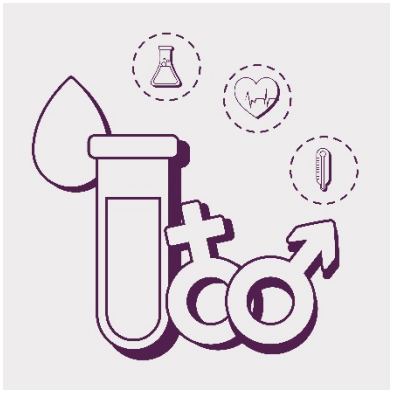

KI Case Studies — Considering sex as a biological variable in basic biomedical research

Major funding agencies around the world are pushing for sex and gender aspects to be meaningfully integrated into all stages of the research they fund, from study design to data analysis and reporting. Likewise, an increasing number of journals require that such aspects be addressed in the papers they publish. This push is not about getting more scientists to undertake sex-difference research or gender studies. It is about getting all scientists to view the work they already do (or propose to do) through a sex-and-gender-conscious lens.

Given that sex differences exist in various traits, genes and biological processes, considering sex as a biological variable (SABV) should be fundamental to biomedical research practice. Yet this is currently far from the case. While clinical researchers are increasingly aware of the importance of including women in clinical studies, basic and preclinical researchers more often than not rely on male (to the exclusion of female) animals, tissues, and cells. This over-reliance on the male can obscure important sex differences in processes of health and disease, as well as in response to treatment. So can failing to spot sex differences when using models or material of both sexes.

SABV in basic research is relevant when the research:

- Uses human tissues, cells or bodily fluids
- Uses animal tissues, cells or bodily fluids
- Uses animal models of human physiology or disease
- May have an impact on diagnosis or treatment
- Will lead to the development of products for human use

Understanding sex differences at the preclinical stage is vital to designing follow-on clinical trials appropriately—and vital, ultimately, to men and women's health. Moreover, taking SABV into account in basic biomedical research can lead to fascinating discoveries.

Here, 3 KI researchers — Jorge Ruas, Elisabet Stener-Victorin and Christopher Cederroth — share their views on considering SABV in their research and explain how doing so has benefitted their research (and why more funding to do so is needed).

KI Case Study 1
Auditory Research

Scientist and Department:
Docent Christopher Cederroth, Dept. of Physiology and Pharmacology (FYFA), section of Experimental Audiology

Relevant publication(s) or grant(s):
Lugo et al 2019. Sex-specific association of tinnitus with suicide attempts. *JAMA Otolaryngol Head Neck Surg.*, published online May 2, 2019. doi:10.1001/jamaoto.2019.0566

Maas et al 2017. Genetic susceptibility to bilateral tinnitus in a Swedish twin cohort. *Genetics in Medicine*, 19(9):1007-1012. doi: 10.1038/gim.2017.4

Schlee et al 2017. Visualization of Global Disease Burden for the Optimization of Patient Management and Treatment. *Frontiers in Medicine*, 4:86. doi: 10.3389/fmed.2017.00086

Hasson et al 2013. Acute stress induces hyperacusis in women with high levels of emotional exhaustion. *PLoS One.* 8(1):e52945. doi:10.1371/annotation/6a09a2e1-5c83-4ae7-859b-454de3e21814.

GenderNet Plus Co-Fund grant 2018: The combined role of genetic and environmental risk factors in the gender-specific development of severe tinnitus (TIGER)

Link to research group:
https://ki.se/en/fyfa/translational-auditory-neuroscience

1. **Please briefly describe your research and explain which parts of it are relevant to SABV as described above.**

My laboratory aims at uncovering the mechanisms underlying tinnitus, the ringing in the ears or the phantom perception of sounds. Tinnitus is a neurological disorder most often emerging from auditory damage or hearing loss. It is quite prevalent but for 2% of the population it has a major impact on life quality. The mechanisms by which tinnitus is triggered and those making it a severe condition are unknown. I perform research on both animal models and humans in order to bridge the knowledge between the two and improve objective diagnostic and therapeutic options. In spite of being a common auditory dysfunction with increasing prevalence, research suggests that it is more frequent in men than in women, while women are more affected by it than men. However, sex and gender aspects have rarely been considered in this research field. As we searched for evidence of a genetic contribution to tinnitus, we found that the heritability of tinnitus is greater in men than in women. In contrast, we have shown that in chronic constant tinnitus, women with an equal loudness perception have a greater level of stress, anxiety, hyperacusis (sensitivity to sounds), and a worse physical and psychological life quality. Following up on these results, we recently discovered that tinnitus increases the risk of suicidal attempts in women but not in men. Altogether, our findings point towards a sexual dimorphism in the pathophysiological mechanisms of tinnitus. This led to the successful funding of our research by the GenderNet Plus co-Fund, which I am delighted to coordinate.

2. **In your research, you show some sex and/or gender-specific differences. Did you plan to look at sex and/or gender differences or did you simply notice them during the project? In case this was not planned, how did you become aware of such differences?**

I was aware of the knowledge gaps on sex and gender aspects in auditory research and, yes, I was curious to see whether anything would emerge from our first analyses. We indeed planned on doing a sex-stratified analysis when we first investigated the concordance of tinnitus in twins to assess its heritability, knowing we had a large enough sample size to do so. However, in a recent adoption study we published, this sex-stratified analysis was not doable due to limited sample size. We however pursue considering sex in our analyses of the Swedish Tinnitus Outreach Project, a study I created and which is the foundation for the research performed in the Gender-Net Co-Plus Fund.

3. **Are you planning to follow up on SABV in your future research? If so, in what way? If not, why not?**

In the research performed in humans, yes, I will definitely address SABV as long as sample size allows me to do so. However, for the research in animals, I strongly believe this depends on the study design and the resources that can be allocated to such projects. In the Focus article you are citing from, Danska refers to a meta-analysis by Prendergast et al. showing that the inclusion of females in neuroscience studies does not add variability and does not require monitoring of the oestrus cycle – in other words, female hormonal fluctuations do not confound biological measures. However, I believe the conclusions of Prendergast et al. are flawed by their methodological approach which has limitations. Instead, I strongly believe this depends on the physiological parameter assessed, the challenge and the read-out.

For instance, in auditory research, we now observe that whereas baseline hearing thresholds do not differ between males and females, the response to a noise challenge differs a lot and, in females, it depends on the oestrus cycle. Thus, in order to address sex effects in our experimental research by including females, it will require knowing which phase of the menstrual cycle they are in. Without such information, a completely biased effect may be obtained and not being representative of a female's hormonal status, ultimately impacting on variability and reproducibility. As a consequence, for research in which we were indeed focusing only on males in the past, we now triple the amount of work to be performed, and its associated costs.

Importantly, I believe that considering SABV in the context of baseline measures is more easily implemented than for experiments with sequential (longitudinal) interventions. Those we perform in auditory research are time consuming and this has a non-negligible impact on productivity, in particular for our students and post-docs who are confronted with a very competitive academic environment. In my view - and in our experimental settings - they would need technical support to address sex and gender aspects in their research. How this applies to research fields other than the auditory field is unclear to me but, having worked on reproduction and metabolism in the past, including SABV was easier (obtaining glycemia measurements from a mouse would take no more than 30 seconds, whereas recording an auditory threshold takes up to 1 hour and a half). Thus, some research fields would be more prone to the inclusion of SABV than others.

4. **In what way did integrating SABV benefit your science?**

In the recent article we have published revealing sex-dependent influences of tinnitus on suicide attempt, such dimorphic aspects would have been masked when simply adjusting for sex and not considering sex-stratified analyses. We now have preliminary data where we identified genetic variants that are associated with tinnitus in men, but not in women (consistent with our heritability study). Here, not having stratified these analyses by sex would have left us with no significant genetic

associations, and thus no insights into molecular mechanisms. Thus, for both the identification of environmental and genetic risk factors in humans, SABV has been essential.

5. **In what ways can your research contribute to sex- and/or gender-specific improvements in medical and biological knowledge, diagnostic tools or therapy (i.e. gendered innovations)?**

There are increasing attempts to classify complex disorders into subtypes but none consider sex as a classification with the common assumption that both men and women would respond to a specific treatment alike. In the case of tinnitus, identifying genes that increase the risk of developing tinnitus in men will provide insights into the biology of tinnitus development, whereas understanding why the impact of tinnitus in women is greater than in men will increase our understanding of the neural mechanisms related to the severity of the disease. Not only will this lead to a greater understanding of the biology of auditory processes, but it will also help designing optimal diagnostics (which might prove effective in one sex but not the other), and gender-specific therapeutic interventions. Regarding the latter, our colleagues in Germany recently found that women are more responsive to sound-based therapies for tinnitus than men. Such findings may redefine the recently published European guidelines for the assessment and treatment of tinnitus, to which I contributed, and significantly improve patient care.

6. **How did you become aware of the importance of addressing SABV (e.g. by attending specific meetings, seminars or other dedicated events; through personal scientific interest; etc.)?**

I have done my PhD thesis in a totally different field, working in a laboratory investigating sexual development, and assessing the impact of endocrine disruptors with estrogen-like activity on male reproduction and metabolism. During this education, I got exposed to researchers investigating risks in male prostate cancer, and in female breast cancer, but from an endocrine perspective. However, we also explored how such compounds could impact male and female development separately. For instance, peri-natal exposure to Bisphenol-A (a monomer used to construct polycarbonate plastics) stimulated adipogenesis in female rats but not in males. Considering SABV has been part of my research from the beginning of my education.

7. **In research involving sexed organisms, do you think that the default option should be to use animals of both sexes? Please explain also by describing your personal experience. [Please do not answer if you have never worked with model organisms]**

No, I do not believe it should be a default option. As mentioned above, SABV cannot be applied in all circumstances, and the researcher – who has expertise in his own field - is best positioned to judge this possibility. For experiments with single interventions, or drug treatments, it is definitely worth considering SABV. In contrast, we are now performing circadian experiments (with collection of organs every 6th hour around the clock) under NIH funding. Including females would have a large impact on the budgeting of such project and wouldn't allow us to address other fundamental questions in the field.

8. **Do you think the same should apply also with regards to cell lines/primary cells? Please explain also by describing your personal experience. [Please do not answer if you have never worked with cell lines/primary cells].**

Time will tell – but according to my own experience, cell lines would be used for screenings or mechanistic insights to complement *in vivo* studies. Since hormones interact in a multi-order feedback system, I would expect fewer insights from cells in a petri dish than from *in vivo* studies.

9. **Common criticisms of the default use of both sexes in experiments is that this would (i) markedly increase the cost of the research and (ii) go against the 3R principle of animal research (i.e. replacement, reduction, refinement). What is your opinion on this?**

In the context of our experimental research, it would definitely have a major impact on research costs (e.g. animal housing, staff, time commitment) and ultimately productivity. I do not think the use of both sexes can be easily applied to all research contexts and research questions.

Regarding 3Rs, the use of both sexes has nothing to do with such principles. If a question aims to address a sex or gender effect, then this would have a scientific value that would justify such experiments.

10. **In your funding applications, have you ever addressed SABV specifically? If yes, for which funding agency?**

- NIH, as the amount of funding allowed us to consider such aspects.
- GENDER-NET, as this was the research focus of this call.

11. **Could you comment on the general awareness of SABV among your peers?**

Low, but our recent findings in humans – and our dissemination activities thereof - have raised awareness among our peers, which is why I am now coordinating a Research Topic in Frontiers to stimulate research on these sex and gender aspects in tinnitus. I predict that, in the long-term, this would lead to an improved knowledge on pathophysiology, improved diagnostics, and finally a medicine adapted to men and women.

In the pre-clinical research area, there is awareness about it. However, there is a clear hurdle to overcome. Methods of automated data extraction and analyses are under way in order to minimize the time spent after data acquisition, but "high-throughput" data collection *in vivo* will be essential to allow such breakthrough in our research field. Until then, consideration of SABV in experimental auditory research will be challenging.

12. **What do you think is the one thing that would markedly increase consideration of SABV among biomedical researchers?**

Project calls like the GENDER-NET are excellent incentives to promote research on sex and gender. While the EU seems to embrace this challenge, each EU country could - for instance – allocate a greater amount of financing for studies aimed at addressing SABV. Specific research programs (at a National or European level) with a focus on SABV could also promote research in this area.

KI Case Study 2
Reproductive Endocrinology and Metabolism

Scientist and Department:
Professor, PhD, Elisabet Stener-Victorin, Dept. of Physiology and Pharmacology (FYFA)

Relevant publication(s) or grant(s):
Fornes et al 2019. Mice exposed to maternal androgen excess and diet-induced obesity have altered phosphorylation of catechol-O-methyltransferase in the placenta and fetal liver. *Int J Obes*. Jan 22. doi: 10.1038/s41366-018-0314-8.

Manti et al 2018. Maternal androgen excess and obesity induce sexually dimorphic anxiety-like behavior in the offspring. *FASEB J*. 32(8):4158-4171. doi: 10.1096/fj.201701263RR.

Hu et al 2015. Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring. *PNAS* 112(46):14348-53. doi: 10.1073/pnas.1507514112.

Link to research group:
https://ki.se/en/fyfa/reproductive-endocrinology-and-metabolism

1. **Please briefly describe your research and explain which parts of it are relevant to SABV as described above.**

The main topic of our research is to investigate the pathophysiology of polycystic ovary syndrome (PCOS), which is the most common endocrine and metabolic disorder in women. It is characterized by elevated levels of sex steroids, in particular androgens. The underlying mechanism is not known, and our hypothesis is that the pregnant PCOS mother affects the growing fetus, causing adult phenotypic changes in both female and male offspring. Currently we investigate whether phenotypic changes can be passed on to subsequent generations, i.e. transgenerational effects.

2. **In your research, you show some sex and/or gender-specific differences. Did you plan to look at sex and/or gender differences or did you simply notice them during the project? In case this was not planned, how did you become aware of such differences?**

Yes, we see gender differences. Some studies are too big to include both genders in the publications. When possible, we include male offspring in all our measurements. However, it is also a matter of time and money as every experiment will be twice as big and the cost increases dramatically.

3. **Are you planning to follow up on SABV in your future research? If so, in what way? If not, why not?**

Yes, see above.

4. **In what way did integrating SABV benefit your science?**

Recent clinical studies indicate that also boys of PCOS mothers may be affected. However, little basic research has been done. Also, the general community shows more and more interest in results where sexually dimorphic changes are shown.

5. **In what ways can your research contribute to sex- and/or gender-specific improvements in medical and biological knowledge, diagnostic tools or therapy (i.e. gendered innovations)?**

Our research demonstrates that not only female offspring, but also male offspring are affected by maternal androgen exposure. We also investigate molecular mechanisms which in the future might be used as biomarkers/predictors.

6. **How did you become aware of the importance of addressing SABV (e.g. by attending specific meetings, seminars or other dedicated events; through personal scientific interest; etc.)?**

Through my research field, working with developmental health and disease.

7. **Do you think the same should apply also with regards to cell lines/primary cells? Please explain also by describing your personal experience. [Please do not answer if you have never worked with cell lines/primary cells].**

Important to use donors of both sexes.

8. **Common criticisms of the default use of both sexes in experiments is that this would (i) markedly increase the cost of the research and (ii) go against the 3R principle of animal research (i.e. replacement, reduction, refinement). What is your opinion on this?**

I already brought up this issue. We are constantly underfunded. We need more funding to have personnel to be able to do the research; also, all molecular analyses are double. As we perform transgenerational experiments and generate our mice via breeding or IVF, it does not go against the 3R. Rather, taking away male pups goes against 3R. In the ideal world, I would like to have funding to be able to follow both sexes in all measures.

9. **In your funding applications, have you ever addressed SABV specifically? If yes, for which funding agency?**

Yes, KAW scholar, KAW project grant and Swedish research project grant and Distinguished Professor applications.

10. **Could you comment on the general awareness of SABV among your peers?**

Good as we investigate this.

11. **What do you think is the one thing that would markedly increase consideration of SABV among biomedical researchers?**

Funding!! I think KI could support those who do this kind of research specifically. Right now, we are starting a big transgenerational experiment. At the moment I don't have funding to take on

one more person that would be needed to follow both sexes, so we will focus only on females. This is directly against the 3R and very sad.

KI Case Study 3
Energy metabolism, obesity and diabetes

Scientist and Department:
Associate Professor Jorge Ruas, Dept. of Physiology and Pharmacology (FYFA)

Relevant publication(s) or grant(s):
Agudelo et al 2018. Kynurenic Acid and Gpr35 Regulate Adipose Tissue Energy Homeostasis and Inflammation. *Cell Metab.* 27(2):378-392. doi: 10.1016/j.cmet.2018.01.004

Bayindir-Buchhalter et al 2018. Cited is a sex-biased mediator of the antidiabetic glitazone response in adipocyte progenitors. *EMBO Mol Med.* 10(8). pii: e8613. doi: 10.15252/emmm.201708613.

Link to research group:
https://ki.se/en/fyfa/the-ruas-lab-molecular-and-cellular-exercise-physiology

1. **Please briefly describe your research and explain which parts of it are relevant to SABV as described above.**

We do pre-clinical research on how the body uses energy (energy metabolism) in the context of physical exercise versus muscle disease, obesity, and diabetes.

2. **In your research, you show some sex and/or gender-specific differences. Did you plan to look at sex and/or gender differences or did you simply notice them during the project? In case this was not planned, how did you become aware of such differences?**

In the broad field of metabolism, sex-specific differences have been appreciated since long. For example, related to hormonal control of metabolism, how individuals respond to different kinds of exercise, or even to the incidence of metabolic disease. We try to run our experiments in males and females, when possible.

3. **Are you planning to follow up on SABV in your future research? If so, in what way? If not, why not?**

We are not specifically trying to identify novel aspects of SABV but are interested in understanding how disturbances in energy metabolism, either positive or negative, affect male and female physiology.

4. **In what way did integrating SABV benefit your science?**

Part of our goal is to detail the molecular mechanisms by which physical exercise is beneficial to human health, so we can propose therapies for metabolic disease. These mechanisms must be validated in males and females, so that they are valid for both sexes.

5. **In what ways can your research contribute to sex- and/or gender-specific improvements in medical and biological knowledge, diagnostic tools or therapy (i.e. gendered innovations)?**

Please see 4.

6. **How did you become aware of the importance of addressing SABV (e.g. by attending specific meetings, seminars or other dedicated events; through personal scientific interest; etc.)?**

It is well known that energy metabolism is different in males and females.

7. **In research involving sexed organisms, do you think that the default option should be to use animals of both sexes? Please explain also by describing your personal experience. [Please do not answer if you have never worked with model organisms]**

Yes. We work mainly with mice and try to use, when possible, males and females.

8. **Do you think the same should apply also with regards to cell lines/primary cells? Please explain also by describing your personal experience. [Please do not answer if you have never worked with cell lines/primary cells].**

For primary cells, we tend to work with mixed populations as they are prepared (in our case) before we know the sex of the animals. We have, on occasion, prepared primary cells from males only but haven't explored these differences yet. Ideally one should move to using homogeneous cell populations.

9. **Common criticisms of the default use of both sexes in experiments is that this would (i) markedly increase the cost of the research and (ii) go against the 3R principle of animal research (i.e. replacement, reduction, refinement). What is your opinion on this?**

Yes, it increases the costs, and funding agencies must take this into consideration. So far there has been no adjustment of grant value for those who include males and females in their research. This should be taken into consideration if we really consider this aspect important. I think this is scientifically necessary.

In terms of the 3R principle, I think it's better to validate findings in both sexes from the beginning, than having to repeat experiments in the future and reach the conclusion that they don't apply in one case or the other.

10. **In your funding applications, have you ever addressed SABV specifically? If yes, for which funding agency?**

Very few funding agencies have ever asked for this, although it has started to change in very recent times.

11. **Could you comment on the general awareness of SABV among your peers?**

Please see 2 and 6.

12. **What do you think is the one thing that would markedly increase consideration of SABV among biomedical researchers?**

Report data on males and females when you present and publish your data. The relevance of the differences people find will encourage others to also look in both sexes.

This questionnaire was adapted (with permission) from a questionnaire used by the EU-funded LIBRA consortium.
If you have questions or would like to share your views on SABV, please contact Tamsin Lindström at KI's Grants Office.