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Factsheet Preclinical Research

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Supplementary: Set of slides for the factsheet Preclinical Research

Preliminary note: The factsheet Preclinical Research, like all other factsheets, presents individual gender differences as examples. It does not claim to be a complete representation of the issue. The Commission is aware of the different, subject-specific perspectives on gender/sex. All factsheets were discussed in the Commission Sex and Gender in Medicine of the Faculty of Medicine of the University of Zurich and approved in the following text. Responsibility for the content lies with the authors. This factsheet summarizes the main conclusions about the importance of sex as a biological variable (SABV) in preclinical research, clarifies the relevant terminology, and describes the methodological aspects that should be considered when factoring sex into *in vivo* and *in vitro* experiments.
Slide numbers refer to the corresponding slideset.

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Preclinical Research

1 Clarification of terminology

Sex refers to a set of biological factors in humans and animals, associated with physical and physiological features such as chromosomes, gene expression, hormone function, and reproductive anatomy, typically resulting in the binary classification of male or female, associated with the production of spermatozoa and oocytes, respectively[1].

Gender refers to the socially constructed roles, behaviors, expressions, and identities associated with women, men, and gender-diverse individuals. It shapes how people perceive themselves and others and influences their actions and interactions. Unlike sex, gender identity is not limited to a binary framework (woman/man) and is not static; it exists on a continuum and can change over time[1].

Sex and gender shape health independently as distinct factors, as well as interactively through the many ways in which they intersect and influence each other[2–4]. In brain research, observed sex differences in biological characteristics cannot easily be separated from gender[5]. Conditions like psychiatric disorders demonstrate this interplay. For instance, women are twice as likely as men to experience depression, with some women experiencing mood symptoms related to hormone changes during puberty, pregnancy, and perimenopause, illustrating the influence of sex as a biological variable[6]. On the other hand, women are more likely to report negative mood states and seek treatment for mental health issues, in contrast to men, which is directly linked to gender norms, directly influencing epidemiological data[7–9].

In addition, it is important to emphasize that environmental factors, through epigenetic changes, influence gene expression, providing a mechanism by which the genome can respond to environmental stimuli. This, in turn, can further shape an individual's phenotype, influencing their personal gender expression. Conversely, gender-related behaviors can also impact hormonal levels and cellular function, demonstrating a bidirectional relationship between sex and gender[3].

Therefore, both sex and gender are applicable when discussing humans, but gender is a trait unique to humans. While non-human animals are social beings, when referring to animal research subjects - particularly in studies involving rats and mice - the term "sex" should be used. Additionally, using the term "gender" in reference to *in vitro* assays, such as those involving cells and tissues, is incorrect. Therefore, in the following text, which focuses to preclinical animal research, the term "sex" will be used.

2 The neglect of sex within in vivo preclinical research

Sex is a fundamental biological variable that significantly impacts physiology, behavior, disease processes, and treatment responses. Although incorporating both sexes is essential for rigorous, reproducible, and responsible research, most preclinical biomedical studies have been conducted without consideration of the animals' sex[10]. This sex bias has been prominent across disciplines, with some fields making better strides than others[9,10]. Where two sexes were included, two-thirds of the time the results were not analysed by sex[9]. This highlights the ongoing need for increased awareness of the importance of sex as a biological variable and for improved guidance on study design and analysis.

3 Consequences of sex (male) bias in preclinical research and drug development

The bias toward using only one sex (typically males) in experiments, while generalizing findings to both sexes without valid evidence, has had particularly negative consequences in preclinical research and drug development[10,11]. Such practices have been a major obstacle to understanding sex-specific disorders and developing effective therapeutics tailored to individual patients[12]. Furthermore, the underrepresentation of both sexes in preclinical studies has been linked to a higher incidence of adverse drug reactions in one sex, likely due to insufficient understanding of sex-specific differences in pharmacokinetics and pharmacodynamics[13].

Even though it is frequently overlooked, conducting single-sex studies is not in line with the 3Rs (Replacement, Reduction and Refinement) principle of animal research for two main reasons: 1) single-sex

studies produce results that are not generalizable to both sexes, meaning animals may be subjected to harm and their lives wasted on inconclusive experiments, and 2) animals of both sexes are born, so using only animals of one sex results in the unnecessary euthanasia of surplus animals[14].

4 Sexual dimorphism in mouse phenotypic traits

Another confirmation that sex should be considered an important variable in the design and analysis of animal studies, regardless of research field or biological system, comes from a multicenter study conducted by the International Mouse Phenotyping Consortium (IMPC)[15]. In this study, IMPC researchers investigated the effect of sex by analyzing up to 234 physical characteristics of more than 50,000 mice (14,250 wild-type and 40,192 mutant from 2,186 single-gene knockout lines). The results showed that sex affected 56.6% of quantitative traits (including metabolic, cardiovascular, bone, behavioral, hematological, and blood clinical chemistry parameters) and 9.9% of qualitative traits (such as head, whisker, and paw shape and coat color), meaning that sex affected the outcome in 2/3 of the studied genes. Additionally, sex modified the genotype effect in 13.3% of qualitative and 17.7% of quantitative traits in mutant mice[15]. Importantly, these sex differences often arose unexpectedly, challenging the assumption that sex effects can be predicted in advance. This study demonstrates that sex differences are common in traits typically assumed to be the same between males and females, and shows that genetic modifications can affect the sexes differently, right down to the underlying genetics[15].

5 Common misconceptions in sex-inclusive research

When considering sex-inclusive preclinical research, two main misconceptions have been reported:
Misconception 1: *Designs that include both sexes require a doubling of the sample size to achieve the same power.* Recent meta-analyses have shown that, for most biologically expected situations (where treatment effects are similar across sexes or there is a baseline sex difference) there is no need to double the sample size[16]. Instead, the intended sample size can be divided between the two sexes for a treatment. Moreover, even when sex is included as an independent variable to formally test for sex differences, it is typically not necessary to use twice as many animals as studies involving only one sex. This is because factorial study designs (i.e., studies that assess the effects of more than one variable, such as treatment and sex) are statistically more powerful.
Misconception 2: *Circulating ovarian hormones make data from female animals more variable than data from males.* As a result, some researchers were concerned that, especially in acute studies, they would need four times the number of experimental animals to assess the outcome variable across the different stages of the estrous cycle, which would be both costly and time-consuming. However, two comprehensive meta-analyses in mice[17] and rats[17] showed that females were actually less variable than males across a range of outcome measures. Thus, in general, sex-dependent variability should be accepted as natural biological variability[18], and assessing the estrous cycle is not always necessary. This does not mean gonadal hormones should never be considered; rather, hormonal variability should be equally evaluated in both sexes based on its potential to influence experimental outcomes[18,19]. Moreover, considering the estrous cycle may enhance the precision of neuroscience studies by revealing hidden sex differences and providing mechanistic insights into identified differences across various neurobehavioral outcomes[20–22].

6 Inclusion of SABV in a single experiment: statistical design, analysis and reporting

Including sex as a biological variable (SABV) does not automatically require specific investigation of sex differences or larger sample sizes[16,23]. This is important because these are common arguments against incorporating females into preclinical studies.
When designing a new study, the default option should always be to start with an exploratory study where each experimental group is composed of both males and females in a 1:1 ratio. As the term suggests, exploratory studies are designed to explore the effect of one or more

factors (e.g., treatment, age, and sex) on one or more outcome variables. Because it is not meant to statistically test a hypothesis, a formal power analysis is not needed but data analysis should be limited to descriptive statistics [24]. Thus, with half males and half females, one may explore (e.g., graphically, descriptive statistics, etc.) whether the data suggest generally a sex difference (i.e., a sex main effect or a significant sex co-variate) and/or a sex difference in the treatment effect (i.e., an interaction between a treatment and sex).

If that is the case, a confirmatory study should be planned to formally test whether a sex difference is present. In this case, sex will become an independent variable (i.e., a fixed effect) and a formal power analysis[24] (there are a number of publicly available programs for conducting power calculations) should be conducted, ideally based on preliminary data or published effect sizes, to determine the adequate sample size. If no data are available, power calculations should be based on a best estimate of the minimum effect size that is considered biologically relevant[10].

Even if sex is included as an independent variable to formally test for a sex difference, in most cases it is not necessary to use twice as many animals compared to studies using only one sex[16,23]. The reason for this is that factorial study designs (i.e., studies in which the effects of more than one variable, e.g., treatment and sex, are assessed) are statistically more powerful[23]. Nevertheless, if the sex difference is very small but the study will be powered to test it statistically (because the sex difference is considered biologically relevant despite being small), a much larger sample size may be needed[23].

If a confirmatory study is statistically powered to detect a relevant sex difference but no sex difference is observed in the results, then it can be reasonably concluded that sex does not seem to influence the outcome measure under these experimental conditions[10]. However, such an outcome does not mean that one should revert to single-sex studies in future experiments. Instead, both males and females should be equally included and treated as a random effect (or blocking factor)[10].

It is worth noting that after implementing the SABV policy, laboratories with existing research focused solely on males may encounter logistical challenges when expanding their studies to include females. In such cases, it is crucial to include a "validation" subgroup rather than directly comparing results between sexes[25]. For instance, if historical data from male subjects indicate certain effects, the new study should replicate these conditions with both male and female subjects, including a validation subgroup of males. By comparing the results of this new male subgroup with the historical data, researchers can confirm consistency. Only after validating that the new male data aligns with the historical data can researchers confidently compare findings between sexes[10].

7 Four types of sex differences

Correctly reporting observed sex effects is crucial for guiding the design of subsequent experiments and for interpreting findings. Four operational categories of sex effects should be used when reporting sex differences in studies:

1) Qualitative Differences: This refers to traits exhibited differently by males and females or traits present in one sex but absent in the other. Examples include the primary sexual organs like testes and ovaries, as well as reproduction-related behaviors like maternal aggression, and male-specific courtship[26].
2) Quantitative Differences: This occurs when an the outcome measures exist along a continuum in both sexes, but the mean value differs between males and females[27]. Examples include body weight, immune responses, stress and anxiety responses, and pain thresholds.
3) Population Differences: This category describes variations in the incidence or distribution of an endpoint between sexes. For instance, in cocaine addiction studies, more females (50%) choose cocaine over palatable pellets compared to males (16%), though their behaviors during cocaine use do not differ[28].
4) Convergent Sex Differences: This refers to endpoints that are similar in both sexes but arise from different underlying mechanisms[29]. For example, estradiol enhances excitatory synaptic transmission in both male and female hippocampi. Despite similar increases in synaptic strength, the mechanisms involving cAMP-regulated protein kinase, internal calcium

stores, and L-type calcium channels differ between sexes[30].

8 Investigating the biological source of sex-based differences

When studying sex-based differences, it is essential to identify their sources, which include: 1) sex hormones´actions during early postnatal development and adulthood, and 2) the sex chromosome complement.

To determine whether sex hormones are responsible for observed differences, researchers should start by examining hormones released from the gonads during adulthood. A preliminary approach involves performing gonadectomy on both males and females and comparing the outcomes. If the sex difference persists after the removal of all gonadal hormones, it may be attributed to organizational effects of steroids during development or differences in sex chromosome complement[26].

An alternative method is to administer exogenous hormones to gonadectomized animals. If the observed sex difference is male-biased, one may mimic the male hormonal profile in both sexes by administering testosterone[26]. Conversely, if the sex difference is female-biased, one should establish a female-typical hormonal profile, such as estradiol or estradiol plus progesterone. If normalizing hormone levels eliminates the sex difference, it suggests that adult gonadal steroid levels are responsible. If the sex difference remains, re-evaluate the roles of developmental hormone exposure or sex chromosome complement[26].

If adult gonadally synthesized hormones are excluded as the source of the sex differences, the next step is to investigate whether the effects originate from developmental factors, such as exposure to gonadal hormones during specific postnatal developmental periods[26]. Following well-established protocols from Becker et al.[25], neonatal rats and mice can be treated with steroid hormones to investigate sex differences. By comparing males, females, males treated with hormone blockers, and females administered masculinizing doses of hormones, researchers can determine if a sex difference in hormone action during development is responsible for observed differences.

If specific endpoints cannot be measured in neonates, animals should be raised to adulthood. Neonatal hormone treatments can alter adult gonadal hormone levels by affecting the hypothalamic-pituitary-gonadal axis, potentially confounding results if the gonads remain. To address this, gonadectomy can be performed to control for hormone levels in adulthood, and subsequent comparisons between males and females can reveal if the sex difference persists. Persistence suggests an organizational effect or genetic basis rather than hormonal activation.

If endpoints are not evident in the absence of hormones, treating males and females with similar hormones and observing their responses can help determine if the sex difference was hormonally organized. If hormone treatment reverses the sex difference, it indicates hormonal organization. If the sex difference remains, it may be due to direct sex chromosome effects. In cases where neonatal hormone blockers have no effect, this may be due to masculinizing effects of prenatal testicular secretions, which are not disrupted by postnatal hormone blockade.

For studies investigating chromosome-related sex differences, the Four Core Genotypes (FCG) model remains a gold standard. This model utilizes a genetically modified mouse line in which the testis-determining gene *Sry* has been translocated from the Y chromosome to an autosome. Consequently, XX mice carrying ectopic *Sry* develop testes, while XY mice lacking *Sry* develop ovaries, although they lose germ cells and cease estrous cycling earlier in life[31,32]. This model effectively separates sex effects due to chromosomal differences from those arising from gonadally-produced sex hormones.

When a sex chromosome effect (XX ≠ XY) is observed in FCG mice, it could be attributed to either the number of X chromosomes (including X dosage, X imprinting, or indirect effects of X inactivation) or the presence/absence of the Y chromosome[33]. As reviewed by Arnold[34], the XY* model is then useful to discriminate between these possibilities. Discovered by Eicher et al[35], XY* mice have an aberrant pseudoautosomal region on the Y chromosome, which recombines abnormally with the X chromosome. XY* fathers, mated to XX females, produce mice that are very similar to XX and XO gonadal females, and XY and XXY gonadal males. The effects of one vs. two X chromosomes is measured by comparing

XO vs. XX females, or XY vs. XXY males. The effects of one vs. no Y chromosome is measured by comparing XY vs. XO, and XXY vs. XX. In the XY* model, mice with a Y chromosome are gonadal males.
Also, it is important to consider that sex differences encoded by the sex chromosomes, can be classified into four groups:
Class I: Y-linked genes that have effects exclusively in males, such as the testis-determining gene *Sry* and other genes necessary for spermatogenesis[36].
Class II: X-linked genes that escape X-inactivation, resulting in higher expression in XX cells compared to XY cells. The number of such genes varies by species, developmental stage, and tissue, with humans showing more escape genes than mice[37].
Class III: X-linked genes that are differentially expressed in XX versus XY cells due to parental imprinting. This results in unequal expression of imprinted genes between the sexes[38]
Class IV: Regions of sex chromosome heterochromatin that act as sex-specific sinks for factors that regulate the amount of euchromatin and/or heterochromatin at interphase and, therefore, epigenetically regulate autosomal gene expression[39,40].

9 Operationalizing sex

Most research is conducted with young adult animals. However, the experiments using both sexes outside of young adulthood range are also important and require special considerations. In experiments involving puberty, age-matching may be complicated by the fact that puberty occurs later in males than in females. The choice between age-matching or assessing a time span covering puberty in both sexes depends on the experimental questions and outcomes[10]. Puberty onset can be tracked by observing physical, hormonal, and behavioral changes: vaginal opening in females[41] and balanopreputial separation in males[37]. Additionally, monitoring physical changes like body weight, reproductive organ growth, external genitalia development, and increased exploratory behavior and scent marking can provide supplementary indicators of puberty.
For neonatal investigations of sex differences (and accurate sexing of mice in general), measuring anogenital distance is a common method[42]. This measurement is best visualized by slightly bending the lower back of the pup, which stretches the genital area. However, this technique has limitations: the extent of the back bend can affect the anogenital distance measurement, potentially leading to incorrect sex assessment. To improve accuracy, the use of the scrotal pigment spot in pigmented mice should be combined with anogenital distance measurements. The scrotal pigment spot is a clearly visible developmental marker and, with an accuracy approaching 100%, provides a more reliable method for determining sex compared to the anogenital distance alone[43].
Studies focusing on fetal sex differences between gestational days 16 and 18 are particularly challenging because reliable methods for sexing mice during this period are limited. In that case, morphological assessment of key genital features-such as the presence or absence of the urethral seam or proximal urethral meatus, the shape of the genitalia, and the presence or absence of an area related to the urethral plate-can be used to identify the sex of the fetuses[44]. This approach can be applied to freshly dissected or fixed fetuses, as well as to still photos, making it possible to examine previously collected samples for sex-specific effects[44].
When fetal or neonatal sex cannot be identified visually, genotyping for sex-specific genes is the primary approach. This method amplifies the Y chromosome to identify XY animals, while the absence of Y chromosome amplification infers XX status. The most commonly used method targets the Y chromosome gene *Sry*[45]. Additionally, other PCR assays employing a single primer pair to amplify fragments from both the X and Y chromosomes, generating distinct size differences between the amplicons, are used for sex determination[46,47].

10 Rodent models of gender-affirming hormone therapies (GAHT)

Although laboratory animals do not have gender, as it is primarily a social construct, recent research has focused on developing rodent models to specifically address the health of transgender individuals and the effects of pharmacological gender-affirming hormone therapy (GAHT)[48]. This research aims to enhance our understanding of several key areas: i) the influence of GAHT on gene expression at different life stages; ii) the

impact of GAHT on neural processes such as learning, memory, and emotional behavior; iii) the interaction between GAHT effects and environmental factors and iv) the effects of GAHT on metabolism, physiology, and associated disease risks[49].
Despite the limitation that rodent species lack the hepatic expression of sex hormone-binding globulin (which binds testosterone in the body) postnatally, impacting hormone metabolism, these animal models can still be valuable[49,50]. They align with efforts to promote gender-inclusive research practices, thereby enhancing the precision of medicine[51].

11 Hormonal influence on sexual behaviors and preferences

The influence of hormones on sexual behavior in rodents is well-documented and highlights how gonadal hormones shape neural structures and behaviors in a sex-typical manner. Research has shown that androgens, acting during critical developmental periods, organize the brain to promote sex-specific behaviors[52]. For instance, early exposure to androgens in females leads to male-typical sexual behaviors in adulthood, as initially observed by Phoenix and colleagues[53] and replicated in various studies with mice and rats. Conversely, the absence or low levels of androgens in males during development result in decreased male-typical behaviors and increased female-typical behaviors when treated with hormones that induce female receptivity. Hormone manipulation studies have also demonstrated that sexual preferences-such as gynephilia (attraction to females) and androphilia (attraction to males)-are mediated by androgen levels, with male-typical levels increasing attraction to female stimuli and low levels or absence leading to a preference for male stimuli[52]. However, ongoing research reveals the complexity of these processes, including the interaction of androgenic and estrogenic pathways, hormone doses, and environmental factors.

12 Sex bias in *in vitro* studies: Consideration for incorporating SABV

Sex bias extends beyond *in vivo* studies and is also a concern in *in vitro* research. Traditionally, the sex of cells in in vitro studies has been overlooked. For example, many studies involving newly generated cell lines fail to specify the sex[54], and when sex is reported, 71% of studies focus solely on males[55]. Additionally, most cell suppliers do not specify the sex of their cells [56]. Although cells may behave differently once removed from the body [4,57], sexually dimorphic differences have been observed in many cell populations[56,58], highlighting the relevance of incorporating sex as a biological variable (SABV) in *in vitro* research.
With the FDA increasingly accepting alternative methods to animal studies for drug approval[59], the reliance on *in vitro* models such as cell cultures, organoids, and microfluidic systems raises concerns about potential sex-specific biases in experimental design and analysis. To address these concerns, two main considerations for incorporating SABV in *in vitro* experiments should be followed.
For primary cells and organoids taken directly from tissues, it is crucial to use samples from both male and female donors. Ideally, other variables such as donor gender, age, ethnicity, and health status should also be considered. Such diverse systems facilitate patient-specific studies on disease progression and treatment responses. Also, it is important to mention that donor sex can influence reprogramming, pluripotency, and differentiation of iPSC lines, introducing experimental variability due to X chromosome dosage/silencing and differential epigenetic signatures[60]. Similar lines of thought should be applied for cells derived from animals.
For commercially available immortalized cell lines, the sex of the cells should be checked, and cells of both sexes should be obtained from the vendor. However, due to the chromosomal instability of these cell lines, it is recommended to confirm the sex of the cells using the PCR.

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