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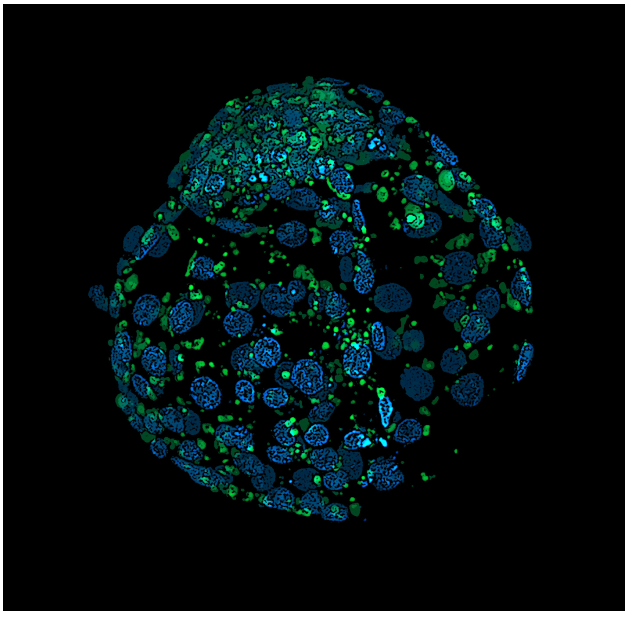
Oocyte maturation in a contaminated environment

Effects of perfluoroalkyl substances on bovine early embryo development *in vitro*

Ida Hallberg



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Cover: Bovine blastocyst captured by confocal microscopy
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Abstract

The content of this thesis is dedicated to female fertility, and more specifically the maturing oocyte. The aim was to investigate chemicals present in the direct environment of the oocyte and more specifically to investigate the sensitivity of the oocyte to perfluoroalkyl substances (PFASs) using a bovine *in vitro* embryo production model.

Human ovarian follicular fluid and serum were subjected to non-target screening. Substances were prioritized according to ubiquitous exposure, calculated hazard, accumulation in follicular fluid, and association to embryo quality. The results showed that the follicular fluid contained a complex mixture of endogenous and exogenous compounds. Standards were used to confirm the identity of selected compounds, of which some were associated with embryo quality.

The effects of oocyte exposure to perfluorononanoic acid (PFNA), perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS) were analysed by combining confocal imaging of nuclei and lipids with microarray-based transcriptomic and epigenomic studies of blastocysts. The results showed how oocyte exposure to high concentrations of PFASs caused embryo lethality. At lower concentrations, the developmental rate and lipid distribution in the blastocyst were affected. The molecular data showed potential pathways related to these findings.

To conclude, the results presented in this thesis highlight the complexity of the environment in which the oocyte develops and furthermore how exposure to PFASs interfere with bovine early pre-implantation embryonic development *in vitro*.

Keywords: PFAS, NTS, Orbitrap, IVP, confocal, transcriptome, DNA-methylation, PFOS, PFNA, PFHxS

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Oocytmognad i en kontaminerad miljö – effekter av högfluorerade ämnen på den tidiga bovina embryoutvecklingen *in vitro*

Sammanfattning

Den här avhandlingen är dedikerad till kvinnlig fertilitet och mer specifikt den mognande oocyten. Målet var att undersöka exponeringen av oocyten för miljökemikalier samt att vidare undersöka oocytens känslighet för högfluorerade ämnen (PFAS) genom att använda en modell av tidig embryoutveckling hos nötkreatur *in vitro*.

Follikelvätska och serum från kvinnor analyserades med ”non-target”-undersökning. Identifiering av ämnen fokuserades till ämnen med utbredd exponering eller beräknad fara, ansamling i follikelvätska och länk till embryokvalitet. Resultaten visade att follikelvätska består av en komplex mix av tusentals ämnen, både av endogent och exogent ursprung. Ett antal kunde identifieras, däribland ämnen som verkade associerade med embryokvalitet.

Effekten av exponering av oocyten på den tidiga embryo utvecklingen studerades *in vitro* med nötkreatur som modell där oocyter exponerades för perfluorononansyra (PFNA), perfluoroktansulfonat (PFOS) och perfluorhexansulfonat (PFHxS). Utvecklade blastocyster studerades med konfokalmikroskopiska bedömningar i kombination med hybridiserings-baserade analyser av genuttryck och DNA-metylering. Resultaten visade hur oocyt-exponering av PFAS bidrar till ökad embryodödlighet i högre doser. I lägre doser påverkades utvecklingshastigheten så väl som fett-distributionen i blastocysterna. De molekylära analyserna kunde identifiera signalkaskader relaterade till dessa förändringar.

Sammanfattningsvis visar resultaten den komplexa miljön oocyten utvecklas i samt hur exponering av PFAS kan störa den tidiga embryoutvecklingen *in vitro*.

Keywords: PFAS, NTS, Orbitrap, IVP, konfokal, genuttryck, DNA-metylering, PFOS, PFNA, PFHxS

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Dedication

Till Thea som ständigt utmanar mig men ger tiofaldigt tillbaka. Och till Harry som ser till att hålla mig på tå.

”A box without hinges, key or lid,
Yet golden treasure inside is hid”
- *J.R.R Tolkien*

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Hallberg, I; Plassmann, M; Olovsson, M; Holte, J; Damdimopoulou, P; Sjunnesson, YCB; Benskin, JP; Persson, S. (2021). Suspect and non-target screening of ovarian follicular fluid and serum – identification of anthropogenic chemicals and investigation of their association to fertility. *Environ Sci: Processes & Impacts,* 2021 Sep 28. doi: 10.1039/d1em00211b. *Online ahead of print*

II. Hallberg, I; Kjellgren, J; Persson, S; Örn, S; Sjunnesson, Y (2019). Perfluorononanoic acid (PFNA) alters lipid accumulation in bovine blastocysts after oocyte exposure during *in vitro* maturation. *Reproductive Toxicology,* 84, 1-8

III. Hallberg, I; Persson, S; Olovsson, M; Sirard, MA; Damdimopoulou, P; Rüegg, J; Sjunnesson, YCB. Perfluorooctane sulfonate (PFOS) exposure of bovine oocytes affects early embryonic development at human relevant levels in an *in vitro* model *(submitted manuscript)*

IV. Hallberg, I; Persson, S; Olovsson, M; Moberg, M; Ranefall, P; Laskowski, D; Damdimopoulou, P; Sirard, MA; Rüegg, J; Sjunnesson, YCB. Phenotypic changes and gene expression profile in bovine embryos after exposure to perfluorohexane sulfonate (PFHxS) during oocyte maturation *in vitro (manuscript)*

Papers I-II are reproduced with the permission of the publishers.

The contribution of Ida Hallberg to the papers included in this thesis was as follows:

I. Took part in planning of the study and funding application, collected the samples, performed extraction, took part in instrumental analysis and performed data interpretation in collaboration with co-authors. Writing of original draft and finalized it together with co-authors.

II. Took part in planning of the study, performed the laboratory work, and performed the confocal imaging, establishing contact for method development of image analysis. Performed statistical analysis in collaboration with statistician. Writing of original draft and finalized it with input from co-authors.

III. Took part in planning of the study and funding application, performed the laboratory work, confocal imaging, statistics, took part in method development for image analysis, molecular laboratory work under supervision, took part in data preparation and performed data interpretation. Writing of manuscript with input from co-authors.

IV. Took part in planning of the study and funding application, laboratory work, confocal imaging, statistics, took part in method development for image analysis, molecular laboratory work under supervision, took part in data preparation and performed data interpretation. Writing of manuscript with input from co-authors.

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Abbreviations

ATM	Serine/threonine kinase
BMI	Body mass index
BPA	Bisphenol A
CL	Confidence level
CTNNB1	β-catenin
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DDT	dichloro-diphenyl-trichloroethane
DEG	Differentially expressed gene
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DRAQ5	Deep red Anthraquinone 5
EDC	Endocrine disrupting chemical
GC-MS	Gas chromatography mass spectrometry
gDNA	Genomic DNA
IETS	International embryo technology society
IPA	Ingenuity Pathway Analysis, Qiagen
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* embryo production

LC-HRMS	Liquid chromatography ultra-high resolution mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
MII	Second metaphase
NTS	Non-target screening
OPU	Ovum pick-up
PCB	Polychlorinated biphenyl
PFAA	Perfluoroalkyl acid
PFAS	Per- and polyfluoroalkyl substance
PFCA	Perfluoroalkyl carboxylate
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFSA	Perfluoroalkyl sulfonate
PPAR	Peroxisome proliferator-activated receptor
QC	Quality control
RELA	Nuclear Factor NF-Kappa-B P65 Subunit
RNA	Ribonucleic acid
STAT3	Signal transducer and activator of transcription 3
TGF	Transforming growth factor
TP53	Tumour protein 53

1. Introduction

Chemicals introduced by humans into the environment may be harmful to humans or wildlife. This has been known for decades. The debate started to shift from *whether* to *which* chemicals when Rachel Carson released her book *The Silent Spring*. The book described a dystopic future where the spring had gone silent following the extinction of insects and by extension birds caused by the widespread use of synthetic pesticides and herbicides (Carson, 1962). The discussion this initiated led to the ban of dichloro-diphenyl-trichloroethane (DDT) in the United States and Europe in the 1970s. The concept of endocrine disruption, however, was introduced decades later (Colborn & Clement, 1992) and was further popularized by the book *Our Stolen Future* by the same author (Colborn, 1996). The book proposed that chemical pollution was threatening the intelligence, fertility, and survival of the human race. Today endocrine disrupting chemicals (EDCs) remain important on the scientific agenda. The international programme on chemical safety defines EDCs as follows: "*an endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*", and this will be the definition used in this thesis (UNEP, 2012).

Birth rates in humans are declining, and globally they are predicted to be below replacement levels by 2100 (Vollset *et al.*, 2020). The main reasons are attributed to increased female education and use of contraceptives. Nonetheless, human infertility is also a common global disease defined as the inability to conceive within 12 months of actively trying to get pregnant. One out of six couples experience some form of infertility at least once, and the current prevalence of infertility is estimated to affect 8–12% of women aged 20–44 years worldwide (ESHRE, 2018). The causes of infertility can be either female (*e.g.,* diseases such as polycystic ovarian syndrome, tubal factor and endometriosis), male (*e.g.,* sperm defects or decreased sperm count), or a combination of both. However, after thorough investigation the

cause of infertility remains unknown in about 25–30% of couples (Evers, 2002; Hull *et al.*, 1985). Sperm count has been and still is used as a biological marker for male fecundity in many species, including humans. Decreased sperm count in humans is associated with decreased male fecundity (Bonde *et al.*, 1998) as well as genital malformations and cancers (Serrano *et al.*, 2013) but also predicts increased overall morbidity and mortality (Jensen *et al.*, 2009). Poor semen quality, undescended testes, testicular cancer, and hypospadias are all symptoms that fall under the term testicular dysgenesis syndrome. Already in the beginning of 21st century testicular dysgenesis syndrome was suspected to be increasing due to environmental influence (Skakkebæk *et al.*, 2001). Other factors such as lifestyle are important, but it is also likely that environmental chemicals are contributing to the decreased sperm count seen during the last 50 years (Levine *et al.*, 2017; Carlsen *et al.*, 1992). This research was the background to the recent release of the book *Count Down* by Shanna Swan aiming to further highlight the subject of EDCs and their negative effect on sperm count and human reproduction in general (Swan & Colino, 2021).

Research on female fertility has been lagging, partially due to the lack of a biological marker for fertility like that of semen quality in men. There are significant knowledge gaps on possible influences of EDCs on fecundity in both sexes, but studies on females are fewer (Green *et al.*, 2020). The concept of ovarian dysgenesis syndrome with evidence of *in utero* origin of gynaecological outcomes such as fecundity impairments, affected folliculogenesis, and steroid signalling has been introduced. This suggests, not surprisingly, similarities between effects on reproduction and subsequent fecundity in males and females (Buck Louis *et al.*, 2011). Over the last decades, increasing efforts have been made to understand the impact of EDCs on female reproduction. There is now evidence of a plausible effect on the reproductive system and negative outcomes of assisted reproductive technologies. Nonetheless, for several groups of chemicals the knowledge is still scarce (Björvang & Damdimopoulou, 2020).

The work within this thesis is dedicated to the female germ cell and especially the final stage of oocyte maturation. We have studied the exposure of the oocyte in the human ovary as well as in the laboratory where the bovine oocyte was studied more closely. In humans, we used a non-target screening approach to evaluate the complex set of chemicals and endogenous substances to which the oocyte is exposed. In comparison to commonly used target approaches, this method attempts to identify substances not driven by previously determined suspects or targets. As the next step, the work was focused on per- and polyfluoroalkyl substances (PFASs) and their potential

effects on final oocyte maturation. Even though they have been manufactured for more than 60 years, information about potential oocyte toxicity of legacy PFASs is scarce. During the course of the work for this thesis, there have been changes in legislation with the aim to reduce the risk of adverse health effects in humans (Schrenk *et al.*, 2020; UNEP, 2009). However, because of their properties making them so-called "everlasting chemicals" they will continue to expose humans and wildlife for decades to come.

1.1 Oocyte development in humans and cattle

In contrast to spermatozoa that are continuously formed during adulthood, the oocytes form during embryogenesis and the pool of oocytes present in the ovary at birth determines the oocytes available throughout adult life. There is no evidence for germline stem cells in adult human ovaries, which supports the dogma of a limited ovarian reserve (Wagner *et al.*, 2020). Oocytes develop within individual follicles in the ovarian cortex, which upon activation mature through the process of folliculogenesis. The oocyte is dependent on a series of modulations of organelles and molecular developments that occur during different stages of folliculogenesis (Sirard *et al.*, 2006; Hyttel *et al.*, 1997). This process takes several months in human and cattle (Britt, 2008; Gougeon, 1986). A schematic illustration of folliculogenesis and parallel oocyte maturation is presented in Figure 1.

1.1.1 Follicular development

In cattle and humans, the process of development within the ovarian follicle from resting stage through pre- and early antral stages takes months under the control of peri-ovarian steroids and gonadotropins (Britt, 2008; Gougeon, 1986). In mammals, such as cattle and humans, preovulatory follicles grow in waves (de Mello Bianchi *et al.*, 2010; Webb & Campbell, 2007; Baerwald *et al.*, 2003). This results in a heterogeneous pool of follicles in different stages of development at any given time in the adult ovary. If not recruited for ovulation, the follicles will undergo atresia. Atresia can occur in all antral follicles, but it is more common in later stages of development (Webb & Campbell, 2007; Gougeon, 1986). At the end of the luteal phase, in uniovulatory species (*i.e.,* humans and cattle) typically one dominant follicle

is recruited from the antral follicles (Britt, 2008; Lussier *et al.*, 1987; Gougeon, 1986).

Surprisingly, we know little about the physiological significance of the follicular fluid during folliculogenesis. However, all mammalian species show similar characteristics that supports a physiological importance. The follicular fluid is formed when the granulosa cells proliferate during follicular growth and eventually form a fluid-filled cavity (antrum) surrounding and nurturing the developing oocyte (Webb & Campbell, 2007). The follicular fluid consists of excretions from granulosa and theca cells as well as transudates from the circulation, and it contains growth hormones and sex-steroids that will affect subsequent oocyte quality (Revelli *et al.*, 2009). Additionally, the follicular fluid also contains exogenous substances, which will be further discussed in the following sections.

1.1.2 Final oocyte maturation

Oocyte nuclear maturation

During early folliculogenesis, the oocyte remains arrested before the first meiotic division. During the antral stages, the oocyte completes its growth and begins nuclear maturation where chromatin becomes more compact in the germinal vesicle in parallel with follicular development (Hyttel *et al.*, 1997). In response to follicle stimulating hormone and pre-ovulatory luteinizing hormone surge, the oocyte will complete maturation. At this point, the oocyte will resume the first round of meiosis and final nuclear maturation where germinal vesicle breakdown and extrusion of the first polar body will occur and the oocyte will arrest at the second metaphase (MII) until fertilisation (Sirard *et al.*, 1989).

Oocyte transcripts and epigenetic programming in germ cells

The maternal transcripts stored in the matured oocyte drive the initial developmental events in the embryo. The first cell divisions occur during transcriptional silencing after final maturation, emphasizing the effect of oocyte quality on subsequent development. The transcripts in the oocyte may predict further embryo development, and deviations during maturation may be correlated with disturbed development (Wrenzycki *et al.*, 2007). In adult tissues, each cell has tissue-specific imprints of the genome that define its morphology and function. During gametogenesis, this imprinting is mostly erased in order to achieve the totipotency needed by the germ cells. The

imprinting is achieved through so-called epigenetic modifications, including DNA methylation, histone modifications and non-coding RNA. These are heritable changes in gene function through mitotic and/or meitotic mechanisms that cannot be explained by changes in DNA sequence. In mammals, DNA methylation is the epigenetic modification most studied. DNA methylation occur by enzymatic addition of a functional methyl group to the DNA, primarily to the cytosine within cytosine-phosphate-guanine-dinucleotides (CpGs). The methylation alter the interaction between DNA and DNA binding proteins (*e.g.,* transcription factors). In general, in promoter-regions, these changes are associated with transcriptional suppression (Jacobs *et al.*, 2017).

Cytoplasmic changes in the oocyte during oocyte maturation

The oocyte undergoes a complex set of cytoplasmic changes in parallel with nuclear and follicular development. In the cow, the organelle distribution is rearranged, and an increasing number of ribosomes represent the high transcriptional activity during the final stage of maturation. Furthermore, the lipid droplets increase in both number and size, as well as elongated mitochondria, which increasingly are positioned alongside the lipid droplets (Hyttel *et al.*, 1989; Hyttel *et al.*, 1986). At final maturation, the organelles are arranged in a way that frees up the most peripheral space (Hyttel *et al.*, 1997).

Metabolism in the oocyte and communication between the oocyte and cumulus cells

Oocyte maturation relies on delicate dynamic fluctuations in metabolic activity, deviations from which are detrimental for development. For example, mitochondrial activity and reorganization as well as ATP levels differ between oocytes with better morphology compared to oocytes of inferior quality (Stojkovic *et al.*, 2001), and higher glycolytic rate in the oocyte is indicative of higher developmental competence (Krisher & Bavister, 1999). However, high concentrations of glucose during oocyte maturation *in vitro* have adverse effects on subsequent embryo development, and this is thought to be due to increased generation of reactive oxygen species and decreased intracellular glutathione pools (Cagnone *et al.*, 2012; Hashimoto *et al.*, 2000). The oocyte is dependent on the cumulus cells for nutrition and support during development (Sutton *et al.*, 2003). The communication between the oocyte and the cumulus cells occurs through gap

junctions and paracrine signalling where also nutrients such as glucose metabolites (pyruvate), amino acids, and nucleotides may pass (Sutton *et al.*, 2003). This is important because the mammalian oocyte has limited capacity to utilize glucose and therefore relies on substrates such as pyruvate and lactate (Downs & Utecht, 1999; Biggers *et al.*, 1967). Pyruvate is provided to the oocyte from glycolysis in the cumulus cells (Richani *et al.*, 2021). During oocyte maturation, metabolism in the oocyte increases and the glucose consumption is doubled compared to the immature oocyte (Collado-Fernandez *et al.*, 2012). Amino acid metabolism supports oocyte maturation and serves as a substrate for the synthesis of proteins and nucleotides, but are not sufficient as a sole source of energy to drive final oocyte maturation (Downs & Hudson, 2000).

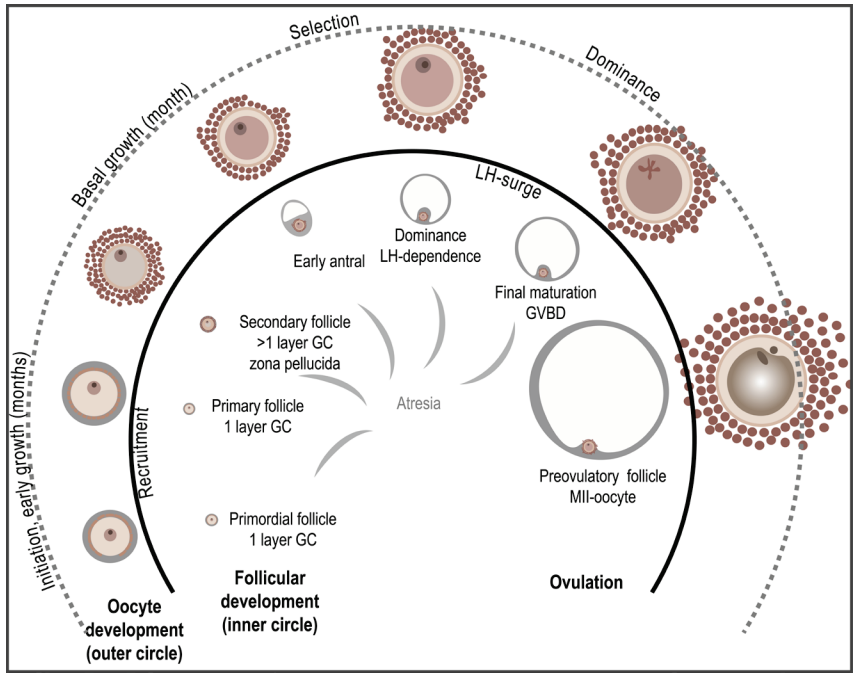


Figure 1. Schematic illustration of follicular (inner circle) and oocyte (outer circle) development in humans and cattle, adapted from Gougeon, 1986 and Hyttel 1997. GC: Granulosa cells, LH: Luteinizing hormone, GVBD: Germinal vesicle breakdown. Illustration is not to scale.

1.2 Early embryonic development in humans and cattle

Most mammals ovulate oocytes that are surrounded by cumulus cells (cumulus-oocyte-complexes) and are capable of fertilisation and subsequent embryonic development. After ovulation, fertilisation occurs in the oviduct where the sperm await ready for fertilisation after the sperm undergo capacitation. Sperm bind to the outer layer of the zona pellucida and undergo the acrosome reaction. At the same time that the first sperm bind to the oocyte cell membrane, the contents of the cortical granules inside the oocyte are discharged prohibiting more than one sperm from penetrating. The male pronuclei fuse with the female forming a zygote, and this event marks the start of the transition from oocyte and sperm to embryo. The first cleavage divisions, which occur within the next days of development, follow a strict scheme of mitoses and cytokinesis (Wong *et al.*, 2010). Deviations during this time have consequence for the developmental potential (Lundin *et al.*, 2001). At this point, the embryo is still dependent on transcripts/active protein synthesis from the oocyte, but at the 4–8 cell stage in humans and at the 8–16 cell stage in cattle embryo genome activation occurs after which the embryo as its own individual will drive further development (Barnes & First, 1991; Braude *et al.*, 1988).

Following the first cell divisions, compaction and cavitation of the early embryo occur. The compaction is referred to as the tight intercellular junctions that develop in the late morula stage, and the cavitation is the formation of the blastocoel where blastomeres will differentiate either into part of the inner cell mass that will constitute the offspring or into the trophectoderm that will eventually develop into the foetal annexes. Blastocyst formation occurs at day 5–6 in humans and day 6–8 in bovines (Hardy *et al.*, 1989; Betteridge & Fléchon, 1988). As for the first cleavage divisions, the timing of events during blastocyst development seems to be important for developmental potential, and even the slightest deviations in timing are associated with implantation failure (Harada *et al.*, 2020; Cruz *et al.*, 2012).

1.2.1 Early embryonic metabolism

Prior to compaction, the embryo has a modest level of metabolism as reflected by low levels of oxidative metabolism. As development progresses and the embryo approaches highly energy-consuming developmental stages such as compaction and blastocoel formation, the demand for energy

increases (Gardner, 1998), leading to increased oxygen as well as energy requirement as the embryo approaches blastocyst stage (Leese *et al.*, 1993). The mammalian blastocyst can metabolize glucose both through oxidative phosphorylation and through glycolysis (Absalon-Medina *et al.*, 2014; Sturmey & Leese, 2003). The oviduct contains a significant amount of free amino acids in relevant concentrations, and the addition of amino acids during embryonic development *in vitro* enhances development in several aspects (Watson *et al.*, 2000; Takahashi & First, 1992). In bovines, the lipid content within the embryo increases during the compaction of the morula stage and decreases when reaching the blastocyst stage (Sudano *et al.*, 2016), which probably reflects the higher energy demand during the preparation for the embryonic transition from morula to blastocyst.

1.2.2 Gene programming in the early embryo

During embryo genome activation, the embryo will initiate individual transcripts, first in parallel with maternal transcripts, and then as the maternal transcripts degrade full embryonic expression takes over (Telford *et al.*, 1990). In the absence of activation of the embryonic genome, embryonic development will cease (Meirelles *et al.*, 2004). After fertilisation, when the genome in both oocyte and sperm is abundant, genome-wide loss of DNA methylation occurs. This mark the second re-programming (the first happening during oogenesis, see section above). The gene imprinting of the zygote is reset before being re-establishing the gene imprinting according to the sex of the embryo (Jacobs *et al.*, 2017). The changes in methylation are dynamic during the early embryo development. There are similarities between species, but also species, sex and lineage specifics (Reik *et al.*, 2001). In humans and bovines, the methylation has only started to be *de novo* methylated at blastocyst stage (Guo *et al.*, 2014; Dobbs *et al.*, 2013), and methylation increase to post-implantation stages.

The plasticity of the epigenome during early development render it susceptible to influences by the environment. In case of negative impacts, this may contribute to an increased risk for disease, due to the epigenetic changes during this period (Baccarelli & Bollati, 2009). In fact, developmental chemical exposure has previously been linked to changes in the epigenetic landscape (Jacobs *et al.*, 2017).

1.3 Human exposure to environmental chemicals

As evidence of adverse outcomes from chemical exposure in humans and animals has increased, compounds previously used in the market have been regulated (Sackmann *et al.*, 2018). However, new compounds are continuously being developed to replace predecessors with known toxic profiles, unfortunately at a speed of production that far exceeds the potential for chemical health risk assessment (so-called "regrettable substitution"). Currently, there are more than 350,000 chemicals and mixtures in global commerce, of which over 50,000 compounds remain publicly unknown due to business confidentiality (Wang *et al.*, 2020). Thus, humans and animals are exposed to a large amount of compounds, which is partly dependent on local environmental pollution as well as individual lifestyle factors and diets. Humans are exposed to chemicals contaminating the environment through ingestion of food and water, inhalation of polluted air, and dermal contact with contaminated soil or other materials (Poothong *et al.*, 2020; Giovanoulis *et al.*, 2018; McKinlay *et al.*, 2008).

Synthetic chemicals found in human tissues

The average population is exposed to a wide range of chemical classes suspected or proven to be EDCs with detectable concentrations in human serum. Many of them are so-called persistent organic pollutants and are regulated in the European market. However, due to their persistent and accumulative properties, they are still regularly reported in human biomonitoring with ubiquitous exposure. *Industrial chemicals* such as polychlorinated biphenyls (PCBs), dioxins, and flame retardants (*e.g.* polybrominated diphenyl ethers and organophosphate flame retardants) are found in human serum (Zong *et al.*, 2015; Sjödin *et al.*, 2008). Another group of industrial chemicals is the per- and polyflouroalkyl substances (PFAS), which are discussed further below. Furthermore, *pesticides* such as DDT (or the metabolite DDE) are still found in human serum even though banned in several parts of the world for many decades (Zong *et al.*, 2015). DDT is still the most effective pesticide against the malaria-causing mosquitoes, and therefore it is still recommended for mosquito control in risk areas for malaria. *Plasticizers* such as phthalates are used in the manufacturing of building materials and consumer products. *Bisphenols* (*e.g.,* bisphenol A, BPA) are used in the manufacturing of plastic articles such as boxes and bottles. In contrast to persistent organic pollutants, bisphenols and phtalates

are not persistent nor do they accumulate in the food chain. However, their use is so widespread that they are commonly detected in humans (Svensson *et al.*, 2021). Another group of chemicals found in human serum today are *parabens*, which have been used for almost a century as a preservative in consumer products such as cosmetics (Ward *et al.*, 2020).

In addition to being detected in human serum and urine, anthropogenic chemicals such as BPA, PCBs, DDT/DDE, PFASs, and polybrominated diphenyl ethers have been found in follicular fluid (Zhu *et al.*, 2015; Petro *et al.*, 2014; Johnson *et al.*, 2012; Petro *et al.*, 2012; Jirsova *et al.*, 2010; Meeker *et al.*, 2009; Yumiko Ikezuki *et al.*, 2002; Trapp *et al.*, 1984). This indicate direct exposure to the maturing oocyte.

1.3.1 Measuring and assessing exposure

Traditionally, highly specific and targeted analytical approaches that offer high sensitivity and selectivity are applied to characterize human exposure to *known substances*. As a result, numerous chemicals and their transformation products are overlooked. To fill this knowledge gap, suspect and non-target screening (NTS) approaches have emerged over the last decade as promising techniques for detecting many hundreds of chemicals – including *unknowns* – simultaneously in one sample. Usually the method of choice to detect environmental compounds is gas/liquid chromatography coupled to mass spectrometry (GC/LC-MS) depending on the chemical properties of the target compounds. The chemical coverage of GC-MS is limited to volatile compounds, whereas LC-MS is able to identify very polar compounds and transformation products. However, LC-MS does not permit the detection of some important compounds such as organochlorine pesticides and PCBs (Hernández *et al.*, 2004). Analytical methods using ultra-high liquid chromatography ultra-high resolution (Orbitrap) mass spectrometry (LC-HRMS) have been available on the market since 2005 and offer high resolving power in combination with high mass accuracy and sensitivity (Krauss *et al.*, 2010). These methods generate mass-spectrometric information on all ionizable, polar compounds present in a sample. This results in many hundreds to thousands of features. Therefore, prioritisation strategies, which reduce the quantity of feature data (*i.e.,* detected compounds), are critical (Hollender *et al.*, 2017). NTS has previously been applied to human serum and follicular fluid with different strategies to cope with this challenge. For example, human serum samples have been

investigated with a focus on increased exposure over time, where filtering of the data was based on time trend ratios as well as database searches for suspects (Plassmann *et al.*, 2018), while case/control studies have been employed for elucidating chemicals specifically associated with occupational exposure (Rotander *et al.*, 2015). Recently, NTS was used as a tool for screening ovarian follicular fluid and serum for emerging as well as legacy PFASs (Kang *et al.*, 2020).

1.4 Per- and polyfluoroalkyl substances (PFASs)

PFASs are a large group of man-made chemicals consisting of over 4700 compounds (OECD, 2018). Perfluoroalkyl substances are aliphatic compounds in which all hydrogen atoms have been replaced by fluoride atoms in *all* carbons of the backbone (*i.e.,* C_nF_{2n+1}-), except for those hydrogen atoms present in any functional groups. Polyfluoroalkyl substances differ from the above mentioned by *at least one* but not *all* carbons having all hydrogens substituted with fluorine atoms (Buck *et al.*, 2011). Common for all PFASs is the C-F bond, which is an extremely stable bond making the compounds resistant to degradation and hence persistent. The perfluoroalkyl acids (PFAAs) may be further divided based on their functional group, namely perfluoroalkyl carboxylic acids/carboxylates (PFCAs, *e.g.,* perfluorononanoic acid, PFNA) and perfluoroalkyl sulphonic acids/sulfonates (PFSAs, *e.g.* perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS)). Based on their carbon chain length they are considered "long-chained" if ≥6 carbons in PFSAs and ≥7 carbons in PFCAs, the difference being due to higher bio-accumulating properties of PFSAs compared to PFCAs. The shorter-chained PFASs usually substitute for longer-chained PFASs in manufacturing for the same reason of bio-accumulative assets.

Because of their water, stain, and grease-repellent properties, PFASs have been used in a range of products, *e.g.* protective clothing, non-stick surfaces, and fire-fighting foam (KEMI, 2015). Originally, the company 3M started manufacturing perfluorooctanoic acid (PFOA) in 1947, which was included in the production of Teflon by DuPont. Shortly after, the grease and water repellent properties of PFOS were accidentally discovered and Scotchguard was subsequently developed. The invention of aqueous film-forming foam followed, which is a fire-fighting foam that contained both PFOS and PFOA.

From industrial manufacturing and consumer products, PFASs leaked into the environment and hence contaminated water and soil. This has led to PFASs being detected worldwide in environmental as well as in wildlife samples, even in remote areas such as the Arctic (Giesy & Kannan, 2001).

1.4.1 Human exposure to PFASs

The main route of human exposure to PFASs is through ingestion of contaminated food or drinking water, but inhalation of dust and dermal contact also contributes to exposure (DeLuca *et al.*, 2021; Poothong *et al.*, 2020; Vestergren *et al.*, 2012). PFASs were first identified in human serum in 2001, when PFOS, PFOA, and PFHxS were identified from commercially available serum samples from biological supply companies (Hansen *et al.*, 2001). Even though local contamination had been detected in fluorochemical industry workers in 1980 (Ubel *et al.*, 1980), this finding of PFASs in non-industrially exposed humans was new. It was further concluded that the fluoride contamination of serum found previously (Taves, 1968) was probably due to PFAS contamination even though not specified at the time. Nowadays, PFAS contamination of human serum shows worldwide distribution (Kannan *et al.*, 2004), and there have been numerous publications reporting PFASs in most samples from the general public in many parts of the world.

The most commonly detected PFASs in humans

During the last seventy years, PFOS and PFOA have been the most manufactured. Because potential health effects have been associated with exposure, PFOS and PFOA have been legislated, and PFOS was included in the Stockholm Convention of persistent organic pollutants in 2009 (UNEP, 2009). This has led to a shift towards other PFASs being used in production, mostly to shorter-chained alternatives. Since the enactment of the legislation, there has been a decreasing trend of concentrations of these PFASs in human serum (Glynn *et al.*, 2012; Kato *et al.*, 2011).

The level of contamination varies significantly between compounds and populations, but PFOS and PFOA have usually been found at the highest concentrations in humans (Kato *et al.*, 2011; Calafat *et al.*, 2007). This is not always the case for species with a diet including much fish. Levels in mink and seal livers in Scandinavia have higher levels of PFNA compared to PFOA (Persson *et al.*, 2013; Kratzer *et al.*, 2011). In communities where

drinking water historically has been contaminated with fire-fighting foam, this is usually associated with higher-than-average exposure also to PFASs such as PFHxS (Gyllenhammar *et al.*, 2019). Local contamination of water may considerably increase exposure for affected individuals or local human populations. For example, a heavily contaminated water depot in Kallinge in Ronneby in Sweden was discovered in 2013 and people living in the area have concentrations about 1000 times higher than "normal background contamination" in their serum, especially of PFOS and PFHxS (Li *et al.*, 2018). There have also been several reports of higher PFAS concentrations after occupational exposure (such as fire fighters or industrial workers) compared to other populations (Fu *et al.*, 2016; Rotander *et al.*, 2015).

Toxicokinetics of PFASs

PFASs are well absorbed after oral intake, they are not metabolized, and they undergo extensive uptake from the enterohepatic circulation. PFASs tend to bind to proteins and subsequently accumulate bound to albumin in blood, but they also accumulate in the liver. PFASs are found in other tissues as well, passing the placenta, thus exposing the growing foetus, and are excreted in milk, thus exposing the nursing child (Mamsen *et al.*, 2017; Kärrman *et al.*, 2007). Not so surprisingly, PFASs are also found in follicular fluid, which suggests direct exposure of the maturing oocyte in the ovarian follicle in similar (or slightly lower) concentrations to those measured in serum (Table 1) (Kang *et al.*, 2020; Kim *et al.*, 2020; Heffernan *et al.*, 2018; McCoy *et al.*, 2017; Petro *et al.*, 2014).

In humans, the half-life of the most common PFASs are in the range of years (Li *et al.*, 2018). The half-life in women compared to men is slightly lower. This is partially due to PFASs being accumulated in blood and hence increased elimination during menstruation. There are also other sex differences in the toxicokinetic properties of PFASs (Wong *et al.*, 2014).

Future exposure

In recent years, there has been a significant movement afoot to ban PFASs as a class of chemicals. Sweden participated in an intention sent to the European Chemicals Agency that is expected to result in a restriction proposal (ECHA, 2021). Because environmental sources (direct and indirect) account for the main route of exposure to PFASs in humans (DeLuca *et al.*, 2021), the exposure will continue as long as environmental contamination persists. Even if new contamination is halted, PFASs will persist in the

environment for decades to come. There are methods being developed aiming to effectively remove PFASs from ground (Xu *et al.*, 2021) and drinking water (Franke *et al.*, 2019). However, more research is needed in order to develop measures to handle PFAS contamination.

Table 1. Concentrations of perfluoroalkyl substances (PFASs) investigated in this thesis in ovarian follicular fluid of women undergoing ovum pick-up for assisted reproductive technologies

PFAS	n	Mean, ng/mL	DF[1] (%)	Sampling year	Reference
PFOS	38	7.5 (0.1-30.4)	100	2008-2009	Petro et al., 2014
	97	4.8 (0.7-22.4)	98	2006-2011	Kim et al., 2020
	31	5.3 (±0.4)		2013-2014	McCoy et al., 2017
	58	2.0 (0.6-4.3)	100	2015	Heffernan et al., 2018
	28	4.8 (0.9-181)	100	2018-2019	Kang et al., 2020
PFNA	38	0.4 (0.2-2.1)	100	2008-2009	Petro et al., 2014
	97	0.5 (0.1-2.0)	97	2006-2011	Kim et al., 2020
	31	0.6 (±0.1)		2013-2014	McCoy et al., 2017
	58	0.4 (0.1-1.4)	100	2015	Heffernan et al., 2018
	28	0.5 (0.1-3.6)	100	2018-2019	Kang et al., 2020
PFHxS	38	0.3 (0.1-1.4)	100	2008-2009	Petro et al., 2014
	97	1.7 (0.2-21.3)	98	2006-2011	Kim et al., 2020
	31	1.8 (±0.3)		2013-2014	McCoy et al., 2017
	58	0.9 (0.1-9.1)	100	2015	Heffernan et al., 2018
	28	0.5 (0.2-1.6)	100	2018-2019	Kang et al., 2020

[1]Reported detection frequency

1.4.2 PFAS toxicity

Mechanisms of toxicity

The long fatty acid chains of PFASs provide hydrophobic chemical properties and are important in the molecular action of imitating endogenous fatty acids. The nuclear receptor family of the peroxisome proliferator-activated receptors (PPARs) seem to be a key player in PFAS toxicity. PFASs mainly activate PPARα (Behr *et al.*, 2020; Takacs & Abbott, 2007). Natural ligands to PPARs are unsaturated fatty acids, but also leukotrienes (PPARα) and prostaglandins (PPARɤ) (Grygiel-Gorniak, 2014). PPARs are nuclear receptors. When bound to a ligand they are translocated to the nucleus and bind to the retinoid X receptor, which together with co-

inhibitors/co-activators regulates gene expression. All PPARs are active in lipid and glucose metabolism. Nevertheless, their functions somewhat differ. PPARα is related to lipid catabolism by stimulating fatty acid oxidation, and it also controls some inflammatory processes. PPARγ, on the other hand, prevents hyperglycaemia by regulating glucose homeostasis and lipid storage (Grygiel-Gorniak, 2014). PPARß/δ is the least investigated, but it also promotes fatty acid oxidation.

PFAS toxicity is not solely attributed to the activation of PPARs as demonstrated by the toxicity of several PFASs in PPAR-null mice (Rosen *et al.*, 2017; Rosen *et al.*, 2010; Rosen *et al.*, 2009). The oestrogen receptor has also been demonstrated to be a target of PFASs (Li *et al.*, 2020; Kjeldsen & Bonefeld-Jorgensen, 2013), and PFASs might also have an antagonistic effect on the androgen receptor (Kjeldsen & Bonefeld-Jorgensen, 2013) and disrupt the thyroid system (Coperchini *et al.*, 2020).

Evidence of adverse health effects related to PFAS exposure

During the past 40 years there have been more than 1000 studies investigating PFAS toxicity, the main body of research being on PFOA and PFOS. The hallmark toxicity of PFASs includes increased liver weight in experimental animals (Cui *et al.*, 2009; Curran *et al.*, 2008) and disturbances in lipid metabolism, including hepatocellular steatosis and hepatotoxic effects (Curran *et al.*, 2008). There is also evidence of decreased circulating thyroid hormones after exposure to PFASs in experimental animal models (Butenhoff *et al.*, 2002). Both PFOS and PFOA have been shown to alter the immune response and by extension cause a reduced response to vaccines (DeWitt *et al.*, 2012; Zheng *et al.*, 2011; Zheng *et al.*, 2009). PFOS has also been associated with reduced resistance to infections (Qazi *et al.*, 2009; Zheng *et al.*, 2009). There are also associations with exposure to PFOS and PFOA to hepatic adenomas in rats suggesting tumour-promoting properties in rodents (Butenhoff *et al.*, 2012). Developmental toxicity is reported in experimental animals and reviewed by Lau *et al.* (2004) and subsequently updated (Lau *et al.*, 2007; Lau *et al.*, 2004). Such effects include increased foetal and/or neonatal mortality and reduced birth weight and/or postnatal growth (Lee *et al.*, 2015; Rogers *et al.*, 2014; Wan *et al.*, 2014; Xia *et al.*, 2011).

In humans, there is an increasing body of evidence suggesting that exposure to PFASs is negatively associated with health outcomes in the general public and in certain exposed groups. There have been studies

associating exposure with reduced immune response to vaccination (Grandjean *et al.*, 2012). Furthermore, even though there is not enough evidence for associations between PFAS exposure and metabolic outcomes such as diabetes and fasting glucose levels (MacNeil *et al.*, 2009), associations have been shown with serum cholesterol in the general population (Nelson *et al.*, 2010) and in a population living near a chemical plant (Frisbee *et al.*, 2010; Steenland *et al.*, 2009). PFOA have also been associated with increased risk of adulthood overweight in women after *in utero* exposure (Halldorsson *et al.*, 2012). The Swedish Environmental, Longitudinal, Mother and child, Asthma and allergy (SELMA) cohort reported last year an association between exposure to PFASs and decreased birth weight in girls but not in boys after maternal exposure during pregnancy (Wikström *et al.*, 2020). This finding was supported by previous studies on PFOS and PFOA (Bach *et al.*, 2015a).

PFAS toxicity on the ovary

The evidence of adverse health effects related to reproductive outcomes in humans is insufficient to draw conclusions (Schrenk *et al.*, 2020). PFASs have been associated with outcomes related to ovarian function, as recently reviewed (Ding *et al.*, 2020). Human cohorts have shown associations with PFASs (specifically PFOS, PFHxS, and PFOA) and longer time to pregnancy and with effects on the menstrual cycle (Zhou *et al.*, 2017; Velez *et al.*, 2015; Lopez-Espinosa *et al.*, 2011; Fei *et al.*, 2009), and recently PFOA was associated with sporadic first trimester miscarriages (Wikstrom *et al.*, 2021). However, there are also inconsistencies and studies reporting no association (Bach *et al.*, 2015b; Vestergaard *et al.*, 2012) or only associations in certain subpopulations (Whitworth *et al.*, 2012).

In rodent models, ovarian cyclicity and follicles seem to be negatively affected by PFOS exposure. Both increased oestrus length as well as decreased pre/ovulatory and antral follicles have been observed (Wang *et al.*, 2018; Feng *et al.*, 2015), which is supported by *in vitro* studies on oocyte maturation in porcine and murine models (Jiao *et al.*, 2021; Martinez-Quezada *et al.*, 2021; Wei *et al.*, 2021; Lopez-Arellano *et al.*, 2019; Dominguez *et al.*, 2016).

1.5 Bovine *in vitro* embryo production (IVP)

Since the birth of the first baby after *in vitro* fertilization (IVF) in 1978, the techniques have continuously evolved. The first IVF calves were born in the early 1980s (Brackett *et al.*, 1982), and not many years later *in vitro* matured oocytes were used to generate live offspring in cattle. Today, reproductive biotechnologies are used routinely in cattle for the benefit of genetic improvement and decreased risks associated with animal contacts. In 2019, there were almost 800,000 transfers of *in vitro*-derived embryos in the global cattle industry, with the majority transferred in South and North America (Viana, 2020). Even though some aspects differ, many of the principles used in human assisted reproduction are adapted from techniques used in cattle and vice versa (Ombelet & Van Robays, 2015).

1.5.1 Oocyte origin for bovine IVP

Bovine oocytes may be retrieved from live animals following ovum pick-up (OPU) after stimulation similar to the techniques used in human assisted reproduction. Followed by *in vitro* maturation (IVM), it is also possible to retrieve oocytes from non-stimulated heifers and cows to generate blastocysts at acceptable rates. In this way, the process of IVM is simulated by removing small antral follicles (3–8 mm) from the ovary for immediate final maturation, thus bypassing the period of follicular dominance and compressing the process to a single day (Hyttel *et al.*, 1997).

1.5.2 Differences between *in vitro* and *in vivo* embryo development

Even though blastocysts develop at decent rates *in vitro*, compared to their *in vivo*-derived counterparts they are generally less viable (Nair, 2008). There are also significant differences in gene expression between pre-implantation embryos developed *in vivo* or *in vitro* (Niemann & Wrenzycki, 2000; Wrenzycki *et al.*, 1996). These changes might correspond to harmful embryo development under laboratory conditions, but some changes might be due to adaptation to the environment rather than damage (Cagnone & Sirard, 2016).

1.5.3 Associations between IVP and offspring health

In the first decades of IVP use in cattle, the increased incidence of large offspring limited the use of the technique. It was long debated whether the

cause of the syndrome (known as 'large offspring syndrome') was caused by the *in vitro* environment, the embryo transfer procedure, or the uterine environment (Young *et al.*, 1998). Indeed it was later established that cellular and molecular deviations caused by the *in vitro* production of embryos in the culture media (and in particular high serum and bovine serum albumin (BSA) content) were associated with the outcome (Lazzari *et al.*, 2002). Events such as negative outcomes and decreased live birth rates after IVP have led to the continuous development of systems for culturing oocytes and pre-implantation embryos.

Today, negative associations after IVP are much less common. In humans, there have been no robust associations seen between IVF and outcomes such as cancers, asthma, or allergies (Hart & Norman, 2013). Nevertheless, there are indications of associations between IVF treatment and cardiovascular and metabolic alterations in adult offspring (Pinborg, 2019; Hart & Norman, 2013).

1.5.4 Evaluation of the bovine blastocyst *in vitro*

To evaluate the developmental competence of the *in vitro*-produced embryo, the best way is to transfer the embryo into a recipient and follow its development into a healthy offspring. However, for several practical and ethical reasons this is not always feasible in a research setting. Therefore, independent of whether the purpose of the embryo is embryo transfer or research, we rely on robust evaluations to predict further developmental potential. As mentioned previously (see section *1.2*), the timing of events during early embryonic development predicts further growth and can be evaluated using a light microscope. The first cleavages can be evaluated at 44 h after fertilization (Betteridge & Fléchon, 1988), and blastocysts can be evaluated according to standardized protocols for bovine IVP (IETS, 2010). The assessment of the blastocyst stage is usually conducted at day seven and day eight of development in the bovine system. At this point blastocysts have reached stages from early blastocysts (the blastocoel and differentiated cell populations are visible), to expanded blastocysts (where the zona pellucida is slightly thinner due to blastocyst expansion), and eventually to hatched blastocysts where the zona pellucida has ruptured (Betteridge & Fléchon, 1988). Further morphological evaluation at the blastocyst stage is also possible and is used in both bovines and humans to select the top-quality embryos for transfer in order to improve IVF outcome (Hardarson *et al.*,

2012; IETS, 2010; Gardner *et al.*, 2000). The quality of the embryo as a prognostic marker for pregnancy is used already in day-two embryos in humans, where blastomere count, proportion of mononucleated blastomeres, and degree of fragmentation are predictive for live births after IVF (Rhenman *et al.*, 2015; Holte *et al.*, 2007). Other non-invasive techniques used to evaluate embryo quality are metabolic assessment in the media used for IVP and detection of specific biological markers produced by the embryo. However, if not using single embryo cultures these evaluations are based on the group of embryos cultured together.

Besides non-invasive evaluation, embryos not intended for transfer to a recipient may be subjected to more invasive techniques evaluating embryo morphology and metabolism. These include but are not limited to fluorescent staining to detect blastomere count, lipid and mitochondria distribution, actin cytoskeleton structure, and apoptotic cells, which allow further evaluation of embryo characteristics (Laskowski *et al.*, 2017; Gonzalez & Sjunnesson, 2013). Embryo gene expression may be associated with blastocyst viability (Wrenzycki *et al.*, 2007), and there are microarray platforms, and more recently RNA sequencing techniques, that have been developed to study the early embryo transcriptome and methylome profile. These methods further contribute to our understanding of embryos' interactions with their microenvironment with the benefit of studying pathways rather than individual genes (Saadi *et al.*, 2014; Robert *et al.*, 2011).

1.5.5 Ethical considerations and bovine IVP as a toxicity model

Studies on rodents have provided the bedrock of our knowledge of preimplantation development as well as the effects of EDCs during early development. However, there are ethical considerations in using experimental animals. Russel and Burch set the foundation for today's research using animal experiments in 1959 (Russell & Burch, 1959). In their work *The principles for humane experimental technique*, they introduced the terms *Replace, Reduce* and *Refine* to minimize the potential for animal pain and distress in scientific studies using experimental animals. The principle is known today by the acronym 3R and is implemented as a framework to develop alternative approaches to animal use in science.

Bovine IVP offers an environment completely without the need to use experimental animals. This applies if the oocytes and sperm are retrieved from non-experimental animal sources (oocytes may be retrieved from

slaughterhouse material), and the embryos are not transferred to a recipient following IVP. When using slaughterhouse material, it is more difficult to consider other characteristics (such as donor breed) that also affect oocyte developmental competence *in vitro* (Abraham *et al.*, 2012). This variation may be counteracted to some extent by an increased number of oocytes used in the *in vitro* setting and a random distribution between experimental groups.

The similarities between human and bovine preimplantation embryo development as well as follicle dynamics provide an attractive model to investigate mechanisms during subsequent events (Adams *et al.*, 2012). Oocyte maturation is a fine-tuned process where there are considerable similarities between mammalian species. However, there are also complex differences, where the bovines show more similarities to humans compared to commonly used rodents (Santos *et al.*, 2014; Ménézo & Hérubel, 2002). Furthermore, the timing of important aspects during early embryo development differ between species where murine embryos have a significantly shorter timeline compared to larger mammals. Murine embryos initiate embryo genome activation already at the two-cell stage (Santos *et al.*, 2014). This implies that there is a decreased demand on the maturing oocyte to sustain development in the mouse (Ménézo & Hérubel, 2002). However, there are also aspects during early preimplantation development where the murine model is more similar to humans. For example the oocyte lipid content is low in both humans and mice with presumed similarities in early embryo metabolism between the species compared to bovines and porcines (Bradley & Swann, 2019).

For exposure studies, the IVP system allows studies of certain windows of exposure, such as oocyte maturation or the early embryo development until blastocyst stage or a combination of both (see specific developmental characteristics in sections *1.1* and *1.2*). Exposure during IVM has been used to study the effect of PCB and resulted in decreased proportion matured oocytes and cleaved embryos (Krogenaes *et al.*, 1998). Cadmium addition during the morula and from the morula to blastocyst stages significantly increased the proportion apoptotic cells in bovine blastocysts as well as altered gene expression related to apoptosis (Jorssen *et al.*, 2015).

2. Aim of the study

The overall aim of this thesis was to investigate chemicals commonly present in follicular fluid and more specifically the effect of PFASs (PFNA, PFOS, and PFHxS) on oocyte maturation using a bovine *in vitro* model.

The specific objectives were to:

- Characterize human ovarian follicular fluid from women undergoing OPU for *in vitro* fertilization using a suspect and non-targeted screening approach to detect environmental pollutants with a special focus on PFASs (Paper I)

- Investigate the effect of selected PFASs detected in human ovarian follicular fluid on developmental competence after exposure during bovine oocyte maturation *in vitro* (Papers II-IV)

    - To describe the phenotypic changes after PFAS exposure during oocyte maturation on blastocyst morphology, cell count, and lipid distribution in day-eight blastocysts (Papers II-IV)
    - To investigate gene expression and DNA methylation pattern changes in blastocysts after selected PFAS exposure during oocyte maturation *in vitro* (Papers III-IV)

3. Material and methods

The material and methods used in the studies are described in detail in the included papers and related supporting information. This section provides an overview of the methods used in the study as well as comparative aspects between the different studies.

3.1 Experimental design

Follicular fluid and serum from women undergoing OPU for IVF were collected and subjected to ultrahigh performance liquid chromatography-high resolution (Orbitrap) mass spectrometry for NTS in an attempt to assess the exposure of the oocyte *in vivo* (I). The method generates data on a wide range of water-soluble semivolatile or nonvolatile organic pollutants. Prioritization of the data aimed to characterize human ovarian follicular fluid based on features with ubiquitous exposure in the cohort, accumulating in follicular fluid, or associated with embryo quality. Special effort was put into identifying exposure to PFASs in humans using an in-house database for suspect screening of the follicular fluid and serum.

For the following studies (II-IV), we wanted to further evaluate the effect of PFAS exposure during IVM. Bovine cumulus oocyte complexes were exposed to selected PFASs (PFNA (II), PFOS (III), and PFHxS (IV)) during 22 h of IVM and subsequently cultured until the blastocyst stage. Developmental competence was followed by recording the proportion of cleaved embryos and embryos cleaved beyond the two-cell stage 44 h after fertilization as well as the blastocyst rate on day seven and day eight of culture. Morphological assessments of the blastocysts (stage, grade) were also performed at day seven and day eight. At day eight, blastocysts were either fixed and stained to detect cells and neutral lipids using confocal

imaging (II-IV) or were later individually snap-frozen (III-IV) and pooled for gene expression and DNA methylation studies using microarray platforms. An illustration of the experimental design of all studies within the thesis is presented in Figure 2.

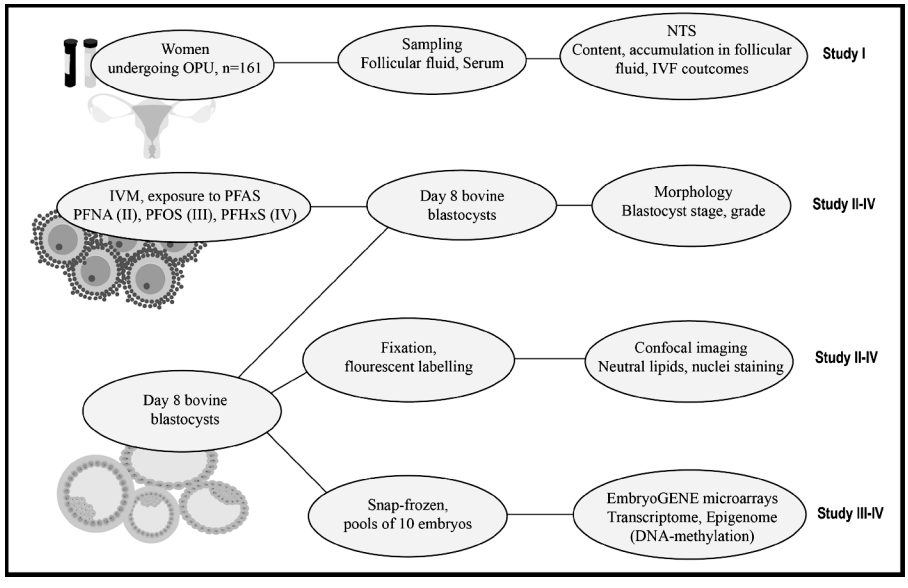


Figure 2 Study design, overview of studies I-IV.

3.2 NTS of human ovarian follicular fluid and serum (I)

3.2.1 Recruitment of patients

Ethical permission was granted from the Swedish Ethical Review Authority (Dnr 2015/798-31/2, 2016/360-32 and 2016/1523-32). During April–June 2016, patients at the private Carl von Linné fertility clinic in Uppsala, Sweden, undergoing OPU for assisted reproductive technologies were asked to participate in the study. Patients received written and oral information about the study by a nurse that was not connected to the project and were given time to consider. Patients were informed about the aim and scope of the project. They were informed that participation was voluntary and they could withdraw their consent at any time and this would not affect their following treatment. They would not receive any compensation and would not be able to trace their results upon participating, but participating would

not contribute to any risks either. The samples collected were from a catheter placed for the IVF-treatment (blood-sample) and fluid that was normally discarded after egg collection (follicular fluid). The samples were anonymized. The patients received contact information and they could ask more questions to the researcher collecting the samples or by phone. Those who agreed to participate signed a written consent form in accordance to the Declaration of Helsinki. Samples were not collected without a signed consent. Data was handled in compliance with relevant laws and institutional guidelines (the Swedish data protection law, PUL, and the general data protection regulation, GDPR), and the biological samples were registered at Uppsala Biobank (IVO 627) following the Swedish law on biobanking in health care. Of the patients asked, 97% accepted, resulting in 185 patients enrolled.

To narrow confounding factors, recent smokers and patients with body mass index (BMI) > 30 were excluded (n = 23). Obstetrical and treatment history (indication for IVF treatment, result of previous IVF treatments if any, and other medical history) were collected. Data on sperm analyses as well as cycle characteristics from routine infertility work-up prior to the treatment were recorded.

3.2.2 Sample processing and instrumental analysis

Sampling

Blood was collected before OPU and centrifuged at 1400 × *g*. Serum was aliquoted and stored at –80°C until further analyses. For NTS, more than 1.5 mL of serum was needed resulting in 116 serum samples being available. Oocytes were aspirated in follicular fluid through transvaginal ultrasound-guided follicular puncture, and oocytes were collected for subsequent IVF. Follicular fluid visibly clear from blood contamination was pooled (one patient was excluded because there was no clear follicular fluid available). The sample was centrifuged to separate cells from the supernatant (follicular fluid) and subsequently aliquoted and stored at –80°C until further analyses. Quality control (QC) samples were prepared by pooling follicular fluid from three random patients and a small volume from all of the serum samples available.

Extraction

Serum and follicular fluid were spiked prior to extraction with an internal standard mixture consisting of isotopically labelled substances with a range of physiochemical properties. Samples were extracted together with sampling blanks and QC samples according to previously developed protocols for human serum (Plassmann *et al.*, 2015).

Instrumental analysis

Instrumental analysis was carried out as previously described (Plassmann *et al.*, 2018). Chromatographic separation of analytes was carried out with an ultra-high performance liquid chromatograph. Detection was carried out on a Q Exactive HF Orbitrap (Thermo Scientific) equipped with a heated electrospray ionization source. A full scan was combined with a data-dependent tandem mass spectrometry. Samples were run in four sequences: follicular fluid and serum in positive and negative mode, respectively.

3.2.3 Data processing and validation

The data from the instrumental analysis were imported into Compound Discoverer (Thermo Scientific) for alignment, peak picking, and feature determination. Peaks and adducts were aggregated to define unique features. The four sequences (follicular fluid positive/negative mode, serum positive/negative mode) were processed separately in Compound Discoverer resulting in four separate feature lists. To be able to compare peak areas present in serum and follicular fluid (i.e., to calculate the ratio), a combined list including extracts of paired samples (follicular fluid/serum) was created. Due to the larger amount of data, the thresholds during peak picking had to be raised compared to the first processing. Blank signals were subtracted (max area sample / area blank >5) and internal standard recoveries in the samples QCs and internal standards were evaluated over the run showing acceptable results with an absence of signal drift. Substances identified through this workflow were assigned a confidence level (CL) 1–5 using the Schymanski scale, where CL = 1 represents a confirmed suspect and CL = 5 defines a tentative identification based on exact mass (Table 2) (Schymanski *et al.*, 2014). Substances identified with CL = 1 were compared to ensure their absence in both the sampling and procedural blanks before being assigned confirmed status.

Table 2 Level of confidence of the identification of features, adapted from Schymanski *et al.,* 2014.

Confidence level [CL]			Description
Structure identification	1		**Confirmed substance** via appropriate measurement of a reference standard with MS, MS/MS, and retention time matching
	2	a	**Probable structure** – library match. Unambiguous spectrum-structure match
		b	**Probable structure –** diagnostic. No other structure fits the experimental information, but without reference match
Substance class, formula	3		**Tentative candidate** with evidence for possible structure(s) but insufficient for one exact structure
	4		**Unequivocal molecular formula** with formula unambiguously assigned using spectral information but insufficient for possible structures
	5		**Exact mass** but with no equivocal information about the formula or structure exist

3.2.4 Prioritization strategy for the identification of features

Database comparisons for tentative identifications

Special effort was put into identifying PFASs in the samples. An in-house exact mass list containing 279 PFASs previously detected in house or in the literature were matched against follicular fluid and serum samples. Furthermore, the Swedish Chemicals Agency Market List (referred to herein as the "Market List") were matched against the samples to generate tentative identifications. The Market List contains approximately 30,000 substances (including industrial chemicals, pharmaceuticals, and pesticides) from different national/regional inventory lists with a focus on the EU market. The Market List prioritizes substances based on both human health risk (score 0–9) and exposure (score 0–27). Tentative assignments were filtered based on high exposure (score ≥15) and unknown or moderate-high hazard (unknown or hazard score ≥3). Filtered features detected in >30% of the samples were included to ensure ubiquitous exposure in our cohort.

Features accumulating in follicular fluid

Based on the premise that features accumulating in follicular fluid may have a greater impact on the maturing oocyte, further prioritization was focused on these features. The ratio of the peak area in follicular fluid relative to the

corresponding area in serum from the same individual was calculated, and features with a ratio ≥20 were then matched to the Market List and a mass spectrum library (*m/z Cloud*).

The internal standard response ratio between follicular fluid compared to serum suggested greater matrix suppression in follicular fluid in positive mode. Generally, all median ratios were below one, which resulted in an underreporting of enriched factors when using peak areas alone. Even though the internal standard mixture covered a wide range of physiochemical properties, we can still not rule out potentially higher or lower matrix-induced ionization effects in unknowns.

Identification of features associated with embryo quality

The identification of suspects was then based on differences in follicular fluid and serum depending on the presence of a top-quality embryo. Embryo quality was assessed on day two by a well-established method considering blastomere count, fragmentation, blastomere size variation, symmetry, and mononuclearity in the blastomeres (Holte *et al.*, 2007). We found that a multivariate statistics approach (orthogonal partial least square – discriminant analysis) resulted in poor separation between the groups. It is possible that the variation in follicular fluid and serum composition alone was not sufficient to describe the differences. Further analyses were therefore based on a fold change threshold of ≥ 1.5 (in >75% of pairs/variable) combined with a t-test with a significance threshold of $p < .05$, which provided significantly different features in follicular fluid and serum of patients with top-quality embryos compared to those with lower quality embryos. Features were matched with the databases for further prioritization. With this strategy, we are willing to accept that some false positives may arise when not applying adjustment for multiple testing, and this was accepted at this point of exploring suspects possibly related to reproductive outcomes and hence of potential interest for future research. Following prioritization, exogenous features identified and associated with embryo quality were further investigated. A logistic regression with confounding variables (age, BMI, anti-Müllerian hormone, infertility cause, and parity) was performed. A brief overview of the NTS approach is shown in Figure 3.

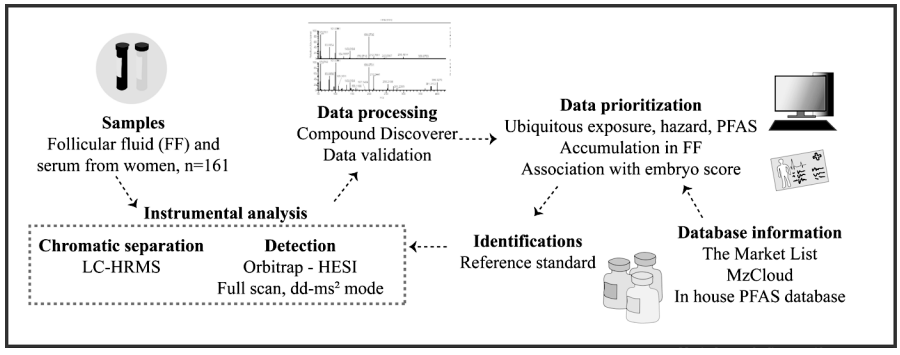
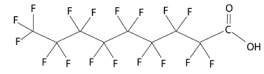
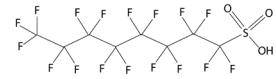
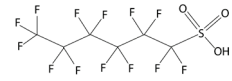


Figure 3. Workflow of the NTS approach (I)

3.3 Bovine IVP (II-IV)

Single PFAS exposure was performed using three different PFASs (Table 3). PFNA (II) was chosen based on its occurrence in humans and in wildlife. In humans it is detected at the highest or second highest concentrations after PFOS and PFOA (Bjerregaard-Olesen *et al.*, 2016). In wildlife such as mink (Persson *et al.*, 2013) and seal (Kratzer *et al.*, 2011), PFNA levels are among the highest. Only PFOS is reported in higher concentrations. The exposure doses were based on previous experiments on zebrafish and amphibians (Liu *et al.*, 2015; Kim *et al.*, 2013; Ulhaq *et al.*, 2013) and concentrations measured in human ovarian follicular fluid (Heffernan *et al.*, 2018; McCoy *et al.*, 2017; Petro *et al.*, 2014), multiplied by a factor of 50–500 to compensate for possible differences between species. PFOS (III) was selected based on the broad knowledge about toxicity mechanisms in other experimental settings. Doses were set to represent human exposure (Heffernan *et al.*, 2018; McCoy *et al.*, 2017; Petro *et al.*, 2014), and the higher dose aimed to represent individuals with higher exposure but within the range of human relevance. PFHxS (IV), one carbon shorter compared to PFOS, was chosen because PFHxS is a major contributor to exposure after contamination with fire-fighting foam and is also of regional relevance (Gyllenhammar *et al.*, 2019). We first used seven doses in a short dose-response experiment ranging from 0.010 µg/mL (representing human exposure) to 100 µg/mL (maximum exposure based on the experimental setup). This was followed by a second experiment with a concentration of 0.1 µg/mL. The exposure concentrations can be found in Table 4. For studies III-IV, the exposure concentrations were validated by mass spectrometry.

Table 3. Perfluoroalkyl substances (PFASs) used in the studies (II-IV)

PFAS	Study	Class	CAS-no	Molecular structure
Perfluorononanoic acid, PFNA 97%	II	PFCA	375-95-1	
Perfluorooctane sulfonate, PFOS potassium salt, ≥98.0%	III	PFSA	1763-23-1	
Perfluorohexane sulfonate, PFHxS potassium salt, ≥98.0%	IV	PFSA	355-46-4	

3.3.1 Media and reagents (II-IV)

Media for IVP were freshly produced at the IVF laboratory facilities on a daily or weekly basis depending on protocols based on standard methods (Gordon, 2003) and modified for use in our laboratory (Abraham *et al.*, 2012). All chemicals and media were purchased from Sigma Aldrich (Stockholm, Sweden) unless stated otherwise. Details on media and preparation can be found in the corresponding papers (II-IV).

3.3.2 Oocyte recovery and origin of the cattle

Bovine ovaries were retrieved from the slaughterhouse after the animals had been killed. Animals were not killed for the sake of this experiment, and hence no ethical permission was needed according to Swedish legislation. Ovaries were transported within 3 h to the IVF laboratory facilities where cumulus-oocyte-complexes were aspirated from 3–8 mm diameter follicles. Acceptable cumulus-oocyte-complexes according to Gordon's morphological criteria were selected for the experiments and were randomly and equally distributed between the experimental groups (Gordon, 1994).

3.3.3 IVM under exposure to PFASs

Cumulus-oocyte-complexes were incubated for 22 h in media for IVM. Experimental groups were incubated with the addition of PFASs, and each replicate contained a control where only vehicle was added (Table 4).

Table 4. Exposure concentrations of PFASs in the experiments performed (II-IV)

PFAS (study)	Intended exposure, µg/mL (µM)	Validated concentration (µg/mL)	Validated concentration (µM)	Vehicle
PFNA (II)	0.1 (2.2)	—	—	TCM199
	10 (21.5)	—	—	TCM199
PFOS (III)	0.02 (0.04)	0.002[a]	0.004	Molecular grade water
	0.1 (0.2)	0.053[a]	0.106	Molecular grade water
PFHxS (IV)	0.01 (0.025)	0.008[a]	0.02	Molecular grade water
	0.1 (0.25)	0.08[b]	0.19[b]	Molecular grade water
	1.0 (2.5)	0.76[b]	1.89[b]	Molecular grade water
	10 (25)	7.96[a]	20.0	Molecular grade water
	20 (50)	15.93[b]	39.8[b]	Molecular grade water
	40 (100)	29.23[a]	73.1	Molecular grade water
	100 (250)	73.01[b]	182.7[b]	Molecular grade water

[a]Validation of concentration using mass spectrometry, [b]Concentration calculated based on stock solutions sent for mass spectrometric validation

3.3.4 IVP

Following IVM, presumed MII oocytes were co-cultured for 22 h with thawed frozen spermatozoa selected by swim-up procedure at a concentration of 10^6/mL. For all experiments, sperm from the same ejaculate from the same bull of Swedish red breed with proven *in vitro* and *in vivo* fertility was used (3-1716 Sörby). The semen was a generous donation from Viking Genetics after breeding with the bull had been discontinued. Presumed zygotes were washed after fertilization *in vitro* to remove excessive cumulus cells and spermatozoa and were moved for *in vitro* culture in serum-free modified synthetic oviductal fluid with wells covered in paraffin oil (OVOIL™, Vitrolife, Göteborg, Sweden).

3.4 Evaluation of bovine embryos

3.4.1 Developmental competence and embryo morphology

During IVP, the developmental competence of the oocytes was recorded by assessing the proportion of embryos developing during culture. At 44 h after fertilization, the proportion of cleaved embryos and embryos cleaved beyond the two-cell stage were recorded. At day seven and eight of culture, the stage and grade as well as the total proportion of fully developed blastocysts were recorded. The grade of each embryo (grade 1–4) was scored according to IETS classification (IETS, 2010) with grade 1 corresponding to a top-quality embryo. Developmental stage was based on IETS classification but modified into three developmental stages (IETS, 2010) including early blastocysts or blastocysts, expanding or expanded blastocysts where the zona pellucida is intact but is thinner due to expansion of the embryo, and hatched or hatching.

3.4.2 Confocal imaging and image analysis

At day eight, replicates including blastocysts were either stained for confocal microscopy and further morphological evaluation (II-IV) or snap-frozen for hybridization studies (see further below). Embryos for confocal imaging were fixed in paraformaldehyde in phosphate buffered saline with 0.1% polyvinyl alcohol. Following fixation, fluorescent labelling for visualization of neutral lipids was performed (HCS LipidTOX™ Green Neutral Lipid Stain H34475, ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. The cells were visualized using fluorescent staining of nuclei with DRAQ5 (4084S, BioNordika, Stockholm, Sweden, II), Hoechst 3342 (Sigma Aldrich, III), or Vectashield containing DAPI (Vector Laboratories Inc., CA, US, IV).

Images were captured using confocal microscopy (Zeiss LSM 510 (II) and 800 (III-IV)). Each embryo was scanned using standard magnification (20×) in sectioned scans (z-stack). Image analysis was developed during the experiments in collaboration with BioImage Informatics Facility in Uppsala. In the first experiment, we were limited by time constraints for the scanning. This resulted in seven sections being scanned from the top of the embryo to the bottom, and three sections were used for image processing (Cell Profiler 2.2.0). Individual sections of the embryos was used to identify lipid droplets and cells (nuclei) (Carpenter *et al.*, 2006). Lipid droplet diameter was measured, and lipid droplet distribution was assessed in treated blastocysts

compared to controls (II). For the following experiments, each section was scanned with a fixed interval of 2 µm and images were evaluated approximating 3D with ImageJ software (Fiji for ImageJ 1.8.0) (III-IV). This approach allowed the approximation of lipid droplet volume, and the parameters that were assessed included total lipid volume, average lipid droplet size, and average lipid volume/cell. The same setting was used to analyse all images within an experiment but was slightly upgraded between the studies. Each cell and lipid were detected using a local adaptive threshold approach, which for each object computes the threshold level that optimizes the ellipse fit (Bombrun *et al.*, 2017; Ranefall *et al.*, 2016), Figure 4.

3.5 Microarray based gene expression and DNA methylation studies of blastocysts after PFAS exposure during IVM (III-IV)

3.5.1 Embryo pools and parallel RNA/DNA extraction

At day eight, blastocysts that developed after exposure to PFOS (0.002 and 0.053 µg/mL, III) and PFHxS (0.1 µg/mL, IV) were subjected to microarray-based analyses. Day-eight embryos were individually snap-frozen in a fixed volume of phosphate-buffered saline with 0.1% polyvinyl alcohol and stored at –80°C until further analyses. For parallel RNA and DNA extraction, embryos of equivalent stage and grade were used to avoid confounding effects due to different embryo characteristics (Rekik *et al.*, 2011).

Embryos were pooled in groups of 10 to obtain the amount of RNA/DNA needed for hybridization. Parallel genomic DNA and total RNA extraction were performed using the AllPrep DNA/RNA micro kit (Qiagen Cat no 80284) according to previously developed protocols by the EmbryoGENE platform in Canada (Robert *et al.*, 2011). The quality and concentration of the genomic DNA and total RNA were evaluated using NanoDrop (NanoDrop Technologies, Wilmington, DE, US) and Bioanalyzer (Agilent technologies, Santa Clara, CA, US). The samples were stored at –80°C.

3.5.2 Microarray-based analysis of the gene expression profile in bovine blastocysts

To yield sufficient antisense RNA for microarray hybridization, amplification was required. RNA was amplified using the two-round

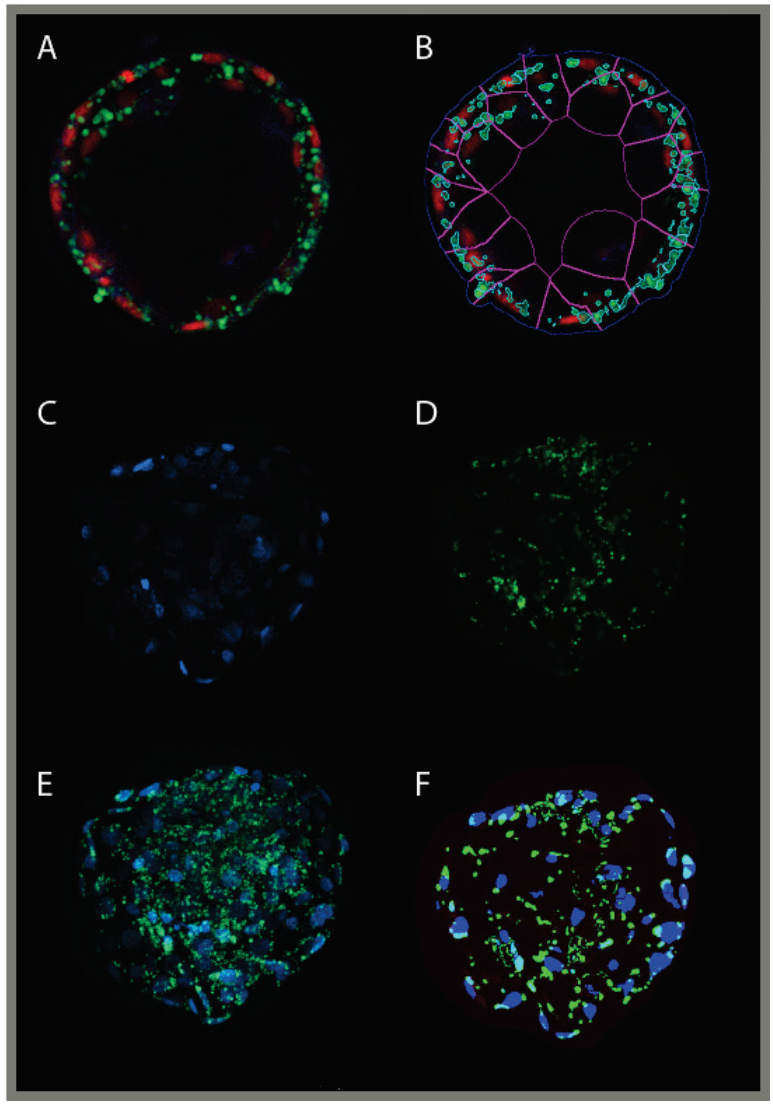


Figure 4. Detection of lipid droplets and cells and image analysis output in day-eight blastocysts (II-IV). A: Overlapping image of a blastocyst with nuclei (red) and lipids (green) (II). B: Output from CellProfiler with nuclei (red) and lipids (green) used to calculate the lipid droplet diameter after exposure (II). C: Single -channel confocal image showing nuclei (blue) in a day-eight blastocyst (III-IV). D: Single-channel confocal image showing lipids in a day-eight blastocyst (green) (III-IV). E: Overlay image of both lipids and nuclei of all z-stacks of the blastocyst (III-IV) F: Representative output from the subsequent experiments with the same embryo (III-IV).

RiboAmp® HS^{PLUS} RNA Amplification kit (Applied Biosystems, Foster City, California, USA), which provides amplification of small amounts of RNA. Exposed groups were compared to the control group using a four-array swap design with labelled samples (Cy3/Cy5). After hybridization, the slides were washed and scanned using a PowerScanner (Tecan Group Ltd., Männedorf, Switzerland). Feature extraction was conducted with Array-pro 6.3 (Media Cybernetics, Rockville, Maryland) as previously described (Robert *et al.*, 2011). Relative transcript abundance was analysed using Flexarray (Blazejczyk *et al.*, 2007). Background was subtracted from the raw fluorescence intensity within and between arrays (Robert *et al.*, 2011). Functional analysis of the gene-expression profile was conducted using Ingenuity Pathway Analysis (Qiagen IPA Software, Qiagen, Redwood City, CA, USA), which enables the analysis of altered pathways and gene-interaction patterns affected in day-eight blastocysts after PFAS exposure during IVM.

3.5.3 Microarray based analysis of methylation patterns in bovine blastocysts

Methylation of DNA was analysed using the bovine EmbryoGENE DNA methylation array as previously described by (Saadi *et al.*, 2014) and (Montera *et al.*, 2013), which contains methylation sites within the bovine genome without topological bias. Genomic DNA was amplified and fragmented before hybridization according to developed protocols (Saadi *et al.*, 2014). Methylation-sensitive enzymes were used for fragmenting, whereby methylated regions were protected from digestion. Subsequently, samples were amplified resulting in exponential amplification of methylated regions. Labelled (Cy3/Cy5) samples from the exposed groups were compared to the controls using the same four-dye swap design as for the transcriptome microarray. Samples were hybridized and washed before scanning with PowerScanner and feature analysis using ArrayPro software. Quantile inter-array scale normalization was performed following loess intra-array normalization. Functional analysis was performed by IPA to analyse altered pathways after PFAS exposure.

3.6 Statistical methods (II-IV)

For statistical comparisons, R software was used (R[1], 3.3.1 and 4.0.5). To calculate the effect of PFAS treatment on the developmental competence of the oocyte, mixed effect logistic regression with binomial distribution was used generating odds ratios for the effect of treatment on developmental parameters (cleaved embryos, embryos cleaved beyond the two-cell stage, and blastocyst development at day seven and day eight after fertilization, all calculated from cultured cumulus-oocyte-complexes) (II-IV). Replicate was added as a random factor, and groups were weighted for size in all models. The proportions of embryos at day seven and eight were considered repeated measurements, hence generating one model for blastocyst development after each treatment. Categorical variables (stage and grade) were analysed using cumulative mixed-effect models with multinomial distribution and with replicate as a random factor. Linear mixed effect models were created to calculate the effect of treatment on cell count (nuclei) and lipid distribution (lipid distribution and lipid droplet size (II), average total lipid volume, average lipid droplet volume, and average lipid/cell volume (III-IV)). Replicate was added as a random factor, and embryo stage was initially added to the model and was removed when appropriate based on likelihood-ratio test and model fit. *P*-values < .05 were considered significant. *P*-values were not adjusted for multiple testing.

For the microarray platform (III-IV), each probe was attributed a probability of fold change between treatment and control by the *limma* package in R using linear models and Bayesian statistics. Differentially expressed genes (DEGs) and differentially methylated regions (DMRs) were defined as fold change > 1.5 and $p < .05$. Applying a Benjamini–Hochberg correction for false discovery rate did not generate enough molecules for pathway analysis, and hence all significant probes ($p < .05$) were considered for pathway analysis by IPA.

[1] http://www.r-project.org

4. Main results

An overview of the main results is presented in this section, whereas details can be found in papers I-IV and in the supporting information of the included papers.

4.1 NTS of ovarian follicular fluid (I)

4.1.1 Patient characteristics

Patients were on average 34.6 years old (range 21–43 years) and had an average BMI of 23.5 (SD 3). The reasons for infertility in the couples had a similar distribution as a larger cohort from the same clinic (Rhenman *et al.*, 2015). No reason for infertility was found in 44% of the couples, male factor infertility accounted for 25% of the patients, and the rest of the patients had female factors as indication for IVF (*e.g.,* tubal factor, endometriosis, ovarian factor, or anovulation). Oocytes retrieved from the procedure were fertilized either with IVF (48.5%), intraplasmatic sperm injection (43.5%), or a combination of both techniques (8%). The majority (53%) had visited the clinic for the first attempt of IVF, whereas the rest had a history of one or more previous IVF treatments that either failed or resulted in previous pregnancies. From the fresh cycles that were included in this study, 85% resulted in an embryo being transferred, 38% in a biochemical pregnancy, and 30% in a live birth.

4.1.2 Detected features in serum and follicular fluid using LC-HRMS

A large number of water-soluble semivolatile or nonvolatile features could be observed in both serum and follicular fluid (20,644 in follicular fluid and 13,740 in serum). The combined list used for calculating the ratios of features

accumulating in follicular fluid contained fewer features (12,162). This was due to the smaller sample size (116 patients compared to 161 in the separate follicular fluid/serum lists) in combination with the difference in Compound Discoverer settings and the specifications used for data processing.

4.1.3 Identification of suspects and non-targets

Our prioritization approach used databases to generate tentative feature identifications (CL = 2–5). In database matches where fragment data or exact masses led us to certain suspected chemicals, reference standards were used for confirmation (CL = 1) and by extension to rule out suspects where the reference standard could not confirm the identity. The Market List could tentatively identify 170 compounds using the prioritization strategy based on estimated hazard, exposure, and ubiquitous presence in our cohort (>30%). Because of endogenous substances also being manufactured (and hence listed in the Market List), both endogenous and exogenous suspects were present in this list.

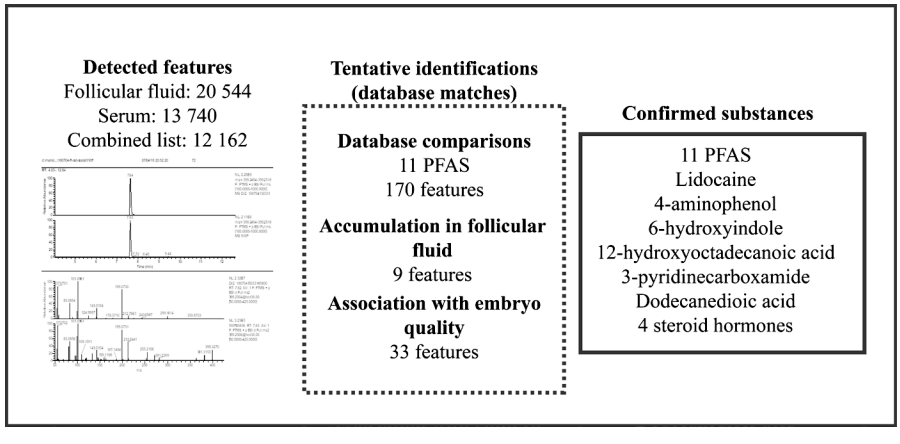


Figure 5. Overview of the tiered prioritization strategy including numbers of features, tentatively identified suspects, and confirmed substances (positive and negative mode combined) (I). Features are defined as the combination of all ions (i.e. adducts, parent ion, in-source fragments, etc.) at a given retention time. Some duplication may exist for substances ionizing in both positive and negative mode.

Using the in-house list of exact mass of PFASs, we could identify 11 PFASs and determine the follicular fluid:serum ratio. The median ratio ranged from 0.64 (PFOS) to 1.04 (PFHxS). These values are comparable to recently

reported data from (Kang *et al.*, 2020) (Table 5). Samples from the same patients, which were also submitted for chemical analyses using a targeted approach in a different project also showed consistent results (Björvang *et al.*, 2021, submitted manuscript).

Table 5. Median ratios of PFASs between follicular fluid and serum

Compound	Cas. no	Median ratio (I)[a]	Comparison ratio[b]
Perfluoroheptanoic acid (PFHpA)	375-85-9	0.65 (0.37-1.00)	0.96
Perfluorooctanoic acid (PFOA)	335-67-1	0.72 (0.33-1.18)	0.76
Perfluorononanoic acid (PFNA)	375-95-1	0.79 (0.31-1.26)	0.71
Perfluorodecanoic acid (PFDA)	335-76-2	0.69 (0.21-1.29)	0.65
Perfluoroundecanoic acid (PFUnDA)	2058-94-8	0.71 (0.05-1.39)	0.59
Perfluoropentane sulfonate (PFPeS)	2706-91-4	0.88 (0.23-1.73)	-
Perfluorohexane sulfonate (PFHxS)	355-46-4	1.04 (0.01-1.65)	0.84
Perfluoroheptane sulfonate (PFHpS)	375-92-8	0.79 (0.04-1.49)	-
Perfluorooctane sulfonate (PFOS)	1763-23-1	0.64 (0.001-1.51)	0.70
9-Chlorohexadecafluoro-oxanonane sulfonate (9Cl-PF3ONS)	756426-58-1	0.94 (0.24-2.15)	-
Perfluoro-ethylcyclohexane sulfonate (PFECHS)	646-83-3	0.88 (0.10-2.66)	-

[a]Median ratios (ranges) determined by calculating the peak area in follicular fluid divided by the peak area in serum from the same individual. [b]Median ratios presented by Kang et al., 2020 who used high-resolution mass-spectrometry for identification of legacy as well as emerging PFASs in follicular fluid from 28 women in China undergoing OPU during 2018-2019.

In the combined list with peak intensities from both follicular fluid and serum, 262 features were found to accumulate at ratios of ≥20, and these were compared to the Market List and mzCloud. One compound, lidocaine, was confirmed with a reference standard (CL = 1); however, this substance is used as a local anaesthetic during OPU and the accumulation in follicular fluid compared to serum (ratio 4559 (0.27-55843)) can be assumed to be from the local injection close to the sampling site. An additional four hormones and hormone derivatives (progesterone, epitestosterone, 17α-hydroxyprogesterone, and 20α-hydroxyprogesterone) could be confirmed using library spectrum data (mzCloud hit >85, CL = 2a) and accumulated in follicular fluid at ratios ≥100. Their occurrence in follicular fluid is

physiologically expected because steroid hormones are locally produced in the follicle (Libersky & Boatman, 1995).

The patients with top-quality embryos did not significantly differ from those with lower quality with respect to age or BMI. NTS detected 252 significantly different features in patients with top-quality embryos compared to those with lower-quality embryos (134 in follicular fluid and 118 in serum). The Market List matched 33 features, and 4-aminophenol (observed in 84% of patients, CL = 1), 6-hydroxyindole (observed in 34% of patients, CL = 2a), and endogenous 12-hydroxydodecanoid acid could be confirmed. Logistic regression models including confounding variables confirmed the association for 6-hydroxyindole with lower embryo quality but could not confirm the association with 4-aminophenol.

4.2 PFAS impact on bovine early embryo development *in vitro* (II-IV)

4.2.1 Developmental competence after exposure to PFASs during IVM

Exposure to PFASs in high concentrations during IVM caused diminished embryo development. Upon PFHxS exposure, diminished embryo development was seen upon exposure to concentrations ≥40 µg/mL. Oocyte exposure to 40 µg/mL PFHxS yielded 8% blastocysts compared to 21% in the controls (p = .05), and 100 µg/mL PFHxS yielded 3% blastocysts (p = .03). Upon exposure to 10 µg/mL PFNA during IVM, the proportion of blastocysts was 8% compared to 26% in the controls (p = .0009) (Table 6, Figure 6). Upon exposure to 10 µg/mL PFNA, this was also evident directly after maturation with the absence or decreased expansion of cumulus cells. Lower concentrations did not seem to cause failure to develop into the blastocyst stage (PFOS 0.002 µg/mL, p =.19; PFOS 0.053 µg/mL p = .10; PFHxS < 40 µg/mL, p > .05; and PFNA 0.1, p = .51). However, delayed development through the first cell divisions was seen as a reduced proportion of cleaved embryos and embryos cleaved beyond the two-cell stage 44 h after fertilization (PFOS 0.053 µg/mL, PFNA 10 µg/mL, and PFHxS 10 µg/mL, 40 µg/mL, and 100 µg/mL). The majority of the embryos that did not proceed to the blastocyst stage ceased development before embryo genome activation (average cell count <8) irrespective of previous treatments (III-IV).

Table 6. Effect of PFAS exposure during oocyte maturation *in vitro* on subsequent early embryo development in bovines.

PFAS	Exposure (µg /mL)	Cleaved embryos	Blastocyst rate	Development rate	Blastomere count	Lipid distribution
PFNA (II)	0.1	—	—	—	—	↑
	10	↓	↓	—	NA[a]	NA[a]
PFOS (III)	0.020	—	—	(↓)	—	↓ / —
	0.053	↓	—	↓	↓	↑
PFHxS (IV)	0.01	—	—	NA[b]	—	—
	0.1	—	—	↑	—	—
	1.0	—	—	NA[b]	—	↑
	10	↓	—	NA[b]	—	↑
	20	—	—	NA[b]	↓	—
	40	↓	↓	NA[b]	↓	—
	100	↓	↓	NA[b]	NA[b]	NA[b]

— $p > .1$, (↓) $.1 > p > .05$, ↓ $p < .05$, NA[a]: variable not assessed, NA[b] : no data due to insufficient number of observations

4.2.2 Phenotypic variation after PFAS exposure during IVM

Concentrations of PFASs that caused diminished embryo development did not seem to affect the morphology of the embryos surviving the conditions during IVM (embryo grade was not affected by PFAS treatment, $p > .05$).

Upon sub-lethal exposure to PFOS and PFHxS during IVM, the developmental rate was affected. The blastocyst development rate was delayed after oocyte exposure to 0.053 µg/mL PFOS ($p = .01$), as evidenced by decreased numbers of blastocysts reaching higher developmental stages and corroborated by decreased blastomere count ($p = .04$). This was not seen upon exposure to 0.002 µg/mL PFOS ($p = .06$). Upon oocyte exposure to 0.1 µg/mL PFHxS, we observed an increased development rate ($p = .04$), which was not observed upon PFNA exposure at the same concentrations ($p = .54$). At higher concentrations, we observed a decrease in blastomere count at concentrations slightly below the concentrations affecting the proportion of developing embryos (20 µg/mL, $p = .01$).

Lipid variation after PFAS exposure during IVM

The lipid distribution was affected after all PFAS treatments but to various extents. After sub-lethal exposure to PFNA during IVM (0.1 µg/mL), the distribution of lipid droplets was different compared to controls (p = .04), and embryos treated with PFNA had an increased proportion of large lipid droplets (p < .0001) and a lower proportion of small lipid droplets (p = .04). A similar response was seen upon PFOS exposure where 0.053 µg/mL resulted in increased amounts of lipids/cells (p < .0001) and increased total lipid volume (p = .0003). This was not observed in the lower dose of PFOS (p > .05), and exposure to 0.002 µg/mL PFOS seemed to have a decreased average lipid droplet size compared to controls (p = .02). After PFHxS treatment, the lipid variations were not consistent with increasing concentration, but we could observe an increased total lipid volume after exposure to 1 µg/mL and 10 µg/mL (p = .01 and p = .02, respectively).

4.2.3 Gene expression variation after PFAS exposure during IVM (III-IV)

To determine the effect of PFAS on gene expression in blastocysts following oocyte exposure, transcriptomic analyses using microarrays on pools of day-eight embryos were performed on blastocysts upon exposure to PFOS (0.002 and 0.053 µg/mL, III) and PFHxS (0.1 µg/mL, IV). Overall, the gene expression analysis showed most abundantly a downregulation upon exposure and showed modest changes. We identified 223 DEGs (fold change > 1.5, p < .05) for 0.053 µg/mL PFOS (5 up and 218 downregulated), 16 DEGs for 0.002 µg/mL PFOS (4 up and 12 downregulated) and 14 DEGs for PFHxS (4 up and 10 downregulated).

Gene sets changes by PFOS or PFHxS (p < .05) were associated with dysregulation of pathways leading to increased apoptosis, *e.g.,* through *tumour protein 53 (TP53)* in the blastocysts. However, enriched pathways leading to this converging point differed between treatments. Upon PFOS exposure, the significant transcripts were associated with pathways important for blastocyst formation and development, such as *β-catenin (CTNNB1)* and *nuclear factor NF-Kappa-B P65 subunit (RELA)* together with *signal-transducer and activator of transcription 3 (STAT3)* (Messerschmidt *et al.*, 2016; Nishikimi *et al.*, 1999). Functions altered in the blastocysts were predicted to be related to, for example proliferation, cell death and differentiation. Metabolic stress response overlapping with lipid

metabolic pathways was also identified. In the blastocysts, these pathways were predicted to lead to altered synthesis and concentration of lipids.

For PFHxS, dysregulation of pathways leading to the production and synthesis of reactive oxygen species was more pronounced. Based on our molecular data, this is predicted to occur through dysregulation of, for example, *serine/threonine kinase (ATM)*, *nuclear factor kappa B subunit 1 (NFKB1)*, and *transforming growth factor (TGF) beta.* The metabolic impact on the blastocysts was mainly attributed to *PPARγ* inhibition. The same was also seen upon PFOS exposure but to a lesser extent. Signs of hormonal impact in blastocysts upon PFHxS exposure were attributed to *beta oestradiol* inhibition.

4.2.4 Methylome changes after PFAS exposure during IVM (III-IV)

In order to address whether PFAS might affect the epigenome in the blastocysts by affecting DNA methylation, the same groups of embryos as for the transcriptome analyses were submitted to a microarray-based analysis of genome-wide methylation patterns. We identified 380 DMRs ($p < .05$, fold change > 1.5) for 0.002 µg/mL PFOS, 189 DMRs for 0.053 µg/mL PFOS and 668 DMRs for PFHxS. Pathway analysis of gene sets affected by both treatments ($p < .05$) again identified dysregulation of *TP53* signalling as an important pathway affected in the blastocysts. This corroborated the transcriptomic data for both PFOS and PFHxS. Upon exposure to both PFOS concentrations, the epigenomic data suggested changes leading to altered *CTNNB1* regulation in the blastocysts. Upon PFHxS treatment, altered *beta-oestradiol* signalling was predicted based on DNA methylation data.

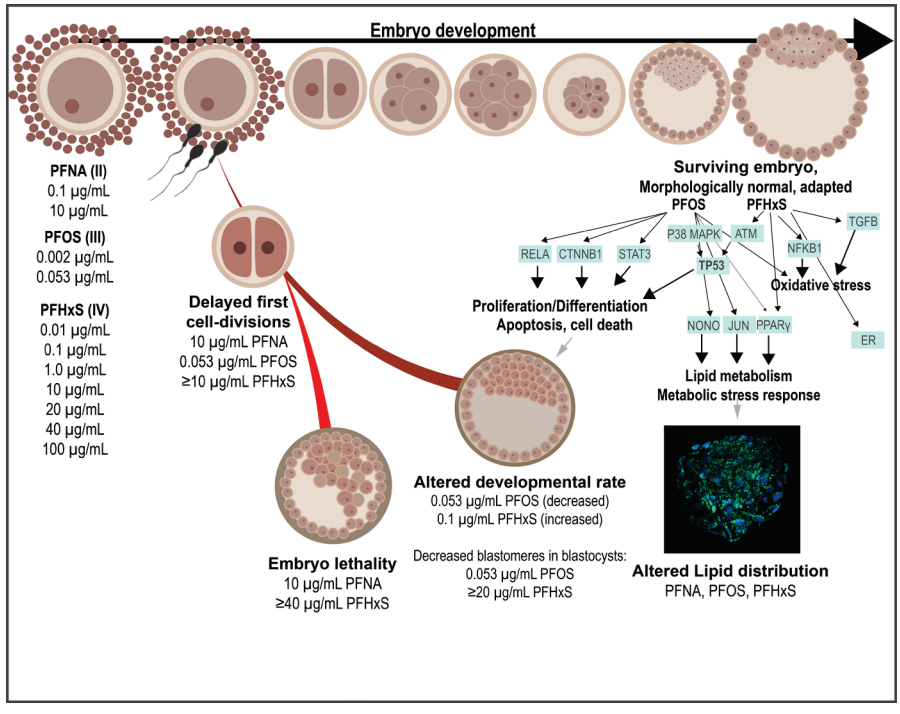


Figure 6. Schematic illustration of the proposed impact of PFASs on bovine early embryo development (II-IV)

5. General discussion

5.1 The complex environment of the maturing oocyte in the ovarian follicle (I)

5.1.1 Applicability of NTS and the prioritization strategy to detect features associated with female fertility

The follicular fluid consists of a mixture of thousands of known and unknown exogenous and endogenous compounds as elucidated using the NTS approach (I). This was one of the first studies using LC-HRMS in paired follicular fluid and serum and from a quite large IVF cohort, providing data as a valuable resource for future use (Hollender *et al.*, 2017). The amount of detected features was not surprising considering recent estimates of the number of chemicals in global commerce (Wang *et al.*, 2020) along with the complex metabolic and endocrine environment of the ovarian follicle. This highlights the knowledge gap between the regularly monitored substances and the live exposure of the human oocyte.

In environmental samples, NTS shows good potential for biomonitoring and effect-directed analyses (Hollender *et al.*, 2017). During the last decades the methods have been continuously developed. Today, NTS is used also in monitoring to detect leakages of unidentified/unanticipated chemicals (Hollender *et al.*, 2017; Sjerps *et al.*, 2016). In human samples, NTS is not yet used as extensively. I believe that the potential shown from environmental samples together with the continuous development of NTS in biological samples adds to the promising future of the method.

Even though a large amount of information was obtained, we were limited to identifying only a small fraction of compounds. Identifications require a substantial amount of time and resources (Hollender *et al.*, 2017), and this is

a challenge. For future research, a combination with strategies for efficient data management, evaluation, and sharing, may contribute to the development (Hollender *et al.*, 2019).

We prioritized substances at a follicular fluid to serum ratio >20 but did not identify any exogenous substances at this level of accumulation except for the locally injected anaesthetic lidocaine. If exogenous substances were accumulated to this extent, this would have been of high importance. From a human health perspective, substances with lower accumulation in follicular fluid could of course also be of interest. Our filtering of the matches (unknown/high hazard and ubiquitous exposure) from the Market List led to the limitation that some presumed bad actors in female fertility were excluded either due to lower distribution in our cohort (*e.g.,* some phthalates detected in < 30 % of the patients) or because current data suggest low hazard (*e.g.,* some PFASs).

5.1.2 Exposure of the maturing oocyte to PFASs

Semi-targeted or suspect screening approaches offer a compromise where data from NTS may be used to scan matrices for certain groups of chemicals, *e.g.,* PFASs, but also chlorinated and brominated substances (Kang *et al.*, 2020; Miaz *et al.*, 2020). We used this strategy successfully to identify PFASs and could estimate their follicular fluid to serum ratio, which was close to one (I). The unknown proportion of extractable organofluorine content in serum is increasing, which might reflect the increasing exposure to novel/unknown PFASs (Miaz *et al.*, 2020). The data from NTS could then be a valuable tool for identifying or monitoring such increases in follicular fluid over time, or to go back to these data if new PFASs of concern are identified in the future.

5.1.3 Prerequisites for the development of a top-quality embryo

The individual features in the follicular fluid were insufficient to explain the chance of obtaining top-quality embryos in our IVF cohort. Hence, our data could not support previous findings suggesting an association between chemical exposure and either embryo quality (Ma *et al.*, 2021; Machtinger *et al.*, 2018; Petro *et al.*, 2014; Petro *et al.*, 2012) or embryos transferred (Governini *et al.*, 2011). This could be due to insufficient power in detecting small decreases in the chances of having a top-quality embryo, which requires large cohorts to detect with this approach. Furthermore, adverse

effects of mixtures should not be neglected without a more thorough risk assessment and other statistical approaches. We chose embryo quality as an endpoint because embryo quality provided a non-invasive measure of oocyte competence and because embryo quality generated better models using multivariate statistical approaches compared to the other variables investigated (*i.e.,* live birth or pregnancy).

Of the suspects identified, we could find both endogenous and exogenous compounds. This is expected. For example, one of the identified compounds associated with embryo quality in our cohort was the fatty acid 12-hydroxydodecanoic acid, which is a derivative of lauric acid. Elevated concentrations of selected non-esterified fatty acids hamper oocyte developmental competence and embryo quality (Van Hoeck *et al.*, 2011). Endogenous compounds in follicular fluid may change composition due to, for example, metabolic disorders, but some chemicals are also potent modifiers of endogenous metabolites and hormones, hence the definition of EDCs.

We found exogenous compounds with possible association with embryo quality, and these could be of interest to study further. 4-aminophenol is used in the manufacturing of paracetamol as well as products such as staining for fur, leather, and textiles. 6-hydroxyindole is used in hair-dyes and was associated with lower embryo quality. A logistic regression model considering confounding variables could also verify this association.

5.1.4 Cohort characteristics and ethical considerations

The IVF situation provides a setting where the follicular fluid is easily accessible after oocyte retrieval and is not intended for other uses. For practical and ethical reasons it is not feasible to access human ovarian follicular fluid from women who are not undergoing OPU. The blood collection did not cause additional procedures, as this was collected when a vein catheter was placed for the IVF-treatment. However, there are other ethical considerations recruiting women to research. Information such as reproductive history is considered sensitive information and personal integrity has to be of highest importance, which is also regulated in the data protection law. It is further possible that research regarding potential effects of chemicals on fertility will cause stress. To minimize possible stress, the patients were informed that they would not benefit be able to trace their individual chemical levels. The reason for participating should be based only

on the personal positive feeling of being able to contribute to research. Of the patients asked to participate, we had a high proportion of acceptance. This is probably due to different reasons. For example, 1: the non-invasiveness character of the study, 2: the interest of the project scope of the public. As a personal reflection during patient recruitment, there were indeed many patients expressing their interest in this area.

IVF patients, might not represent the general public in some respects. For example, cohorts recruited from fertility clinics seem to be older, to have higher annual household incomes, and to be more likely to have received previous fertility treatment (Stanford *et al.*, 2016). Furthermore, the women who accepted enrolment also seem more likely to have had a previous live birth compared to those who did not want to participate (Stanford *et al.*, 2016). The latter could lead to a bias in studying sub-fertility endpoints, but our high response rate should minimize this.

We cannot exclude that the population tested might be more or less exposed to certain compounds than to others. However, the quantification of target compounds in serum and follicular fluid in our cohort (Björvang *et al.*, 2021, submitted manuscript) confirms that PCBs and PFASs are in the same range as the levels measured in a larger cohort of pregnant women in Sweden (Svensson *et al.*, 2021) implying that any bias might be small.

5.2 Consequences of PFAS exposure during oocyte maturation on bovine early embryonic development *in vitro* (II-IV)

PFHxS has been considered for inclusion in the Stockholm Convention for global elimination, and PFOS was included already in 2009 (UNEP, 2009). The EU is moving toward a ban for the entire group of PFASs (ECHA, 2021). These measures can reduce future environmental contamination but will not make the exposure go away in the near future because PFAS properties as "forever chemicals". All three selected PFASs are included in the new European Food Safety Authority threshold for total tolerable intake, which was significantly reduced in 2020. This reduction was based on the lowest concentrations where adverse effects on vaccine response could be seen in children, which was 17.5 ng/mL of the sum of PFASs (Abraham *et al.*, 2020). This corresponded to a calculated long-term maternal tolerable intake of 4.4 ng/kg body weight (Schrenk *et al.*, 2020). The calculated sum of the mean of

eight PFASs from the target analytical approach was 8.4 ng/mL in our cohort of adult women (Björvang *et al.*, 2021, submitted manuscript).

5.2.1 Bovine oocyte exposure concentrations

The concentrations used were validated for PFOS and PFHxS, but not for PFNA. Because we discovered that filtering the media decreased the PFOS concentration, we changed the methodology for the last study and the validated concentrations were closer to the intended exposure. For PFNA, the media concentration was not validated, but because the same method was used as for PFOS experiments I would expect the exposure concentration to also be significantly lower compared to the intended concentration in this experiment. For the final study (IV), the PFHxS concentrations for the microarray experiments were based on results from the initial dose-response data. These data had quite large variations possibly due to small groups and few replicates. It might therefore have been interesting to use a slightly higher concentration for the microarray studies with increased phenotypic changes.

5.2.2 Bovine environmental exposure to PFASs

To my knowledge, there is only one study assessing PFAS contamination from environmental exposure in cattle in Sweden (Vestergren *et al.*, 2013). In that study, the authors suggested minimal contamination of PFHxS in bovines. Both PFOS and PFNA were found in cow tissue and water and feed, but at considerably lower concentrations compared to in humans (Table 1, assuming an approximate ratio between follicular fluid to serum to be close to one, I). The cow blood contained 14 ± 7.3 ng/kg PFNA and 110 ± 19 ng/kg PFOS, and this can be compared to the PFAS concentrations in blood from women in our cohort with a mean of 0.32 ± 0.17 ng/ml PFNA, 4.5 ± 2.6 ng/ml PFOS, and 1.1 ± 1.7 ng/ml PFHxS (Björvang *et al.*, 2021, submitted manuscript). The lower exposure in bovines is likely due to different exposure sources. Cows are lower in the food chain compared to humans and are not exposed to high-risk feed such as fish in Sweden. Cattle tissue also seem less contaminated with other compounds (such as PCBs and dioxins) compared to humans (Petro *et al.*, 2010).

Local contamination of the water supply might result in significant exposure considering the large water consumption in dairy cattle, which could then affect farms locally. Sampling of the follicular fluid prior to the

experiments was not possible due to practical reasons and limited resources. However, with the background stated above, I do not suspect the PFAS contamination of the oocytes prior to the experimental setting to be substantial. The studies on final oocyte maturation were conducted after washing the cumulus oocyte complexes of most of the remaining follicular fluid.

5.2.3 Oocyte maturation as a window of exposure

We chose the exposure to take place during oocyte maturation. This means that we limited the exposure to exclude further development, including fertilisation and by extension exposure to the sperm. This differs from the *in vivo* situation. One could argue, though, that this mimics the human IVF situation when the *in vivo*-exposed oocytes are moved to *in vitro* culture with minimal presumed exposure (Iwasaki *et al.*, 2012). The environment during oocyte maturation in humans can be assessed by sampling the follicular fluid, something that is not as easily assessed from the environment during early embryonic development.

Transient, elevated stress is normal during early embryogenesis. The embryo may be most susceptible to exposure to stress/environmental insults during windows where the developmental programme require large amounts of energy. This could include oocyte maturation, but could also correspond to, for example, embryo genome activation and blastocyst formation (Puscheck *et al.*, 2015). It is possible that one week of culture after the exposure would allow the oocyte and the subsequent embryo to compensate or adapt. Persistent effects on early embryonic development are interesting, it would also have been interesting to investigate variables directly after maturation. When using slaughterhouse material, it is however difficult to overcome the fact that independent of exposure, not all oocytes will survive (Sirard, 2019). This could be the reason that endpoints after oocyte maturation seem to be less sensitive to environmental challenges compared to variables related to early embryonic development after oocyte exposure. For example, PCB exposure during oocyte maturation caused effects on further embryo development at doses where no effects could be seen directly after IVM (Krogenaes *et al.*, 1998).

5.2.4 Bovine early pre-implantation embryonic development upon exposure to PFASs during oocyte maturation *in vitro*

Oocyte developmental competence upon PFAS exposure

Exposure to concentrations of PFASs well above what have been measured in human follicular fluid during IVM caused a detrimental effect on early embryo development in bovines. The bovine oocyte seems to be more sensitive to PFNA compared to PFHxS. Diminished embryonic development was not observed after PFOS exposure, but embryo lethality was not expected because the concentrations used reflect human levels.

The lowest concentration at which we could observe negative effects differed considerable between the compounds. This is not surprising and is supported by other studies where PFAS toxicity is known to differ between compounds, in general with higher toxicity of sulfonates compared to carboxylates and increasing toxicity with increasing chain lengths (Oseguera-Lopez *et al.*, 2020; Jantzen *et al.*, 2016; Ulhaq *et al.*, 2013). Porcine oocytes studied upon exposure to PFOS *in vitro* showed decreased maturation at a concentration of 22 µM, but no adverse outcomes were observed at concentrations ≤12.5 µM (Dominguez *et al.*, 2016). The highest concentration tested in our studies was significantly lower (0.053 µg/mL = 0.1 µM). This might imply that embryo development is a more sensitive outcome compared to studying the oocyte directly after exposure.

Developmental rate in blastocysts upon PFAS exposure during IVM

At sub-lethal concentrations, we could see effects on the embryo developmental rate. The mature oocyte drives the first cell divisions without further transcripts or control of the embryo. This period is of great importance, and studies show how embryo quality at day two of culture predicts implantation in humans (Holte *et al.*, 2007), and this indicates how the success or failure of embryonic development is in large part determined at early stages (Wong *et al.*, 2010). This is supported by our and others' observations that embryos that fail to develop to the blastocyst stage usually halt before embryo genome activation (III, IV).

Delayed development through the first cell divisions was observed for all PFAS tested (10 µg/mL PFNA/PFHxS and 0.053 µg/mL PFOS). Upon PFOS exposure, this delayed development was corroborated by reduced developmental rate to the blastocyst stage and reduced cell count in blastocysts. This was also the case upon PFHxS exposure, although at higher

concentrations. A reduced cell count in blastocysts was observed at doses below that causing embryo lethality (20 µg/ml). However, at lower concentrations not affecting the first cell divisions (0.1 µg/mL) PFHxS seems to cause an increased developmental rate at the blastocyst stage. This might imply different mechanisms of toxicity between the different compounds or different mechanisms depending on concentrations (further discussed below).

5.2.5 Lipid distribution in bovine blastocysts upon oocyte exposure to PFASs during IVM (II-IV)

PFASs caused lipid alterations in bovine blastocysts upon oocyte exposure in all treatments, but to various extents. Lipid variation upon PFAS exposure has previously been observed in different experimental settings using different model species (Sant *et al.*, 2021; Seyoum *et al.*, 2020; Yi *et al.*, 2019). The hallmark toxicity observation for PFASs is liver enlargement due to liver steatosis (Chang *et al.*, 2018; Bijland *et al.*, 2011), which is *de facto* related to lipid metabolism and has been attributed to PPAR agonist activity in the liver (Khazaee *et al.*, 2021; Behr *et al.*, 2020; Takacs & Abbott, 2007; Shipley *et al.*, 2004). In bovine embryos, the storage of lipids in droplets increases at the morula stage and decreases at the blastocyst stage (Sudano *et al.*, 2016) when the energy demand is high, which stresses the importance of lipids as an energy source in bovine embryo development. Metabolic challenges or stress might result in the accumulation of lipids, and it is well established that the *in vitro* situation challenges the embryo compared to the situation *in vivo* (Cagnone & Sirard, 2016). The increased volume of lipids seen in cultured pig embryos compared to the *in vivo* counterpart (Romek *et al.*, 2009) is most probably a result of this environmental challenge. Furthermore, serum addition during culture is known to be detrimental for normal embryo development and to promote lipid accumulation (Ordonez-Leon *et al.*, 2014). In our experiments, PFASs caused changes in lipid distribution upon oocyte exposure. This was mostly related to lipid accumulation (0.1 µg/ml PFNA, 0.053 µg/ml PFOS, 1-10 µg/ml PFHxS). We could not observe any changes in higher or lower concentrations of PFHxS implying a non-monotonic dose response of the effect on lipid distribution. At human-relevant levels, PFHxS did not seem to cause an alteration in lipid distribution. PFOS, on the other hand, resulted in lipid accumulation at the higher concentration (0.053 µg/ml) and smaller lipid

droplets upon exposure to the lower concentration (0.002 µg/ml). This could imply different effects on lipid distribution at higher or lower concentrations, within the range where both concentrations are relevant to humans. In summary, we can conclude that lipid deviations occurred at lower concentrations of PFOS compared to PFHxS and (at least at the tested concentration) to PFNA.

We successfully established methods for the objective analysis of lipid content in bovine embryos at our facilities, and this might provide a valuable tool for characterization of the bovine embryo. The 2D technique used for the second study (II) resulted in a blunter assessment of lipid content compared to the more detailed view on volumes in the other experiments (III-IV). I would therefore not encourage comparisons of lipid volumes between treatments. This also applies between the last experiments when the method was slightly updated. Because the same settings were used between experimental groups, the differences within treatments and their respective controls should be as robust as possible under the circumstances. Lipid content measured as total fluorescence intensity correlates with volume estimated using lipid droplet size (Barcelo-Fimbres & Seidel, 2011) and might have increased the comparability between the studies included in this thesis. However, the measurement of lipid droplet size might provide extra valuable information. We could observe this when changes in lipid droplet size were evident without changes in total volume (III). In a previous study examining differences between cattle breeds, they could correlate an increased amount of lipids, but decreased average lipid droplet volume, with lower mitochondrial activity (Baldoceda *et al.*, 2015).

5.2.6 Gene expression and DNA methylation in blastocysts upon oocyte exposure to PFASs (III-IV)

Microarray analysis

Gene expression and DNA methylation microarrays have been used to provide a holistic view of the pathways that are affected in the bovine embryo under different metabolic conditions (Desmet *et al.*, 2016; Laskowski *et al.*, 2016; Girard *et al.*, 2015) and environmental insults (Page-Lariviere *et al.*, 2017; Page-Lariviere *et al.*, 2016). However, RNA sequencing techniques have recently been developed also for small samples and are now available for analysing single cells in embryos (Lavagi *et al.*, 2018). The main difference between hybridisation based techniques (microarrays) and RNA

sequencing is that the latter allow a full sequencing of the RNA transcriptome while hybridisation techniques rely on predetermined profiles in the microarray. This lead to a clear advantage in coverage with RNA sequencing. More insight into toxicity mechanisms may be achieved by the greater number of transcripts identified and this also imply that RNA sequencing may identify more transcripts of toxicological interest such as non-coding transcripts (microRNA). Even so, the results between RNA sequencing compared to microarray-based platforms are quite coherent, and a study exploring the differences concluded that the altered pathways and by extension, the conclusions drawn, are similar between the methods (Rao *et al.*, 2018).

Changes in gene expression after PFAS exposure

Overall, the treatments resulted in the majority of transcripts being downregulated and only a few significant transcripts were upregulated. The fact that the PFHxS concentration was two times higher than the higher concentration of PFOS but the number of transcripts were fewer, suggest that the bovine oocyte is less sensitive to PFHxS compared to PFOS also on the transcriptomic level. Gene expression changes were modest. This is most likely due to the fact that we look at subtle effects, also on the phenotypic side, *i.e.,* not mortality and gross morphological changes. Small changes in gene expression could still be important, especially when they belong to the same pathways.

Early embryos have limited defence against insults from the environment, and it is only when the embryonic genome is activated that the embryo can respond to environmental factors (Van Soom *et al.*, 1997; McKiernan & Bavister, 1994). Although having no opportunity to change their surroundings, embryos can modulate their transcriptome and activate adaptive mechanisms in order to survive (Puscheck *et al.*, 2015). However, we do not know how these early effects translate into later effects, such as possible influence on later development or offspring health.

Potential mechanisms of PFAS toxicity in the bovine embryo

The transcriptome data predicted dysregulation of pathways associated with proliferation and differentiation. This could provide a mechanistic explanation for the delayed first cleavages and the altered developmental rate at the blastocyst stage. The molecular data suggested further how apoptotic pathways and embryo stress response were affected in the blastocysts. As the

in vitro situation per se increase stress response it is interesting to compare the results from our molecular studies with the situation *in vitro* vs. *in vivo* where the embryos show activation of similar mechanisms (Cagnone & Sirard, 2016). There are also similarities between our findings and those after exposure to ethanol during embryo culture (Page-Lariviere *et al.*, 2017). It could be speculated that PFAS exposure during IVM increases embryo stress and adds to the stressors already present in the *in vitro* system. *TP53* activation has further been demonstrated to be the key player in the increased apoptosis observed after PFAS exposure (Chen *et al.*, 2017; Shi *et al.*, 2008).

We did not find clear associations with PPARα-dependent effects in the blastocyst after exposure during IVM in the bovine model (III-IV). Instead, our molecular data suggested that other pathways related to cell metabolism are dysregulated after PFOS exposure. This predicted decreased levels of lipid synthesis and storage. It seems as though the embryo adapted and tried to counteract the lipid accumulation that was seen in the phenotype. As previously discussed, PFAS are known PPAR activators and *PPARα* contributes to most transcriptional changes (>75%) in the murine liver (Rosen *et al.*, 2017). This discrepancy might be explained by the embryo coping with the environmental challenge during oocyte maturation one week previously. This is different from measuring the effect directly on the oocyte. Furthermore, there are obvious differences between the oocyte and the liver. For example, in the maturing oocyte, PPARγ seems to be more important compared to PPARα. The addition of a PPARγ activator during IVM decreases functional fatty acid oxidation while upregulating genes involved in fatty acid activation. This ultimately results in negative effects on embryo development (Dunning *et al.*, 2014). Furthermore, perfluoroalkyl sulfonates (*e.g.,* PFOS, PFHxS) appear to have gene expression profiles more similar to PPARγ in differentiating adipocytes. Perfluoroalkyl carboxylates (*e.g.,* PFNA, PFOA), on the other hand, alter genes in more modest ways and more similarly to PPARα in this system (Watkins *et al.*, 2015). PFHxS seem to be more prone to activate pathways independent of PPARα compared to other PFAS (Rosen *et al.*, 2017).

DNA methylation changes

Our molecular results of changes in DNA methylation are interesting, and the experimental setting leading to these changes has to be emphasized. During the exposure period (*i.e.*, final oocyte maturation), the embryonic DNA is highly methylated (Kafri *et al.*, 1992). During further development,

the genome will go through reprogramming and *de novo* methylation. During the studied period (day 8 of culture), the bovine genome is only starting to become methylated again after this large restructuring. This process is not yet finished but will continue for the following period, which means that the methylation at day 8 is significantly lower compared to day 12 in the bovine genome (Montera *et al.*, 2013). Thus, the changes that we observed after PFAS treatment during oocyte maturation are potentially persistent beyond the reprogramming process. However, we do not have the means to test this hypothesis in this experimental setting and can thus not state whether these early effect would translate into later development or disappear. The changes in DNA methylation were modest, and the observed alterations have to be interpreted with the absence of adjustment for multiple testing or fold change threshold in mind (similar to the gene-expression array). Even so, the strength of the analysis lies in the inclusion of several molecules for each pathway analysed. Furthermore, changes seen consequently in a dose-dependent manner (III) or in both the microarrays for gene expression and methylation changes (III-IV) further strengthen the reliability of these findings.

Epigenetic changes have been associated with disease later in life (Baccarelli & Bollati, 2009), and chemical exposure has been linked to changes in the epigenetic landscape (Jacobs *et al.*, 2017). For all tested compounds, the molecular data predicted similarities in dysregulated pathways in both the transcriptomic and the epigenomic microarrays that were related to embryo stress or apoptosis, *e.g.,* through altered *TP53* signalling (III, IV). The DMRs further predicted biological functions related to cell death, apoptosis, and proliferation (III) and dysregulation of *beta-oestradiol* signalling in the blastocysts (IV). PFASs has previously been associated with epigenetic changes in women (Xu *et al.*, 2020), but to my knowledge the studies included in this thesis are the first to report epigenetic changes in bovine blastocysts upon oocyte exposure to PFAS.

5.3 Implication for subsequent development

5.3.1 The right developmental stage at the right time

Even though the processes are complex, delayed development as early as the time of the first divisions of the zygote is predictive of later blastocyst quality

in humans (Wong *et al.*, 2010) and successful live birth (Rhenman *et al.*, 2015). This suggests that the delayed cleavage seen in our studies during early cleavages (II-IV) is of potential relevance for later development. In humans, the implantation window is narrow and the blastocyst needs to be at the right developmental stage at the right time for successful implantation to occur (Franasiak *et al.*, 2016). The decreased developmental rate seen after PFOS exposure at the blastocyst stage (III) and the decreased number of cells in blastocysts after PFHxS exposure (IV) could hamper this. One might speculate that such disruption of timing of early events might lead to decreased chance of implantation, which could be one mechanism explaining the reports of delayed time to pregnancy associated with PFAS exposure in human cohorts (Velez *et al.*, 2015; Fei *et al.*, 2009). This delayed development at the blastocyst stage could also provide insights into the mechanisms involved in reduced intrauterine growth thus associating PFOS (and PFNA but not PFHxS) with lower birth weight in humans (Wikström *et al.*, 2020).

5.3.2 Altered lipid distribution of the embryo and possible impact on the offspring

There is evidence suggesting that lipid accumulation during embryogenesis is detrimental for blastocyst viability and further development in bovines. Increased lipid accumulation in blastocysts, *e.g.,* from serum addition during culture (Ordonez-Leon *et al.*, 2014) decreases cryotolerance in bovine embryos (Abe *et al.*, 2002). Hence, lipid accumulation seems to decrease the viability of the embryo or the capacity of the embryo to respond to environmental challenges. This implies that the altered lipid distribution we observed in our experiments is probably not beneficial for subsequent embryonic development.

In human cohorts, PFAS exposure has been linked to increased serum lipids and cholesterol (Nelson *et al.*, 2010; Steenland *et al.*, 2009) and is suggested to have obesogenic effects on the offspring (Halldorsson *et al.*, 2012). These findings have been corroborated by experiments in mice, where altered glucose and lipid metabolism have been observed upon exposure *in utero* (Lee *et al.*, 2015; Wan *et al.*, 2014). There is also evidence to suggest that lipid accumulation in zebrafish fry persists also after a period of recovery (Du *et al.*, 2009). Even if no causal relationship has been established, I am

intrigued to think that our findings in the early embryo fit well with the changes seen in offspring and adults.

5.3.3 Evidence of affected female fertility

To extrapolate the findings in our experiments to human risk assessment, it is important to consider the exposure concentrations. For PFHxS and PFNA, the concentrations clearly exceeded the exposure of the general public and were only in range for some extremely highly contaminated populations (Li *et al.*, 2018). For PFOS, however, both concentrations represented the general human situation. Embryo lethality occurred at higher concentrations. More subtle negative outcomes such as altered phenotype or dysregulation of gene pathways and DNA methylation that we could observe at human-relevant levels are more difficult to assess in regards of human relevance.

Nevertheless, with the corroborated findings in phenotype, gene expression, and DNA methylation taken together and with the evidence already present for mechanisms of PFAS toxicity, I would personally think that these results provide further evidence for the negative effects of PFASs on female fertility. Furthermore, we have only considered single exposures during a short exposure window. It seems as though exposure to mixtures with PFASs does not increase the potency of the individual chemicals (Menger *et al.*, 2020). Nevertheless, in combination with other chemicals, the mixture with PFAS may cause changes when the compounds alone do not (Deng *et al.*, 2020; Ramhøj *et al.*, 2020), implying that the adverse effects of mixtures cannot be neglected.

6. Conclusion

The studies included in this thesis investigated the composition of human ovarian follicular fluid in humans and the effects of exposure of the maturing oocyte to selected PFASs *in vitro* using a bovine model.

Our results demonstrate that:

- NTS using LC-HRMS is applicable to ovarian follicular fluid and serum, and the data obtained can be valuable for future research.
- The ovarian follicular fluid consists of a complex environment of endogenous and exogenous compounds, where thousands of compounds are unidentified.
- Over 200 features accumulate in follicular fluid at a ratio >20, and another 252 features might be associated with embryo quality.
- PFASs may be detected using suspect screening, and PFASs are transferred to follicular fluid at approximately the same ratio as in blood.

The developmental competence of the bovine oocyte is affected upon PFAS exposure during oocyte IVM.

- Exposure of the bovine oocyte to high concentrations of PFHxS and PFNA caused diminished capacity to develop into the blastocyst stage.
- Exposure of the bovine oocyte during IVM to PFASs concentrations relevant to the human general or certain exposed populations interferes with bovine early embryo development by

affecting developmental rate, cell count, and lipid distribution in developing blastocysts, but does not cause embryo lethality.

- Blastocysts show an overall decrease in gene expression upon oocyte exposure to PFOS that is more pronounced at the higher concentration. Differently expressed genes are associated with pathways related to cell death, proliferation, and metabolic stress response and suggest a possible molecular explanation for the phenotypic changes seen in the blastocysts.
- Upon oocyte exposure to PFHxS, gene expression patterns in blastocysts show an overall decrease in expression. Differentially expressed genes are associated with pathways related to increased oxidative stress and dysregulation of pathways related to metabolic and hormonal response.
- The transcriptomic data from blastocysts exposed to PFOS and PFHxS showed similarities but also differences, implying that the mechanisms of toxicity differ between the compounds in the bovine model, and some of the pathways associated with the transcriptomic data could also be corroborated by DNA-methylation, which might imply more persistent changes in embryos.

7. Future perspectives

During the work presented in this thesis, we were able to answer some questions. This contributed to our knowledge of the exposure of the developing oocyte in humans and the effects of certain PFASs on early embryo development *in vitro*. Further studies are encouraged to increase our knowledge about potential health effects and implications for human health. Questions raised during this work include:

- Does the composition of human follicular fluid affect outcomes of assisted reproductive technologies?

  The first study using LC-HRMS data to investigate the composition of follicular fluid did not establish a model where single features explained the embryo quality. Suggestions for moving forward could include further modelling with the data to, for example, consider mixture exposure or the association between exposures to groups of compounds (*e.g.,* total fluorocarbon compounds).

- How does the contamination of human follicular fluid change over time?

  I would encourage future research to investigate how the contamination in human ovarian follicular fluid differs over time. This would include samples taken previously or in the future in relation to the data obtained from our cohort with the aim of identifying unknowns with increasing exposure.

- Can the *in vitro* embryo production model be used for screening of chemicals for reproductive toxicity on a larger scale?

  Based on the results of our studies, there is potential to increase the use of the bovine model in toxicity testing. Other species such as porcines may also provide a valuable tool. Something that might limit the use is the interpretation between effects on early embryo development and live offspring, and this would also be interesting to study further.

- Can the effects on blastocyst stage be predicted by studying the oocyte after IVM?

  It would consume less time (and resources) if endpoints directly after IVM would predict effects seen at blastocyst stage, and this would be interesting to study further, especially non-invasive methods that would allow further development of the evaluated oocytes.

- Does long-term exposure imply a more pronounced embryo toxicity?

  There are many questions in this area that would be interesting to explore, including longer or other windows of exposure in the bovine IVP system more similar to the exposure in real life. Within the scope of experimental studies, it would also be interesting to model the development for a longer period. Such work could include follicular cultures and/or models of implantation in different species.

- Can the findings from the molecular studies be corroborated by the blastocyst phenotype upon PFAS exposure?

  The gene-expression and DNA-methylation microarrays suggested that the altered pathways are related to embryo stress, increased apoptosis, and metabolic alterations. It would be interesting to investigate if these changes are corroborated by the phenotype of the embryo. Suggestions would be to use, for example, fluorophores to

detect mitochondria and apoptotic cells and to assess reactive oxygen species production.

- What is the effect of mixtures on oocyte developmental competence?

  To mimic the human exposure, one has to consider mixture effects. This is possible using the bovine IVP, and the challenge is mainly in composing the relevant mixtures and evaluating the results. There is a possibility to use the NTS approach "the other way around". One could prepare extracts from pools of women (*e.g.,* high/low embryo quality or other variables) and expose oocytes to these extracts in an effect-directed analysis using LC/GC-MS or a combination of both techniques.

- What doses are relevant to study in the *in vitro* setting?

  A safety margin is usually applied in experimental studies to account for the possibility that humans are more sensitive compared to the experimental animal, but the optimal composition is not yet known. Lower doses might exert different/other effects, and humans might be more or less sensitive compared to the animal used.

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Popular science summary

Millions of couples worldwide suffer from involuntary childlessness. In many couples, the reason cannot be explained even with thorough examinations. The content in this thesis focuses on female fertility and more specifically the egg cell. In women, fertility is dependent on the egg cell. The quality of the egg further affects early development as well as the chance of achieving pregnancy and even the development to come after that. Many groups of chemicals are found in the fluid surrounding the egg in the female ovary that might affect subsequent developmental potential. One of these groups are perfluoroalkyl substances (PFASs). These chemicals are found in products with oil, stain, and water-repellent surfaces, and humans are mainly exposed through food and water. PFASs have been found in the fluid around the egg. There are studies suggesting that PFASs might affect female fertility, but there is currently not enough evidence to draw firm conclusions.

In this thesis, we have investigated the complex environment to which the egg is exposed and how this might interfere with the development of the egg cell. We used a novel approach of screening of organic compounds in blood and the fluid surrounding the egg (the follicular fluid) in women. This approach allowed us to gather information on a large amount of compounds. We identified 11 PFASs, which surrounded the egg in similar concentrations as were found in the blood. Furthermore, we could identify substances where the concentration surrounding the egg was connected to the embryo quality as well as substances that were in higher concentrations around the egg compared to the blood. The work was applied to identify some compounds, but all of the retrieved data can be used in future research.

We wanted to investigate more in detail what happens during the early development after the egg cell has been exposed to certain chemicals. For this purpose, we used a laboratory cow model, and we retrieved eggs from

cow ovaries at the slaughterhouse. Therefore, we could perform experiments on the eggs without using experimental animals. We treated eggs with three different PFAS chemicals, fertilized the eggs, and followed the embryo development after the treatment. In this way, we could investigate if the compounds were harmful for egg development.

We found that high concentrations of PFASs caused embryo death. We also found that eggs exposed to PFASs developed at a slower pace. The early development is very strictly regulated, and small changes might affect the chance of achieving a pregnancy. The fat content in the embryos was changed after the eggs had been exposed to PFASs. Most commonly the fat content was increased. We do not know what increased fat content in the embryos means for the offspring, but it is most probably not a beneficial signature in the embryo. The gene expression was investigated and we could find signalling cascades related to the effects in the embryos. We could also see how the DNA had been "programmed" differently in the embryos after the eggs were exposed, which could persist during subsequent development.

The knowledge obtained through the work in this thesis can contribute to the increasing body of evidence suggesting that chemical exposure can affect female fertility. We could show how the egg cell is exposed to thousands of compounds, both produced in the body and from chemical exposure. We could further show how one single chemical exposure (PFAS) has the power to affect cow early embryo development after a short time of exposure of the egg. Some of the changes we saw in the cow model occurred at concentrations as low as those that can be measured in the average human population.

Populärvetenskaplig sammanfattning

Miljontals par lider av ofrivillig barnlöshet i världen. Även efter noggranna undersökningar förblir orsaken till den minskade fruktsamheten okänd i många fall. Arbetet i den här avhandlingen handlar om kvinnlig fruktsamhet och mer specifikt äggcellen. För kvinnor är fruktsamheten helt beroende av en äggcell som är kapabel att driva den tidiga embryoutvecklingen. Äggets kvalitet påverkar inte bara den tidigare utvecklingen utan har även betydelse för embryots förmåga att fästa i livmodern och till och med utvecklingen senare än så. Under tiden ägget mognar i äggstocken exponeras det för kemikalier som kan påverka äggets kvalitet. En grupp av dessa kemikalier är högfluorerande ämnen (PFAS) som finns i produkter som vatten- fett- och smutsavvisande material. Människor får i huvudsakligen i sig ämnena via vatten och mat och hos de flesta finns flera PFAS i vätskan runt ägget i äggstocken. Det finns tidigare forskning där man misstänker att PFAS kan ha negativa effekter för kvinnlig fruktsamhet, men ännu är ingen definitiv slutsats dragen.

I den här avhandlingen har vi undersökt den komplexa miljön i vilken ägget utvecklas. Vi har använt en ny metod med en sållning av organiska föreningar i prov från vätskan runt ägget (follikelvätskan) samt i blod från kvinnor. Vi identifierade 11 PFAS, som fanns i liknande koncentration i vätskan kring ägget som i blodet. Vidare kunde vi identifiera ämnen som misstänks kunna vara associerade med embryokvalitet och ämnen som återfinns i högre koncentration runt ägget jämfört med i blodet. Med den här metoden kunde vi samla in data från en stor mängd ämnen, en del kunde helt identifieras, men informationen kan också användas i framtida forskning.

Vi ville vidare undersöka mer i detalj vad som händer med den tidiga utvecklingen efter att ägget exponerats för olika kemikalier. För att undersöka detta använde vi ko som modelldjur. Äggceller från kor hämtades

från slakteri, vilket gjorde att vi kunde utföra experimenten utan att använda försöksdjur. Äggen behandlades med selekterade PFAS och därefter följde vi embryoutvecklingen på laboratoriet med syfte att undersöka om exponeringen kunde påverka embryoutvecklingen.

Resultaten visar hur höga koncentrationer av PFAS är direkt dödligt för embryon. I lägre doser kunde vi se hur deras utvecklingshastighet var påverkad. Varje delning av celler är strikt reglerad under den tidiga utvecklingen och endast små avvikelser kan bidra till minskade chanser att bli en graviditet. Vidare var fettinnehållet i embryot ändrat efter att ägget blivit exponerat för PFAS. Det vi främst såg var att innehållet fett ökade. Vi vet inte exakt vad detta innebär för avkomman, men däremot är det med stor sannolikhet inte positivt för embryots utveckling. Undersökning av genuttryck och DNA gjordes för att försöka förstå mekanismen bakom förändringarna vi sett i embryona. Dessa analyser kunde identifiera signalvägar som var påverkade och skulle kunna förklara dessa. Programmeringen av DNA var också förändrad, vilket är något som kan kvarstå även under den fortsatta utvecklingen.

Sammanfattningsvis kan resultaten i denna avhandling bidra till den ökade kunskapen om kemikalieexponering och kopplingen till kvinnlig fertilitet. Vi kunde se hur ägget under mognaden exponeras för tusentals ämnen, både sådana som är producerade i kroppen och direkt gynnsamma, men också miljögifter. Vidare visar resultaten hur exponering av enstaka kemikalier (PFAS) kan påverka den tidigare embryoutvecklingen även efter en kort exponering av äggcellen på laboratoriet. Vissa förändringar vi observerade under embryoutvecklingen sågs vid koncentrationer som är samma som de som är uppmätta i människor.

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I

Environmental Science Processes & Impacts

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Suspect and non-target screening of ovarian follicular fluid and serum – identification of anthropogenic chemicals and investigation of their association to fertility†

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In this work, ultra-high performance liquid chromatography-high resolution (Orbitrap) mass spectrometry-based suspect and non-target screening was applied to follicular fluid ($n = 161$) and serum ($n = 116$) from women undergoing *in vitro* fertilization in order to identify substances that may be associated with decreased fertility. Detected features were prioritized for identification based on (i) hazard/exposure scores in a database of chemicals on the Swedish market and an in-house database on per- and polyfluoroalkyl substances (PFAS); (ii) enrichment in follicular fluid relative to serum; and (iii) association with treatment outcomes. Non-target screening detected 20 644 features in follicular fluid and 13 740 in serum. Two hundred and sixty-two features accumulated in follicular fluid (follicular fluid: serum ratio >20) and another 252 features were associated with embryo quality. Standards were used to confirm the identities of 21 compounds, including 11 PFAS. 6-Hydroxyindole was associated with lower embryo quality and 4-aminophenol was associated with higher embryo quality. Overall, we show the complexity of follicular fluid and the applicability of suspect and non-target screening for discovering both anthropogenic and endogenous substances, which may play a role in fertility in women.

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Environmental significance

Millions of couples worldwide suffer from involuntary childlessness. The role of anthropogenic chemicals in ovarian follicular fluid is important to assess as they may affect the maturing oocyte and its competence to form a viable embryo. In this work, suspect and non-target screening of follicular fluid and serum together with a 3-tiered prioritization strategy were used to identify substances that may be associated with decreased fertility in women undergoing IVF treatment. These data show the applicability of suspect and non-target screening for discovering both anthropogenic and endogenous substances, which may play a role in fertility in women.

Introduction

Human infertility is defined as the inability to conceive within 12 months of actively trying, and is estimated to affect up to one out of six couples,[1–6] comprising approximately 25 million citizens in the European Union alone.[7] In 25–30% of fully investigated couples, the reason for infertility remains unknown.[5,6] The quality, or developmental competence, of the oocyte affects the early survival of the embryo as well as the establishment of pregnancy and subsequent fetal development.[8] Oocyte quality is in turn dependent on follicle growth and oocyte maturation, which is achieved during folliculogenesis, a process that takes at least half a year in humans from primordial to ovulatory stage. Primordial follicles develop through primary and secondary stages and further to antral follicles which contain follicular fluid. The follicular fluid (FF) surrounding the maturing oocyte consists of secretions from granulosa and theca cells as well as transudates from the circulation, including for example growth factors and sex steroids, but also exogenous substances.[1,9–12]

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Growing evidence indicates that exposure to endocrine disrupting chemicals (EDCs) may negatively impact human fertility by affecting the oocyte and early embryo development,[13–17] which is supported by animal studies and findings *in vitro*.[18–24] Chemicals including bisphenol A (BPA), polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), per- and polyfluoroalkyl substances (PFAS), and polybrominated diphenyl ethers (PBDEs) have been found in ovarian FF exposing the oocyte,[13,25–31] with the possibility of interfering with oocyte maturation. PFAS are of particular concern due to their widespread occurrence in human blood. However, there are conflicting results regarding the effects of PFAS on fertility in epidemiological studies,[25,27,32–37] which stresses the need for further investigations, especially since PFAS seem to target the ovary in different ways.[38] For example, experimental *in vitro* studies suggest adverse effects of PFAS on oocyte maturation.[39,40]

According to recent estimates, over 300 000 chemicals are or have been in global chemical commerce and now constitute potential environmental pollutants.[41] Traditionally, highly specific and targeted analytical approaches are applied to characterize human exposure to known substances. As a result, numerous chemicals and their transformation products are overlooked. To fill this knowledge gap, suspect- and non-target screening (NTS) approaches have emerged over the last decade as promising techniques for capturing novel anthropogenic substances. Prioritization strategies, which reduce the quantity of data from tens of thousands of features, are critical to the success of these methods.[42] For example, time trends have been used to prioritize anthropogenic chemicals over endogenous substances in human serum[43] and whole blood[44,45] while case/control strategies have been employed for elucidating chemicals specifically associated with occupational exposure.[46,47] Other studies have employed suspect screening approaches, focusing on matching features to mass spectral databases of anthropogenic substances.[43,48]

In this work, we applied an NTS approach to human ovarian FF and serum in order to identify substances that could potentially affect fertility in women. A 3-tiered feature prioritization approach was utilized, involving (i) matching to substances with high exposure and unknown- or moderate-high hazard scores in the Swedish Chemicals Agency Market List and further an in-house PFAS database; (ii) enrichment in FF relative to serum; and finally, (iii) association with *in vitro* fertilization outcomes with a focus on embryo quality. To the best of our knowledge, this is among the first studies to use NTS to investigate ovarian FF. In addition to providing insight into associations between chemicals and reproductive outcomes, the acquisition of non-target data offers the opportunity for retrospective mining, as new chemicals become available.

Material and methods

Patient recruitment

This study was approved by the Swedish Ethical Review Authority (Dnr 2015/798-31/2, 2016/360-32 and 2016/1523-32). Patients that visited the Carl von Linné clinic in Uppsala, Sweden, for assisted reproductive technology (ART) procedure consisting of ovum pick-up (OPU) for *in vitro* fertilization (IVF) were invited to participate in the study. Participation was voluntary; the patients could withdraw their consent at any point and they were informed that their participation would not affect their IVF-treatment. Patients received written and oral information about the study from a nurse and those agreeing to participate signed a written consent form in accordance to the Declaration of Helsinki.

From April 23 to June 16, 2016, 244 patients visited the clinic for OPU. Fifty-four patients were not invited to participate due to lack of time ($n = 36$), non-Swedish speaking patients ($n = 14$), freezing of oocytes for storage and later use ($n = 1$), and avoiding pressure on emotionally stressed patients ($n = 3$). This resulted in 190 invited patients, of whom five declined and 185 accepted to participate in the study. All patients enrolled in the study were assigned a random 3-digit code for pseudonymization of the samples. Information regarding reproductive history, cause of infertility as well as other health parameters were collected from the patients' records. Data was handled in compliance with relevant laws and institutional guidelines (the Swedish data protection law, PUL, and the general data protection regulation, GDPR), and the biological samples were registered at Uppsala Biobank (IVO 627) following the Swedish law on biobanking in health care. In the final analysis, we included patients who were non-smokers with body mass

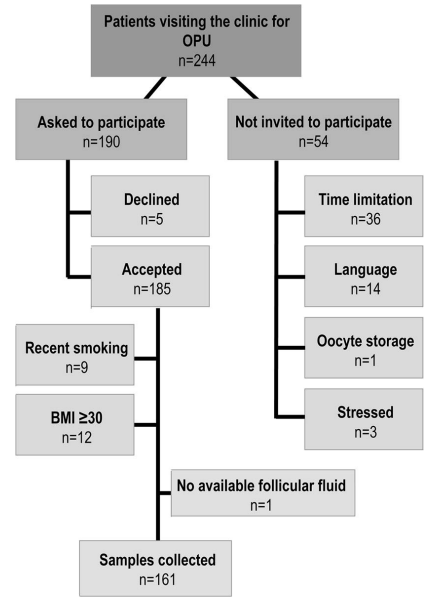

Fig. 1 Flow chart of patients visiting the clinic for ovum pick-up from April 23 to June 16, 2016. Excluded patients and reason for exclusion are presented in the chart. Sampling from one patient resulted in no available follicular fluid because of blood contamination, resulting in 161 samples analyzed.

indices (BMIs) of <30 and ongoing treatment with OPU during the recruitment period. Nine patients were excluded due to smoking during the last 12 months and 14 were excluded due to BMIs ≥30 resulting in 162 patients for sample processing (Fig. 1).

Sample collection

Clear FF with no visible blood contamination ($n = 161$) was freshly collected after OPU in 50 mL test-tubes (559 001, Sarstedt, Nümbrecht, Germany) on ice, discarding the first aliquot due to possible contamination with wash-fluid used in the OPU-tubing system. All FF aliquots from a single patient were pooled together into one sample. The samples were centrifuged at 500×*g* to separate cells from the supernatant FF, and the supernatant was subsequently aliquoted and stored at −80 °C until analysis. Serum collection was carried out as follows: prior to OPU, patients were given an intravenous catheter and blood was drawn into two tubes (GREI456089, VWR, Stockholm, Sweden). The first tube was discarded in order to avoid contamination from the catheter and the second was kept for processing. The blood was centrifuged within 30 minutes at 1400×*g* (5 minute duration) and serum was separated and stored at −80 °C until analysis. For NTS, >1.5 mL was needed for analysis, resulting in 116 samples.

Standards and reagents

A full list of 28 authentic- and 11 isotopically labelled substances can be found in Table S1 and 2 of the ESI.† The internal standard mixture was ready available in the laboratory and included substances with a wide range of physiochemical properties (*e.g.* K_{ow} ranging from ∼ −1 [sucralose] to 5.7 [tonalide]).

Extraction

The extraction procedure was originally developed for human whole blood samples and is described in detail elsewhere.[44,49] Prior to extraction, samples (1.5 mL of serum or 2 mL of follicular fluid) were thawed at room temperature and then spiked with 10 μL of internal standard mixture (50–125 ng of each substance; see Table S1 in the ESI† for a full list). Two mL of acetonitrile, 200 mg NaCl and 800 mg $MgSO_4$ were added to 2 mL of FF while 2 mL of acetonitrile, 150 mg NaCl and 600 mg $MgSO_4$ to 1.5 mL of serum. After addition of steel beads, a bead-blender (1600 Mini-G, SPEX Sample Prep, Metuchen, NJ) was used to homogenize the samples for 5 min at 1500 rpm. The mixture was centrifuged for 10 min at 2200×*g* and the supernatant was removed. This extraction was repeated with another volume of acetonitrile and the combined extracts were concentrated under nitrogen to 150 μL. After freezing overnight, the samples were centrifuged (5 min at 8000×*g*), and 100 μL of extract was transferred to a LC-vial containing 100 μL LC-MS grade water.

Instrumental analysis

Instrumental analysis was carried out using a previously developed method.[44] Briefly, chromatographic separation of analytes was carried out with a Dionex UltiMate 3000 ultra-high performance liquid chromatograph equipped with a Hypersil GOLD aQ analytical column (2.1 mm × 100 mm, 1.9 μm I.D.) and a prefilter (2.1 mm, 0.2 μm) (Thermo Scientific, USA). The mobile phases consisted of LC-MS grade water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient started at 5% B with a linear increase over 10 min to 99% B, followed by a 5.5 min hold and re-equilibration at 5% B for 2 min. The injection volume was set to 5 μL and the column temperature was held at 40 °C. Detection was carried out on a Q Exactive HF Orbitrap (Thermo Scientific, USA), equipped with a heated electrospray ionization (HESI) source. The capillary temperature was set to 350 °C with a spray voltage of 4.5 kV (positive mode) and 3.7 kV (negative mode), sheath gas (nitrogen) at 30/45 arbitrary units (pos/neg mode), auxiliary gas at 10/5 au (pos/neg) and auxiliary gas heater at 350 °C. A full scan was combined with a data-dependent MS2 (ddMS2) fragmentation on the top five. The full scan was run with a resolution of 120 000 Full Width at Half Maximum (FWHM) at 200 *m*/*z* and a scan range of 100–1000 Da. ddMS2 scans were run with a resolution of 15 000 FWHM at 200 *m*/*z*, normalized collision energy of 30%, an intensity threshold of 1×10^5, a dynamic exclusion for 10 seconds and an apex search between 1–10 seconds. Samples, quality controls (QCs) and blanks were run in random order with one internal standard repeated every 15 samples. Samples were run in four sequences: FF-positive mode, FF-negative mode, serum-positive mode, and serum-negative mode. A mass calibration of the Orbitrap was performed before each sequence of samples.

Data processing

Alignment, peak picking and feature determination were carried out using Compound Discoverer, versions 2.0 and 3.1 (Thermo Scientific, USA). Aggregation of peaks and adducts was implemented to define unique features. All Compound Discoverer parameters are listed in Table S3 and 4 in the ESI.† The four sequences (FF pos/neg, serum pos/neg) were processed separately in Compound Discoverer 2.0 (parameters listed in Table S3, ESI†), resulting in four separate feature lists (containing peak areas in all samples for each feature). For tier 2 (see below) we wanted to directly compare feature peak areas in FF to those in serum, which was done by reprocessing with Compound Discoverer 3.1 (this new version just became available when starting to work on this part; parameters are listed in Table S4, ESI†). This time only two feature lists were generated, one for positive and one for negative mode, each including both FF and serum samples from 116 patients (including procedural blanks for blank subtraction). For these combined feature lists the intensity thresholds during peak picking had to be raised compared to the first processing, due to the larger number of samples that needed to be processed (and by extension, the amount of time this would take). All calculated ratios between FF and serum for detected suspects were taken from these combined feature lists.

Following blank subtraction (max area sample/area blank > 5), feature prioritization was carried out using a three-tier approach (Fig. 2) which is described in detail below.

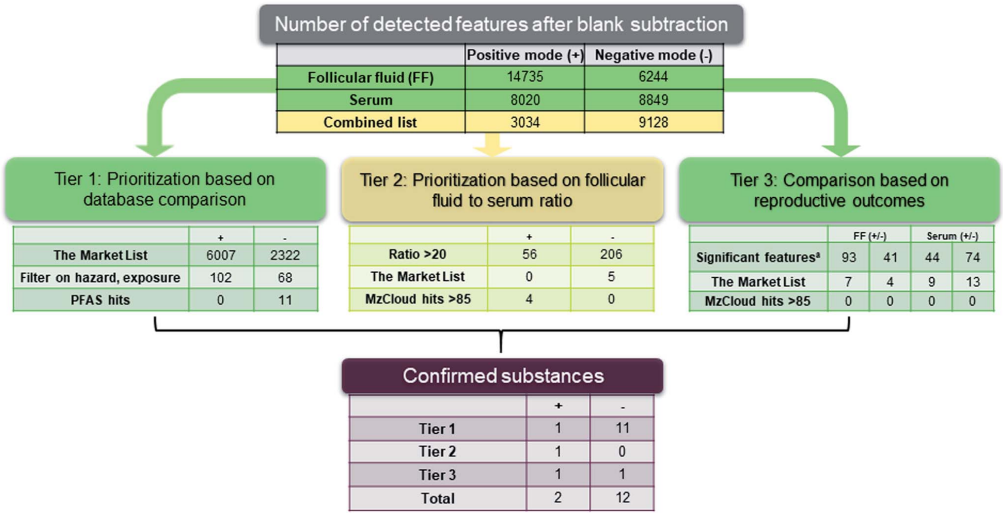

Fig. 2 Overview of the tiered prioritization strategy including numbers of features and tentatively identified suspects identified using the databases the Market List and MzCloud, and confirmed substances measured in positive (+) and negative (−) ionization mode presented for all tiers. Features are defined as the combination of all ions (*i.e.* adducts, parent ion, in-source fragments, *etc.*) at a given retention time. Some duplication may exist for substances ionizing in both positive and negative mode. The combined list (yellow box) was generated re-processing the data using different thresholds (see Data processing for details) and applied in Tier 2. The Market List matches were filtered on both high exposure and unknown-to-high hazard score (see Data processing for details). [a]significantly ($p < 0.05$) different (fold change > 1.5) suspects in the two groups.

Substances identified through this workflow were assigned a confidence level using the Schymanski scale.[50] In brief, suspects identified through databases based on exact mass (±5 ppm) but with insufficient information for one exact structure were defined as a tentative identification and assigned a confidence level [CL] from 3–5 depending on the available data. With further information such as MS2 matches to a library spectrum the suspect was defined as a probable identification with CL 2a or as 2b when there was additional experimental evidence like homologue series. Suspects confirmed with an authentic standard were considered CL 1. Further details can be found in the ESI, Table S5.†

Quality control

In order to avoid false-positives, both sampling blanks and procedural blanks were processed together with samples. Sampling blanks ($n = 5$) were prepared immediately prior to follicular fluid collection by rinsing the sample collection tubing with buffer using enough volume to fill the system with fluid and collect a volume of ~0.5 mL after the rinse (G-RINSE, Vitrolife, Göteborg, Sweden), which was then saved for analysis. Serum was collected using vene-catheters (Venflon PVK 20 G, BD Medical Surgical Systems, Stockholm, Sweden) and sampling blanks prepared and processed the same as for follicular fluid, by rinsing vene-catheters ($n = 3$) prior to patient sampling. Each extraction batch of FF ($n = 16$ samples/batch) and serum ($n = 19$ samples/batch) included one procedural blank consisting of 1 mL of MilliQ water (in total $n = 10$ procedural blanks for FF samples and $n = 6$ for serum samples). During the initial stage of data processing, features with signal intensities within 5-fold of the average procedural blank intensity were removed from the dataset and were not considered thereafter. This removed 13–54% of the features in the datasets (see Table S6, ESI† for exact numbers). Moreover, once a feature was identified using an authentic standard, we re-confirmed its absence in both the sampling and procedural blanks.

In order to account for procedural losses and confirm that the extraction procedure was suitable across a wide range of substances, a suite of 11 internal standards (Table S1, ESI†) were spiked into all samples prior to extraction. Internal standard recoveries in both FF and serum samples were acceptable, ranging from 61 to 107% for all substances (relative standard deviations (RSDs) ranging from 13–30% in serum and 13–23% in FF for all samples; see Fig. S1 in the ESI†). Each extraction batch of FF or serum contained a pooled QC sample prepared from $n = 3$ samples of FF or 150 μL portions of all serum samples which were extracted the same way as patient samples. Internal standard recoveries in both FF and serum QCs ranged from 45–119% with RSDs of 5.4–15.9% in serum QCs and 4.5–14.2% in FF QCs (Fig. S1 and Table S8, ESI†). Finally, during instrumental analysis, internal standard solutions prepared in acetonitrile were analysed every 15 samples to monitor

instrumental drift over the course of the run. RSDs for internal standard peak areas in these solutions ranged from 1.8 to 21% and showed an absence of signal drift over the course of the run (Table S8, ESI†).

Tier 1: prioritization based on database comparisons

Applying the four separate feature lists, features detected in FF or serum samples were compared to two different databases: the Swedish Chemicals Agency Market List (KEMI Market List, referred to herein as the "Market List") and an in-house exact mass list of PFAS containing 279 PFAS previously detected in house or the literature. The Market List is available on the NORMAN Suspect List Exchange website[51] and contains 30 000 substances (industrial chemicals, pharmaceuticals, pesticides, *etc.*) from different national/regional inventory lists with a focus on the EU market. The Market List prioritizes substances based on both human hazard and exposure scores, which are based on confidential data from the importer/manufacturer supplied to the Swedish Chemicals Agency. The exposure score is calculated based on risk of environmental contamination, such as the quantity and degree of uncontrolled release during use and the extent of use on the market (range 0–27, where 27 represent the greatest exposure). The hazard score is calculated based on hazard classifications described by the EU's Classification, Labelling and Packaging (CLP) regulation, using toxicological information regarding carcinogenic, mutagenic and reproductive toxicity (range 0–9 where 9 represent the greatest hazard) (for details, see Market List documentation).[52] The entire feature list (*i.e.* exact masses with 5 ppm tolerance) in serum and FF was compared to the Market List and resulted in 18 462 matches. Tentative assignments were filtered based on high exposure score ($\geq$15) and also unknown or moderate- to high hazard scores (unknown or $\geq$3) in FF. Filtered features occurring in >30% of the FF (n = 170) were prioritized for further investigation to limit the search to more common exposures.

Additionally, an in-house database containing PFAS exact masses were matched with the feature lists of FF and serum. Afterwards reference standards were used to confirm the detected suspects of 11 PFAS (see ESI Table S2†).

Tier 2: prioritization based on follicular fluid to serum ratio

This prioritization strategy was based on the premise that features enriched in FF may have a greater impact on the maturing oocyte. To prioritize these features, we used the combined FF/serum datasets (see data processing section) to determine the ratio of the peak area of a given feature in FF to its corresponding peak area in serum from the same individual. The features with a ratio >20 in at least 110 out of 116 patients were then searched against the full Market List and the mass spectral library mzCloud. In order to assess the impact of matrix-induced ionization effects on the calculated ratios, we compared internal standard responses between FF and serum. The ratios of IS areas in FF to serum ranged from 0.015 to 0.89 (see Table S7 in the ESI†), with a median ratio in negative mode of 0.73 and in positive mode of 0.22 suggesting more matrix suppression in FF compared to serum in positive mode. Generally, all median ratios were below 1, which results in an underreporting of enrichment factors when using peak areas in FF relative to serum. We note, however, that matrix effects could only be assessed for internal standards; we cannot rule out the possibility of higher or lower matrix-induced ionization effects for non-targets.

Tier 3: prioritization based on reproductive outcomes

Tier 3 suspects were prioritized using statistical comparisons between features in FF and serum depending on assisted reproductive technology (ART) outcomes based on (i) the live birth of an offspring, (ii) a positive pregnancy determined by a home urine hCG-test executed by the patient, and (iii) embryo quality (at least one top quality embryo $\geq$9.1, range 1–10) assessed on day 2 by a well-established method.[53] In brief, embryo score incorporates cleavage stage with the information of embryo variables associated with higher implantation-rates at day two, *i.e.* variation in blastomere size and the number of mononucleated blastomeres.

Features associated with differences in reproductive outcomes were identified using MetaboAnalyst.[54,55] Prior to processing, missing values (*i.e.* below detection limit) were replaced by a small value and the data were filtered by variance to remove excessive noise[56] before normalization of the data by mean centering. The difference between the ART outcomes was investigated using multivariate statistic approaches. However, we found that orthogonal partial least squares discriminant analysis (OPLS-DA) resulted in poor separation between the groups (top embryo quality: $R2 = 0.14$, pregnancy test $R^2 = 0.10$, live birth $R^2 = 0.07$), possibly due to the massive amount of data and influence of other parameters, so that the variation in FF and serum composition alone was not sufficient to describe differences in ART outcome. Further analyses were therefore focused on embryo quality, which had the highest R^2 of the three ART outcome models and, more importantly, allowed a more focused assessment of the direct effect of chemicals in the follicular fluid on the maturing oocyte and embryo development. A fold change threshold between peak area in the two ART groups of $\geq$1.5 (in >75% of pairs/variable) combined with t-test significance threshold of $p < 0.05$ provided significantly different features in FF and serum of patients with top quality embryos compared to those with lower quality. Since we are using this approach as a prioritization strategy, we were willing to accept some false positives which may arise when not adjusting for multiple testing. The significantly different features were compared to the databases mzCloud and the Market List for further prioritization. Logistic regression (glm model of CRAN package, R 3.6.1) was used assess if the groups (high *vs.* low embryo quality) differed in age or BMI as these are possible confounding factors. Difference in ovarian reserve estimated by the biomarker anti-müllerian hormone (AMH) in serum was investigated using the same method. P-values < 0.05 were considered significant. After prioritization, the association between the identified exogenous substances and high/low embryo quality was investigated using a logistic regression model with age, BMI and AMH as explanatory variables. AMH

and features were log-transformed to reduce skewness. The variables "fertility cause" (male/female origin) and "parity" were considered for inclusion in the model but removed based on goodness of fit of the model and significance.

Results and discussion

Recruited patients

Baseline statistics of patients and the fresh cycles included in the study are presented in Table 1. The reasons behind the patients' infertility as stated in the medical records was unknown (43.8% of the cases), male infertility (24.8%), tubal factor (6.5%), endometriosis (3.9%), ovarian factor (10.4%), anovulation (9.8%) or sterilized (0.5%). The distribution of the diagnoses is similar to larger cohorts from the same clinic.[57] Oocytes were fertilized either with IVF (48.5%), intraplasmatic sperm injection (ICSI, 43.5%) or a combination thereof (8%). For treatment resulting in developing embryos, 49% resulted in at least one top quality embryo. The average score of the top embryo was 8.6 ranging from 0.3–10 in all patients.

Number of detected features

In the individual feature lists for FF, a total of 20 644 features were detected (14 474 in positive and 6170 in negative mode; features ionizing in both modes would be counted twice), with an average of 3141 (standard deviation (SD) ±380) unique features per patient. Of these features, 745 features were present in >90% of the patients and 2349 in >50%. In addition, 6385 features were patient-specific, *i.e.* detected in only one (but not necessarily the same) patient. In serum, we detected 13 740 features (6493 in positive and 7247 in negative mode), with an average of 2508 (SD ± 228) unique features per patient. A total of 2049 features occurred in >50% of patients, of which less than half (885 features) were observable in >90% of patients. In serum, 4254 features were detected in no more than one (but not necessarily the same) patient.

Table 1 Characteristics of patients enrolled ($n = 161$)

Parameter	Value
Age in years, mean (SD)	34.6 (4.6)
AMH[a], μg L^{-1} (SD)	3.25 (2.9)
Previous IVF-treatments, *n* (%)	
No previous	85 (52.8)
One previous	33 (20.5)
Two or more previous	43 (26.7)
Body mass index (BMI), mean (SD)	23.5 (3.04)
Eggs for insemination, mean (range)	10.2 (1–36)
Parity, *n* (%)	
Nulliparous	109 (68)
Primiparous	44 (27)
Multiparous	8 (5)
Pregnancy rate[b], %	37.9
Live birth rate[b], %	29.6

[a] Ovarian reserve estimated by the biomarker anti-müllerian hormone (AMH), measured in μg L^{-1}. [b] Results from fresh IVF cycles where 85% of the cycles resulted in an embryo transfer.

The combined lists (including FF and serum) comprised 3034 features in positive mode and 9128 features in negative mode (166 was detected in >50% of patients in both follicular fluid and serum and 41 in >90%). The inconsistency in the number of features between the individual and combined feature lists is due to (a) differences in the number of samples used for data processing (individual feature lists included all 161 patients for FF, while the combined feature lists only included data from 116 patients where both FF and serum data was collected); (b) the version of Compound Discoverer used; and (c) differences in settings in Compound Discoverer used for data processing (more specifically, different thresholds during peak picking and alignment, see materials and methods for more details).

Tier 1: prioritization based on database comparison

Of the 3306 features occurring in ≥30% of the FF samples, 170 matched substances with high exposure scores and unknown- or moderate-to-high hazard scores in the Market List (CL 3–5). Of these 170 tentatively identified substances, analysis of authentic standards for 14 chemicals confirmed the identities of five substances (and at the same time ruled out nine identities). Substances identified at CL 1–2 are presented in Table 2 and the remaining tentative identifications (CL 3–5) can be found in Table S9 and 10 in the ESI.† 3-pyridinecarboxamide (CL = 1; also known as nicotinamide or vitamin B3) was confirmed. Two additional substances, tris(2-butoxyethyl) phosphate (TBEP, CL = 1) and dibutylamine (CL = 1; also known as *N*-butyl-1-butanamine) were observed frequently in follicular fluid but was also observable at similar intensities in the sampling blanks; consequently, these substances were not considered further.

Although our focus was on anthropogenic chemicals, several endogenous compounds were identified, including 12-hydroxyoctadecanoic acid (CL = 1, detected in 57% of the patients) and dodecanedioic acid (CL = 1, detected in 67% of the patients). In addition to their natural occurrence, these substances are manufactured commercially and were prioritized in Tier I due to their presence on the Market List and unknown or unrecorded hazards.

Eleven PFAS were tentatively identified by matching the combined FF and serum feature list with the in-house database on PFAS; their identities were subsequently confirmed using reference standards (Table 2). The median FF:serum ratio ranged from 0.64 (PFOS) to 1.04 (PFHxS). These values are comparable to previously reported data,[58] a comparison of which can be found in Table S11 in the ESI.† Some PFAS are known to cause developmental toxicity in experimental animals[59] and disturb lipid metabolism *in vitro*[39,60] and in humans.[61] Some PFAS are also associated with affected ART outcomes according to previous studies.[25,34] However, despite clear connections with effects reported in the peer-reviewed literature, PFAS did not appear using the other prioritization strategies in this study (*i.e.* enrichment in FF and connection to reproductive outcomes). In addition, several EDCs commonly investigated in relation to human health outcomes were not

Table 2 Summary of substances identified using the various prioritization strategies

		Compound	Mass[a]	CAS no	Ratio[b]	RSD[c]	Conf. Level[d]
Tier 1	Identified based on database identifications	12-Hydroxyoctadecanoic acid[e]	300.2664	106-14-9	0.55 (0.016–7.61)	215.60	1
		3-Pyridinecarboxamide	122.0480	98-92-0	1.13 (0.01–71.94)	590.54	1
		Dodecanedioic acid	230.1518	693-23-2	9.06 (1.64–152.72)	243.40	1
		Perfluoroheptanoic acid (PFHpA)	363.9769	375-85-9	0.65 (0.37–1.00)	20.60	1
		Perfluorooctanoic acid (PFOA)	413.9737	335-67-1	0.72 (0.33–1.18)	17.00	1
		Perfluorononanoic acid (PFNA)	463.9705	375-95-1	0.79 (0.31–1.26)	18.80	1
		Perfluorodecanoic acid (PFDA)	513.9673	335-76-2	0.69 (0.21–1.29)	25.94	1
		Perfluoroundecanoic acid (PFUnDA)	563.9641	2058-94-8	0.71 (0.05–1.39)	35.34	1
		Perfluoropentane sulfonate (PFPeS)	349.9470	2706-91-4	0.88 (0.23–1.73)	23.70	2b
		Perfluorohexane sulfonate (PFHxS)	399.9439	355-46-4	1.04 (0.01–1.65)	19.54	1
		Perfluoroheptane sulfonate (PFHpS)	449.9407	375-92-8	0.79 (0.04–1.49)	31.00	2b
		Perfluorooctane sulfonate (PFOS)	499.9375	1763-23-1	0.64 (0.001–1.51)	82.94	1
		9-Chlorohexadecafluoro-oxanonane sulfonate (9Cl-PF3ONS)	531.9029	756 426-58-1	0.94 (0.24–2.15)	41.60	1
		Perfluoro-ethylcyclohexane sulfonate (PFECHS)	461.9407	646-83-3	0.88 (0.10–2.66)	32.20	1
Tier 2	Accumulation in follicular fluid	Progesterone	314.2246	57-83-0	255.8 (78.77–646.78)	47.48	2a
		Lidocaine	234.1732	137-58-6	4559 (0.27–55 483)	202.87	1
		Epitestosterone	288.2089	481-30-1	50.29 (9.24–163.86)	52.14	2a
		17α-Hydroxyprogesterone	330.2195	68-96-2	100.18 (9.59–2302.09)	236.9	2a
		20α-Hydroxyprogesterone	316.2402	145-14-2	25.47 (2.40–75.55)	49.27	2a
Tier 3	Associated with embryo quality	4-Aminophenol	109.0528	123-30-8	0.18 (0.02–2.51)	171.76	1
		6-Hydroxyindole	133.0528	2380-86-1	0.88 (0.01–2.73)	45.07	2a

[a] Neutral monoisotopic mass (Da). [b] Median ratios were determined by calculating the peak area in FF/peak area in serum from the same individual. [c] RSD, Relative standard deviation of the ratios. [d] Confidence level of confirmation, see section Data processing, Materials and methods for description.[50] For aall substances at CL 1 and 2b chromatograms (and, where high enough intensities were present in the samples, also MS2 spectra) are listed in Fig. S2 A–R in the ESI. [e] Hydroxyoctadecanoic acid found through both tier 1 & 3.

prioritized for identification using the approach in tier 1. Some of these compounds are not included as they do not fulfill the criteria of ubiquitous exposure even though mentioned in the Market list (some phthalates for example) and other were given low hazard score in the Market List and thus not included in the prioritization used in this study (some PFAS and parabens).

Tier 2: prioritization based on follicular fluid to serum ratio

The identification of substances enriched in FF (Tier 2) is particularly important for facilitating reproductive risk assessment based on exposure of the oocyte during maturation – a specific and sensitive period during development.[62] In Tier 2, twenty-two percent of all features in the combined feature lists (2709 of 12 162 as sum of pos and neg mode) were found in both FF and serum. A total of 262 features with FF:serum ratios of ≥20 in 110 patients were compared to the full Market List and mzCloud. Suspects including identification level can be found in Table S9 and 10 (ESI).† One compound, lidocaine, was confirmed with a reference standard (CL = 1); however, this substance is used as a local anesthetic during OPU and the accumulation in FF compared to serum can be assumed from the local injection close to the sampling site. An additional four hormones and hormone derivatives (progesterone, epitestosterone, 17α-hydroxyprogesterone, and 20α-hydroxyprogesterone) could be confirmed using library spectrum data (mzCloud hit >85, CL = 2a). This result is not surprising considering that steroids such as progesterone, as well as androgens and estrogens are produced in the follicle and their concentrations are therefore higher in FF compared to plasma or serum.[63–65]

The ratio that was chosen as a prioritization limit in this study (>20) reflects a substantial enrichment in follicular fluid. Enrichment of anthropogenic chemicals at lower ratios are also of importance to investigate, but were not included here in an effort to reduce the number of features to a reasonable amount for identification.

Tier 3: prioritization based on reproductive outcomes

Multivariate statistical models for ART outcomes (positive pregnancy test, live birth) resulted in poor group separation. Further analysis was therefore only implemented for embryo quality. The patients with top quality embryos did not significantly differ from those with lower quality with respect to age (intercept 35.6, estimate −1.07, $p = 0.07$) or BMI (23.27, 0.32, $p = 0.55$). However, the patients with high embryo quality also had higher AMH levels (2.58, 1.17, $p = 0.027$). NTS detected 252 significantly different features in patients with top quality embryos compared to those with lower quality (134 in FF and 118 in serum). Features associated with patients with different embryo quality may represent substances that play a role in oocyte developmental competence. The significantly different features were further analysed and compared to The Market List and mzCloud. The Market List matched 11 suspects in FF and 22 in serum, while hits in mzCloud all scored <85% which indicate insufficient data for a probable confirmation (CL 3–5, presented in Table S9 and 10 in the ESI†). Authentic standards

resulted in three confirmations: 12-hydroxydodecanoid acid (CL = 1; endogenous, discussed in Tier 1), 4-aminophenol (or isomers, see below; CL = 1) and 6-hydroxyindole (CL = 2a). The aggregated list of identified compounds is presented in Table 2.

When using a fold change threshold of ≥1.5 between peak areas of high/low embryo quality patients combined with *t*-test significance threshold ($p < 0.05$), 4-aminophenol was associated with higher embryo quality. However, this could not be confirmed with logistic regression that included the confounding factors age, BMI and AMH (Table S12 in the ESI†). 4-Aminophenol was observed in 83% of the patients but we cannot rule out that this assignment may correspond to aminophenol isomers. This chemical is found in consumer products such as personal care products and cosmetics and is used in the industry for staining fur, leather and textiles as well as in the manufacturing of pharmaceuticals such as paracetamol. In humans, the chemical aniline is metabolized into paracetamol/acetaminophen and 4-aminophenol is in turn a minor metabolite of paracetamol that is nephrotoxic.[66] In this study, 4-aminophenol was present in lower concentration in serum of women with lower quality embryos and it was also detected in FF. This suggests that 4-aminophenol might have effects on oocyte maturation. An epidemiological study found no association between acetaminophen or 4-aminophenol urine concentrations in females and time to pregnancy, but a significant association for males.[67]

Both the tier 3 prioritization method and a logistic regression model that included confounding factors (age, BMI and AMH) found an association between 6-hydroxyindole and low embryo quality ($p = 0.02$, Table S12 in the ESI†). 6-Hydroxyindole was detected in serum in 34% of the patients and was found in FF. 6-Hydroxyindole is commonly used in hair dyes. Gut bacterial degradation of certain amino-acids produces indole that is absorbed from the gut and further metabolized into 6-hydroxyindole by CYP11A1.[68] CYP11A1 regulates androgens and has been shown to be important in the etiology of polycystic ovary syndrome.[69]

Compounds previously associated with IVF outcomes such as PCBs, phthalates and bisphenols were not identified using this approach. This should not be interpreted as a negative result but rather a function of the analytical technique (these substances are analyzed by GC-MS rather than LC-MS) and/or prioritization method or outcome (embryo quality).

Conclusion

The results from this study show that FF contains a complex mixture of endogenous and anthropogenic substances. It is possible that the occurrence of anthropogenic substances inside the follicle could disrupt the composition of the FF, which in turn could lead to difficulties in conceiving. For example, studies using omics-approaches to define FF composition and the effect on fertility parameters have shown that endogenous substances differ between high or low oocyte-yielding cows[70] and there are differences in protein patterns in FF that determine if the oocyte could be fertilized or not.[71] By using *in vitro* models, exposure effects on oocyte maturation and the pre-implantation embryo can be further explored, for example in the bovine or porcine model.[39,62] Only a fraction of the detected features in FF and serum were prioritized for identification in our study (Tiers 1–3), but further identifications using data from this study can be used retrospectively as new concerns arises or new compounds are discovered.[72]

Author contributions

IH: Methodology, Formal Analysis, Investigation, Visualization, Writing – Original Draft, Review & Editing, MP: Methodology, Validation, Formal Analysis, Investigation, Supervision, Writing – Original Draft, Review & Editing, SP: Conceptualization, Methodology, Supervision, Writing – Original Draft, Review & Editing, JB: Methodology, Resources, Validation, Supervision, Writing – Original Draft, Review & Editing, YS: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – Review & Editing, PD: Resources, Methodology, Supervision, Writing – Review & Editing, MO: Supervision, Writing – Review & Editing, JH: Resources, Writing – Review & Editing. All authors reviewed and approved the manuscript before submission.

Conflicts of interest

There are no conflicts to declare.

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Perfluorononanoic acid (PFNA) alters lipid accumulation in bovine blastocysts after oocyte exposure during *in vitro* maturation

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ABSTRACT

Perfluorononanoic acid (PFNA) is one of the perfluoroalkyl acids present in human tissues. In this study, effects on early embryo development after PFNA exposure were investigated using the bovine *in vitro* production system. Oocytes were exposed to PFNA during maturation *in vitro* (10 μg mL^{-1} and 0.1 μg mL^{-1}), and then fertilized and cultured in parallel with control groups. Developmental parameters (cleavage, blastocyst formation) were followed and embryo quality evaluated (stage, grade). Embryos developed after exposure to 0.1 μg mL^{-1} were stained to distinguish nuclei, active mitochondria and neutral lipids. 10 μg mL^{-1} of PFNA had a severe negative effect on blastocyst formation (OR: 0.27 $p < 0.05$), an effect not observed at 0.1 μg mL^{-1}. However, lipid droplet distribution was significantly altered in embryos exposed to 0.1 μg mL^{-1}, suggesting a disturbance of lipid metabolism after exposure to sublethal levels of PFNA during oocyte maturation *in vitro*.

1. Introduction

Per- and polyfluoroalkyl substances are highly fluorinated aliphatic chemicals characterized by their extremely strong and stable C–F bond [1]. The subgroup perfluoroalkyl acids (PFAAs) contains compounds that, because of their durability and hydrophobicity, have been used widely since the 1950s in water and stain repellent materials, cookware, food packaging, insulating agents, and flame-retardants [1–3]. As a consequence of this widespread use and their stability, PFAAs can be found in both nature and wildlife, accumulating at the top of the food chain [1,4,5]. Because these chemicals are known to be toxic, there is legislation restricting their use (for examples, see Buck et al. [1]). Environmental decrease in PFAA levels is expected to be slow because of their persistence.

In humans, PFAAs can be detected in human tissue and have been found to pass through the placenta, exposing the growing embryo [6,7]. The PFAAs usually studied are the sulphonic acid perfluorooctane sulfonic acid (PFOS, detected in the highest concentrations in human tissue) and the carboxylic acid perfluorooctanoic acid (PFOA, the second most concentrated in human tissue). Perfluorononanoic acid (PFNA) has also been frequently observed [3,8–11] and, in contrast to PFOS and PFOA, PFNA levels have been increasing in humans in the first decade of this century [9,10,12]. PFNA is detected in human serum at mean concentrations of 0.8–1.49 ng mL^{-1}, with demographic, geographic and temporal differences [3,6–8,11]. For instance, remarkably higher levels are displayed in certain regions of Japan (mean 3.54–6.7 ng mL^{-1}) [13,14]. PFNA has also been detected in human placenta, foetal organs [7] and follicular fluid (0.2–2.1 ng mL^{-1} [6]) indicating exposure to the maturing oocyte.

The developmental toxicity of PFAAs has been demonstrated in animal studies (reviewed by Lau et al. [15]), with severity varying between species and compounds. Generally, the perfluorinated sulphonic acids and the chemicals with longer carbon chains have higher toxicity compared to the carboxylic acids [16–18].

PFAAs have been studied in relation to subfecundity or infertility, although with contradictory results [19–24]. In some studies, PFNA (but not PFOS and PFOA) were associated with pregnancy loss and increased time to pregnancy [25,26].

Safety assessment and toxicity testing of chemicals, and investigations of reproductive toxicity, include *in vivo* tests, most commonly using small rodents as model organisms. With the European REACH Legislation [27], the use of experimental animals has increased [28]. Even though the *in vitro* models of today can not completely replace the *in vivo* models used, the further improvement of *in vitro* techniques is

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essential for minimizing the need for experimental animals [28,29] in line with the 3Rs and the scientific concept.

For female reproductive disorders, no validated laboratory *in vitro* tests are available [30]; however, early embryo development in cows resembles human early embryo development more than the commonly used rodent models (reviewed by Santos et al. [28]), suggesting that bovine *in vitro* embryo production might be a useful complement for assessment of reproductive toxicity (with the added advantage of not relying on experimental animals). Further, the technique enables the study of certain windows of exposure including the late stages of oogenesis, a specialized process where many critical events occur [28]. The half-lives of PFAS in human serum takes years [31] compared to rodents that process the compounds faster, resulting in an elimination half-life of PFNA in mice serum of 26–69 days [32]. The pharmacokinetics is less understood in other species such as monkeys and bovines but is slower compared to rodents [33,34].

The aim of this study was to investigate the effect of PFNA exposure during oocyte maturation on the development of early-stage bovine embryos *in vitro*. Bovine oocytes were exposed during maturation *in vitro*, and the bovine *in vitro* produced (IVP) embryos were used as a model for assessing reproductive toxicity.

2. Materials and methods

2.1. Experimental design

Production of bovine embryos through maturation, fertilization, and culture *in vitro* were conducted using cumulus-oocyte-complexes (COCs) from bovine ovaries. Two experiments were run exposing COCs (n = 846) to PFNA during maturation *in vitro* in two different concentrations. Following COC selection, experimental and control groups were kept separate during all subsequent *in vitro* procedures. In the first experiment, COCs were exposed to 10 μg mL^{-1} PFNA (PFNA 10, n = 200) cultured in parallel with a control with no addition of PFNA (C 10, n = 201), and in the second experiment, the COCs were exposed to 0.1 μg mL^{-1} PFNA (PFNA 0.1, n = 223) and cultured in parallel with a control (C 0.1, n = 222). The experiment was run in n = 6 replicates for PFNA 10 and n = 8 replicates for PFNA 0.1.

Embryo development was evaluated (see below, section 2.4) after the primary cleavage divisions at 44 h post fertilization (pf), and at days 7 and 8 pf. At day 8 pf, embryo morphology and stage of development (grade, stage) were evaluated using a stereomicroscope. For the lower PFNA concentration, day 8 pf blastocysts were fixed and stained for nuclei, neutral lipids, and active mitochondria and further analyzed morphologically using confocal microscopy (Fig. 1).

2.2. Media and reagents

Chemicals and reagents were obtained from Sigma Chemical Company (Sigma Aldrich, Stockholm, Sweden) if not stated otherwise. Media were produced at the IVF laboratory at the Department of Clinical Sciences (Swedish University of Agricultural Sciences, Uppsala, Sweden) as described by Gordon, 1994 [35]. Completed media were adjusted for pH and osmolality, filtered through a 0.2 μm filter unit, stored at 4 °C and used within one week according to protocols. Before use, media were pre-heated or equilibrated in a humidified atmosphere of 5% CO_2 at 38.5 °C for at least one hour. Incubations were conducted in 500 μL wells of medium in a humidified atmosphere of 5% CO_2 at 38.5 °C if not stated otherwise.

Search-medium for selection of COCs consisted of pre-heated HEPES-buffered tissue culture medium 199 (TCM199, M7528) supplemented with 0.2% w/v bovine serum albumin (BSA), Fraction V (A3311) and 50 μg mL^{-1} gentamicin sulphate (G1264). For maturation *in vitro*, maturation-medium consisting of bicarbonate-buffered TCM 199 (M2154) supplemented with 0.68 mM L-glutamine (G8540), 50 μg mL^{-1} gentamicin sulphate (G1264), 0.4% w/v BSA, Fraction V (A3311), 0.1 μg mL^{-1} FSH and 0.03 μg mL^{-1} LH (Stimufol, PARTNAR Animal Health, Stoumont, Belgium) were used. PFNA (perfluorononanoic acid 97%, 394459-5 G) was dissolved in TCM 199 without HEPES (TCM199, M2154) and added in the maturation-medium of the treatment groups until final concentrations of 10 μg mL^{-1} and 0.1 μg mL^{-1} respectively were reached.

Maturation of COCs were recorded and matured oocytes were prepared for fertilization in modified HEPES-buffered Tyrode's medium without glucose [35], with addition of 0.3% w/v BSA, Fraction V (A3311) and 50 μg mL^{-1} gentamicin sulphate (G1264) (wash-medium) and sperm prepared in modified Ca^{2+}-free Tyrode's medium [35] with 6.9 mM glucose (G6152), 16 mM Sodium DL-lactate (L7900) and an addition of 50 μg mL^{-1} gentamicin sulphate (G1264) (capacitation medium). Fertilizations were conducted in modified HEPES-buffered glucose-free Tyrode's medium [35] with 16 mM Sodium DL-lactate (L7900) complemented with 50 μg mL^{-1} gentamicin sulphate (G1264), 3 μg mL^{-1} heparin (H3184), 3 μg mL^{-1} penicillamine, 3 μg mL^{-1} epinephrine and 1.1 μg mL^{-1} hypotaurine (pencillamine-hypotaurine-epinephrine (PHE)-solution [35]) (fertilization-medium). Final cultures were conducted in modified synthetic oviductal fluid (mSOF [35]) with addition of 0.4% w/v fatty acid free BSA (A7030), 50 μg mL^{-1} gentamicin sulphate (G1264), 20 μl mL^{-1} BME amino acids solution (50x) (B6766) and 10 μl mL^{-1} MEM non-essential amino acids solution (100x) (M7145), wells covered in OVOIL™ (Vitrolife, Göteborg, Sweden).

2.3. Oocyte collection and in vitro maturation (IVM)

Abattoir-derived ovaries from non-stimulated heifers and cows were collected after the animals had been killed. As the animals were not sacrificed for the sake of these experiments, no ethical permission was needed according to Swedish legislation. Ovaries were transported approximately 3 h to the IVF laboratory at the Department of Clinical Sciences (Swedish University of Agricultural Sciences, Uppsala, Sweden) in insulated containers with sterile 0.9% saline-solution, with an arrival temperature of about 30 °C (range 28–33 °C). COCs were aspirated using a 5 mL syringe and a 1.8-gauge needle from 3 to 8 mm diameter follicles. In search-medium, the COCs were selected according to Gordon's [29] morphological criteria. COCs with compact multilayer cumulus investment and homogeneous ooplasm were selected for the

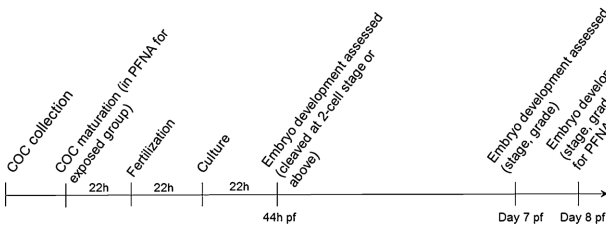

Fig. 1. Experimental design.
Bovine cumulus oocyte complexes (COCs) were selected for the experiment and exposed to PFNA in two concentrations during maturation *in vitro*. Embryo development was evaluated after the primary cleavage divisions at 44 h pf and at days 7 and 8 pf. For the lower concentration (PFNA 0.1), day 8 blastocysts were stained for nuclei, neutral lipids and active mitochondria.

experiments and divided randomly and evenly into two groups (group size range; 20–40), treated experimentally, and cultured in parallel along with control groups. For *in vitro* maturation, COCs were incubated in maturation-medium for 22 h with the addition of PFNA for the treated groups.

2.4. In vitro fertilization and culture

After maturation *in vitro* the COCs were transferred to pre-heated wash-medium. Oocytes were prepared for fertilization through manual removal of excessive cumulus cells until 3–5 layers remained surrounding the oocyte. Semen from a single bull of Swedish red dairy breed with proven field and *in vitro* fertility was used (3–1716 Sörby). Motile sperm were selected after 45 min swim-up in capacitation medium and added to the oocytes at a concentration of 1×10^6 spermatozoa mL^{-1}, and sperm and oocytes were incubated in fertilization media for 22 h to facilitate fertilization. After fertilization, the remaining cumulus cells and sperm were removed through manual pipetting and the presumed zygotes were cultured until day 8 pf in mSOF, covered in OVOIL™ to prevent extensive evaporation and oxidation.

2.4.1. Evaluation of embryos

Developing embryos were first evaluated 44 h pf using a stereomicroscope (SteREO Discovery.V8, Carl Zeiss Microscopy GmbH, Jena, Germany) for primary cleavage division, and the cleavage level (*i.e.* the proportion of oocytes cleaved at the 2-cell stage or higher) was determined. At days 7 and 8 pf, the proportions of oocytes that had developed into embryos were recorded, and embryo morphologies and developmental stages were further assessed. Developmental stages were scored according to IETS classification with modification into 3 stages (Fig. 2): early blastocysts or blastocysts (stage 1), expanding or expanded blastocysts where the zona pellucida are intact but thinner due to expansion of the embryo (stage 2), and hatching or hatched embryos (stage 3) [36]. The quality of each embryos (grade 1–4) was assessed according to IETS classification, where grade 1 (excellent/good) refers to a spherical and symmetrical embryo mass consistent with the stage of development with blastomeres uniform in size, shape and color and only minor irregularities; grade 2 (fair) refers to embryos with at least 50% of the embryo showing an intact, viable embryonic mass with moderate irregularities in shape, mass or size; grade 3 (poor) refers to embryos with at least 25% of the embryo showing an intact, viable embryonic mass but with severe irregularities in shape, mass or size of the embryo; and grade 4 (dead or degenerating) in the classification for non-viable embryos or oocytes [36,37].

2.4.2. Staining and image analysis

Staining and confocal microscopy for further analysis of the embryos were applied to pf day 8 blastocysts after treatment with 0.1 μg mL^{-1} PFNA during maturation *in vitro*, a method previously used to detect deviations during early embryo development [38,39].

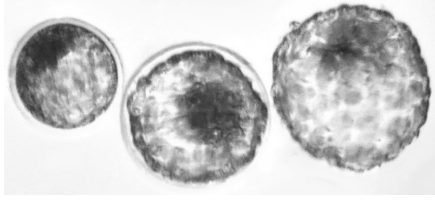

Fig. 2. Classification of blastocysts 8 days pf for stage and grade.
Left: blastocyst of excellent/good quality (stage 1, grade 1); Middle: expanding blastocyst with intact but thinner zona pellucida than stage 1, excellent/good quality (stage 2, grade 1); Right: hatched blastocyst of excellent/good quality (stage 3, grade 1).

Fluorescent labeling for visualization of active mitochondria was conducted through incubation with 200 nM MTO (Mitotracker® Orange CMTMRos, ThermoFisher Scientific, Waltham, USA). Fixation followed incubating the embryos in 2% paraformaldehyde (PFA) in phosphate-buffered saline with 0.1% polyvinyl alcohol (PBS-PVA) overnight at 4 °C or at room temperature (RT) for one hour, followed by rinsing in PBS-PVA. For visualization of nuclei, embryos were incubated with 5 μM DRAQ5® (Deep red Anthraquinone 5, 4084S, BioNordika, Stockholm, Sweden) for 20 min at RT. After rinsing in PBS-PVA, embryos were stained for neutral lipids using LipidTOX™ (HCS LipidTOX™ Green Neutral Lipid Stain H34475, ThermoFisher Scientific, Waltham, USA) for 30 min at RT according to the manufacturer's instructions.

Stained blastocysts were mounted in approximately 2 μl of fluid on a well of a microscope slide (ER-201B-CE24, Thermo Fisher Scientific, Portsmouth, NH) in Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were captured using an LSM 510 laser scanning microscope (Zeiss LSM 510, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with He/Ne 543, He/Ne 633 and Ar 450–530 nm lasers. Each embryo was scanned using standard magnification (20x) in single scans and seven levels of sectioned scans (z-stack).

Images taken as single channel images were converted to overlay images and saved in TIFF format. Only z-stack levels 2, 4 and 6 were used for image analysis to prevent nuclei and lipid droplets being counted twice. Image analysis was performed using CellProfiler (Cell Profiler 2.2.0 (rev ac0529e), http://cellprofiler.org/, accessed at 2017-02-06, [40]) and manually.

The blastocysts were identified by segmentation using a pixelwise classifier trained in Ilastik [41] and confirmed manually, and non-blastocysts were excluded. Nuclei and lipid droplets were identified using the IdentifyPrimaryObjects module in CellProfiler where an initial Maximum Correlation Threshold [42] was applied followed by Watershed separation [43]. Cell outlines were estimated by expanding the nuclei by a maximum of 50 pixels. For image analysis, 88 embryos were included in 264 images. Embryos for which one or more image showing poor quality regarding fluorescent labeling for nuclei (n = 31) and lipids (n = 8) were excluded from the analysis. Lipid droplet (n = 32975) sizes were measured at maximum diameter (Fig. 3). Two researchers independently assessed mitochondria distribution based on 3D images of the z-stack by using scores of 1 through 3 (1: even distribution without distinct aggregations of mitochondria, 2: mild asymmetry in distribution with some aggregations, 3: uneven distribution with pronounced aggregations, see [38]) (Fig. 4). Blastocysts were excluded from the analysis (n = 9) if the assessments deviated or if image quality was inadequate for making a determination.

2.4.3. Statistics

Mixed-effect logistic regression was performed to calculate the odds ratio (OR) for the effect of treatment on developmental competence at three time points (cleaved, cleaved higher than the 2-cell stage level 44 h pf, and blastocyst development at day 7 and 8 pf). OR results are presented in terms of the treatment effect compared to the relevant control, where $OR < 1$ indicates a negative effect of treatment on developmental competence. Replicates were added as a random factor and weighted depending on group-size. Data on embryo development on blastocysts day 7 and 8 pf were treated as repeated measurements (glmer model of the lme4 package, R i386, 3.3.1, http://www.r-project.org). Categorical variables (stage, grade, and mitochondrial distribution) were analyzed using cumulative link mixed-effect models with multinomial distribution (clmm model of the ordinal package, R i386, 3.3.1) with replicate as a random effect. Dose group (PFNA 0.1 or 10) was initially added as a random effect to the models due to significant differences in developmental parameters between the two experiments (lm model, R i386), but was removed based on AIC and likelihood-ratio tests between models ($p > 0.05$) indicating that the variation was accounted for through the variable replicates and individual controls. No differences were found regarding parameters associated with laboratory

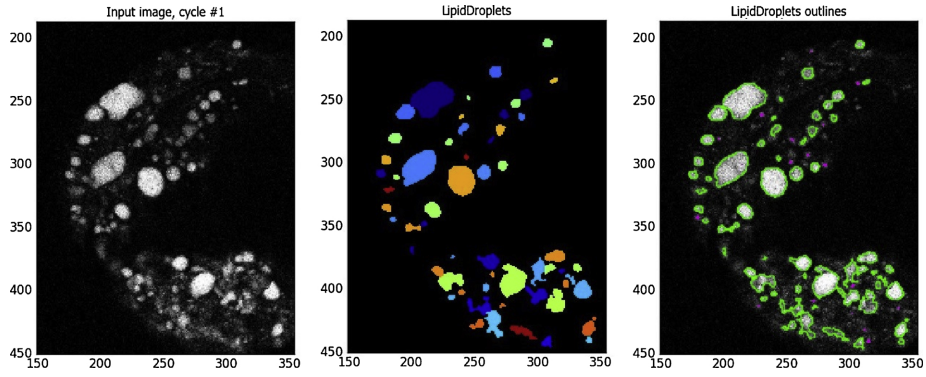

Fig. 3. Lipid droplet identification in confocal images of day 8 pf blastocysts.
Fluorescent labeling for visualization of neutral lipids were applied and images captured in sectioned scans in seven levels (z-stack) using a confocal microscopy. Images taken as single channel-images (left) from z-stack levels 2, 4 and 6 were used for lipid-identification. Lipid droplets were identified using CellProfiler using Maximum Correlation Threshold followed by Watershed separation (middle, right). Lipid droplet (n = 32975) sizes were measured at maximum diameter.

work between the different concentrations (time for aspiration, maturation and amount of ovaries, $p > 0.05$).

Linear mixed effect models was performed to calculate the effect of PFNA 0.1 on nuclei and lipid droplet size (lmer model of the lme4 package, R i386, 3.3.1) with replicate as a random factor. For lipid droplet size, log-transformed values were used to assume normal distribution of the response variable. The difference in lipid droplet size between groups was significant but did not show a monotone distribution (Fig. 5). Thus, assuming the normal distribution was insufficient to explain changes between the treatments, bin values were added and mixed effect logistic regression with lipid droplet size as a response variable was used according to cut-off value (< 3 µm, 3–6 µm, 6–9 µm, 9–12 µm and > 12 µm in diameter).

P-values < 0.05 were considered to be significant. Data are presented as mean ± standard deviation (SD) if not otherwise stated.

3. Results

3.1. Effect of PFNA on developmental competence of immature oocytes in vitro

Treatment with 10 µg mL^{-1} PFNA (PFNA 10) during oocyte maturation had a negative effect on developmental competence. The effect could be seen after maturation *in vitro* where the cumulus cloud of the treated group did not expand as it did in the control (Fig. 6). The risk of impaired development was increased with PFNA exposure, both regarding the proportion of cleaved oocytes 44 h pf (OR 0.59, p = 0.01) and the proportion of blastocysts developed from cultured immature oocytes (OR 0.27, $p < 0.001$). No significant effect was seen on cleaved oocytes more advanced than the 2-cell stage at 44 h pf (p = 0.09) (Table 1). This developmental toxicity at 10 µg mL^{-1} PFNA was not observed at 0.1 µg mL^{-1} PFNA exposure during oocyte maturation *in vitro*. Results of logistic regression analyses are presented in

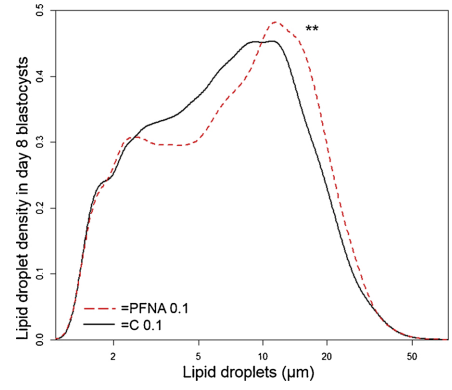

Fig. 5. Densities of lipid droplets in day 8 pf blastocysts. Lipid droplets of different size (diameter) in treatment (PFNA 0.1) and control (C 0.1) showing a non-monotonic distribution. ** indicates a significant difference (p = 0.04).

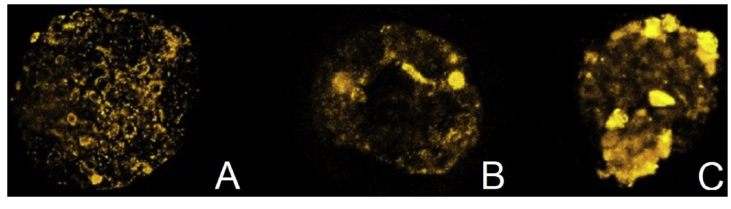

Fig. 4. Grading of distribution of active mitochondria. A: Grade 1, even distribution without distinct aggregations of mitochondria; B: grade 2, mild asymmetry in mitochondria distribution with some aggregations; C: grade 3, uneven mitochondria distribution with pronounced aggregations.

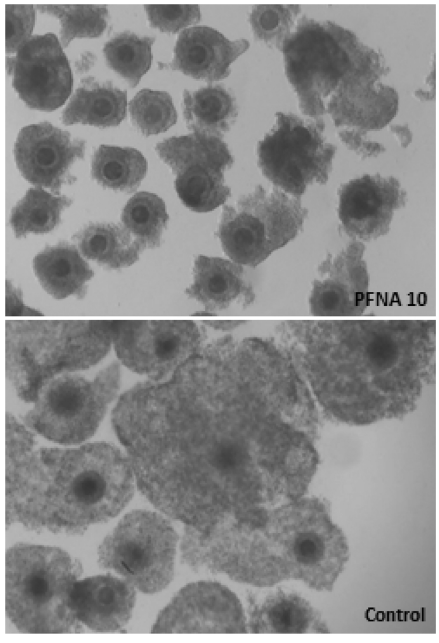

Fig. 6. Cumulus oocyte-complexes after maturation *in vitro* treated with 10 μg/mL PFNA and Control. Note the change in expansion of the cumulus cloud between treated group (PFNA 10) and control.

Table 1
Result of PFNA exposure during maturation *in vitro* on developmental competence of the immature oocytes.

	PFNA 0.1^ *n=223*	C 0.1^ *n=217*	PFNA 10^ *n=199*	C 10^ *n=200*
Cleaved[a]	0.81 ± 0.09	0.80 ± 0.07	0.75 ± 0.10*	0.85 ± 0.03
Cleaved above 2[a]	0.59 ± 0.10	0.60 ± 0.05	0.60 ± 0.14	0.71 ± 0.07
Day 7 blastocysts[b]	0.12 ± 0.10	0.11 ± 0.05	0.05 ± 0.03*	0.15 ± 0.06
Day 8 blastocysts[b]	0.20 ± 0.09	0.19 ± 0.05	0.08 ± 0.05*	0.26 ± 0.08

^Results presented as mean of batch results ± SD after exposure with 0.1 μg mL-1 in PFNA 0.1 and 10 μg mL-1 in PFNA 10 and relevant control groups (C0.1 and C10).

[a] Cleaved oocytes and cleaved above the 2-cell stage of cultured immature oocytes 44h post fertilization (pf).

[b] proportion of blastocysts days 7 and 8 pf.

* Indicates a significant difference between treatment and control.

Table 2 and developmental parameters are presented in Table 1.

3.2. Effect of PFNA exposure during maturation in vitro on blastocyst morphology day 8 pf

Developed blastocysts after treatment with PFNA during *in vitro* maturation in the 0.1 μg mL^{-1} group (PFNA 0.1: n = 35, C 0.1: n = 34) and 10 μg mL^{-1} (PFNA 10: n = 16, C 10: n = 50) were morphologically assessed for stage score (1–3) and grade classification (1–4). In these experiments, no risks of changes in stage score in developed blastocysts 8 days pf were observed after PFNA exposure during oocyte maturation *in vitro* either at 0.1 μg mL^{-1} PFNA (1.26 (-0.19-2.71), p = 0.54) or 10 μg mL^{-1} PFNA (1.43 (-0.28-3.14), p = 0.51). No changes in grade classifications of formed blastocysts 8 days pf were observed (PFNA 0.1: 2.04 (0.47–3.61), p = 0.11, PFNA 10: 2.19 (0.44–3.94), p = 0.16).

Table 2
Logistic regression of the effect of PFNA treatment during maturation *in vitro* on developmental parameters presented as odds ratio (confidence intervals) compared to controls.

	PFNA 0.1		PFNA 10	
Variable	Odds ratio (CI)	P-value	Odds Ratio (CI)	P-value
Cleaved 44 h pf[a]	0.87 (0.57–1.37)	0.54	0.59 (0.39–0.90)	0.01
Cleaved above 2 cells 44 h pf[a]	0.73 (0.51–1.05)	0.09	0.72 (0.49–1.04)	0.09
Blastocyst rate[b]	0.85 (0.53–1.37)	0.51	0.27 (0.14–0.53)	0.0009

[a] Cleaved embryos and Cleaved above 2 cell stage of cultured immature oocytes 44 h pf.

[b] blastocyst rate after 8 days pf.

3.3. Effect of 0.1 μg mL^{-1} PFNA exposure on nuclei count, mitochondria, and lipid distribution

There was no observed difference in number of nuclei in formed blastocysts 8 days pf in PFNA0.1 during maturation *in vitro* compared to the control group (PFNA 0.1: 91.7 ± 33, C 0.1: 99.2 ± 40, p = 0.40). In addition, PFNA 0.1 did not affect the distribution of mitochondria within the blastocysts (OR (CI): 1.15 (0.52–2.59), p = 0.73, median grade (range): PFNA 0.1: 2 (1–3), control: 2 (1–3)).

However, there was a significant difference in lipid droplet distribution in PFNA 0.1 during oocyte maturation, with a higher proportion of larger lipid droplets in this treatment compared to the control (Fig. 5, p = 0.04). Blastocysts in this group also had a higher proportion of very large lipid droplets (diameter > 12 μm, p < 0.0001) but a lower proportion of small lipid droplets (diameter ≤ 9 μm) compared to control (Table 3). In contrast, no statistical difference was found in lipid droplets with a diameter of 9–12 μm (p = 0.09, Table 3).

4. Discussion

4.1. Effect of PFNA on developmental competence of maturing bovine oocytes in vitro

Previous studies have investigated the developmental toxicity of PFNA using laboratory animals like rodents and zebrafish [16–18,44–47]. In this study, we have examined endpoints of developmental toxicity using the bovine IVP as a model, exposing oocytes during 22 h maturation *in vitro* to either 10 μg mL^{-1} PFNA (PFNA 10) or 0.1 μg mL^{-1} PFNA (PFNA 0.1).

PFNA 10 exposure was based on previous *in vitro* studies on zebrafish [16,18,47] and levels measured in mouse serum after oral exposure while PFNA 0.1 was based on levels measured in human follicular fluid [6] multiplied 50-500x to compensate for the short exposure time. No comparable studies using the IVP model are available. Exposure in PFNA 0.1 is consistent with low-dose *in vitro* exposure [17] and significantly lower than the dose measured in mouse serum after oral administration [44,46,48].

Signs of severe developmental toxicity, including a decreased proportion of cleaved embryos at 44 h pf and impaired blastocyst development at 7 and 8 days pf, could be seen after exposure to 10 μg mL^{-1} PFNA during *in vitro* maturation, exceeding the effects observed in zebrafish [16,18,47]. In mice, similar maternal serum concentrations did not affect litter size, pup weight or live pups/litter [46,48]. This toxicity difference could be due to different species responses to PFAAs [15] (specifically, the lower chemo-permeability of the zebrafish

Table 3
Logistic regression of the effect of exposure to 0.1 μg mL-1 PFNA (PFNA0.1) during maturation *in vitro* on the distribution of lipid droplet size in day eight blastocysts after fertilization.

Lipid droplet size[c]	C 0.1	PFNA 0.1	Odds ratio (CI)	p-value
Sum[k]	395.51 (154.23)	431,57 (223.82)	1.13 (1.00-1.27)	0.04
< 3 μm[d]	83.93 (44.46)	90.16 (88.24)	0.95 (0.89-1.00)	0.04
3-6 μm[d]	95.56 (43.78)	89.43 (58.67)	0.82 (0.78-0.87)	2.28e-12
6-9 μm[d]	68.09 (32.07)	64.62 (26.92)	0.87 (0.82-0.93)	9.55 e -06
9-12 μm[d]	51.86 (25.28)	58.19 (23.23)	0.94 (0.99-1.13)	0.09
> 12 μm[d]	96.07 (85.19)	129.16 (80.19)	1.32 (1.25-1.39)	< 2.0e-16

[c] Lipid droplet (n = 32975) size measured as maximum diameter in day eight embryos (n = 88) in PFNA exposed group (PFNA0.1) and control-group (C0.1).
[k] Sum (SD) and overall effect of PFNA0.1 on distribution of lipid droplet size.
[d] Effect of PFNA0.1 on the proportion of lipid-droplets of size < 3, 3–6, 6–9, 9–12 and > 12 μm in maximum diameter.

chorion might have reduced uptake [49] and different metabolism of PFAAs in mice [32]). A similar level of toxicity was not observed in embryos exposed to 0.1 μg mL^{-1} PFNA where no difference could be seen in the proportion of embryos cleaved or developed into day 7 or 8 pf blastocysts.

Embryos were assessed morphologically after 7 and 8 days pf by evaluating endpoints of developmental toxicity, such as impaired development, malformations, or degradation of the early embryo (stage, grade). No differences were observed between controls and PFNA exposed groups. However, the extensive toxic effects of PFNA 10 resulted in a very small number of embryos available for evaluation (day 8 pf blastocysts, n = 16).

4.2. Effects of 0.1 μg mL^{-1} PFNA on nuclei count, mitochondria, and lipid distribution

Bovine oocytes exposed to PFNA 0.1 μg mL^{-1} (PFNA 0.1) did not show any signs of developmental toxicity when assessed using non-invasive morphological criteria (stage, grade). These findings were confirmed through staining procedures for visualizing nuclei and mitochondria, which is not surprising because nuclei count is a measure of the number of cell-divisions, and changes in mitochondria distribution can be a sign of embryo degradation or cell death [50,51].

Lipid accumulation has previously been evaluated in bovine embryos produced by IVP and is known to affect the viability or the susceptibility of cryopreservation in bovines [52,53]. Different lipid storage in the early embryo is associated with changes in lipid metabolism in bovines, seen as differences in lipid content between breeds [54]. In this study, lipid droplet sizes were studied as an endpoint for lipid metabolism [54–56]. A change in the size distribution of lipid droplets in PFNA 0.1 was observed, suggesting a disturbance of lipid metabolism during the early embryo development. This result is supported by the mode of action of PFNA, which disrupts endocrine activation of a peroxisome proliferator-activated receptor (PPAR). PPARs are nuclear ligand-activated transcription-factors associated with lipid metabolism and involved in catabolism and oxidation of fatty acids [57,58]. Specifically, PFNA has been shown to disrupt activation of PPAR-alpha (PPARα), demonstrated by both by direct effects on the receptor [46] and the lack of effect in knock-out mice [48]; other PFAAs have shown similar activation of PPARα [59]. Lipid metabolization through fatty acid oxidation is important for the oocyte meiotic maturation and early embryonic development [57,60], and changes in lipid metabolism during this period might be especially problematic for a developing blastocyst [60].

Lipid metabolism has also been investigated in human cohorts, and epidemiological studies suggest that exposure to PFAAs during embryo development may cause adverse outcomes later in life [61,62]. However, the results are contradictory. An increase in BMI of female offspring has been associated with *in utero* PFNA exposure [61], and *in utero* exposure to PFOS, PFOA, and PFNA has been positively associated with higher levels of total and non-high density cholesterol [62]. Other results associate obesity in children with PFNA exposure [63]. Finding possible negative effects of PFAAs on human health is difficult, because the effects might be present only in the context of certain metabolic conditions [64].

4.3. The usefulness of bovine IVP as a model for developmental toxicity

The IVP setup enables developmental-specific windows of exposures including the final stages of the oocyte maturation. The oogenesis is an especially sensitive period where many critical events take place and mimicking this period with exposure during the *in vitro* maturation might result in changes visible first later during the embryo development [28] without confounders of exposure of the male gamete. Further, the system might be a useful tool investigating concerns raised from results connecting exposure of the female and impaired results after IVF-treatment [65], especially within the group of couples with unknown infertility reasons.

Species differences in toxicological sensitivity suggest that models used for risk-assessment of developmental toxicity should resemble the target species as closely as possible. Bovine early embryo development is more similar to human embryo development than are small rodents or zebrafish, so mechanistic studies of developmental toxicity using bovine IVP may provide valuable information for human risk assessment [28]. Without resorting to the use of experimental animals, we have demonstrated developmental toxicity of PFNA to bovine embryos, and also demonstrated differences in lipid metabolism that appear to have been caused by disturbances to the same endocrine pathway demonstrated in other studies [46,48].

Although it is unlikely that *in vitro* tests will soon be able to completely replace *in vivo* models, clearly further *in vitro* models are needed to assess potential adverse effects to human fertility [30]. The bovine IVP system seems to be a potentially very useful *in vitro* model for studying the adverse effects of PFNA and possibly other per- and polyfluoroalkyl substances as well. Although, consideration is needed regarding the period of exposure during oocyte maturation *in vitro* as the first meiotic divisions of the gametes *in vivo* are conducted during fetal development and thus this *in vitro* method cannot handle effects of life-long exposure.

4.4. Conclusion

In this study, we investigated the effect of PFNA during oocyte maturation *in vitro* using bovine IVP as a model for reproductive toxicity. We observed acute toxicity after exposure to 10 μg mL^{-1} PFNA as well as changes in lipid droplet distribution in embryos exposed to 0.1 μg mL^{-1} PFNA during maturation *in vitro*. More studies exploring long-term effects and lower-dose exposures are needed for further health risk assessment.

Conflict of interest

The authors declare no conflict of interest.

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This thesis was dedicated to female fertility, and more specifically the maturing oocyte. A non-target approach was used to investigate and characterize the complex environment of the maturing oocyte in humans. Bovine oocytes, exposed to perfluoroalkyl substances during oocyte maturation, were cultured until blastocyst stage *in vitro*. Impact on embryo development, morphology and molecular patterns were investigated. The results contribute to knowledge about potential effects of environmental chemicals on female fertility.

Ida Hallberg received her postgraduate education at the Department of Clinical Sciences, Swedish University of Agricultural Sciences. She obtained her degree in veterinary medicine in 2014, at the Faculty of Veterinary Medicine and Animal Science at the same university.

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