

that could impact the differential effects of cellular sex.
- Consideration of biological sex of the source of tissue in human tissue–based translational models and consideration of SABV in multicellular complex 3D models.

In vitro lung models, including 2D and 3D cell cultures, organoids derived from primary or pluripotent stem cell cultures, and lung-on-a-chip systems, provide invaluable tools for investigating the molecular mechanisms driving chronic lung diseases (96, 97). However, these models often fail to account for sex-specific biological responses, leading to incomplete or biased data that may not translate to real-world clinical outcomes. Incorporating SABV into routine and advanced *in vitro* lung models is essential for translational research to understand sex-based differences in lung biology and pathology. When integrating male and female cell lines, cell-based models can reveal differential gene expression and functional responses. Organoids and lung-on-a-chip technologies will allow us to study sex-specific microenvironmental cues in detail.

The biological sex of cells plays a critical role in determining their phenotype and function. Frequently used lung cell lines for *in vitro* studies of various lung diseases are summarized in Table 2. These include epithelial, fibroblast (FB), and immune cell lines, with details on the name of cell line, cell type, source, sex of the donor, and age. Additional details about these cell lines are included in Table E1 in the online supplement. Cells derived from males and females differ in sex chromosomal complement and responses to environmental stimuli, growth factors, and disease. These sex-based differences manifest in various ways, including differential gene expression, immune responses, and susceptibility to injury or disease in lung tissues. Failing to account for cellular sex in experimental designs can lead to incomplete or inaccurate conclusions, limiting the generalizability of findings. If cells are isolated from animals or humans, the donor's sex should be specified. Ideally, the sex of primary cell lines obtained from commercial sources should be confirmed. If the sex of the cells under study is unknown, assessing for the presence of X and Y chromosomes or X- and Y-chromosome–encoded genes (e.g., *SRY*) is an easy way of deciphering the sex of the cells under investigation. If the functions of genes on the X- or Y-chromosome are being studied, the above-mentioned factors and consideration of escape from X chromosome inactivation (XCI) may become significant. XIST expression may be increased in certain male cancers and lung diseases, such as pulmonary arterial hypertension (98, 99).

Several factors related to cell culture can further influence sex-specific differences in phenotype. Variables such as isolation techniques, culture conditions, subculturing duration, and the timing of measurements can introduce variability that masks or amplifies sex-based differences. Prolonged passaging may also lead to cellular senescence or altered gene expression and (in the case of pluripotent stem cells) variable X chromosome inactivation or reactivation, thus differentially affecting male and female cells. Therefore, carefully controlling these variables is crucial for accurately interpreting sex-based differences in *in vitro* studies. In human tissue–based translational models, the biological sex of the tissue donor must be considered, as sex differences extend to tissue architecture and responses to injury. Incorporating SABV is equally important in complex 3D models like organoids and multicellular coculture systems, which simulate the physiological microenvironment of the lung. For instance, female-derived lung organoids may exhibit different repair mechanisms than male-derived counterparts, highlighting the need to include both sexes in lung disease modeling. By systematically integrating SABV into *in vitro* lung models, researchers can uncover critical sex-based differences that may influence disease progression and drug efficacy. These findings could inform the development of more personalized and effective therapeutic interventions tailored to the needs of both sexes. In the sections below, we highlight the key sex-based differences in major lung cell subpopulations.

Consideration of SABV in Cell-based Model Systems

Mesenchymal cells and smooth muscle cells. Sex differences in airway diseases manifest throughout the lifespan, influencing airway

Table 2. Commonly Used Cell Lines as *In Vitro* Models of Lung Disease

Cell Line or Source	Cell Type	Sex of Donor and Age
A549 (ATCC)	Epithelial cell	Male (White, 58 yr)
BEAS-2B (ATCC)	Epithelial cell	Male
NCI-H1299 (ATCC)	Epithelial cell	Male (White, 43 yr)
Calu-3 (ATCC)	Epithelial cell	Male (White, 25 yr)
NCI-H441 (ATCC)	Epithelial cell	Male (30 yr)
NCI-H292 (ATCC)	Epithelial cell	Female (Black, 32 yr)
16HBE14o- (SIGMA)	Epithelial cell	Male (1 yr)
MLE12 (ATCC)	Epithelial cell	Female FVB/N strain (5-mo-old mouse lung)
WI-38 (ATCC)	Fibroblast	Female (3-mo-old embryo)
MRC-5 (ATCC)	Fibroblast	Male (White, 14-wk-old embryo)
HLF1 (ATCC)	Fibroblast	Male (White, 13-wk gestation fetus)
IMR-90 (ATCC)	Fibroblast	Female (White, 16-wk gestation fetus)
RLE-6TN (ATCC)	Alveolar cell type II	Male Fischer 344 rat lung (56 d)
MH-S (ATCC)	Alveolar macrophage	Male BALB/c (7-wk-old mouse lung)
RAW 264.7 (ATCC)	Macrophage	Male BALB/c (adult)
THP-1 (ATCC)	Monocyte	Male (1-yr; peripheral blood)
HL-60 (ATCC)	Promyeloblast	Female (White, 36 yr; peripheral blood)

Definition of abbreviation: ATCC = American Type Culture Collection.

reactivity and remodeling processes (proliferation, fibrosis), which involve airway smooth muscle (ASM) cells and FBs. In adults without asthma, ASM cells from females show comparable Ca^{2+} responses to bronchoconstrictors at baseline compared with males but exhibit heightened responses under inflammatory or asthmatic conditions (100). In addition, FBs from adult females produce more cytokines than those from males (101).

Neonatal male mice exhibit greater baseline ASM proliferation and collagen deposition, exacerbated by hyperoxia, highlighting SABV in sensitivity to perinatal insults that contribute to neonatal asthma (102). Fetal androgens support airway branching in males and increase ASM presence and airway thickness (103). Human fetal ASM and FBs isolated from 18- to 22-week fetal lungs serve as relevant models to study intrinsic SABV and the effects of perinatal insults during critical airway development. These models reveal sex differences in ASM's sensitivity to oxygen and regulatory mechanisms like clock genes (102) and antioxidants (104). Because of potential chromosomal abnormalities in spontaneous abortions, chromosomal analysis is essential to confirm their relevance to normal perinatal airway biology.

In adults, sex differences in ASM and FBs are influenced by sex steroids and their receptors, which are critical to asthma progression at life stages such as puberty, pregnancy, menopause, and aging, as well as in hormonal dysregulation seen in obesity. Both male and female ASM cells express estrogen receptors (105, 106), with females exhibiting stronger $[Ca^{2+}]_i$ responses to estrogen. Estrogen receptors also differentially affect extracellular matrix production by ASM, particularly in females with asthma. In addition, ASM cells from both sexes express androgen receptors, with notable expression in individuals with asthma, and both respond to testosterone $[Ca^{2+}]_i$ levels (100). In pulmonary fibrosis, sex differences in airway thickness and fibrosis suggest SABV in FB biology, with estrogen showing reciprocal interactions with profibrotic factors. Estrogen receptor (ERα) was increased in male IPF myofibroblasts, and their activity contributed to fibrosis (69). This underscores the investigation of sex-steroid receptors in both sexes.

Immune cells. Sex is frequently overlooked in lung immunological research despite significant sex biases in various lung diseases (107). Evidence of sex differences in the immune system spans many species, indicating that this may be an evolutionarily conserved trait (108). Although mechanisms driving these sex differences remain inadequately characterized, evidence suggests that both sex chromosomes and gonadal hormones modulate immune cell functions (109–111). Genes on the X chromosome also play a significant role in immune regulation and contribute to sex differences in immune-related diseases. In contrast, Y chromosome polymorphisms can influence susceptibility to viral infections (112). Further exploration of sex-based chromosomal differences, alongside hormone-mediated functional studies, is needed to elucidate these mechanisms fully.

Some immune sex differences are consistent across the lifespan, whereas others emerge after puberty and diminish with reproductive senescence, implicating both genetic and hormonal influences (109). Transcriptomic and functional studies, such as those by Gupta and colleagues, reveal that young adult females have neutrophils with a more activated profile than males, characterized by heightened type I IFN activity and proinflammatory responses influenced by sex hormones (113). In contrast, Aomatsu and colleagues found that male neutrophils release more TNFα and exhibit more significant MAPKs (mitogen-activated protein kinases) and PI3K (phosphatidylinositol 3-kinase) activation in response to LPS. Male neutrophils and macrophages also show higher TLR expression and lower phagocytic capacity than females (114). Molloy and colleagues demonstrated that female sex hormones delay neutrophil apoptosis and increase reactive oxygen species production in females (115).

Sex has been suggested to influence macrophage differentiation, and sex hormones have been shown to modulate gene expression and macrophage proliferation directly (116–119). Sexual dimorphism significantly impacts the phenotype and function of tissue-resident macrophages, influencing their roles in immune surveillance, inflammation, and disease pathology (120–124). Studies have shown that male and female macrophages differ in gene expression profiles and responses to inflammatory stimuli. Furthermore, sex differences extend to macrophage responses in pathological conditions (121, 123, 124), with male macrophages showing a higher propensity for migration, inflammation, and tissue remodeling.

The balance between the pro- and antiinflammatory roles of macrophages is critical in the progression of lung injury. Biological sex influences alveolar macrophage behavior and programming, potentially impacting the pathophysiology of various lung diseases differently in males and females. Studies have shown that alveolar macrophages from female mice express greater levels of immunomodulatory genes, suggesting a sex-dependent variation in macrophage polarization (125). In a group B streptococcal–induced pneumonia model in mice, male alveolar macrophages had a greater proinflammatory phenotype than females (126).

Endothelial cells. Sex differences in endothelial cell physiology are shaped by hormonal and nonhormonal factors, including genetic and chromosomal mechanisms, contributing to sex-specific vulnerabilities in cardiopulmonary diseases (127). Studies consistently demonstrate that endothelial cells retain sex-specific functional, growth, and stress response biases, even in low-passage cultures or in the absence of sex hormones, emphasizing the significance of intrinsic sex differences (128).

For instance, female neonatal pulmonary microvascular endothelial cells exhibit enhanced migration, angiogenesis, and resilience to hyperoxia compared with male cells. In contrast, male cells show greater susceptibility to endothelial-to-mesenchymal transition under hyperoxic conditions, contributing to their higher vulnerability to bronchopulmonary dysplasia (85). *In vitro* experiments with neonatal human pulmonary microvascular endothelial cells, both in standard and hormone-free media, confirmed that biological sex modulates endothelial cell function (85, 129–131). Similarly, female pulmonary artery endothelial cells (PAECs) demonstrate greater proliferative responses mediated by intersectin-1–driven activation of p38–ELK1 signaling and long noncoding RNA Xist, which regulates genes such as ELK1 and KLF2, potentially explaining the sexual dimorphism observed in diseases like pulmonary arterial hypertension (132). The transcriptome and the secretome of the human pulmonary endothelium are distinct by sex (133, 134). Sex hormones modify several aspects of PAEC and lung microvascular cell function

(e.g., angiogenesis, regulation of vasomotor tone, proliferation) in health and disease (36, 135–137). Genes on the X chromosome and the long noncoding RNA *Xist* may also modulate PAEC proliferation (132).

Epithelial cells. The lung epithelium, the first line of defense against environmental insults and pathogens, exhibits significant sex-related differences in function and response to injury. These differences, influenced by hormonal variations and sex chromosomes, contribute to the observed disparities in respiratory disease susceptibility and severity between males and females. A meta-analysis of lung single-cell RNA sequencing studies found that ACE2 and TMPRSS2, proteins essential for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry into host cells, are more highly expressed in males, particularly in airway secretory cells and alveolar AT2 cells (138). In acute respiratory distress syndrome, males exhibit greater shedding of the alveolar epithelial glycocalyx, a layer critical for surfactant function (139). Similar differences have been reported in the neonatal lung (88, 89, 140). Although additional studies of alveolar epithelial cells have revealed sex-specific differences in alveolar fluid transport, oxidative stress responses, and ozone-induced inflammation, in general, the impact of SABV on alveolar epithelial type 2 and type 1 cells remains understudied (141–144). Regarding the airway epithelium, sex steroids also impact ciliary function (145), with additional studies of sex-specific differences studied in the air–liquid interface culture model, detailed below. Exposure to cigarette smoke incites a distinct transcriptomic and proteomic response from male and female lung epithelial (146–148) cells. The influence of sex hormones on lung epithelial function is evident in studies investigating the effects of androgens and estrogens (141).

Air–liquid interface culture models. Primary human airway epithelial cell cultures are widely used to assess airway responses to various stimuli, gaining popularity because of ease of growth, versatility for physiological and pharmacological manipulation, and flexibility to model exposures (e.g., viral infections, cigarette smoke, air pollution particles, etc.). The most straightforward systems are airway monocultures, which provide a quick and cost-effective method to study cellular responses. However, monocultures generally consist of basal cells, which do not fully represent the diverse cell types within the mature airway epithelium. Air–liquid interface models better replicate *in vivo* airway mucosa, typically comprising pseudostratified columnar cells, multiciliated cells, secretory (goblet) cells, basal cells, and rare cell types like tuft/brush cells, neuroendocrine cells, and pulmonary ionocytes.

Despite the utility of these *in vitro* systems, sex modifiers remain underexplored. Important sex differences in the epithelial ACE-2 expression (a receptor for SARS-CoV-2) (149) and proresolving lipid profiles between cells from male and female donors have been reported (150). Sex hormones also influence airway epithelial cell behavior: estradiol enhances mucus production, barrier function, and mucus flow through post-translational fucosylation of mucins, whereas testosterone may reduce mucus production, improve barrier function, and downregulate proinflammatory cytokine synthesis in airway epithelial cells (151). There is a clear need to evaluate sex differences in airway epithelial cells *in vitro* and to understand how sex hormones impact cell proliferation and maturation. Future studies should specify donor sex and document the hormonal composition of culture media to ensure accurate modeling of sex-based differences in 2D or 3D airway culture systems.

Experimental models with ESCs and iPSCs. When designing experiments using pluripotent stem cell model systems, such as ESCs or iPSCs, it is crucial to consider how donor sex can introduce variability in the cells' reprogramming, pluripotency, and differentiation (152–156). This variability is partly the result of variable X chromosome dosage and silencing (155, 156). A broad literature has established that two X chromosomes are initially active in female mouse blastocyst embryos *in vivo* and female mouse embryonic stem cells (mESCs) *in vitro* (Xa) (152–154). To adjust for X chromosome copy number, during early embryonic female development, one X chromosome undergoes transcriptional silencing (Xi) through a process known as XCI, initiated upon exiting the pluripotent state (157). Unlike mESCs, female human ESCs (hESCs) have been shown to exhibit variable XCI, with the majority of female hESCs in the undifferentiated state displaying one Xi with complete XCI (155, 158, 159). However, the epigenetic and silenced state of the Xi can vary between lines or even between passages of the same line, particularly with increasing time in culture, where *XIST* becomes irreversibly silenced and portions of the Xi become aberrantly reactivated (160).

Differences in XCI between mouse and human iPSCs (miPSCs and hiPSCs, respectively) have also been reported (152, 153, 155, 156). Reprogramming of mouse female somatic cells to generate miPSCs results in reactivation of Xi (152). Hence, most miPSCs resemble mESCs, with two active X chromosomes, one of which silences upon differentiation (152–154). In contrast, Tchieu and colleagues reported that reprogramming human female somatic cells, such as FBs, does not reactivate Xi, with the majority of female hiPSCs retaining complete XCI with preservation of the same active Xa and inactive Xi as their somatic cell of origin (155). Despite the lack of initial reactivation, this team found female hiPSCs after prolonged culture did not indefinitely preserve a pristine state of XCI (153, 155). Female hiPSCs/hESCs typically exhibit *XIST* expression, unlike mouse iPSCs/ESCs (161); however, subsets of female hiPSCs/hESCs lacking XIST expression also lack H3K27me3 and H4K20me heterochromatic modifications on the Xi (162) and exhibit aberrant X-linked gene expression (163).

Caution should be taken when using female pluripotent stem cell lines for regenerative medicine applications, as some hESCs/hiPSCs lacking XIST expression exhibit phenotypes of reduced differentiation potential (163, 164). In addition, variable XCI and potential X chromosome reactivation in female hiPSCs may complicate the application of hiPSCs for modeling human diseases, particularly when attempting to understand the role of sex-biased gene expression. For example, Topa and colleagues published a detailed study of X chromosome potential escape from XCI, derepression, and allele-specific gene dosage in 165 female hiPSC lines profiled in the undifferentiated state (156). They reported that female hiPSCs retain patterns of XCI shared with human tissues *in vivo*. However, there can be erosion of XCI in some lines with time in culture. Importantly, this resulted in hiPSC line-to-line variation in the number of depressed genes and their degree of biallelic expression. The authors reported that derepression was uncommon and nonrandom, favoring epigenetically variable genes prone to derepression in human tissues *in vivo*. Derepression of XCI,

occurring in a subset of the lines studied, could also impact expression from autosomes. Of relevance to those using hiPSCs for lung-directed differentiation via definite endoderm, Topa and colleagues also found that lines with XCI erosion exhibited slightly less efficient endodermal differentiation *in vitro*. However, line-to-line variation was very high in these experiments, leading the authors to conclude that factors other than XCI were likely responsible for these differences (156). These authors also emphasized that variations in sex chromosome–related gene expression in iPSCs can be powerful models to help understand the mechanisms and consequences of this varying gene dosage (156). This approach was pursued in one report using isogenic hiPSC lines with different sex chromosome complements to discover sex differences and their linkage to X versus Y chromosomes (165).

Taken together, variations between male and female ESC or iPSC lines and potential variability in XCI erosion and effects on gene dosage and/or expression in female hiPSCs necessitate careful monitoring of hiPSC study design and monitoring of *XIST* expression (156, 164). Also, it is important to reproduce key findings in multiple hiPSC lines, preferably of multiple genetic backgrounds and representing both male and female sexes (166).

3D model systems (organoids, lung-on-chip). 3D cell culture systems, including those derived from primary cells, ESCs, or iPSCs, aim to replicate lung tissue's histological and functional aspects in health and disease. Advances have been reported in investigating sex-specific differences using 3D models of the human brain (167), cardiac tissue (168), and reproductive tissue (169). The organoid platform of the lung has been rapidly developing over the last decade (170). Recent advances in lung organoid development and applications in disease modeling have been reviewed (171) in highlighting many benefits of using 3D organoids to shed light on the complexity of lung mesenchymal–epithelial interactions to model various aspects of lung biology (172) and diseased states, including chronic obstructive pulmonary disease, IPF, cystic fibrosis, lymphangioleiomyomatosis (173), and host–microbe interactions.

However, sex-specific differences using 3D lung organoids have not been well understood or experimentally addressed. Lung organoids derived from female iPSCs or ESCs may be uniquely impacted by variable X inactivation or reactivation in the starting stem cell preparations, as discussed in detail above, but these effects on resulting lung lineages have yet to be profiled. Although the biomimetic microsystem of lung-on-a-chip has been developed, the sex-specific differences remain to be addressed (174). Similarly, lung organoids modeling pulmonary fibrosis (175–179) have been reported without specifically addressing sex-specific differences.

Thus, key issues and challenges in 3D modeling of lung organoids and lung-on-a-chip remain. These include developing concrete and measurable sex-related variables in assessing lung function, structure, and response to injury and repair or therapeutic interventions. For example, there is a lack of data demonstrating the expression of estrogen and progesterone receptors and their ratio in male and female lung cell lineages in *in vivo* and *ex vivo* experimental models and understanding how those parameters change during the menstrual cycle and pregnancy.

Fundamental methodological limitations, including cost, lack of standardized methodology, relevance of nonhuman model systems, prolonged time frame for experimental comparative analysis, and inherent limitations of tools and/or techniques remain to be established, together with standardized methodology to systematically address sex-specific differences in lung organoids and lung-on-a-chip. To produce comparable experimental data requires an establishing a standard for male and female human (mouse) key lung cell lineages (lung FBs, alveolar epithelial and endothelial cells), including *1*) basic cell characterization and maintenance conditions; and *2*) identification, characterization, and monitoring the expression of estrogen and progesterone receptors and their ratios. Donor sex should be accounted for as an experimental variable during the generation of these models (180). In multilineage-type models (models incorporating different lung cell types), the sourced cells could be from male and female patients in these multicell models and thus behave like a chimeric model system. Cells sourced from the same sex to make multilineage models may be needed to study the role of SABV in these model systems. Sex chromosome regulation of cellular phenotype may warrant changes to bioengineering approaches to investigate the role of SABV in lung diseases (181–183). Researchers should document these experimental variables clearly in their methods and results.

Additional* in vitro *experimental design considerations. Given the influence of cellular sex and interactions with sex steroids, *in vitro* studies should confirm the cellular sex of the donor sources. Commercially sourced adult human cells often lack this information, necessitating laboratory verification and classification before conducting experiments. For cells derived from patient samples (e.g., bronchoscopy, surgical resections), detailed documentation of donor sex and age is essential. If institutional review board-compliant, cellular sex can be determined and verified from primary tissues and monitored for consistency across subcultures. Data on menopausal status, hormone therapy use, and pregnancy history in female donors further help put data derived from female donors into context. Such data should be collected in biorepositories.

In addition, cataloging sex hormone and receptor levels in cells may be important, as these cells contain pathways for local sex steroid production (184), which can create autocrine or paracrine effects *in vitro*. Culture media can inadvertently affect steroidal pathways (e.g., phenol red as a proestrogenic agent (185, 186) and growth factor interactions, as seen with neurotrophins and estrogens. Therefore, culture media should be formulated to control for hormonal and growth factors, as informed by current literature (187–189). Serum-free and charcoal-stripped media allow for studying cellular behavior in low- or no-hormone environments (188, 189).

To balance SABV considerations with practical laboratory needs, one approach could involve the research community performing a targeted set of experiments using human lung cells across the age spectrum in carefully curated media. Such studies would establish a reference platform with genomic, proteomic, and functional data, enabling reliable exploration of SABV without dismissing prior foundational research. Key takeaways for consideration of SABV in *in vitro* models are summarized in Figure 3.

Research Recommendations for *In Vitro* Models

- Verify and document the cellular sex of commercially sourced cell lines and primary cells before conducting

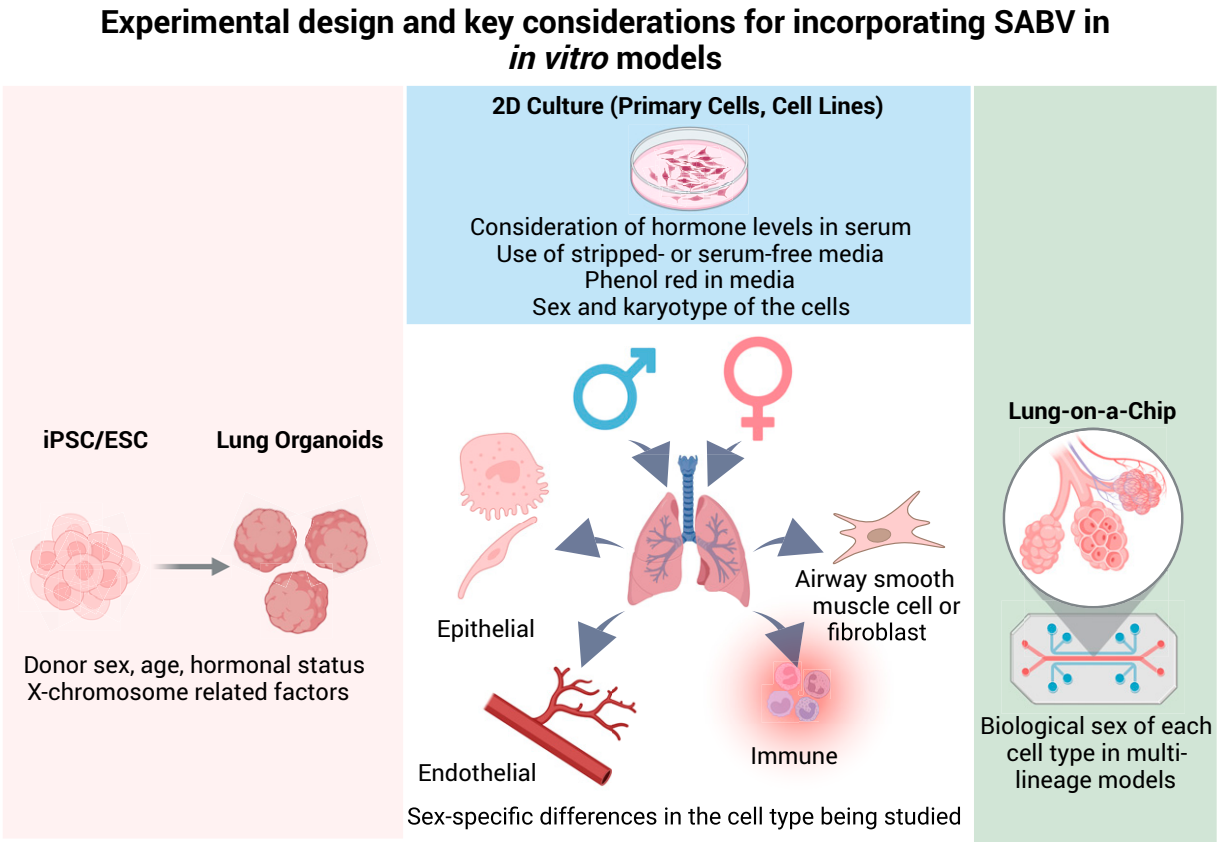

Figure 3. Experimental design and key considerations for incorporating SABV in *in vitro* models. SABV = sex as a biological variable. Lingappan K. (2025) https://BioRender.com/w19b887.

experiments. If cells are derived from patient samples, report the donor's sex, ethnic background, age, menopausal status, hormone therapy use, and pregnancy history. These data help researchers contextualize findings from female donors and should be collected in biorepositories.

- Document culture conditions and avoid media components that can activate sex steroid receptor signaling. Use phenol red–free and charcoal-stripped media if experimental conditions allow.
- Consider the potential autocrine or paracrine effects of sex steroids. Catalog sex hormone and receptor levels in cells. Culture media should be formulated to control hormonal and growth factors, as some media components can affect steroidal pathways and growth factor interactions. Replicate sex-specific physiologic conditions.
- Consider how donor sex can introduce variability in the reprogramming, pluripotency, and differentiation in models using iPSCs and ESCs.
- Consider the sex of cells when building complex 3D models, recognizing the increased potential for sex-based variability.

Statistical Design, Analysis, and Reporting

Power Analysis

Researchers should determine minimum group sizes and sex allocations based on primary outcomes and effect sizes from preliminary studies. Ideally, an equal number of male and female biological replicates should be used; at a minimum, the sex of the biological replicates should be recorded *a priori*. Preliminary findings can guide the use of two-way ANOVA to assess sex as an independent variable and its interaction with other factors. In addition, incorporating existing sample sizes and power analyses will aid in designing future studies focused on sex differences. Reporting effect size, statistical power, and predicted sample sizes for each sex is essential for accurately calculating the necessary sample size to examine SABV (190).

To ensure adequate statistical power for detecting sex-specific effects, sample sizes must be calculated separately for each sex and then combined, accounting for sex differences in disease incidence and effect size. The ideal study design for evaluating sex differences should be powered to detect statistically significant sex-by-treatment interactions. Notably, such a study requires a larger sample size than one designed solely to detect the main effect or sex alone (191).

In a preclinical study, investigating sex differences may not be the primary focus, and the sample sizes needed to detect sex differences may be unknown. In this situation, the researcher can be guided by "The **Four C's** of Studying Sex to Strengthen Science" outlined by the National Institutes of Health (178, 179): *1*) **Consider**—design studies that account for sex or justify its exclusion; *2*) **Collect**—gather sex-based data; *3*) **Characterize**—analyze sex-specific data; and *4*) **Communicate**—report and publish

Table 3. Major Recommendations for Incorporating Sex as a Biological Variable in Preclinical Models of Lung Disease

In vivo models
- Consider the stage of the estrous cycle, the timing of experimental interventions, circadian rhythms, endpoint assessments, dietary factors, use of tamoxifen, strain differences, and environmental factors that can lead to or confound sex-based differences in endpoints and phenotypes.
- Consider the organizational and activational effects of gonadal hormones. Consider local production of sex steroids in the lung as a possible modulator of outcomes.
- Consider the role of sex chromosomes in contributing to sex-biased findings in lung diseases.
- Consider the differential sex-biased effects of puberty, menopause, and aging on animal models of lung disease.
- Consider the appropriate experimental design and power to accurately and transparently report findings based on biological sex.

In vitro models
- Verify and document the cellular sex of commercially sourced cell lines and primary cells before conducting experiments. If cells are derived from patient samples, report the donor's sex, ethnic background, age, menopausal status, hormone therapy use, and pregnancy history. These data help researchers contextualize findings from female donors and should be collected in biorepositories.
- Document culture conditions and avoid media components that can activate sex steroid receptor signaling. Use phenol red–free and charcoal-stripped media if experimental conditions allow.
- Consider the potential autocrine or paracrine effects of sex steroids. Catalog sex hormone and receptor levels in cells. Culture media should be formulated to control hormonal and growth factors, as some media components can affect steroidal pathways and growth factor interactions. Replicate sex-specific physiologic conditions.
- Consider how donor sex can introduce variability in the reprogramming, pluripotency, and differentiation in models using iPSCs and ESCs.
- Consider the sex of cells when building complex 3D models, recognizing the increased potential for sex-based variability.

Definition of abbreviations: 3D = three-dimensional; ESCs = embryonic stem cells; iPSCs = induced pluripotent stem cells.

Table 4. SABV: Checklist for Preclinical Studies

☐ **Was available literature reviewed for the influence of biological sex?**
- Does SABV modulate the incidence, pathophysiology, severity, outcome, and/or response to therapy?
- Is there a clear articulation that the phenomenon or condition disease under study does or does not have a different incidence or prevalence based on sex or gender?
- Is there published literature describing known mechanisms explaining sex or gender differences, or lack thereof, in the research area under study?
- Are there sex-based differences in cell line or primary cell phenotype, including response to treatments, stress, and differentiation potential?

☐ **Was the influence of sex considered while formulating the research question?**
- If disease prevalence is skewed by sex, was SABV taken into consideration during formulation of the primary research question?
- Is a strong justification provided for studies or applications proposing to study only one sex?
- Is the goal to identify, explain (mechanisms), or study sex or gender as a confounder or interaction variable while testing the main study hypothesis?
- Please bear in mind: absence of data regarding sex differences in an area of research does not, by itself, constitute strong justification to study only one sex.

☐ **Was the influence of sex considered in the study design?**
- Were the effects of sex hormones, sex chromosomes, hormonal cycles, and reproductive stages considered and addressed?
- Were both male and female animals and/or cells *in vitro* model systems included in the study? Male and female animals should be strain- (or strain and genotype) and age-matched and reared under identical conditions (cages, bedding, diet). Donor sex and age should be reported for primary cells and cell lines.

☐ **Was the influence of sex considered in the study design?**
- Were there potential biological confounders (such as dietary phytoestrogens, tamoxifen, estrus cycle, phenol red, use of serum, special media, exogenous hormones) that may have affected experimental results?
- Did the study need to be powered taking SABV into account? Sex-based powering: tests hypothesis in both males and females and powers each to determine effect.
- Was a factorial study design adopted to address SABV in the study?

☐ **Were research findings appropriately generalized?**
- No sex-based effect should also be reported.
- To reduce publication bias, researchers should report when sex differences (main or interaction effects) are not detected or when data regarding sex differences are statistically inconclusive.
- Reporting null results is crucial for meta-analysis.

☐ **Was data analyzed and reported disaggregated by sex?**
- Are the terms "sex" and "gender" used correctly?
- Do the results report if sex differences are or are not detected in analyses?
- Are data disaggregated by sex (whether significant in effect or not), which may be valuable for future research and meta-analysis?

Definition of abbreviation: SABV = sex as biological variable.

sex-related findings. Even if adequately powered statistical analysis indicates that a phenotypic sex difference is absent, it remains possible that the disease phenotype may have sexually divergent underlying mechanisms.

Importance of the Factorial Design

In a 2 × 2 factorial design (192), each sex is represented within the two treatment or intervention groups. A factorial design is a simple yet powerful way to incorporate both sexes into a single experiment. Factorial designs incorporate at least two factors, with at least two levels each, so the experimental units incorporate all combinations. This design allows us to answer the following three questions: Does the outcome variable differ between treated and control groups? Does the outcome variable differ between males and females? Is there an interaction between biological sex and treatment or intervention?

Reporting Sex Differences

When reporting study findings, it is essential to provide specific information to ensure accuracy and minimize bias, as described in the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines (193). In the methods section, it is crucial to state the sex of biological replicates and how that was assessed, the respective sample size, and a justification of power ensuring the ability to detect sex-based effects. If available, researchers should include sex-specific biological variables, such as estrous cycle status, developmental stage (e.g., whether animals are pre- or postpubertal), and hormone levels. In addition, the choice and justification of the statistical analysis used to assess sex differences and interactions of sex with other variables should be provided. For *in vitro* studies, the sex of the cells used and the culturing conditions used (e.g., use of phenol red–free and/or charcoal-stripped media) should be documented.

The figures and tables in the manuscript should display all data disaggregated by sex (included in supplemental data), using distinct visual markers for male and female data points, including both significant and nonsignificant findings and effect sizes for sex differences. The description of results should also clearly outline the presence and absence of sex differences in measured outcomes and sex-stratified analyses, considering chromosome- or hormone-dependent mechanisms and sex-specific molecular pathways. Finally, the discussion section should place findings and conclusions within the context of sex-based biological mechanisms, specifying if findings apply to one or both sexes and acknowledging potential limitations on their applicability and relevance to both sexes. Table 3 summarizes the major recommendations for incorporating SABV in preclinical models of lung disease. A checklist (Table 4) for the inclusion and analysis of SABV in the model, which researchers and reviewers can use, has been included in the online supplement.

Conclusions

Females and males have variable manifestations of major acute and chronic lung diseases across the life course. Preclinical and *in vitro* models of lung diseases have been critical for understanding pathophysiology, but modeling sex differences in pulmonary processes has lagged and lacked rigor. Study designs need to include experimental approaches that support the disaggregation of data by sex and investigate the role of sex chromosomes and gonadal influences. Given the importance of inbred mice to lung disease research, careful cataloging of sex differences by inbred strain will create an invaluable resource for the lung community. Across all models, modeling the hormone milieu will more closely approximate the human system; this is an area of challenge and opportunity. Careful study design focused on powered analyses in a 2 × 2 factorial design may yield the most detailed data to address outcomes between treated vs. controls, males vs. females, and interactions between biological sex and interventions. This research statement has provided support that SABV needs to be considered in preclinical and cellular models of lung diseases. In addition to understanding lung pathobiology, modeling sex differences will motivate sex-aware approaches to therapeutic innovation. Standardized approaches to experimental design and reporting of sex differences are integral for accelerating the discovery for all pulmonary diseases. ■

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