Executive Summary

Context and overall objectives of the project

The FREIA project aims to provide better test methods to identify human-made chemicals that disturb hormones and their actions on development and function of the reproductive system in women. Currently available test methods are not fit for purpose, which is partly the reason why the effects of such endocrine disrupting chemicals (EDCs) on female reproductive health are often overlooked in regulatory chemical safety assessments. This means that women's reproductive health is at risk globally. Our EU-funded project FREIA will increase our understanding of how EDCs can harm female reproductive health. We will use this information to provide better test methods that enable fit-for-purpose chemical regulation. We will also share our knowledge widely to improve the reproductive health of women globally.

Main results of the work performed between January 2019 and December 2021

In the FREIA project, we first looked for biological characteristics (biomarkers) for female reproductive toxicity using two well understood EDCs, diethylstilbestrol (DES, a potent estrogen receptor activator) and ketoconazole (KTZ, a blocker of steroid hormone production). Next, we will assess how well our test methods and potential novel endpoints can identify EDCs that cause female reproductive toxicity. Here, we briefly describe the results from the first 36 months of the FREIA project, from January 2019 until December 2021.

Identification of EDCs in a regulatory context still relies heavily on rat studies. We showed that the endpoints that are currently being assessed in regulatory toxicity studies are not sufficiently sensitive to detect an endocrine disrupting effect (https://doi.org/10.1016/j.reprotox.2021.01.003). We found that a delay in activation of the brain to produce Gonadotropin Releasing Hormone (GnRH) is more sensitive to mark a delay in pubertal onset than the standard examination of vaginal opening (VO) in female rats that were exposed in the womb to DES and KTZ. Strikingly, the effect on GnRH was not detected when rat brain cells were exposed to DES or KTZ in a culture dish. This underlines the importance to focus on endocrine axes in the whole animal. We are investigating several additional hormone-sensitive endpoints, such as mammary gland development. A proposal to investigate this was submitted to the Organisation for Economic Co-operation and Development (OECD), a platform for international standard-setting. In addition, we found that the pups had increased blood levels of hormones like progesterone, pregnenolone, androsterone and estradiol after exposure to KTZ in the womb, and to a lesser extent DES.

Studies with cell cultures of fetal and adult human ovaries, bovine oocytes and immortalized ovarian cells all indicate an effect on steroid hormone formation and oocyte ripening by DES and KTZ, albeit in different directions. Ongoing gene expression analyses are designed to elucidate whether similar genes and pathways are affected in cell cultures and how this relates to effects in rats and humans. Differences in susceptibility towards effects of KTZ and DES may partly be explained by the presence of different cell types in the ovary at different ages (https://eovary.ki.se), or the presence of different cell types in our experimental models. Nonetheless, both the rat study and the human ovary cultures show that exposure to KTZ had a stronger effect than DES, suggesting that chemicals targeting steroid hormone formation (steroidogenesis) may have worse effects on oocyte maturation and quality than those targeting the estrogen receptor (ER).

In current regulatory toxicity testing, the gold standard to study interaction with steroidogenesis is the H295R steroidogenesis assay (OECD test guideline 345). The last step in sex hormone formation, the conversion of testosterone to estradiol, is mediated by the aromatase enzyme. We have developed a computational model that can predict inhibition of aromatase with high precision and accuracy (https://qsar.food.dtu.dk), complementary to the existing H295R assay. Nevertheless, our studies showed that effects of EDCs can also

occur earlier in the steroidogenic pathway, or via alternative routes. We are now performing a study with other labs to investigate whether the H295R assay can be improved by measuring more steroid hormones. A project plan for this was submitted to the OECD. Moreover, we showed that steroid hormone profiles from human adult ovary cultures are clearly different from H295R profiles. The implication of this will be investigated further in the next phase of the FREIA project.

Considering the limited effects of known ER activator, DES, on ovarian function during early life, the ER does not seem to be a high impact target for (developmental) female reproductive effects. On the other hand, ER-beta is known to play an important role in differentiation of estradiol-producing cells surrounding the maturing oocyte. We showed that a wide variety of potential EDCs had an ER-beta activating or deactivating effect in our ER-beta assay. Notably, the chemicals that were studied for ER-beta interaction were also detected in the biological fluids surrounding oocytes, the follicular fluids, in Swedish and Estonian women undergoing fertility treatment. The total exposure to these chemicals and some chemicals specifically, decreased a woman's response to ovarian stimulation by hormones. Moreover, some chemicals in the follicular fluids of these women were associated with a reduced chance of the treatment resulting in live birth. Further studies are undertaken to investigate molecular pathways that are affected by the exposures.

Identification of molecular targets and pathways will guide the development of test methods and test strategy, provided that the steps from molecular interaction to female reproductive toxicity are defined. For example, we have developed a computational model to predict peroxisome proliferator-activated receptor (PPAR)-gamma activation, which is linked to female infertility. We have described 16 additional possible pathways (doi: 10.1007/s00204-020-02834-y and 10.1159/000515478). We will evaluate with additional studies and different compounds which of these pathways and targets are suitable additional endocrine-sensitive endpoints to test for female reproductive toxicity by EDCs.

FREIA is one of the eight projects on test method development for EDC identification within the EURION cluster (www.eurion-cluster.eu). On the FREIA website (www.freiaproject.eu), general background information on EDCs and female reproductive health can be found as well as project specific information, including webinar recordings, peer-reviewed scientific publications and databases, and the FREIA factsheet (5 languages) and infographic (14 languages).

Progress beyond the state of the art and potential impacts

FREIA uniquely provides the opportunity to investigate hormonal processes in human ovaries from fetal to adult age in order to improve scientific knowledge on the causes of female reproductive toxicity. Our committed collaborators for policy, advocacy and communicating actions to promote women's health and a healthy society allow FREIA to have a huge societal impact. The FREIA approach will strongly support the work of European regulatory agencies, or even globally through the EURION cluster activities. The tools we are developing perfectly fit the needs of modern-day toxicity testing with a clear regulatory application in mind. Together, the FREIA outcomes will support testing, identification and assessment of EDCs that are toxic for female reproduction.