KI Case Studies, Series 2 — 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.

In this second series of the KI SABV Case Studies, 4 KI researchers — Bertrand Joseph, Rochellys Diaz Heijtz, Rikard Holmdahl, and Ivan Nalvarte— share their views on considering SABV and explain how doing so has benefitted their research.

Any questions?
Please contact Tamsin Lindström

Please see next page for the questionnaire

KI Case Study 1
Life and death decisions at the cellular level

Scientist and Department:
Bertrand Joseph, Professor of Molecular Cancer Biology at the Institute of Environmental Medicine

Relevant publication(s) or grant(s):
Arg1^{+}microglia are critical for shaping cognition in female mice. Vassilis Stratoulias, Rocío Ruiz, Shigeaki Kanatani, Ahmed M. Osman, Jose A. Armengol, Antonio Rodríguez-Moreno, Adriana-Natalia Murgoci, Irene García-Domínguez, Lily Keane, Guillermo Vázquez-Cabrera, Isabel Alonso-Bellido, Nathalie Vernoux, Dario Tejera, Kathleen Grabert, Mathilde Cheray, Patricia González-Rodríguez, Eva M. Pérez-Villegas, Irene Martinez-Gallego, David Brodin, Javier Avila-Cariño, Mikko Airavaara, Per Uhlén, Michael T. Heneka, Marie-Ève Tremblay, Klas Blomgren, Jose L. Venero, Bertrand Joseph.
https://www.biorxiv.org/content/10.1101/2021.08.15.456225v1

Link to research group:
https://ki.se/en/imm/bertrand-joseph-research-group

1. Please briefly describe your research and explain in what way sex as a biological variable (SABV, as defined on the previous page) is relevant.
Our team investigate the role of microglia, resident immune cells of the central nervous system, in the development of the brain, as well as their contribution to brain disorders, ranging from Alzheimer's disease (AD) to glioblastoma (GBM) and autism spectrum disorders (ASD). Both the healthy brain and the diseased brain exhibit sex differences and, thus, SABV must be considered. AD, GBM and ASD all have sex differences in humans in terms of prevalence, response to treatment and so on.

2. In your research, you show some sex-specific differences. Did you plan to look at sex differences or did you simply notice them during the project?
The response is a mixed one: when possible, we include both sexes in our *in vivo* analysis or analysis of human tissue samples from biobanks. However, I must admit that we do not systematically expect that we would see a sex difference.

In the above study (Stratoulias *et al*), as the animals to be analysed were difficult to obtain, we decided to pool male and female data, and we could not see any significant effects of microglial deficiency in Arg1+. When we thereafter analysed the data taking SABV into account, we found that female mice, but not male mice, exhibited a robust phenotype.

3. Do you plan to follow up on SABV in your future research? If so, in what way? If not, why not?
Definitely, and more as a routine for all projects. We also now aim at developing new *in vitro* models that include SABV. As an illustration, most microglial cell lines are 'defined' as sex unknown.

4. In what way did integrating SABV benefit your research? Can your research contribute to sex-specific improvements in medical or biological knowledge, diagnostic tools, or therapy?
Integrating SABV lead to the discovery of microglial cellular and molecular mechanisms that would not have been uncovered without addressing SABV. Further understanding of sex differences in microglial cellular functions could lead to better understanding of brain dysfunction and potentially open new avenues for therapeutic approaches that include SABV.

5. In research involving vertebrates, 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, no doubt about it. For example, if we had not done so, we would not have uncovered the function of *arg1*$^{+}$ microglia. Furthermore, the conditions we study have sex differences, and it makes little sense to not include that parameter in our investigations.

6. Do you think the same should apply also with regards to cell lines or primary cells? Please explain also by describing your personal experience. [Please do not answer if you have never worked with cell lines or primary cells].
As mentioned above, we are now trying to develop male and female cell lines from primary microglia.

7. 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).

a. What is your opinion on this?
Yes, you can "save" on number of animals by looking only at one sex. But if you do not include both sexes, 1) you are not taking into account SABV, which could be essential for your model, and 2) you do not get any significant results if you picked the "wrong" sex, and you will have sacrificed animals unnecessarily.

b. Are you aware that research funders do not necessarily expect basic-science studies to be powered to detect sex differences, but that they do expect you to collect data, if possible, in a way that allows you to tabulate and disaggregate analysis by sex?
Not aware of it

c. Are you aware that the 'reduction' part of the 3R definition has evolved from 'methods which minimise the number of animals used per experiment' to 'appropriately designed and analysed experiments that are robust and reproducible, and truly add to the knowledge base', including allowing 'the information gathered per animal in an experiment to be maximised in order to reduce the use of additional animals'?
Yes

8. In your funding applications, have you ever addressed SABV specifically? If yes, for which funding agency, and do you think addressing SABV helped your application?
Yes, EU, Vetenskapsrådet, Hjärnfonden.
No idea if the reviewers took that aspect into account. I included it as it is relevant for our investigations.

9. Could you comment on the general awareness of SABV among your peers?
Sex differences are rather accepted in the field of microglia.

10. What do you think are the key steps that could be taken to substantively increase consideration of SABV among biomedical researchers, e.g., raising awareness, providing evidence of value, online tools and resources, or hands-on support (such as project-specific guidance or statistical support with research design)?

a. In general?
   It will come; as more publications include SABV, you will have no choice but to follow.

b. At KI specifically?
   Courses on SABV in your research?

KI Case Study 2
Nuclear receptor signaling in neurodevelopment and neurodegeneration

Scientist and Department:
Ivan Nalvarte, Associate Professor at Biosciences and Nutrition

Relevant publication(s) or grant(s):
Grant: Understanding the role of menopause and estrogen receptor activation for Alzheimer's disease risk, US National Institutes of Health R01, prime award

Publications:
Maioli S, Leander K, Nilsson P, Nalvarte I. Estrogen receptors in the ageing brain. *Essays in Biochemistry*, Oct 8, 2021
https://pubmed.ncbi.nlm.nih.gov/34623401/

Li X, Zhong H, Wang Z, Xiao R, Antonson P, Liu T, Wu C, Zou J, Wang L, Nalvarte I, Xu H, Warner M, Gustafsson JA, Fan X. Loss of liver X receptor β in astrocytes leads to anxiety-like behaviors via regulating synaptic transmission in the medial prefrontal cortex in mice. *Molecular Psychiatry*, 2021
https://pubmed.ncbi.nlm.nih.gov/33963286/

Brundin PMA, Landgren BM, Fjällström P, Shamekh MM, Gustafsson JÅ, Johansson AF, Nalvarte I. Expression of Sex Hormone Receptor and Immune Response Genes in Peripheral Blood Mononuclear Cells During the Menstrual Cycle. *Frontiers Endocrinol*, Sep 22, 2021
https://pubmed.ncbi.nlm.nih.gov/34630328/

Nalvarte I. Sex stratified treatment of neurological disorders: Challenges and perspectives *Brain Sci*, Feb14, 2020, 10(2)
https://pubmed.ncbi.nlm.nih.gov/32075025/

Varshney M and Nalvarte I. Genes, gender, environment, and novel functions of Estrogen Receptor beta in the susceptibility to neurodevelopmental disorders. *Brain Sci,* Feb 2017, 7(3), 24 https://pubmed.ncbi.nlm.nih.gov/28241485/

Link to research group:
https://ki.se/en/bionut/nuclear-receptor-signaling-in-neurodevelopment-and-neurodegeneration-ivan-nalvarte

1. **Please briefly describe your research and explain in what way sex as a biological variable (SABV, as defined on the previous page) is relevant.**

My research group study the role of the female sex hormone estrogen in reproduction, neurodevelopment, and neurological diseases. In particular, we are interested in the contribution of estrogen and the estrogen receptors in sex differences in neurological diseases. Alzheimer's disease (AD), depression, and anxiety disorders are more common in women, whereas Parkinson's disease and neurodevelopmental disorders, such as autism spectrum and attention-deficit disorders, are more common in males. Lately, our focus has been on understanding the contribution of estrogen signalling to the sex differences observed in AD. Sex hormones have neuroprotective properties and their levels differ between ageing men and women. While levels of the male sex hormone testosterone decline slowly in ageing men, estrogen levels decline more abruptly in women at menopause. Therefore, it is suggested that the loss of estrogen may result in some degree of loss of resilience to neurological disorders. Why some women are more resilient to this loss than others is unknown. By integrating epidemiologic data on menopause, hormone therapy, and AD outcome with experimental studies in rodent and cell models, as well as in post-mortem human brains, we want to decipher the contribution of sex hormones to AD risk and progression. Thus, our research incorporates sex as a biological variable on several levels: from population-based cohorts to mouse and cell models. In this way, we think our research can contribute to better understanding AD pathogenesis, to identifying particular risk groups among men and women, and to developing new, more personalized recommendations or treatments to combat AD in men and women.

2. **In your research, you show some sex-specific differences. Did you plan to look at sex differences or did you simply notice them during the project?**

This was planned. It is well known that women run a 2-3-fold increased risk of being diagnosed with AD. There is a wealth of scientific literature anchoring this increased female risk; nevertheless, the underlying causes and mechanisms are largely unknown.

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

Yes. We will use population-based cohorts of postmenopausal women of which half have been prescribed hormone therapy, and integrate the data gathered in rodent male and female models, as well as in male and female cell models (e.g., induced pluripotent stem cells). Cell models are important in discriminating between effects of genetic sex (e.g., XX vs XY chromosome) and sex-hormone signalling.

4. **In what way did integrating SABV benefit your research? Can your research contribute to sex-specific improvements in medical or biological knowledge, diagnostic tools, or therapy?**

Our research revolves around SABV since we study sex differences in neurological diseases. We hope that our research can contribute to identifying particular risk groups among men and women as well

as to tailoring more personalised preventive recommendations or treatment options to these risk groups.

5. **In research involving vertebrates, 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.]**

The default option should be to ask the questions, "Can it be assumed that my research affects males and females differently? If so, is this due to biological sex differences or to societal gender differences?". If biological sex differences can be assumed, then one should use animals of both sexes. From my personal experience in the field of neuroendocrinology, we almost always see that male and female animal models show different neurological experimental outcomes.

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

Not regarding cell lines (since these are usually available only in one sex), but probably when using primary cells or animal- or human-derived stem cells. It depends on the research question. If you expect or see sex differences in animal or human studies, these sex differences could be due to genetic sex differences (XX vs XY chromosome), and then the cells originating from the female sex (XX) may behave differently to the cells originating from the male sex (XY).

7. **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).**

    1. What is your opinion on this?

    I think this criticism is valid. One should weigh the pros vs cons. What would one gain from including both sexes in animal research and what would one gain in not doing this.

    2. Are you aware that research funders do not necessarily expect basic-science studies to be powered to detect sex differences, but that they do expect you to collect data, if possible, in a way that allows you to tabulate and disaggregate analysis by sex?

    Yes

    3. Are you aware that the 'reduction' part of the 3R definition has evolved from 'methods which minimise the number of animals used per experiment' to 'appropriately designed and analysed experiments that are robust and reproducible, and truly add to the knowledge base', including allowing 'the information gathered per animal in an experiment to be maximised in order to reduce the use of additional animals'?

    Yes

8. **In your funding applications, have you ever addressed SABV specifically? If yes, for which funding agency, and do you think addressing SABV helped your application?**

Yes. We addressed this in our application to the NIH (USA). Since the application revolved around sex differences in AD, SABV was an integral part of the application.

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

When I started working on sex differences in AD, most clinicians and even some renowned Swedish researchers in the field did not know about these sex differences. This was just a few years back. I am very happy that nowadays it is well accepted that sex differences exist in AD and that understanding these differences has become a priority focus area of some large funding agencies (such as the US National Institute of Aging).

10. **What do you think are the key steps that could be taken to substantively increase consideration of SABV among biomedical researchers, e.g., raising awareness, providing evidence of value, online tools and resources, or hands-on support (such as project-specific guidance or statistical support with research design)?**

    1. In general?

    Raising awareness by asking for SABV considerations from granting agencies and scientific journals.

    2. At KI specifically?

    Raising awareness by organizing workshops and seminars on the topic (e.g., from grants office?) and by including SABV in education at all levels.

11. **Any other comments?**

Great initiative!

KI Case Study 3
Microbiota-gut-brain axis and neurodevelopmental disorders

Scientist and Department:

Rochellys Diaz Heijtz, Associate Professor at Neuroscience (also Professor at the University of Rouen Normandy, France)

Relevant publication(s) or grant(s):

Grant: Role of Gut Microbiota in Typical and Atypical Brain Development, Research Environment Grant Within Interdisciplinary Research, Swedish Research Council (2018-2024)

Publications:

1. Arentsen, T., Qian, Y., Gkotzis, S., Femenia, T., Wang, T., Udekwu, K., Forssberg, H., Diaz Heijtz, R., 2017. The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. *Mol Psychiatry* 22, 257-266. Doi: 10.1038/mp.2016

2. Arentsen, T., Khalid, R., Qian, Y., Diaz Heijtz, R., 2018. Sex-dependent alterations in motor and anxiety-like behavior of aged bacterial peptidoglycan sensing molecule 2 knockout mice. *Brain Behav Immun* 67, 345-354. Doi: 10.1016/j.bbi.2017.09.014

3. Femenia, T., Qian, Y., Arentsen, T., Forssberg, H., Diaz Heijtz, R., 2018. Toll-like receptor-4 regulates anxiety-like behavior and DARPP-32 phosphorylation. Brain Behav Immun 69:273-282. Doi: 10.1016/j.bbi.2017.11.022

4. Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., Pettersson, S., 2011. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A* 108, 3047-3052. Doi: 10.1073/pnas.1010529108

Link to research group:
https://ki.se/en/neuro/diaz-heijtz-laboratory

1. **Please briefly describe your research and explain in what way SABV (as defined on the previous page) is relevant.**

My laboratory is primarily interested in understanding the biological basis of neurodevelopmental disorders such as autism spectrum disorder (ASD). There is currently no cure for ASD, and existing medications are only partly effective. A key goal is thus to identify novel pathophysiological pathways of ASD, as well as new potential strategies to reduce ASD-associated symptoms. In recent years, considerable interest has been devoted to the potential role of the gut microbiota (the trillions of microorganisms inhabiting our gastrointestinal tract) in the pathogenesis of ASD. New scientific discoveries showing a role for the gut microbiota in the modulation of brain development and behavior have triggered a paradigm shift in our conceptualization of the origin of human brain disorders, including ASD. Currently, my laboratory is investigating the role of the gut microbiota in typical and atypical brain development in both humans and animal models, as well as the cellular and molecular pathways involved in the crosstalk between the microbiota and the developing brain.

There are important sex differences in the prevalence and manifestation of ASD and related neurodevelopmental disorders, including attention- deficit/hyperactivity disorder (ADHD). It is thus important to consider sex as a biological variable to better understand the underlying biology of ASD and to identify better treatment strategies.

2. **In your research, you show some sex-specific differences. Did you plan to look at sex differences or did you simply notice them during the project?**

Given the fact that that most neurodevelopmental disorders exhibit a sex bias, I have always been interested in investigating potential sex-specific differences in my research work.

I became aware early in my career about the importance of addressing sex as a biological variable in my research work. I was very much inspired by the work of Prof. Roger Gorski at the University of California Los Angeles (UCLA), who passed way in October 2021. Prof. Gorski was a pioneer in the field of brain sex differences. He helped establish the concept of the hormone-dependent sexual differentiation of the structure and function of the brain. I was fortunate to visit his lab at UCLA during my research training in neuroendocrinology.

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 research? Can your research contribute to sex-specific improvements in medical or biological knowledge, diagnostic tools, or therapy?**

We have learned from our own work that some effects of the gut microbiota on the brain are age- and sex-dependent, and therefore, these host factors must be considered when developing new

microbiota-based therapeutic strategies for the treatment of ASD and other neurodevelopmental disorders.

5. **In research involving vertebrates, 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 with both males and females.

For example, we showed that the absence of the bacterial peptidoglycan-sensing molecule Pglyrp2 leads to major sex-dependent alterations in motor and anxiety-like behavior. Pglyrp2-deficient female mice, but not males, show better motor performance. However, they display increased levels of anxiety-like behavior, suggesting that the modulatory effects of peptidoglycan-sensing molecules in the brain are highly dependent upon multiple host factors including sex, and type of neuronal circuits.

6. **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).**

   a. What is your opinion on this?

   b. Are you aware that research funders do not necessarily expect basic-science studies to be powered to detect sex differences, but that they do expect you to collect data, if possible, in a way that allows you to tabulate and disaggregate analysis by sex?

   c. Are you aware that the 'reduction' part of the 3R definition has evolved from 'methods which minimise the number of animals used per experiment' to 'appropriately designed and analysed experiments that are robust and reproducible, and truly add to the knowledge base', including allowing 'the information gathered per animal in an experiment to be maximised in order to reduce the use of additional animals'?

We need to have more active discussions about all the above points. This will help clarify "myths" about the use of males and females in biomedical research.

7. **In your funding applications, have you ever addressed SABV specifically? If yes, for which funding agency, and do you think addressing SABV helped your application?**

Grants from the Swedish Brain Foundation, the Swedish Medical Council (VR), and the Horizon 2020 Framework Programme of the European Union.

Currently, all major funding agencies around the world require scientists to meaningfully integrate sex and gender aspects into the research they support.

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

Many of my peers are very aware of SABV, but they have valid concerns about the extra costs and efforts involved when studying both sexes.

9. **What do you think are the key steps that could be taken to substantively increase consideration of SABV among biomedical researchers, e.g., raising awareness, providing evidence of value, online tools and resources, or hands-on support (such as project-specific guidance or statistical support with research design)?**

   a. In general?

   Presenting and discussing sex-differences in various venues (e.g., meetings, workshops, websites) and their potential clinical implications.

   b. At KI specifically?

   KI will benefit by having regular workshops about SABV policy and resources available to help our biomedical researchers.

   In addition, we need to bring together our clinical and preclinical scientists at KI. This will facilitate the dissemination of knowledge and new findings to the broader scientific community, a key aspect for rapid implementation of biomedical findings into practical use.

KI Case Study 4
Chronic Inflammatory Disorders

Scientist and Department:

Rikard Holmdahl, Professor at Medical Biochemistry and Biophysics

Relevant publication(s) or grant(s):

1. Holmdahl R, Jansson L, Andersson M. Female sex hormones suppress development of collagen-induced arthritis in mice. *Arthritis Rheum*. 1986;29:1501-9.
2. Holmdahl R, Jansson L, Meyerson B, Klareskog L. Oestrogen induced suppression of collagen arthritis: I. Long term oestradiol treatment of DBA/1 mice reduces severity and incidence of arthritis and decreases the anti type II collagen immune response. *Clin Exp Immunol.* 1987;70(2):372-8.
3. Holmdahl R, Jansson L. Estrogen-induced suppression of collagen arthritis. III. Adult thymectomy does not affect the course of arthritis or the estrogen-mediated suppression of T-cell immunity. *Brain Behav Immun.* 1988;2(2):123-32.
4. Holmdahl R, Carlsten H, Jansson L, Larsson P. Oestrogen is a potent immunomodulator of murine experimental rheumatoid disease. *Br J Rheumatol.* 1989;28:54-8.
5. Holmdahl R. Estrogen exaggerates lupus but suppresses T-cell-dependent autoimmune disease. *J Autoimmun*. 1989;2(5):651-6.
6. Jansson L, Holmdahl R. Oestrogen induced suppression of collagen arthritis. IV: Progesterone alone does not affect the course of arthritis but enhances the oestrogen-mediated therapeutic effect. *J Reprod Immunol.* 1989;15:141-50.
7. Holmdahl R, Andersson M, Goldschmidt TJ, Gustafsson K, Jansson L, Mo JA. Type II collagen autoimmunity in animals and provocations leading to arthritis. *Immunol Rev.* 1990;118:193-232.
8. Jansson L, Mattsson A, Mattsson R, Holmdahl R. Estrogen induced suppression of collagen arthritis. V: Physiological level of estrogen in DBA/1 mice is therapeutic on established arthritis, suppresses anti-type II collagen T-cell dependent immunity and stimulates polyclonal B-cell activity. *J Autoimmunity.* 1990;3:257-70.

9. Mattsson R, Mattsson A, Holmdahl R, Whyte A, Rook GA. Maintained pregnancy levels of oestrogen afford complete protection from post-partum exacerbation of collagen-induced arthritis. *Clin Exp Immunol.* 1991;85(1):41-7.
10. Holmdahl R, Jansson L, Andersson M, Jonsson R. Genetic, hormonal and behavioural influence on spontaneously developing arthritis in normal mice. *Clin Exp Immunol.* 1992;88(3):467-72.
11. Jansson L, Holmdahl R. Oestrogen-induced suppression of collagen arthritis; 17 beta-oestradiol is therapeutically active in normal and castrated F1 hybrid mice of both sexes. *Clin Exp Immunol.* 1992;89(3):446-51.
12. Mattsson R, Mattsson A, Hansson I, Holmdahl R, Rook GA, Whyte A. Increased levels of prolactin during, but not after, the immunisation with rat collagen II enhances the course of arthritis in DBA/1 mice. *Autoimmunity*. 1992;11(3):163-70.
13. Jansson L, Holmdahl R. Genes on the X chromosome affect development of collagen-induced arthritis in mice. *Clin Exp Immunol*. 1993;94(3):459-65.
14. Jansson L, Holmdahl R. The Y chromosome-linked "autoimmune accelerating" yaa gene suppresses collagen-induced arthritis. *Eur J Immunol.* 1994;24(5):1213-7.
15. Jansson L, Olsson T, Holmdahl R. Estrogen induces a potent suppression of experimental autoimmune encephalomyelitis and collagen-induced arthritis in mice. *J Neuroimmunol.* 1994;53(2):203-7.
16. Holmdahl R. Female preponderance for development of arthritis in rats is influenced by both sex chromosomes and sex steroids. *Scand J Immunol.* 1995;42(1):104-9.
17. Jansson L, Holmdahl R. Estrogen-mediated immunosuppression in autoimmune diseases. *Inflamm Res.* 1998;47(7):290-301.
18. Jansson L, Holmdahl R. Enhancement of collagen-induced arthritis in female mice by estrogen receptor blockage. *Arthritis Rheum*. 2001;44(9):2168-75.
19. Fernandez Lahore G, Förster M, Johannesson M, Sabatier P, Lönnblom E, Aoun M, et al. Polymorphism in estrogen receptor binding site causes CD2-dependent sex bias in T cell autoimmune diseases. *Nat Com*. 2021. Sep 22;12(1):5565.

Link to research group:
https://ki.se/en/mbb/mir-current-research-and-publications

1. **Please briefly describe your research and explain in what way SABV (as defined on the previous page) is relevant.**

Sex-linked effects influence essentially all basic biology to a variable extent. A good example are autoimmune diseases, which are the focus of our research. Females are more prone to developing certain autoimmune diseases, e.g., rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), whereas males rather run the risk of developing others, e.g., ankylosing spondylitis. The basis for these sex differences is not known.

2. **In your research, you show some sex-specific differences. Did you plan to look at sex differences or did you simply notice them during the project?**

We have intentionally tried to address sex differences. Sex differences is an old observation for autoimmune diseases, and we wanted to address the cause. Sex hormones and their regulation are highly conserved across species; thus, it is adequate to use mouse models for these issues. However, sex differences are the sum of a range of different genetic and environmental factors and are not easy

to understand. Several years ago, we made some of the basic findings that showed that sex hormones have a fundamental role in regulating RA- and SLE-like diseases in animal models. We found that several of the female sex hormones, in particular the different variants of oestrogens, suppress the development of arthritis but promote the development of lupus, in contrast, in fact, to the dogma prevailing at the time (1, 2)(3-7)(8-18). We could also confirm that oestrogens during pregnancy protect against arthritis, and that the postpartum relapse of the disease was due to the rapid drop in levels of oestrogens, rather than other factors.

Animal models are very useful for addressing sex differences through controlled experiments; for example, sex hormones can be blocked pharmaceutically, genetically, or surgically.

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

We are always aware of sex differences, and we address the influence of both hormones and genetic differences in all projects. We are currently also directly addressing sex differences in a project in which we (for the first time) positioned a polymorphic oestrogen-receptor DNA-binding site (19), which profoundly regulates surrounding genes, thus, with an impact only in females.

4. **In what way did integrating SABV benefit your research? Can your research contribute to sex-specific improvements in medical or biological knowledge, diagnostic tools, or therapy?**

Our goal is to understand why we develop autoimmune diseases, with the goal to prevent these diseases. Understanding sex differences is a part of the solution.

5. **In research involving vertebrates, 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.]**

Most important is to use controlled experiments with groups matched, with only the issue that is under study differing. This is, of course, the basic fundament in science but is often not followed. It also includes sex differences. Sex must be matched between the experimental groups, but it gives more power if sex difference is the issue under investigation, of course. But using both sexes in mouse experiments has pros and cons relating to both genetic and environmental issues. However, sex differences are not the main issue for understanding biology or autoimmune disease as most of the biology is the same between sexes—but it is important to keep the possibility of sex differences in mind and to consider.

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

In principle, yes, though in most cases sex might not have a major influence. In some cases, however, it does; for instance, transferring cells of male origin into a female mouse is not a good idea as the male cells express proteins that do not exist in females, and which therefore trigger a strong immune response in the female.

7. **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).**

a. What is your opinion on this?

The whole area of 3R is very confusing and often misleading. The only reason to use animals in research is to better understand biology. This is mainly based on curiosity and will lead humans to understand themselves and the surrounding in a more correct way. In addition, it could also help to develop better treatment and prevention of diseases. So, the main question is how to do research better. It requires controlled experiments and enough statistical power, enough mouse numbers. The implementation of 3R, at least in Europe, has lowered the quality of science in Europe and led to a tendency to do unethical experiments on humans; a recent example is the experiments performed by Paolo Macchiarini.

b. Are you aware that research funders do not necessarily expect basic-science studies to be powered to detect sex differences, but that they do expect you to collect data, if possible, in a way that allows you to tabulate and disaggregate analysis by sex?

I am not aware of such regulations. However, I think it is very important to understand the biology of both sexes. Our main problem in doing this is the increasing difficulties in doing experimental research, with higher costs and regulations prohibiting research.

c. Are you aware that the 'reduction' part of the 3R definition has evolved from 'methods which minimise the number of animals used per experiment' to 'appropriately designed and analysed experiments that are robust and reproducible, and truly add to the knowledge base', including allowing 'the information gathered per animal in an experiment to be maximised in order to reduce the use of additional animals'?

I am reviewing quite a number of papers in the experimental field, and the trend is that quality in animal research has decreased, owing to new so-called ethical regulations. Experiments are usually under-powered and, when the investigators are asked why, they refer to restrictions they must follow as well as economic costs. Often, factors they must follow owing to their 'ethical' regulations include having enriched material in the cages, which has behavioural effects; killing animals before experiments end, which skews the results; or even using historic controls, which is obviously not correct. According to results from anonymous questionnaires, such practices are common and increasing. The result is that many published animal experiments cannot be reproduced. It is bad for science and will, in the end, lead to the use of more animals, or alternatively to doing experiments on humans, a practice which led to the Helsinki declaration agreement.

8. In your funding applications, have you ever addressed SABV specifically? If yes, for which funding agency, and do you think addressing SABV helped your application?

We usually address SABV, for the simple reason that it is included in our research.

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

In our research group it is a research issue. Among clinicians and the general population, it is often quite a simple-minded view in that it is the sex per se, and not the myriad of different underlying factors, that is of importance. An example are mouse models of RA, in which males are more susceptible to experimental arthritis—the opposite to human RA, to which females are more susceptible. However, this effect could simply depend on stress resulting from the grouping of animals after puberty in a nonphysiological way, an effect that is enhanced by having enriched material in the cages, allowing animal hierarchies to be built.

10. **What do you think are the key steps that could be taken to substantively increase consideration of SABV among biomedical researchers, e.g., raising awareness, providing evidence of value, online tools and resources, or hands-on support such as project-specific guidance or statistical support with research design)?**

    a. In general?
    Prioritize experimental animal research so that there are possibilities to address these issues.

    b. At KI specifically?
    Change the organisation of animal facilities with the goal to have them serving science and scientists instead of implementing controls according to animal rights issues and organisations. This will lead to a dramatic reduction of cost and better research AND ethical care of the animals.

11. **In taking SABV into account in your research, study designs have you used?**

The only way to address SABV is to study sex by using a 'criss-cross' design, which requires a substantial number of animals, a design that can provide strong statistical power. This can seldom be done owing to the reasons explained above. Therefore, we usually choose one sex (male or female) to make our studies consistent.
We could earlier address the impact of sex differences in a more complex setting as we did experiments with heterogeneous stocks. It requires many animals, which is today not possible because of economic and 'ethical' regulatory constraints. Which means we have had to kill mice specifically designed for this approach.
In humans, sex differences are commonly included as co-variables, but there is no way to understand the biological background of sex differences with epidemiology. An example is the attempts to study the influence of oestrogens on RA in female patients, which cannot provide any explanation but do, after all, indicate that in humans (unsurprisingly) the effect of oestrogen on disease is the same as in animal models, as was earlier shown in mouse experiments.

12. **Any other comments?**

Addressing SABV is a scientific issue that requires in-depth experimental research. Unfortunately, strong stakeholders work to minimize these possibilities. Addressing these issues with epidemiology is a political and not a scientific issue.