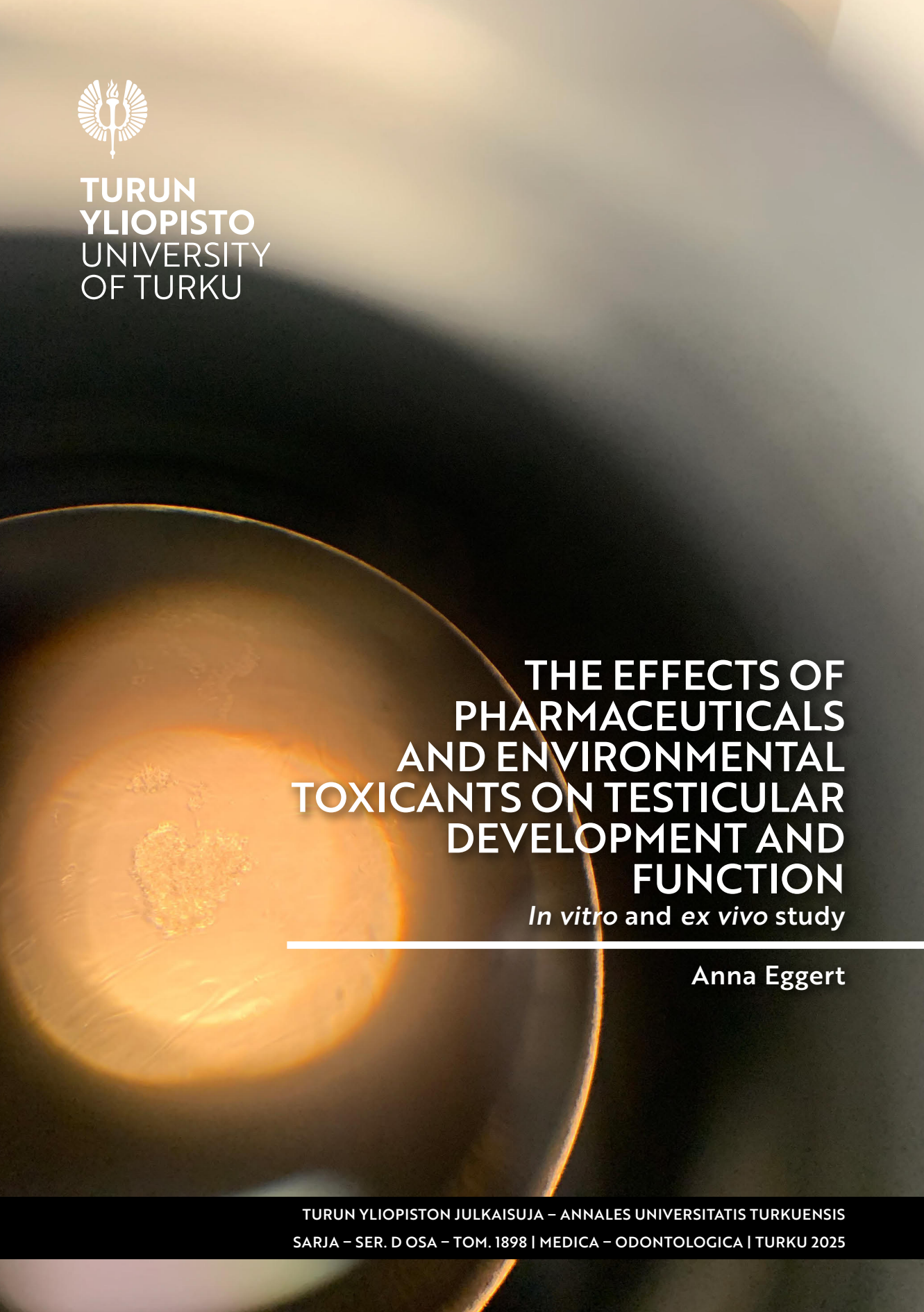

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In vitro and *ex vivo* study

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The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

Cover Image: Anna Eggert

ISBN 978-952-02-0270-5 (PRINT)
ISBN 978-952-02-0271-2 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2025

"Alterius non sit,
qui suus esse potest"

-Paracelsus-

To my children

UNIVERSITY OF TURKU
Faculty of Medicine
Institute of Biomedicine
Physiology
EGGERT ANNA: The effects of pharmaceuticals and environmental toxicants on testicular development and function – in vitro and ex vivo study
Doctoral Dissertation, 119 pp.
Turku Doctoral Programme of Molecular Medicine
August 2025

ABSTRACT

Disorders of male reproductive health are becoming increasingly prevalent due to unknown reasons. Pharmaceutical agents and environmental chemicals can cause adverse effects on the reproductive system, but reproductive toxicity often remains unnoticed. Although the testing of chemicals and pharmaceuticals is increasing and regulated by EU legislation, data on reproductive toxicity is still very limited. Investigating the mechanisms underlying the increased rates of male reproductive health disorders is crucial when trying to stop this growing trend. Furthermore, the methods used in toxicology require continuous development, and a shift towards *in vitro/ex vivo* methods is highly desirable. My doctoral research focuses on analyzing the testicular toxicity of commonly used anticancer drugs (imatinib and dasatinib) and a ubiquitous environmental contaminant, perfluorooctanoic acid (PFOA), in rodent models, utilizing *in vitro* and *ex vivo* toxicological methods. Rodents, particularly mice and rats, are the most commonly used animal models in male reproductive toxicity research due to *e.g.* their similarities to human spermatogenesis and its regulation.

Male gonads are vulnerable to external harmful factors, especially during embryonic development, but they remain vulnerable also later in life. Consequently, we analyzed the effects of PFOA both on fetal and adult rat testes in the first part of my doctoral studies. The main results were that PFOA exposure *ex vivo* inhibits fetal rat steroidogenesis and induces germ cell apoptosis in adult rat testes. Therefore, exposure to PFOA poses a risk to male fertility, particularly through pregnant females carrying male embryos.

The growing population of cancer survivors at fertile age represents a significant challenge for clinicians and researchers. It is essential to recognize the risks associated with both cancer and its treatments on fertility, and to identify the least harmful yet most effective treatment options. In the second part of my doctoral studies, we analyzed the effects of imatinib and dasatinib on male rodent germ cells and germline stem cells. We showed that imatinib exposure, both *in vitro* and *ex vivo*, adversely affects the proliferation and survival of male rodent germ cells. The plausible mechanism of imatinib action in spermatogenic cells involves the inhibition of c-KIT/SCF signaling and reduced expression of c-KIT. However, dasatinib did not show to cause adverse effects at clinically relevant doses *ex vivo*, but inhibited male germline stem cell colony growth *in vitro*.

KEYWORDS: Dasatinib, endocrine disruption, imatinib, Leydig cells, male germ cells, male germline stem cells, male reproductive health, PFOA, Sertoli cells, spermatogenesis, steroidogenic pathway

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Biolääketieteen laitos
Fysiologia
ANNA EGGERT: Lääkeaineiden ja ympäristökemikaalien vaikutus kehittyvään ja täysiaikaiseen kivekseen sekä sen toimintaan – *in vitro* ja *ex vivo* tutkimus
Väitöskirja, 119 s.
Turun molekyylilääketieteen tohtoriohjelma
Elokuu 2025

TIIVISTELMÄ

Miesten lisääntymisterveyden häiriöt ovat useiden tutkimusten mukaan lisääntyneet ympäri maailmaa. Tiedetään, että lääkeaineet ja ympäristökemikaalit voivat olla haitallisia lisääntymisterveydelle, mutta tiedot niiden lisääntymistoksisuudesta voivat silti olla puutteellisia, vaikka lääkeaineiden ja kemikaalien testaaminen on EU-lainsäädännöllä säädeltyä. Lisääntymistoksikologinen tutkimus on tärkeää miesten lisääntymisterveysongelmien taustasyiden selvittämisessä. Lisäksi lisääntymistoksikologiset menetelmät vaativat jatkuvaa kehittelyä ja *in vitro/ex vivo* -menetelmien suosiminen tutkimuksissa on toivottavaa. Väitöskirjatyössäni selvitin moderneina syöpälääkkeinä käytettyjen tyrosiinikinaasi-inhibiittoreiden (imatinibi ja dasatinibi) sekä ympäristölle haitallisen yhdisteen, perfluoro-oktaanihapon (PFOA), kivestoksisia vaikutuksia jyrsijöillä solu- ja kudosviljelymalleja käyttäen. Jyrsijät ovat yleisin eläinmalli ihmisen lisääntymistoksisuuden tutkimuksessa mm., koska siittiöiden tuotanto ja sen säätely on samankaltaista.

Miehen sukupuolirauhaset ovat alttiita ulkoisten tekijöiden haittavaikutuksille läpi eliniän, mutta etenkin sikiöiässä. Tämän vuoksi analysoimme PFOA:n vaikutuksia sikiöaikaiseen ja aikuiseen rotan kivekseen väitöskirjatyöni ensimmäisessä osatyössä. PFOA-altistus *ex vivo* vaikutti haitallisesti sikiöaikaisen rotan kiveksen hormonituotantoon sekä aikuisen rotan kivessoluihin. Tulokset osoittavat PFOA altistuksen olevan riski miesten hedelmällisyydelle etenkin poika-alkiota odottavien naisten kautta.

Syövästä selviäminen on yhä todennäköisempää modernien syöpähoitojen ansiosta, joten syöpälääkkeiden vaikutus potilaiden hedelmällisyyteen halutaan välttää. Imatinibi ja dasatinibi parantavat syöpiä tehokkaasti eikä niiden ilmoiteta aiheuttavan totaalista hedelmättömyyttä kuten esim. alkyloivat sytostaatit ovat tehneet tuhotessaan kiveksen itusolujen kantasoluja. Toisessa väitöskirjani osatyössä analysoimme imatinibin ja dasatinibin lisääntymistoksisia vaikutuksia jyrsijäperäisiin kivessoluihin ja siittiöntuotannosta vastaaviin kantasoluihin. Imatinibi, mutta ei dasatinibi, lisäsi urosrotan itusolujen apoptoosia kliinisesti relevanteilla annoksilla. Sekä imatinibi että dasatinibi häiritsevät uroshiiren itusolujen kantasoluryhmittymien kasvua *in vitro*. Osoitimme, että imatinibin apoptoosia aiheuttava vaikutus erilaistuvissa itusoluissa johtuu c-KIT/SCF-signaloinnin inhibitiosta sekä vähentyneestä c-KIT:n ilmentymisestä.

AVAINSANAT: Dasatinibi, hormonihäirikkökemikaalit, imatinibi, Leydigin solut, miehen itusolujen kantasolut, miehen itusolut, miesten lisääntymisterveys, PFOA, Sertolin solut, spermatogeneesi, steroidogeeninen polku

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Abbreviations

ab = Antibody
AGD = Anogenital distance
ALL = Acute Lymphoblastic Leukemia
AR = Androgen receptor
BSA = Bovine Serum Albumin
cAMP = Cyclic adenosine monophosphate
CML = Chronic Myeloid Leukemia
c-RET = REarranged during Transfection
CV = Coefficients of variation
DAB = Diamino benzidine
DMEM = *Dulbecco's Modified Eagle Medium*
DMSO = Dimetylsulfoxide
ED = Embryonic day
EDC = Endocrine disrupting chemicals
EDTA = EthyleneDiamineTetraaceticAcid
GAPDH = Glyceraldehyde 3-phosphate dehydrogenase
GDNF = Glial cell line-Derived Neurotrophic Factor
GFRα1= GDNF Family Receptor Alpha 1
GIST = GastroIntestinal Stromal Tumor
FBS = Fetal Bovine Serum
Fk = Forskolin
hCG = Human chorionic gonadotropin
HSCT = Hematopoietic stem cell transplantation
KI67 = Protein, associated with cell proliferation
LH = Luteinizing hormone
mGSC = male Germline Stem Cell
NSCLC = Non-small cell lung cancer
PBS = Phosphate-Buffered Saline
PFAS = per- and polu-fluoroalkyl substances
PFHxS = Perfluorohexanesulfonic acid
PFNA = Perfluorononanoic acid

PFOA = Perfluorooctanoic acid
PFOS = Perfluorooctane sulfonate
PFS = Progression free survival
PDGFR= Platelet-Derived Growth Factor Receptor
Ph+ = Philadelphia-Chromosome-positive
Ph+ ALL = Philadelphia-chromosome-positive acute lymphoblastic leukemia
P4 = Progesterone
RA = Retinoic Acid
RIA = Radioimmunoassay
RT = Room Temperature
SCF = Stem Cell Factor
SEM = Standard error of the mean
SSC = Spermatogonial Stem Cells
SOX-9 = SRY (Sex-Determining Region Y)-Box 9
StAR = Steroidogenic acute regulatory protein
STRA8 = Stimulated By Retinoic Acid 8
T = Testosterone
TKI = Tyrosine Kinase Inhibitor
Tunel = Terminal deoxynucleotidyl transferase dUTP nick end labeling

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Eggert A, Cisneros-Montalvo S, Anandan S, Musilli S, Stukenborg JB, Adamsson A, Nurmio M, Toppari J. The effects of perfluorooctanoic acid (PFOA) on fetal and adult rat testis. *Reproductive Toxicology*. 2019; Dec;90:68-76.

II Eggert A, Laasanen S, Nurmio M, Wahlgren A, Jahnukainen K, Eerola K, Nieminen M, Olotu O, Kotaja N, Mäkelä JA, Toppari J. Imatinib decreases germ cell survival and germline stem cell proliferation in rodent testis *ex vivo* and *in vitro*. *Andrology.* 2024; Oct 18: Online ahead of print.

1 Introduction

The problems of male reproductive health, such as decreased semen quality, cryptorchidism, and testicular germ cell cancer, have increased globally during recent decades (Le Cornet et al., 2014; Toppari et al., 2010; Helena E. Virtanen et al., 2017, 2024). The reason behind this may be environmental exposure to antiandrogenic compounds (Skakkebaek, 2016; Helena E. Virtanen et al., 2024). For example, pharmaceutical agents and environmental chemicals can cause adverse effects on the reproductive system, and reproductive toxicity often remains unnoticed despite the fact that testing of chemicals and pharmaceuticals is regulated by EU legislation. However, the methods used in toxicology need continuous development, and a shift from *in vivo* towards *in vitro/ex vivo* methods is desirable.

PFOA is a synthetic and persistent chemical that bioaccumulates in living organisms, and due to its stain- and water-resistant properties, it has been widely used as a surfactant (Lau et al., 2007). PFOA can cross the placenta, and it has a long half-life (Lau et al., 2007; Mamsen et al., 2019). PFOA has been reported to cause reproductive toxicity, but the mechanisms of action have not been thoroughly studied. The origin of male reproductive health problems may partly lie in fetal life. *In utero* exposure to environmental toxicants can cause adverse effects on the reproductive system that appear in adulthood (Juul et al., 2014). Therefore, we have analyzed the effects of PFOA on both fetal and adult rat testes in the first part of my doctoral studies (Eggert et al., 2019).

Inhibition of protein tyrosine kinases is one of the current approaches to targeted cancer therapy. Imatinib mesylate is a first-generation tyrosine kinase inhibitor (TKI), and it is widely used as a treatment for *e.g.* chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). Imatinib treatment is efficacious against these cancers, and therefore most patients treated with imatinib live longer than 10 years after treatment. Moreover, CML among patients at fertile age is increasing (Höglund et al., 2015). There is also evidence to show that imatinib crosses the blood-testis barrier (X. Chang et al., 2021), and according to Chang *et al*. (2017) imatinib-treated CML chronic-phase (CML-CP) patients have reduced semen quality (X. Chang et al., 2017; Mariani et al., 2011; Nicolini et al., 2016).

Dasatinib is a second-generation multitargeting kinase inhibitor, and it is used as a treatment to imatinib-resistant or -intolerant CML, Ph-positive acute lymphoblastic leukemia (ALL), and also for newly diagnosed CML-CP. Notably, evidence of imatinib resistance and intolerance has been recognized for a long time and it is emerging (Millot et al., 2011; Quintás-Cardama et al., 2009). Meanwhile, dasatinib has shown to be approximately 300 times more potent inhibitor of main target tyrosine kinase (BCR-ABL) than imatinib in preclinical models (Kantarjian et al., 2010; Lombardo et al., 2004). However, the effects of dasatinib on testicular cells and male reproductive health are largely unknown.

Both imatinib and dasatinib inhibit the signaling of essential receptor tyrosine kinases in testicular cells (Abbaspour Babaei et al., 2016) and are used for both children and adults at fertile age. The impact of imatinib and dasatinib on male germ cells and fertility is a relevant concern and worthy of investigation. Hereby, in the second part of my doctoral thesis, we have analyzed the effects of the common tyrosine kinase inhibitors imatinib and dasatinib on rodent testis and male germline stem cells (mGSCs) (Eggert et al., 2024).

2 Review of the Literature

2.1 Development of testis

Developing into a biological male is determined at fertilization when the fertilizing sperm cell carries a Y chromosome (Lukusa et al., 1992). Androgen and sperm production are the main functions of the testis, and normal development of the male reproductive system is needed for this function.

2.1.1 In fetal life

Male reproductive health is established early in the development of the testis. The development of the male reproductive system starts from bipotential gonads, which develop at the site of the genital ridge on the mesonephros during the 5th and 6th post-fertilization weeks in humans (Helena E. Virtanen & Toppari, 2014). There are two pairs of unipotential genital ducts: mesonephric (Wolffian) and paramesonephric (Müllerian) ducts. The epididymides, vas deferens, and seminal vesicles arise from the Wolffian ducts. Most parts of the female reproductive system arise from the Müllerian duct. In the 7th week after fertilization, the gonads of the embryo start their differentiation into either an ovary or testes. In male embryos, normal Sex-determining region Y (SRY) expression in the bipotential gonad directs the differentiation of somatic cells into Sertoli cells. The Sertoli cells are the first somatic cells to be specified in the developing testis. When the development of a biological male begins, the Sertoli cells guide the primordial germ cells toward spermatogenic origin, and the germ cells accumulate to form the first stage of the testis together with the somatic cells (J. A. Mäkelä et al., 2019). More exact, primordial germ cells migrate into the germinal ridge and from there into testicular cords approximately on 6th week after fertilization (De Felici, 2013; Himelreich Perić et al., 2023). The migration, proliferation and apoptosis of primordial germ cells is also regulated by c-KIT/SCF during fetal life (Mauduit et al., 1999). The Sertoli cells also coordinate the differentiation of other somatic cells in the developing testis, such as Leydig cells. Testis development does not arise from germ cells, but spermatogenic cell development is dependent on somatic cells (J.-A. Mäkelä et al., 2019). Embryonic Sertoli cells inhibit germ cells from entering meiosis (Griswold,

1998). The number of Sertoli cells is determined during fetal development, and their quantity determines sperm production capacity later in life (J.-A. Mäkelä et al., 2019). This means that only immature Sertoli cells proliferate, and Sertoli cell proliferation is no longer observed in normal conditions of adult mammalian testes (Schulz et al., 2005; Sharpe, 2003).

In order to achieve full masculinization, the Leydig cells in the developing testis begin secreting testosterone under the influence of hCG (human chorionic gonadotropin). In rats, an early common masculinization programming window (MPW) is defined from embryonic day (ED) 15.5 to ED 18.5, when testosterone concentrations rise. According to Welsh *et al*. (2008), this window is particularly susceptible to external influences, because androgens ensure the correct development of the male reproductive tract during this time window. A similar MPW is thought to occur in humans between weeks 8 and 14 of pregnancy (Welsh et al., 2008). In humans, a similar MPW is estimated to occur at 8-14 weeks of gestation (Sharpe, 2020).

Under the influence of anti-Müllerian hormone (AMH) Müllerian ducts regress approximately on 6.5th post-fertilization week and differentiation of the Wolffian ducts start. Due to the testosterone induction, the Wolffian ducts form the epididymides, vas deferens, and seminal vesicles and these are completed by 10^{th} week after fertilization. During this stage of development, the testis moves from the genital ridge across the pelvis to lie at the internal inguinal ring, which is the opening of the processus vaginalis (Barteczko & Jacob, 2000). There are two phases in testicular descent: the first phase occurs before mid-gestation, during which the gubernaculum enlarges to hold the testis near the inguinal region. The second phase is called the inguinoscrotal phase, and it is dependent on proper secretion of testicular androgens; it is usually completed by the time of birth. The gubernaculum is essential for testicular descent, and in the inguinoscrotal phase, the gubernaculum migrates across the pubic region to reach the scrotum (Favorito et al., 2014; H. E. Virtanen & Toppari, 2008). The testis may not descend into the scrotum if the abdominal muscles cannot increase intra-abdominal pressure. The epididymis precedes the testis into the processus vaginalis at weeks 26 to 36. These structures descend into the scrotum and become fused with the posterior layers of the scrotum, providing an anchor that prevents the testis from rotating. The processus vaginalis closes when the fetus is full term (from week 37 to 40) by eliminating communications between the peritoneum and the inguinal canal or scrotum (Lukusa et al., 1992; J.-A. Mäkelä et al., 2019; Helena E. Virtanen & Toppari, 2014).

2.1.2 In prepubertal life

After birth, the rapid decrease of placenta-derived steroids (especially estrogens) is considered to cause temporary activation of the hypothalamic-pituitary-gonadal axis. In boys, FSH and LH levels are high during the first 3 months of life and decline at 6 months of age to the low levels that are present until the onset of puberty. In humans, this phase is called minipuberty (Grumbach, 2005; Kuiri-Hänninen et al., 2014). The prepubertal testis has classically been defined as a quiescent organ. However, the male gonad significantly enlarges its volume during minipuberty, but after that, the size of the testis remains more or less stable until the onset of puberty (Main et al., 2006; Rey, 1999).

2.1.3 In puberty

Testicular growth in puberty occurs rapidly. Early in puberty, the first testicular volume growth can be explained by the increase in Sertoli cell number and the length of seminiferous tubules. The largest and fastest reason for rapid growth is the spermatogonial proliferation and the expansion of meiotic and haploid germ cells, which together will increase the diameter of seminiferous tubules. FSH stimulates the Sertoli cells and spermatogonial proliferation, while LH/testosterone is essential for normal spermatogenesis. On the other hand, FSH and LH/testosterone are both needed for normal spermatogenesis, and they work in synergy. (Koskenniemi et al., 2017) The increased secretion of testosterone also causes the darkening of the skin and the development of pubic hair on the scrotum (Ebling, 1987; Van De Graaff & Fox, 1992).

2.2 Spermatogenesis

The production of sperm (spermatogenesis) is a complex and ongoing differentiation process in the seminiferous tubules of the testis. During spermatogenesis, tens to hundreds of millions of sperm are produced per day (J. Mäkelä & Toppari, n.d.). The formation of the most specialized cell type in the human body, completed sperm, takes approximately 74 days in male humans (Neto et al., 2016). Two types of cells are found inside the seminiferous tubules: germ cells, including spermatogenic cells and male germline stem cells (mGSCs), and somatic Sertoli cells, which nurture and guard the germline. (J. Mäkelä & Toppari, 2017).

2.2.1 Male germline stem cells

A population of undifferentiated type A spermatogonia forms the stem cell population, male germline stem cells (mGSCs) aka spermatogonial stem cells

(SSCs). These cells are located on the basement membrane of seminiferous tubules, and they will ensure life-long spermatogenesis and fertility by going through self-renewal and differentiation (J. A. Mäkelä & Hobbs, 2019).

2.2.2 Maturation of germ cells

Male germ cells mature in three phases: the proliferative phase (mitosis), the meiotic phase (meiosis), and the spermiogenic phase (spermiogenesis). The differentiating progeny first multiplies mitotically, then enters and completes meiosis, and finally undergoes spermiogenesis to produce sperm. (J. Mäkelä & Toppari, 2017). During the **mitotic phase**, diploid spermatogonia undergo several mitotic divisions in rodents (rats/mice) whereas in human there are only 3 generations of spermatogonia. mGSCs transform after 2-16 rounds of mitosis, producing the first generation of differentiating or cycling spermatogonia called type A1 spermatogonia. These cells will then undergo six mitotic divisions, resulting in generations of more differentiated spermatogonia: type A2-A4 spermatogonia, intermediate spermatogonia, and type B spermatogonia. (De Rooij & Grootegoed, 1998; J. Mäkelä & Toppari, 2017)

The meiotic phase starts with preleptotene spermatocytes, daughter cells of type B spermatogonia after the final mitotic division. Preleptotene spermatocytes then undergo meiotic DNA replication and proceed to meiotic prophase, which is a lengthy process lasting over two weeks in rats. The nuclei of primary spermatocytes increase in size during prophase, and here the genetic recombination between homologous chromosomes takes place. It is possible to recognize leptotene, zygotene, pachytene, diplotene, and diakinesis phases in the several days-long prophase by the morphology of the nucleus. The pachytene phase is very long, whereas the diplotene phase is rather short. During diakinesis, meiotic divisions start to take place. As a result of meiosis I, two secondary spermatocytes are generated. These cells live a short time and enter the second meiotic division soon, and each produces two haploid spermatids . (Handel & Schimenti, 2010).

The early round spermatids develop into mature, motile spermatozoa during **spermiogenesis**, *i.e.*, spermatid differentiation. During spermiogenesis, dramatic morphological changes take place as round spermatids evolve into spermatozoa with specialized structures (complete with a head, midpiece, and tail) that enable independent movement and makes the fertilization possible. The major morphological transformations during spermiogenesis are the development of the acrosome on one side and a flagellum on the other side, followed by chromatin compaction and head shaping (Hermo et al., 2010).

2.2.3 Stages of the seminiferous epithelial cycle

Inside the seminiferous tubules are germ cells and Sertoli cells. The differentiating germ cells have multiple forms. There are phases with predictable and recognizable compositions during spermatogenesis called stages of the seminiferous epithelial cycle in seminiferous tubules. In rats, 14 stages have been defined (Russell, L.D., Ettlin, R., Sinha Hikim, A. and Clegg, 1990), and in mice and humans, the cycle of the seminiferous epithelium is divided into 12 stages (Caldeira-Brant et al., 2020; Kotaja et al., 2004). Sertoli cells, one or two generations of spermatocytes in the middle, and one or two generations of spermatids nearest to the lumen of the tubule are present in each stage. Stages can be recognized by morphological differences in the acrosomes and nuclei of the spermatids. One region of the seminiferous tubule develops in a cyclic fashion at regular time intervals, resulting in the cycle of the seminiferous epithelium. The defined cell associations along the length of the seminiferous tubule form wave-like fashions, and one complete wave is the tubule segments between two identical stages (J. A. Mäkelä et al., 2020; J. Mäkelä & Toppari, n.d.; Parvinen, 1982; Toppari & Parvinen, 1985). These waves and stages can be visualized by transillumination from isolated seminiferous tubules (Ant & Vanha-Perttula, 1972; J. A. Mäkelä et al., 2020).

2.2.4 Sertoli cells

Sertoli cells extend from the basal lamina to the lumen of the seminiferous tubules. They have a columnar shape, and they are relatively long cells. Sertoli cells nurse immature germ cells enabling them to divide, undergo meiosis, and differentiate (Clermont et al., 1987; Clermont & Harvey, 1965; Griswold, 1998; J. Mäkelä & Toppari, n.d.; Russell, L.D., Ettlin, R., Sinha Hikim, A. and Clegg, 1990). Sertoli cells also offer physical support, form the blood-testis barrier by specialized junctions between each other near the basement membrane, and regulate the energy metabolism of germ cells (Griswold, 1998; Rato et al., 2012).

2.2.5 Leydig cells

The key elements of male fertility - testosterone production and spermatogenesis - are considerably connected, and both processes are endocrine-regulated by hormones secreted from the hypothalamus and pituitary. When a biological male reaches sexual maturity, the successful function of the testis is dependent on the hypothalamic gonadotropin-releasing hormone (GnRH) to stimulate the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. In response to LH stimulation, the Leydig cells produce testosterone, which is needed for production of sperm and also for the development

and maintenance of many physiological functions in the male body *e.g.* regulation of libido, bone mass, fat distribution, muscle mass and strength. (Haider, 2004; Huhtaniemi, 2021; Witherspoon & Flannigan, 2022)

2.2.6 Endocrine and paracrine regulation of spermatogenesis

FSH and androgens are the main regulators of spermatogenesis. LH, estradiol, activins, inhibins, follistatin, and other paracrine factors also play a role in spermatogenesis. LH induces testosterone biosynthesis in Leydig cells, which causes high intratesticular testosterone levels. In this way, LH regulates spermatogenesis. LH production is regulated via testosterone and GnRH by negative feedback. FSH is important for optimal testicular development and maximal sperm production. (Haider, 2004; J. Mäkelä & Toppari, 2017; Witherspoon & Flannigan, 2022)

2.2.7 The c-KIT/Stem Cell Factor system

Stem cell factor (SCF) is produced by Sertoli cells, and it is an important cytokine in the regulation of spermatogenesis and cancers. c-KIT, the receptor for SCF, is important for male fertility, but mutated forms of c-KIT have a role in many cancers (Dubreuil et al., 1990; Hakovirta et al., 1999; Sheikh et al., 2022; Yan, Suominen, et al., 2000). Stem cell factor (SCF) signaling through the c-KIT receptor has been shown to have an anti-apoptotic effect on germ cells, and it is essential for spermatogonial proliferation (Hakovirta et al., 1999; Orth et al., 1996, 1997; Yan, Kero, et al., 2000).

2.3 External toxic effects of testis

Male gonads are vulnerable to external harmful factors that start from embryonic age and continue during all the growth phases (Foresta & Gianfrilli, 2021). Many studies globally have shown increasing rates of male reproductive disorders like cryptorchidism, hypospadias, poor semen quality, decreased testosterone levels, and testicular germ cell cancer during recent decades (Helena E. Virtanen et al., 2017). Genetics cannot explain this phenomenon because changes in prevalence have been so rapid. Therefore, it is suggested that the reason behind the increased problems in male reproductive health may be environmental exposure to antiandrogenic compounds or other environmental toxicants (Skakkebaek, 2016; Helena E. Virtanen et al., 2024) and their direct or epigenetic disadvantages on testicular cells and male fertility. During testis development, there are three main periods that are particularly sensitive: the intrauterine phase, the neonatal phase including the so-called

minipuberty, and puberty. However, also during childhood, when the testes are apparently non-active, adverse effects with irreversible consequences on testicular function can occur (Foresta & Gianfrilli, 2021).

Spermatogenesis in adulthood itself requires proper participation of many cell types, including the Leydig cells, the Sertoli cells, the germ cells, the germline stem cells, as well as the regulation of various hormones, paracrine factors and epigenetic regulators (J. Mäkelä & Toppari, 2017; Neto et al., 2016). Any changes in cellular homeostasis, in hormone levels and in the epigenetic effects during spermatogenesis may lead to the decline of male reproductive capacity (Han & Huang, 2021).

2.3.1 Endocrine disrupting chemicals

Among different environmental risk factors affecting male fertility, endocrine-disrupting chemicals (EDCs) have gained particular attention in the last decades. EDCs have the ability to disrupt male reproductive organs and hormone levels at different ages and grades, with long-term consequences and possibly also transgenerational effects by affecting the regulators of male reproductive health, such as DNA methylation, histone modification and noncoding RNAs. (Han & Huang, 2021)

Endocrine disruptors are "exogenous substances or mixtures that alter the function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations" (IPCS, 2002). EDCs may interfere with the synthesis, action, and metabolism of sex steroid hormones, which may lead to developmental and fertility problems, as well as infertility and hormone-sensitive cancers in women and men. Bisphenol A, phthalates, and perfluoroalkyl substances (PFAS) are well-known EDCs, and they have been proven to have strong experimental evidence of effects on hormone nuclear receptors, the hypothalamus-pituitary-testis axis, and direct action on spermatogenesis and steroidogenesis (Foresta & Gianfrilli, 2021; Yilmaz et al., 2020).

"Sola dosis facit venenum" stated Paracelsus, the 16th-century famous physician who brought chemistry to medicine (Borzelleca, 2000; Evans, 2023). With these words, "the father of toxicology and the brother of patient-centered care" meant that "all things are poison, and nothing is without poison", signifying that the dosage alone makes the substance toxic or not. In reproductive toxicology, especially with EDCs, this presumption is not valid due to three main reasons. Firstly, as also mentioned above, the sensitivity to EDCs varies with age. For example, many environmental substances only cause minor or reversible effects in adulthood, but the same dose per body weight during sensitive developmental periods can result in irreversible consequences; *e.g.,* male fetuses are most vulnerable to permanent toxic

effects on reproductive health during gestational weeks 8-14 (Welsh et al., 2008). Secondly, the exposure combination of many EDCs or other external toxicants at the same time may lead to deleterious effects on overall health, even when the single dose of an individual toxicant has been stated as "safe" (Hass et al., 2007; Howdeshell et al., 2008; Kortenkamp, 2014). Thirdly, there are variations in vulnerability to EDCs between individuals because of differences in the metabolism of EDCs based on genetic or lifestyle factors. However, both factors can strengthen each other's effects on, *e.g.,* fertility (Toppari et al., 2016; White et al., 2009).

Because it was beneficial in industrial usage, many EDCs were meant to have long half-lives; however, from a human and wildlife point of view, this has turned out to be harmful. Due to their long biological half-lives, many EDCs and some other environmental toxicants accumulate in living organisms and in food chains. (Diamanti-Kandarakis et al., 2009).

A wide array of experimental and animal data shows strong evidence for negative effects of exposure to phenols, phthalates, pesticides, and per- and poly-fluoroalkyl substances (PFAS) on male reproductive health (Mínguez-Alarcón et al., 2023; Rodprasert et al., 2021). Even if epidemiological studies carried out on humans have shown controversial and inconsistent results, an overall conclusion points toward a positive association between exposure to EDCs and alteration of the male reproductive system (Foresta & Gianfrilli, 2021). According to recent reviews, and when all information is taken into consideration, there is cumulative evidence showing associations between occupational and environmental pesticide exposure in relation to male reproductive health (Martenies & Perry, 2013; Mínguez-Alarcón et al., 2023; Whorton et al., 1977). For instance, organophosphate pesticides have been reported to cause antiandrogenic activity (Kitamura et al., 2003; Tamura et al., 2001, 2003). Additionally, the effects of phthalates on humans have strong negative evidence. However, limited and inconclusive relationships remain for the other examined EDCs. Reasons for the irregularity in results may be explained by differences in study populations, exposure concentrations, the amount of collected samples, study and experimental design, and residual confounding. Additional studies are needed, especially for newer phenols and PFAS (Mínguez-Alarcón et al., 2023; Rodprasert et al., 2021; Shi et al., 2024; Tarapore & Ouyang, 2021).

2.3.2 PFAS

PFAS are a large group of synthetic per- and polyfluoroalkyl substances used, *e.g.*, in furniture textiles, technical clothing, non-stick pans, firefighting foams, and electroplating. The C-F bonds in PFAS are very stable chemical structures, and for this reason, PFAS disintegrate slowly and are persistent in the environment. Therefore, PFAS cause global concern. Many PFAS are used as surfactants or

surface protectors due to their perfluorocarbon moieties, which are both hydrophobic and lipophobic (Buck et al., 2012). The surface tension of water in PFAS-based fluorosurfactants can be less than 16 mN/m (Alexander et al., 2014; Buck et al., 2012). Also, the surfaces of fluorinated polymers have about half the surface tension compared to hydrocarbon surfaces. For instance, a close-packed, uniformly organized array of trifluoromethyl (-CF3) groups creates a surface with a solid surface tension as low as 6 mN/m (FOWKES, 1964). As a comparison, the normal surface tension of water at 20 °C is 72.75 mN/m (International Tables of The Surface Tension of Water | PDF | Surface Tension | Temperature, n.d.), and *e.g.* modified cotton fabric has showed resistance to water and liquids with tension of 47 mN/m (Chauhan et al., 2019). The most widespread PFAS are PFOS (perfluorooctane sulfonate), PFNA (perfluorononanoic acid), PFHxS (perfluorohexanesulfonic acid) and PFOA (perfluorooctanoic acid) (Schrenk et al., 2020). Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the most studied PFAS (Tarapore & Ouyang, 2021).

Because the use of PFAS is very common, exposure to PFAS occurs mainly via daily life equipment, diet, and the environment. In humans, exposure to PFAS occurs through food and water intake, which accounts for approximately 40-100% of all PFAS intake. The main food sources of PFAS are fish, fruits, meat, and eggs (Airaksinen et al., 2018; Uhl et al., 2023). A minor source of PFAS exposure is room dust and respiratory air. PFAS substances can be released into the environment during the manufacturing and storage of these chemicals, as well as from surrounding areas. PFAS bioaccumulate in humans and wildlife, mainly in the liver, kidneys, and in the proteins of blood (Schrenk et al., 2020; Uhl et al., 2023).

When PFAS interact with serum transport proteins and hepatic proteins, accumulation is easily allowed more widely in other tissues of the body, such as the spleen, brain, testes, and ovaries (Chaparro-Ortega et al., 2018; Lau et al., 2007). Due to their ability to resist natural metabolism in the body, PFAS are not excreted via urine or feces (Schrenk et al., 2020; Uhl et al., 2023).

The European Union has regulated PFAS since 2008. The organization of 27 European countries is currently considering a ban on the manufacture, sale, and use of over 10,000 different types of PFAS, albeit with exemptions meant to protect key industries. (ECHA 2023; Uhl et al., 2023). It is worth mentioning that PFOA has been banned since July 2020 under the EU´s Persistent Organic Pollutants (POPs) regulation, but some specific and temporary exemptions have been included. The use of PFOS has been restricted since 2009 in EU under the POP regulation. (Uhl et al., 2023) However, the well-known fact about the chemical industry is that when some compounds are banned, chemical companies often just replace the banned ones with new substances having similar chemical properties like newer PFAS. Nevertheless, the manufacturing and marketing of PFAS substances will be

restricted more extensively in EU now and in the future (ECHA 2023; Uhl et al., 2023).

Human exposure to PFAS has been shown to be linked with an increased risk for thyroid disease, hypercholesterolemia, colitis ulcerosa, kidney, testicular and prostate cancer, and pregnancy-induced hypertension, according to epidemiological studies (Blake et al., 2018; Crawford et al., 2017; Domingo & Nadal, 2017). In addition, there has been an association between PFAS exposure and adverse effects on the immune (Liang et al., 2022; Tarapore & Ouyang, 2021), endocrine, metabolic, and reproductive systems, including fertility and pregnancy outcomes (Benbrahim-Tallaa et al., 2014; Gilliland & Mandel, 1993; Shi et al., 2024; Tarapore & Ouyang, 2021). More specifically, exposure to PFAS may be involved in the increased prevalence of male reproductive health problems. Epidemiological studies conducted to date suggest that young adulthood exposure to some PFAS may increase the risk of testicular cancer and disrupt male reproductive hormone levels. The impact of PFAS on semen parameters is not yet clear. There is increasing evidence that prenatal exposure to individual PFAS and their mixtures is associated with disturbed reproductive hormones and reduced semen quality. Additional studies are needed to determine if and when exposure to PFAS affects semen parameters and the risk of testicular cancer, whether alterations in reproductive hormones regulate this association, and to conclude the impact of exposure to a combination of many PFAS at the same time on reproductive health (Mínguez-Alarcón et al., 2023; Shi et al., 2024).

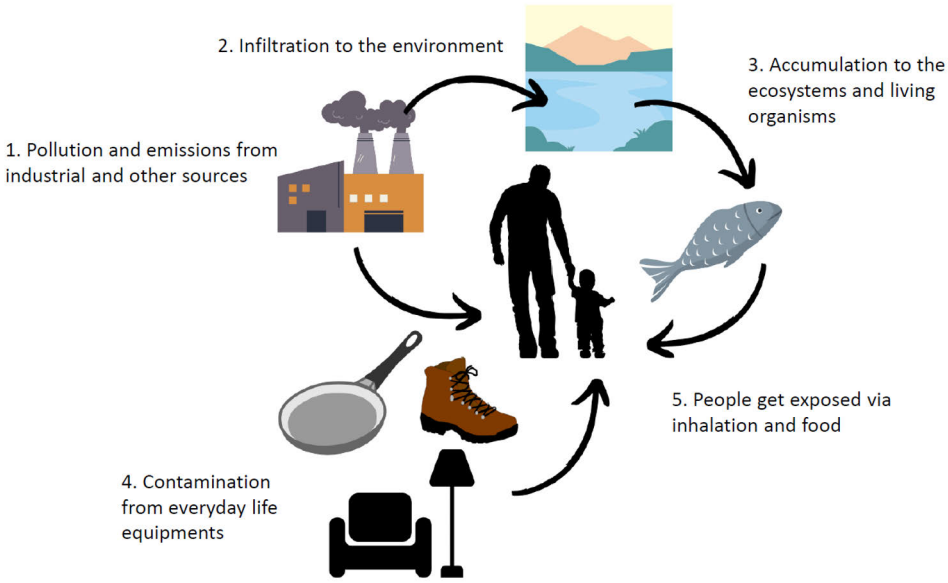

Figure 1. How PFAS drift from environment to humans.

2.3.2.1 PFOA

PFOA (perfluorooctanoic acid) is a fluorosurfactant that has been manufactured since the 1940s, and it is widely used in industrial products due to its stain, fat, and water-resistant features (Bergman et al., 2015; Lau et al., 2007; Olsen et al., 2007; Russell et al., 2015). The chemical structure of PFOA consists of a perfluorinated alkyl chain of 7 carbons and a terminal carboxylate group (FIGURE 2). A strong carbon-fluorine bond makes PFOA stable and non-degradable. Accordingly, PFOA is a persistent and widely dispersed chemical in the environment, and it bioaccumulates in living organisms with a half-life of approximately 3.5 years in humans (Lau et al., 2007; Olsen et al., 2007).

PFOA has constantly been detected in the blood of human and animals worldwide (Lau et al., 2007). It has been shown that PFOA can cross the placenta and it has been found in umbilical cord blood denoting a risk to *in utero* exposure for the fetus. PFOA has also been found in breast milk and in newborns contributing postnatal exposure in humans and rats. (Cariou et al., 2015; Hinderliter et al., 2005; Lau et al., 2007; Lindstrom et al., 2011) In adults, PFOA has been found also in seminal plasma (Guruge et al., 2005; La Rocca et al., 2015), and some studies show that PFOA disturbs the function of blood testis barrier *in vivo* and *in vitro* (Dankers et al., 2013; Lindeman et al., 2012; Y. Lu et al., 2016).

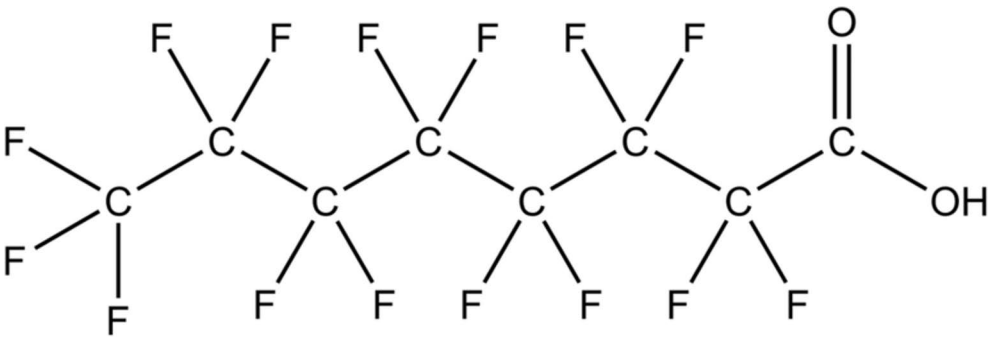

Figure 2. Chemical structure of PFOA.

Animal studies demonstrate that exposure to PFOA is associated with small birth weight and size, delays in physical development and neonatal mortality. In rodent studies PFOA has shown to cause weight loss, liver enlargement, changes in lipid metabolism and increased incidence of liver, pancreas, and testicular tumors. (Lindstrom et al., 2011) A meta-analysis from Bartell *et al.* (2021) indicates that for humans there is an average 16 % increase in cancer risk per 10 ng/mL increase in serum PFOA for kidney cancer and 6 % increase in cancer risk per 10 ng/mL increase

in serum PFOA for testicular cancer (Bartell & Vieira, 2021). In adult male rats PFOA may reduce testosterone levels and increase estradiol levels (Lau et al., 2007). In humans, it is suggested that *in utero* exposure to PFOA may affect semen quality and reproductive hormone levels in adult men (Vested et al., 2013).

While the general toxicity of PFOA has been proofed in many studies, there is still limited evidence available about *in utero* or perinatal exposure effects of PFOA. (Bach et al., 2022; Bergman et al., 2015) Also, the effects of PFOA on male reproductive health and testicular cells need additional studies (Shi et al., 2024; Tarapore & Ouyang, 2021). Due to the toxicological evidence and characteristics mentioned previously, European Chemicals Agency (ECHA) has recognized PFOA as a substance of very high concern based to its persistent, bioaccumulative, and toxic (PBT) properties. It is also classified as carcinogenic, mutagenic, or toxic for reproduction (CMR) (ECHA, 2025). The industrial production of PFOA has decreased in Europe and USA, while there are statistics which show that in some areas in Asia the production has increased (E. T. Chang et al., 2016). PFOA has even been eliminated from several trademarks *e.g.* Gore-Tex eliminated the use of PFOA from their outdoor clothes already in 2013. EU has banned PFOA since July 2020, but temporary exemptions existed until July 2023 and July 2025. Nowadays PFOA is not used in commercial products anymore, but the chemical is still found in the environment and manufacturers have turned to less regulated PFAS to create the same effects. However, the persistence in the environment and the relatively long estimated intrinsic elimination half-life of PFOA in humans highlight the concerns about its potential health effects.

2.4 The effects of cancer treatments on male fertility

When a person receives a cancer diagnosis, she/he often needs to be treated with burdensome cancer therapy. In addition, they may face the threat of future infertility due to the gonadotoxicity of many anti-cancer drugs (Duffin et al., 2024; Lambertini et al., 2016; Wallace, 2015). The population of cancer survivors is dramatically increasing. Meanwhile, a trend towards postponing pregnancy later in life is reported. As a result, many patients do not have their own biological children by the time they are diagnosed with cancer. Therefore, cancer survivors of fertile age are a growing population. Understanding the effects of both cancer and all kinds of anticancer treatments on fertility has become important since approximately 5% of current cancer survivors were diagnosed before 40 years of age. Hence, more efforts are focused on researching the effects of all kinds of cancer treatments on fertility (Lorenzi et al., 2021).

As with the other possible cancer treatment complications, oncologists have a responsibility to inform patients about the risk of potential treatment-related infertility and about fertility preservation options before the onset of cancer treatments. However, existing data about anti-cancer therapies and male fertility are still poor and heterogeneous; also, complicating issues exist, such as patient-related factors that influence fertility (Lambertini et al., 2016; Lorenzi et al., 2021; Ramstein et al., 2017).

Maturing male germ cells and male germline stem cells are sensitive to external harmful effects like radiation or cytotoxic agents throughout whole lifespan. For instance, spermatogenesis ends rapidly after pelvic irradiation or cytotoxic treatment. If the spermatogonial stem cells, aka male germline stem cells, survive these traditional cancer treatments without irreversible damage, the spermatogenesis will start again after treatments (Van Alphen, Van De Kant, et al., 1988). In young boys, the effects of childhood chemotherapy and irradiation on fertility emerge first at the age of puberty when the testes do not grow normally. However, traditional cancer treatment, particularly alkylating substances, can cause irreversible male infertility if the treatment destroys male germline stem cells. Thus, nowadays many young cancer patients survive the anti-cancer treatments without permanent effects on fertility (Jahnukainen, 2012).

2.4.1 Traditional cancer treatments and male fertility

Traditional chemotherapy and radiation therapy are highly effective in treating cancer because they target all dividing cells, but their risks for fertility for both men and women are well-established in an agent- and dose-dependent way (Lorenzi et al., 2021; Ramstein et al., 2017). Especially high-dose cytotoxic treatment and total body or pelvic area irradiation are associated with a high risk of damage to gonads and male germ cells in patients of all ages (Jahnukainen, 2012; Jahnukainen et al., 2015; Ramstein et al., 2017). Alkylating agents used in traditional chemotherapy represent the greatest danger to fertility (Lorenzi et al., 2021). In men, the most susceptible cell types are proliferating germ cells during spermatogenesis. Permanent or long-term impairment occurs if the treatment affects male germline stem cells, which are responsible for lifelong spermatogenesis. For instance, irradiation destroys both maturating male germ cells and male germline stem cells (Van Alphen, Van de Kant, et al., 1988).

The earliest developed anti-cancer drugs were alkylating agents (Hall & Tilby, 1992). Originally, they were used as a weapon during World War I *i.e.* an alkylating agent mustard gas, which was named for its yellow-brown and mustard-like odor, was found to cause a remarkable decrease in rapidly dividing white blood cells in exposed soldiers during World War I (DeVita & Chu, 2008; Goodman et al., 1946).

When this property of mustard gas was recognized, animal studies were performed, which proved that nitrogen mustard induced regression of lymphoid tumors in mice and subsequently in patients with lymphoma (DeVita & Chu, 2008). Based on these findings, cyclophosphamide was discovered, and it represents one of the most commonly used chemotherapeutics in children and adults with cancer. Other well-known alkylating agents are busulfan, chlorambucil, melphalan, ifosfamide, and procarbazine (Duffin et al., 2024; Ramstein et al., 2017).

Alkylating agents, like cyclophosphamide, kill the male germline stem cells, which means that these male patients often suffer from impaired sperm quality and infertility (Jahnukainen, 2012; Van Alphen, Van De Kant, et al., 1988). Other traditional anticancer drugs that are associated with male infertility and decreased sperm quality are cisplatin and procarbazine (K et al., 2011; Meirow & Schenker, 1995). Patients with high-risk and relapsed hematopoietic malignancies need treatment with allogeneic hematopoietic stem cell transplantation (HSCT) to be cured. HSCT is associated with a significant risk of germ cell disruption, with azoospermia in 85% of men and oligozoospermia in the rest of the treated men. The effects of HSCT on fertility depends on intensity of regimen and which co-treatments are involved (Anserini et al., 2002; Rovó et al., 2006; Sarafoglou et al., 1997).

2.4.2 Targeted cancer therapy

Since the early 2000s, targeted therapies have started to overtake other treatment options in oncology. Targeted cancer therapy is considered a type of cancer treatment that targets proteins that control how cancer cells grow, divide, and spread. One of the first widely used targeted therapies was imatinib, a tyrosine kinase inhibitor (TKI), which revolutionized the treatment of chronic myeloid leukaemia (CML) and was approved for medical use for the first time in 2001 (M. H. Cohen et al., 2002; Druker et al., 2001; Duffin et al., 2024). Accordingly, the inhibition of protein tyrosine kinases is one of the current approaches to targeted cancer therapy. Targeted cancer therapy may include approaches such as targeting the PI3K pathway, targeting the classical MAP kinase cascade, targeting angiogenesis, and combining kinase inhibitors with checkpoint inhibitors like surface expressed programmed cell death ligands (PDL1/PD1 pathway) (P. Cohen et al., 2021).

The early selection of kinase targets for therapeutic intervention in oncology broadly followed two strategies. The first strategy was to focus on patients with mutations in the protein kinases that could be targeted by specific tyrosine kinase inhibitors. The second strategy was to develop more effective next-generation versions of approved TKIs against the most crucial oncogenic kinase targets (P. Cohen et al., 2021). Early examples of next-generation drugs include, for example,

nilotinib (Breccia & Alimena, 2010) and dasatinib (Kantarjian et al., 2006, 2010), which are more potent BCR-ABL inhibitors than imatinib and provide a more complete cytogenetic response (Mealing et al., 2013).

The use of kinase inhibitors has revolutionized the treatment guidelines of multiple malignancies, including CML, gastrointestinal stromal tumor (GIST), melanoma, and non-small cell lung cancer (NSCLC). In CML, the use of allogeneic bone marrow transplantation (HSCT) as a first-line treatment for patients with chronic phase disease has been supplanted by the use of inhibitors of BCR–ABL. The clinical use of systemic chemotherapy as a first treatment for GIST or BRAF mutant melanoma has vanished or is reserved for last-line treatment. In NSCLC, the use of genotype-directed therapies, including kinase inhibitors, has replaced the use of systemic chemotherapy as a first-line therapy for patients with advanced NSCLC (P. Cohen et al., 2021). Several clinical trials have also demonstrated that the use of genotype-directed therapy for EGFR mutation and ALK-rearranged NSCLC results in improved response rates and progression-free survival (PFS), as well as reduced adverse side effects compared with chemotherapy (P. Cohen et al., 2021; Maemondo et al., 2010; Oizumi et al., 2012; Solomon et al., 2014).

Despite the fact that kinase inhibitors have undoubtedly made a breakthrough in cancer therapy, these agents are not curative. Kinase inhibitors usually just prevent disease progression. Therefore, these drugs are often used as chronic therapy, which also highlights the need to study their side effect profile well. The resistance of malignant cells to specific kinase inhibitors is a recognized challenge. There are three types of resistance to kinase inhibitors: innate, primary, or acquired resistance (P. Cohen et al., 2021). Drug developers try to profile the resistance challenges of kinase inhibitors well and discover new next-generation TKIs that bind more effectively and target more selectively. Extensive kinase inhibitor profiling has also brought new clinical opportunities to be discovered and unwanted side effects to be understood — and in some cases even eliminated (Klaeger et al., 2017). Despite the impact that protein kinase inhibitors have had on the treatment of cancer, relatively few protein kinase inhibitors have been approved so far for the treatment of other diseases. It is predicted that breakthroughs in the investigation of small-molecule inhibitors will continue to take place (P. Cohen et al., 2021).

Whereas the risk of cytotoxic drugs for fertility is well-known, the effects of targeted cancer therapy on fertility parameters, potential teratogenicity, pregnancy outcome, and management in case of accidental exposure are not established. (Lorenzi et al., 2021)

2.4.2.1 Imatinib

Imatinib is a small molecule agent and a first-generation TKI. Originally, imatinib was designed to inhibit BCR-ABL tyrosine kinase activity in malignant Philadelphia-positive (Ph+) cells in hematological malignancies (Waller, 2018). The BCR/ABL tyrosine kinase system activates numerous downstream pathways leading to increased proliferation, reduced apoptosis, abnormal adhesion and migration, and genetic instability. Imatinib treatment results in the blockage of BCR-ABL downstream signal transduction pathways, leading to apoptosis and reduced cell growth of targeted cells. BCR/ABL is both necessary and sufficient for the development of CML. The majority of CML patients nowadays enjoy long-term remissions and have a normal life expectancy after the onset of diagnosis (Osman & Deininger, 2021). Imatinib is also widely used as a treatment for, e.g., Philadelphia-positive acute lymphoblastic leukemia (ALL, Ph+) and GIST, because it inhibits not only BCR–ABL but also other protein kinases (KIT, ARG, and PDGF receptors), which can be overexpressed or mutated in these and other cancers, too. Most of the imatinib-treated patients live longer than 10 years after the onset of diagnosis (Mughal et al., 2016; Osman & Deininger, 2021; Sacha, 2014; Waller, 2018). Thus, imatinib treatment is usually not a cure, but rather an effective way to control the malignancy from progressing and is therefore often used as chronic therapy (Waller, 2018).

It is known that stem cell factor (SCF) signaling through the c-KIT receptor is essential for normal male reproductive function and germ cell survival (Orth et al., 1996, 1997; Yan, Suominen, et al., 2000) and mutant mice which have abnormalities in c-KIT/SCF gene expression are infertile (Mauduit et al., 1999). The downstream activity of the c-KIT/SCF gene expression system regulates basic biological processes, including cell survival, proliferation, differentiation, and migration (Rönnstrand, 2004), and it is known that this signaling is crucial, e.g., for normal hematopoiesis, fertility, and pigmentation. Deregulated c-KIT kinase activity has therefore been found in many pathological conditions, including cancer and allergy (Lennartsson & Rönnstrand, 2012). Moreover, platelet-derived growth factor (PDGF) and its tyrosine kinase receptor (PDGFR) are important for the development of Leydig cells and peritubular myoid cells in the testis (Basciani et al., 2010; Gnessi et al., 1995).

There is evidence that imatinib crosses the blood-testis barrier (X. Chang et al., 2021), and it has been shown that imatinib-treated patients have reduced semen quality (Mariani et al., 2011; Nicolini et al., 2016; X et al., 2017), which represents a risk of imatinib treatment for testicular physiology and male reproductive health. Meanwhile, there is evidence that imatinib-treated men have been able to father children after or during imatinib treatment (Hensley & Ford, 2003; Nicolini et al., 2016).

The prevalence of CML is predicted to increase, leading to an increased number of CML patients of fertile age as well (Höglund et al., 2015; Huang et al., 2012; Osman & Deininger, 2021). Imatinib has shown promising results when tested on patients with several inflammatory diseases (Bernal-Bello et al., 2020; Cahill et al., 2017; De Backer & Lefebvre, 2008; Magro & Costa, 2006). Imatinib treatment for patients with recent-onset type 1 diabetes has also been studied (Gitelman et al., 2021) and most recently tested on a pediatric patient with diabetes (Lavelle et al., 2024), *i.e.*, an increasing number of young people may be treated with imatinib in the future.

The large use of imatinib has highlighted the need to pay attention to the effects of imatinib on reproductive health, especially because imatinib inhibits the signaling of essential receptor tyrosine kinases that also exist in testicular cells (Abbaspour Babaei et al., 2016; Gnessi et al., 1995, 2000; Waller, 2018; Yan, Suominen, et al., 2000).

The effects of imatinib on adult testicular cells and male germline stem cells (mGSCs) are largely unknown. Considering the relatively high number of patients receiving imatinib and the fact that its clinical use is extending beyond oncology, its effects on spermatogenic cells and adult male reproductive health require a deeper investigation.

2.4.2.2 Dasatinib

In oncology, imatinib resistance and intolerance are clinical challenges (Millot et al., 2011; Quintás-Cardama et al., 2009). Dasatinib is a second-generation TKI that inhibits BCR-ABL, the SRC family kinases (SRC, LCK, HCK, YES, FYN, FGR, BLK, LYN, FYN-related), receptor tyrosine kinases (c-KIT, PDGFR, DDR1 and 2, c-FMS, ephrin receptors), and TEC family kinases (TEC and BTK) (Lindauer & Hochhaus, 2014). Because dasatinib inhibits c-KIT and PDGFR, it poses, like imatinib, a potential risk for male germ cells (Yan, Suominen, et al., 2000). Dasatinib is used for BCR-ABL-driven diseases, such as CML and Philadelphia-chromosome-positive acute lymphoblastic leukemia (Ph+ ALL), especially when imatinib resistance or intolerance occurs (Lindauer & Hochhaus, 2014).

Dasatinib has been shown to be over 300 times more effective as a BCR-ABL inhibitor than imatinib in preclinical and clinical studies (Kantarjian et al., 2010; Lombardo et al., 2004). In combination with the natural flavonoid quercetin, dasatinib is considered a senolytic drug. Therefore, it has been tested in clinical trials aiming to eliminate senescent cells and, consequently, to treat diseases where cell senescence is involved (Meiners et al., 2024). Like imatinib, dasatinib is also used in both children and fertile adults (Lindauer & Hochhaus, 2014). In studies about the effects of TKIs on spermatogenesis, dasatinib has interestingly shown milder effects

than those of imatinib (Yassin et al., 2012). The effects of dasatinib and quercetin treatment on male mice *in vivo* have recently been studied, and they did not observe adverse effects on mouse spermatogenesis after 5 months of exposure (Garcia et al., 2023). However, the cell-level effects of dasatinib on spermatogenic cells and mGSCs have largely remained unknown.

3 Aims

1. Aim To analyze the effects of PFOA on fetal rat testis, fetal steroidogenesis and adult rat testicular cells *ex vivo*. (Publication I.)

2. Aim To analyze the effects of imatinib on adult rodent testis and male germline stem cells *in vitro* and *ex vivo*. (Publication II.)

3. Aim To analyze the effects of dasatinib on adult rodent testis and male germline stem cells *in vitro* and *ex vivo*. (Publication II.)

4 Materials and Methods

4.1 Experimental animals and treatments

Young adult Sprague-Dawley male rats (60-80 days old) were obtained from the Animal Center of Turku University (Turku, Finland) or B&K Universal (Sollentuna, Sweden). Young adult wild-type DBA/2J mice (> 60 days old) were purchased from the Central Animal Laboratory of the University of Turku (Turku, Finland). All animals were housed in a 12 h light/12 h dark cycle in a climate-controlled room at 21±3°C with a relative humidity of 55% ±15%. Water and standard food (Rat and Mouse Breeder and Grower, SDS diets/LBS Biotech, UK) were distributed *ad libitum.* All animal experiments were carried out according to Finnish and Swedish laws and following the guidelines of Ethics of Animal Experimentation at the University of Turku in accordance with the Guide for Care and Use of Laboratory Animals. The use of experimental animals in this study was approved by the University of Turku Ethics Committee for animal experiments. Nineteen pregnant female rats, 40 male rats and 13 mice were sacrificed for this study.

4.2 Chemicals

Perfluorooctanoic acid (PFOA), 96% purity, CAS No. 335-67-1, and dimethylsulfoxide (DMSO), > 99.7% purity, CAS No. 67-68-5, were purchased from Sigma-Aldrich (Saint Louis, USA). Catalogue numbers were 171468 for PFOA and S-002-D for DMSO (Sigma-Aldrich). DMSO was used as a vehicle for PFOA and dasatinib; therefore, control conditions in PFOA and dasatinib experiments included DMSO in the same ratio as used as a vehicle in the highest concentration of each substance.

4.3 Imatinib and dasatinib

Imatinib (#ab142070) was purchased from Abcam (Amsterdam, Netherlands) or received as a generous gift from Novartis (STI571, Glivec®). Dasatinib (#ab142050) was ordered from Abcam, Netherlands. The imatinib doses of 1, 3, 5, and 10 μM and dasatinib doses of 0.01, 0.1, 0.3, and 1 μM used in this study were chosen because

they are near clinical plasma concentrations detected in patients (Chien et al., 2022; IJzerman et al., 2020; Miura, 2015; Peng et al., 2005; Shah et al., 2008; Yoo et al., 2013; Yu & Badhan, 2023). A dose of 100 µM imatinib simulates an overdose scenario; however, individuals exposed to this dosage have survived it (Bhargav et al., 2007; Dehours et al., 2010). Likewise, a dasatinib dose of 10 µM is an overdose, but at least experimental animals have tolerated it without apparent toxic effects (Lombardo et al., 2004).

4.4 Extraction of mouse embryonic fibroblasts

A pregnant female wild-type DBA/2J mouse was sacrificed by CO2 asphyxiation and cervical dislocation at gestational day 13.5, counting the day of the plug as 0.5. The uterus was sterilely dissected and moved to a Petri dish containing sterile PBS. The embryos were released by making an incision in the uterus. Heads of the embryos were cut off, and internal organs were removed under a stereomicroscope. Remaining tissue material was transferred to 6-well plates (one embryo per well), cut into small pieces, and enzymatically digested using trypsin/EDTA (1 ml per embryo) for a total of 15 minutes at 37°C, then dissociated by pipetting. The cell suspensions were mixed with culture medium and transferred to Petri dishes for culture. MEFs were harvested using trypsin/EDTA when the cultures were confluent. MEFs from passages 3-15 were used as feeders for mGSCs.

4.5 Extraction of mouse male germline stem cells

Adult (>60 days old) male mice from the DBA/2JRccHsd strain were sacrificed by CO2 asphyxiation and cervical dislocation. The testes were dissected and decapsulated. Germline stem cells were isolated and cultured, as previously described (La et al., 2018). Briefly, single-cell suspensions were prepared by enzymatic digestion: collagenase type I (0.1 mg/ml; Worthington) for 10 min at 37°C, followed by trypsin (0.6 mg/ml; Worthington) and DNase I (4.5 U/ml; Worthington) for 5 min at 37°C. Seminiferous tubule fragments were dissociated by pipetting in 10% FBS in PBS and passed through a 70-µm cell strainer. Following centrifugation at 600 x g for 5 min, the cell pellets were resuspended in 2% FBS in PBS, and spermatogonia (including mGSCs) were isolated by biotinylated CD9 antibody (Biolegend clone MZ3, 1:500) selection using an EasySep biotin selection kit (Stem Cell Technologies) according to the manufacturer's instructions. Finally, the CD9-selected cells were resuspended in mGSC culture medium consisting of StemPro-34 media (Thermo Fisher Scientific) supplemented with 5 mg/ml BSA (Sigma-Aldrich, A9418), 6 mg/ml glucose, L-glutamine (1x, Gibco), Pen/Strep (1x), MEM vitamin solution (1x, Gibco), MEM nonessential amino acid solution (1x,

Gibco), 0.5 µM 2-mercaptoethanol (Gibco), 60 µM putrescine, 0.1 mM ascorbic acid (Stem Cell Technologies), 340 µM pyruvate (Sigma), 1 µl/ml lactic acid (Sigma), 10 µg/ml biotin (Sigma-Aldrich), 25 µg/ml insulin (Sigma), 100 µg/ml transferrin (Sigma-Aldrich), 30 nM sodium selenite, 30 ng/ml beta-estradiol (Sigma), 60 ng/ml progesterone (Sigma), 1% FBS (Gibco), StemPro-34 supplement (1x), 20 ng/ml human EGF (Gibco), 10 ng/ml murine bFGF (Peprotech), and 10 ng/ml human GDNF (Peprotech) and plated on mitomycin-inactivated (10 µM, 4 h) MEFs.

4.6 Cell culture and *in vitro* exposure

mGSC cultures were maintained at 37°C, 5% CO2, and passaged every 3-5 weeks using trypsin/EDTA. Passages 4-6 from two independent mGSC lines were used for experimentation. For proliferation analysis, mGSCs were seeded in 24-well plates at low density and incubated in 0, 1, 3, 5, and 10 µM imatinib or 0, 0.01, 0.1, 0.3, and 1 µM dasatinib for 5 days, 7 days, or 4 weeks. Control conditions in dasatinib experiments included 1 µl of DMSO/1 ml of mGSC culture medium. The number of cells was quantified using a TC20 automated cell counter (Bio-Rad). Trypan Blue was used to detect dead cells in the samples. For apoptosis, the Annexin V-FITC Early Apoptosis Detection Kit (Cell Signaling, #6592) was used according to the manufacturer's instructions. For differentiation analysis, mGSCs were subcultured until they formed grape-like colonies, followed by exposure to 0, 1, 5 and 10 µM imatinib for 3 days. Retinoic acid (1 mM; Sigma-Aldrich R2625) was applied for the last 48 h. Cells were treated with trypsin/EDTA, reconstituted in 2% FBS in PBS, stained with c-KIT-APC (1:200; eBioscience, 17-1171-82) and MCAM-FITC antibodies (1:200; BioLegend, 134708). DAPI (1:10,000) used for live/dead discrimination. The samples were analyzed using using a flow cytometer (ACEA) and FlowJo v10.9 software.

4.7 Transillumination-assisted microdissection of seminiferous tubules

Adult (> 60 days old) Sprague-Dawley rats and C57/Bl6J mice (> 60 days old) were euthanized by CO2 asphyxiation and cervical dislocation. The testes were dissected and decapsulated. Seminiferous tubule segments representing specific stages of the seminiferous epithelial cycle were cut under a stereomicroscope using the transillumination-assisted microdissection method, as described previously (J. A. Mäkelä et al., 2020; Parvinen, 1982). The microdissection time was limited to less than 1 hour from euthanasia. Four to six seminiferous tubule segments from individual animals were collected for each dose in ex vivo culture experiments. The

number of experimental repeats in each tubule culture experiment corresponds to the number of animals used ("n").

4.8 Tissue culture and *ex vivo* exposure

Staged seminiferous tubule segments of rat and mouse were incubated at 34°C in a humidified atmosphere containing 5% CO_2. For reproductive toxicological analysis, tubule segments were incubated for 24, 48, or 72 h in the presence or absence of PFOA, imatinib, or dasatinib in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham containing 1x L-glutamine, 15 mM HEPES, 0.1% bovine serum albumin (BSA), and 100 µg/ml gentamicin. The tubule cells were exposed to PFOA in concentrations of 0, 10, 50, and 100 µg/ml; to imatinib in concentrations of 0, 1, 10, or 100 µM; and to dasatinib in concentrations of 0, 0.01, 0.1, 1, and 10 µM. DMSO (Sigma-Aldrich, Saint Louis, USA) was used as a vehicle for PFOA and dasatinib and was therefore added at 1 µl/ml to the control culture medium in PFOA and dasatinib experiments.

Two types of fetal testis culture were used in this study. In the first condition (Adamsson et al., 2008; Brokken et al., 2009), testes were placed in a 4-well plate, each well plate containing 1 ml of Dulbecco's modified Eagle's medium (DMEM high glucose, pyruvate, and GlutMAX; Invitrogen Co., Paisley, UK) supplemented with 0.1% bovine serum albumin (BSA) and 0.1 g/l of gentamycin (Biological Industries, Betha Emek, Israel) in each well. The control culture contained the vehicle, 1 µl of dimethylsulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA), and PFOA (Sigma Chemical Co., St. Louis, MO, USA) concentrations of 10, 50, or 100 µg/ml in the culture. Fetal testes were cultured for 24 h at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Testes were stimulated by 7 IU/ml of human chorionic gonadotropin (hCG; Pregnyl®, Shering Plough, N.V. Organon, Oss, Holland) or forskolin 50 µM (LC Laboratories, USA) 3 h before ending the culture. One hour before the hCG and forskolin stimulation, 0.1 mM of 3-isobutyl-1-methyl xanthine (IBMIX; Sigma-Aldrich Finland Oy, Helsinki, Finland) was added as described previously (Nikula et al., 1999). hCG and forskolin stimulation increase the cAMP, testosterone and progesterone levels in the fetal testes culture compared to the basal condition. Hereby, we wanted to study whether PFOA blocks the natural increase of cAMP and hormone levels by hCG and forskolin stimulation, which further on provides us with information about the level of the disturbance (gonadotrophin level or receptor level) (Nikula et al., 1999). At the end of the exposure, the culture medium was collected, warmed up to 60 °C for 5 min, and frozen at −20 °C, while the testes were stored at −80 °C. The cultured fetal testes samples were used for Western blot analysis, and the cyclic AMP (cAMP), progesterone, and testosterone measurements were done from the culture

medium, respectively. In the first fetal testis culture setup, five replicates for each condition were used (n = 5). The fetal testes for each replicate were derived from different litters; thereby, each replicate represents one pregnant rat dam. Another set of experiments was carried out on Millicell filters (pore size 0.4 µm; Millipore, Billerica, MA, USA) as described previously (Habert et al., 1991; Livera et al., 2006). Filters were placed in a 24-well plate containing 320 µl of medium containing PFOA as described above and incubated for 24 h without hCG and forskolin stimulation, because no hormonal measurements were performed from these testis cultures. After the culture, testes were collected for morphological analysis, fixed in 4% paraformaldehyde, and embedded in paraffin, while the culture medium was stored at −20 °C. Four rat dams were sacrificed, and the collected fetal testes (n = 4) for the immunofluorescence and TUNEL were from different litters.

4.9 Squash preparation

After 24 hours of tissue culture, the tubule segments were transferred into 20 µl of culture medium and pipetted onto a microscope slide. Each tubule segment was gently squashed between a coverslip and a microscope slide until a cloud-like cell monolayer spread on the microscope slide (Toppari & Parvinen, 1985). This step was monitored under a Leica DMR light microscope (Leica Microsystems, Wetzlar, Germany), and the desired spermatogenic stages VIII and IX were verified by inspecting the polarization of the nucleus and the shape of the acrosome. After squashing, the samples were dropped into liquid nitrogen for freezing. The coverslip was flipped off with a scalpel, and the sample was first dipped in ice-cold 96% EtOH and then fixed in 4% PFA for 10 minutes. After fixation, the samples were stored in PBS at 4°C until the next day.

4.10 Immunohistochemical detection of Cleaved-Caspase 3

Apoptosis analysis by immunohistochemistry was done for 24 h cultured and squashed seminiferous tubule segments of stages VIII and IX. The squashed tubule segments were first treated with 1% H2O2 in PBS for 15 min and then washed for 5 min in PBS twice. Treatment with 0.1% Triton-X in PBS at RT was done for 15 min, and then blocked with 0.5% BSA in 0.1% Triton-X PBS for 30 min in a humid chamber. The first antibody (apoptosis marker), Cleaved-Caspase 3 (rabbit ab; 9661S, Cell Signaling, Leiden, Netherlands), was added in a 1:100 dilution with 0.5% BSA and 0.1% Triton-X for 1 h at RT in a humid chamber and washed three times in PBS. The second antibody, EnVision+System-HRP-labeled polymer anti-rabbit ab (K4003, Dako North America, California, USA), was used at a 1:1 dilution

for 30 min and washed three times in PBS. The samples were stained with diaminobenzidine (DAB) (Dako North America, California, USA) for 2 min and counterstained slightly with hematoxylin and eosin. Afterwards, they were mounted with Aquamount. The microscope slides were scanned with a Pannoramic 250 slide scanner and analyzed with Pannoramic viewer software. The cleaved caspase-3 positive cells were quantified per 1 mm of seminiferous tubule. The length of each tubule segment was measured with Pannoramic software.

4.11 Whole Mount preparation and detection of GFRα1, KI67, STRA8, c-KIT and LIN28A

Mouse seminiferous tubule segments (5 mm long) from stages IX-I were dissected and collected for tissue culture in 0 and 10 µM imatinib and dasatinib to analyze whether imatinib or dasatinib disturbs the mGSC proliferation ex vivo. Exposure time was 48 h. Control conditions in dasatinib experiments included 1 µl of DMSO per ml of tubule culture medium. Adult male mice (> 60 days) from a C57Bl/6J background were used for imatinib (n=4) and dasatinib (n=3) experiments, respectively. After tissue culture, the tubule segments were washed three times in ice-cold PBS, fixed, and stained, as previously described (Faisal et al., 2019; J. A. Mäkelä et al., 2020). Briefly, the tubules were fixed (4% PFA) for 6 h at +4°C on a rotating table, followed by two washes with PBS. A 1 h blocking was done in 2% BSA + 10% FBS in 0.3% Triton X-100 in PBS. Afterwards, the samples were stained overnight at +4°C on a rotating table with primary antibodies: GFRα1 (1:250; RnD Systems, AF560) and KI67 (1:200; Invitrogen, 14-5698-82). After staining, the samples were washed three times in PBS and then incubated for 2 h at RT on a rotating table with respective secondary antibodies (1:500; Invitrogen, A11056, A21208). Finally, after three washes in PBS, the tubules were poured and mounted onto a microscope slide. Forty randomly chosen areas were selected for 3i CSU-W1 spinning disk confocal microscopy imaging (3i) and systematically quantified for GFRα1-positive/KI67-positive or negative cells. On average, 766 (±118) GFRα1-positive cells were quantified for KI67-positivity/negativity per sample.

Mouse seminiferous tubule segments from stages II-VI were dissected for an ex vivo differentiation induction experiment. The tubule segments were first incubated for 2 hours in 0, 1, and 10 µM imatinib. RA (1 µM) was then added for 22 hours. At the end of the culture, the tubule segments were collected by pipetting and washed twice in ice-cold PBS. The samples were prepared for whole-mount immunofluorescence staining using STRA8 (1:500; Abcam, ab49405), c-KIT (1:300; Invitrogen, 17-1171-82), and LIN28A (1:500; R&D Systems, AF3757) antibodies as described above, or for flow cytometry using the c-KIT antibody (1:100; eBioscience, 17-1171-82). For quantitation of LIN28A-positive/STRA8-

positive or negative cells, 40 randomly chosen areas were imaged (3i CSU-W1 spinning disk confocal microscope; 200x magnification; 3i Intelligent Imaging Innovations) and systematically quantified. On average, 644 (±153) LIN28A-positive cells were quantified for STRA8 positivity/negativity per sample (n=2 per group). The difference was so clear in these two first repeats that we did not sacrify the third animal for this experiment. In flow cytometry analysis, c-KIT positive cells were divided into c-KIT+ and c-KIT-high subsets (n=3 per group).

4.12 Western blot of fetal testes

A pool of 5–6 fetal testis (ED 17.5) was homogenized in 35–40 µl of ice-cold lysis buffer (0.2% Nonidet P-40 in phosphate-buffered saline (PBS) and protease inhibitor tablet; Complete mini, Roche Diagnostics, Mannheim, Germany), and the homogenate was centrifuged at 13,000 rpm for 15 min at 4 °C. The total protein concentration of each supernatant was measured via the bicinchoninic acid detection method (BCA protein assay kit, Pierce, Rockford, USA). For each sample, 20 µg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Hybond-P, Amersham Biosciences, Buckinghamshire, UK). To block the unspecific antibody binding, the membrane was incubated for 1 h with PBS supplemented with 5% fat-free milk and 0.2% Tween 20. The protein immunodetection was performed by incubating the membrane overnight at room temperature with a polyclonal rabbit StAR antibody (Abcam, Cambridge, UK) diluted to 1:2000 in PBS with 1% fat-free milk and 0.2% Tween 20. Monoclonal mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (HyTest, Turku, Finland) diluted to 1:5000 was used as a loading control. The antibody binding was detected using corresponding horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies (Amersham Life Science, Buckinghamshire, UK) diluted to 1:10,000 for 1 h at room temperature (RT) and revealed by ECL chemiluminescence (Western Lighting® ECL Pro, Perkin Elmer, Inc., USA) on Fujifilm LAS 4000. ImageJ 1.46r (Wayne Rasband, National Institutes of Health, USA) was used to analyze the Western blots. The StAR was normalized with the GAPDH, and the results are represented as relative protein abundance. At least 4 separate samples were analyzed in each experiment, which were repeated four times (n = 4).

4.13 cAMP, progesterone and testosterone assays in fetal testis culture

The cAMP measurements from fetal testis culture after PFOA exposure were carried out using a radioimmunoassay that detected femtomole amounts of cAMP by

acetylating the cyclic nucleotides at the 2´0 position with acetic anhydride, as described previously (Habert et al., 1991). Secreted levels of progesterone (P4) and testosterone (T) from fetal testes to the medium were measured by time-resolved fluoroimmunoassays (DELFIA; Perkin-Elmer Life and Analytical Sciences, Turku, Finland), as described earlier (Haavisto et al., 2001). The sensitivity of the assay was 100 pg/ml and 250 pg/ml for progesterone and testosterone, respectively. The intra- and inter-assay coefficients of variation (CV) were below 6% and 12%.

4.14 Immunofluoresence detection of TUNEL, KI67 and SOX9

Four fetal testes per experimental condition (n=4) at ED 17.5 after 24-h culture were fixed in 4% PFA and embedded in paraffin. Four 1 µm-thick sections were cut, and every tenth section was attached to glass slides for staining. Double indirect immunofluorescence assessment of proliferation and apoptosis of somatic cells was performed. Sertoli cells were detected by using goat polyclonal anti-SOX-9 primary antibody (ab5535, Merck Millipore, Frankfurt, Germany; final concentration 2.1 µg/ml) coupled to Alexa 594 donkey anti-rabbit (A-21207, Thermo Fisher, Waltham, USA; final concentration 0.4 µg/ml). Rat monoclonal anti-KI67 primary antibody (14-5698-82, Thermo Fisher, Waltham, USA; final concentration 1 µg/ml) coupled to Alexa 488 donkey anti-rat (A-21208, Thermo Fisher, Waltham, USA; final concentration 1.7 µg/ml) was used for the detection of proliferative cells. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was done as described previously (Rotgers et al., 2014). Briefly, deparaffinized and rehydrated sections were permeabilized in a pressure cooker in 0.1 M citrate buffer, pH 6. Quenching of autofluorescence was performed by submerging the slides in 100 mM NH_4Cl. Then, sections were incubated with the TUNEL reaction mixture for 60 min at 37 °C, and the reaction was stopped by using 300 mM NaCl and 30 mM NaCitrate. To detect the TUNEL-positive cells, an incubation with streptavidin Alexa 488 (S32354, Thermo Fisher, Waltham, USA; final concentration 0.4 µg/ml) was performed. The positive control was performed by adding 1 U/µl DNAse for 30 min at 37 °C, and for the negative control, terminal deoxynucleotidyl transferase (TdT) was omitted. All (100–300) Sertoli cells in proliferation (Ki67-positive, SOX-9-positive) and undergoing apoptosis (TUNEL-positive, SOX-9-positive) were quantified in 1–3 non-consecutive sections from four fetal testes (n=4). All (300–400) Leydig cells (interstitial TUNEL-positive) were quantified in 1–3 non-consecutive sections from four fetal testes (n=4). Graphs are shown as percentages of single or double positive cells. All immunofluorescence samples were imaged using a Pannoramic MIDI FL slidescanner with the 40x Plan Apochromatic objective

(Zeiss), and all images were processed with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

4.15 Flow cytometry analysis for seminiferous tubules

Two types of flow cytometry analysis were performed in my doctoral studies. The first analysis determined which cell types are most vulnerable to the effects of PFOA in the seminiferous tubules, following a protocol previously described (Rotgers et al., 2015). Briefly, 20 individual seminiferous tubule segments (2 mm) of stages VII-VIII were pooled and placed in digestion medium (1 mg/ml collagenase/dispase 10269638,001; Roche, Basel, Switzerland; 1 mg/ml hyaluronidase H3506; Sigma-Aldrich, Saint Louis, MO, USA; and 1 mg/ml DNAse1 DN-25; Sigma-Aldrich, Saint-Louis, MO, USA). Tubule segments were disintegrated using McPherson-Vannas scissors for 1 min. The samples were incubated at 37 °C for 15 min, and they were mechanically disrupted at 10- and 5-minutes intervals. The obtained cell suspensions were filtered through a 40 μm pore strainer (FlowMI, H13680-0040, New Jersey, USA) and washed with PBS by centrifugation. Single cells were fixed with 4% paraformaldehyde followed by permeabilization with 90% methanol. The samples were stored at –20 °C prior to analysis.

To evaluate the value of somatic and germ cells after PFOA exposure, Vimentin antibody detection and DNA staining were performed. Monoclonal rabbit anti-vimentin antibody conjugated to Alexa 488 (9854; Cell Signaling Technologies, final concentration 4 μg/ml) was used to detect Sertoli cells. FxCycle Far-Red DNA stain (F-10348; Life Technologies Ltd, Paisley, UK, final concentration 200 nmol/ml per 1 x 1000000 cells) was used to assess the germ cells on the basis of their DNA contents. A defined volume of suspension was analyzed by the flow cytometer (BD LSRII, Becton Dickinson, Franklin Lakes, NJ, USA), and the number of cells was normalized to cells/mm of seminiferous tubule. Analyses were normalized to cells/mm of seminiferous tubule. A defined volume of suspension was analyzed by the flow cytometer (BD LSRII, Becton Dickinson, Franklin Lakes, NJ, USA), and the number of cells was normalized to cells/mm of seminiferous tubule. Analyses were performed with the noncommercial Flowing Software 2.5 (Perttu Terho; Turku Centre for Biotechnology, Finland; www.flowingsoftware.com) as previously described in detail (Rotgers et al., 2015).

The second flow cytometry method was used to analyze the effects of imatinib on different testicular cell populations in seminiferous tubules. Twelve 5-mm long seminiferous tubule segments from stages VII-VIII were dissected from three rats (n=3) and cultured in 0, 1, 10, and 100 μM imatinib under the conditions described above for 24 h. The cultured tubule segments were collected by pipetting and washed

twice with PBS. Single-cell suspensions were prepared by enzymatic digestion using Collagenase type IV (Sigma C5138; 1 mg/ml in DMEM) at 37°C for 15 min. Following pelleting at 600 x g for 5 min at 4°C and a wash with 2% FBS in PBS, the cells were resuspended in 500 µl of 2% FBS in PBS and incubated with Hoechst 33342 (1:1000) at room temperature for 10 min. Finally, the samples were analyzed with a NovoCyte® Flow Cytometer (ACEA Biosciences Inc.) and FlowJo v10.9 (BD) software.

4.16 Incorporation of ^{3}H-thymidine

Six 2-mm-long rat seminiferous tubule segments from stages V, VIIa, and VIII-IX were microdissected and cultured in 0, 1, 10, or 100 µM imatinib, and 100 µM imatinib/100 ng/ml recombinant mouse SCF (Genzyme Transgenics Corp., Cambridge, MA) under the conditions described above for 24 h (n=4), 48 h (n=3), or 72 h (n=3). Tubule segments were labeled for the last 4 h of culture with 3H-thymidine (Amersham Pharmacia Biotech, UK, specific radioactivity 5.0 Ci/mmol, final concentration 0.5 µCi). The samples were harvested (Söder et al., 1992) using a Skatron Instruments Cell Harvester. This included flushing with distilled water and collection of segments on glass fiber filter papers (Skatron, Lier, Norway). Filter discs containing high molecular weight material, including DNA, were punched out and transferred to scintillation minivials, and Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA) was added. Incorporated radioactivity was measured by counting in a Beckman LS5000CE scintillation spectrometer as counts per minute (cpm).

4.17 Presentation and statistical analysis of the data

All data were analyzed for statistically significant differences using one-way ANOVA, T-test, or non-parametric tests (Kruskal-Wallis), followed by Dunnett's, Tukey's, or Dunn's Multiple Comparison tests on GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The graphs for data presentation were created using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The significance level was set at $p < 0.05$.

Table 1. Summarizing table of the methods.

METHOD	WHAT IS ANALYZED?	WHY?
Fetal testis culture in 4 different PFOA concentrations: 0, 10, 50 and 100 µg/ml	The effects on fetal rat testis after 24 h exposure *ex vivo*	To simulate the effects of *in utero* exposure to PFOA in developing testis
Rat seminiferous tubule culture in different doses of PFOA, imatinib or dasatinib for 24-72 h	To study the effects of these compounds on adult rat male germ cell survival and DNA synthesis	To simulate the situation when adult testicular cells are exposed to PFOA, imatinib and dasatinib
Mouse male germline stem cell (mGSC) culture, different exposure times for imatinib and dasatinib: 3, 5, 7 and 27 days	mGSC colony growth during cell culture, viability (Trypan blue and Annexin V) and differentiation capacity (Retinoic acid induced differentiation)	To address whether imatinib and dasatinib inhibit mGSC proliferation and differentiation, and increase cell death or apoptosis
Immunofluorescence on cultured fetal rat testis (24 h) or mouse seminiferous tubule segments (24-48 h)	Sertoli cells (SOX-9), mGSCs (GFRα1, SALL4, MCAM), proliferation (KI67), apoptosis (TUNEL), differentiation (LIN28, STRA8, c-KIT)	To analyze the effects of PFOA on fetal Sertoli cell proliferation and whether PFOA induces fetal Sertoli cell and Leydig cell apoptosis. To analyze whether imatinib and/or dasatinib adversely affect mGSC proliferation and differentiation
Immunohistochemistry for squashed rat seminiferous tubules	Cleaved caspase-3	To detect apoptotic cells in the seminiferous tubules following exposure to PFOA and TKIs
Western blot for cultured and PFOA exposed fetal rat testes	StAR	To analyze the effects of PFOA on fetal rat steroidogenesis
cAMP, progesterone and testosterone measurements from the culture media of fetal rat testis	Culture media with and without hCG and forskolin stimulations	Determine the effect and level of disturbance on steroidogenesis
Flow cytometry	Fxcycle, Vimentin, Hoechst, DAPI, Annexin V-PI, CD9, MCAM and c-KIT	To analyze the effect of PFOA and TKIs on different testicular cell populations and apoptosis. Determine the number of c-KIT positive cells after TKI exposure (effects of TKIs on mGSC differentiation).

5 Results

5.1 PFOA exposure decreases cAMP, progesterone and testosterone levels and the expression of StAR in fetal rat testis culture (I)

Male fetuses are vulnerable to external toxic effects, especially during masculinization, which in rats occurs at ED 15.5 – 18.5 (Welsh et al., 2008). To study the effects of PFOA on fetal steroidogenesis, we measured the levels of cAMP, progesterone, and testosterone from fetal testes culture after 24 hours of exposure. We also wanted to further investigate at which level the possible disturbance of PFOA on steroidogenesis occurs: on the receptor level or by disturbing the G-protein-mediated signaling. Therefore, fetal cultures were stimulated with forskolin or hCG 3 hours before ending the culture, as described previously (Nikula et al., 1999). The levels of cAMP decreased significantly at all PFOA concentrations (10, 50, and 100 μg/ml) in fetal testis cultures, both in basal and hCG-stimulated conditions (I, Fig. 1). In the forskolin-stimulated culture, there was a significant decrease in cAMP levels at PFOA 50 and 100 μg/ml (I Fig. 1). Progesterone levels in hCG-stimulated fetal testis culture conditions were significantly reduced at PFOA 100 μg/ml, but the hormone levels did not change in basal conditions (I Fig. 2). Furthermore, in the forskolin-stimulated fetal testis cultures, the levels of progesterone were significantly lower at PFOA 50 and 100 μg/ml (I Fig. 2). PFOA decreased testosterone levels in hCG-stimulated fetal testes cultures at doses of 50 and 100 μg/ml and in forskolin-stimulated cultures at 100 μg/ml (I Fig. 3). In order to study whether early steroidogenesis was influenced by the action of PFOA, fetal testicular expression of StAR was analyzed. In the basal conditions of Western blot results (I Fig. 4), there was no clear difference in the levels of StAR between the PFOA-exposed and control fetal testes. However, a significant decrease in StAR protein expression was observed in hCG-stimulated fetal testes at PFOA 50 and 100 μg/ml (I Fig. 4 A). The expression of StAR protein decreased significantly at PFOA 100 μg/ml in the forskolin-stimulated fetal testes (I Fig. 4 A). Because PFOA decreased cAMP levels both in hCG- and forskolin-stimulated conditions, this indicates that PFOA disturbs fetal steroidogenesis at least at the gonadotrophin

signaling level, but other mechanisms are also possible, such as disturbance at the receptor level.

5.2 PFOA does not have an effect on proliferating fetal Sertoli cells (I)

In order to assess whether PFOA affects the proliferation of fetal Sertoli cells, we performed immunofluorescence staining with SOX9 and KI67 for 24-hour cultured fetal rat testes (ED 17.5) (I Fig. 5A). There was no significant difference in the number of KI67 positive Sertoli cells in PFOA-exposed fetal rat testes compared to the control (I Fig. 5B). Co-stained SOX9 and TUNEL positive cells were absent in both PFOA-exposed and control cultured fetal testes, indicating that there were no detectable apoptotic fetal Sertoli cells after 24-hour culture with exposure to PFOA. However, interstitial TUNEL positive fetal Leydig cells were detectable by their polyhedral or round morphology (I Fig. 6A), and quantification showed an increased tendency of TUNEL positive fetal Leydig cells in PFOA-exposed fetal rat testes (I Fig. 6B), albeit the difference was not significant.

5.3 PFOA induces apoptosis and decreases the number of 2C, proliferating and 4C cells in adult seminiferous tubules (I)

The relatively long half-life of PFOA and its ability to bioaccumulate in humans and animals represent a risk for adult testicular cells and spermatogenesis as well. In addition, real-life co-exposure to environmental chemicals makes it difficult to study the direct toxicological effects of only one chemical. Therefore, we used seminiferous tubule culture and *ex vivo* PFOA exposure to analyze the effects of PFOA on the adult testis by microdissecting seminiferous tubule segments from adult rat testes, stages VII-VIII. These stages were selected because they are relatively easy to dissect in large amounts, and within the time window of a 24-hour culture, many key spermatogenic events are expected to occur, including spermatogonial differentiation followed by S phase, preleptotene-to-leptotene transition, meiotic DNA synthesis, onset of spermatid elongation, and spermiation. The flow cytometry analysis based on Fx-cycle DNA staining showed that all seminiferous tubule cell types decreased after 24 hours of PFOA exposure at 100 µg/ml, except for haploid spermatids (I Fig 7). In the cell distribution analyzed by flow cytometry, we were able to discriminate 1C (haploid spermatids), 2C (consisting of somatic cells and spermatogonia, preleptotene spermatocytes), and 4C (leptotene and pachytene spermatocytes) populations. The decrease in almost all cell populations in seminiferous tubules prompted us to further investigate whether

PFOA induces apoptosis in seminiferous tubules. We prepared squash samples to detect apoptosis in seminiferous tubule segments (stage VIII) by using an early apoptosis marker, cleaved Caspase-3 (Nicholson et al., 1995). Images of immunohistochemistry staining with cleaved caspase-3 (I Fig 8A) and the quantification show that PFOA at 100 µg/ml induced apoptosis in cultured seminiferous tubule segments at stage VIII (I Fig 8B).

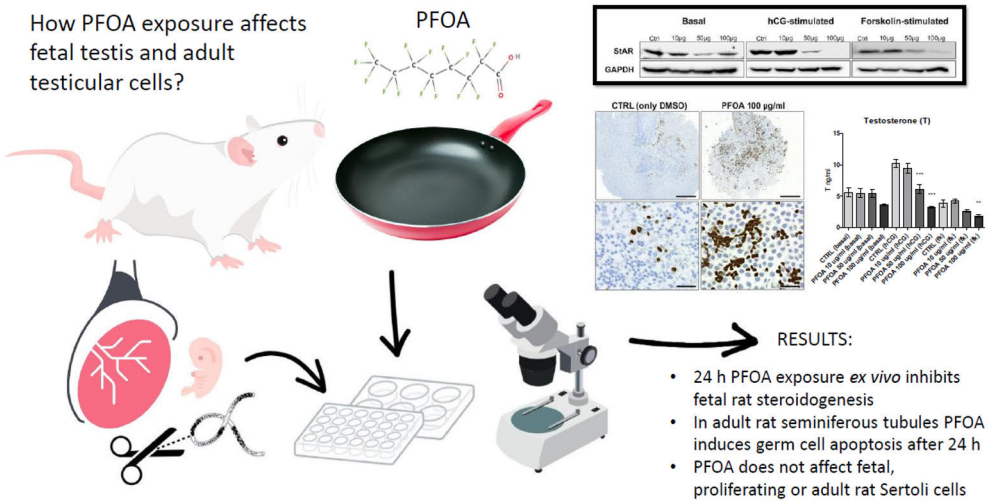

Figure 3. Graphical abstract of the effects of PFOA on fetal and adult rat testis.

5.4 Imatinib decreases the proportion of DNA-synthesizing cells in cultured rat seminiferous tubules (II)

As introduced in Chapter 2.4.2.1, imatinib inhibits many tyrosine kinases that are essential for testicular development and spermatogenesis. However, very little is known about the direct effects of imatinib on adult male germ cells and other testicular cells. Therefore, we studied the effects of imatinib on rat seminiferous tubule segments from epithelial stages VII-VIII in tissue culture with and without imatinib (0, 1, 10, and 100 µM). These stages were selected because they are relatively easy to dissect in large amounts, and within the time window of a 24-hour culture, many key spermatogenic events are expected to occur, including spermatogonial differentiation followed by S phase, preleptotene-to-leptotene transition, meiotic DNA synthesis, onset of spermatid elongation, and spermiation. Single-cell suspensions made from the cultured tubule segments were stained with Hoechst 33342 chromatin dye, which also penetrates living cells. The cell

suspensions were analyzed by flow cytometry. Again, the same cell populations were identified: 1C (spermatids), 2C (consisting of somatic cells and spermatogonia, preleptotene spermatocytes), and 4C (leptotene and pachytene spermatocytes) populations (II, Supplemental Fig. 1D) in all samples. Significant changes between control and imatinib-treated samples were observed only in the number of cells in the S phase of the cell cycle (II, Fig. 1A, B), which dose-dependently decreased in the presence of imatinib (II, Fig. 1C). These results suggest that imatinib affects DNA-synthesizing cells (spermatogonia and early spermatocytes) after 24 hours of imatinib exposure *ex vivo*.

5.5 Imatinib reduces ^{3}H-thymidine incorporation in cultured rat seminiferous tubules (II)

The observed decrease of cells in the S phase after imatinib exposure can be rationalized by two explanations: either DNA synthesis in spermatogonia or early spermatocytes is disturbed and/or delayed due to exposure to imatinib, or imatinib induces apoptosis in these cells. Therefore, we first dissected rat seminiferous tubule segments from stages I, V, VIIa, and VIII–IX for culture in imatinib under the conditions mentioned in chapter 4.8 and in the presence of ^{3}H-thymidine, as described in more detail in chapter 4.16. After 24 h and 72 h of culture, we were able to see a dose-responsive decline in the amount of ^{3}H-thymidine incorporation in stages VIII-IX at 24 h and VIIa at 72 h (II, Fig. 2 A-C). These data suggest that imatinib impinges on both meiotic DNA synthesis and mitotic DNA synthesis (CLERMONT & HARVEY, 1965; Russell, L.D., Ettlin, R., Sinha Hikim, A. and Clegg, 1990). In the results presented, meiotic DNA synthesis occurs in preleptotene spermatocytes during the 24-h culture of stages VIII-IX (II, Fig. 2 A) and during the 72-h culture of stage VIIa, respectively (II, Fig. 2 C). Mitotic DNA synthesis occurs in type B spermatogonia during the 72-h culture of stage V (II, Fig. 2 B).

5.6 Imatinib induces apoptosis dose-dependently in cultured rat seminiferous tubules (II)

In order to assess whether imatinib-induced apoptosis might explain the cellular changes in cultured rat seminiferous tubules, we cultured rat seminiferous tubules from stages VIII and IX-XI in 0, 1, 10, and 100 µM imatinib for 24 h, processed them to squash preparations, and stained for cleaved Caspase-3 (II Fig. 3 A-E). Quantification showed that clinically relevant doses of imatinib increased the number of cleaved Caspase-3-positive cells dose-dependently. These results demonstrate that, like in cancer cells (Kim et al., 2019; Ujvari et al., 2022), imatinib induces apoptosis in spermatogenic cells (II, Fig. 3 F, G).

5.7 Imatinib blocks the effect of stem cell factor (SCF) in cultured rat seminiferous tubules (II)

As one possibility is that apoptosis in imatinib-exposed seminiferous tubules (II, Fig. 3 F-G) could explain the reduction in DNA synthesis (II, Fig. 2), we wanted to further investigate whether imatinib blocks the function of SCF, a critical survival factor for germ cells (Hakovirta et al., 1999; Yan, Suominen, et al., 2000). To get the answer, we dissected rat seminiferous tubule segments from stage XII for culture with imatinib and SCF. Stage XII was selected because we have previously shown that SCF promotes the survival of DNA-synthesizing spermatogonia in that stage (Hakovirta et al., 1999). After a 24-h culture, we observed a small but significant decrease in DNA synthesis caused by imatinib alone, confirming our earlier results (II, Fig. 2 A-C). Notably, SCF administered at 100 ng/ml caused a greater than 3-fold increase in ^{3}H-thymidine incorporation, which was abolished by imatinib (Fig. 3 H). Together, these results suggest that the primary effects of imatinib on differentiating male germ cells (type A1 to B spermatogonia and preleptotene spermatocytes) are mediated through inhibition of c-KIT/SCF signaling, resulting in increased cell death and disturbed DNA synthesis, which further can impair spermatogenesis.

5.8 Dasatinib does not induce apoptosis in rat seminiferous tubules after 24-h culture with clinically relevant doses (II)

Dasatinib also inhibits c-KIT (Lindauer & Hochhaus, 2014). In differentiating germ cells, dasatinib may also disturb the c-KIT/SCF signaling. Therefore, we wanted to explore whether dasatinib induces apoptosis in rat seminiferous tubule segments at stages VIII and IX-XI. Tubule segments of these stages were cultured for 24 h with or without dasatinib (0, 0.01, 0.1, and 10 μM), then squashed and stained for cleaved Caspase-3 (II, Fig. 3 I-L). Only the highest dose of dasatinib (10 μM) induced apoptosis in rat seminiferous tubules at stages VIII and IX-XI (II, Fig. M-N).

5.9 Imatinib and dasatinib decrease mGSC colony growth *in vitro* (II)

Given the fact that the feasibility of seminiferous tubule culture for reproductive toxicological experiments is limited to short-term cultures (TOPPARI & PARVINEN, 1985), we moved to study imatinib's effects also on male germline stem cells (mGSCs), which can be propagated *in vitro* (Kanatsu-Shinohara et al., 2003). Moreover, while the effects on spermatogenic cells are temporal and reversible when the stressor is removed, the effects on mGSCs are more permanent

because they are the foundation of lifelong spermatogenesis and are present in the testis throughout the whole lifespan. In these experiments, we decided to use the mouse as a model organism because mGSC culture has been refined better in mice than in any other mammal. We established mouse mGSC cultures on mitomycin-inactivated mouse embryonic fibroblasts (MEFs) by selecting CD9-positive cells from adult mouse testes using MACS, as previously described (La et al., 2018). mGSCs in culture were verified by characteristic grape-like colony morphology (Kanatsu-Shinohara et al., 2003) and MCAM (melanocyte cell adhesion molecule) (Garbuzov et al., 2018) and SALL4 (Spalt-like 4) (Hobbs et al., 2012) expression (II, Supplemental Fig. 3).

We investigated the effects of imatinib on mGSC proliferation, apoptosis, and differentiation capacity. A second-generation TKI, dasatinib, was used as another TKI in the mGSC experiments. We used two experimental setups to study the outcome of imatinib and dasatinib exposure on mGSCs. The imatinib and dasatinib concentrations in these experiments were decided based on meticulous investigation of the effects on MEF feeders, allowing the direct effects of imatinib and dasatinib on mGSCs to be studied (II, Supplemental Fig. 4 A-O and Fig. 5 A-M). Doses of 1-10 μM imatinib and 0.01-1 μM dasatinib were chosen because these doses did not significantly affect the viability, cell growth, or morphology of MEFs (II, Supplemental Fig. 5 I, J, O and Fig. 5 K-M). Firstly, we passaged mGSCs on 24-well plates at a standard cell density and cultured them for 7-15 days until they formed small colonies (II, Supplemental Fig. 6 A-B and Fig. 7 A-D). Imatinib (0, 1, 5, and 10 μM) or dasatinib (0, 0.01, 0.1, 0.3, and 1 μM) was then added for 7 days, followed by quantification of the cell amount. Trypan Blue was used to detect cell death alongside the cell calculation. Interestingly, there was a dose-dependent decline in the number of mGSCs (II, Fig. 4 A and B), showing that both imatinib and dasatinib adversely affected mGSC colony growth.

Secondly, because cell density in mGSC cultures affected the composition of mGSC populations by promoting progenitor-like characteristics at higher densities (La et al., 2018), we discovered the effects of imatinib and dasatinib treatment before mGSC colony formation, *i.e.*, that imatinib and dasatinib exposure was started when mGSCs were plated as single cells on feeder layers. mGSCs were cultured in 24-well plates at a low cell density (3-4 thousand mGSCs per well) and cultured with or without imatinib for 4 weeks, corresponding to the standard subculturing interval for mGSCs in our laboratory. Control wells reached high confluence in 4 weeks, but there was a clear decline in the number and size of mGSC colonies in imatinib-exposed wells (II, Supplemental Fig. 6 I-L). This was confirmed by cell counting, showing a 70-90% dose-dependent decline in mGSC number in the presence of imatinib (II, Fig. 4 C), providing further evidence that mGSC propagation and/or survival are compromised in the presence of imatinib. With dasatinib, a pronounced

reduction in mGSC proliferation and colony growth was evident already after 5 days, which led us to prematurely terminate the experiment (II, Fig. 4 D and Supplemental Fig. 7 I-P). For unknown reason the proliferation and colony formation after plating in the presence of dasatinib was disturbed earlier and more clearly than in the presence of imatinib. Therefore, we did not continue this type of mGSC exposure to dasatinib for longer time. This phenomenon needs further investigation *e.g.* how dasatinib affects mGSCs before colony formation and does dasatinib affect mGSC survival.

The number of mGSCs in culture is premised on their proliferation rate and the presence of cell death. To discover the possible cell death and apoptosis in more detail, we studied the cell viability of mGSCs cultured in the presence or absence of imatinib (0, 1, 5, and 10 mM) using the Annexin V cell death analysis flow cytometry kit. Based on Annexin V (AV; early apoptosis marker) staining and propidium iodide (PI; for dead cell discrimination) treatment, the cultured mGSCs were divided into healthy (AV-neg/PI-neg), early apoptotic (AV-pos/PI-neg), late apoptotic (AV-pos/PI-pos), and necrotic cells (AV-neg/PI-pos). The proportion of healthy cells remained at 95-98% in all studied conditions (II, Fig. 4 E), although the reduction in the percentage of healthy cells in 10 mM imatinib was significantly higher than in 0, 1, and 5 mM imatinib.

5.10 Imatinib impinges on mGSC differentiation *in vitro* (II)

When stimulating mGSCs in culture with retinoic acid (RA), mGSCs respond by starting to express markers of differentiating spermatogonia, such as c-KIT and STRA8 (stimulated by retinoic acid gene 8), emulating the shift of undifferentiated spermatogonia to type A1 differentiating spermatogonia at stages VII-VIII of the seminiferous epithelial cycle *in vivo* (La et al., 2018; Zhou et al., 2008). To address whether imatinib disturbs this decisive cell fate shift, we passaged mGSCs at a standard density in 24-well plates and allowed them to grow for two weeks. We then treated the cells with varying doses of imatinib (0, 1, 5, and 10 μM) and 1 μM RA. The samples were then processed into single-cell suspensions and analyzed with flow cytometry for c-KIT expression (II, Supplemental Fig. 8). Approximately 83% of spermatogonia were found to be c-KIT-positive under control conditions, and a dose-dependent decrease was observed in the proportion of c-KIT-positive spermatogonia in the presence of imatinib (II, Fig. 4 F). These results suggest that imatinib inhibits mGSC differentiation *in vitro*.

5.11 Imatinib does not block mGSC differentiation but decreases c-KIT expression in differentiating spermatogonia (II)

We then studied whether imatinib can suppress the differentiation-inducing effect of RA in cultured mouse seminiferous tubules. For these experiments, we collected segments from stages II-VI by microdissecting mouse seminiferous tubules. We chose these stages because it has been previously shown that progenitor Aundiff become sensitive to RA between stages II-VI (Endo et al., 2015; Ikami et al., 2015). Following a 24-h culture in the presence or absence of imatinib (0, 1, and 10 μM) and 1 μM RA, the tubule segments were fixed and stained with LIN28A, which displays increased expression in 4-16-cell syncytia of Aundiff (A-aligned 4-16) primed for differentiation (Chakraborty et al., 2014; McAninch et al., 2020), and STRA8 and c-KIT differentiation markers (II, Fig. 5 A-F). Intriguingly, unexposed tubule segments of stages II-VI that were not exposed to RA were without STRA8 and c-KIT positive cells, whereas in RA-treated tubules, the clear majority of LIN28A-positive A-aligned 4-16 were positive for STRA8 and c-KIT irrespective of the presence or absence of imatinib (II, Fig. 5 B-C and E-G), suggesting that imatinib does not inhibit spermatogonial differentiation induction *ex vivo*.

5.12 Imatinib, but not dasatinib, reduces the number of KI67-positive A_{undiff} spermatogonia *ex vivo* (II)

A_{undiff} spermatogonia are the *in vivo* counterparts of cultured mGSCs (La et al., 2018). It is known that *in vitro* culture of mGSCs does not fully correspond to the stem cell niche in the testis. Therefore, we wanted to discover the effects of imatinib and dasatinib on A_{undiff} proliferation and differentiation *in situ* using mouse seminiferous tubule culture. We first microdissected mouse seminiferous tubule segments from stages IX-I for a 48-h culture in 0 or 10 μM imatinib and dasatinib. We chose these stages because A_{undiff} are considered to self-renew in stages X-II (Sharma & Braun, 2018; Tagelenbosch & de Rooij, 1993). After 48-h culture and *ex vivo* exposure, the tubule segments were fixed and stained with GFRα1 (GDNF family receptor alpha 1), a specific marker of the self-renewing A_{undiff}, and KI67, a proliferation marker (II, Fig. 6 A-B and C-D). In control samples, around 37% of GFRα1-positive cells was found to be KI67-positive, whereas in the presence of 10 μM imatinib, the percentage fell to 29% (II, Fig. 6 E), confirming our *in vitro* results concerning the anti-proliferatory effect of imatinib on stem and progenitor cells of the male germline. In contrast, in dasatinib-exposed tubule segments, there was no significant difference in the proportions of KI67-positive GFRα1-positive cells as compared to control (32% in CTRL vs. 33% in dasatinib 10 μM) (II, Fig. 6 F).

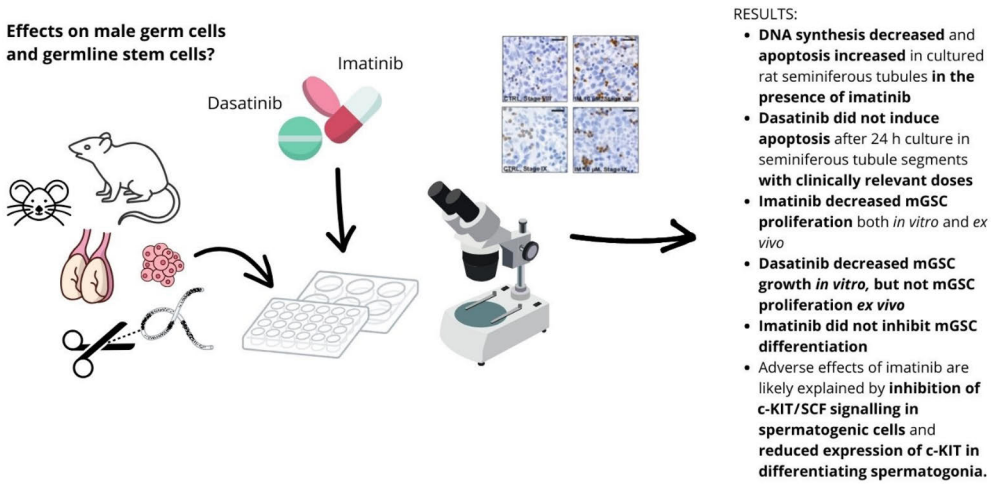

Figure 4. Graphical abstract of the results of imatinib and dasatinib on rodent (mouse and rat) male germ cells and mouse male germline stem cells.

6 Discussion

6.1 The effects of PFOA on fetal and adult rat testis (Study I)

The endocrine-disrupting properties of perfluorooctanoic acid (PFOA) present an increasing concern about the effects of PFOA on humans and wildlife. In my first doctoral study, we discovered the effects of a persistent environmental pollutant, PFOA, on young adult (60-80 days) and fetal (ED 17.5) male rats by using *ex vivo* exposure in tissue culture. Our results show that 24 h PFOA exposure *ex vivo* inhibits steroidogenesis in fetal rat testis (ED 17.5). This conclusion is based on the results where PFOA exposure decreased the accumulation of cAMP in fetal testes cultures in all doses and culture conditions. PFOA doses of 50 μg/ml and 100 μg/ml reduced progesterone and testosterone secretion in hCG- and forskolin-stimulated fetal testes cultures. Since reduced levels of cAMP, progesterone, and testosterone due to PFOA exposure were observed, we wanted to analyze whether early events of steroidogenesis are also impaired by the action of PFOA. Therefore, we measured the expression of steroidogenic acute regulatory protein (StAR) in fetal testes after culture with and without PFOA. Western blot results showed that the levels of StAR in the fetal testis were significantly lower after hCG and forskolin stimulation in PFOA-exposed testes than in controls. StAR protein is involved in the early phase of steroidogenesis, and it is synthesized in response to LH (D. M. Stocco, 2001). StAR protein transfers cholesterol to the inner mitochondrial membrane and to the cholesterol side chain cleavage enzyme system (D. M. Stocco, 2000).

The impact of PFOA on male steroidogenesis or androgen levels has been shown in many studies (Bach et al., 2016; Di Nisio et al., 2019; Han et al., 2022; Han & Huang, 2021; Lau et al., 2007; H. Lu et al., 2019; Song et al., 2018; Tian et al., 2019; D. Zhang et al., 2024; H. Zhang et al., 2014). However, the results are somewhat controversial since some studies have shown PFOA to increase testosterone levels or stimulate steroidogenesis (Bach et al., 2016; Han & Huang, 2021; Louvet et al., 2008; Sun et al., 2018; Tian et al., 2019), while in other studies, including our results, the secretion of steroid hormones and/or corresponding protein or gene expression in rodent models has decreased due to the action of PFOA (H. Lu et al., 2019; Song et al., 2018; D. Zhang et al., 2024; H. Zhang et al., 2014). An interesting example of

controversial findings is that Han *et al*. (2022) observed upregulation of StAR mRNA but a decrease in other steroidogenic genes after *in vivo* PFOA exposure in male rat testes, while increasing overall serum hormone levels were observed (Han et al., 2022). The differences between Han *et al.*'s results compared to ours may be explained by differences in the methods used and PFOA doses. Han *et al*. administered relatively low oral PFOA doses for adult male rats, while in our study, we used fetal testes, relatively high doses of PFOA, and only 24 hours of *ex vivo* exposure in tissue culture conditions. Supporting this argument, Tian *et al.* observed that low doses of PFOA increased testosterone levels, while high doses of PFOA reduced testosterone levels in mouse Leydig cells *in vitro* (Tian et al., 2019). Kabacki *et al.* (2024) observed decreased testosterone and progesterone levels after PFOA exposure in bovine granulosa cells, but they didn't see decreased StAR expression. Kabacki *et al.* used lower PFOA concentrations than we did, which may explain the differences in StAR expression results. However, the decreased hormone secretion levels in their study were in line with our results (Kabakci et al., 2023). One study by Song *et al.* (2018) showed that orally administered PFOA during pregnancy caused reduced testosterone levels in male mice offspring (Song et al., 2018). In contrast, Jensen *et al.* (2020) studied maternal serum PFAA concentrations, including PFOA, in early human pregnancy and serum concentrations of androgens, their precursors, and gonadotropins during mini-puberty in human infancy. In boys, no significant association was found between PFAAs and concentrations of androgens, their precursors, and gonadotropins during mini-puberty (Jensen et al., 2019). However, to our knowledge, my first doctoral study is the only research so far that has investigated the direct effects of PFOA on fetal rodent testis by using *ex vivo* exposure.

The window ranging from ED 15.5 to 18.5 in rats has previously been identified as a sensitive masculinization programming window, where androgens ensure the correct development of the male reproductive tract. The fetal rat testis produces testosterone from ED 15.5 to ED 21.5, and during this period, masculinization occurs. Disruptions of hormonal levels during this time range may lead to male reproductive health disorders like cryptorchidism, hypospadias, poor semen quality, decreased testosterone levels, and testicular structural damage (Juul et al., 2014; Welsh et al., 2008). We chose this exposure period (ED 17.5) and 24-hour exposure time for two reasons. First, because it covers this critical developmental period, which is sensitive to external toxicants possessing, *e.g.*, antiandrogenic effects, and secondly, because the fetal testes are reliably identified and dissected from the mesonephros at this developmental point.

Exposure time in tissue cultures is limited to short-term cultures. The PFOA doses used in this study were selected based on the highest reported serum PFOA levels in occupational workers (Hekster et al., 2003) and the short exposure time was

taken in to consideration in the selection of PFOA doses. However, the PFOA doses used in this study are relatively high and exceptional since the general population in the USA has about 5-8.5 µg/l PFOA in their blood, while highly exposed people have 1000 µg/l (=1 µg/ml = 2.4 µM). The highest concentration in our study is 100 µg/ml of PFOA (241.5 µM) representing an extreme overdose. Since the production and use of PFOA have declined, the levels in blood have gone down as well. From 1999 to 2014, blood PFOA levels among the general U.S. population have decreased by more than 60% (Cheng et al., 2022), (https://www.epa.gov/pfas; Agency for Toxic Substances and Disease Registry's ATSDR).

Previous studies have shown that PFOA does not function as an antagonist or agonist for the androgen receptor (AR) (Rosenmai et al., 2013; Vinggaard et al., 2008). By contrast, there is evidence that PFOA can act as an agonist to AR. Results of a human study from Di Nisio *et al.* have shown increased levels of PFOA in plasma and seminal fluid correlating positively with higher testosterone and LH levels in serum and a reduction in semen quality, testicular volume, penile length, and AGD (Di Nisio et al., 2019). The clinical results from Di Nisio *et al*. are in line with results from some preclinical studies where exposure to environmentally relevant PFOA doses were associated with increased testosterone levels (Bach et al., 2016; Han & Huang, 2021; Sun et al., 2018; Tian et al., 2019). However, our measurements from fetal rat testis culture do not agree with the clinical results from Di Nisio *et al*., where PFOA-exposed men had increased testosterone and LH levels in serum and there are controversially results from another human studies too (Bach et al., 2016; Lopez-Espinosa et al., 2016; Tarapore & Ouyang, 2021; Tsai et al., 2015; Vested et al., 2013).

In our study, 24 hours of PFOA exposure inhibited the increase of cAMP both in the hCG- and forskolin-stimulated culture mediums. In light of these results, we can conclude that PFOA affects steroidogenesis at the gonadotropin signaling level, but other mechanisms are also possible, such as disturbances at the receptor level. Our results are in line with other studies in which PFOA has been shown to decrease testosterone and progesterone levels by inhibiting the biosynthesis of these hormones in rodent Leydig cells *in vitro* (Tian et al., 2019; D. Zhang et al., 2024; Zhao et al., 2010). Our results are also consistent with a study where gestational exposure of mice to 5 mg/kg/day of PFOA caused reduced testosterone levels in male offspring (Bao et al., 2021). Based on the results of Tian *et al.* (2019), PFOA can disrupt cholesterol transport into mitochondria and then alter steroid synthesis. Meanwhile, Tian *et al.* (2019) suggest that PFOA-regulated nuclear (membrane) receptor response and steroidogenesis result in the disruption of sex hormone secretion, meaning that PFOA may have a biphasic effect on testosterone and progesterone production. (Tian et al., 2019) Similarly, Zhang *et al.* (2023) suggest that PFOA inhibits androgen biosynthesis by selectively targeting key enzymes in the synthesis

pathway (D. Zhang et al., 2024). Our results are in agreement with the findings of Zhang *et al.*, since in our experimental design, PFOA did not inhibit the hCG-, and Fk-stimulated increase of cAMP, suggesting that PFOA might affect the steroidogenic enzymes and thereby inhibit the hormone biosynthesis. However, more studies to investigate the underlying mechanisms of PFOA on steroidogenesis are needed.

We also observed an increased tendency of apoptosis in fetal Leydig cells after 24 h of PFOA exposure, yet with relatively high doses compared with the detected plasma concentrations of PFOA (Cheng et al., 2022). By contrast, the current study shows that PFOA does not affect either apoptosis or proliferation of fetal Sertoli cells after 24 hours of exposure in tissue culture. Our results confirm that PFOA affects Leydig cells, as shown in previous studies as well (Biegel et al., 1995; Tian et al., 2019; D. Zhang et al., 2024; Zhao et al., 2010).

We also studied the effects of PFOA on adult rat testis. PFOA exposure *ex vivo* induced apoptosis dose-dependently in seminiferous tubules after 24 hours, presenting a risk for normal sperm quality in PFOA-exposed male humans and animals. The flow cytometric analysis of tubule segments of stages VII-VIII showed that PFOA significantly decreased the amounts of cells in the 2C (diploid cells), proliferating cells, and in the 4C (cells in meiosis and in G2/M-phase of the cell cycle) populations in adult rat testis. PFOA did not have a significant effect on the 1C (haploid germ cells) population of rat testicular cells. It is known that the most vulnerable testicular cells to undergo apoptosis in adult rat testis are spermatogonia, early and meiotically dividing spermatocytes. Secondary spermatocytes, mid to late spermatocytes, and spermatids seldom undergo apoptosis (Henriksen et al., 1995; Yan, Suominen, et al., 2000). This is in agreement with our flow cytometry and immunohistochemistry data. Therefore, we can conclude that the affected testicular cells during PFOA exposure in rat seminiferous tubules were mostly spermatogonia in the 2C population and primary spermatocytes in the 4C cells.

In epidemiological studies, PFOA has been associated with causing adverse effects on male reproductive functions in humans (Di Nisio et al., 2019; Joensen et al., 2009, 2013; Vested et al., 2013), and several animal studies support this association (Bach et al., 2016; Biegel et al., 1995; Di Nisio et al., 2019; Lau et al., 2007; Wan et al., 2011). PFOA has, for example, been shown to cause direct toxicological effects on male reproductive physiology, such as affecting sperm motility, the number of morphologically normal sperm, rates of sperm aneuploidy, DNA fragmentation index, and androgen levels in men. However, the exact observed effects vary between different studies, and dissonance exists (Bach et al., 2016; Vested et al., 2013). Since the direct effects of PFOA on adult rat seminiferous tubules have not been studied before, my first doctoral study brings new information about the ability of PFOA to target male germ cells and induce apoptosis in

spermatogonia and spermatocytes. These results may further explain the associations of PFOA with sperm abnormalities and decreased sperm count in PFOA-exposed men.

Main limitations of this study are the short exposure time (24 h) and relatively high PFOA doses. The exposure time was chosen to be short, because tissue cultures are limited only to short-term cultures. In the future more mechanistic studies about the effects of PFOA on steroidogenesis and testicular cells should be performed.

Taken together, our results and the results of other studies suggest that PFOA exposure represents a risk for male reproductive health. Caution to avoid PFOA exposure especially during pregnancy is justifiable. In practise this means avoiding particularly damaged or overheated non-stick cooking tools, avoiding *e.g.* microwave pop-corn packages and other food packages containing PFOA, avoiding vegetables planted in the PFOA-contaminated areas, avoiding water from PFOA-contaminated municipal sources or private wells and avoiding water- and stain-resistant clothes which have been made by using PFOA or any type of PFAS.

6.2 Imatinib decreases germ cell survival and germline stem cell proliferation in rodent testis *ex vivo* and *in vitro* (Study I and II)

Traditional anticancer treatments like radiation and cytotoxic chemotherapies are recognized to significantly and even permanently affect fertility in both men and women (Ramstein et al., 2017). A deeper understanding of the molecular signaling involved in carcinogenesis has led to the discovery of more targeted anticancer treatments like tyrosine kinase inhibitors (TKIs), which have also been considered to cause less infertility and other undesired side effects. Despite the widespread use of TKIs, only limited data on their effects on male reproductive health exist (Elsabagh et al., 2024; Rambhatla et al., n.d.; Suzan et al., 2021). However, there is evidence that there might be an association between TKIs and impaired male fertility, but the underlying cellular-level effects are mainly unknown (Elsabagh et al., 2024).

Imatinib is a first-generation TKI, and it is often used as a chronic therapy. Its clinical use is increasing, and it is used for both children and adults of fertile age, representing a risk for testicular physiology and male reproductive health. The approach of my second doctoral study was to recognize the dose-specific effects of imatinib on male mouse germline stem cells (mGSCs) and other spermatogenic cells, both *ex vivo* and *in vitro*. Since we know that the therapeutic doses of imatinib also inhibit c-KIT tyrosine kinase, which is essential for healthy spermatogenesis, our hypothesis was that imatinib might have a harmful effect on testicular cells. We also evaluated the *ex vivo* and *in vitro* effects of imatinib on male mouse germline stem

cells (mGSC) for the first time from the above presented approach. mGSCs maintain the lifelong fertility of men, and if the cancer treatments destroy mGSCs, a permanent infertility is possible for male patients (Jahnukainen, 2012).

By our novel and well-developed *in vitro* and *ex vivo* reproductive toxicological techniques, we demonstrated that imatinib decreased stage-specific DNA synthesis and induced apoptosis in cultured rat seminiferous tubule segments. Imatinib also had an adverse effect on mGSC colony growth both *in vitro* and *ex vivo* but did not induce cell death in cultured mGSCs. Imatinib did not impinge on the induction of spermatogonial differentiation but suppressed c-KIT expression in nascent differentiating spermatogonia, giving a convincing explanation for its pro-apoptotic property in spermatogenic cells.

The most significant finding of my second doctoral study is that imatinib decreases mGSC colony growth dose-dependently *in vitro* and *ex vivo* with clinically relevant imatinib doses of 1, 3, 5, and 10 μM. To our knowledge, only one study has been conducted where the effects of imatinib on cultured mGSCs have been investigated (Heim et al., 2011). While Heim and colleagues concluded that imatinib decreases mGSC expansion *in vitro*, which is in line with our findings, their study had a limitation given the enriched expression of differentiation markers c-KIT and SOHLH1 (Spermatogenesis and Oogenesis specific basic Helix-Loop-Helix 1) in cultured mGSCs. Notably, only a small subset of cells (0.1%) was able to form spermatogenic colonies following transplantation (Heim et al., 2011). These data suggest that their mGSC cultures consisted of a mixture of stem and differentiating spermatogonia. This is different from our mGSC cultures, which were devoid of c-KIT expression, making the comparison of these two studies difficult. Moreover, Heim *et al*. used only a single dose of imatinib (1 μM), whereas we studied the effects of imatinib using three different doses.

In our laboratory, Nurmio *et al*. (2007) previously showed that administration of imatinib to 5- to 7-day-old male rats interfered with postnatal testicular development (Nurmio et al., 2007). Despite the changes in the prepubertal testis, when these males were allowed to age, they hosted normal spermatogenesis but sired 20% smaller litters than the controls (Nurmio et al., 2007). These data suggest that while the effects of imatinib on the developing testis are adverse, they can be largely resolved when the stressor is removed. In the present study, we extended that aspect developmentally and used adult rodent models to study the effects of imatinib on spermatogenic cells and mGSCs, using *ex vivo* seminiferous tubule culture and mGSC propagation *in vitro*. A relatively new publication from Suzan *et al.* (2021) reported the effects of imatinib exposure in the prenatal period. Imatinib was administered intraperitoneally to dams. They observed a decrease in total sperm counts as well as histopathological and biochemical changes in the testis tissue of prenatally imatinib-exposed rats, highlighting the need for further studies on the

effects of imatinib on a cellular and molecular level (Suzan et al., 2021). Chang *et al.* (2021) showed that imatinib induced apoptosis in mouse spermatogonia, while we performed apoptosis analysis in rat seminiferous tubules. Long-term treatment of adult male mice with clinical doses of imatinib led to plummeting sperm counts, suggesting that TKs targeted by imatinib are crucial for spermatogenesis (X. Chang et al., 2021). However, the cell-level effects of imatinib on spermatogenic cells and mGSCs have remained understudied.

Previous studies have shown that imatinib-treated male patients have decreased sperm counts (Nicolini et al., 2016; X et al., 2017). Our observations suggest that the reduced sperm counts in imatinib-treated male patients may occur due to decreased mGSC proliferation and interference with c-KIT/SCF signaling in spermatogenic cells, leading to fewer differentiating germ cells, a gradual reduction of stem cells, a decline in proliferating spermatogonia, and increased germ cell apoptosis. Therefore, my second doctoral study provides plausible new cellular-level explanations for how imatinib might disturb spermatogenesis and essential male germline stem cells from the perspective of fertility preservation. However, our results and clinical evidence support the fact that the effects of imatinib on male fertility may not be permanent, as confirmed by the fact that imatinib-treated male patients have been able to father their own biological children (Hensley & Ford, 2003; Shash et al., 2011).

Since imatinib intolerance and resistance have emerged, new generation TKIs have been developed. Dasatinib, a second generation TKI, which among its other target TKIs also inhibits c-KIT (Lindauer & Hochhaus, 2014), a crucial survival factor during spermatogenesis (Yan, Suominen, et al., 2000). Therefore, in my third doctoral study, we investigated whether the newer TKI and more potent BCR-ABL-targeting dasatinib has similar adverse effects on spermatogenic cells and mGSCs as imatinib.

Differently from imatinib, clinically relevant doses of dasatinib did not induce apoptosis in rat seminiferous tubules (stage VIII and IX) after 24 h of exposure *ex vivo*. Only the highest dose of dasatinib (10 μM), representing an overdose, caused apoptosis at both studied stages of the epithelial cycle. In dasatinib-exposed mGSCs, we observed a dose-dependent decline in colony growth with clinically relevant doses. When we further wanted to corroborate those findings using an *ex vivo* seminiferous tubule culture setting, we did not observe a decline in proliferating mGSCs in the presence of dasatinib.

Interestingly, there are some indications that the effects of dasatinib on spermatogenesis are milder than the effects of imatinib (Yassin et al., 2012). Garcia DN *et al.* (2023) studied the effects of dasatinib and quercetin on male mice *in vivo*, and they did not observe adverse effects on mouse spermatogenesis after 5 months of exposure (Garcia et al., 2023). Furthermore, male patients treated with dasatinib have demonstrated a less pronounced impact on spermatogenesis compared to those

treated with imatinib or nilotinib (Yassin et al., 2012). These results align with our observations about dasatinib on seminiferous tubules and mGSCs *ex vivo.* My doctoral studies about the effects of dasatinib on rodent testicular cells and mGSCs provide reasonable explanations for why dasatinib might have less impact on spermatogenesis than imatinib.

Most recently, imatinib has been advocated as a potential therapeutic agent for pediatric patients with type 1 diabetes (Lavelle et al., 2024). Before imatinib is started to be used as a treatment for new indications, the side effect profile needs to be well investigated, including possible adverse effects on fertility. Based on our findings, caution is necessary when planning to treat young patients with imatinib and dasatinib beyond oncology.

Our findings indicate that these two TKIs have partially distinct effects on adult male germ cells and mGSCs. Limitation of the study is that this data is from rodents and may not be directly comparable to findings in humans. Therefore, we cannot draw direct conclusions on *e.g.* clinical drug selection. Mechanistic studies on the effects of imatinib and dasatinib on mGSCs are needed.

7 Summary/Conclusions

This doctoral research brings new detailed information about how the studied pharmaceuticals and environmental chemicals can affect male reproductive health. The first part of this doctoral thesis also highlights the vulnerability of male fetuses to *in utero* exposure to environmental toxicants.

The first study showed that a ubiquitous environmental contaminant, perfluorooctanoic acid (PFOA), exposure inhibits fetal rat steroidogenesis by decreasing cAMP, progesterone, and testosterone levels after 24 hours of exposure *ex vivo*. Furthermore, the expression of StAR was decreased in fetal testes after 24 hours of exposure to PFOA as well. We also observed an increased tendency of apoptosis in fetal Leydig cells, albeit the difference was not significant. Thus, we can conclude that PFOA affects fetal Leydig cells. In adult rat seminiferous tubules, PFOA induces apoptosis. Based on our results of the experiments, PFOA does not affect fetal, proliferating, or adult rat Sertoli cells.

The second study of this doctoral thesis investigated the effects of first- and second-generation tyrosine kinase inhibitors (imatinib and dasatinib) on adult rat spermatogenic cells and mouse male germline stem cells (mGSCs) using *ex vivo* and *in vitro* culture. The results show that clinically relevant doses of imatinib, but not dasatinib, increase germ cell apoptosis, and clinical doses of both drugs disturb the self-renewal of mGSCs *in vitro*. Our data indicate that imatinib's pro-apoptotic effect on differentiating germ cells is partly due to inhibition of c-KIT/SCF signaling and the reduced expression of c-KIT, a survival factor in differentiating spermatogonia.

Pharmaceutical agents and environmental chemicals can cause adverse effects on the reproductive system, and reproductive toxicity often remains unnoticed despite the fact that testing of chemicals and pharmaceuticals is regulated by EU legislation. However, the methods used in toxicology need continuous development, and a shift towards the use of *in vitro/ex vivo* methods and the inclusion of fewer animals is wanted. Hereby, this doctoral study also offers desired methodological insights and novel tools for male reproductive toxicity research.

Taken together, the results of this thesis indicate that PFOA has adverse effects on both adult and fetal rat testis, and that imatinib and dasatinib affect the self-renewal of male germline stem cells *in vitro*. The outcomes of imatinib and dasatinib on germ cell apoptosis and male germline stem cells *ex vivo* differ. Therefore, the results concerning one tyrosine kinase inhibitor may not be universally applicable to all tyrosine kinase inhibitors. These findings underscore the importance of ongoing research and development in toxicology to better understand and mitigate the risks posed by environmental and pharmaceutical agents to reproductive health.

Acknowledgements

The research described in this PhD thesis was carried out at the Centre for Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku.

Firstly, my deepest gratitude goes to my supervisor, Professor, MD Jorma Toppari, for entrusting me with a fascinating research project when I was a medical student and later on giving me an interesting and important doctoral research project. Your wisdom, social and general intelligence, passion for science and your communication skills are something I admire, and which have left eternal footprints on my heart. I also highly appreciate that you have created such a pleasant working environment, granted me financial support, and given me the freedom to plan my own working schedule. These key aspects made my thesis possible.

Next, my sincerest thanks go to Docent Juho-Antti Mäkelä. Under your supervision, I grew as a scientist. In the beginning, you were always available when I needed guidance and support in the lab. Thank you! I am also grateful for your biomedical creativity and intelligence that contributed to the progression of my second research project. We even did scientifically cool things together such as extracting male germline stem cells (mGSCs) from mouse testis, and you encouraged me to extract MEFs by myself. Overall, your contribution has been crucial during my pathway to a PhD.

I am grateful to Professor Rod Mitchell and Senior Researcher PhD Sofie Christensen for the careful review of my thesis and the helpful suggestions for improvement. In addition, I am thankful to Professor Tiina Santonen for agreeing to be my opponent for the public examination.

My deep gratitude goes to Professor Noora Kotaja as well. Thank you for "adopting" me into your group's weekly Monday meetings. It has been a pleasure to get to know you during these years and I warmly thank you for all the scientific discussions which have inspired me and also developed the quality of my research. I sincerely admire your passion for science; you are definitely an inspiring role model for young scientists. Thank you also for your support and encouragement during the final steps of this work.

I am grateful to my thesis follow-up committee members, Professor, MD Kirsi Jahnukainen and Professor Rod Mitchell. You have pointed out critical questions,

and your suggestions improved the quality of my scientific work. I want to thank especially you, Kirsi, for your wisdom and support during the review process of my second manuscript. Your determined attitude, clinical and scientific expertise, and enthusiasm for science continue to inspire me.

I also want to thank PhD Mirja Nurmio and PhD Annika Adamsson. Under your supervision, my advanced study project developed to an original peer-reviewed publication during my medical studies. Your friendly guidance at the beginning of my research career and all your encouragement have been invaluable on my path to a PhD.

I kindly acknowledge all my co-authors Sathyavathy Anandan, Sheyla Cisneros-Montalvo, Stefania Musilli, Jan-Bernd Stukenborg, Aida Wahlgren, Opeyemi Olotu, Sini Laasanen, Kim Eerola, and Miisael Nieminen for their contribution to this work. I would like to thank Erica Nyman and Taina Kirjonen for the technical help in the first publication. I am grateful to Eliisa Löyttyniemi for her willingness to help and give professional advice regarding the statistical analysis of research presented in this thesis, and for being my teacher in biostatistics at medical school. I also extend my gratitude to Markus Peurla for his support with the slide scanner microscope and 3i.

My special thanks go to the past and present members of "Toppari group" including Helena Virtanen, Mirja Nurmio, Annika Adamsson, Jaakko Koskenniemi, Sergey Sadov, Wiwat Rodprasert, Emmi Rotgers, Sheyla Cisneros-Montalvo, Heidi Elamo, Johanna Järvi and Kreeta Viitala. I have been fortunate to spend these years in such a great company, filled with laughter and a hilarious sense of humor regarding our study subject.

The whole Kotaja research group deserves my gratitude as well. The weekly meetings every Monday morning, filled with brainstorming ideas, troubleshooting, and scientific discussions in a supportive atmosphere, have contributed to my success today. Thank you, everyone!

I particularly want to thank Ammar Ahmedani, Lin Ma, Opeyemi Olotu, Mari Lehti, Hanna Heikelä, Michael Gabriel, Wiwat Rodprasert, Konrad Patyra, Jaakko Koskenniemi and Gabriela Martínez Chacón for your friendship, for all the scientific and non-scientific conversations, and all peer support during these years.

I have enjoyed working in the Department of Physiology (C5 floor) and therefore I express my gratitude to all past and present professors, principal investigators, researchers, students and technicians for making the working atmosphere so nice: Ilpo Huhtaniemi, Matti Poutanen, Harri Niinikoski, Nafis Rafman, Pirjo Pakarinen, Petra Sipilä, Leena Strauss, Jukka Kero, Manuel Tena-Sempere, Antti Perheentupa, Ashutosh Trehan, Oliver Meikar, Hanna Hyssälä, Janne Hakkarainen, Milena Doroszko, Henriette Undeutsch, Heli Jokela, Heidi Kemiläinen, Ram Prakash Yadav, Henna Joki, Luís Crisóstomo, Tiina Lehtiniemi, Matthieu Bourgery, Kristiina Makkonen, Mari Vähä-Mäkilä, Marika Kariskoski, Kamila Pulawska-Moon, Donata Ponikwicka-Tyszko, Krisztina Kukoricza, Riikka Huhtaniemi, Arttu Junnila, Laura

Mairinoja, Guillermo Martinez Nieto, Saori Itoshima, Elina Louramo, Samuli Laasanen, Inka Raimoranta, Jasmin Lassila, Raktim Hati, Kreeta Viitala, Riikka Palimo, Maiju Rantamäki, Oliver Mehtovuori, Leena Karlsson, Mona Niiranen, Jonna Palmu, Heli Niittymäki, Katri Hovirinta, Nina Messner, Madeleine Latvala, Elina Valkonen, Clara-Theresia Kolehmainen, Pauliina Toivonen, and Jenni Airaksinen.

I am grateful to Tuula Hämäläinen, Taina Kirjonen, Marko Tirri, Minna Lindroth, Satu Tölkkö, and Johanna Järvi for making everything work efficiently in the department and always being willing to help. My special thanks go to Ida Hyötyläinen for organizing such a well-working cell culture department. Thank you all for these years and for the good spirit you have created around you!

I would like to thank all my colleagues in TYKS Acute for creating such a nice working environment including precious coffee breaks filled with laughter and collegial support even beyond medicine. Throughout my PhD journey, I have appreciated your interest in my research and your encouragement.

Moreover, I am grateful to all my dear friends and soul sisters for your friendship and support over the years. You have truly made my life much more enjoyable.

I express my heartfelt gratitude to my parents, Ari and Vilhelmiina, for their unconditional love and support. You offered me a childhood in which my creativity had a chance to flourish, and you always encouraged me to choose my own path in life. Minni, my dear little sister, thank you for your support and for standing by my side. Likewise, I also want to extend my gratitude to my grandparents. I will always feel your love and support in my heart.

Finally, I want to thank my spouse. You have encouraged me to pursue my dreams and you have always understood my enthusiasm for science and medicine. Thank you for your love and support. With you, I am truly grateful that life has blessed us with our delightful and so lovable children. To my children, I want to say: I am proud to be your mother, and my love and support for you will never end. I encourage you to follow your dreams and passions. True success comes from hard work, but also from enjoying the ride.

This PhD study was financially supported by Turku Doctoral Programme of Molecular Medicine (TuDMM), Sigrid Jusélius Foundation, Finnish Cultural Foundation, AAMU Finnish Children's Cancer Foundation, Emil Aaltonen Foundation, Turunmaa Duodecim Society, The Finnish Medical Foundation, The Finnish Endocrine Society, University of Turku Research Grant Fund, TYKS Foundation, Turku University Foundation, Finnish Physiological Society and Tremendo

28.7.2025

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TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2025

ISBN 978-952-02-0270-5 (PRINT)
ISBN 978-952-02-0271-2 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)