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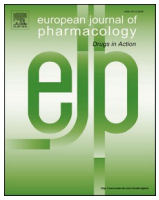

# Let's consider sex contextualism within sex-aware preclinical pharmacological research

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ABSTRACT

It is now well established that consideration of sex and gender is essential to deliver effective healthcare and preventive strategies for all individuals, as well as to reduce health disparities. A sex-based perspective is indispensable in the context of drug research. This review focuses specifically on the concept of sex contextualism, which recognizes that sex encompasses a range of dynamic variables. It also reports some of the biological factors that must be considered in building sex contextualism. This approach provides a constructive framework for addressing sex-related biological differences, but its proper implementation requires the involvement of well-trained researchers with different expertise. To achieve truly sex-aware research it is necessary to adopt appropriate experimental designs, carefully select pre-analytical and analytical procedures, precisely define experimental variables, and rigorously report standards. Additionally, the gender of the investigators must be considered, since everyone is gendered, and numerous studies have demonstrated that the gender of the experimenter can influence research outcomes. In this review, we discuss strategies to incentivize sex-aware preclinical pharmacological research. Although this approach is more time-consuming and costly than those that ignore sex differences, it is essential for ensuring therapeutic appropriateness for all individuals.

## 1. Introduction

Since ancient times, humanity has subscribed to the belief that the male body is the norm. This has led to a persistent sex bias in both preclinical and clinical research. Historically, in preclinical studies male young models (mainly mice and rats) in highly controlled environments have been used (Hägg and Jylhävä, 2021). The use of male animals predominated even when the prevalence of the studied disease was greater in women than in men (Franconi et al., 2007; Yoon et al., 2014). However, there are numerous differences between male and female bodies, some of which are summarized in Tables 1 and 2 for humans and mice, respectively.

Enrollment of women was and continues to be neglected, although some progress has been achieved over the years (Allegra et al., 2023; Mauvais-Jarvis et al., 2021; Sosinsky et al., 2022; Yoon et al., 2014). Researchers often fail to reproduce promising preclinical results. A recent report estimated that about 50% of these failures were due to various factors, including study design, data analysis and reporting (Freedman et al., 2015), and other variables (*e.g.*, the sex, age, storage, fasting status etc of experimental subjects) not being considered

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appropriately (Campesi et al., 2021a,b; Franconi et al., 2015, 2019).

The primary goal of pharmacological preclinical investigations is to individuate the pharmacokinetics and pharmacodynamics of drugs to anticipate the safe initial dose and dose escalation plan for phase 1 studies and drug safety (Beery and Zucker, 2011; Prendergast et al., 2014). This review focuses on the influence of sex on different aspects of preclinical research. It discusses *in vivo* and *in vitro* studies that address concerns about this issue and suggests some practical solutions for performing real sex-aware pharmacological research. Sex-related factors (*e.g.*, hormonal changes, typically occurring over the course of life in females, genetic and epigenetic factors, environmental factors) are not often considered in preclinical research, contributing to the sex gap (Brady et al., 2021; Campesi et al., 2021a,b; Franconi et al., 2019; Kundakovic and Tickerhoof, 2024; Woitowich et al., 2020). Nevertheless, despite numerous international institutions and funding agencies (*e.g.*, Food and Drug Administration (FDA), Canadian Institutes of Health Research, National Institute of Health of United State, the European Commission, UK Research and Innovation (UKRI)) issuing mandates to incorporate sex as an important variable in preclinical research (Greaves et al., 2023; UKRI, 2022), researchers are still struggling to put these into practice. The situation is gradually changing, although very slowly, and several scientific journals have started following Sex and Gender Equity in Research (SAGER) guidelines (Peters et al., 2021).

The use of sex as a biological variable increases the scientific rigor of data and improves the translational values of preclinical findings. However, to reach these goals, it is necessary to ensure appropriate experimental design and pre-analytical and analytical procedures as well as accurate operationalization of experimental variables and accurate reporting (Campesi et al., 2021a,b; Garcia-Sifuentes and Maney, 2021; Pape et al., 2024; Rich-Edwards and Maney, 2023). Moreover, it is important to study the sex factors and their interactions. It is clear that sex-aware pharmacology is crucial to producing appropriate pharmacokinetics and pharmacodynamics data as well as the activity and safety profile of medicines for all individuals (Campesi et al., 2021a,b; Madla et al., 2021; Mauvais-Jarvis et al., 2021). Yet, the application of sex-aware pharmacology presents several concerns that will be discussed in this review.

**Table 2**
Features showing constitutive sexual dimorphism between male and female mice.

| Trait category | Parameters |
|---|---|
| **Morphology** | Fat mass |
| | Bone mineral content (excluding skull) |
| | Bone area |
| | Heart weight |
| | Spleen weight |
| **Physiology** | Fasted blood glucose concentration |
| | Initial response to glucose challenge |
| | Area under glucose response curve |
| | Sodium, potassium, creatinine, albumin, phosphorus and iron levels |
| | Alanine aminotransferase and alkaline phosphatase levels |
| | Total, high density lipoprotein and low-density lipoprotein cholesterol |
| | Triglycerides, free fatty acids, fructosamine, lactate dehydrogenase |
| | Alpha amylase, unsaturated iron binding capacity |
| | Heart rate, heart rate variability, multiple electrocardiographic parameters |
| | Forelimb and hindlimb grip strength |
| | White blood cells, red blood cells, platelets, and large unstained cells |
| **Behavior** | Locomotor activity |
| | Whole arena average speed |
| | Center and periphery distance traveled |
| | Center average speed |
| | Total distance traveled |

Within each category, traits with significant differences between male and female animals are reported. Data from (Mank and Rideout, 2021; van der Bijl and Mank, 2021).

**Table 1**
Some of the most well-known human biological differences between sexes.

| Parameters | Sex differences | Parameters | Sex differences |
|---|---|---|---|
| **Oral absorption** | M > W | **Body surface** | M > P > W |
| **Gastric secretion** | M > W > P | **Organ size** | M > W |
| **Gastric emptying** | M > W > P | **Organ flow** | Liver and skeletal muscle M > W<br>Adipose tissue: W > M (it increases in P) |
| **Intestinal transit time** | M > W > P | **Total water** | M > P > W |
| **Cardiac output** | M > P > W | **Plasma volume** | M > P > W (it changes during menstrual cycle) |
| **Heart Rate** | W > M | **Body fat** | W > M |
| **Q-T interval** | W > M | **Resting and systolic blood pressure** | M > W |
| **Organ dimension (heart, liver, kidney, lung)** | M > W | **Urethra length** | M > W |
| **Pulmonary function** | M > P > W | **Bone mass** | M > W |
| **Plasma Albumin** | M = W<br>P and OC reduce it | **CYP2B6** | W > M |
| **Distribution volume** | W > M for lipophilic and M > W for hydrophilic drugs | **CYP1A2** | M > W<br>P and OC reduce it |
| **Plasma α1-acidic glycoprotein** | M > W<br>P and OC reduce it | **CYP2C9** | M = W<br>P and OC reduce it |
| **Liver P-glycoprotein** | M > W | **CY2C19** | M = W |
| **Organic cation transporters 2** | M > W | **CYP3A4** | M > W<br>OC increase it |
| **Organic anion-transporters polypeptide** | M > W | **CYP2D6** | M > W<br>OC reduce it |
| **Renal flow** | M > W | **CYP2E1** | M > W; |
| **Glomerular filtration rate** | M > W | **Uridine Diphosphate glucuronosyltransferases** | M > W |
| **Tubular secretion/reabsorption** | M > W | **Cathecol-O-methyltransferase** | M > W |
| **Skin pore size** | M > W | **Acetyl and Butyryl choline esterases** | M > W |
| **Gastric alcohol dehydrogenase** | M > W | **Xanthine-oxidase** | W > M |

M: men; W: women, P: pregnancy; OC: oral contraceptives. Data from (Mauvais-Jarvis et al., 2021; Tamargo et al., 2017).

## 2. Critical points for the development of sex-aware pharmacological research

### 2.1. Not just sex and gender concepts

A critical point when integrating sex and gender into pharmacological research is the selection of terminology because there are imprecision and confusion in the use of the two terms, *i.e.*, sex and gender (Keogh and Boerner, 2024). Sex and gender are often used interchangeably, and gender is inappropriately used in the context of non-human investigations (Boerner et al., 2018; Keogh and Boerner, 2024). The imprecision is also present in documents of International Conference on Harmonization, National Academies of Sciences, Engineering, and Medicine (Bates et al., 2022), FDA and European Medicine Agency (EMA) (Greaves et al., 2023). Because of this ambiguity, bibliographic research is challenging. Some define *sex* as variations in biological factors between males and females, whereas *gender* is a multifaceted concept that refers to psycho-socio-cultural factors involving self-identity, social interactions, behaviors, expressions, roles, norms, relations, power etc. (Bates et al., 2022). In human studies, participants are requested to identify their sex or gender category, whereas in investigations performed in animals, they are assigned to a sex category based on genital anatomy (Ritz and Greaves, 2024).

The assumption that '*sex is biological and gender is social*' serves as a reminder that not all differences are rooted in biology. Biological processes, in fact, are shaped by psycho-socio-environmental factors (*e.g.,* epigenetics), and, in turn, psychological processes (*e.g.,* gender self-identity) can be influenced by biological factors. However, both *sex* and *gender* are not fixed concepts. Typically, the term *sex* identifies male, female, or intersex subjects, but sex-related factors and traits, such as anatomy, physiology, hormones, gene expression, reproduction and metabolism, are quite numerous. Therefore, the sex phenotype is the result of a complex interplay of different factors. According to Richardson (Richardson and Richardson, 2022), it is time to include the concept of "*sex contextualism*" to investigate sex factors in research. The notion of *sex contextualism* comes from multiple variables included in the term sex, as well as the dynamic interactions of these variables (Pape et al., 2024). Following recent mandates of sex as a biological variable, sex contextualism offers constructive guidance for biomedical researchers in addressing sex-related biological variations. Conversely, *intersectionality* refers to the complex interactions of individual identities and social positions (*e.g.,* race, socioeconomic status, nationality, and ability) influencing their health outcomes (Crenshaw, 2013). Nevertheless, *sex* and *gender* have distinct meanings; they are strongly interrelated and very often form Gordian nodes (Boerner et al., 2018; Campesi et al., 2021a,b; Fishman et al., 1999). These interactions can change over the course of life and complicate the research. Finally, given the difficulties in separating the biological (sex) and environmental (gender) influences, numerous authors use both terms to avoid ambiguity (Campesi et al., 2021a,b; Keogh and Boerner, 2024; Mauvais-Jarvis et al., 2021).

### 2.2. Are researchers sufficiently trained to perform sex-aware pharmacological research?

Sex-aware investigations may present concerns. A recent survey showed that numerous publications based on sex differences lack statistical evidence, suggesting that researchers may not be adequately trained (Garcia-Sifuentes and Maney, 2021). Courses on this topic are available online at the National Institute of Health (NIH) and the Canadian Institute of Health Research websites (Gompers et al., 2024). Training would also be helpful in reducing implicit biases related to sex and gender, which typically prevail in men (Garcia-Sifuentes and Maney, 2021). Researchers should be aware that each person is gendered, including the researcher himself or herself.

### 2.3. How can we encourage the recruitment of researchers in sex-aware pharmacology?

Sex-aware research is more expensive both in terms of time and economic costs. Globally, the value of researchers is assessed by their scientific production (manuscripts and patents); therefore, the practice of sex-aware research may slow his/her career. These considerations highlight the need to provide both economic and career incentives for researchers who focus on this topic. Furthermore, incentivizing sex-aware research would make the field more attractive and encourage the involvement of new and early-career researchers.

### 2.4. New regulations and mandates

New and stricter regulations and mandates should be enforced in pharmaceutical and medical device industries toward the inclusion of female cells and animals because the current guidelines and recommendations have not led to sufficient progress (Sosinsky et al., 2022; Yoon et al., 2014). Agencies should devote greater attention and resources to ensuring that sex contextualism is indispensable for drug registration. Funding agencies should require sex contextualism and gender intersectional analysis.

## 3. Experimental design for sex-aware pharmacology

Advances in high-throughput techniques and the generation of functional genomic datasets have enabled the development of *in silico* models that may facilitate the identification of sex-dependent genes and the prediction of sex-related drug responses (Cui et al., 2018). However, it is unclear whether these computational approaches can accurately predict sex differences (Cui et al., 2018). Conventional *in vitro* and *in vivo* models are, therefore, still necessary for studying sex differences in biological systems, as well as for developing innovative therapeutic and medical approaches.

To improve the reproducibility and translatability of results, attention must be paid to various factors, which will be reviewed in the following sections. In addition to experimental design and statistical data analysis, several environmental variables, related to animal husbandry and laboratory backgrounds, could be controlled by applying shared rules across different test centers. We will specifically discuss the impact of age, stress and hormones, which have been clearly demonstrated to affect multiple parameters in a sex-dependent manner (Bale and Epperson, 2015; Fritz García et al., 2024; Handa et al., 2022; Konstandi et al., 2022).

### 3.1. How to design experiments to properly include sex as a biological variable

Despite an overall increase in the number of studies including both male and female animals (Woitowich et al., 2020), the design of experiments and the statistical analysis of data were still unfair in the large majority of these publications, possibly masking - but also overreporting - sex differences (Garcia-Sifuentes and Maney, 2021). Simply adding female cells or animals to experiments does not ensure an accurate evaluation of sex differences. Rather, experiments must be designed to allow the appropriate statistical analysis of such differences. To this end, the so-called factorial design provides the opportunity of analyzing the impact of more than one variable on experimental results. Thus, choosing a 2x2 factorial design approach allows to study the effect of treatment (variable 1) and sex (variable 2) but, especially, the interaction between treatment and sex by applying a 2-way analysis of variance (ANOVA) (Fig. 1). When a statistically significant interaction is observed, evidence for a sex difference in the response to treatment is strongly supported (Barris et al., 2024; Buch et al., 2019; Garcia-Sifuentes and Maney, 2021; NIH, 2015; Vorland, 2021). Moreover, the addition of a supplementary experimental variable in a factorial design

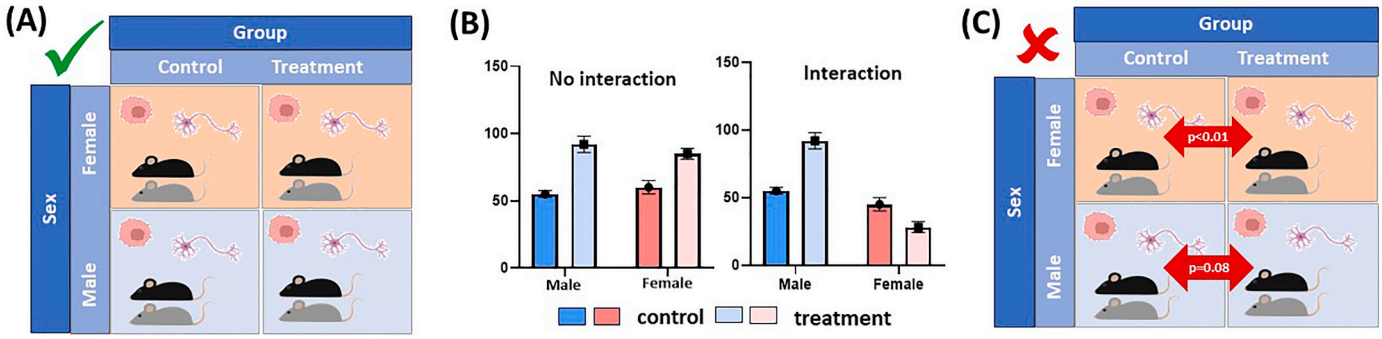


**Fig. 1.** (A). 2x2 factorial design to test sex differences. Both female (pink boxes) and male (light blue boxes) cells or animals are exposed to treatment, e.g., a drug, a diet, the silencing of a gene, or to the appropriate control. (B) Sex differences between experimental groups are evaluated by means of the 2-way analysis of variance (ANOVA), considering treatment as column factor and sex as row factor. Left panel shows no significant interaction detected between sex and treatment. In contrast, a statistically significant interaction is observed between sex and treatment in the right graph, supporting evidence for a difference between male and female cells/animals in response to treatment. (C) Performing a statistical test between treatment and control groups within each sex, and comparing the nominal statistical significance, is not an effective method to detect sex differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

produces further information without requiring a main increase in the number of animals (Buch et al., 2019). At variance, using a 1x1 factorial design, independent experiments for each sex need to be performed, doubling the number of animals used, without providing any information about interaction. Moreover, performing a statistical test between control and treatment within each sex, and comparing the nominal statistical significance, is not an accurate method to search for sex differences (Barris et al., 2024; Buch et al., 2019; Garcia-Sifuentes and Maney, 2021; NIH, 2015; Vorland, 2021).

### 3.2. *When pilot experiments are needed*

Historically, physiology, biochemical tests, and standard basal behavior have been characterized in males. Conversely, the physiology, biochemistry and behavior of females are largely understudied. Therefore, pilot experiments must be performed to investigate sex-dependent reference values when disaggregated male and female data are unavailable (NIH, 2024; Tannenbaum et al., 2017).

### 3.3. *Intersections between age and sex*

Sex differences begin in prenatal life and persist throughout the lifespan (Bale, 2016; Caterino et al., 2021). Age-specific metabolic signatures have been measured in both males and females (Caterino et al., 2020, 2021; Costanzo et al., 2022). Age also influences cell biochemistry and physiology, representing an important risk factor for the initiation and progression of many diseases, as well as affecting the individual's hormonal status that can, in turn, alter their response to pharmacological treatments (Fritz García et al., 2024). Although it is well known that drug metabolism is influenced by sex and age (Anthony and Berg, 2002; Cotreau et al., 2005; Milsap and Jusko, 1994; Soldin and Mattison, 2009), the effects of these factors on pharmacodynamics are less well understood. For example, levels of the postsynaptic serotonin 5HT1A receptor increase with age in women whereas they tend to decrease in men (Moses-Kolko et al., 2011). Therefore, well-designed studies should enroll sufficient numbers of males and females in different age groups, and the relationship between age and sex should be considered in advance, to prevent false positive or negative results. Sex stratification in every age group is highly advisable for validating reference values and increasing their diagnostic and therapeutic appropriateness.

### 3.4. *Sex-linked response to stress and hormonal milieu*

Stress is a significant regulatory factor in pharmacological research as it affects both pharmacokinetics and pharmacodynamics. Accordingly, some authors have recently defined stress as "*a potential regulatory factor in the outcome of pharmacotherapy*" (Konstandi et al., 2022). The first evidence that sex influenced the stress response was provided by Selye in 1937 (Selye, 1937), who found that the adrenal glands of female rats were larger than those of male rats. Indeed, sex differences in the function of the hypothalamic–pituitary–adrenal (HPA) axis have been reported in rodents, and some of the underlying mechanisms have been elucidated (Handa et al., 2022; Selye, 1937). For instance, females exhibit greater and more prolonged secretion of the adrenocorticotropic hormone and glucocorticoids, indicating greater reactivity to stress and reduced negative feedback regulation (Bale and Epperson, 2015). Furthermore, testosterone and estradiol may both modulate HPA axis function in adult and ageing rats (Bale and Epperson, 2015; Handa et al., 2022). During proestrus, when estrogens are high, basal and stress-responsive corticosteroids are also high (Selye, 1937). It is therefore important to consider the phase of the estrous cycle of animals when testing them. Several studies have suggested that androgens may inhibit the HPA axis (Handa et al., 2022; Selye, 1937). However, it should be noted that gonadal steroids can affect the HPA axis differently from early life through adulthood and that stress-induced HPA activation in men and women diverges from that observed in rodents (Handa et al., 2022).

### 3.5. *Good laboratory practice (GLP) in sex-related preclinical studies*

GLP is a quality system encompassing the organizational procedures and environment in which non-clinical laboratory studies are planned, executed, supervised, documented, disclosed, and stored. Its primary objective is to ensure the quality and credibility of safety test data submitted to regulatory authorities for the purpose of obtaining research permits. However, the GLP mandates of the EMA (European Union, 2024) and the US FDA (FDA, 2016) lack precise indications for studying the influence of sex and do not consider sex contextualism.

The ARRIVE.2 (*Animal Research: Reporting In Vivo Experiments*) guidelines (du Sert et al., 2020) outline ten "essentials", one of which, n. 8 - Experimental Animals, mentions sex. It recommends that studies "*provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage*". However, little emphasis has been placed on sex as biological variable (SABV) globally (du Sert et al., 2020).

## 4. Pre-analytical questions

To ensure samples of suitable quality, it is necessary to choose the right sample to be processed. Critical decisions regarding the

measurement of drugs and their metabolites must be made during the study design phase, taking into account circadian rhythms, and specimen collection, processing, and storage. All of these processes must be conducted in a sex-aware manner (Franconi et al., 2015, 2019). It is important to recall that even minor changes in handling and housing of experimental animals can produce significant biological variations, underlying the relevance of standardized and validated animal procedures in the pre-analytical phase of experiments.

### 4.1. Sex differences and animal diet

Different standard commercial diets can cause various physiological changes in experimental animals (Robertson and Rowland, 2005; Sabbatini et al., 2006). In particular, diets derived from soybeans, which are used as a source of crude proteins, lead to exposure to high doses of isoflavones (Rietjens et al., 2017). Soy-derived diets can contain different amounts and types of isoflavones, with levels varying between formulations and even between batches (Heindel and vom Saal, 2008), which can cause variability in experimental outcomes. In addition, the timing (*e.g.*, gestation, infancy, growth, or maturation) and duration of exposure can result in apparently contrasting outcomes (Jensen and Ritskes-Hoitinga, 2007). It is interesting to note that the elimination half-life and area under the curve of genistein (a phytoestrogen belonging to the isoflavone class) and daidzein (an isoflavone extracted from soybeans), as well as their glycosides, differ between males and females (Messina et al., 1994). Given the pleiotropic properties of estrogens, dietary isoflavones could significantly impact estrogen-sensitive endpoints, potentially interfering with them. A soy-derived diet requires monitoring of isoflavones to avoid hidden biases (Sabbatini et al., 2006). Furthermore, different protein sources (*e.g.,* soy, casein) can lead to variations in amino acids levels in the blood, which may affect experimental outcomes in male and female animals (Westmark et al., 2023). Finally, it is important to recall that diets can alter bile acid homeostasis in a sex-dependent manner, as sex, along with atherogenic and calorie-restricted diets, is among the factors that most strongly affect bile acid homeostasis (Ma et al., 2021). Taken together, these findings suggest that rodent diets may affect experimental outcomes and should therefore be considered when designing and reporting studies focused on sex differences.

### 4.2. Collection and storage of samples

Sample collection should be approved by the ethics committee and be painless and stress-free as possible. It should be conducted under anesthesia if possible, or by a well-trained operator if anesthesia is not possible (Parasuraman et al., 2010). The frequency of collection depends on the size of the animal (Parasuraman et al., 2010), but the timing is also important because some biomarkers can fluctuate yearly, seasonally, monthly, daily, or even hourly. The menstrual or estrous cycle, with fluctuating levels of sex hormones over time, should be taken into account when analyzing female samples (Fig. 2). Conversely, the circadian rhythm of testosterone should be considered for male rodents (Fig. 2), as it may significantly affect research data when using males (Ning et al., 2025). For example, experimental evidence in male rats shows that testosterone influences the circadian rhythm of core body temperature: neonatal castration alters the timing of the circadian rise in body temperature, and perinatal testosterone replacement can restore normal rhythmicity (Zuloaga et al., 2009).

Some biomarkers and analytes require immediate processing and should be refrigerated or cooled, as they can degrade or become elevated if left at room temperature. However, others are unaffected if the sample remains unprocessed for 24 to 48 h (Parasuraman et al., 2010). As delayed processing is usual, long-term storage is a routine procedure, with samples being maintained in both mechanical and nitrogen freezers. In mechanical freezers, however, temperature can vary substantially depending on the location within the freezer (Su et al., 1996).

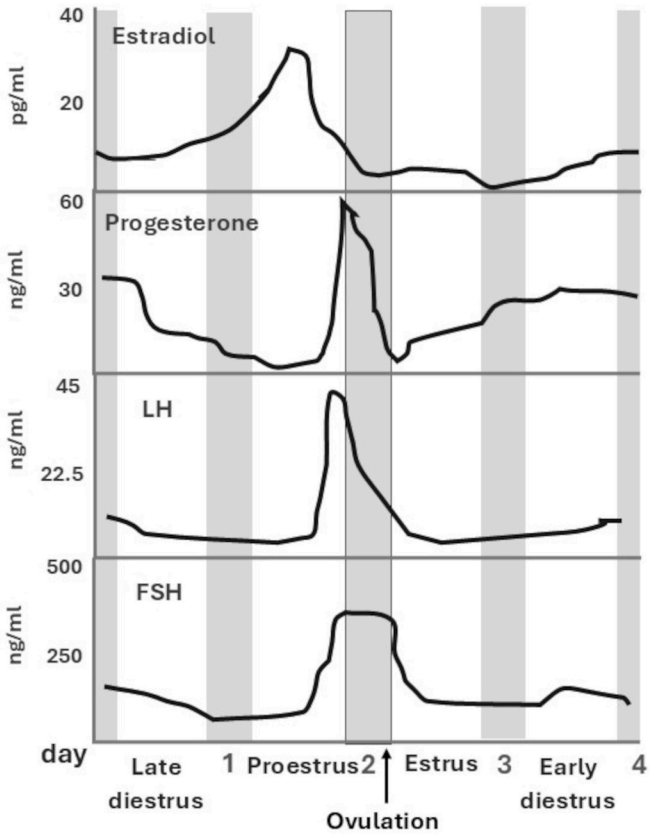


**Fig. 2.** The rodent reproductive cycle. The average fluctuations of estradiol, progesterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) during the 4-day estrous cycle of female rats. Gray bars indicate night (6 p.m. to 6 a.m.). In mice, the estrous cycle is similar but varies in length (4-7 days).

Clearly, degradation over time is an important issue, especially for samples stored for extended periods, because storage conditions and temperature will affect sample quality. Samples should therefore be stored safely and aliquoted to avoid sample degradation. Finally, the storage process may be influenced by the anticoagulant used (Evans et al., 2001).

### 4.3. Blood, serum, and plasma collection

Numerous variables can influence blood sampling, including the choice of collection tubes and anticoagulants, waiting time before centrifugation, and temperature (Evans et al., 2001; Rai and Vitzthum, 2006). Blood collection, handling, anesthesia, and storage may affect the results in a sex-dependent manner (Evans et al., 2001; Rai and Vitzthum, 2006; Su et al., 1996) and cause stress in laboratory animals due to handling, restraint and the invasiveness of the procedures (Champy et al., 2004; Toft et al., 2006). Therefore, operators should minimize stress to preserve animal welfare and produce rigorous scientific results (Champy et al., 2004). In rats and mice, single and repeated blood collection is more problematic in females, because they weigh less and have lower total blood volume than males (Rai and Vitzthum, 2006), and blood cell damage occurs more often in blood obtained from females (Arunachalam and Sasidharan, 2021). The frequency of collection is relevant and depends on animal sizes. If blood collection is not associated with fluid replacement, it should be limited to 10% of total circulating blood volume in healthy, normal, adult animals. In case of repeated blood samples at short intervals, a maximum of 1.0% of an animal's total blood volume can be collected every 24 h (Morton et al., 2001).

Although the cannulation technique decreases the stress caused by

repeated blood collections and allows access to deep vessels for infusions and drug administration (Morton et al., 2001; Toft et al., 2006), it can also cause pain and therefore may require analgesic drugs and adequate monitoring. There is insufficient evidence regarding the influence of sex on cannulation; however, some sex differences could be plausible due to the smaller diameter of female blood vessels. Despite the use of sterile equipment, infections can occur (Morton et al., 2001). The site of blood sampling influences the concentration of cells and metabolites in a sex-dependent manner (Arunachalam and Sasidharan, 2021; Schnell et al., 2002). Indeed, a study conducted on mice and rats demonstrated that cardiac puncture leads to a lower total peripheral leukocyte count than tail vein sampling, and that this effect appears to be sex-dependent (Schnell et al., 2002; Singh, 2012). The effect of temperature and storage time on the stability of blood plasma/serum samples can induce pre-analytical variability depending on the specific analyte (Nemzek et al., 2001), as also discussed in paragraph 4.2 ("Collection and storage of samples").

In conclusion, blood collection can change biologically relevant variables; therefore, using standardized protocols for blood samples collection is of fundamental importance.

### 4.4. Urine collection

Urine collection and processing are important *in vitro* diagnostic screening tests, especially in pharmacokinetics and toxicological studies. Using metabolic cages to collect urine for animals may induce sex-linked changes in urinary and blood levels, which return to baseline values within three to four days of training (Silva-Santana et al., 2020). Furthermore, as discussed in paragraph 4.8 ("Housing conditions of male and female animals"), substances with estrogenic activity can be released from cage materials over time or through regular use (Narayanan, 2000). From a sex-aware perspective, it is important to note that female rodents have a lower glomerular filtration rate and a greater urinary volume, resulting in less concentrated urine. They also drink more than males (Kuo et al., 2004; Mauvais-Jarvis et al., 2017; Stechman et al., 2010). Female spiny mice excrete more urine than males, although amounts of urinary sodium and protein are similar (Schmidt et al., 2001). Differences between males and females decrease when renal parameters are corrected for body weight, which is lower in females (Blantz et al., 1988; Dickinson et al., 2013; Wang et al., 1994).

### 4.5. Equipment

Globally, male animals tend to be larger than females. For example, fully adult female rats typically weigh 250–300 g, whereas fully adult male rats typically weigh 300–500 g (Dalla et al., 2024). These ranges represent fully grown adults under standard laboratory conditions and may require adjustments of equipment such as surgical instruments and needles.

The height of the infrared beams in the Open Field boxes, which are used to evaluate locomotor activity and anxiety-like states, should differ between males and females (Dalla et al., 2024). Importantly, when the same equipment is used for male and female animals, it is essential to clean it thoroughly, as residual odors may affect behavioral outcomes. If cleaning is not possible (*e.g.,* for operating boxes), it is advisable to use two different sets (Dalla et al., 2024). Odors may also influence the results in the testing room, as demonstrated by the finding that female mice exhibit more rearing behavior when they smell male urine (Dalla et al., 2024).

### 4.6. Fasting and food restriction

Fasting is a variable inducer that depends partly on sex. Interestingly, more metabolites differ between the sexes in the fed state than in the fasting state (Saravanan et al., 2023). Short-term fasting can increase or decrease the expression of P-glycoprotein (an ATP-dependent efflux pump with a broad and diverse range of substrates) in male and female animals, respectively. After food intake, P-glycoprotein levels are significantly higher in females, leading to altered drug bioavailability (Dou et al., 2020). The overnight fasting of nocturnal animals cannot be compared with that of humans (Jensen et al., 2013). Given the sex differences observed in the digestive system (Tamargo et al., 2017), it is biologically plausible that fasting is influenced by sex; females may require longer fasting periods than males prior to sampling. The duration of fasting before oral drug administration should therefore be accurately evaluated in both sexes (Brauer et al., 2011). Food restriction is a widespread practice in neuroscience, but male and female animals may require different regimens due to their different sizes (Dalla et al., 2024). Indeed, when age- and sex-matched animals are compared with *ad libitum*-fed animals, they should lose less than 15% of their weight. Additionally, the effects of food restriction may differ depending on the age of the animal (Dalla et al., 2024).

### 4.7. Sex differences in biological rhythms

Several physiological and behavioral phenomena exhibit strong rhythmicity that is regulated by circadian (approximately 24 h) and infradian (biological cycles that last longer than 24 h, such as the female menstrual cycle, or annual such as hibernation patterns) rhythms (Oliveira et al., 2024). These biological rhythms are a fundamental feature of all organisms, governing several aspects of physiology and behavior, and they can occur in a sex-dependent manner (Walton et al., 2022). For instance, circadian rhythms play a part in maintaining cardiovascular function and metabolic homeostasis; a night-time meal, for example, induces a significant rise in blood triacylglycerol levels compared to a daytime meal (Lal et al., 2024). Therefore, it is not surprising that disruption of metabolic circadian rhythms can lead to metabolic disorders, including obesity and neurological disorders (Lal et al., 2024; Sun et al., 2023; Takahashi et al., 2008). Awareness of biological rhythms and the factors that may influence them is crucial when designing and reporting experiments. The time of day is critical when studying sex differences, as circadian rhythms have been observed in cytochrome P450 enzymes (CYPs), drug transporters, and drug metabolism, among others (Lévi et al., 2024). Neglecting these factors may result in inconsistent findings and misinterpretation of data. In fact, dedicated clinical trials demonstrated that drug response depends on circadian timing (Lévi et al., 2024) and that biological rhythms may be influenced by sex (Walton et al., 2022). For example, administration of ramipril (an angiotensin-converting enzyme inhibitor) at bedtime reduced blood pressure for 24 h, whereas administration in the morning reduced blood pressure for only 16 h (Hermida and Ayala, 2009). Sex differences in circadian rhythms may have significant implications for biomedical research, particularly with regard to pharmacological manipulation from the perspective of either pharmacokinetics or pharmacodynamics.

### 4.8. Housing conditions of male and female animals

The housing conditions may affect biological parameters differently in male and female animals. Depending on the type of rodent chow (*e.g.,* containing soy phytoestrogens) or rodent bedding (*e.g.,* corncob bedding), the *vivarium* environment might provide exogenous sources of estrogens. Exogenous estrogens may also be induced by plastic cages and water bottles, which can release endocrine disruptors such as bisphenol A and phthalates. These disruptors can affect estrogen-sensitive metabolic parameters in a sexually dimorphic manner (Thigpen et al., 2013), but they can also be released from polystyrene tubes (Soto et al., 1991).

To avoid high or low physiological levels of corticosterone and minimize the stress effects of housing, rats (both male and female) should be housed in groups of three to five (depending on the size of the cage). For example, crowding increases corticosterone levels in male rats and decreases them in females, compared to animals housed

individually (Brown and Grunberg, 1995). Indeed, male rats have the lowest level of corticosterone when housed individually, while female rats have the highest (Arndt et al., 2009; Brown and Grunberg, 1995). Notably, the effect of housing seems less evident in male mice (Brown and Grunberg, 1995). Housing conditions also modify the acetylcholine release in a sex-dependent manner (Masuda et al., 2005).

The above data suggest that research must consider housing conditions as critical variables that affect the behavior of male and female rats differently.

### *4.9. Effects of environmental enrichment in male and female laboratory animals*

Environmental enrichment is another critical variable that can significantly impact the behavior, brain structure and function of animals (Fox et al., 2006; Nithianantharajah and Hannan, 2006). Notably, some of the changes induced by environmental enrichment may depend on sex (Kondo et al., 2008; Martinez-Cue et al., 2002; Pena et al., 2006). Environmental enrichment can also affect emotionality in a sex-dependent manner (Lin et al., 2011), although studies often report conflicting results (Abramov et al., 2008; Belz et al., 2003; Klein et al., 1994; Zhu et al., 2006). Given this and other evidence, environmental enrichment is not ideal for studying sex differences as it could lead to misinterpretations.

## 5. Sex aspects in reference values

It is essential to present and validate reference values for each species, sex and age group. Unfortunately, the influence of sex on reference values has received limited attention so far. A recent study of *Macaca mulatta* anesthetized with ketamine found sex differences in biochemical blood parameters such as red blood cells, albumin, creatinine and glucose, many of which were influenced by age (Zhu et al., 2023). Blood lipids also vary in Meishan pigs depending on sex and age (Kojima, 2008). Sexual dimorphism in the proteomics of serum, plasma, urine, cerebrospinal fluid and bronchoalveolar fluid has been observed in rats, mice, dogs and primates, both in physiological conditions and during diseases (The Jackson Laboratory, n.d.).

Numerous drugs and their metabolites can interfere with analytes, resulting in false positive or false negative values. This may occur in a sex-dependent manner, given that drug distribution and metabolism can be sex-dependent. For instance, anesthetics such as methoxyflurane and isoflurane alter physiological parameters in rats: they reduce erythrocyte, hemoglobin and hematocrit in female rats and increase glucose in male rats (Deckardt et al., 2007). Reference values are sex-dependent and may vary according to the specific parameter. In some cases, the effect of sex is related to age, suggesting that determining the reference value of each parameter is crucial to reducing errors. Normal values should be obtained for each sex at a single age, taking into account the estrous and menstrual cycles, as well as reproductive phase of the female life. When designing experiments, it is necessary to establish the normal range of analytes for both males and females. Laboratories should also create a database of reference ranges to minimize environmental interference.

## 6. Anesthesia in male and female animals

The first case of sex differences described in pharmacology occurred in 1932 and was associated with barbiturates (Nicholas and Barron, 1932). However, in anesthesia research, the majority of animal studies largely neglected sex despite clear clinical evidence supporting sex differences in drug sensitivity (Campesi et al., 2012; Moody et al., 2021). Studies on animals have reported significant sex differences in sensitivity to sodium pentobarbital and to steroid anesthetics, with female rats being more sensitive to their effects than male rats despite equal weight-based dosing (Joksimovic et al., 2021). However, sevoflurane alters short-term cognitive function and hippocampal neuronal apoptosis in rats, with males experiencing more severe cognitive dysfunction than females (Xie et al., 2015). Furthermore, sensitivity to isoflurane in male mice is bidirectionally modulated by testosterone. Castration increases resistance to the anesthetic, whereas acute administration of testosterone enhances sensitivity. In contrast, ovariectomy in female mice does not alter sensitivity to isoflurane (Wasilczuk et al., 2024). Following propofol and isoflurane anesthesia, male rats recover more quickly than females (Mansouri et al., 2019). It is important to note that anesthetic drugs may also modify some biochemical reference values (Deckardt et al., 2007).

## 7. Cells and cellular organelles as experimental models for sex studies

In somatic tissues, the expression of around 40% of genes differs between males and females (Idda et al., 2021; Oliva et al., 2020), with certain genes becoming sex-dimorphic in response to stress (Penaloza et al., 2009). Another source of sex differences is the epigenetic regulation of gene expression, whereby the genome responds to *environmental stimuli* (Lafta et al., 2024). Notably, cellular sex differences are dependent on the cell type and parameters and include housekeeping genes and proteins that can be differentially expressed in male and female cells and tissues (Campesi et al., 2013). This requires pilot experiments to identify a suitable normalization protein, particularly in Western blot analysis.

Furthermore, the role of phenol red, a common pH indicator with estrogenic activity, should be investigated in advance (Mauvais-Jarvis et al., 2017).

Available data suggest that sex differences could be better studied in primary cultures rather than in cell lines (Mauvais-Jarvis et al., 2017; Vallabhajosyula et al., 2020), as the former allows the investigation of factors such as age, reproductive history, and lifestyle-. Cell lines are unique lineages that may exhibit chromosomal mutations and other irregularities compared to primary cells (Richardson et al., 2015). Conceptually, it is questionable whether cell lines truly retain a sex, particularly since several cell lines derived from an XY donor may lose Y-chromosomal gene expression (Richardson et al., 2015). The choice of passage at which the analysis is performed also appears to be critical. For instance, differences in sexual hormone receptor expression disappear after 14 passages from isolation in rat vascular smooth muscle cells (Pellegrini et al., 2014).

Sexual dimorphism has also been reported in subcellular organelles. Indeed, mitochondria obtained from different animals and tissues exhibit significant sex differences in mitochondrial gene expression, redox state, calcium handling, mitophagy, and apoptosis (Campesi et al., 2013; Silaidos et al., 2018; Ventura-Clapier et al., 2017). Similarly, lysosomes exhibit sex differences in macroautophagy and mitophagy (Franconi et al., 2025; Shang et al., 2021; Triolo et al., 2022).

In summary, sex is an important biological variable at both cellular and subcellular levels, affecting gene expression, protein abundance, and organelle function. While immortalized cell lines are convenient, they often undergo chromosomal and epigenetic alterations that can obscure sex-dependent features and are frequently uncharacterized with respect to sex (Mauvais-Jarvis et al., 2017; Richardson et al., 2015; Vallabhajosyula et al., 2020). Therefore, primary cells or well-characterized sex-annotated models are preferable when studying sex differences *in vitro*, as they better reflect physiological variability, including factors such as age and reproductive history. Researchers should also carefully consider experimental conditions, including passage number and culture reagents, to preserve sex-dependent characteristics (Celac et al., 2026; Franconi et al., 2015; Karp, 2025; Mazure, 2016). Overall, rigorous attention to cell sex and experimental design is essential to generate reproducible and translationally relevant findings.

## 8. Specific characteristics of human samples

For investigations involving human cells, it is important to collect information on factors such as age, lifestyles, level of education, socio-economic status, drug use and exposure to xenobiotics, through a questionnaire (Table 3), as previously suggested (Franconi et al., 2015, 2019). Using a questionnaire ensures the reproducibility of experiments and prevents the results obtained from samples of one sex from being over-generalized to the other.

Interestingly, gender and sex can influence the development of cells and tissues before they are removed from a donor. For example, bone development is affected by sex hormones, but also by gender factors such as diet and exercise patterns (Carson and Manolagas, 2015). Furthermore, cigarette smoking and alcohol consumption can have different epigenetic effects in men and women (Meyer-Bockenkamp et al., 2023; Skov-Jeppesen et al., 2023). Moreover, several studies have demonstrated that exercise - has a different impact on males and females (Wiley et al., 2024), and that there are differences in the levels of certain biomarkers (*e.g.,* acylcarnitine) (Costanzo et al., 2022; Lee et al., 2023). To date, the omission of sex and gender is common when using human samples, particularly when human primary cells or purchased cells are used (Kim et al., 2021). When purchasing human cells, vendors should provide detailed information about the sex of the cells. If this information is not provided, the researcher should determine it (Morikawaa et al., 2011). When using samples from women, information on hormonal status (*e.g.,* pre- or post-menopausal status, cycle phase), use of oral contraceptives or hormone replacement therapy, number of pregnancies, and type of birth should be reported. If samples need to be taken from multiple donors, it is advisable that all fertile women be in the same phase of their menstrual cycle to limit variability.

When the donor is a human being, race/ethnicity is another characteristic that must be considered. Representation from different ethnic groups is essential because inter-racial/ethnic sex differences in pharmacokinetics and pharmacodynamics may vary in subjects of different populations (Chen, 2006; Ramamoorthy et al., 2015). According to sex contextualism (Boerner et al., 2018), because cells may recall their life experiences (Harper, 2024), it is crucial to evaluate, define and analyze sex-related biological variables alongside environment, age and ethnicity. Studies employing one sex only may be useful for closing research gaps, investigating differences among cell types within a sex, or studying diseases or interventions that affect females or males differently. However, in these cases, findings cannot be generalized to all populations. Furthermore, authors should report how sex and gender were considered in the study design, ensure adequate representation of both sexes, or justify the use of one sex only (Bøttern et al., 2023). If only one sex/gender is included in the study, the sex of the sample donors should be specified in the title and the abstract.

**Table 3**
Questionnaire for human donors.

| | |
|---|---|
| **Anatomical and physiological information** | Sex |
| | Age |
| | Weight, height and body mass |
| | Race/ethnicity |
| | Place of house |
| | Diseases (present and in the previous 3 months) |
| | Drug use (present and in the previous 3 months) and washout |
| **Woman aspects** | Age of first menstruation |
| | Date of last menstruation and menstrual cycle phase |
| | Pregnancies (number, complications, type of delivery) |
| | Abortions (number and which month of pregnancy) |
| | Oral contraceptive use (type and how long time) |
| | Medically assisted reproduction |
| | Age of menopause |
| | Pre-menopausal or post-menopausal status |
| | Hormonal replacement therapy (type of hormone and duration of use) |
| **Social and cultural information** | Occupation |
| | Level of education |
| | Socioeconomic status |
| | Caregiver (h/day) |
| | Single, married, widows, divorced |
| **Lifestyles** | Diet |
| | Smoke (how long and how many cigarettes/day) |
| | Alcohol use (type and how many drinks/day) |
| | Physical activity (type and how many times a week) |

Modified from (Franconi et al., 2015).

## 9. Experimental models

### 9.1. Physiologically based pharmacokinetic (PBPK) modeling

The EMA defines PBPK as "*a mathematical model that simulates the concentration of a drug over time in tissue(s) and blood, by taking into account the rate of the drug's absorption into the body, distribution in tissues, or metabolism and excretion (ADME) on the basis of interplay between physiological, physicochemical and biochemical determinants*" (EMA, 2019), and it can be useful for studying pharmacokinetics (Chu et al., 2022; El-Khateeb et al., 2021). In view of sex differences, it is critical to include sex-dependent characteristics in PBPK modeling, which are often not considered (Naji-Talakar et al., 2021).

### 9.2. Humanized computational models

Humanized computational models enable the translation of relationships across species by adapting computational models derived from preclinical experiments (Brubaker and Lauffenburger, 2020). These studies compare high-throughput DNA and RNA sequencing datasets from animals and humans. It has been suggested that most sex-biased gene expression is species-dependent, and that the molecular iron-regulated surface determinants observed in humans are often not mirrored in non-human mammals (Naqvi et al., 2019). These results suggest that caution should be applied when generalizing sex-dependent molecular findings to humans by comparing gene expression profiles among various mammalian species. In conclusion, despite the progress made over the past 60 years, most humanized computational models of disease-related phenotypes remain poorly studied with regard to sex differences.

### 9.3. Three-dimensional (3D) models

Three-dimensional models still suffer from a lack of sex inclusivity and male bias. A 2022 review showed that, of studies involving organ-on-a-chip and organoid technologies, 27% and 5% used male and female cells, respectively; 53% did not specify sex, and 20% used both female and male cells but did not examine sex effects (McClain et al., 2024). It is clear that sex-based variability must be considered at every level when constructing 3D models, particularly in areas where sex differences are relevant. This is essential for obtaining relevant data to improve the accuracy of physiological models and enhance their applicability to the biology of all individuals.

### 9.4. Transgenic mouse models

Since 1989, genetically modified models have been widely used in biomedical research. To quantify the influence of sex on phenotype, data from 14,250 wildtype and 40,192 mutant mice were analyzed, revealing a significant number of sex-dependent mammalian traits in both wild-type and mutant mice (Karp et al., 2017; Mank and Rideout, 2021; Mohammed et al., 2021; van der Bijl and Mank, 2021). For example, only male animals with depleted renal androgen receptor showed

significant alterations of the ammonia cycle (Harris et al., 2021). Additionally, 8-month-old female p53-heterozygous mice exhibited a 50% higher incidence of spontaneous lymphoma than males (Mahler et al., 1998). Similarly, female Tg. AC mice (transgenic mice carrying the v-Ha-ras oncogene, used for skin carcinogenicity studies), were found to have a 50% higher incidence of spontaneous lung tumors than males (Mahler et al., 1998). In contrast, 26-week-old male rasH 2 mice exhibited a 16% incidence of forestomach tumors, whereas no tumors were observed in females (Yamamoto et al., 1996). Knowledge of the fact that genetic modification produces sex differences is a crucial factor for translating preclinical research into clinical practice. Despite the genetic modifications found in different mouse strains, the above data indicate that differences in sex-related physiological and pathological mechanisms still represent a critical aspect when designing preclinical experiments rigorously.

### 9.5. *In vivo and ex vivo models*

It is important to know the translatability of the model because cells and animal models often fail to reflect the sex differences observed in humans. As they focus solely on the biological aspects of human diseases (Miller, 2014), these models should be considered as a proxy for human biological phenomena (Mazure, 2016). Although appropriate animal models can be useful for screening sex differences, which can be highly variable within species (Parsch and Ellegren, 2013), the resulting data often do not inform human sex differences (Justice, 2024).

Male animals have predominantly been used so far because it is believed that females are more variable due to fluctuations in their estrous cycle. However, significant individual variability has also been demonstrated in male rodents, suggesting that females may not be more variable than males (Prendergast et al., 2014). Indeed, sexual hormones, primarily testosterone, exhibit circadian variations in male rodents too (Fig. 3) (Coquelin and Desjardins, 1982), and these fluctuations can significantly impact their behavior (Celec et al., 2015). However, the estrous cycle may affect some experimental results in a strain- and context-dependent manner (Chari et al., 2020; Lovick and Zangrossi, 2021), so its effects on the parameters under investigation should be tested preliminarily. Fig. 3 shows circadian variations of estradiol and testosterone in humans and rodents (Coquelin and Desjardins, 1982), which may affect male behavior (Celec et al., 2015).

Beyond hormones, genes also play an important role in sex differences. Many genes on sexual and autosomal chromosomes are sex-biased, depending on the species, organs, and often the cell type (Rodríguez-Montes et al., 2023). Humans have the highest number of sex-biased genes, followed by rabbits, mice, and rats. However, only a few of these genes are conserved across the species (Rodríguez-Montes et al., 2023). Notably, sex differences vary across species and lifespan (Beck and Meyerholz, 2020; Sabolic et al., 2011). Some of these differences are quite evident (*e.g.,* body dimensions) while others are less visible (or invisible) but no less important (*e.g.,* drug clearance and immune responses) (Mauvais-Jarvis et al., 2021).

Sexual dimorphism is widely present in mammals of the same species. However, there are no currently animal models available to investigate human male and female phenotypes (Rodríguez-Montes and Kaessmann, 2024). Investigating human sex differences in non-human models is very challenging because differences between the sexes vary

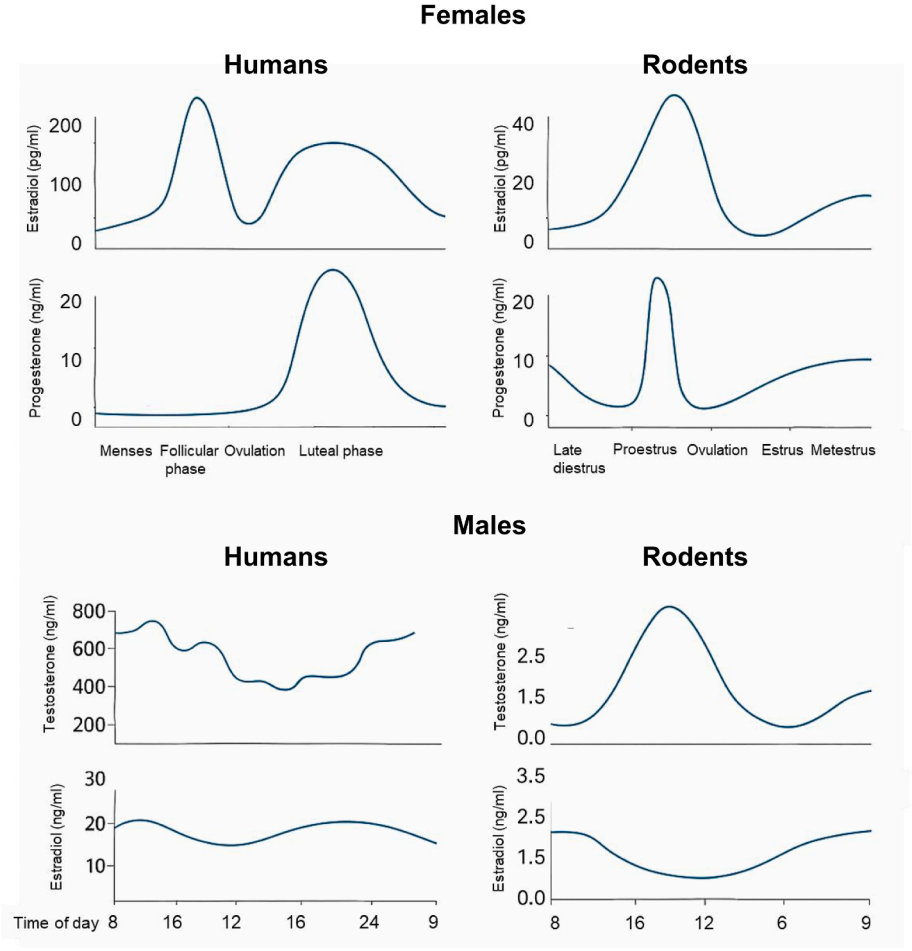


**Fig. 3.** Comparison of hormone fluctuations in humans and rodents. Upper panels show female estradiol and progesterone across the menstrual (humans) and estrous (rodents) cycles, while lower panels show male testosterone and estradiol across the day. Modified from (Dalla et al., 2024).

among common laboratory species and human life-cycle traits are not well represented in animal models (Richardson et al., 2015). While rats and mice predominate in preclinical research, other species can also be used to study sex differences, including fish, nematodes, and Drosophila (Rodríguez-Montes et al., 2023). The choice of experimental animal will depend on the hypothesis to be tested.

A multitude of human diseases present numerous sex differences (Campesi et al., 2021a,b; Franceschini and Fattore, 2021; Legato, 2017; Regitz-Zagrosek et al., 2016). From this perspective, achieving consistent results in preclinical research is necessary to ensure a translational approach in clinical research. This is currently quite difficult due to the predominant use of male animals (Beery and Zucker, 2011). The significant sex disparity displayed by human pathologies can also be seen in some animal models of human disease, particularly in terms of the incidence and progression of symptoms (Buoncervello et al., 2017). However, in other cases, the sex differences observed in animals do not mimic human diseases. For example, animal models of myocardial infarct and diabetes mellitus do not accurately reflect human conditions (Franconi et al., 2008; Seifirad and Haghpanah, 2019). For example, mice deficient in the insulin receptor die within seven days of birth (Accili et al., 1996), while the same mutation generates mild hyperglycemic conditions in humans (Ailhaud et al., 1992). Additionally, female rodents with type 2 diabetes mellitus did not show an increased risk of developing cardiac disease, whereas the risk was higher in diabetic women (Campesi et al., 2017; Campesi et al., 2021a,b; Franconi et al., 2008). Notably, despite the promising results of pharmacological and mechanical therapies in rodent models of myocardial infarction, their clinical translation in trials of patients with acute myocardial infarction has generally been disappointing (Geerkens et al., 2025). Therefore, sex differences in animal models cannot always be directly translated to humans (Mauvais-Jarvis et al., 2017).

Table 4 illustrates variations in sex differences across species, using renal transporters as a representative example.

Existing differences between animals and humans should not prevent researchers from studying animals; rather, they should emphasize the need for greater attention in choosing the animal model. Researchers should be aware that sex bias is one of the factors contributing to the poor translation of data into human terms and to the low replicability of results, and they should carefully evaluate the relevance and applicability of findings found in animals to human health.

## 10. Administration and dosage of drugs

Sex plays a key role in drug response and dosage formulation, and sex differences exist in the main four pharmacokinetic areas: absorption, distribution, metabolism, and elimination. Despite the differences in drug pharmacokinetics between males and females, it is surprising that sex-related dose recommendations do not exist for most drugs. In both preclinical and clinical research, it is now well recognized that body composition, metabolic profile, blood components, and behavioral traits affect drug safety and activity (Zucker and Prendergast, 2020). In particular, adverse drug reactions appear to be influenced by sex differences in body weight, among other factors. It is often assumed that adjusting drug doses according to body weight allows proportional reductions in females, potentially reducing adverse reactions. However, this is not always the case, as not all differences between males and females can be attributed to body weight. Even though females generally weigh less than males, many physiological and morphological traits do not scale proportionally with body mass (Wilson et al., 2022). Consequently, dose calculations based solely on body mass may overestimate the actual requirements in females, highlighting the risk of significant dosing errors when sex-dependent biological scaling is not considered.

Therefore, the differences between males and females (Tables 1 and 2) cannot be generalized, and females cannot simply be considered smaller versions of their male counterparts (Wilson et al., 2022).

Moreover, for most drugs, concentrations depend on both the volume of distribution and clearance. These, in turn, depend on body weight, independently of sex. Typically, females have a higher percentage of body fat than males, which can impact the volume of distribution of certain drugs. Additionally, glomerular filtration is reduced in females, resulting in decreased renal drug clearance (Table 1). Sex differences in metabolism are believed to be the main reason for different pharmacokinetic outcomes observed between males and females. Several different activities have been associated with specific isoforms of CYPs. Sex differences in CYP and uridine diphosphate glucuronosyltransferase activities, as well as renal excretion, imply differences in clearance and, consequently, in drug elimination (Yang et al., 2012). These considerations are valid for any route of drug administration, although the oral route has specific characteristics (Soldin et al., 2011). Based on the available literature, we can conclude that sex influences the safety and activity of several drugs.

Conversely, very little is known about excipients included in a given formulation. Interestingly, research has begun to identify sex differences in the effects of excipients on drug bioavailability. This suggests that the role of excipients in pharmaceutical formulations should not be underestimated. For example, it has been demonstrated that the widely used excipient polyethylene glycol (PEG) 400, which is a well-established solubility-enhancing molecule used to increase the bioavailability of poorly soluble drugs, modifies the bioavailability of ranitidine in a sex-dependent manner in rats (Afonso-Pereira et al., 2016). Notably, low doses of PEG 400 increased ranitidine bioavailability in male rats but not in females (Afonso-Pereira et al., 2016). In humans, sex-related differences were demonstrated when PEG 400, at doses relevant to pharmaceutical formulations, was added to ranitidine in healthy volunteers. Notably, PEG 400 increased ranitidine bioavailability in males, but not in females, with no differences observed between the sexes in the absence of PEG 400 (Ashiru et al., 2008). It is notable that excipients could have a significant influence on drug bioavailability, leading to reductions and/or increases in drug regimens for one sex but not the other, a phenomenon that warrants further investigation.

**Table 4**
Sex differences of renal transporters in different animal species.

| Transporters | Rats | Mice | Rabbits | Humans |
|---|---|---|---|---|
| **Oat 1/OAT1** | M > F | M > F | M = F | M = F |
| **Oat 2/OAT2** | M < F | M < F | M = F | M = F |
| **Oat 3/OAT3** | M > F | M < F | M = F | M = F |
| **OAT4** | ND | ND | ND | M = F |
| **Oat 5** | M < F | M < F | ? | ND |
| **Mrp4** | M > F | M < F | ? | ? |

M: male; F: female; OAT: Organic Anion Transporter, MRP4: Multidrug Resistance-associated Protein; ND: not detected. Data from (Celec et al., 2015; Coquelin and Desjardins, 1982; Miller, 2014).

## 11. The sex and gender of the experimenter influence experimental animals

Several studies have found that the sex and gender of the experimenter influence several outcomes in humans, including physical performance (Rikli, 1976), performance in intelligence testing (Back and Dana, 1977), autobiographical memory narratives (Grysman and Denney, 2017), hormone levels (Elliott et al., 2017), social behavior (Borden, 1975), and pain sensitivity (Kallai et al., 2004). Notably, men report less pain in response to nociceptive stimulation when tested by a female experimenter (Alabas et al., 2012).

There is compelling evidence that rodents can also differentiate the sex and gender of human experimenters, which can influence their behavioral and/or biological responses, as well as their response to drug treatment. For instance, the odor of a male researcher has been shown to evoke place aversion and increase stress-induced analgesia and anxiety-related behaviors in both male and female mice (Sorge et al., 2014). Interestingly, in mice and rats, the presence of a male researcher caused

a stress response comparable to that induced by 15 min of restraint in a tube or 3 min of forced swimming, a stress-induced reaction that renders animals of both sexes less sensitive to pain (Sorge et al., 2014). As this male-experimenter-induced response diminishes over time, it is recommended that the experimenter remain in the room with the animals during the habituation period prior to testing. The sex and gender of the researcher also affect the response of mice to ketamine, with mice "preferring" female researchers because they are more susceptible to stress in the presence of male researchers (Georgiou et al., 2022).

In addition, it has been reported that male rats are more susceptible than female rats to the presence of a male experimenter during transiently stressful experiences. Female rats, on the other hand, display intensified anxiety-related behaviors associated with higher or lower circulating levels of corticosterone and oxytocin, respectively, when tested by a male experimenter (Faraji et al., 2022). As these psychophysiological changes may significantly affect the outcomes of preclinical studies, policy papers have begun to emphasize the importance of reporting the gender of the experimenter in future studies (Stewart et al., 2015).

Notably, the sex and gender of the experimenter may affect replicability in rodent-based experiments, whereas it seems to have little influence on behavioral responses in the zebrafish model (Lieggi et al., 2020; Stewart et al., 2015). This is likely because zebrafish are less exposed to experimenter-specific odor and pheromones than rodents due to living in water tanks.

Additionally, male and female researchers focus and interpret results differently (Gowaty, 1997). Beyond considerations of animal sex, these data suggest the need to consider and report the sex and gender of researchers to increase the accuracy of data and interpretation.

## 12. General conclusions

It is time to acknowledge the critical role of sex in preclinical research, starting from the earliest phases of investigation, in order to increase the scientific rigor of biomedical studies. The persistent global sex bias in research may undermine the validity and generalizability of biological knowledge. Indeed, sociological investigations have shown that researchers recognize the importance of sex as a biological variable, yet actual changes in research practices have remained limited (Gaskill et al., 2025). Changing the status quo requires overcoming implicit biases and aligning research practices with researchers' stated beliefs and values (Gaskill et al., 2025). In other words, adopting best practices in sex-based research demands a substantial cultural shift, leading to changes in perceived norms and the removal of existing barriers. These barriers include ethical, financial, methodological, and practical constraints (Karp and Reavey, 2019; Medical Reasearch Council, 2021).

Moreover, it is essential to ensure adequate training in sex-based research and to foster the development of a new generation of researchers with expertise in this area. Sex research requires more time and economic resources, so it should be incentivized with economic (and non-economic) measures for all involved. A good incentive for the pharmaceutical industry, which requires sex-aware research in all phases of research, could be a small extension of patent time, as is the case for pediatric drugs. Academic researchers are valued based on their scientific output. If they conduct sex-aware research, which is more expensive than other biomedical research, they should receive greater financial support. Additionally, given that sex-aware investigations are more time-consuming, they should be evaluated accordingly. Offering incentives could make this topic more attractive and encourage new and young researchers to engage in this area after receiving the appropriate training.

The pitfalls of sex investigation require a multidisciplinary research team which includes both men and women (see above). Importantly, the integration of all factors that sex variable that can change over time (*i.e.,* sex contextualism) requires is still a challenge. In addition, in view of the fact that sex comprises multiple variables, it is necessary to adopt rigorous study design, as well as pre-analytical and analytical methods, to improve research accuracy. Indeed, understanding the intricate influences of sex has become paramount for deciphering its roles in health and disease. It is also necessary to accurately and appropriately analyze any significant sex differences, including stress.

A peculiar attention is required in the choice of animal species and in the experimental models of diseases because animal sex differences may not always be applicable to humans, as well as not all sex differences found in experimental models of diseases can translate to human populations.

As current guidelines and recommendations have not led to sufficient progress, new and stricter regulations and mandates regarding the inclusion of female cells, animals, and women should be enforced in the pharmaceutical industry. Agencies could contribute more by requiring that sex-aware research should be an indispensable part of drug registration, as it is a pillar of personalized and holistic medicine.

Finally, the application of sex-aware research is a mandatory step in personalized medicine and it could give a great contribution to promote equity in health though information and education of decision-maker.

## CRediT authorship contribution statement

**Flavia Franconi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Data curation, Conceptualization. **Vincenzo Brancaleone:** Writing – review & editing, Writing – original draft. **Ilaria Campesi:** Writing – review & editing, Writing – original draft. **Maria Grazia Cattaneo:** Writing – review & editing, Writing – original draft. **Liana Fattore:** Writing – review & editing, Writing – original draft. **Armando Genazzani:** Writing – review & editing. **Maria Grazia Morgese:** Writing – review & editing, Writing – original draft. **Antonietta Rossi:** Writing – review & editing, Writing – original draft. **Luigia Trabace:** Writing – review & editing, Writing – original draft, Conceptualization.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2026.178804.

## Data availability

No data was used for the research described in the article.

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