Sex in basic research – Concepts in the cardiovascular field

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Abstract

Women and men, female and male animals and cells are biologically different, and acknowledgement of this fact is critical to advancing medicine. However, incorporating concepts of sex-specific analysis in basic research is largely neglected, introducing bias into translational findings, clinical concepts and drug development. Research funding agencies recently approached these issues but implementation of policy changes in the scientific community is still limited, probably due to deficits in concepts, knowledge and proper methodology.

This expert review is based on the EUGenMed project (www.eugenmed.eu) developing a roadmap for implementing sex and gender in biomedical and health research. For sake of clarity and conciseness, examples are mainly taken from the cardiovascular field that may serve as a paradigm for others, since a significant amount of knowledge how sex and estrogen determine the manifestation of many cardiovascular diseases (CVD) has been accumulated. As main concepts for implementation of sex in basic research, the study of primary cell and animals of both sexes, the study of the influence of genetic versus hormonal factors and the analysis of sex chromosomes and sex specific statistics in genome wide association studies (GWAS) are discussed. The review also discusses methodological issues, and analyses strength, weaknesses, opportunities and threats in implementing sex-sensitive aspects into basic research.

Key words: sex, basic research, chromosomes, hormones, animal models, cardiac cell models

Introduction

Women and men are biologically different at the level of the cells, the organs and the organism. While sex refers to biological differences between males and females, in terms of genetics, epigenetics and endocrinology, gender refers to sociocultural status. Gender aspects are specific to humans, while sex differences can be studied in animal models and isolated cells. Knowledge on sex specificity in animal models, on different metabolic pathways and physiology is needed for interpretation of human diseases. Yet, in many research fields the proportion of studies utilizing male and female animals favors males.[1] This bias occurs even in the majority of transgenic mouse strains with cardiovascular or immunological phenotypes where significant sex differences are obvious. Furthermore, there is ongoing scientific debate about the benefits of preclinical studies of sex differences, when balanced against the potential harm of introducing conceptual and empirical errors into research.[2]

Drug development is getting more and more difficult and costly, and new approaches are needed. The philosophy of precision medicine asks us to replace the “one size fits all” paradigm by more targeted approaches. Understanding sex specific mechanisms and deciphering why preferentially one sex or age group is protected or affected shall lead to opportunities of developing better therapies for all. All the sex specific differences impact understanding of physiology, pathophysiology and response to therapy.

The impact of sex and gender is particularly well studied in the field of CVD (Fig 1). Sex and gender influence CVD by their effects on heart, brain, heart /brain interaction, their effects on the vasculature and the peripheral muscle, liver and kidney, drug metabolism and excretion. This has recently been reviewed elsewhere by our Eugenmed group.[3] Therefore, we also chose CV research as a main area for the present review and analyze how introducing sex specific aspects in basic research will open new paradigms in understanding human disease.

The aim of this review is not to cover in a comprehensive manner all approaches to analyze sex in basic CV research and we refer to previous work for this purpose.[4, 5] In contrast, we aim at presenting concepts, mechanisms and best practice examples mainly from Europe but including also leading scientists from other areas of the world, as they were identified in the FP 7 funded project EUGenMed (www.eugenmed.eu). Not only research findings are discussed but also resources (Table 1)[6] and principles for basic research on sex differences with their strength, weaknesses, opportunities and

threats.

Methods

The present materials have been gathered within the interdisciplinary EU funded project EUGenMed (FP 7, www.eugenmed.eu/). EUGenMed aimed at building a roadmap for implementation of sex and gender in European biomedical and health research. This expert review is part of this road map.[3] It is built on a systematic collection of the literature in our database "*gendermeddb*" that contains more than 13,000 references on sex and gender in medicine and basic research, including major reviews on research strategies and educational resources (Table 1) and the analysis of this database in the EUGenMed project. We also screened PubMed with the same search terms for most recent publications that were not yet included in the database.[7]

The selection of the main focus, cardiovascular research, is based on the result of the EUGenMed process (www.eugenmed.eu). Legitimation of the writing group has been achieved by selecting this group of experts from a large set of European stakeholders in gender medicine. This was done at the EUGenMed kick-off conference in an open, transparent process. Experts were invited to 4 conferences and a workshop held in Berlin and developed together the present paper.

Table 1:

Resources on sex in basic research
http://www.eugenmed.eu/
http://gendermeddb.charite.de/
http://sgbmeducationsummit.com/
https://genderedinnovations.stanford.edu/
http://sgwhc.org/#sthash.T25i3nzd.dpbs
http://www.cihr-irsc-igh-isfh.ca/
https://www.sexandgendercourse.org/
https://gender.charite.de/en/education/elective_courses/
http://www.isogem.com/

Mechanisms for sex differences: Sex chromosomes, sex hormones

Primary factors causing sex differences are sex chromosomes, which are present in every cell type and differ between males and females, followed by maternal and paternal imprinting, by incomplete X-inactivation and epigenetic modification (Fig. 2).[8] They induce early in embryogenesis gonad development and the synthesis of sex hormones.

Sex hormones, synthesized in the gonads or extragonadal tissues, interfere with the effects of sex chromosomes. Notably, testosterone is converted to estradiol by aromatase in many organs. Activational effects of sex hormones, that requires presence of the hormone and organizational (delayed) effects that result frequently from epigenetic modifications and persist in absence of hormones must be separated. Sex differences in transcriptomic regulation may arise from purely genetic differences XX vs XY, from maternal or paternal imprinting, but also from secondary epigenetic modifications and effects of hormones. The brain plays a major role as it controls hormone production via the Hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal axes, the growth hormone system, and finally behavior.

Developmental origin of disease

In line with the new paradigm of the Developmental Origins of Health and Disease (DOHaD), and throughout the life cycle of ancestors, parents and offspring, the environmental factors to which an individual is exposed throughout life can leave an epigenetic footprint on the genome that dictate the coordinate expression of genes.[9] Non-genetic and non-cultural heritability of susceptibility/resilience to

common chronic diseases often show sex-specific differences. This is due not only to the chromosomal sex (XX or XY) before gonad differentiation, but later on, to a complex intermingling of both hormones and X/Y genes regulating autosomal genes through epigenetic processes. Crucial periods are gametogenesis and the early development, where the individual's epigenome is particularly sensitive to the effects of the environment, building up the individual's health capital to respond more or less well to the vagaries of life and most often in a sex-specific manner.[10] Changes in sex differences for epigenetic marks and modifiers also revealed the existence of different adaptation mechanisms in males and females.

Hypothalamic-pituitary-adrenal axis

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is associated with increased risk of depression, the metabolic syndrome and accelerated cognitive decline as a person ages. Activity of the HPA axis is 'programmed' *in utero*: overexposure of the developing fetus to excess glucocorticoids is associated with low birth weight and increased reactivity of the HPA axis with associated adverse health including cardiovascular risk factors, cardiovascular diseases (CVD), asthma and poorer cognitive function.[11] Sex-specific differences in early life programming of the HPA axis in humans may underpin the observed sex differences in these diseases. Psychosocial stress and glucocorticoid medications affect placental glucocorticoid biology and HPA axis function in early- and later-life. Female offspring have increased diurnal cortisol secretion and HPA axis reactivity, compared to males.[12] Further, permeability of the female placenta to maternal glucocorticoids increases following maternal stress. Changes in placental permeability are associated with changes in the expression of 11β-hydroxysteroid dehydrogenase enzymes in the newborn. Thus, sex differences in the effects of maternal stress and in the placental handling of glucocorticoid hormones may be a mechanism underlying sex differences in diseases later in life including depression and cardiometabolic disease.

Sex hormones and the brain

Sex differences in brain morphology have been described in both rodents and humans in many different areas such as hypothalamus, amygdala, hippocampus, cortex and others. Differences are present in the volumes of brain nuclei, cell numbers, synapse number, and expression of genes/proteins.[13] However, for the majority of these sex differences it is still not clear how they exactly develop, and what is the connection between particular sexually dimorphic brain structure and behavior, diseases of peripheral organs or psychiatric illnesses. Although majority of sex differences in the past have been attributed to the action of sex steroid hormones, recent studies suggest that brain sexual differentiation is not simply a consequence of masculinization of male fetal brain by testosterone.[13] Prepubertal exposure to estrogens might be responsible for active feminization of mouse female brain, and several studies in rodent models have shown contribution of sex chromosomes to the sexual differentiation of certain behaviors such as aggressive and parental behavior, social interaction, and others. Epigenetic regulation also contributes to the sexual differentiation of the brain.[14, 15] The effect of these differences affects disease related behavior and thereby outcome of diseases in the human.

The X chromosome and Genome wide association studies

Genome wide association studies (GWAS) have advanced our understanding of the genetics of complex diseases. However, most of the GWAS analyzed the 22 autosomal chromosomes only so that, although the X chromosome constitutes 5% of the genome and underlies almost 10% of Mendelian disorders, it harbors only 15 of the 2,800 associations reported by GWAS of nearly 300 traits.[16] There are various reasons for not including the X chromosome in GWAS: i) poor coverage, ii) increased workload owing to sex-specific quality control, iii) power issues owing to a smaller sample size, and iv) the requirement for specific tools.

Such specific tools are needed because males and females have unequal numbers of X chromosomal loci. This needs to be addressed in the genotype-calling step and has consequences for genotype imputation and association analyses.[17] Additionally, in the process of X-inactivation, large parts of one of the female X chromosomes are silenced, so that one copy in males and two copies in females have equal effects.[8] X-inactivation is incomplete, and it is estimated that about three-quarters of X chromosomal genes are silenced in one female X chromosome in some individuals. This is important when deciding

how to test for associations with X chromosomal variants as described recently.[17]
In future GWAS, the inclusion of X chromosomal data might partly explain the missing heritability of complex diseases, especially those with sex-specific features.

Epigenetic control of gene regulation

Sexual dimorphisms arise due to a combination of genetic determinants and environmental cues which are frequently transmitted by epigenetic regulation. Including DNA methylation, non-coding RNAs and histone modifications, epigenetic regulation is essentially involved in S&G-specific gene regulation.[18, 19] Imprinting is a well-known epigenetic process of allele-specific gene regulation dependent on the parent of origin. Whether the maternal or paternal alleles of imprinted gene clusters are expressed is independent of the underlying sequence, but mainly determined by DNA-methylation and certain histone modifications. Another epigenetic control of gene expression is the X-chromosome inactivation that is specific to females and describes the random inactivation of one X-chromosome by an lncRNA.[20]
More recently, studies have been addressing the question of whether there are sex-specific epigenetic modifications of both alleles. Indeed, several autosomal sex-dimorphic DNA methylation sites as well as histone modifications have been identified in different mouse organs and were often linked to sexually dimorphic expression patterns.[21] Since most studies so far are limited on single epigenetic marks in one tissue and mouse strain, it would be advantageous in the future to integrate data from studies of epigenetics, gene expression and protein abundance.

Sex differences in transcriptomic regulation

The limited approaches for genome-wide expression profiling of the heart under physiological conditions indicate that there are relatively few genes with a sexually dimorphic expression, which actually seem to be sex chromosome-linked.[22] The situation changes dramatically under pathological conditions. In pressure overload-induced hypertrophy, the response of the cardiac transcriptome significantly differs between men and women.[23] In response to pressure overload, fibrosis and inflammatory pathways are increased, while those associated with energy-producing processes are decreased in hearts from males. In contrast, in heart from females, pathways associated with energy production are increased and those associated with fibrosis-related and inflammatory processes are decreased. Other whole-genome profiling studies reported sex-specific transcriptomic differences in end-stage heart failure and in new-onset heart failure.[24] Sex and age interact on cardiac protein expression, with an upregulation of pro-inflammatory and pro-apoptotic proteins in males and angiogenetic and cytoskeletal proteins in females and a downregulation of cytoskeletal proteins in males and of integrin signaling in females (Fig 3).[25-27] Moreover, there is good evidence that estrogen affects gene expression in the heart in a sex-specific manner, as discussed below for collagen synthesis.[28-30]

Sex hormone receptors

Key component in expression of sex differences are the signaling pathways activated by the estrogen and androgen receptors (ERs, AR). ER and AR belong to the family of nuclear receptors and are important regulators of a plethora of cellular events and strong epigenetic modulators. Two ERs, ERα and ERβ, bind to the DNA and function as ligand-induced transcription factors thereby regulating gene expression and cell function.[31] In addition, activation of ER that are localized to the plasma membrane results in signaling cascade activation, such as ERK/MAPK and PI3K.[32] ERα and ERβ can regulate gene expression differentially within the same tissue or cell[33] and they can exert different effects in females and males.[28] These differences may be attributed to either sex differences in DNA and histone modifications, in co-factor expression or different levels of ERα relative to ERβ. Therefore, the preponderance of one of these ER over the other, and their expression at the cell surface (mER) and access to nuclear DNA might change the impact of estrogen activity, as discussed below in more detail. Estrogen can also bind to a newly described orphan G-protein coupled receptor (GPR30), which is located at the cell membrane and can acutely activate signaling kinases.[34]

Sex differences in major cellular functions

Sex and estrogen exert a plethora of effects in all CV cells and on almost all cellular functions. As these have been reviewed in detail recently[4, 5] (Fig 3) we focus in this review on 3 best practice examples for mechanisms that affect almost all CV cells, cardiomyocytes, fibroblasts, endothelial and smooth muscle cells.

Sex differences in cell death and survival

XX and XY cells have different susceptibility to undergo apoptosis, anoikis, autophagy or senescence. The response of cells from males and females to the same stress, e.g. oxidative, leads to a different fate, i.e. XX cells are more resistant to microenvironmental injury and to death insults than cells from males, and survive better, e.g. undergoing autophagic cytoprotection.[35] Estrogen, through nuclear and surface estrogen receptors, modulates cell survival and death signaling pathways.[36] (Fig 4) In particular, the activation of the extracellular signal–regulated kinase (ERK) pathway, i.e. ERK phosphorylation, after non -nuclear ERα ligation, appears capable of activating an autophagic cytoprotection cascade. Furthermore, some pumps at the cell surface, able to maintain intracellular milieu, are as well up-regulated by estrogen signaling pathways.[37] It can be hypothesized that these two mechanisms can partially explain the higher propensity of cells from females, in which the estrogens-ER binding predominantly occurs, to counteract exogenous stress activating an autophagic cytoprotection response.[38]

Mitochondrial function

Mitochondria exhibit a strong gender-specific behavior as they are exclusively maternally inherited and exert differential effects in males and females. Because of this exclusive maternal transmission, the interest in the role of mitochondria and sex determination is growing. Most of the mitochondrial proteins are encoded by the nucleus; therefore, mitochondrial structure and function are tissue-specific and subjected to sex-specific influences. In addition, ERs are also present in mitochondria, promoting mitochondrial biogenesis, respiratory activity and signaling pathways for protection against oxidative stress which is related to a number of CV pathologies.[39]

Sex differences in mitochondria potentially include energy production, defenses against oxidative stress, substrate utilization, calcium regulation, mitochondrial biogenesis and mitophagy and mechanisms of apoptosis (Fig 4)(for review[4, 5]). For example, mitochondria from females have higher resistance to ischemia/reperfusion injury because they produce less reactive oxygen species (ROS) and have higher antioxidant capacity. Female rodents have altered posttranslational modification of several mitochondrial proteins, including ALDH2, a protein that is involved in cardioprotection, suggesting that altered phosphorylation of mitochondrial proteins alters ROS handling in female mitochondria.[40] Genes involved in metabolism and mitochondrial biogenesis show different patterns of regulation in female compared to male mouse hearts that might contribute to the lower severity of heart failure in females.[28] Female rats are much less sensitive to the cardiotoxic effects of anthracyclines by mechanisms involving mitochondria.[41] Whether a similar difference is present in human heart remains to be explored.

Fibrous tissue synthesis

Cardiac fibrosis leads to global heart dysfunction and is a major predictor of heart failure. In humans, sex differences in cardiac fibrosis exist under specific pathological conditions. For example, in aortic stenosis, men show higher collagen deposition associated with higher activation of pro-fibrotic markers compared with women.[42, 43]

Similar to the human condition, hearts from male mouse show more cardiac fibrosis under pressure overload, correlated with higher activation of pro-fibrotic genes, compared to hearts from females.[44] 17β-Estradiol, through activation of ERα and ERβ, decreases the development of fibrosis in hearts of female mice. Only few studies compared ER signaling on cardiac fibrosis in both sexes. In a mouse model with pressure overload induced myocardial hypertrophy (MH), ERβ limited fibrosis in hearts from females, but promoted it in males.[28] Possible mechanisms include activation of ERK signaling and control of collagen synthesis via ERα or sex specific phosphorylation of ERα and ERβ (Fig. 5). Hearts of female mice show significantly less ERβ-modulated miRNA induction compared with those from males.[29] *In-vitro* studies, using rat cardiac fibroblasts from both sexes, delineate the sex-dimorphic regulatory role

of E2/ER on pro-fibrotic gene expression.[30]

Translational approaches

Translational approaches, i.e. studies spanning the bridge from experimental model systems to the human, or vice versa, often do not consider sex or sex differences. There are a few exceptions: first, sex differences in DNA methylation predict sex differences in CV phenotypes in animal and cell systems and in the human. Second, sex differences in cardiac metabolism and related phenotypes may be translated from mice to men. Third, studying the interaction between pregnancy and CVD in experimental systems and in the human may be considered a translational approach.

Sex differences in epigenetics

Epigenetic modifications represent the mechanism by which the environment influences the genome and gene expression. Intrauterine undernutrition leads to sex specific promoter methylations in metabolic and cardiovascular genes.[45] In an experimental study, intrauterine hypoxia led to greater PKCepsilon depression in male than in female hearts of fetuses and adult offspring. Hypoxia-induced methylation of SP1 sites in the PKCepsilon promoter was significantly greater in males than in females, and this was associated with greater depression of PKCepsilon and sensitivity to ischemic injury in the males.[46] Patients with heart failure present an altered promoter methylation in genes involved in contractility, fibrosis and apoptosis;[47] however it remains to be established whether DNA methylation state participate in the gender-specificity of these genes.[22, 23] Lower global leukocyte DNA methylation was associated with higher cardiovascular risk in postmenopausal women.[48] Sex specificity in DNA methylation may be mediated by the fact that DNA modifying enzymes, i.e. histone acetyl transferases CBP and p300 are recruited to the DNA by estrogen and androgen receptors and that DNA de/methylases are expressed in a sex-specific manner.[8]

Lipid and glucose metabolism in the myocardium

In a number of models, based on studies in mainly male rodents, HF shifts myocardial metabolism away from fatty acid and towards glucose metabolism. Since glucose is a more oxygen-efficient fuel than fatty acids, this was first considered to be beneficial, in particular in ischemic conditions. However, it now becomes apparent that this shift leads to insulin resistance and earlier functional deterioration. Female animals did better in non-ischemic HF models than males and this was associated with better preservation of mitochondrial metabolism and fatty acid utilization.[28, 49] Translation of this sex difference to humans has recently been accomplished. In human left ventricular remodelling under pressure overload, sex-dependent regulation of metabolic pathways occured with a less severe decrease in mitochondrial gene expression in the female than in the male heart.[23] Moreover, healthy women have a greater capacity for myocardial fatty acid oxidation than men a characteristic that is preserved in HF.[50]

Pregnancy complications and later CVD: focus on vascular function

A woman's reproductive history serves as a predictor for later risk of CVD. Preeclampsia (PE), a disorder peculiar to human pregnancy, is characterized by concomitant occurrence of hypertension and proteinuria.[51, 52] Women with a history of PE have higher CVD risk if compared to women with normal pregnancy. PE women delivering preterm and mothers with recurrent PE carry even greater risks for later CVD and kidney failure. Being the mother of growth restricted baby or a preterm infant also increase the risk of CVD later in life. PE and CVD share risk factors such as diabetes, obesity or hypertension, and pathogenetic mechanisms such as oxidative stress, endothelial dysfunction and insulin resistance. In women who develop PE, the threshold for clinical CVD is breached during pregnancy and subsequently again later in life, as increasing age is added to the already present and/or newly acquired CVD risk factors. In this way, adverse pregnancy outcomes may reveal women at increased risk of CVD in later life.[3]

Drug development

More and severe adverse effects of drugs in women than men led to drugs withdrawn from the US market between 1997 and 2000 (US general accounting office 2011 Drug Safety). Indeed, new drugs

often fail in the phase 3 studies. Deficits in correspondence of animal models to the human study settings, i.e. participant selection, may play a role. The new technical possibilities to study the "omics" help to select sex-specific targets. Recently, sex differences in omics have been evidenced also in adult and neonates of humans.[53] However, sex differences appear to be organ- and stimulus specific, and these variables have to be considered in the experimental approaches.[54]

Different life phases of women and men are not sufficiently considered in drug development. The decline of the endogenous production of hormones, in particular, estrogen at menopause, often leads to functional disorders. In a more general manner, it will be mandatory to study the interaction of sex with age in women and men. Finally, it is relevant to recall that the pharmacodynamic aspects should be considered more intensely in sex-specific drug design.[55]

Sex differences in preclinical research

Most preclinical research in drug development is done using male animals and cells with unidentified sex.[56, 57] However, significant differences exist in the outcomes of male and female mice in models of myocardial infarction, pressure overload and genetic CVDs, diabetes mellitus, multiple sclerosis or other diseases that are often not considered by the researchers.[54] As extreme consequences, a drug or gene modification may be effective in a male animal model and completely ineffective in females on some outcome parameters, or vice versa.[58] For example, transgene overexpression of melusin, a muscle-specific chaperone protein capable of ERK1/2 signaling activation in the heart, reduced early mortality after myocardial infarction in male mice but failed to do so in female animals.[58, 59] (Fig. 6)

Structure-function of estrogen receptor in vivo: optimization of its modulation in medicine

Estrogens display protective effects on the development of atherosclerosis and type 2 diabetes in animal models.[60, 61] ERα, but not ERβ, is necessary for most of the arterial and metabolic actions of E2. Estrogens also elicit deleterious effects on the uterus and breast as well as increase risk of venous thromboembolism. These two deleterious actions represent the main limitation and Achille's heel of classic estrogen therapies and may have contributed to the negative results of the Women Health Initiative.

The full length ERα is composed of 6 domains containing the 2 independent activation functions AF-1 and AF-2. Owing to specific transgenic mouse models, the respective roles of AF-1 and of AF-2 activation functions, and the «membrane initiated steroid signalling» (MISS) could be elucidated as well as their physiological roles in the proliferative effects of E2 on sex target, arteries and metabolism.[62, 63]

Selective estrogen receptor modulators (SERMs) have a highly tissue-specific action. Indeed, SERMs are molecules that retain some desired/beneficial actions of estrogens (on bone for instance) and oppose some deleterious effects particularly on breast (ER positive breast cancer proliferation and recurrence). A challenge is thus to develop new SERMs based on the uncoupling between the beneficial effects of E2 and its proliferative effects on reproductive targets and/or its venous pro-thrombo-embolic effects. For this purpose new SERMs or combination of estrogens with a SERM with potentially greatly improved safety profile have been developed.[64]

Cardiac function, testosterone and PDE5-inhibitors

Sex-specific clinical characteristics have been discussed related to estrogen levels. However, several studies have also found relationships with varying levels of testosterone. For example, lower testosterone and higher E2 levels correlate with increased risk of CVD and CV mortality in men. Testosterone replacement therapy (TRT) in hypogonadism moderates metabolic components associated with CV risk, but it remains unclear whether low testosterone is an actual cause-effect relationship.

The androgen receptors are present in cardiac myocytes from multiple species, including men and women. Androgen exerts a hypertrophic effect via a direct AR-mediated pathway, while loss of androgens due to castration in men or AR antagonist remarkably reduces cardiac hypertrophy and fibrosis. In clinical setting, male patients with heart failure present deficiencies in circulating androgens, including testosterone, and the androgen level is an independent predictor of poor outcome.[65]

Androgens regulate the cGMP-specific phosphodiesterase 5 (PDE5) expression and functional activity in cardiac tissue. PDE5 is overexpressed in cardiac hypertrophy and in ischemic cardiomyopathy. PDE5 inhibitors have provided cardioprotection against a broad range of heart diseases in experimental and

clinical studies and are discussed as new treatment options for heart failure.[66] However, a large clinical trial testing the efficacy of PDE5 inhibitors in patients with heart failure, RELAX, mainly enrolled male patients and failed. After the failure of the RELAX trial, animal experimental work revealed the reason why the trial design was less than optimal. The PDE5 inhibitor sildenafil ameliorates cardiac failure caused by Gαq overexpression or pressure overload through an estrogen-dependent mechanism in female but not male mice.[67] This observation shows the importance of quality pre-clinical work and the need for sex-specific consideration in general and in the use of PDE5 inhibitors in heart failure. The registered "RECOGITO" trial (NCT01803828) has subsequently been designed to measure gender differences in response to PDE5i in cardiac remodeling occurring in patients with type 2 diabetes.

Principles for basic research on sex differences

Study primary cells of both sexes

Cultured cells are largely used to identify molecular-signaling pathways. Nonetheless, recent surveys of the literature report poor acknowledgement of the sex of the cells. In a review of the ten cardiovascular journals with impact factor, only ≈20-28% reported the sex of cells.[68] In a survey of a recent issue of the American Journal of Physiology Cell Physiology, 75% of all publications did not report the sex of cell lines or animals.[69] Studying differences in primary cell lines would be of valuable interest to decipher hormonally driven from intrinsic differences in male and female cells unrelated to hormonal exposure.[69]
The development of high-throughput screening assays to identify and develop drugs for various human diseases is largely based on the use of cell lines or primary cells. Considering the sex disparity in disease severity and response to drugs, the question of whether the screening should be made on male or female cells or on both sexes is important and must be included in the interpretation of results.[69] Indeed, many stroma cells produce sex hormones, express their receptors and change during culture. Estrogen receptors vary during culture passage at least in rat aortic vascular smooth muscle cells.[70] Permanent cell lines are reported to lose their sex chromosomes. Therefore, sex chromosome complement of the cells and production and expression of sex hormones in the cells under study needs to be determined before analysis.

Study animals of both sexes

The large majority of studies using experimental animals including transgenic ones use only males. Most male biases are encountered in pharmacology, physiology and neuroscience, and female bias in immunology.[1, 56] For example, some of heart failure animal models present major sex differences and similar differences are found in other diseases.[5] Today, animal testing is commonly used in preclinical studies for drug development. It is therefore of extraordinary relevance and importance to understand and to validate these tests for each sex. However, inclusion of sex needs caution when extrapolating to humans. For example, in contrast to humans, in some mouse strains, male animals are more susceptible to type 2 diabetes mellitus and have more severe disease than females.[71] This is however not true for all strains and some studies indicate that tissue injury in diabetes in females may occur with less pronounced hyperglycemia and glucose intolerance.[72] Additionally, particularly in the rat, females show less ischemia–reperfusion injury; however, this is not observed in all animal studies.[73]
The argument that females are more variable due to estrus cycle and thus increase variability has been questioned.[1, 56, 74, 75] Indeed, females are less variable than males for several endpoints and estrus cycle related variability does not need in general to be controlled in female mice.[74, 75] On the opposite, variability may be increased when male and female sexes are mixed. Regular reassessment of animal models can help to identify sex differences and human relevance of each model for sex specific research. Finally, the international differences in the usage of soy in fabrication of experimental animal diets have sex specific effects on expression of cardiac pathology in particular.[76]
In conclusion, accounting for sex (as well as other biological variables such as age and hormonal status) increases transparency and enhances reproducibility in results among laboratories.[77]

Study genetic versus hormonal influence and include sex chromosomes in GWAS

In recent years, two genetic mouse models have been developed to provide insights into the interaction of sex chromosomes and sex hormones. This is first the four core genotype (FCG) mice, with the

translocation of SRY gene on an autosome. This translocation results in two extra geno/phenotype combinations.[78] In addition to WT females (XX) and males (XY), there are animals with two X chromosomes and testes (XX^{sry+} males) and animals with X and Y chromosomes with ovaries (XY^{sry-} females). In these mice, the genetic sex does not correspond to their phenotypic sex, although they are still exposed to sex steroid hormones during development, but not appropriate for their karyotype. Another mouse model, steroidogenic factor 1 knockout mice (SF-1 KO), completely lack gonads due to gonadal agenesis early during development.[79] Both of these models, FCG mice and SF-1 KO mice, have shed important information, e.g. about the contribution of sex chromosomes to the sexual differentiation of the brain and other organs.

To detect genetic bases for sex differences, all chromosomes, including the sex chromosomes, must be included in genetic analysis. To overcome the hurdles of X chromosomal analyses, pipelines for analyzing X or Y chromosomal data within a standard GWAS have been established. By selecting specific algorithms and parameter settings, the analysis of X and Y chromosomal SNPs is manageable and gives new clues as to the genetics of complex diseases.[17]

Strengths, weaknesses, opportunities and threats of present approaches

At a time of personalized medicine and precision medicine, a special attention to sex specific mechanisms to unravel the impact of cellular XX vs XY chromosomes, and their interaction with effects of estrogens versus androgens during the fetal period and lifetime is needed for defining homogenous target groups. Strengths of sex specific approaches include the power to detect new pathways in females and males, and to describe better the effects of sex hormones and their interaction with age, ethnicity, and environmental conditions, to reduce variability in animal models by analyzing homogenous groups with well-defined sex and sex hormone status. (Fig. 7)

Weaknesses arise from extrapolating reductionist findings from animal models to complex human beings. Naturally, the relevance of mice or rats for extrapolation to humans must be questioned. Sex differences interfere with genetic, i.e. strain differences. Moreover, adequate animal models for menopause transition are lacking. Surgical ovariectomy in young female mice eliminates all ovarian tissues and ovarian hormones, LH, FSH and progesterone, including testosterone synthesizing stroma cells, and not only ovarian follicles as is the case in natural menopause.[80]

Problems arise since isolated cells and particularly permanent cell lines may modify or loose sex chromosomes, which can lead to very specific behavior and limit their usefulness. Thus, confirmation of the sex chromosome content of a cell line under investigation is mandatory. However, all preclinical research is subject to criticism for reductionist approaches and it may be overcome by careful and critical selection of models.

Opportunities include the power to detect new drugs that fit women or men better, that may even act in females or males only and to understand new and hormone-driven mechanisms in pathophysiology.

Threats arise from the misconception of researchers, and deficits in knowledge of suitable models and specific research tools, on the cost-effectiveness of the approach, and the limitations of the *in vitro* settings for modeling sex.[2, 81] However, these questions are far not confined to sex differences but rather address all preclinical research. It must be acknowledged that studying sex requires expertise and knowledge to develop significant research hypotheses and highly specific tools to answer these questions.

Views from non-European countries

Views from Canada

In 2010 the Canadian Institutes of Health Research (CIHR) began to require all grant applicants to answer questions about whether and how they address S&G in basic science research.[82] CIHR's Institute of Gender and Health recognizes that sex differences in the occurrence of pathologies and therapeutics is a complex interaction between biological factors (sex) and social, historical, psychological and environmental (gender) parameters.[83] In 2010, less than 20% of basic scientists in Canada reported consideration of sex or gender. This number has since doubled, but remains unacceptably low as the

inclusion of sex in basic research drives discovery of disease mechanisms.[84] For instance, Canadian scientists recently discovered that different immune cells mediate mechanical pain hypersensitivity in male and female mice, opening the door for new drug development that targets microglial pathways in males and T lymphocyte pathways in females.[85]

In coming years, two measures will hold basic scientists to higher levels of accountability. Mandatory peer reviewer training will enable assessment of the appropriate integration of S&G in funded basic science protocols. Second, science journal editors will start adopting S&G reporting requirements in their editorial policies as per the Sex and Gender Equity in Reporting (SAGER) guidelines. Both of these levers will ensure that research results are accurate, reproducible and applicable to both sexes.

Views from US

In 1993, the National Institutes of Health (NIH) Revitalization Act mandated inclusion of women in clinical trials. However, in the legislation, there was no mention of basic human physiological functional studies or mechanistic studies utilizing isolated cells or tissues. In 2001, the Institute of Medicine, "Exploring the Biological Contribution of Human Health: Does Sex Matter?" focused attention on the need to consider sex as a biological variable from basic to translational research (Table 1). However, acceptance and consideration of sex as a biological variable was not embraced by the scientific community, a shortcoming which prompted the NIH to implement policies requiring investigators to account for S&G in the design and data analysis with sound scientific justification to study only one sex (NOT-OD-15-102: Consideration of Sex as a Biological Variable in NIH Funded Research and NOT-OD-15-103: Enhancing Reproducibility through Rigor and Transparency). Implementation of these policies began in 2016.[86] Long-term success of these policies will require careful monitoring and education to embed concepts of S&G into all levels of science education. Basic and clinical scientists continue to partner with advocacy groups such as the Society of Women's Health Research and professional societies (e.g. Organization for the Study of Sex Differences, the American Physiological Society and the Endocrine Society) to increase research and reporting of data on S&G differences in basic and translational research. Online resources and methodological guides continue to be developed and are available to facilitate learning for undergraduate, graduate and health care professionals. A report of the National Heart, Lung, and Blood Institute Working Group on Sex Differences Research in Cardiovascular Disease has been launched recently that points the scientific questions and challenges for future research.[87]

Views from Japan

S&G differences on cardiovascular diseases were recognized in Japan at the annual meeting of Japanese College of Cardiology in 1999. The promoting members founded the predecessor of the Japanese Association for Gender-Specific Medicine that consisted of clinical and basic researchers among various fields in 2003. In 2010, the "Guidelines for Gender –Specific Cardiovascular Disease (JCS 2010)" has been issued by the Japanese Circulation Society. Another initiative in Japan that began in 2001 was the increase in number of outpatient clinics for women which are staffed by female physicians.

On the other hand, S&G researches in basic and clinical science for disciplines other than cardiology are not substantially present in Japan. One reason is that there is not a suitable application category for S&G themes for grants funded by the Japanese Ministry of Education, Culture, Sports, Science and Technology. Another reason is themes and judges and funds for women's health still favor gynecology and gynecologists.

In addition to gynecology, S&G aspect of medicine affect all areas of women's health. Likewise, S&G aspects of men's health need to expand beyond urology. Japan is at a turning point in promoting S&G research. It is the time to take action and edify governmental granting agencies to fund S&G research.

Options for the future

For promoting sex-specific basic research, the definition of scientific excellence is a critical issue. Depending on the scientific culture, dominant thinking may be that excellent science is to define a new pathway per se and not to characterize, in which human subjects, females or males, young or old, it may be effective. This attitude may however change since scientists acquire more societal responsibility and

society requests pay-back from its investment in biomedical research. Consideration of S&G is a cornerstone for improving quality and reproducibility of basic and translational science.
There is rising public, professional and regulatory awareness related to the importance of S&G Specific Medicine. Paradigms are being changed, research in the area of S&G topics is expanding, and high standard scientific meetings on the topic are being held worldwide and in many medical schools S&G Specific Medicine has been introduced into the curriculum. The International Society for Gender Medicine (www.isogem.com) includes currently eight national societies. S&G Specific Medicine is now being perceived as a major step in the improvement of the quality of medical care for men and women. Continuous efforts need to be invested in order to keep and increase this momentum and to increase our fundamental knowledge. Table 2 highlights the recommendations for future research in the field.

Table 2

Recommendations for future basic research

- Consider sex in experimental design of basic research projects
- Study both sexes in animal studies
- Consider primary cells from both sexes and identify sex of cell lines
- Study genetic, epigenetic and hormonal modifiers
- Include sex chromosome in GWAS studies
- Study pregnancy and related specific disorders specially CVD
- Integrate data from studies of epigenetics, gene expression and protein abundance
- Consider S&G in pharmacology and specific drug design
- Sex as well as species should be mentioned in the titles of articles
- Scientific journals should consider introducing S&G in their editorial policy
- Specific calls from each country and EC should be dedicated to S&G issues
- S&G consideration should be included in biology and medicine university courses

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Figure legends

Figure 1: Sex and estrogen dependent mechanisms, affected organs and disease entities in CVD, as reviewed recently.[3]

Figure 2: Mechanisms that contribute to sex differences during development and throughout life in experimental animals and humans. Sex hormones, including gonadal and extra-gonadal sex hormones change in their activity during lifetime (yellow bars) and exert direct effects at different developmental stages of life. They also interact with genetic and epigenetic mechanisms (yellow/blue arrow). Genetic and epigenetic factors may contribute to sex differences in the absence of sex hormones (blue bars) during lifetime.

Figure 3: Effect of sex and estrogen in cardiovascular cells. Figure depicts the organelles of the cell where sex differences are apparent: in signaling from receptor tyrosine kinase (RTK) and G-protein coupled receptor (GPCR) to the nucleus, in sarcoplasmic reticulum Ca^{2+} handling, at the contractile elements, in the mitochondria, in nuclear gene transcription, ribosomal function, in autophagy and protein degradation. For details see text and ref. [4, 5]
Abbreviations: ER, estrogen receptor; GSK3β, glycogen synthase kinase 3β; HSL, hormone-sensitive lipase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NOS, nitric oxide synthase; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; Glut-1, glucose transporter 1; RTK, receptor tyrosine kinase, GPR30, G protein-coupled receptor 30; GPCR, G-protein-coupled receptor, Akt.

Figure 4. Schematic representation of sexual-dimorphism in mitochondria.
Estrogen by binding to the estrogen receptors (ERα, ERβ, GPR30) can activate mitochondrial biogenesis by upregulating the co-activator of mitochondrial biogenesis PGC-1α and its downstream cascade, Estrogen receptors α and β are also present in mitochondria and may directly activate mitochondrial DNA (mtDNA) transcription and replication. ERs can also modify mitochondrial function by non-genomic effects (dotted line) involving known (MAPK, PI3K) and unknown signaling pathways. Female mitochondria produce more energy, utilize more fatty acid and are able to handle more calcium and to undergo increased autophagy (in red) than their male counterparts. Male mitochondria release more free radicals and proapoptotic signals (in blue).

Figure 5: Summary of 17β-Estradiol (E2) and estrogen receptor (ER)-mediated effects on pro-fibrotic mechanisms.
In female sex (in red, left side), **A)** E2-activated ERα inhibits RhoA/ROCK/cofilin pathway leading to attenuated cardiac fibrosis. **B)** In addition, E2 and ERβ signal through protein kinase A (PKA) and AMP kinase (AMPK) to inhibit Rho-kinase activation of TGFβ-1-mediated pro-fibrotic actions. **C)** Further, E2 bound ERα activates extracellular signal-regulated Kinase (ERK) 1/2, leading to phosphorylation of transcription factor Elk-1 resulting in down-regulation of Matrix-metalloproteinase-2 (MMP-2) by co-repressor recruitment, observed in cardiac fibroblasts from both sexes. **D)** Moreover, in female cardiac fibroblasts, E2 activated ER downregulates collagen I, III and pro-fibrotic micro RNA (miRNA) network expression.
In male cells (in blue, right side), **E)** in contrast, E2/ER up-regulate collagens and miRNA, leading to higher expression of pro-fibrotic miRNA network, inhibition of Sprouty 1 (SPRY1), rasa1 and rasa2 leading to higher activation of ERK1/2 and further down-stream pro-fibrotic signaling. Grb2: growth factor receptor-bound protein 2; Co-R: Co-repressor; Co-A: Co-activator; RTK: Receptor protein-tyrosine kinase. See [4] for review and references.

Figure 6: **Example of sex differences in preclinical research.** Survival of melusin overexpressing (OE) mice after myocardial infarction in comparison with untreated controls. A) whole group, males and females, b) males only, c) females only. Survival in the whole mixed sex group is significantly improved, even though females do not benefit.[58]

Figure 7: Strengths, weaknesses, opportunities and threats – SWOT analysis for including sex specific aspects in basic research.

References

1. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. *Neurosci Biobehav Rev* 2011;**35**:565-572.
2. Richardson SS, Reiches M, Shattuck-Heidorn H, Labonte ML, Consoli T. Opinion: Focus on preclinical sex differences will not address women's and men's health disparities. *Proc Natl Acad Sci U S A* 2015;**112**:13419-13420.
3. Eugenmed Cardiovascular Clinical Study Group, Regitz-Zagrosek V, Oertelt-Prigione S, Prescott E, Franconi F, Gerdts E, Foryst-Ludwig A, Maas AH, Kautzky-Willer A, Knappe-Wegner D, Kintscher U, Ladwig KH, Schenck-Gustafsson K, Stangl V. Gender in cardiovascular diseases: impact on clinical manifestations, management, and outcomes. *Eur Heart J* 2016;**37**:24-34.
4. Regitz-Zagrosek V, Kararigas G. Mechanistic Pathways of Sex Differences in Cardiovascular Disease. *Physiol Rev* 2017;**97**:1-37.
5. Blenck CL, Harvey PA, Reckelhoff JF, Leinwand LA. The Importance of Biological Sex and Estrogen in Rodent Models of Cardiovascular Health and Disease. *Circ Res* 2016;**118**:1294-1312.
6. Mcgregor AJ, Hasnain M, Sandberg K, Morrison MF, Berlin M, Trott J. How to study the impact of sex and gender in medical research: a review of resources. *Biol Sex Differ* 2016;**7**:46.
7. Oertelt-Prigione S, Gohlke BO, Dunkel M, Preissner R, Regitz-Zagrosek V. GenderMedDB: an interactive database of sex and gender-specific medical literature. *Biol Sex Differ* 2014;**5**:7.
8. Berletch JB, Yang F, Xu J, Carrel L, Disteche CM. Genes that escape from X inactivation. *Hum Genet* 2011;**130**:237-245.
9. Hochberg Z, Feil R, Constancia M, Fraga M, Junien C, Carel JC, Boileau P, Le Bouc Y, Deal CL, Lillycrop K, Scharfmann R, Sheppard A, Skinner M, Szyf M, Waterland RA, Waxman DJ, Whitelaw E, Ong K, Albertsson-Wikland K. Child health, developmental plasticity, and epigenetic programming. *Endocr Rev* 2011;**32**:159-224.
10. Kuroki S, Matoba S, Akiyoshi M, Matsumura Y, Miyachi H, Mise N, Abe K, Ogura A, Wilhelm D, Koopman P, Nozaki M, Kanai Y, Shinkai Y, Tachibana M. Epigenetic regulation of mouse sex determination by the histone demethylase Jmjd1a. *Science* 2013;**341**:1106-1109.
11. Reynolds RM. Glucocorticoid excess and the developmental origins of disease: two decades of testing the hypothesis--2012 Curt Richter Award Winner. *Psychoneuroendocrinology* 2013;**38**:1-11.
12. Carpenter T, Grecian S, Reynolds R. Sex differences in early life programming of the hypothalamic-pituitary-adrenal axis in humans suggest increased vulnerability in females. *Psychoneuroendocrinology* 2015;**61**:32.
13. Majdic G, Tobet S. Cooperation of sex chromosomal genes and endocrine influences for hypothalamic sexual differentiation. *Front Neuroendocrinol* 2011;**32**:137-145.
14. Mccarthy MM, Nugent BM. At the frontier of epigenetics of brain sex differences. *Front Behav Neurosci* 2015;**9**:221.
15. Nugent BM, Wright CL, Shetty AC, Hodes GE, Lenz KM, Mahurkar A, Russo SJ, Devine SE, Mccarthy MM. Brain feminization requires active repression of masculinization via DNA methylation. *Nat Neurosci* 2015;**18**:690-697.
16. Hindorff L, Macarthur J, Morales J, Junkins H, Hall P. A Catalog of Published Genome-wide Association Studies. 2013. http://www.genome.gov/gwastudies/
17. Konig IR, Loley C, Erdmann J, Ziegler A. How to include chromosome X in your genome-wide association study. *Genet Epidemiol* 2014;**38**:97-103.
18. Delaval K, Govin J, Cerqueira F, Rousseaux S, Khochbin S, Feil R. Differential histone modifications mark mouse imprinting control regions during spermatogenesis. *EMBO J* 2007;**26**:720-729.
19. Li E, Beard C, Jaenisch R. Role for DNA methylation in genomic imprinting. *Nature* 1993;**366**:362-365.
20. Augui S, Nora EP, Heard E. Regulation of X-chromosome inactivation by the X-inactivation centre. *Nat Rev Genet* 2011;**12**:429-442.

21. Penaloza CG, Estevez B, Han DM, Norouzi M, Lockshin RA, Zakeri Z. Sex-dependent regulation of cytochrome P450 family members Cyp1a1, Cyp2e1, and Cyp7b1 by methylation of DNA. *FASEB J* 2014;**28**:966-977.
22. Kararigas G, Bito V, Tinel H, Becher E, Baczko I, Knosalla C, Albrecht-Kupper B, Sipido KR, Regitz-Zagrosek V. Transcriptome characterization of estrogen-treated human myocardium identifies Myosin regulatory light chain interacting protein as a sex-specific element influencing contractile function. *J Am Coll Cardiol* 2012;**59**:410-417.
23. Kararigas G, Dworatzek E, Petrov G, Summer H, Schulze TM, Baczko I, Knosalla C, Golz S, Hetzer R, Regitz-Zagrosek V. Sex-dependent regulation of fibrosis and inflammation in human left ventricular remodelling under pressure overload. *Eur J Heart Fail* 2014;**16**:1160-1167.
24. Heidecker B, Lamirault G, Kasper EK, Wittstein IS, Champion HC, Breton E, Russell SD, Hall J, Kittleson MM, Baughman KL, Hare JM. The gene expression profile of patients with new-onset heart failure reveals important gender-specific differences. *Eur Heart J* 2010;**31**:1188-1196.
25. Isensee J, Witt H, Pregla R, Hetzer R, Regitz-Zagrosek V, Noppinger PR. Sexually dimorphic gene expression in the heart of mice and men. *J Mol Med (Berl)* 2008;**86**:61-74.
26. Diedrich M, Tadic J, Mao L, Wacker MA, Nebrich G, Hetzer R, Regitz-Zagrosek V, Klose J. Heart protein expression related to age and sex in mice and humans. *Int J Mol Med* 2007;**20**:865-874.
27. Dworatzek E, Baczko I, Kararigas G. Effects of aging on cardiac extracellular matrix in men and women. *Proteomics Clin Appl* 2016;**10**:84-91.
28. Fliegner D, Schubert C, Penkalla A, Witt H, Kararigas G, Dworatzek E, Staub E, Martus P, Ruiz Noppinger P, Kintscher U, Gustafsson JA, Regitz-Zagrosek V. Female sex and estrogen receptor-beta attenuate cardiac remodeling and apoptosis in pressure overload. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:R1597-1606.
29. Queiros AM, Eschen C, Fliegner D, Kararigas G, Dworatzek E, Westphal C, Sanchez Ruderisch H, Regitz-Zagrosek V. Sex- and estrogen-dependent regulation of a miRNA network in the healthy and hypertrophied heart. *Int J Cardiol* 2013;**169**:331-338.
30. Mahmoodzadeh S, Dworatzek E, Fritschka S, Pham TH, Regitz-Zagrosek V. 17beta-Estradiol inhibits matrix metalloproteinase-2 transcription via MAP kinase in fibroblasts. *Cardiovasc Res* 2010;**85**:719-728.
31. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Strom A, Treuter E, Warner M, Gustafsson JA. Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* 2007;**87**:905-931.
32. Simoncini T, Mannella P, Genazzani AR. Rapid estrogen actions in the cardiovascular system. *Ann N Y Acad Sci* 2006;**1089**:424-430.
33. O'lone R, Knorr K, Jaffe IZ, Schaffer ME, Martini PG, Karas RH, Bienkowska J, Mendelsohn ME, Hansen U. Estrogen receptors alpha and beta mediate distinct pathways of vascular gene expression, including genes involved in mitochondrial electron transport and generation of reactive oxygen species. *Mol Endocrinol* 2007;**21**:1281-1296.
34. Prossnitz ER, Arterburn JB, Sklar LA. GPR30: A G protein-coupled receptor for estrogen. *Mol Cell Endocrinol* 2007;**265-266**:138-142.
35. Malorni W, Campesi I, Straface E, Vella S, Franconi F. Redox features of the cell: a gender perspective. *Antioxid Redox Signal* 2007;**9**:1779-1801.
36. Ortona E, Gambardella L, Barbati C, Malorni W. Membrane-associated functional estrogen receptors alpha are upregulated in cardiomyocytes under oxidative imbalance. *IJC Metabolic & Endocrine* 2014;**5**:67-69.
37. Matarrese P, Colasanti T, Ascione B, Margutti P, Franconi F, Alessandri C, Conti F, Riccieri V, Rosano G, Ortona E, Malorni W. Gender disparity in susceptibility to oxidative stress and apoptosis induced by autoantibodies specific to RLIP76 in vascular cells. *Antioxid Redox Signal* 2011;**15**:2825-2836.
38. Clocchiatti A, Cora E, Zhang Y, Dotto GP. Sexual dimorphism in cancer. *Nat Rev Cancer* 2016;**16**:330-339.
39. Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. *Cell* 2012;**148**:1145-1159.
40. Lagranha CJ, Deschamps A, Aponte A, Steenbergen C, Murphy E. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res* 2010;**106**:1681-1691.

741 41. Moulin M, Piquereau J, Mateo P, Fortin D, Rucker-Martin C, Gressette M, Lefebvre F, Gresikova M,
742 Solgadi A, Veksler V, Garnier A, Ventura-Clapier R. Sexual dimorphism of doxorubicin-mediated
743 cardiotoxicity: potential role of energy metabolism remodeling. *Circ Heart Fail* 2015;**8**:98-108.
744 42. Petrov G, Dworatzek E, Schulze TM, Dandel M, Kararigas G, Mahmoodzadeh S, Knosalla C, Hetzer R,
745 Regitz-Zagrosek V. Maladaptive remodeling is associated with impaired survival in women but not in
746 men after aortic valve replacement. *JACC Cardiovasc Imaging* 2014;**7**:1073-1080.
747 43. Petrov G, Regitz-Zagrosek V, Lehmkuhl E, Krabatsch T, Dunkel A, Dandel M, Dworatzek E,
748 Mahmoodzadeh S, Schubert C, Becher E, Hampl H, Hetzer R. Regression of myocardial hypertrophy after
749 aortic valve replacement: faster in women? *Circulation* 2010;**122**:S23-28.
750 44. Witt H, Schubert C, Jaekel J, Fliegner D, Penkalla A, Tiemann K, Stypmann J, Roepcke S, Brokat S,
751 Mahmoodzadeh S, Brozova E, Davidson MM, Ruiz Noppinger P, Grohe C, Regitz-Zagrosek V. Sex-specific
752 pathways in early cardiac response to pressure overload in mice. *J Mol Med (Berl)* 2008;**86**:1013-1024.
753 45. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, Slagboom PE, Heijmans BT. DNA
754 methylation differences after exposure to prenatal famine are common and timing- and sex-specific.
755 *Hum Mol Genet* 2009;**18**:4046-4053.
756 46. Patterson AJ, Chen M, Xue Q, Xiao D, Zhang L. Chronic prenatal hypoxia induces epigenetic
757 programming of PKC{epsilon} gene repression in rat hearts. *Circ Res* 2010;**107**:365-373.
758 47. Movassagh M, Vujic A, Foo R. Genome-wide DNA methylation in human heart failure. *Epigenomics*
759 2011;**3**:103-109.
760 48. Ramos RB, Fabris V, Lecke SB, Maturana MA, Spritzer PM. Association between global leukocyte DNA
761 methylation and cardiovascular risk in postmenopausal women. *BMC Med Genet* 2016;**17**:71.
762 49. Regitz-Zagrosek V, Oertelt-Prigione S, Seeland U, Hetzer R. Sex and gender differences in myocardial
763 hypertrophy and heart failure. *Circ J* 2010;**74**:1265-1273.
764 50. Kadkhodayan A, Lin CH, Coggan AR, Kisrieva-Ware Z, Schechtman KB, Novak E, Joseph SM, Davila-
765 Roman VG, Gropler RJ, Dence C, Peterson LR. Sex affects myocardial blood flow and fatty acid substrate
766 metabolism in humans with nonischemic heart failure. *J Nucl Cardiol* 2016;
767 51. Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and
768 cancer in later life: systematic review and meta-analysis. *BMJ* 2007;**335**:974.
769 52. Mcdonald SD, Malinowski A, Zhou Q, Yusuf S, Devereaux PJ. Cardiovascular sequelae of
770 preeclampsia/eclampsia: a systematic review and meta-analyses. *Am Heart J* 2008;**156**:918-930.
771 53. Ruoppolo M, Scolamiero E, Caterino M, Mirisola V, Franconi F, Campesi I. Female and male human
772 babies have distinct blood metabolomic patterns. *Mol Biosyst* 2015;**11**:2483-2492.
773 54. Franconi F, Rosano G, Campesi I. Need for gender-specific pre-analytical testing: the dark side of the
774 moon in laboratory testing. *Int J Cardiol* 2015;**179**:514-535.
775 55. Regitz-Zagrosek V. *Sex and Gender Differences in Pharmacology.* Springer Verlag, 2012;214
776 56. Zucker I, Beery AK. Males still dominate animal studies. *Nature* 2010;**465**:690.
777 57. Yoon DY, Mansukhani NA, Stubbs VC, Helenowski IB, Woodruff TK, Kibbe MR. Sex bias exists in basic
778 science and translational surgical research. *Surgery* 2014;**156**:508-516.
779 58. Unsold B, Kaul A, Sbroggio M, Schubert C, Regitz-Zagrosek V, Brancaccio M, Damilano F, Hirsch E,
780 Van Bilsen M, Munts C, Sipido K, Bito V, Detre E, Wagner NM, Schafer K, Seidler T, Vogt J, Neef S,
781 Bleckmann A, Maier LS, Balligand JL, Bouzin C, Ventura-Clapier R, Garnier A, Eschenhagen T, El-
782 Armouche A, Knoll R, Tarone G, Hasenfuss G. Melusin protects from cardiac rupture and improves
783 functional remodelling after myocardial infarction. *Cardiovasc Res* 2014;**101**:97-107.
784 59. Sbroggio M, Bertero A, Velasco S, Fusella F, De Blasio E, Bahou WF, Silengo L, Turco E, Brancaccio M,
785 Tarone G. ERK1/2 activation in heart is controlled by melusin, focal adhesion kinase and the scaffold
786 protein IQGAP1. *J Cell Sci* 2011;**124**:3515-3524.
787 60. Kassi E, Spilioti E, Nasiri-Ansari N, Adamopoulos C, Moutsatsou P, Papapanagiotou A, Siasos G,
788 Tousoulis D, Papavassiliou AG. Vascular Inflammation and Atherosclerosis: The Role of Estrogen
789 Receptors. *Curr Med Chem* 2015;**22**:2651-2665.
790 61. Gupte AA, Pownall HJ, Hamilton DJ. Estrogen: an emerging regulator of insulin action and
791 mitochondrial function. *J Diabetes Res* 2015;**2015**:916585.
792 62. Billon-Gales A, Fontaine C, Filipe C, Douin-Echinard V, Fouque MJ, Flouriot G, Gourdy P, Lenfant F,
793 Laurell H, Krust A, Chambon P, Arnal JF. The transactivating function 1 of estrogen receptor alpha is

794 dispensable for the vasculoprotective actions of 17beta-estradiol. *Proc Natl Acad Sci U S A*
795 2009;**106**:2053-2058.
796 63. Billon-Gales A, Krust A, Fontaine C, Abot A, Flouriot G, Toutain C, Berges H, Gadeau AP, Lenfant F,
797 Gourdy P, Chambon P, Arnal JF. Activation function 2 (AF2) of estrogen receptor-alpha is required for
798 the atheroprotective action of estradiol but not to accelerate endothelial healing. *Proc Natl Acad Sci U S*
799 *A* 2011;**108**:13311-13316.
800 64. Abot A, Fontaine C, Buscato M, Solinhac R, Flouriot G, Fabre A, Drougard A, Rajan S, Laine M, Milon
801 A, Muller I, Henrion D, Adlanmerini M, Valera MC, Gompel A, Gerard C, Pequeux C, Mestdagt M,
802 Raymond-Letron I, Knauf C, Ferriere F, Valet P, Gourdy P, Katzenellenbogen BS, Katzenellenbogen JA,
803 Lenfant F, Greene GL, Foidart JM, Arnal JF. The uterine and vascular actions of estetrol delineate a
804 distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation.
805 *EMBO Mol Med* 2014;**6**:1328-1346.
806 65. Jankowska EA, Drohomirecka A, Ponikowska B, Witkowska A, Lopuszanska M, Szklarska A, Borodulin-
807 Nadzieja L, Banasiak W, Poole-Wilson PA, Ponikowski P. Deficiencies in circulating testosterone and
808 dehydroepiandrosterone sulphate, and depression in men with systolic chronic heart failure. *Eur J Heart*
809 *Fail* 2010;**12**:966-973.
810 66. Giannetta E, Feola T, Gianfrilli D, Pofi R, Dall'armi V, Badagliacca R, Barbagallo F, Lenzi A, Isidori AM.
811 Is chronic inhibition of phosphodiesterase type 5 cardioprotective and safe? A meta-analysis of
812 randomized controlled trials. *BMC Med* 2014;**12**:185.
813 67. Sasaki H, Nagayama T, Blanton RM, Seo K, Zhang M, Zhu G, Lee DI, Bedja D, Hsu S, Tsukamoto O,
814 Takashima S, Kitakaze M, Mendelsohn ME, Karas RH, Kass DA, Takimoto E. PDE5 inhibitor efficacy is
815 estrogen dependent in female heart disease. *J Clin Invest* 2014;**124**:2464-2471.
816 68. Taylor KE, Vallejo-Giraldo C, Schaible NS, Zakeri R, Miller VM. Reporting of sex as a variable in
817 cardiovascular studies using cultured cells. *Biol Sex Differ* 2011;**2**:11.
818 69. Shah K, Mccormack CE, Bradbury NA. Do you know the sex of your cells? *Am J Physiol Cell Physiol*
819 2014;**306**:C3-18.
820 70. Pellegrini M, Bulzomi P, Lecis M, Leone S, Campesi I, Franconi F, Marino M. Endocrine disruptors
821 differently influence estrogen receptor beta and androgen receptor in male and female rat VSMC. *J Cell*
822 *Physiol* 2014;**229**:1061-1068.
823 71. Franconi F, Seghieri G, Canu S, Straface E, Campesi I, Malorni W. Are the available experimental
824 models of type 2 diabetes appropriate for a gender perspective? *Pharmacol Res* 2008;**57**:6-18.
825 72. Reichelt ME, Mellor KM, Bell JR, Chandramouli C, Headrick JP, Delbridge LM. Sex, sex steroids, and
826 diabetic cardiomyopathy: making the case for experimental focus. *Am J Physiol Heart Circ Physiol*
827 2013;**305**:H779-792.
828 73. Murphy E, Steenbergen C. Gender-based differences in mechanisms of protection in myocardial
829 ischemia-reperfusion injury. *Cardiovasc Res* 2007;**75**:478-486.
830 74. Prendergast BJ, Onishi KG, Zucker I. Female mice liberated for inclusion in neuroscience and
831 biomedical research. *Neurosci Biobehav Rev* 2014;**40**:1-5.
832 75. Becker JB, Prendergast BJ, Liang JW. Female rats are not more variable than male rats: a meta-
833 analysis of neuroscience studies. *Biol Sex Differ* 2016;**7**:34.
834 76. Harvey PA, Leinwand LA. Dietary phytoestrogens present in soy dramatically increase cardiotoxicity
835 in male mice receiving a chemotherapeutic tyrosine kinase inhibitor. *Mol Cell Endocrinol* 2015;**399**:330-
836 335.
837 77. Clayton JA. Studying both sexes: a guiding principle for biomedicine. *FASEB J* 2016;**30**:519-524.
838 78. Arnold AP, Chen X. What does the "four core genotypes" mouse model tell us about sex differences
839 in the brain and other tissues? *Front Neuroendocrinol* 2009;**30**:1-9.
840 79. Grgurevic N, Budefeld T, Spanic T, Tobet SA, Majdic G. Evidence that sex chromosome genes affect
841 sexual differentiation of female sexual behavior. *Horm Behav* 2012;**61**:719-724.
842 80. Guo Y, Flaherty MP, Wu WJ, Tan W, Zhu X, Li Q, Bolli R. Genetic background, gender, age, body
843 temperature, and arterial blood pH have a major impact on myocardial infarct size in the mouse and
844 need to be carefully measured and/or taken into account: results of a comprehensive analysis of
845 determinants of infarct size in 1,074 mice. *Basic Res Cardiol* 2012;**107**:288.

81. Tannenbaum C, Schwarz JM, Clayton JA, De Vries GJ, Sullivan C. Evaluating sex as a biological variable in preclinical research: the devil in the details. *Biol Sex Differ* 2016;**7**:13.
82. Johnson J, Sharman Z, Vissandjee B, Stewart DE. Does a change in health research funding policy related to the integration of sex and gender have an impact? *PLoS One* 2014;**9**:e99900.
83. Ritz SA, Antle DM, Cote J, Deroy K, Fraleigh N, Messing K, Parent L, St-Pierre J, Vaillancourt C, Mergler D. First steps for integrating sex and gender considerations into basic experimental biomedical research. *FASEB J* 2014;**28**:4-13.
84. Klein SL, Schiebinger L, Stefanick ML, Cahill L, Danska J, De Vries GJ, Kibbe MR, Mccarthy MM, Mogil JS, Woodruff TK, Zucker I. Opinion: Sex inclusion in basic research drives discovery. *Proc Natl Acad Sci U S A* 2015;**112**:5257-5258.
85. Sorge RE, Mapplebeck JC, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin JS, Sotocinal SG, Chen D, Yang M, Shi XQ, Huang H, Pillon NJ, Bilan PJ, Tu Y, Klip A, Ji RR, Zhang J, Salter MW, Mogil JS. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat Neurosci* 2015;**18**:1081-1083.
86. Miller VM, Reckelhoff JF. Sex as a Biological Variable: Now What?! *Physiology (Bethesda)* 2016;**31**:78-80.
87. Maric-Bilkan C, Arnold AP, Taylor DA, Dwinell M, Howlett SE, Wenger N, Reckelhoff JF, Sandberg K, Churchill G, Levin E, Lundberg MS. Report of the National Heart, Lung, and Blood Institute Working Group on Sex Differences Research in Cardiovascular Disease: Scientific Questions and Challenges. *Hypertension* 2016;**67**:802-807.