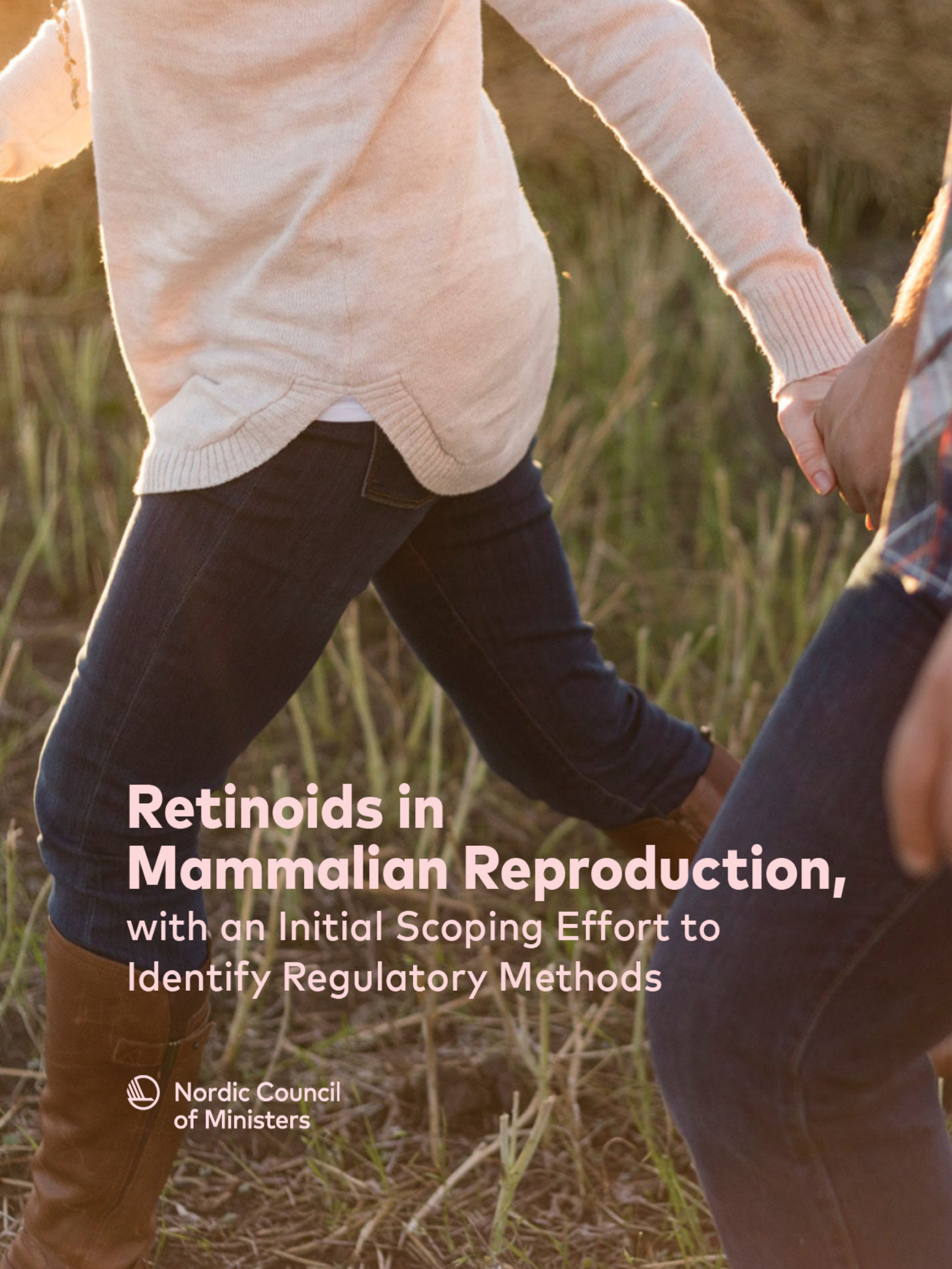

Retinoids in Mammalian Reproduction,

with an Initial Scoping Effort to Identify Regulatory Methods

Nordic Council of Ministers

Contents

Preface

Terms of reference and Scope of report

This Nordic report represents part of the outcome of a project within the Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme (TGP). The project (4.97) was initiated by Sweden in 2015 (Helen Håkansson, Institute of Environmental Medicine, Karolinska Institutet, Sweden) and coordinated by the Swedish Chemicals Agency under supervision of both a steering committee from the funding body (Nordic Co-ordination for the Development of Test Methods in Toxicology and Ecotoxicology, Nord-UTTE) and by the OECD Endocrine Disrupters Testing and Assessment Advisory Group (EDTA-AG).

The starting point of this project were ideas brought forward in the OECD Detailed Review Paper (DRP) 178 on endocrine disrupter testing[1], which addressed multiple aspects of the endocrine system, beyond estrogens, androgens, thyroid and steroidogenesis (EATS). The OECD DRP 178 suggests projects in several areas, and particularly mentions assays relevant to the retinoid system, such as retinoid X receptor (RXR) and retinoic acid receptor (RAR) reporter assays, aryl hydrocarbon receptor (AhR) reporter assays, adipocyte differentiation, and retinoid serum levels. As identification of retinoid system modulation is not presently included in any OECD test guideline it is urgent to cover this knowledge gap.

The long-term aim of the present project, is to develop methods that facilitate early screening, to enhance existing *in vivo* test guidelines and to identify markers of biological effects for use in population studies, based on information from retinoid biology and disturbed retinoid signalling in several organ systems. The EU Commission funded the development of a draft-DRP[2] on the retinoid system that covered *e.g.* overall biology of the retinoid system in human health and the environment. A section on retinoic acid and the reproductive system as well as a substantial annex on the retinoid system in male reproduction were also included. At that stage, it became apparent for all parties involved that the scope of the DRP was too broad, and that science was, in certain aspects, not mature enough for regulatory purposes. Consequently, the scope was narrowed down, and priority was given to reproduction, as female reproduction had been highlighted in the recommendations from the EU Commission prioritisation workshop 2017[3]. In addition, it was decided that the annex on male reproduction should be further developed. Broad support was also given to the decision of the lead country Sweden, to publish this TemaNord report as a first step in the Retinoid DRP process. This TemaNord report will be followed up in the upcoming DRP on retinoids, due to be published by the OECD in 2020–21.

1. OECD (2012), *Detailed Review Paper on the State of the Science on Novel In vitro and* InVivo *Screening and Testing Methods and Endpoints for Evaluating EndocrineDisrupters*, OECD Series on Testing and Assessment, No. 178, OECD Publishing,Paris, http://dx.doi.org/10.1787/9789264221352-en
2. OECD (2017), "Detailed review paper on the retinoid system (draft)", not published, OECD, Paris.
3. Setting priorities for further development and validation of test methods and testing approaches for evaluating endocrine disruptors, in Brussels 2017 (Final Report European Commission Directorate-General for Environment Directorate B. (ISBN 978-92-79-83076-1), Available at https://publications.europa.eu/en/publication-detail/-/publication/6b464845-4833-11e8-be1d-01aa75ed71a1/language-en

In this report, the focus has been on mammalian organisms, with the majority of research data originating from studies in rodents. The report does not cover retinoid biology or retinoid disruption in non-mammalian organisms. In the present report, the term "reproduction" refers to the formation and development of the reproductive organs and their normal function. Teratogenic effects are only briefly covered in this report.

Disposition

The report covers an overview of retinoid biology, the role of retinoids in the reproductive organs, the impact of chemicals on reproduction via the retinoid system, potential adverse outcome pathways, and an initial scoping effort for the possible role of chemical-induced retinoid disruption of the male and female reproductive systems.

Financing and workforce

The Nordic Working Group for Chemicals, Environment and Health (NKE) (in NKE Contracts 2016-023, 2017-003, 2018-026, 2019-003) and The Swedish Chemicals Agency, provided the main funding for this report. Furthermore, the European Commission (Framework Contract ENV.A.3/FRA/2014/0029) provided funding for the draft-DRP reported in 2017. The draft-DRP was authored by Alice Baynes and Edwin Routledge, Brunel University, London, and Sofie Christiansen and Ulla Hass, Technical University of Denmark. Parts of the draft-DRP have, with the permission of the European Commission, been incorporated in this report.

The main author of this TemaNord report is Dr. Charlotte Nilsson, RISE Research Institutes of Sweden. Dr. Miriam Jacobs (Public Health England) is the main author of the sections *Heterodimerisation partners and cross-talk* and *Epigenetics and its role in the retinoid system*. The in-kind contributions from scientific experts involved in writing and reviewing this document is gratefully acknowledged (see list of contributors).

This report is publicly available via the NKE website[4], and describes the status as of December 2019.

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4. https://www.norden.org/en/organisation/nordic-working-group-chemicals-environment-and-health-nke

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Summary

Retinoids are essential for vision, embryonic development, adult growth and development, as well as for reproduction in both males and females. In addition to being a nutrient-derived vitamin (vitamin A), retinoids are also considered as hormones, based on the hormonal-like signaling of retinoid-specific nuclear receptors which affect gene transcription. In general, all-*trans* retinoic acid (RA) is viewed as the physiologically active form. Tissue levels of RA are maintained via tightly regulated enzymatic synthesis and catabolism, and are also dependent on proper uptake, transport and storage of different form of RA precursors.

RA acts mainly by interacting with nuclear retinoid acid receptors, RARs, which forms heterodimers with retinoid X receptors, RXR. These heterodimers bind to specific DNA response elements. The RXR receptor type also heterodimerizes with other nuclear receptors (*e.g.* PPARs, VDR, CAR, PXR, LXR and FXR). Thus, extensive cross-talk between nuclear receptor pathways depend on RXR. RA has also been implicated in epigenetic regulation.

During embryonic formation of reproductive organs and sex differentiation of germ cells, RA is initially available from several possible enzymatic sources in or near the gonad. RA is believed to have an important role in meiosis initiation, however, this is currently a very active research field. Meiosis initiation occurs *in utero* in the female, and postnatally in males. Investigations in rodents have shown that temporal and spatial expression of specific enzymes, involved in RA metabolism, is critical for avoiding premature meiosis in males.

RA has been reported to be of importance for ovarian somatic cell development and function, as well as for the implantation of the fertilized embryo into the endometrium. In addition, altered retinoid signaling has been associated with endometriosis, as well as polycystic ovarian syndrome in women.

In the adult male, RA signaling is important for proper spermatogenesis. More specifically, correct RA levels must be maintained inside the seminiferous tubule for proper spermatogonia differentiation, meiosis initiation and release of spermatozoa. In addition, RA has been suggested to play a role in the formation and maintenance of secondary male reproductive organs (seminal vesicles, epididymis, prostate). However, many of these processes depend also on other endocrine pathways, and extensive cross-talk between these pathways exist. Consequently, for many of the processes described above, more research is needed to elucidate the exact role of RA signaling, the mechanisms controlling spatial and temporal availability of RA in reproductive tissues, and the influence of RXR-cross-talk in retinoid homeostasis.

Some chemicals have been demonstrated to interfere with the retinoid pathway. Chemicals, for which there are at least some data, include pharmaceutical compounds, conazole fungicides and organotins. However, there is a lack of studies investigating effects of chemicals, except a few pharmaceutical compounds, on reproduction, while simultaneously examining effects on retinoid related parameters in reproductive organs. This is a major data gap.

Four different visualizations of possible adverse outcome pathways, using available research, between effects on retinoid homeostasis and reproductive adversity, in males or females, is presented in this report. These visualization pathways can be used as starting points for future AOP development of retinoid disruption.

The initial scoping effort presented in this report identified that the RA-catabolizing CYP26 enzymes and the RA-synthesizing RALDH enzymes could be integrated at (CF) Level 1 and 2 in the OECD Conceptual Framework[5]. This could be possible with development of *in silico* methods, such as QSARs or molecular docking models for these enzymes, and/or *in vitro* assays. In the current *in vivo* test guidelines (CF level 3-5), already implemented histopathological analyses covers important reproduction-related parameters. However, as discussed in the present report, the regulation of reproduction involves many other endocrine modalities, and the interpretation of the observed effects is further impeded by extensive cross-talk between nuclear receptors. Consequently, no endpoints specifically relevant for retinoid disruption that could be added to the existing OECD test guidelines have been identified in this report.

In addition, no suitable reference chemicals, except for a few pharmaceutical compounds, known to affect fertility specifically *via* the retinoid pathway have been identified. Such chemicals will be needed in a future validation step.

In spite of these challenges, selected *in vitro* and/or *in silico* retinoid-related endpoints, presented in this report, could be part of a broader screening test battery aimed at developmental and reproductive toxicity. For *in vivo* studies, histopathological readouts of for example ovaries and testes, already included in the OECD test guidelines, can provide information to support regulatory decision-making, without depending on knowledge of exactly which pathway(s) that have been disturbed.

5. OECD (2018), "Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption", OECD Series on Testing and Assessment, No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en

Sammanfattning

Retinoider är viktiga för synen, embryonal utveckling, tillväxt och utveckling hos barn och unga, liksom för till exempel reproduktion hos både män och kvinnor. I allmänhet anses all-*trans* retinsyra (RA) som den fysiologiskt aktiva formen. Retinoider kallas i vardagligt tal för vitamin A, och betraktas också vara ett hormon. Vävnadsnivåer av RA upprätthålls *via* en noggrant reglerad balans mellan enzymatisk bildning och nedbrytning, och är också beroende av korrekt reglerat upptag, transport och lagring av olika former av retinoider (RA-prekursorer).

RA utövar huvudsakligen sina effekter genom att binda till retinalsyrareceptorer, RARs, som bildar heterodimerer med retinoid X receptorer. Dessa heterodimerer binder till specifika gensekvenser (DNA-responselement). RXR-receptorerna heterodimeriserar också med andra nukleära receptorer (t.ex. PPAR, VDR, CAR, PXR, LXR och FXR). Alla dessa receptorer är inblandade i kärnreceptorsignalering, och således kan retinoidsignalering också påverkas av, och påverka, signalering *via* dessa andra kärnreceptorer. Detta kallas för cross-talk. RA har också visats vara inblandat i epigenetisk reglering.

Under utvecklingen av reproduktionsorganen, och den könsdifferentiering av könsceller som sker samtidigt, finns RA initialt tillgängligt från flera olika källor i, eller i närheten av, gonaden. RA anses vara viktigt vid initiering av meiosen, dock, är detta ett mycket aktivt forskningsfält. Meiosinitiering sker under fosterutvecklingen hos kvinnor och efter födseln hos män. Studier på gnagare har visat att reglering av det temporala och rumsliga uttrycket av specifika enzymer, involverade i RA-metabolism, är avgörande för att meiosen ska initieras embryonalt hos honor, men också för att förhindra för tidig meios hos hannar.

RA har även rapporterats vara av betydelse för somatisk (det vill säga, ej könsceller) cellutveckling i reproduktionsorganen, för äggstockarnas funktion, och för implantering av embryot i livmoderslemhinnan (endometriet). Hos kvinnor, har dessutom förändrad retinoidsignalering associerats med endometrios, liksom med polycystiskt ovariesyndrom.

Hos den vuxne individen är RA-signalering viktig för korrekt spermiebildning. Mer specifikt måste korrekta RA-nivåer upprätthållas inuti de sädesförande kanalerna (seminiferous tubules) för korrekt spermatogonie-differentiering, meios-initiering och frisättning av spermier (spermatozoer). Dessutom har RA föreslagits vara viktigt för de sekundära manliga reproduktionsorganens utveckling och funktion (sädesblåsan, bitestiklarna, prostata). Många av dessa processer styrs också av andra endokrina system, och mellan dessa system finns det omfattande samreglering. Mer kunskap behövs om många av de ovan beskrivna processerna, för att förstå vilken betydelse RA-signalering har för reproduktionen i förhållande till andra endokrina signalvägar, och på vilket sätt cross-talk mellan olika kärnreceptorer påverkar RA-signalering.

Några få kemikalier har visats störa retinoidsignalering. Kemikalier, för vilka det finns åtminstone vissa data, inkluderar farmaceutiska retinoider, konazol-fungicider och

organiska tennföreningar. Dock saknas studier som undersökt en specifik kemikalies effekter, förutom några läkemedel, på honlig och/eller hanlig fortplantning, samtidigt som man för samma kemikalie undersökt effekten på retinoidsystemets reglering i reproduktionsorganen. Avsaknad av sådan information är en stor kunskapslucka, som behöver fyllas.

I denna rapport presenteras fyra olika visualiseringar av möjliga ogynnsamma utfallsvägar (possible adverse outcome pathways; AOPs), som beskriver möjliga samband mellan effekter på retinoidbalansen i fortplantningsorganen, och påverkan på fortplantningen. Vår förhoppning är att dessa visualiseringar kan användas som utgångspunkt vid framtida arbete med att utveckla och förfina dessa AOP-utkast.

Det inledande arbetet med inhämtning av kunskap för identifiering av möjliga metoder (initial scoping effort) som presenteras i denna rapport identifierar att de RA-nedbrytande CYP26-enzymerna och de RA-syntetiserande RALDH-enzymerna skulle kunna integreras på nivå 1 i OECDs konceptuella ramverk (CF nivå 1 och 2). Detta kan vara möjligt med hjälp av utveckling av *in silico*-metoder, såsom (Q)SAR (kvantitativa struktur-aktivitetssamband) eller molekylära dockningsmodeller, och/eller *in vitro*-metoder, för dessa enzymer. I de nuvarande riktlinjerna för *in vivo*-testmetoder (CF nivå 3-5), omfattas viktiga reproduktionsrelaterade parametrar, identifierade som känsliga för störningar av retinoidbalansen, redan nu av befintliga histopatologiska undersökningar. Som diskuteras i denna rapport regleras dock reproduktionen av många andra endokrina signaleringsvägar, och tolkningen av observerade effektsamband försvåras dessutom av omfattande cross-talk mellan nukleära receptorer. Följaktligen har inga utvärderingsmått identifierats i denna rapport som är specifikt relevanta för retinoidstörning, och som kan läggas till OECDs befintliga testriktlinjer för *in vivo*-studier.

Inga lämpliga referenskemikalier, med undantag för ett fåtal läkemedelssubstanser, kända för att påverka fertiliteten enbart *via* påverkan på retinoidsignalering, har identifierats i denna rapport. För framtida validering av retinoidspecifika metoder, kommer sådana kemikalier att behövas.

Trots dessa utmaningar kan utvalda *in vitro*- och *in silico*-metoder utgöra en del av ett bredare screeningtestbatteri som syftar till identifiering av substanser som potentiellt, *via* retinoidsignalering, kan påverka reproduktionen. För regulatoriskt beslutsfattande kan till exempel histopatologiska undersökningar av äggstockar och testiklar i *in vivo*-studier, som redan beskrivs i OECDs testmetoder, ge tillräcklig information, även utan vetskap om exakt vilken endokrin signaleringsväg som har påverkats.

List of abbrevations

ADH	Alcohol dehydrogenase
AhR	Aryl hydrocarbon receptor
ALDH	Aldehyde dehydrogenase
AMH	Anti-müllerian hormone
AO	Adverse outcome
AOP	Adverse outcome pathway
ARAT	Acyl CoA:retinol acyltransferase
BMP	Bone morphogenic protein
BTB	Blood-testis barrier
CAR	Constitutive androstane receptor
CF	Conceptual framework
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
CYP	Cytochrome P450 hydroxylase
CYP17	17,20-lyase, 17α-hydroxylase
Cyp2b10	Cytochrome P450, Family 2, subfamily b, polypeptide 10
CYP26	Cytochrome P450, Family 26
Dazl	Deleted in azoospermia-like
DEHP	Diethyl hexyl phthalate
DHRS3	Retinaldehyde reductase 3
DMRT1	Doublesex and mab-3 related transcription factor 1
dpc	Days post coitum
DRP	Detailed Review Paper
Foxl2	Forkhead box protein L2
FSH	Follicle stimulating hormone
FXR	Farnesoid X receptor

GR	Glucocorticoid receptor
GW	Gestational week
HSD	Hydroxysteroid dehydrogenase
KE	Key event
LH	Luteinizing hormone
LRAT	Lecithin:retinol acyltransferase
LXR	Liver X receptor
MEHP	Monoethyl hexyl phthalate
MIE	Molecular initiating event
MMP	Matrix metalloproteinases
Nanos2	Nanos C2HC-Type Zinc Finger 2
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PCOS	Polycystic ovarian syndrome
PGC	Primordial germ cells
PND	Post-natal day
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
(Q)SAR	(Quantitative) structure-activity relationship
RA	All-trans retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAMBA	RA metabolism blocking agents
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP4	Retinol-binding protein 4
RDH10	Retinol dehydrogenase 10
RDH11	Retinol dehydrogenase 11
RE	Retinyl esters
Rec8	Meiotic recombination component gene Rec8
REH	Retinyl ester hydrolase
RIP140	Nuclear receptor interacting protein 1

ROH	Retinol
RXR	Retinoid X receptor
P450scc	Cytochrome P450 cholesterol side-chain cleavage enzyme
SDR	Short-chain dehydrogenase/reductase
Sox9	SRY -Box Transcription Factor 9
SRC-1; Ncoa1	Nuclear receptor coactivator 1
SREBP-1c	Sterol regulatory element binding protein-1c
Sry	Sex-determining region Y protein
Stra6	Stimulated by retinoic acid, gene 6
Stra8	Stimulated by retinoic acid, gene 8
TG	Test guideline
TTR	Transthyretin
VAD	Vitamin A deficiency
VDR	Vitamin D receptor
Wnt4	Wnt Family Member 4

1. Introduction to the retinoid system

1.1. History and general background

Retinoids[6], a chemically related group of compounds which includes vitamin A, can exist in several different forms (see Figure 1). As the name "vitamin" implies, they are essential micronutrients that must be supplied from the diet, either in the form of carotenoids (orange, red and yellow fat-soluble pigments) from vegetable sources, or retinol (ROH) and retinyl esters (RE) from animal sources (reviewed by Harrison 2012, Al Tanoury *et al.* 2013). Retinoids from animal sources are originally derived from carotenoids (reviewed in O'Byrne and Blaner 2013). The liver, an organ found in all vertebrate organisms, can be a rich source of retinoids and may have been used by the ancient Egyptians as a cure for night blindness, a typical symptom of retinoid deficiency (Wolf 1996). Conversely, Arctic cultures have long known to avoid eating polar bear liver, which can contain extremely high levels of retinoids, in order to avoid adverse effects such as blurred vision, nausea, skin loss, coma and even death (Rodahl and Moore 1943). Thus, both hypovitaminosis A and hypervitaminosis A can be detrimental.

In addition to being a nutrient-derived vitamin, retinoids can also be considered as hormones, based on their nuclear receptor signaling (Giguère *et al.* 1987, Petkovich *et al.* 1987). In contrast to classical hormones, there is no endocrine gland that synthesizes retinoids, controlled *via* feedback by the hypothalamus and pituitary gland. Instead, the levels of the active form of vitamin A (all-*trans* retinoic acid; RA) are tightly regulated via local cellular enzymatic mechanisms; a regulation that is critical for correct signaling *via* the nuclear receptors (reviewed in Ghyselinck and Duester 2019).

The requirement for retinoids in normal physiological functions has been studied for more than a century, by examining the effects of retinoid deficiency or excess in different species, and, more recently, in genetically modified mice (reviewed in Clagett-Dame and Knutson 2011). The importance of retinoids and the retinoid signaling pathways is reflected in both the ancestry and the conservation of genes and pathways among both vertebrates and invertebrates (André *et al.* 2014).

Retinoids are essential for vision, reproduction, embryo-fetal development, adult growth and development, and maintenance of immunity and epithelial barriers (reviewed in O'Byrne and Blaner 2013). Most diets contain sufficient amounts of retinoids, and the most fat-soluble forms, retinyl esters, can be stored within the body at relatively high levels, thereby counteracting periods of low dietary retinoid intake (reviewed in O'Byrne and Blaner 2013). In spite of this, vitamin A deficiency (VAD) is common in some parts of the world (WHO 2009), and VAD is the main

6. The term "retinoids" originally had a structural basis, referring to isoprene-derived compounds (IUPAC 1982), but currently the term refers to compounds (natural or synthetic) capable of activating a set of receptors (Sporn and Roberts 1985).

cause of preventable blindness in the world (West 2003, Bastos Maia *et al.* 2019). It has also been hypothesized that one reason for hearing loss among humans in the developing world is gestational VAD (Emmett and West 2014). In addition, new metabolic functions for retinoids have been reported, in *e.g.* lipid metabolism and insulin response (reviewed in Napoli 2017, Cione *et al.* 2016).

For several decades, retinoids have been used for pharmaceutical purposes (reviewed in Theodosiou *et al.* 2010). For example, retinoids can be prescribed for treatment of cystic acne, where they have favorable effects on epithelial cell differentiation. In acute promyelocytic leukemia, retinoids can induce terminal differentiation in cancerous cells, and thereby proliferation ceases.

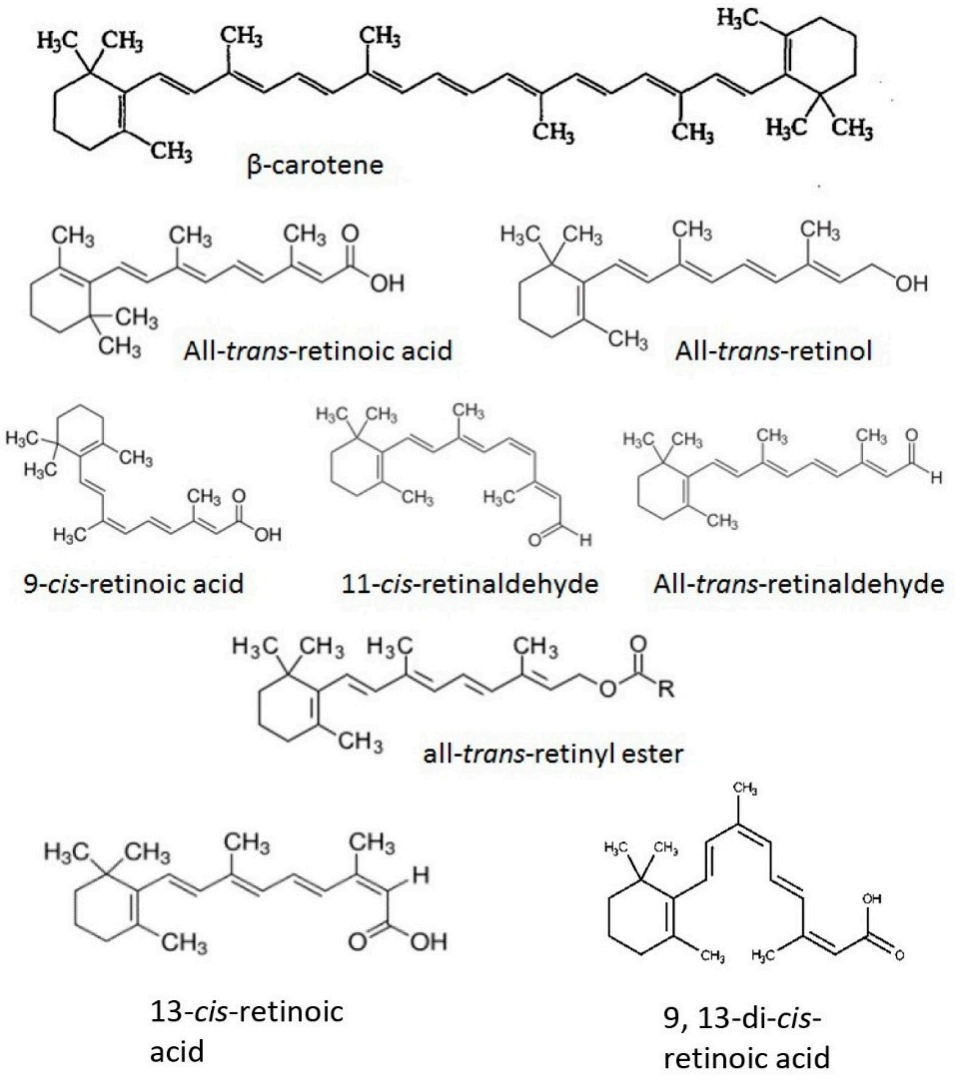

Figure 1: Chemical structures of various forms of naturally occurring retinoids and retinoid precursors. R (in the retinyl ester) represents an acyl chain of variable length (reviewed in Goodman 1984

1.2. Retinoid storage, uptake, transport and metabolism

1.2.1. Storage

The vast majority, approximately 80–85%, of retinoids in the body are stored in the liver (reviewed in O'Byrne and Blaner 2013). Two main cell types, hepatocytes and hepatic stellate cells, have been identified as being central to metabolism and storage of retinoids. Hepatocytes contain 10–20% of hepatic retinoids, and they are also involved in the initial uptake of retinoids into the liver (Blomhoff *et al.* 1982). Hepatocytes are also the site of synthesis and secretion of the serum transport protein of ROH; retinol-binding protein type 4 (RBP4; Sauvant *et al.* 2001). Thus, hepatocytes are important both in the uptake and the mobilization of vitamin A into, and out of, the liver. Hepatic stellate cells have been found to contain 80–90% of hepatic retinoids (reviewed in Blaner and Li 2015). Studies in mice using radiolabeled RE have shown dietary retinoids to be initially taken up by the hepatocytes and then rapidly transferred to hepatic stellate cells for storage (Blomhoff *et al.* 1982). Smaller RE stores are also found in a number of other organs or tissues, including the lung, brain, skin, muscle, kidney, spleen, white and brown adipose tissue, and testis (reviewed in Blaner and Li 2015).

Another retinoid storage organ is the eye (reviewed in Palczewski 2012). Retinaldehyde has long been known to play a vital role in vision and eye function. The retina and retinal pigmented epithelium contain 11-*cis* and all-*trans*-isomers of retinaldehyde, but also ROH and RE. The retinal pigmented epithelium is the main ocular site of RE storage, while the photoreceptors of the retina contain large amounts of retinaldehyde (reviewed in Palczewski 2012).

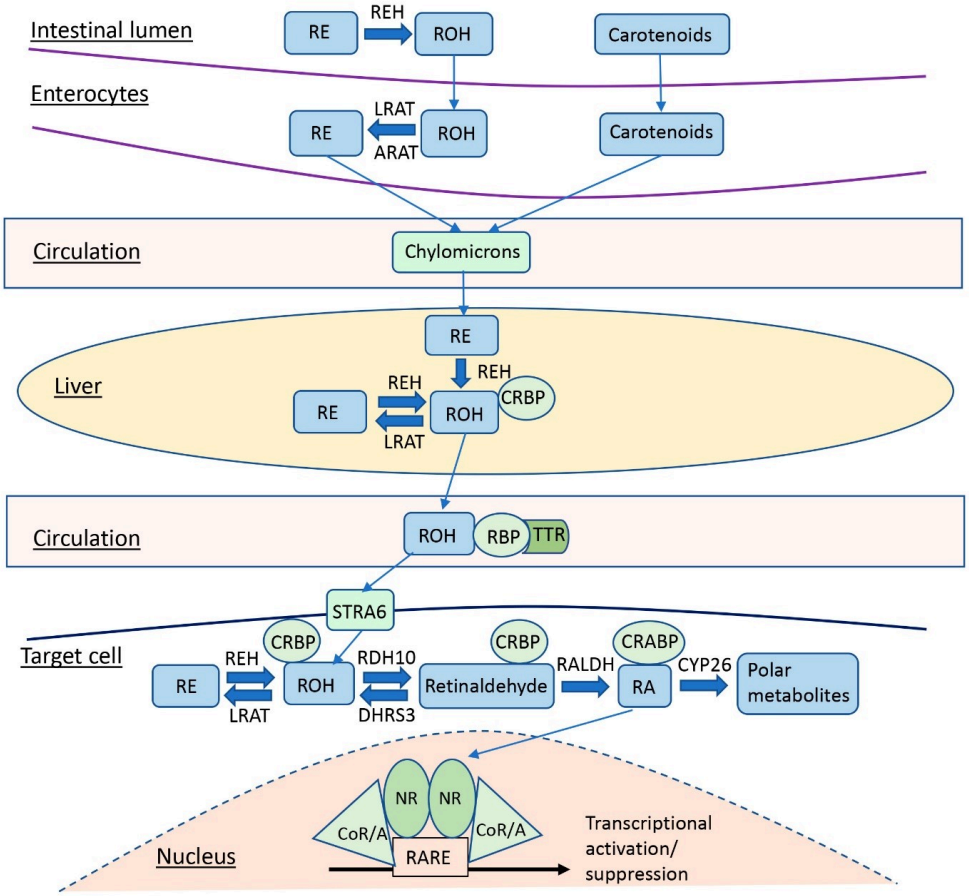

Figure 2: Overview of retinoid uptake, transport, metabolism, and nuclear signaling (as described in detail in the note below)

Note:

In the intestinal lumen, RE are hydrolyzed into ROH, which after uptake into enterocytes is re-esterified to RE. RE together with carotenoids are incorporated into chylomicrons and exported to the circulation. Eventually, the chylomicron remnants are taken up by the liver. Here, RE are again hydrolysed into ROH. ROH is either re-esterified for hepatic storage or bound to RBP4 for release into the circulation. In the bloodstream, the ROH-RBP4 complex associates with transthyretin (TTR). After uptake (possibly aided by STRA6) into target cells, ROH is reversibly oxidized to retinaldehyde by RDH10. Retinaldehyde may then be irreversibly oxidized to RA by retinal dehydrogenases (RALDHs), or be reduced back to ROH by DHRS3. RA is degraded to non-active polar metabolites by CYP26 enzymes, but can also be shuttled to the nucleus by cellular RA-binding proteins (CRABP), where RA acts as a ligand to nuclear receptors (NR). Non-liganded receptors interact with co-repressors (CoR), repressing transcription, while RA liganded receptors- binds co-activators (CoA), subsequently activating transcription, thereby modulating expression of Retinoic Acid Response Element (RARE)-regulated target genes. Additional abbreviations: ARAT, acyl CoA:retinol acyltransferase); CRBP, cellular retinol-biding protein; CYP26; cytochrome P450 hyroxylase type 26; DHRS3, retinaldehyde reductase 3; LRAT, lecithin:retinol acyltransferase; RA, retinoic acid; RE, retinyl esters; REH, retinyl ester hydrolase; RBP, retinol-binding protein 4; ROH, retinol; SDR, short-chain dehydrogenase/reductase; Stra6, stimulated by retinoic acid, gene 6; RDH10, retinol dehydrogenase 10.

A number of enzymes are required for the balance of storing and re-mobilizing of retinoids during times of abundance and insufficiency, respectively (see Figure 2). The most important enzymes are those synthesizing RE (the physiologically important enzyme is LRAT; lecithin:retinol acyltransferase) and hydrolyzing RE to ROH (REH; retinyl ester hydrolase). These enzymes, along with accumulation of RE in lipid droplets (chylomicrons) within cells and tissues, manage the natural fluctuations in dietary retinoid intake (O'Byrne and Blaner 2013). The process of remobilization of stored RE into circulating ROH seems reliant on interactions with RBP4, based on observations in RBP4-deficient mice (Quadro *et al.* 1999).

1.2.2. Uptake and transport

Intestinal uptake of retinoids in the diet (reviewed in Harrison 2012) involves hydrolysis of RE (by REH) into ROH in the intestinal lumen, followed by re-esterification of ROH into RE after absorption of ROH into the enterocytes. In addition to LRAT, the enzyme acyl CoA:retinol acyltransferase (ARAT) can play an important role when the levels of ROH exceed normal physiological levels (as after a meal rich in retinoids). The resulting RE, together with carotenoids, are released into the circulation as part of chylomicron particles.

In the circulation, a range of retinoid forms are found in fasting and/or non-fasting states. ROH bound to RBP4 and chylomicron-RE are considered the most important transport forms. Circulating RE levels are highly dependent on the dietary content of RE and carotenoids. During fasting, ROH is the main circulating form of retinoids. Serum ROH levels are maintained within a narrow range, unless the RE levels in liver and other tissues are very low (Green and Green 1994, and reviewed in Tanumihardjo *et al.* 2016).

Both circulating and intracellular ROH is generally bound to chaperone proteins. Of these, RBP4 transports ROH in the blood, and cellular retinol-binding protein types 1 and 2 (CRBP1, CRBP2) and inter-photoreceptor retinoid-binding protein are believed to manage intracellular transport (reviewed in Blaner and Li 2015). In contrast, there is no specific binding protein for the transport of RA in the plasma, as it is synthesized locally in target cells. Intracellular retinoic acid-binding proteins (CRABPs) are involved in RA transport from the cytoplasm to the nuclear retinoic acid receptors (RAR), and also play a role in determining intracellular RA levels by controlling the amount of RA that is available for degradation (reviewed in Napoli 2012, and in Napoli 2017).

Retinoids are delivered to tissues in a similar manner as vitamin D and thyroid hormone (reviewed in Blaner and Li 2015), *i.e.*, by transport of large quantities of the biologically-inactive form (in this case ROH), and relatively low quantities of the biologically-active form (RA) in the blood (reviewed in Blaner and Li 2015). ROH, bound to RBP4, forms a larger complex with the T4 transporting protein transthyretin (TTR) in the blood (See Figure 2). Both TTR and RBP4 are mainly synthesized in the liver and choroid plexus, secreted into plasma by the liver, and into cerebrospinal fluid by the choroid plexus, respectively (Shirakami *et al.* 2012).

Transport of ROH (bound to RBP4) across membranes may be facilitated in both directions by the membrane protein, Stimulated by retinoic acid, gene 6 (STRA6)

(reviewed in Kelly and von Lintig 2015). ROH uptake by STRA6 seems to be enhanced by the presence of LRAT and CRPB1 on the intracellular side of the membrane, in a manner that appears to depend on the availability of ROH inside the cell. Alternatively, with the exception of the eye, the role of STRA6, even under VAD conditions, may not be mandatory for ROH availability to tissues (Berry *et al.* 2013).

1.2.3. Metabolism

The most abundant forms of retinoids in the body are ROH and RE. A number of conversion steps are required to transform relatively inactive forms of retinoids into the biologically active RA form within target cells (reviewed in Shannon *et al.* 2017). Once ROH-RBP4 has been bound to STRA6 and taken up into a target cell, ROH is converted to RA via a two-step oxidation process (See Figure 2). ROH is first converted into retinaldehyde; this conversion is carried out by a short-chain dehydrogenase/reductase (SDR; a complex consisting of retinol dehydrogenase 10 [RDH10], and retinaldehyde reductase 3 [DHRS3]), and possibly also by the less specific cytosolic alcohol dehydrogenases (ADH) (reviewed in Shannon *et al.* 2017). The predominant fraction of intracellular ROH is bound to CRBP and directed towards SDR (reviewed in Napoli 2017). The formed retinaldehyde can subsequently either be reduced back to ROH by SDR, or, under VAD conditions, by retinol dehydrogenase 11 (RDH11) (Belyaeva *et al.* 2018). In a second RA-conversion step retinaldehyde can be irreversibly oxidized into RA by retinaldehyde dehydrogenases RALDH 1, 2 or 3 (also known as aldehyde dehydrogenases ALDH1A1-3, reviewed in Shannon *et al.* 2017). The ratio of holo-CRBP to apo-CRBP[7] appears to signal whether retinaldehyde should be reduced back to ROH or further oxidized to RA (reviewed in Napoli 2017). The fate of the retinaldehyde also depends on a negative feedback response to available RA (reviewed in Shannon *et al.* 2017).)

RA has a short half-life (approximately one hour), and RA tissue levels are governed in a spatially and temporally controlled manner, mainly by a balance of local synthesis and metabolic breakdown with RA feedback loops (reviewed in Kedishvili 2013, and Teletin *et al.* 2017). Some tissue-specific uptake of RA from serum appears to take place, *via* a still unestablished mechanism (Kurlandsky *et al.* 1995). RA is catabolized mainly by cytochrome P450 hydroxylases (CYP) 26A1, B1 and C1 (Topletz *et al.* 2015). A large number of other CYP enzymes have also been shown to degrade RA *in vitro*, although the relevance of these enzymes in normal retinoid homeostasis *in vivo* is unclear (reviewed in Laudet *et al.* 2015). In the adult human, CYP26A1 is mainly expressed in the liver, and is responsible for more than 90% of the hepatic clearance of RA (Thatcher *et al.* 2010). CYP26A1 is also expressed in *e.g.* testis, epididymis, uterus, endometrium and placenta, while CYP26B1-expression is more dispersed and found *e.g.* in the brain, testes, placenta, ovaries and endometrium (Human Protein Atlas[8]). In the human fetus, CYP26A1 is the form predominantly expressed in the brain, whereas CYP26B1 is expressed in all tissues except the brain (reviewed in Kedishvili 2013). At least in humans, CYP26C1 is mainly expressed during embryonic development, but is also expressed at low levels in adult tissues, *e.g.* in testis (reviewed in Ross and Zolfaghari 2011).

The physiological role of RA in cell differentiation has motivated its use in cancer treatment, including reproductive organ malignancies (Siddikuzzaman *et al.* 2011).

7. Ratio: holo-CRBP (CRBP-ROH/retinaldehyde) to apo-CRBP (CRBP without ROH/retinaldehyde).
8. https://www.proteinatlas.org/ (accessed in May 2019).

The treatment increases the normal serum nM (nanomolar) levels of RA up to μM (micromolar) levels, which over time leads to autoinduction of RA clearance *via* upregulation of CYP26 enzymes (Jing *et al.* 2017). Attempts have been made to counteract therapy resistance by developing *e.g.* CYP26 inhibitors (Nelson *et al.* 2013). However, side effects and low potency has limited the use of these inhibitors (Jing *et al.* 2017). Similar efforts to maintain RA homeostasis has also been observed in models of teratogenicity, and it has been speculated that some of the teratogenic effects of RA may be due to a prolonged local RA deficiency, caused by differential induction of genes coding for enzymes synthesizing (*Raldh1-3*) or breaking down (*Cyp26a1* and *Cyp26b1*) RA (Lee *et al.* 2012).

1.3. Retinoid receptors and gene regulation

RA acts mainly by interacting with nuclear retinoic acid receptors, RARs, which form heterodimers with retinoid X receptors (RXRs) and ultimately regulate gene transcription, thereby influencing a variety of cellular processes (reviewed in Germain *et al.* 2006). The receptor dimers bind to retinoic acid response elements (RARE), and, in the absence of ligand, recruit a co-repressor complex that suppress transcription (Vilhais-Neto and Pourquié 2008). In the presence of ligand, the co-repressor complex dissociates and is replaced by a co-activator complex, leading to transcription of the target gene (Germain *et al.* 2002). While RAR can be activated both by RA and 9-*cis*-RA as well as synthetic ligands, RA is considered to be the only endogenous RAR ligand (Allenby *et al.* 1993, Mouchon *et al.* 1999). RARs in an unliganded state are also known to act as transcriptional repressors (reviewed in Weston *et al.* 2003). The endogenous ligand for RXR was initially suggested to be 9-*cis*-RA, and other forms such as 9-*cis*-13,14-dihydro-RA have been put forth more recently (de Lera *et al.* 2016). However, the physiological relevance of the suggested ligands is still unclear (reviewed in Krężel *et al.* 2019). Non-enzymatic isomerization between different forms is likely to be important (reviewed in Blaner 2001), which impedes conclusions regarding endogenous ligands. In RAR-RXR heterodimers, RXR is a "silent partner", meaning that the RAR ligand is both necessary and sufficient for dissociating the corepressor complex (Mangelsdorf and Evans 1995, le Maire *et al.* 2019).

RARs and RXRs belong to the same nuclear hormone receptor family as steroid hormones, thyroid hormone and vitamin D receptors, as well as various orphan receptors and receptors activated by intermediary metabolites (*e.g.* PPAR; peroxisome proliferator-activated receptor, LXR; liver X receptor, FXR; farnesoid X receptor and PXR; pregnane X receptor) (Szanto *et al.* 2004). RXR is also the essential heterodimerization partner to these receptors. Some of these nuclear receptors may also be involved in retinoid signaling responses. For example, PPARα, β/δ and γ heterodimerize with RXR and function as transcription factors (Mangelsdorf *et al.* 1995; see also section on cross-talk below). RA has been reported to serve as a ligand for PPAR β/δ, but with a much lower affinity than for RAR (Al Tanoury *et al.* 2013).

Three of the retinoid receptors (RARα, RXRα and RXRβ) have widespread expression patterns, whereas RARβ, RARγ and RXRγ show a more restricted, tissue-specific expression. Therefore, most tissues are potential targets of retinoid signalling, although different heterodimeric complexes can transduce the RA signal (reviewed in Rhinn and Dollé 2012). Many tissues will also be indirect targets *via* RXR

heterodimerization with receptors (see Figure 3). Receptors mediate and interpret the information provided in the chemical structure and energy of a nuclear receptor ligand, in the context of the cell and its physiology, converting it into a sequence of receptor-protein and receptor DNA interactions. This can be *via* ligand binding, receptor phosphorylation, induction of allosteric changes in receptor docking surfaces including subunits of transcription, epigenetic machinery and enzyme induction. The next section explores these latter molecular aspects of retinoid mechanisms related to receptor cross-talk.

In addition to the classical genomic effects, RA has been found to have a number of non-genomic mechanisms such as kinase activation (reviewed in Rochette-Egly 2015, Park *et al.* 2019). More specifically, these effects may include activation by RA of phosphatidylinositol 3-kinase(PI3K)/Akt signalling during neural differentiation, rapid activation of p38 mitogen activated protein kinase (p38MAPK)/mitogen and stress-activated kinase 1 (MSK) pathways (reviewed in Laudet *et al.* 2015). Such effects are not further discussed in this report.

1.4. Heterodimerisation partners and receptor cross-talk

Members of the same nuclear receptor family share a common heterodimerisation partner, RXR. There is cross talk with other nuclear receptors and with a broad range of intracellular signaling pathways. Consequently, there may be competition for RXR for the dimerization stage of receptor activation of DNA. There may even be a cascade effect, in which metabolites produced through the activities of one receptor act as specific signaling molecules and ligands to modulate the next receptor, a link in the nuclear receptor intercommunication web of the body (See Figure 3).

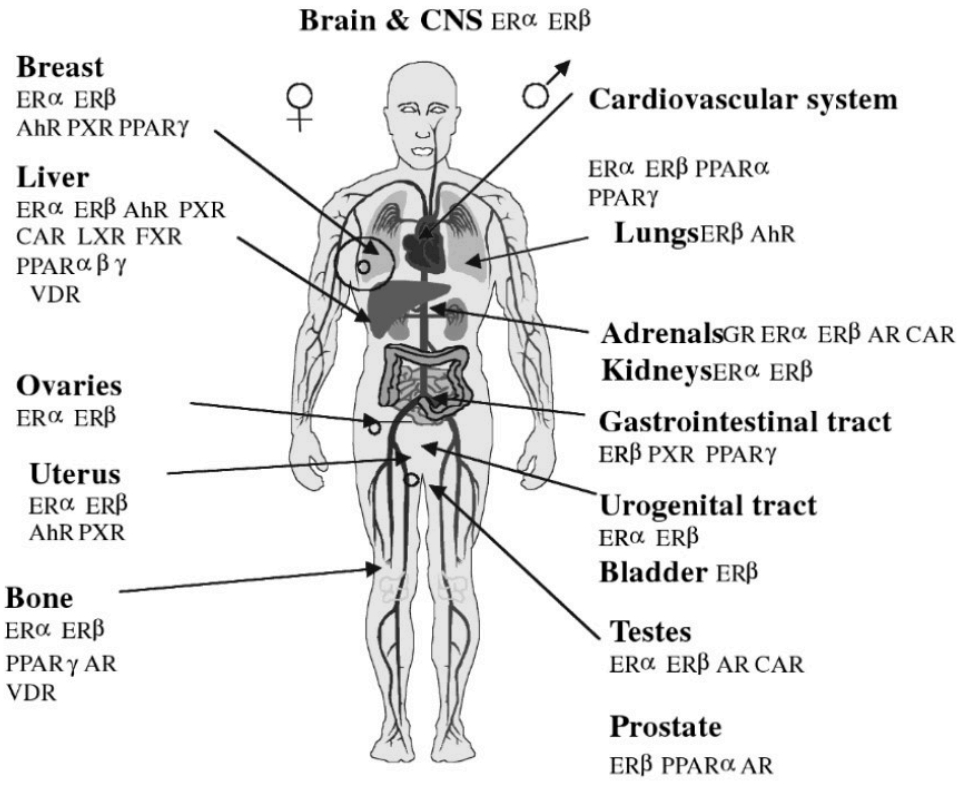

Figure 3: Schematic diagram showing differences in tissue distribution of nuclear receptors

Note:

RXR; retinoid X receptor, is the heterodimerisation partner essential to the normal functioning of the main xenobiotic metabolizing receptors including CAR, PXR, PPARs, LXR, FXR. Furthermore, cross-talk with ERα and ERβ is established. In addition, RXR is also the heterodimerization partner for VDR and the thyroid stimulating hormone receptor (TSHR) (not included in the Figure). Abbreviations: *AhR; Aryl hydrocarbon receptor, AR; androgen receptor, CAR; constitutitive androstane receptor, ERα and ERβ; estrogen receptors α and β, FXR; farnesoid X receptor, GR; glucocorticoid receptor, LXR; liver X receptor, PPARs; peroxisome proliferator activated receptors, PXR; pregnane X receptor, VDR; vitamin D receptor. (Jacobs 2005, reprinted with kind permission from the publisher: Taylor and Francis Inc.)*

The ubiquitous RXRα is the necessary heterodimerization partner for many receptors, and is essential for xenobiotic metabolism *in vivo*. The receptors include the thyroid receptor, PXR, CAR, where retinoic acid has also been noted to repress CAR induction of the Cytochrome P450, family 2, subfamily b, polypeptide 10 (*Cyp2b10)* gene in mouse hepatocytes (Kakizaki *et al.* 2002), as well as LXR, FXR, GR, PPARα (Cai *et al.* 2002) and PPAR gamma (Dubuquoy *et al.* 2002) to bind to DNA. Crystal structure data of the PPARγ and RXRα heterodimer shows the asymmetric conservation heterodimerization interfaces between both receptors (Gampe *et al.* 2000). RXRα dimerizes through a 40-amino acid subregion within the ligand binding domain, known as the 'identity box'. Mutation of two important determinants (alanine 416 and arginine 421) within this box has been shown to impair

the actions of receptor dimerization partners. RXRα is well established as a heterodimeric integrator of multiple physiological processes in the liver, and is a regulatory component of cholesterol, fatty acid, bile acid steroid and xenobiotic metabolism and homeostasis.

The retinoid ligands of RXR have distinct effects in different contexts and have been reported to significantly alter the response of the CAR-RXR heterodimer to CAR ligands (Tzameli *et al.* 2003) for example. Suppression of RXRα has a concomitant effect upon the heterodimerization partner. For example LXR is reported in this way to inhibit PPARα signaling in the nutritional regulation of fatty acid metabolism. PPARα has a counter-inhibitory action repressing LXR/RXR binding through the sterol regulatory element binding protein-1c (SREBP-1c) (Ide *et al.* 2003, Yoshikawa *et al.* 2003).

RXRα also has cofactors in common with other nuclear receptors, for instance, over 20 years ago Wiebel and co-authors described a competitive element between nuclear receptor interacting protein 1 (RIP140) and nuclear receptor coactivator 1 (SRC-1; Ncoa1) in binding with OR-1 with RXR to heterodimers of a novel orphan receptor (Wiebel *et al.* 1999). RIP140 is also implicated in the potentiation of endocrine disrupting compounds *in vitro* (Sheeler *et al.* 2000). SRC-1 RXR phosphorylation can also be induced through stress pathway activation (Lee *et al.* 2000), which would reduce RXR availability for other receptor heterodimerization partners. Hua and co-authors have demonstrated competition between RAR/RXR heterodimers and ERα for binding sites in a breast cancer MCF-7 cell line. Indeed, the experimental work suggested that there may be antagonistic transcriptional regulation for up to 71% of the target genes that they evaluated (Hua *et al.* 2009).

As with many nuclear receptors, ERα- and RAR-binding sites appear to have co-evolved on a large scale throughout the human genome, often resulting in competitive binding activity at nearby or overlapping *cis*-regulatory elements. The intersection between these two critical nuclear hormone receptor signaling pathways is highly coordinated to give a unifying mechanism for balancing gene expression output via local regulatory interactions dispersed throughout the genome. This selection or competition of dimerization partners determines tissue/organ and biological system level outcomes. It can be affected by genetic, nutritional, and environmental factors, which for the latter can include both beneficial and adverse nutrient and chemical exposure.

An example for the RXR-PXR downstream activation of CYP3A4 is given in Figure 4.

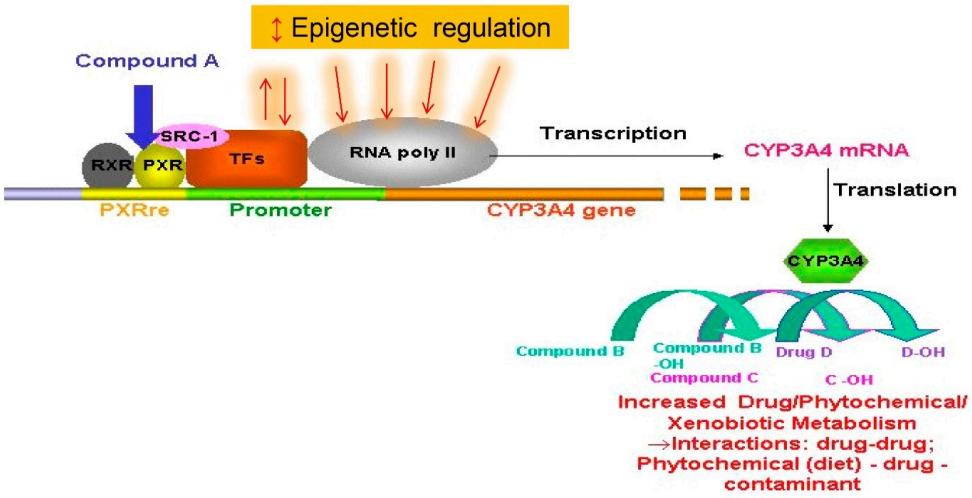

Figure 4: Illustration of chemical-nuclear receptor-, and epigenetic, transcription factor regulation

Abbreviations:
PXRre; pregnane X receptor response element, RNA poly II; RNA polymerase II, TFs; transcription factors. (Adapted from Jacobs et al. 2005.)

Not only are the heterodimerization receptor cross-talk aspects of retinoid biology, via RXR, essential for many xenobiotic metabolic processes in the body, but they are also essential in the steroidogenic pathway, for the production of sex steroids that are prototype ligands for androgen and oestrogen receptor activity, and thus affect the pool of circulating sex steroid hormones (See Figure 5).

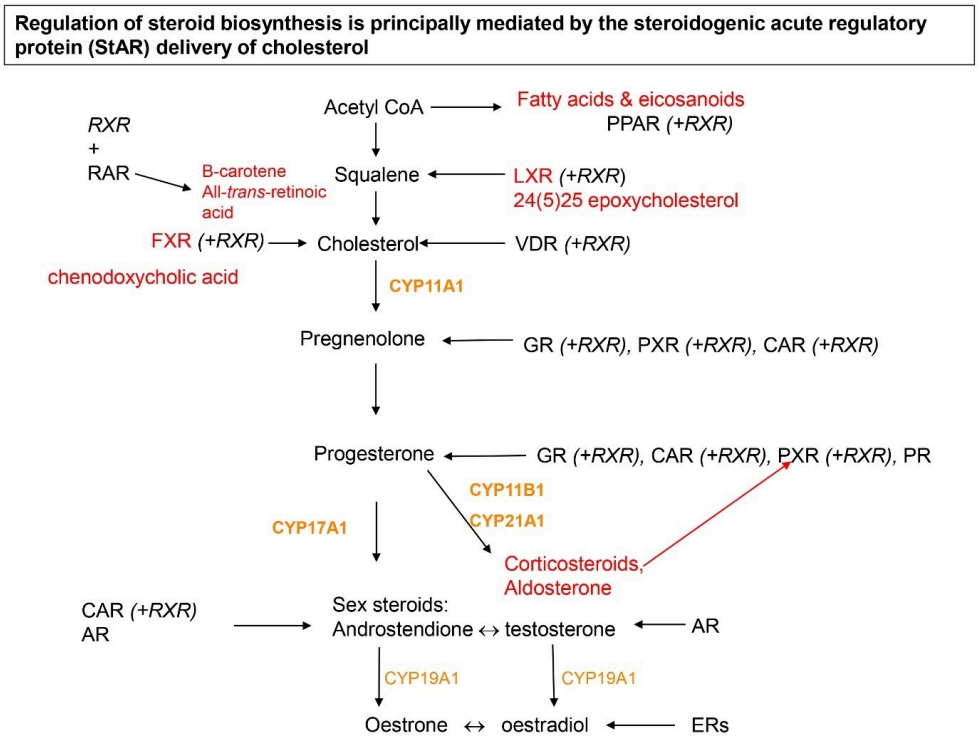

Figure 5: The steroidogenic pathway indicating RAR; retinoic acid receptor, RXR; retinoid X receptor, and heterodimerization partner interactions

Note:

RXR and RAR play a pivotal role at the outset of the pathway, that affects the entire subsequent cascade, whilst RXR also play a key role in the subsequent pathway steps as a heterodimerization partner for PPAR, LXR, FXR, GR, PXR and CAR.

Abbreviations: *AhR; Aryl hydrocarbon receptor, AR; androgen receptor, CAR; constitutitive androstane receptor, ER; estrogen receptor, FXR; farnesoid X receptor, GR; glucocorticoid receptor, LXR; liver X receptor, PPARs; peroxisome proliferator activated receptors, PXR; pregnane X receptor, VDR; vitamin D receptor, PR; progesterone receptor. (Adapted from Jacobs 2004.)*

Furthermore, these molecular initiating event level processes each have pathway outcomes that in the case of the PPARs is associated with lipid homeostasis, adiposity and obesity. For example, the delivery of retinoic acid to either RAR or PPARβ/δ/VDR determines its biological effects on adipose development (Wang B *et al.* 2016). In fibroblasts for example, binding with a ligand-activated VDR stimulates non-adipogenic gene transcription, whilst in adipocytes ligand activation of PPAR gamma together with heterodimerization with RXR stimulates adipogenic gene transcription.

Other retinoic acid regulation consequences include insulin stimulated glucose secretion, regulation of continuous asynchronous spermatogenesis (Chung *et al.* 2004, Chung and Wolgemuth 2004, Hogarth and Griswold 2013), and immunomodulatory roles in inflammation and cancer (Stevison *et al.* 2015), as well as RXR and RAR expression in tumours (Li *et al.* 2014).

In addition, cross-talk has also been reported with androgenic signaling (Long *et al.* 2019), as well as with xenobiotica-related receptor pathways, *via e.g.* the constitutive androstane receptor (CAR), PXR and AhR, has been demonstrated;

possible responses include triggering or suppression of induction of xenobiotica-metabolizing enzymes (Murphy *et al.* 2007, Shmarakov *et al.* 2019).

Cross-talk has additionally been shown on the level of regulating expression of *e.g.* RA-catabolizing enzymes in the CYP26 family. It has been demonstrated that in human liver cells, PPARγ agonists rosiglitazone and pioglitazone induce CYP26A1 as well as the normally less abundant CYP26B1 (Tay *et al.* 2010). The non-endocrine sonic hedgehog (SHH) pathway is reported to regulate expression of the *Cyp26a1* and *Cyp26b1* genes, thereby preventing excessive RA levels during mouse embryonic development (El Shahawy *et al.* 2019). In the testicular Leydig cells, cross-talk between the retinoid signaling system and testosterone signaling has been shown to be crucial for steroidogenic cell function (Jauregui *et al.* 2018). Mechanistically this can be understood through the steroidogenic pathway, shown in Figure 5.

1.5. Epigenetics and its role in the retinoid system

Epigenetic changes are changes in gene expression that a) do not involve gene sequence alterations and b) may persist after the initial trigger is long gone (reviewed in Greally and Jacobs 2013, Villota-Salazar *et al.* 2016, and Jacobs *et al.* 2017). Epigenetic processes regulate gene expression without mutating DNA, and the dynamics are essential for normal development. They can be modified by environmental chemicals, potentially leading to developmental and later life adverse health outcomes across multiple generations. Epigenetic processes include DNA methylation, histone modifications and microRNA (miRNA) signaling.

Histone deacetylation/methylation and DNA methylation are usually associated with repression of gene transcription, while histone acetylation/demethylation and lack of DNA methylation instead leads to transcriptional activation.

miRNAs are small noncoding RNAs that also act as endogenous regulators of gene expression. RA regulates the expression of many different miRNAs, with multiple fundamental biological roles. miRNAs have been extensively studied as targets and mediators of the biological activity of RA during embryonic development, as well as in normal and neoplastic cells. However, a recent review article reports that relatively few studies have experimentally explored the direct contribution of miRNA function to the RA signalling pathway. The tissue-specific roles of miRNAs modulated by RA include stem cell pluripotency, maintenance and regeneration, embryonic development, hematopoietic and neural differentiation, therefore playing a major general role in human disease pathogenesis (reviewed in Nervi and Grignani 2014).

Environmental factors, including nutrients and diet, can alter the epigenetic cell signaling pathways, including the recruitment of transcription factors which regulate epigenetic modifications, and a good and topical example for this, is adipogenesis. Retinoic acid enhances adipogenic commitment in progenitor cells through altering epigenetic modifications in the promoters of key adipogenic genes, such as Zinc Finger Protein 423 (*Zfp423*), Extracellular signal-regulated kinase (*ERK*), Delta Like Non-Canonical Notch Ligand 1 (*Dlk1*)/Pre-adipocyte factor 1 (*Pref1*), SRY-Box Transcription Factor 9 (*Sox9*) and Kruppel Like Factor 2 (*Klf2*) in the development of preadipocytes. Epigenetic regulation of PPARγ and CCAAT Enhancer Binding Protein Alpha (*C/EBPα*) expression during adipogenesis has been reported (Ngo *et al.* 2014), and the PPARγ2 promoter for DNA demethylation has been detected in *in vitro*

studies of a chemical flame retardant, BDE 47, using a 3T3-L1 model of adipogenesis (Kamstra *et al.* 2014).

As noted in the cross-talk section, retinoic acid can alter the partnership of RXRs with other nuclear receptors, and this has been mechanistically demonstrated for the regulation of adipogenesis in the current scientific literature.

RA is known to mediate cell differentiation also *via* epigenetic mechanisms (reviewed in Urvalek *et al.* 2014). Epigenetic changes are also involved in, *e.g.,* the process of transient induction of the *Cyp26a1* gene by RA (Yuan *et al.* 2012). RA itself is a significant regulator of miRNA expression, and there are several recent relevant studies using RA for the induction of proliferation or differentiation, or as a treatment, that also reveal which miRNAs RA can up- or down-regulate (*e.g.* Shen *et al.* 2016, Czaika *et al.* 2016, Wang JH *et al.* 2016, Ouimet *et al.* 2015). Identification of pivotal miRNA markers are presently being used in diagnostic clinical treatment, and such markers have potential for possible development of *in vitro* assay study designs and inclusion in *in vivo* test methods. However, the resulting downstream consequences do not necessarily then go through the retinoid pathway, but have multiple roles in various metabolic activities of the body such as ERK Mitogen-Activated Protein Kinase (MAPK) signaling (Shen *et al.* 2016), apoptosis (Wang B *et al.* 2016) and macrophage metabolism (Oiumet *et al.* 2015) in specific and highly varied disease outcomes. Additionally, it has been experimentally confirmed that miRNA-34-1 down regulates CYP3A4 by targeting RXR alpha (Pan *et al.* 2009), whilst miR-30c-1-3p (Vachirayonstien *et al.* 2016) and miR-27b (Oda *et al.* 2014) downregulate CYP3A4 *via* PXR and VDR, as both receptors need RXR as their heterodimer partner. In some cases, epigenetic actions of RARs appear to be independent of the ligand, *i.e.*, RA (Laursen *et al.* 2012).

2. Formation of female and male reproductive organs, and the role of retinoids

The formation of the female and male reproductive organs is initiated early in fetal life.

The genital ridges (the presumptive gonads) first appear around halfway through gestation in mice, or at 10.5 days post coitum (dpc) (Spiller *et al.* 2017), whereas they appear during the first trimester in humans (Johansson *et al.* 2017, Mamsen *et al.* 2017). The somatic cell progenitors of the genital ridges are initially bipotential, *i.e.*, they are capable of developing into either Sertoli and Leydig cells (in males) or granulosa and theca cells (in females) (reviewed in Svingen and Koopman 2013). The Sertoli and granulosa cells are the first somatic cells to differentiate in testes and ovaries, respectively, and they are important for supporting the germ cells and also for orchestrating the subsequent differentiation of somatic cell progenitors into the steroidogenic Leydig and theca cells (Rotgers *et al.* 2018).

Soon after they are formed, the genital ridges begin to differentiate either as testes, in the presence of the Y-chromosomal gene, Sex-determining region Y protein (*Sry)*, or as ovaries, in the absence of *Sry* (reviewed in Svingen and Koopman 2013). The transcription factor SRY upregulates the expression of the gene SRY-Box Transcription Factor 9 (*Sox9)*, which in turn governs the expression of several male-specific genes, leading to differentiation of the bipotential somatic cell progenitors into Sertoli cells (Kashimada *et al.* 2011). In the absence of *Sry*, as in females, a different set of genes are expressed by default, such as Wnt Family Member 4 (*Wnt4)* and Forkhead box protein L2 (*Foxl2)*, leading to differentiation of somatic progenitor cells into granulosa cells and subsequently to ovarian development (Kashimada *et al.* 2011). During early testis differentiation, Sertoli cells encircle clusters of gonocytes and form testis cords; the future seminiferous tubules. Shortly thereafter, fetal Leydig cells emerge in the interstitial space and start producing testosterone, which is essential for masculinization of the male fetus (reviewed in Svingen and Koopman 2013).

At the time of gonad formation, RA appears to be available from several possible sources. In mice, the adjacent mesonephros has been suggested as an important source, where both dehydrogenase 10 (*Rdh10*) and retinaldehydrogenase 2 (*Raldh2*) are expressed, as well as *Raldh3* at a lower level, (Niederreither *et al.* 2002a, Niederreither *et al.* 2002b, Bowles *et al.* 2006, Spiller and Bowles 2015, Bowles *et al.* 2018). Using a RARE-controlled *LacZ* reporter gene system, RA was demonstrated to be localized in the anterior part (nearest the mesonephros) of the mouse ovary (Bowles *et al.* 2006).

An alternative RA source is the coelomic epithelium, which covers the embryonic gonad, and where Raldh2 is also expressed (Niederreither *et al.* 1997, Teletin *et al.*

2017). Species differences appear to exist: RA-producing (retin)aldehyde dehydrogenases are present in both female and male fetal gonads in humans (Childs *et al.* 2011, Le Bouffant *et al.* 2010), suggesting a capacity for *de novo* RA synthesis in the gonad proper. Gonadal RA synthesis in the rabbit appears similar to the one in the human gonad (Diaz-Hernandez *et al.* 2019). Species differences are evident when it comes to gonadal architecture; the human and rabbit gonads both develop a well-defined cortex and medulla, where somatic and germ cells can interact differently than in the mouse, for example, which has a different gonadal architecture (Diaz-Hernandez *et al.* 2019).

In mice, Cyp26-dependent degradation of endogenous RA appears to be critical for somatic testis development, as evident from observations of mild ovotestes, impaired steroidogenesis and a feminized reproductive tract in *Cyp26b1*-null C57/BL6 13.5 dpc/14.5 dpc male mouse embryos (later time-point not examined as the embryos are not viable after ~15 dpc) (Bowles *et al.* 2018). However, in an earlier study using *Cyp26b1*-null mice on a different genetic background, testis formation and somatic cell differentiation in neonatal pups appeared normal, although some germ cells had prematurely entered meiosis while others appeared apoptotic. Germ cells were essentially absent in testes from neonatal *Cyp26b1*$^{-/-}$ pups (MacLean *et al.* 2007). In an *ex vivo* model using rat fetal testis, exogenous RA disrupted the proper formation of seminiferous cords, as well as the maintenance of the testicular cell fate of the somatic cells; following RA exposure, expression of ovarian-specific marker Foxl2 was observed (Spade *et al.* 2019a). In a human *ex vivo* testis model, exogenous RA appeared to disrupt the seminiferous cords and altered the expression of somatic cell markers (Jørgensen *et al.* 2015). Thus, there appears to be differences between rodents and humans with regard to where and how RA is synthesized and regulated in the developing gonads, although it is clear that RA influences both germ and somatic cell lineages in both species.

Even in adulthood, the sexual fate of the male gonads must be maintained, probably *via* the active presence of the transcription factor Doublesex and mab-3 related transcription factor 1 (DMRT1) (Minkina *et al.* 2014). The role of DMRT1 in the testes appears to be to prevent RA-initiated activation of specific potential feminizing genes (*Foxl2* and Estrogen receptor β; *Esr2*) in Sertoli cells; without DMRT1 present, male-to-female transdifferentiation of somatic cells was observed in the pre- and postnatal mouse testes (Minkina *et al.* 2014), or even complete male-to-female sex reversal (Zhao *et al.* 2015).

In contrast, based on observations in mice with deletions/disruptions of either RARs or RA-synthesizing enzymes, suggest that RA signaling is not critical for correct ovarian development in the female mouse embryo (Minkina *et al.* 2017), although earlier studies have suggested that RA might maintain ovarian differentiation and development (Minkina *et al.* 2014, Suzuki *et al.* 2015). Additional studies suggest that abnormal endogenous RA levels may be able to influence somatic cell differentiation and/or function in mice (Bowles *et al.* 2018). Further research is needed to clarify the role of RA in ovarian development and function.

3. Germ cells, meiosis and the role of retinoids

Most of our current understanding of meiosis initiation and early sex differentiation is derived from studies in mice. Data from humans are sparse (See Table 1 and text below).

Table 1: Sensitive windows in males and females, comparing rodents and humans (information obtained from Le Bouffant *et al.* 2010, Grive and Freiman 2015, Johansson *et al.* 2017, Mamsen *et al.* 2017, Teletin *et al.* 2017)

Process	Time period in females	Time period in males
Early gonadal development	Mice: 11.5 dpc[a]. Rats: 13.5 dpc. Humans: just beyond GW[b] 7	Mice: just beyond 11 dpc. Rats: just beyond 12 dpc. Humans: just beyond GW 7
Meiosis initiation	Mice: 13.5 dpc. Rats: 16.5 dpc. Humans: GW 10 -12	Mice: end of first post natal week at puberty. Humans: at puberty
Follicular assembly	Rodents: post-natally (mice: primordial follicle formation initiated 2-3 days before birth, follicle assembly continues until PND 6). Humans: pre-natally (during mid-gestation stage)	-
Early follicle recruitment[c]	Rodents post-natally. Humans: initiated pre-natally	-
Spermatogenesis	-	Mice: beginning at puberty. Humans: beginning at puberty

Note:

[a] *dpc: days post coitum.*

[b] *GW: gestational week.*

[c] *Takes place immediately after follicular assembly.*

In both male and female mouse embryos, a cluster of pluripotent primordial germ cell (PGC) precursors arise under the influence of bone morphogenic protein (BMP) at 6.25 dpc at the base of the allantois of the embryo (reviewed in Yadu and Kumar 2019). Subsequently, the PGCs migrate through the presumptive hindgut to the genital ridges (formed at 10.5 dpc), which will differentiate into testes or ovaries (see section 5). During the migration and colonization period, epigenetic reprogramming (genome-wide demethylation) occur in PGCs, which allows transcription of genes that are suppressed epigenetically in somatic cells. This may explain why only germ cells are capable of responding to the meiosis-inducing signal from RA (reviewed in Yadu and Kumar 2019).

In both males and females, the haploid gametes are produced from primordial germ cells *via* meiosis in the gonads, following a similar process of events, although the timing differs. In the mouse ovaries, germ cells enter the prophase of the first meiotic division (at around 13 dpc), whereas in the testes, germ cells (now situated in the testis chords) instead enter quiescence by 12.5 dpc; they slow down their proliferation towards mitotic arrest as G_0/G_1-arrested gonocytes (Kashimada *et al.* 2011). Thus, in the fetal gonad the commitment of germ cells towards oogenesis involves entry into meiosis, whereas commitment to spermatogenesis involves the inhibition of meiotic initiation, suppression of pluripotency and mitotic arrest. This mitotic arrest is maintained until after birth (Spiller and Bowles 2015, Spiller *et al.* 2017) and consequently meiosis initiation occurs postnatally in males. Thus, all oocytes are produced before birth, while spermatocytes are produced continuously during post-pubertal life in males.

Meiotic entry during fetal development seems to be regulated by two master genes: Stimulated by retinoic acid, gene 8 (*Stra8*), required for the initiation of meiosis in female fetal germ cells, and Nanos C2HC-Type Zinc Finger 2 (*Nanos2),* expressed in male fetal germ cells (Rossitto *et al.* 2015). Nanos2 is required to prevent *Stra8* expression and meiosis initiation in male fetal germ cells (Rossitto *et al.* 2015). Other genes, such as the one coding for Fibroblast growth factor 9 (*Fgf9)*, have been implicated as being of importance for proper control of meiotic entry; Figure 6 is an attempt to summarize this information. STRA8 appears to be critical for several meiotic cellular processes such as DNA replication, condensation of chromosomes and double-stranded DNA breaks (reviewed in Yadu and Kumar 2019). The role of *Stra8* in meiosis is also evident in *Stra8*$^{-/-}$ mice, as both females and males are infertile, while heterozygotes of both sexes are fertile (Baltus *et al.* 2006). *Stra8*$^{-/-}$ females display smaller ovaries lacking oocytes and follicles, and in *Stra8*$^{-/-}$ males, testes are smaller and testicular germ cell numbers are severely reduced (Baltus *et al.* 2006). In addition, spermatocytes in *Stra8*$^{-/-}$ males undergo apoptosis before reaching the leptotene and zygotene stages of meiotic prophase (Baltus *et al.* 2006). The *Stra8* gene contains putative retinoid acid response elements (RAREs) and its expression is activated by RA (Spiller *et al.* 2017). RA-signaling is implied in Stra8 signaling *in vivo*, as evidenced by the lack of Stra8 induction in *ex vivo* cultured mouse fetal ovaries, exposed to the RAR antagonist BMS-204493. (Koubouva *et al.* 2006). In line with these hypotheses, it has been demonstrated that exogenous RA added to *ex vivo* cultures of 12.5 dpc mouse testes induces expression of the meiois marker *Stra8* (Bowles *et al.* 2006, Koubouva *et al.* 2006). Very low concentrations of RA (10 nM) has been shown to induce *Stra8* expression in 11.5 dpc germ cells *in vitro* (Bowles and Koopman 2007). The *Stra8* locus in non-germ cells appears to be epigenetically silenced, thus, only pre-meiotic germ cells respond to RA by *Stra8* induction (Wang and Tilly 2010, Spiller and Bowles 2015).

The sex-specific timing of *Stra8* expression is conserved between mice and humans (Childs *et al.* 2011). If STRA8 has other roles, they are still largely unknown (Griswold 2016). Interestingly, in humans, spermatogenic impairment, evident as azoospermia or oligozoospermia, was associated with a specific *STRA8* single nucleotide polymorphism (Lu *et al.* 2013).

Another gene essential for meiosis, *Rec8* meiotic recombination component gene *(Rec8)*, also contains a RARE, and is activated by RA independently of *Stra8* (Kuobova *et al.* 2014). *Rec8* encodes a meiosis-specific component of the cohesion complex, and is required for several steps in meiotic chromosomal activities, *e.g.*

chromatid cohesion and chiasmata formation (Kuobova *et al.* 2014). Neither female nor male *Rec8*$^{-/-}$ mice that survive to reach sexual maturity are fertile (Xu *et al.* 2005). At 18.5 dpc, fetal ovaries of female *Rec8*$^{-/-}$ mice displayed apparently normal prophase I germ cells, but also abnormal germ cells with compacted chromosomes, while at PND5 and older (up to adult), no oocytes or ovarian follicles were present (Xu *et al.* 2005). Findings of involuted genital tracts in the same females were interpreted as being a consequence of ovarian hormone failure, due to the lack of (steroidogenic) follicles.

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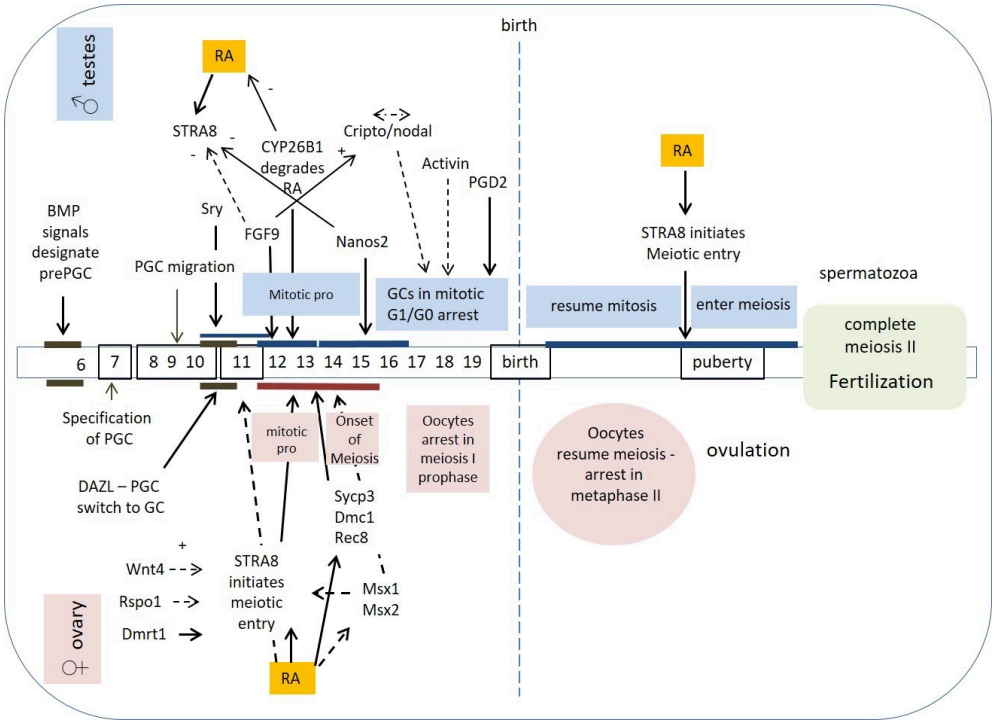

Figure 6: Comparison of male and female germ cell development and meiotic progression in mice

Note:

The summary is adapted from Figure 3 inClagett-Dame & Knutson, 2011; Figure 1 in Feng and co-authours, 2014; Rossitto and co-authours, 2015 and Figure 1 in Spiller & Bowles, 2015. Blue indicates male-specific events, red/pink indicates female-specific events and grey, events shared by both sexes. Numbers indicate days post coitum (dpc). Arrows and bars indicate rough timing of gene expression, solid arrows indicate direct effect, and broken arrows indicate an incompletely characterized or likely indirect effect. Plus (+) or minus (-) indicate supportive/additive or inhibitory action on other genes. Abbreviations: GC; germ cell, PGC; primordial germ cells, RA; retinoic acid, DAZL; deleted in azoospermia-like, BMP; bone morphogenic protein, mitotic pro; mitotic proliferation. (Printed, with kind permission of the EU Commission; OECD 2017; draft DRP, not published).

RA has been implicated in controlling the onset of meiosis in both males and females (Bowles *et al.* 2006, Koubova *et al.* 2006). RA-treatment of cultured fetal mouse ovaries from 14.5 dpc, increases the number of meiotic cells (Livera *et al.* 2000b). Any response to RA in both male and female germ cells must be preceeded by expression of the gene Deleted in azoospermia-like (*Dazl*), which is present in the post-migratory germ cells after their arrival in the gonad (Lin *et al.* 2008). The RA necessary for meiosis initiation is synthesized locally in both fetal testes and ovaries. As mentioned in Section 5, several sources for RA in mouse fetal gonads have been suggested: the mesonephros (near the anterior part of the fetal gonads), the coelomic epithelium, and the fetal gonads themselves. In fact, an anterior-posterior pattern of *Stra8* expression has been demonstrated in mouse female fetal gonads (reviewed in Bowles *et al.* 2006), implying that the mesonephros may be the most important source of RA for the mouse gonad. Germ cell meiosis initiation in human gonads is asynchronous, as compared to the anterior-posterior wave observed in mice, which suggests a less important role for the mesonephros in humans (Le

Bouffant *et al.* 2010, Childs *et al.* 2011). In the rabbit, contrary to the mouse, meiosis onset occurs after termination of the connection between the gonad and the mesonephros (Hayashi *et al.* 2000). It should also be noted that in a human fetal testis *ex vivo* model, RA was capable of inducing *Stra8* but not other meiosis-associated genes (Childs *et al.* 2011). Species differences are also evident from studies using rabbits, where both meiosis initiation and mitotic arrest occurs postnatally, and the gonadal expression profiles of *e.g. Raldh1*, *Raldh2*, *Stra8* and *Cyp26b1* suggest both similarities and differences when compared to humans and mice (Diaz-Hernandez *et al.* 2019).

An important difference between female and male mice is that the RA-catabolizing enzyme Cyp26b1, which is expressed in mouse fetal gonads of both sexes at 11.5 dpc, is no longer expressed in the female gonad from 12.5 dpc (Bowles *et al.* 2006). Consequently, RA is degraded in the fetal testis, and thereby meiotic entry is blocked (MacLean *et al.* 2007). In contrast, RA levels are maintained in the fetal ovary, *Stra8* and *Rec8* is expressed, and female gonocytes will subsequently enter meiosis I (Rossitto *et al.* 2015).

Anomalous meiosis initiation takes place in fetal mouse testes as a result of exogenous RA exposure (Bowles *et al.* 2006, Koubova *et al.* 2006). The expression of *Stra8* is upregulated in *Cyp26b1*$^{-/-}$ fetal testis (Bowles *et al.* 2006). Furthermore, increased *Stra8* expression has been observed in *ex vivo* cultured 12.5 dpc mouse fetal testes treated with either ketoconazole (a non-specific CYP inhibitor) or R115866 (a more CYP26-specific inhibitor) (Koubova 2006). Ketoconazole has no effect in the presence of the RAR antagonist BMS-204493, which further proves the role of RA in Stra8 induction (Koubouva *et al.* 2006). In concordance with these *ex vivo* studies, the absence of *Cyp26b1* in male mouse embryos would lead to sustained concentrations of RA in the testes, as well as subsequent induction of *Stra8* expression followed by meiosis. In line with this, suppressed induction of meiosis is observed when *Stra8* is knocked-out concomitantly with Cyp26b1 (Saba *et al.* 2014).

Species differences may exist for the expression of gonadal CYP26B1, as suggested by the higher than expected expression in human fetal ovaries at GW 14-16 (Childs *et al.* 2011). It has also been suggested that in the human fetal gonad, RA levels are regulated primarily *via* synthesis mediated by RALDH-subtypes rather than by CYP26B1 catabolism (Le Bouffant *et al.* 2010). This could explain the asynchronous initiation of meiosis in human ovaries. It has been noted that while the roles of RA and STRA8 in meiosis initiation appear to be conserved between humans and several animal species, the role for CYP26B1 differs between mice and other species such as humans and marsupials (Hickford *et al.* 2017). In mice, ovarian *Cyp26b1* expression is known to be downregulated prior to meiosis initiation, while such downregulation is not observed in the human ovaries (Hickford *et al.* 2017).

As discussed in the previous paragraph, RA levels are maintained in both male and female mouse gonads alike in the absence of Cyp26b1. In a *Cyp26b1*$^{-/-}$ mouse model, germ cell development was studied in ovaries and testes from embryos at 13.5 dpc and from neonatal pups (MacLean *et al.* 2007). In that study, ovarian germ cells at both stages appeared unaffected by the lack of Cyp26b1. In the embryonic *Cyp26b1*$^{-/-}$ testes, some germ cells had prematurely entered meiosis while others appeared apoptotic. Increased levels of RA were demonstrated in the embryonic *Cyp26b1*$^{-/-}$ testes. Germ cells were essentially absent in testes from neonatal *Cyp26b1*$^{-/-}$ pups. Premature germ cell meiosis could also be observed in male

wildtype genital ridges cultured in the presence of a synthetic retinoid (Am580, which is resistant to Cyp26b1 metabolism), suggesting that excess RA was responsible for the effect (MacLean *et al.* 2007).

RA has been widely accepted to be important), for the induction of meiosis (Bowles *et al.* 2016, Spiller *et al.* 2017, Teletin *et al.* 2017. However, it has been suggested that the role of RA in the induction of meiosis may be facilitating rather than critical (Kumar *et al.* 2011, Teletin *et al.* 2019, Bellutti *et al.* 2019). Kumar and coworkers reported that meiosis occurred normally in mouse fetal ovaries lacking RA due to ablation of *Aldh1a2* and *Aldh1a3* (Kumar *et al.* 2011). It was subsequently shown that in 11.5 dpc cultured mouse urogenital ridges with chemically inhibited Raldh2 and Raldh3, levels of the third RA-producing enzyme, Raldh1, were elevated (Bowles *et al.* 2016). Thus, at least in mouse ovarian germ cells, RA appears indeed to be an inducer of meiosis (Yadu and Kumar 2019). This is currently a very active area of research and the exact role of RA in meiosis induction needs to be further clarified.

4. Female reproductive organ development, function and health and the role of retinoids

The ovary is the site for differentiation and release of mature oocytes for fertilization. It is also where sex hormones (necessary for follicle development, estrous cyclicity, maintenance and function of the reproductive tract) are synthesized and released (Barnett *et al.* 2006).

Retinoids are known to play an important role in the reproductive organs of females, including sex differentiation (as previously described in Chapters 5 and 6), and oogenesis/folliculogenesis during embryogenesis, and potentially also in adult pathologies such as endometriosis.

4.1. Oogenesis

Oogenesis is the formation of haploid mature gametes from diploid oocytes, and the entire process begins *in utero* with primary oocytes eventually arresting at prophase I (the first phase in meiosis) in primordial follicles, which then can grow to full maturity after puberty (Barnett *et al.* 2006, Teletin *et al.* 2017). In more detail, PGCs differentiate into oogonia upon arrival at the developing female gonad and then undergo mitotic proliferation to create a stock of millions of oogonia that form germ cell nests/cysts, *i.e.*, clusters of cells connected by intercellular bridges (reviewed in Johansson *et al.* 2017). These nests break down when some of the oogonia within the nest undergo apoptosis, thus breaking the connections between the cells and thereby allowing somatic cells to enter and surround the germ cells. This rearrangement results in the creation of primordial follicles each containing a primary oocyte arrested in prophase I and surrounded by a single layer of flattened somatic (granulosa) cells. The breakdown of germ cell nests, constituting the beginning of primordial follicle formation (initiating folliculogenesis, see next section), results in a decrease in oocyte number. The oogonia will enter meiosis I around 13.5 dpc in mice, and between 10 and 12 weeks of gestation in humans (Grive and Freiman 2015). By entering meiosis I, the cells can no longer divide mitotically. Thus, when all oogonia have entered the first meiotic division, the size of the ovarian reserve is fixed.

4.2. Ovarian somatic cells and folliculogenesis

As the genital ridges begin to differentiate into ovaries in female fetuses (at approximately 11 dpc in mice), a subset of gonadal somatic cells will differentiate

into granulosa cells, after which meiosis will commence in the oogonia (Minkina *et al.* 2017). The germ cell nests with the proliferating oogonia will break down and somatic cells will enter and surround the germ cells, thereby forming the primordial follicles. The processes of nest breakdown, germ cell death and formation of primordial follicles are similar in mice and humans, but more sequentially organized in mice (Johansson *et al.* 2017). Nest breakdown takes place around the time of birth in mice and begins during mid-gestation (around GW 16) in humans (Grive and Freiman 2015). In addition, follicle assembly takes place prenatally in humans and postnatally in rodents (Johansson *et al.* 2017). These differences may suggest different initiating mechanisms. The primordial follicles, each of which contains an oocyte surrounded by a single layer of somatic pre-granulosa cells, represent the adult ovarian reserve (Grive and Freiman 2015).

Folliculogenesis, the process of follicle maturation (See Figure 7), primarily takes place from the onset of puberty (Johansson *et al.* 2017). The first (primordial) follicles each consist of a single oocyte surrounded by a layer of flat granulosa cells. Granulosa cells progressively form several layers around the oocyte with an outer layer of androgen-producing theca cells. The granulosa cells subsequently convert androgens to estrogens. As granulosa cells continue to proliferate, the antrum is formed. At this stage, selection occurs between growing follicles, so that only one or a limited number of follicles continue growing to the preovulatory stage, while others undergo atresia. After ovulation, luteinized theca cells and mural granulosa cells produce progesterone.

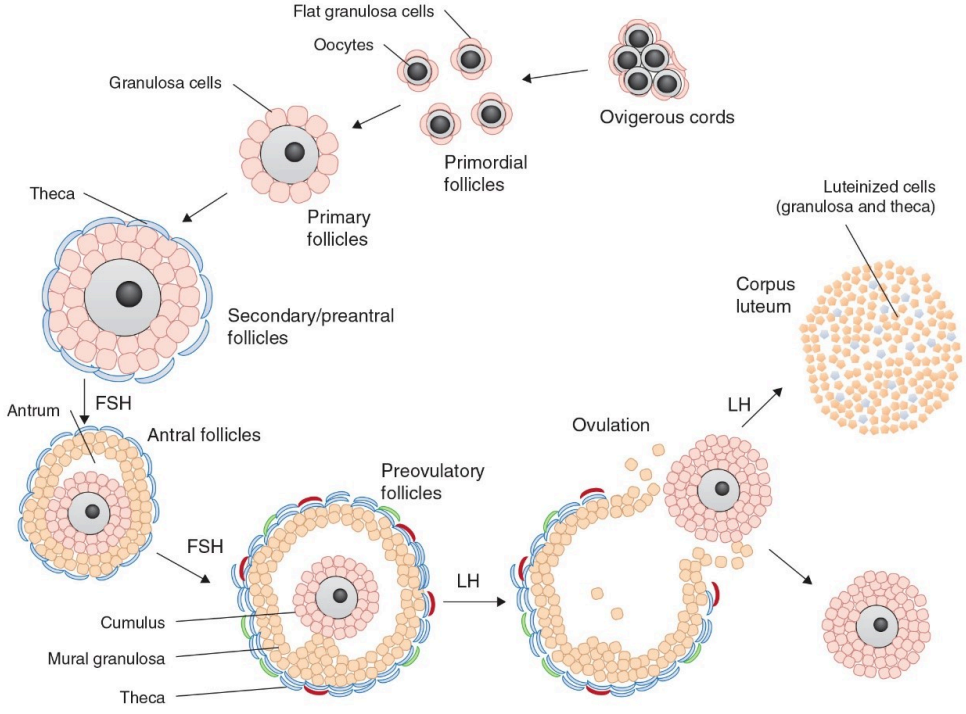

Figure 7: Overview of folliculogenesis

Note:

After breakdown of the germ cell nests (in the figure referred to as ovigerous cords), flat granulosa cells form a single layer around oocytes, thereby forming primordial

follicles; this step is considered as the start of folliculogenesis. More layers of granulosa cells form around the oocytes and eventually also an outer layer of theca cells surrounds the follicles. The granulosa cells continue to proliferate and are separated by the antrum into two populations: cumulus and mural granulosa cells. A small selection of these antral follicles grow (while others undergo atresia) and are eventually released by ovulation. Up until ovulation, theca cells synthesize androgens that are metabolized into estrogens by granulosa cells. After ovulation (triggered by FSH; Follicle-Stimulating Hormone, and LH; Luteinizing Hormone), progesterone is produced in the corpus luteum by theca cells and mural granulosa cells. (Georges et al. 2014, reprinted with kind permission from the publisher: Bioscientifica Ltd.)

RA has been considered to be required to maintain ovarian differentiation and development (Minkina *et al.* 2014, Suzuki *et al.* 2015). RA is also believed to regulate mouse ovarian follicle development in the adult; this phenomenon has been studied e.g. in vitro by stimulating granulosa cell proliferation (Demczuk *et al.* 2016). Using cat ovarian cortices in an *ex vivo* experiment, RA was shown to activate the development of primordial follicles into primary and secondary follicles, possibly *via* differential regulation of matrix metalloproteinases (MMP) (Fujihara *et al.* 2018). It has been shown that mouse theca cells and granulosa cells express the enzymes necessary for conversion of ROH to RA (different forms of ADH and RALDH), or degradation of RA (Cyp26b1). Further, in these experiments by Kawai and co-workers, these enzymes were demonstrated to be differentially regulated after injection of equine chorionic gonadotropin, an analogue of Luteinizing hormone (LH)/Follicle stimulating hormone (FSH) (Kawai *et al.* 2016, Kawai *et al.* 2018). As demonstrated *in vitro*, ovarian *de novo* synthesis of RA is required for follicular expression of the LH receptor in granulosa cells of mouse ovaries and for their ability to respond to the ovulatory LH surge; oocytes appear to negatively regulate RA synthesis in pre-ovulatory follicles, impacting LH receptor expression in follicular somatic cells possibly *via* an epigenetic mechanism (Kawai *et al.* 2016, Kawai *et al.* 2018). Cultured human ovarian cumulus granulosa cells (obtained during oocyte retrieval during the course of *in vitro* fertilization) produce RA from ROH in the media. RA causes de-phosphorylation of connexin 43, involved in gap junction intercellular communication (GJIC) between the granulosa cells in the cumulus-oocyte complex. De-phosphorylation of connexin 43 increases GJIC, which plays an important role in oogenesis and successful fertilization (Best *et al.* 2015).

Minkina and co-authors however demonstrated that RA may not be required for ovarian granulosa cell specification, differentiation or function. No significant effects were found on ovarian differentiation, follicle development or female fertility in geneticially manipulated mouse models, in which all three RARs were deleted in the female somatic gonad at the time of sex determination (Minkina *et al.* 2017). Nor did the knockout of all three RA-producing aldehyde dehydrogenase genes (*Aldh1a1-3*) at the same timepoint appear to masculinize the mouse ovaries. Additionally, an RAR antagonist (BMS-189453) was capable of blocking meiotic initiation in the germ cells of 10.5 dpc wild-type female mouse gonads cultured *ex vivo,* but had little effect on the expression of markers for either granulosa or Sertoli cells; thus, disruption of RA signaling does not appear to disrupt early somatic differentiation in the female fetus (Minkina *et al.* 2017). Notably, the triple knockout model was based on tamixofen-

inducible Cre, whereby the mice are administered tamoxifen to silence the genes; however, tamoxifen is itself an estrogenic compound known to affect gonadal sex differentiation (Patel *et al.* 2017).

In mouse ovaries, RBP4 is expressed before puberty but increases significantly in the peripubertal period. In adult mice, RBP4 expression increased at pro-estrous and peaked at estrous and was localized mainly in the granulosa and theca cells of follicles. Expression is also induced by FSH, alone or in combination with LH, while LH alone had no effect (Jiang *et al.* 2018b).

4.3. Ovarian steroidogenesis

Ovarian steroidogenesis, or the production of sex steroid hormones, is regulated by the pituitary hormones LH and FSH; LH stimulates the ovarian thecal cells to produce androgens and FSH stimulates the granulosa cells to convert these androgens to estrogens (Hannon and Flaws 2015). The process of synthesizing the ovarian steroids from cholesterol is complex, and requires the action of several enzymes (see Figure 8).

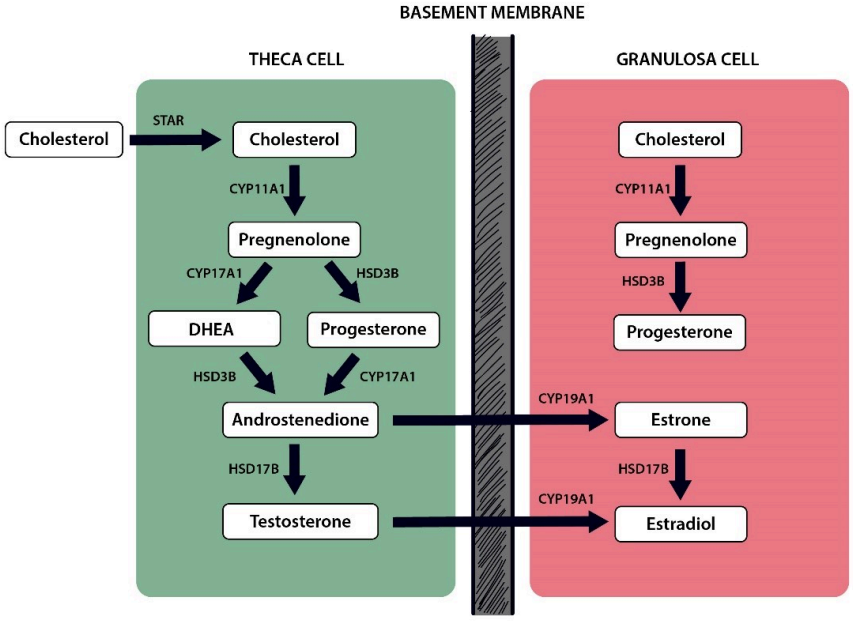

Figure 8: Overview of ovarian steroidogenesis

Note:

Prior to ovulation, enzymatic conversion of cholesterol to estradiol occurs initially in the theca cells and subsequently in the granulosa cells, primarily in the mature antral follicles. After ovulation, conversion of cholesterol to progesterone occurs in the corpus luteum. Hormones: listed in the white text boxes; steroidogenic enzymes: listed adjacent to the arrows. Abbreviations: *DHEA; dehydroepiandrosterone, STAR; steroidogenic acute regulatory protein. (Hannon and Flaws 2015, reprinted under the terms of the Creative Commons Attribution License (CC BY), from frontiers in Endocrinology.)*

Ovarian steroidogenesis is reported to be regulated by RA in several systems (recently reviewed in Damdimopoulou *et al.* 2019). For example, in rats, low levels of RA and ROH stimulate the formation of progesterone (Bagavandoss and Midgley 1987). In a human ovarian surface epithelium cell line, RA significantly induces production of progesterone (Papacleovoulou *et al.* 2009). In women, ROH in plasma is associated with higher estradiol and testosterone levels (Mumford *et al.* 2016). It is likely that RA regulates either the expression or the activity (or both) of the steroidogenic enzymes (Wickenheisser *et al.* 2005). This hypothesis is supported by data from retinoid-deficient rats, in which ovarian activity of the cytochrome P450 cholesterol side-chain cleavage enzyme (P450SCC) decreased along with the progression of retinoid deficiency (Jayaram *et al.* 1973). In addition, RA may modulate the pituitary gonadotropins, which in turn regulate steroidogenesis (Minegishi *et al.* 2000).

In the endometrial epithelial cells, estradiol is locally inactivated through conversion to the less potent estrone by the enzyme 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2). RA produced in the stromal cells may act as a paracrine factor capable of stimulating the production of HSD17B2 (Taylor *et al.* 2015, Jiang *et al.* 2018a).

A connection between ROH/RA and ovarian function has been hypothesized based on the observation that serum ROH levels in women vary with the stages of the estrous cycle (highest levels in the proestrus and estrus phases), and that RBP4 serum levels are positively correlated with gonadotropin levels (such as FSH and LH) (Jiang *et al.* 2017). Similarly, ovarian expression of RBP4 mRNA and protein in adult female mice increased at proestrus and peaked at estrus (Jiang *et al.* 2018b). In ovaries from mice treated with FSH, increased levels of RA (and other retinoid forms) and RBP4, as well as increased expression levels of RA-synthesizing enzymes (*Adh1*, *Raldh1*), were observed (Jiang *et al.* 2018b, Liu *et al.* 2018). Similar effects were observed in FSH-treated mouse granulosa cells, in addition to increases in *e.g.* STRA6 (the specific receptor for RBP4) and CRBP1, suggesting that gonadotropin FSH modulates the pathways for ROH uptake and its metabolism to RA in the mouse ovary (Liu *et al.* 2018).

4.4. Other female reproductive organs and pregnancy

In the female fetus, the Müllerian ducts differentiate into the oviduct, uterus and upper vagina, with the resulting epithelia having distinct and separate organ-specific morphology and function (Nakajima *et al.* 2016). At least in the mouse, RA signaling *via* RAR during embryo development may determine the fate of Müllerian duct stroma into either uterus or upper vagina, as seen *ex vivo* after treatment of Müllerian ducts with RA (leading to uterine epithelial differentiation) or treatment with RAR-inhibitors (leading to vaginal epithelial differentiation) (Nakajima *et al.* 2016).

The structure and function of the mucosal inner lining of the uterus, the endometrium, is regulated by the ovarian sex steroid hormones estradiol and progesterone. In humans, the endometrium undergoes major changes during the

menstrual cycle. During the proliferative phase, ovarian estrogen stimulate proliferation and growth, and during the secretory phase, progesterone prepares the endometrium for potential implantation. A number of retinoid-related genes (ALDH1A1, ALDH1A2, CYP26A1, CRABP2, RARα, RARγ, and RXRα) are expressed in the endometrium of both rodents and humans, and their expression is regulated by estrogen and/or progesterone (Jiang *et al.* 2017). As reviewed by Jiang and co-workers, expression of these and other retinoid-related genes have been noted in either stromal or epithelial cells (Jiang *et al.* 2017).

Following fertilization, the implanting blastocyst first attaches to the epithelial cells and then invades the endometrium by displacing the epithelial cells. It is finally embedded in the endometrium, where the formation of the placenta is initiated (Su and Fazleabas 2015). The details of these processes can be species-specific.

RA regulates expression of MMPs, which are produced by endometrial stromal cells during decidualization (*i.e.,*the process of endometrial changes in preparation for pregnancy). RARs are expressed in the uterine stroma of mice and uterine epithelium of rats. In rats, RAR protein expression is influenced by ovarian steroids; RAR expression increases under the influence of estradiol, suggesting involvement of retinoids in growth and proliferation of endometrial epithelia. In postmenopausal women taking estrogen, uterine RAR expression is reported to increase. Increased RAR levels have also been observed in premenopausal women during the proliferative phase; a phase which is associated with elevated estradiol levels (Sayem *et al.* 2018).

In the mouse uterine epithelium, Cyp26a1 (both mRNA and protein) is expressed during the blastocyst implantation period. Removing or inactivating Cyp26a1 in mice led to a decreased pregnancy rate, and reduced numbers of implantation sites (Han *et al.* 2010). It appears that the Cyp26a1 enzyme, by degrading RA, might prevent the otherwise inhibitory effects of RA on implantation-related genes in the endometrial epithelium during mouse embryo implantation (Ma *et al.* 2012). RA treatment of mouse blastocysts *in vitro* inhibited cell proliferation and caused retarded growth (Huang *et al.* 2005). Following implantation, these RA-treated blastocysts were resorbed to a greater degree than untreated blastocysts (Huang *et al.* 2005). During preimplantation development of the blastocyst, RA signaling appears not to be involved (Rout and Armant 2002).

Via the PPAR/RXR heterodimer, the retinoid signaling pathway is involved in human placental processes such as the invasion of the uterine epithelium by extraembryonic trophoblasts. Both trophoblasts and decidual cells appear to be capable of synthesizing RA as well as the presumed RXR agonist ligand, 9-*cis*-RA (Tarrade *et al.* 2001). At least *in vitro*, trophoblast invasion is inhibited by PPAR and RXR agonists, while PPAR and RXR antagonists increases invasion. Functional retinoid signaling pathways have also been demonstrated in human amniotic membranes (Marceau *et al.* 2006).

RA concentration in follicular fluid (obtained during oocyte retrieval during the course of *in vitro* fertilization) was found to be positively correlated with embryo quality (scored on day 3 after fertilization). RA levels in follicular fluid also correlated moderately with plasma RA. Women with endometriosis had significantly lower concentration of RA in follicular fluid and in plasma than women with no endometriosis (Pauli *et al.* 2013).

The genital tubercle is a tissue present during embryonic development of the reproductive system; it later develops into either the glans clitoris or the glans penis in humans. RA appears to be involved in genital tubercle development, along with Rarb, Raldh2 and Cyp26b1 (Liu *et al.* 2012).

4.5. Female reproductive pathologies and retinoid involvement

4.5.1. Endometriosis

Endometriosis is defined as the presence of endometrial tissue outside of the uterus, usually on the ovaries, fallopian tubes, and the peritoneum; a state which can cause severe pain. In addition, endometriosis is associated with fertilization problems (Taylor *et al.* 2015, Jiang *et al.* 2018a).

Several studies have demonstrated altered retinoid pathway signaling associated with endometriosis. In human normal premenopausal endometrial tissue, mRNA expression of the RA-catabolizing enzyme CYP26A1 increases substantially during the progesterone-dominated secretory phase (leading to degradation of RA and therefore diminished RA signaling) when compared to the estrogen-dominated proliferative phase, during which RA-synthesizing enzymes such as RALDHs are increased (Deng *et al.* 2003). At least *in vitro*, RA has been shown to inhibit the decidualization of stromal cells; thus, reduced concentration of RA in the endometrial tissue during the secretory phase could be necessary for successful implantation (Deng *et al.* 2003). In the mouse uterus, endogenous RA levels appear to be controlled both by estrogen-dependent expression of RALDH enzymes and by progesterone-dependent expression of CYP26A1 (Fritzsche *et al.* 2007). Expression of CYP26A1 is down-regulated in both the secretory and proliferative phases in endometrial biopsies from women with moderate or severe endometriosis, when compared to healthy women (Burney *et al.* 2007). The availability of RA may therefore be increased in the endometrial tissue of women with endometriosis. In endometriosis, there is evidence for progesterone resistance, and cultured stromal fibroblasts originating from endometriotic lesions have decreased ability to decidualize (which is necessary for a successful blastocyst implantation) (Burney *et al.* 2007).

Reduced STRA6, CRBP1 and ALDH1A2 expression have been demonstrated to reduce RA in endometrial stromal cells (Jiang *et al.* 2018a). Transcriptional activation *via* the RA-CRABP2-RAR pathway has been reported to trigger cell cycle arrest and apoptosis; thus, reduced signaling can cause endometrial cells to escape apoptosis and contribute to survival of ectopic cells (Jiang *et al.* 2018a). Since RA appears to stimulate the production of HSD17B2, which inactivates estradiol in the endometrium, an abnormal RA pathway in endometriosis may explain the aberrant HSD17B2 expression and the high local estradiol concentrations in endometriosis. In addition, altered retinoid action may cause decreased expression of gap junctional protein connexin 43 along with decreased gap junctional intercellular communication, reducing the decidualization capacity of the stromal cells in endometriosis, which could contribute to progression of endometriotic lesions and the associated subfertile uterine phenotype (Taylor *et al.* 2015, Jiang *et al.* 2018a).

4.5.2. Polycystic ovarian syndrome

Polycystic ovarian syndrome (PCOS) is a heterogenous disorder, characterized both by signs of androgen excess and ovarian dysfunction (such as irregular ovulation and/or polycystic ovarian morphology) (Escobar-Morreale 2018). In PCOS, an increased ovarian androgen production from theca cells, an elevated LH:FSH hormone ratio, and enlarged ovaries containing many antral follicles are often observed (reviewed in Jiang *et al.* 2017). The associated arrest in follicular growth and anovulation can cause subfertility or infertility.

When comparing the response to RA, 9-*cis*-RA and ROH in cultured theca cells isolated from normal-cycling women and women with PCOS, only RA led to increased testosterone production (possibly *via* increased expression of 17,20-lyase; *Cyp17*) in normal theca cells, while in the theca cells of PCOS patients, all three tested retinoid variants (RA, ROH, and 9-*cis*-RA) had the same effect (Wickenheisser *et al.* 2005). Thus, in PCOS, theca cells may be sensitized to retinoid signaling stimulation. Moreover, mRNA expression of RA-synthesizing enzymes retinol dehydrogenase (RoDH2) and ALDH6 was increased in PCOS theca cells (Wood *et al.* 2003), and PCOS ovaries show enhanced expression of RoDH2 (Marti *et al.* 2017). These data suggest an increased rate of RA synthesis in the thecal cells of PCOS women (reviewed in Jiang *et al.* 2017).

In women of reproductive age with acne and PCOS, treatment with oral isotretinoin (13-*cis*-retinoic acid) decreased ovarian volume (Acmaz *et al.* 2019).

4.6. Retinoid deficiency/excess in female reproduction

As reviewed in Clagett-Dame and Knutson 2011 (citing publications going back to the 1920s), retinoids are required for successful fertilization, implantation, placentation, embryogenesis and full-term pregnancy: in severely retinoid-deficient female rats, reproduction fails prior to implantation, while in less severe deficiency, fertilization and implantation occur, but embryonic death at mid-gestation is often observed. Retinoid deficiency has adverse effects on placental morphology in rats (Noback and Takahashi 1978). In female pregnant VAD rats, RA supplementation maintains normal implantation and early embryogenesis (White *et al.* 1998), but unless supplementation is sufficiently high by 8.5 dpc, fetuses will be reabsorbed (White *et al.* 2000). Retinoids are also required for the normal onset of meiosis in the developing embryo (discussed in detail in Chapter 6), and germ cells in rat embryos with severe retinoid deficiency fail to enter meiosis. This is accompanied by an observed failure of *Stra8* induction. Supplementation of small amounts of RA to dams was sufficient to initiate meiosis (Li and Clagett-Dame 2009). Retinoid-deficient mice have a prolonged estrous cycle, and display a decreased rate of oocyte maturation and number of ovulated oocytes after gonadotropin treatment (Kawai *et al.* 2016).

Pregnancy is an absolute contraindication for all oral treatment with retinoid drugs in the EU[9] as it is teratogenic (Lammer *et al.* 1985). The pattern of birth defects that can be observed in several organ systems, however, does not appear to specifically

9. https://www.ema.europa.eu/en/documents/referral/retinoid-article-31-referral-prac-assessment-report_en.pdf (accessed in November 2019).

target the female reproductive organs (Azais-Braesco and Pascal 2000, Pennimpede *et al.* 2010), although RA signaling has been shown to be involved in development of the genital tubercle (Liu *et al.* 2012).

5. Male reproductive organ development, function and health, and the role of retinoids

The testis produces large numbers of gametes (sperm, or spermatozoa) throughout the reproductive life of the male, and is also the primary source of androgens, which are required for spermatogenesis and the development and maintenance of male secondary sex characteristics throughout the body (reviewed in Bittman 2015).

RA plays several critical roles in the development and/or function of both Sertoli and Leydig cells (Jauregui *et al.* 2018, and reviewed in Lucas *et al.* 2014) as well as in spermatogenesis (reviewed in Mark *et al.* 2015, Griswold 2016, Teletin *et al.* 2017). In addition, RA signaling appears to be necessary for proper development of the testis itself (Spade *et al.* 2019a). As will be discussed below, testicular RA is not derived from the circulation, as a catabolic barrier is formed by Cyp26 enzymes present in the peritubular myoid cells (Vernet *et al.* 2006). The testicular site of RA synthesis (using ROH taken up from the circulation) varies with age (see Spermatogenesis section below). *Stra6* expressed in Sertoli cells may play a role in ROH uptake from the circulation, at least under VAD conditions (Kelly *et al.* 2016).

5.1. Testicular somatic cells and steroidogenesis

In the adult testis, LH stimulates the production of testosterone in Leydig cells. Steroidogenesis itself takes place in both fetal and adult Leydig cells and depends on several steroidogenic enzymes, such as CYP11A1, HSD3B1 and CYP17A1 (Jauregui *et al.* 2018). Testosterone secreted from the Leydig cells regulates the end of meiosis, the establishment and maintenance of the blood-testis barrier (BTB), and spermiation (Jauregui *et al.* 2018). Sertoli cells both create the BTB (*via e.g.* tight junctions and gap junctions) and play an active role in translocating the male germ cells within the seminiferous tubule epithelium (reviewed in Xiao *et al.* 2014).

RA signaling, via RAR/RXR, appears to be necessary for development and function of the Sertoli cells (Lucas *et al.* 2014). In primary rat Sertoli cells isolated on PND 10 and 20, RA suppressed proliferation and initiated tight junction formation (Nicholls *et al.* 2013). Leydig cells express proteins for RA synthesis, breakdown and signaling (Griswold and Hogarth 2018). Based on impairments observed in VAD mice, proper differentiation of Leydig cells appears to depend on sufficient retinoid levels (Yang *et al.* 2018). Fertility studies using conditional transgenic adult mice lacking functional Leydig cell RARα, show that RA signaling via RAR/RXR is required for normal Leydig cell function (Jauregui *et al.* 2018). The same conditional knockouts also had altered steroidogenic enzyme expression levels in Leydig cells, increased BTB permeability, as well as apoptotic pachytene spermatocytes, and

were infertile (Jauregui *et al.* 2018). The observed phenotype is similar to mice with low or no levels of testosterone (discussed in Jauregui *et al.* 2018). Increased BTB permeability has also been demonstrated in neonatal mice after treatment with the RALDH2 inhibitor WIN 18,466 (Amory *et al.* 2011, Kent *et al.* 2016). The mechanism behind effects on the BTB, and the possible role of RA on its maintenance, still needs to clarified.

In an *ex vivo* model using mouse fetal testes, RA treatment increased testosterone production (Bellutti *et al.* 2019). In contrast, RA has been shown to decrease testosterone production in the developing rat testis (Livera *et al.* 2000a). In *ex vivo* cultured human fetal testes, RA treatment increased testosterone production and expression of steroidogenic enzymes such as cholesterol side-chain cleavage enzyme (P450scc), Cyp17, and steroidogenic acute regulatory protein (StAR) (Lambrot *et al.* 2006). Interestingly, testicular P450scc activity in retinoid-deficient rats decreased concomitantly with the progression of retinoid deficiency (Jayaram *et al.* 1973). Thus, RA appears to be capable of influencing steroidogenesis in both rodents and humans.

5.2. Spermatogenesis

Spermatogenesis (described in Figure 9, and in more detail in Figure 10) is the formation of haploid gametes (spermatozoa) from the diploid stem spermatogonia, and includes the process of spermatogonia differentiation, meiosis, differentiation of spermatids (spermiogenesis) and spermatid release (spermiation) (reviewed in Mark *et al.* 2015). Spermatogenesis takes place inside the seminiferous tubules in a rigidly structured process ensuring continuous, life-long sperm production (reviewed in Griswold 2016). As will be described below, the process in pubertal animals differs from that in adult animals. The human testis produces sperm in a continuous manner similar to rodents, and it is reasonable to assume that similar regulatory patterns of sperm production also exist in the human testis (Jørgensen *et al.* 2015, Griswold 2016) although more research is needed for a better understanding of human spermatogenesis.

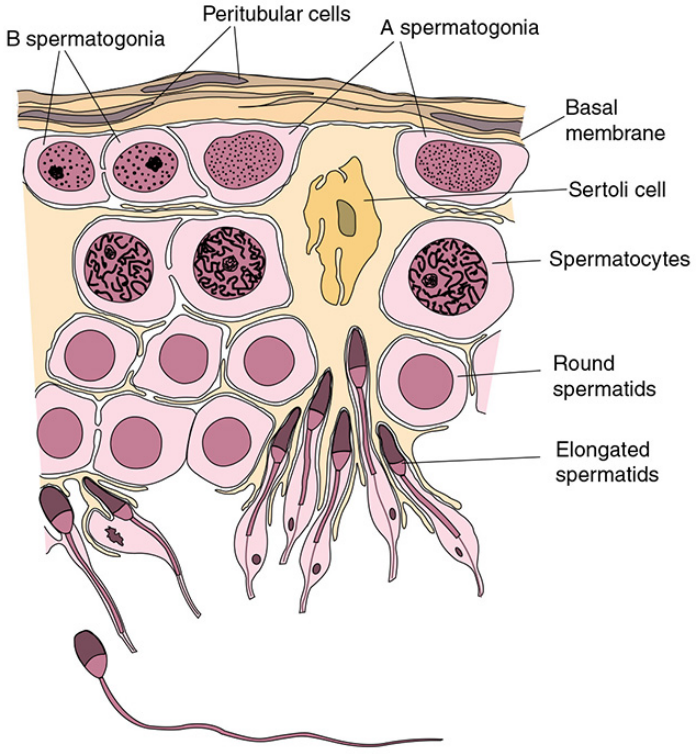

Figure 9: Schematic of a cross-section of an adult mouse seminiferous tubule, surrounded by peritubular cells

Note:
The spermatogonia are located on the inside of the basal membrane of the seminiferous tubule, and are completely surrounded by Sertoli cells. The primary spermatocytes and round spermatids are located closer to the lumen. The elongated spermatids will eventually be shed into the lumen as spermatozoa. (de Rooij and Mizrak 2008, reprinted with kind permission from the publisher: The Company of Biologists Ltd., Cambridge UK.)

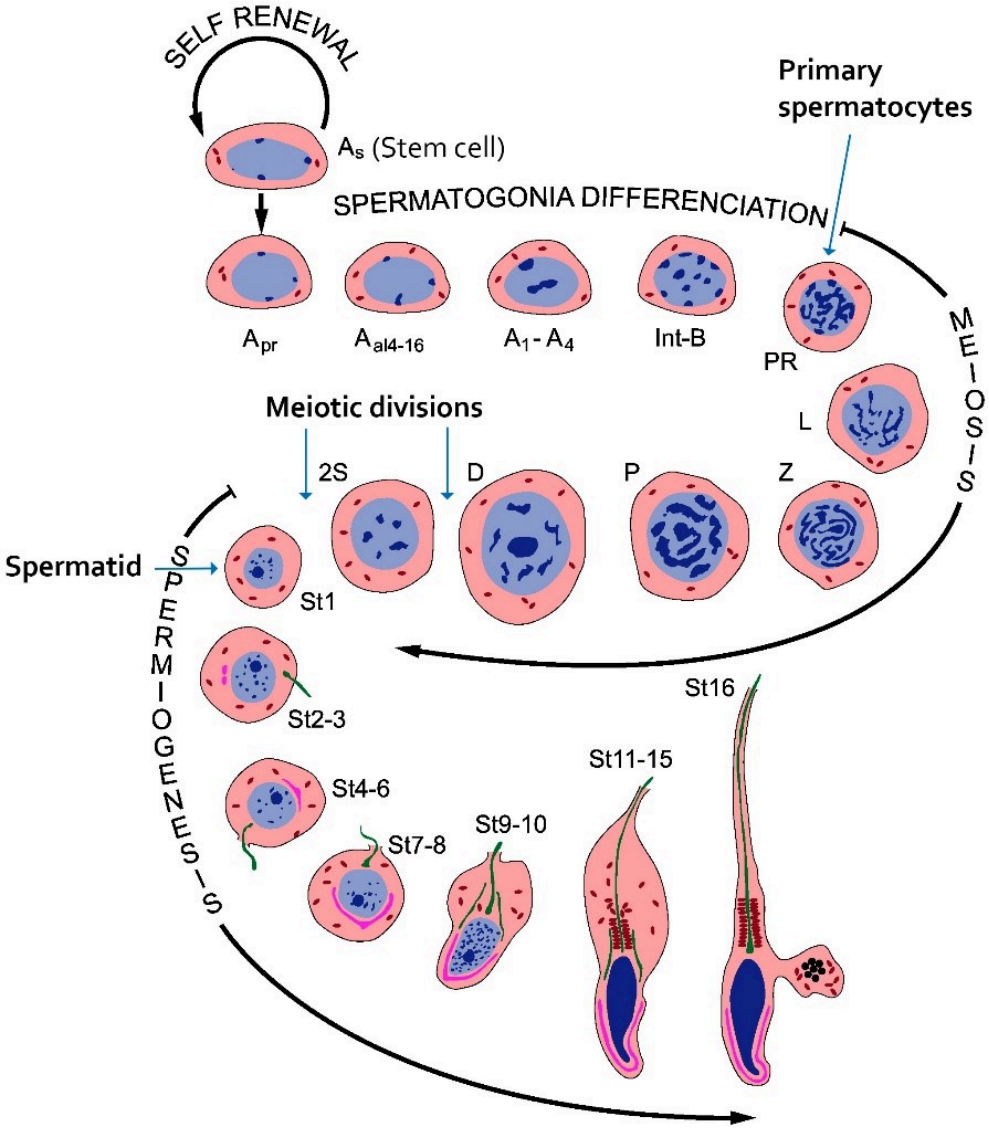

Figure 10: Spermatogenesis in the adult mouse

Note:

In mice, spermatogonia in the single-cell state (A_S) are considered as the true stem cells of the spermatogenic lineage. A_s spermatogonia divide to maintain a stem cell population (A_s) and expand the population of cells (A_{pr} and $A_{al4–16;}$) which enter the differentiation pathway (Stages A_1–A_4, Int and B, collectively referred to as differentiating spermatogonia (the previous stages, A_s – A_{al16}, are collectively also referred to as undifferentiated spermatogonia). In the meiotic phase, the primary spermatocytes undergo recombination and segregation of homologous chromosomes during the meiotic divisions to generate secondary spermatocytes (2S) and subsequently step 1 spermatids (St1). The spermiogenesis phase, is subdivided into 16 steps based on morphological criteria (round spermatids, steps 1 to 8; elongating spermatids, steps 9 to 16). The first 12 steps span the entire cycle of the seminiferous epithelium. Step 16 spermatids are released into the lumen of the seminiferous tubules as spermatozoa, during a process called spermiation. Abbreviations: PR; preleptotene, L; leptotene, Z; zygotene, P; pachytene, D; diplotene (the stages of the first meiotic prophase), 2S; secondary spermatocytes. (Mark et al. 2015. Reprinted with kind permission from the publisher: Elsevier.)

At least in mice, RA is considered to be critically involved in several steps of spermatogenesis: spermatogonia differentiation specifically during the A_{al} – A_1 transition, for spermiation, and in regulation of the seminiferous epithelium cycle (see Figure 11 and *e.g.* Teletin *et al.* 2019). RA is indispensable *in vivo* to trigger the A_{al} – A_1 transition (Teletin *et al.* 2019) and is also required for the survival of some A_{undiff} spermatogonia (reviewed in Teletin et al. 2017). Synthesis of both mRNA and protein STRA8 occurs exclusively in two distinct phases of spermatogonia differentiation; in differentiating type A spermatogonia (A_1-A_4), as well as in spermatocytes in the preleptotene (PR) and leptotene (L) phase at meiosis entry (Griswold 2016). See section *Meiosis in the post-natal male* below, for further discussion about meiotic regulation.

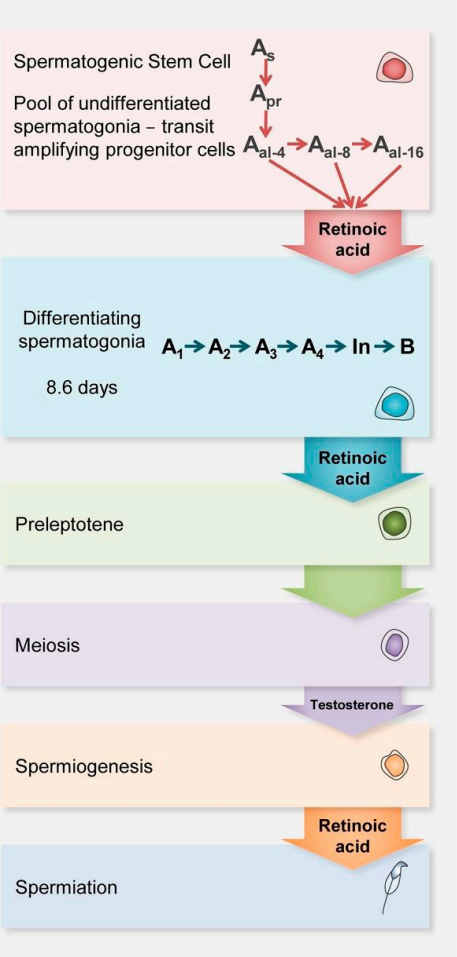

Figure 11: Steps in germ cell differentiation and spermatogenesis thought to be controlled by RA in mice

Note:
Once the first/pubertal wave (during which differentiating spermatogonia develop directly from prospermatogonia) has been initiated, the subsequent rounds of differentiating spermatogonia arise from a subset of A single-state spermatogonia (A_s) acting as spermatogenic stem cells. Stem cells divide and form A paired spermatogonia (A_{pr}) that in turn divide and form an aligned cell syncytia of 4, 8, and 16 cells (also called "transit amplifying progenitor cells"), which transition without cell division into A_1 differentiating spermatogonia. After five cell divisions (synchronized to the cycle of the seminiferous epithelium), B spermatogonia are

formed, followed by another mitotic division resulting in the formation of preleptotene spermatocytes. The preleptotene spermatocytes proceed through the rest of the meiosis, forming haploid spermatids that are eventually elongated. (Griswold 2016, reprinted with kind permission from the publisher: American Physiological Society, Rockville, MD, USA.)

5.2.1. Spermatogonia differentiation and spermiogenesis – first and subsequent waves

In the juvenile mouse, the mitotically quiescent gonocytes (also termed pro-spermatogonia) re-enter the cell cycle at approximately PND 1-2, and turn into spermatogonia at PND 3-6 as they migrate to the periphery of the testis cords, where they are surrounded by Sertoli cells (reviewed in Teletin *et al.* 2017 Teletin *et al.* 2019). During this first post-natal week in mice, a sub-population of gonocytes differentiates directly (without passing through the undifferentiated spermatogonia stages) into differentiating spermatogonia that support the so-called first wave of pubertal spermatogenesis. After the first wave of spermatogenesis (completed by PND 35 in the mouse), subsequent waves derive from the undifferentiated spermatogonia that have acquired self renewal capacity, the spermatogonial stem cells (Yoshida *et al.* 2006).

During the onset of puberty in mice, sperm development is initiated by RA produced in Sertoli cells, since these, at this stage, are the only cells in the seminiferous epithelium with RALDH activity (Raverdeau *et al.* 2012). The earliest reported presence of a germ cell derived source of RA in the postnatal mouse is at PND 9 in zygotene spermatocytes (Teletin *et al.* 2019). The RA produced in the Sertoli cells acts in a paracrine manner on the spermatogonia, to regulate the A_{al} – A_1 transition. Spermatogenesis is blocked in juvenile male mice deficient in RDH10 in Sertoli cells and germ cells, leading to a local lack of RA (Tong *et al.* 2013).

In juvenile male mice with arrested spermatogonia differentiation, due to either vitamin A deficiency or genetic knockout of Sertoli cell *Aldh1a*, a single injection of RA will resume the A_{al} – A_1 transition, with preleptotene spermatocytes expressing Stra8 and Rec8 appearing synchronously in all seminiferous tubules (reviewed in Griswold 2016). The same effect is observed in mice first treated with the RALDH inhibitor WIN 18,446, and subsequently treated with RA (Hogarth *et al.* 2013). RA administration to vitamin A-deficient mice or rats further results in truncation of the normal 12 (mouse) or 14 (rat) stages of the seminiferous epithelium cycle to only three or four stages (still enabling sperm production). Additionally, the normally asynchronous wave of spermatogenesis is synchronized, leading to a pulsatile rather than continuous sperm production (Teletin *et al.* 2017).

5.2.2. Post-pubertal RA synthesis

Once the first wave of spermatogenesis has progressed beyond a certain point, it appears, based on data from mice in which all three *Aldh1a* genes were deleted in Sertoli cells, that Raldh activity in Sertoli cells is no longer needed for spermatogonia differentiation to proceed (Raverdau *et al.* 2012). Thus, Raverdeau suggested that RA must be produced elsewhere, most likely by RALDH2 in spermatocytes and spermatids. Interestingly, Beedle and co-workers reported that in the testis of mice genetically engineered to have a postnatal severe deficiency of *Aldh1a2* gene

expression in germ cells (*via Stra8-Cre*), or globally (*via* a tamoxifen-inducible *Cre*), no adverse effects on male fertility or health are noted (Beedle *et al.* 2018). In addition, in other mouse studies, where *Aldh1a1-3* genes have been ablated, it is suggested that spermatocyte-synthesized RA is dispensable for spermatogenesis, and that the Sertoli and spermatocyte RA sources are redundant in maintenance of spermatogenesis (reviewed in Ghyselinck and Duester 2019). Species differences may exist; studies using human testis biopsies suggest that RALDH1 is the predominant form that synthesizes RA in the Sertoli cells, while RALDH2 has the corresponding role in developing sperm (Arnold *et al.* 2015). As mentioned above, blocked spermatogenesis is observed in juvenile male mice deficient in RDH10 in Sertoli cells and germ cells; however, in adult age, these mice appear to have normal spermatogenesis (Tong *et al.* 2013), suggesting that in the adult animal, RDH10 is not important for control of RA availability in the testis.

5.2.3. The seminiferous epithelium cycle and the spermatogenic wave

The seminiferous epithelium cycle covers the development from spermatogonia to spermatozoa, as they move from the basal compartment of the seminiferous tubule to the lumen. The stages are usually illustrated using Roman numerals (See Figure 12). The number of stages are species specific; *e.g.*, mice have twelve stages, and humans six. In the mouse, four seminiferous epithelium cycles are required to complete the development from spermatogonia to spermatozoa.

The timing of the progenitor cell commitment and subsequent differentiation and maturation of spermatogonia along the seminiferous tubule is staggered, forming a spermatogenic wave (Griswold 2016). The cycle of the seminiferous epithelium is initiated by the precisely timed transition of undifferentiated A_{al} spermatogonia into A_1 spermatogonia, which subsequently will divide to generate successively differentiating A_2, A_3, and A_4 spermatogonia (Figure 11 and *e.g.* Griswold 2016, Teletin *et al.* 2019).

The spermatogonia continue to divide mitotically to produce cells that replenish the stem cell pool and cells that undergo a series of mitotic divisions to increase the number of spermatocytes, that subsequently progress through meiotic sub-stages. In addition, in mice, it takes 8.6 days for the A_1 spermatogonia to become preleptotene spermatocytes and enter meiosis and an additional 8.6 days ×3 to form elongated spermatids ready for spermiation. The net result is that once the spermatogenic cycle is fully established, the same cell associations or the same group of cell types appear every 8.6 days (Griswold 2016). The length of the spermatogenic wave is species-specific, *e.g.*, 8.6 days in mice, 13 days in rats and 16 days in humans (reviewed by Bittman 2016, Griswold 2016).

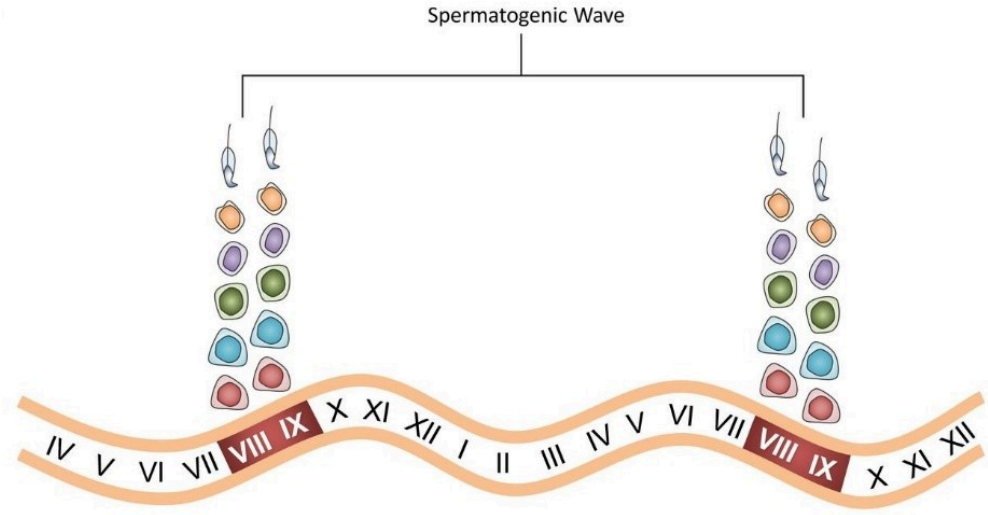

Figure 12: Overview of the spermatogenic wave

Note:

Under normal physiological conditions, spermatogenesis is asynchronous, with a wave generated by RA pulses (red patches, stages VIII and IX in mice) along the tubules. These RA pulses drive the spermatogonial transition from A_{al} to A_1.

Red, *undifferentiated A spermatogonia;* teal, *differentiating A_1 spermatogonia;* green, *preleptotene spermatocytes*; purple, *pachytene spermatocytes;* orange, *round or elongating spermatids;* blue, *elongated spermatids. (Griswold 2016, reprinted with kind permission from the publisher: American Physiological Society Rockville, MD, USA.)*

During the seminiferous epithelium cycle, pulses of RA are observed at stages VIII-IX (Griswold 2016; see Figure 12). During these stages the three RA-dependent steps of spermatogenesis is described to occur, namely the transition from undifferentiated to differentiated spermatogonia, meiosis initiation, and spermiation (Griswold 2016). Further, RA may play a role in the initiation of spermatid elongation, which also occurs at stage VIII (Endo *et al.* 2017). The expression of the RA regulated genes *Stra8* (in spermatocytes) and *Stra6* (in Sertoli cells) peak at stages VII-VIII of the seminiferous epithelium cycle (Teletin *et al.* 2017, Griswold and Hogarth 2018). However, at least in adult mice, RALDH enzymes (RALDH1A1-3 and ALDH8A1) were not expressed in a stage-specific manner (Kent *et al.* 2016).

5.2.4. Regulation of RA signaling

For the regulation of spermatogonial exposure to RA, two hypotheses have been presented: 1) all spermatogonia are primed to respond to RA, but the exposure to RA is periodic and tightly controlled, and 2) all spermatogonia are exposed to RA but only some can respond (Busada and Geyer 2016). The differential expression of RARγ between spermatogonia subpopulations could explain how the A_s spermatogonia (with no RARγ expressed) remain undifferentiated and maintain self-renewal capabilities even in the presence of high RA concentrations, which induce differentiation in the (RARγ-expressing) A_{al} progeny populations (Teletin *et al.* 2017).

Observations from cell-specific conditional knockout mouse studies of RA receptor isomers suggest that the RA signal in spermatogonia is transduced via RAR/RXR heterodimers (Gely-Pernot *et al.* 2015). Another suggestion is that RA signaling also operates via Sertoli cells, as germ cell differentiation can proceed in the absence of functional RAR and RXR isotypes in spermatogonia (Teletin *et al.* 2017). RA may also act via non receptor-mediated pathways, such as *via* kinase signaling (Busada and Geyer 2016).

Conditional knockout studies in mice show that despite the critical role of RA in spermatogonia differentiation and in male germ cell meiosis, both differentiation and meiosis can occur in germ cells where RAR or RXR are absent, although a fraction of the A_1 spermatogonia are adversely affected (Gely-Pernot *et al.* 2015). Initiation and progression of meiosis also proceeded in these mice. The conclusion was that the RA signaling pathway was not autocrine, but rather operated in Sertoli cells (Gely-Pernot *et al.* 2015; see also previous sections *Post-pubertal RA synthesis and The seminiferous epithelium cycle and the spermatogenic wave*).

5.2.5. RA metabolism: Role of Cyp26

Spermatogonia exposure to RA is also regulated by the presence of the RA-catabolizing CYP26 enzymes; Cyp26a1 and Cyp26b1 form a catabolic barrier against any RA present in the immediate environment of the seminiferous tubules. All three CYP26 isoforms are present in the mouse postnatal testis, although observations after conditional deletion of the Cyp26a1 and/or Cyp26b1 genes in germ and/or Sertoli cells reveal that Cyp26b1 is the critical isoform (Hogarth *et al.* 2015). In the fetal mouse testis, elimination of RA by Cyp26b1 is necessary not only to prevent premature meiosis, but also for normal mitotic arrest of male PGCs and for preventing germ cell apoptosis (Rossitto *et al.* 2015). Cyp26b1 is initially produced by the Sertoli and Leydig cells and/or interstitial somatic cells, although after birth, Cyp26b1 transcripts are confined to the peritubular myoid cells (Rossitto *et al.* 2015). No variations in the expression of Cyp26 enzymes exist across the seminiferous epithelium cycle, unlike several proteins involved in retinoid storage (*e.g.* LRAT) and RA synthesizing Raldh1 in Sertoli cells and Raldh2 in germ cells) which are regulated in a periodic manner along the seminiferous tubule (Teletin *et al.* 2017).

5.2.6. Meiosis in the male

Just as for oocytes, RA may play a role in the entry of the spermatocytes into meiosis (Raverdeau *et al.* 2012), possibly via control of replication-dependent core histone gene expression necessary for entry into S phase (Chen *et al.* 2016). However, recent findings by Teletin and co-workers suggest that RA may act only as a facilitator in the initiation of meiosis (Teletin *et al.* 2019). See chapter 6 for further discussion on the role of RA in meiosis.

5.2.7. Spermiation

Cell-cell junctions between spermatozoa and late-stage spermatids are degraded in a process facilitated by Sertoli cells, and thereby immature spermatozoa (step 16 spermatids) are released into the tubular lumen. The released spermatozoa are

subsequently transported by peristaltic movements of the tubule, *via* rete testis to epididymis, where the spermatozoa acquire motility and fertilization potential (reviewed in Xiao *et al.* 2014).

In early studies using VAD rats, a delayed release of late spermatids was observed (Huang and Marshall 1983). In the mouse, RA is now known to be required to disengage spermatozoa from the Sertoli cell cytoplasm during spermiation; in male mice genetically modified to lack RALDH1-3 in their Sertoli cells, spermatids were retained in the seminiferious epitithelium (Raverdeau *et al.* 2012). Spermiation failure was also observed in *Rbp4*-null mice rendered VAD (Ghyselinck *et al.* 2006). Similar observations made in Rarα-null mice (Chung *et al.* 2005) suggest that the effect of RA on spermatid release is mediated by RARα.

5.3. Secondary male reproductive organs

The prostate produces slightly alkaline prostate fluid that makes up approximately 30% of the volume of semen in humans and is critical for male reproductive health (Verze *et al.* 2016). The retinoid signaling pathway has several functions in differentiation and maintenance of secondary male reproductive organs. RA is necessary for prostate formation from the urogenital sinus during sexual differentiation (Vezina *et al.* 2008, Bryant *et al.* 2014). Genetic deletion studies in mice have demonstrated the importance of RA signaling via *Rarγ* in the prostate and in the seminal vesicles (Lohnes *et al.* 1993).

RA signaling appears to be necessary for the function of epididymis, into which sperm are released after spermiation, undergo maturation, and are stored until ejaculation (Jauregui *et al.* 2018). In a conditional transgenic model with a dominant negative form of RARα expressed in Leydig cells and in the epididymis, the resulting abnormal epididymis phenotype may contribute to the infertility observed in these mice (Jauregui *et al.* 2018). These observations support previous findings that lack of RA signaling in the epididymis results in squamous metaplastic epididymal epithelium; alternatively, the abnormal epididymal phenotype may be due to lack of testosterone (Jauregui *et al.* 2018).

5.4. Male reproductive pathologies

Impaired RA signaling may play a role in several male reproductive pathologies. The transition from PGCs to differentiating spermatogonia is impaired in the absence of RA in the testis cords (Teletin *et al.* 2017). PGCs that fail to differentiate into spermatogonia may be the source of carcinoma *in situ*, which in humans may develop into testicular germ cell cancer (Busada and Geyer 2016, Teletin *et al.* 2017).

In prostate tumor tissue, RA concentrations are lower than in normal prostate tissue (reviewed in Nelson *et al.* 2013). Since it has been hypothesized that this is due to increased RA catabolism, inhibitors of CYP26 enzymes, called RA metabolism blocking agents (RAMBAs) have been used in the treatment of prostate cancer (Nelson *et al.* 2013). RAMBA therapy promotes differentiation and inhibits proliferation by increasing endogenous RA in tumors (Denis *et al.* 1998). Some RAMBAs also inhibit estrogen synthesis *via* inhibition of aromatase/CYP19

and testicular androgen synthesis via inhibition of 17,20-lyase/CYP17 (Bryson and Wagstaff 1996). The RALDH2 enzyme expression is also altered in prostate cancer. The expression of the *ALDH1A1* gene is lower and its promoter region is hypermethylated in epithelia from malignant prostate tumors (Kim *et al.* 2005).

Cryptorchidism, or non-descended testis, is a congenital malformation that is associated with an increased risk of testicular cancer and infertility in adult life (Bay *et al.* 2011). Complete testicular descent is necessary for normal testicular function in adult males, and the process of testis descent is regulated by the Leydig cell hormones insulin-like peptide 3 (INSL3) and testosterone (Bay *et al.* 2011). The spectrum of reproductive system malformations caused by VAD in rats includes cryptorchidism (See *et al.* 2008). Both RA levels and *Stra8* expression were significantly lower in the rat cryptorchid testis (induced by *in utero* exposure to the anti-androgen flutamide) compared to the normal testis (Peng *et al.* 2016). In an *in vitro* system, RA upregulated the expression of the gene Relaxin family peptide receptor 2 (*RXFP2*; *LGR8*), which encodes the receptor for INSL3 (Klonish *et al.* 2005).

5.5. Retinoid deficiency/excess in male reproduction

Animal studies measuring effects of experimentally increased or decreased levels of retinoids have demonstrated the importance of narrowly regulated retinoid levels for normal male reproduction. Adverse effects on the male reproductive system following experimental limitations of RA have been demonstrated in multiple *in vivo* studies (discussed in previous sections of this report, and reviewed in Clagett-Dame and Knutson 2011). In rodents maintained on a vitamin A-deficient diet, testicular degeneration with impaired spermatogenesis and a complete disappearance of all meitotic and postmeiotic cells has been observed (Coward *et al.* 1969, Morales and Griswold 1987, van Pelt and de Rooij 1990). Spermatogenesis is arrested at the preleptotene spermatocyte stage in VAD rats and at the spermatogonia stage in VAD mice; however, spermiation failure is observed in both species under VAD conditions (Ghyselinck *et al.* 2006). Vitamin A deficiency also leads to replacement of normal glandular epithelium in the epididymis, prostate and seminal vesicles, by a stratified squamous keratinizing epithelium, resulting in inhibited seminal fluid production (Wiseman *et al.* 2017).

It has been known for almost a century that male rats maintained on a retinol-deficient diet supplemented with RA will be sterile, possibly because the testis cannot take up RA from the circulation (Kurlandsky *et al.* 1995).

In human populations with retinoid deficiency, symptoms such as blindness are well-known, but information on reproductive parameters such as sperm production or fertility is lacking (Hogarth and Griswold 2010). However, observations made in testicular tissue samples obtained in different clinical situations, suggest a correlation between adverse reproductive parameters and disturbed retinoid signaling. Significantly lower levels of in *13-cis*-RA was observed in testis tissue biopsies in men with abnormal sperm production undergoing scrotal surgerydue to various benign indications (Nya-Ngatchou *et al.* 2013). In a different study, lower levels of the RALDH2 enzyme in testicular tissue were associated with male infertility (Amory *et al.* 2017). In follow-up studies, treatment with 13-*cis*-RA (used in the treatment of acne) appeared to increase sperm production (Çinar *et al.* 2016,

Amory *et al.* 2017).

6. Impact on female and male reproduction by compounds acting via the retinoid system

Exposure to chemicals early in life may affect both female and male reproductive organs and their functions, most likely *via* several mechanisms, with effects *via* estrogen, androgen and/or steroidogenic pathways being the most frequently discussed. A testicular dysgenesis syndrome (Skakkebaek *et al.* 2001) as well as an ovarian dysgenesis syndrome (Buck Louis *et al.* 2006, reviewed in Johansson *et al.* 2017) have been described. Both dysgenesis syndromes are defined as early (fetal) alterations in testicular or ovarian structure or function that cause an impairment of reproductive parameters in adulthood. One of the mentioned fetal alterations in the ovarian dysgenesis syndrome was disruption of the RA-dependent meiosis initiation (reviewed in Johansson *et al.* 2017). For embryonic males, it has been suggested that testicular toxicity could result from disruption of local RA homeostasis or signaling (Spade *et al.* 2019b). In the adult organism, chemicals may interfere with *e.g.* normal sperm production (Sharpe 2010) and normal function and morphology of female reproductive tissue (reviewed in Johansson *et al.* 2017).

Table 2 summarizes some observed effects and mechanisms by which compounds, or chemicals, could interfere with the retinoid pathway in (and also in *e.g.* Nilsson and Håkansson 2002, Novak *et al.* 2008, Shmarakov 2015). In brief, chemicals have been shown to deplete tissue retinoid levels by affecting retinoid metabolism, also after *in utero* exposure. Chemicals can also cause activation or inactivation of retinoid receptors. As described in this report, the retinoid pathway is important, or even critical, in several aspects of both female and male reproduction and during fetal development. It is thus conceivable that chemicals capable of interfering with the retinoid pathway may, as a result, cause adverse effects on reproductive parameters, assuming that such chemicals reach target cells during critical windows.

There were publications retrieved, during the course of this project, describing a general effect by compounds on RA-content and enzyme or receptor expression (*e.g.* in the liver), but none of these animal studies (with the exception of some pharmaceutical compounds) also describe effects on retinoid parameters in reproductive organs, such as RA-synthesizing/metabolizing enzymes. For the male reproductive system, there are *in vivo* studies, where brominated flame retardants causes decreased liver retinoid stores in male Wistar rats, and where slight effects on reproductive organ weights is reported. However, no adverse effects on reproductive organ histopathology, or on reproductive outcome, was found, and no retinoid-related parameters were measured in the reproductive organs (see van der Ven *et al.* 2008, and van der Ven *et al.* 2009 in Table 2).

While most data originate from animal studies, there is human data as well. For example, in the 1960s, it was demonstrated that the pharmaceutical compound WIN

18,446 could reversibly inhibit spermatogenesis in men (Heller *et al.* 1961). More recently, the same compound was shown to inhibit the conversion of retinal to RA, most likely by inhibiting ALDH1A2 (Paik *et al.* 2014), and this finding has led researchers to suggest the use of of retinoid metabolism inhibition as an approach to male contraception (Hogarth *et al.* 2011). Acne treatment with 13-*cis*-RA (isotretionin) appeared to increase sperm production (Çinar *et al.* 2016, Amory *et al.* 2017), which is in line with earlier observations of reduced testicular concentrations of 13-*cis*-RA in men with low sperm production (Nya-Ngatchou *et al.* 2013). An important species difference between humans and rodents is that male rodents produce a large surplus of sperm, so decreases in sperm count or quality may not lead to decreased fertility. The situation is very different in humans, where sperm counts/quality are often so low, that any additional decrease would have direct adverse effects on fertility (reviewed in Working 1988).

Table 2: Examples of chemicals interfering with the retinoid system in different models

Chemical(s)	Model system	Endpoint	Observed effect	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Male rats, single dose	Retinoid levels in several organs, including testes and epididymis	↓Retinyl esters in liver, testes, epididymis. ↑Retinyl esters in kidney	Håkansson *et al.* 1991
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Pregnant rats and PND 7 pups, single dose in utero	Retinoid levels in liver, lung and kidney	↓Retinyl esters in maternal and perinatal liver and lung. ↑Retinyl esters in maternal and perinatal kidney	Kransler *et al.* 2007
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Male rats, single dose	RA and retinyl ester levels and LRAT expression in liver and kidney	↑ RA in liver and kidney. ↓Retinyl esters in liver. ↑Retinyl esters in kidney. ↑ LRAT in kidney	Hoegberg *et al.* 2003
Technical pentabromodiphenyl ether mixture	Female and male rats (enhanced 28d TG407 study)	Retinoid levels in liver	↓Retinyl esters in liver	van der Ven *et al.* 2008[a]
Hexabromocyclodecane	Female and male rats (enhanced 1-gen TG415 study)	Retinoid levels in liver	↓Retinyl esters in liver	van der Ven et al. 2009[b]
Fluconazole	Mice (exposed in utero)	mRNA induction of CYP26A1 and CYP26B1. Organ development (not repro)	Upregulation of CYP26A1 and CYP26B1. Abnormal branchial arch development.	Tiboni *et al.* 2009
Ketoconazole	Mouse testis organ cultures	Meiosis markers	Meiosis induction in fetal testes	Bowles *et al.* 2006
Bisphenol A	Mice (exposed in utero)	Retinoid and mRNA levels in liver	↑RA. ↓RXRβ	Esteban *et al.* 2019

Organochlorine pesticides (chlordane, dieldrin, aldrin, endrin, endosulfan)	Reporter cell lines (RARE & RARα, β, γ). CYP26A1	Activation of RARs. Induction of CYP26A1	Activation was observed. CYP26A1 induction was observed.	Lemaire *et al.* 2005
543 environmental chemicals	Yeast cells transfected with the human RARγ	RARγ agonistic activity	85 of the 543 chemicals had RARγ agonistic effects (especially monoalkylphenols and styrene dimers).	Kamata *et al.* 2008
309 environmental chemicals	HepG2 cells transfected with cis/trans-reporter transcription units for RARα, β, γ	Transcription factor activity	Maximum responders were lindane, oxadiazon and imazalil	Martin *et al.* 2010
28 environmental and other compounds	Human uterus and prostate cytosol	Chemical displacement; inhibition of ^{3}H-RA binding to cytosol	A number of the chemicals could displace ^{3}H-RA. MEHP most potent.	Paganetto *et al.* 2000
PPARγ agonists rosiglitazone and pioglitazone	HepG2 cells	mRNA induction of CYP26B1 and CYP26A1	mRNA induction of CYP26B1 and (to a lesser extent) CYP26A1	Tay *et al.* 2010
Phenobarbital	Human hepatocytes	mRNA induction of CYP26B1	Weak induction of CYP26B1	Finkelstein *et al.* 2006
Tributyltin	Human and mice ovarian theca cells	Cholesterol homeostasis via RXR pathway	Impaired cholesterol homeostasis	Pu *et al.* 2019
Tributyltin, triphenyl tin	Mouse adipocytes	Cell differentiation into adipocytes	Agonist activity via PPARγ/RXR	Kanayama *et al.* 2005

Note:

[a] Also observed in males: decreased weight of epididymis, and an increased weight of the seminal vesicles, however, no histopathological changesRE were noted; in females: induced adrenal activity of the steroidogenic CYP17 enzyme.

[b] Also observed in F1-males: decreased weight of testis and prostate (concomitant with a reduction in body weight, however, no histopathological changes were observed). Epididymal sperm count or sperm morphology were not affected, except for the observation of a decreased ratio of separated sperm heads. Note that no significant dose–response effects on endpoints of reproduction, i.e. mating success, time to gestation, gestation duration, number of implantation sites and litter size, were observed.

It should be pointed out that effects of chemicals on retinoid homeostasis can be indirect, since some metabolic enzymes are used both for detoxification and for retinoid homeostasis, such as RALDH/ALDH class 1 (Alnouti and Klaassen 2008). Induction of *Aldh* isomers, as studied in mouse livers, was isomer- and activator-specific after *in vivo* administration of several activators of different nuclear

receptors (such as CAR, PXR, PPAR, AhR) (Alnouti and Klaassen 2008). The well-known induction of CYP enzymes via AhR after exposure to dioxins and dioxin-like polychlorinated biphenyls (PCBs) may be relevant since some of these P450 enzymes are believed to be involved in either synthesis or oxidation of RA (Murphy *et al.* 2007). If the end result is altered RA concentrations in fetal or adult reproductive organs, correct development and/or function of these organs could be compromised.

Since RXR can heterodimerize with several other nuclear receptors, it is also conceivable (and has indeed been shown; see *e.g.* Tarrade *et al.* 2001) that any interference with the retinoid system can also have effects on other signaling pathways, *via e.g.* PPAR, PXR, CAR and VDR (see earlier section on cross-talk). Also, interactions with AhR signaling pathways on several levels have been shown (Murphy *et al.* 2007, Vezina *et al.* 2008). From a functional/endpoint perspective, cross-talk with the other endocrine systems (estrogen, androgen, thyroid) is important to consider.

6.1. Compounds affecting female reproduction

6.1.1. Analgesics

Exposure to the analgesic drugs **acetaminophen** (paracetamol) and **indomethacin** appeared to give rise to delayed entry into meiosis in female rats after *in utero* exposure, with changes in ovarian *Stra8* levels reflecting this delay (Dean *et al.* 2016). Other effects, not currently linked to *Stra8* or RA, included decreased fetal ovarian germ cell numbers and (in adult females exposed *in utero*) reduced ovarian size and female fertility (measured as number of pups per litter) were observed (Dean *et al.* 2016).

In a mouse study with *in utero* exposure to **paracetamol**, both fertility, follicle numbers and germ cell numbers (as indicated by decreased mRNA levels of the germ cell marker mouse Vasa homologue; Mvh) decreased (Holm *et al.* 2016). In ovaries dissected at 12.5 dpc and exposed to 100 µM paracetamol for three days in culture, no effect of paracetamol on ovarian Stra8 levels was observed. Paracetamol did not cause the same decrease of the germ cell marker Mvh ex vivo as it did in vivo; the authors suggested that the sensitive window for the paracetamol effect occurred earlier than 12.5 dpc (Holm *et al.* 2016).

6.1.2. Bisphenol A

In mice, in utero exposure to bisphenol A has been associated with a delay in meiotic prophase I, hypothesized to be due to a decreased expression of *Stra8* in fetal oocytes (Zhang *et al.* 2012). In the same experiment, it was observed at PND 3 that an increase in bisphenol A dose levels were associated with an increased number of oocytes in germ cell cysts and fewer oocytes in primordial follicles (Zhang *et al.* 2012). Increased DNA methylation may be a mechanism for how bisphenol A affects Stra8 expression meaning that the effect of bisphenol A appears to be independent of any direct effect on retinoid metabolism or homeostasis, although it exerts (one of) its effects on the RA-regulated gene *Stra8* (Zhang *et al.* 2012).

Not all data support an effect of bisphenol A on *Stra8*. In human fetal oocytes

cultured in bisphenol A-containing media, the expression pattern of *Stra8* was similar to that in control cultures (Brieño-Enriquez *et al.* 2012). In fetal ovaries originating from pregnant mice exposed to bisphenol A, the observed increase in *Stra8* expression did not differ from that in unexposed mice (Lawson *et al.* 2011).

6.1.3. Phthalates

Diethyl hexyl phthalate (DEHP) causes a delay in meiosis in mouse fetal germ cells following *in utero* exposure, and the concomitant decrease in mRNA and protein expression of *Stra8* was suggested to be related (Zhang *et al.* 2015). The DNA methylation level of *Stra8* in oocytes of the F1 generation increased as a result of DEHP exposure, and these changes were inherited by the F2 generation (Zhang *et al.* 2015).

DEHP may also exert effects *via* PPAR-RXR. *In vitro*, the DEHP metabolite monoethylhexyl phthalate (MEHP) suppressed expression of CYP19 , the rate-limiting enzyme for conversion of testosterone to estradiol, via activation of PPAR-RXR heterodimers in rat ovarian granulosa cells (Lovekamp-Swan *et al.* 2003).

Phthalates can target many aspects of ovarian development and normal function (reviewed by Hannon and Flaws 2015); the exact mechanisms are unknown.

6.1.4. Organotin compounds

In marine gastropods, tributyltin, found as a contaminant of dibutyltin in vinyl plastics and also used in antifouling paints for ships and fishing nets, induces imposex (the development of male genitals in females) via binding RXR, thereby activating the RXR-RAR heterodimer (Nishikawa *et al.* 2004).

In human placental choriocarcinoma cells, trialkyltins stimulate human chorionic gonadotropin production and CYP19/aromatase activity by acting as RXR agonists (Nakanishi *et al.* 2005).

The RXR signaling pathway may be involved in the observed increase in progesterone production in human placental cells (in vitro) following organotin exposure (reviewed in Macejova *et al.* 2016).

No RXR activation was observed in rodent or human placenta tissue in which ng-mg/kg levels of organotin levels were present (de Araújo *et al.* 2018).

In ovarian theca cells from humans, mice and other mammalian species, tributyltin stimulated cholesterol extracellular efflux via the RXR pathway (Pu *et al.* 2019).

6.1.5. R115866

This triazole-containing molecule is a CYP26 inhibitor, more potent than liarozole, and largely without the inhibitory ability of liarozole on the CYP-dependent formation of estradiol and testosterone (Stoppie *et al.* 2000). R115866 administration leads to increased endogenous levels of RA, with subsequent RA-like effects such as inhibition of vaginal keratinization in estrogen-stimulated rats (Stoppie *et al.* 2000).

6.1.6. Isotretinoin/13-cis-RA

This retinoid, used for *e.g.* treatment of acne, has been found to lead to reduced antral follicle count, ovarian volume and levels of anti-Müllerian hormone (AMH; a marker of ovarian follicle number) in humans (Aksoy *et al.* 2015). Similar effects have been observed in rats (Abali *et al.* 2013). The effects appear to be transient both in humans (Çinar *et al.* 2017) and in rats (Korkmaz *et al.* 2017) once treatment ceases. Isotretinoin administered to rats at doses up to five times higher than clinical doses used for acne treatment reportedly had no adverse effects on fertility, conception rate, gestation or parturition, as summarized in an US FDA Pharmacology Review of isotretinoin[10]. The ICH[11] reproductive toxicology guideline for registrations of pharmaceuticals for human use does not require histopathological examination of ovaries in reproductive toxicity studies, and therefore it is not possible to draw any firm conclusions on possible effects of isotretinoin on ovarian volume or follicle count from the US FDA summary of this study.

6.2. Compounds affecting male reproduction

6.2.1. Thiocarbamate herbicides

Molinate, a known testicular toxicant in the rat, is also an inhibitor of RALDH and has been shown to inhibit the conversion of retinal to RA; decreased testicular levels of RA were observed in rats dosed with molinate (Zuno-Floriano *et al.* 2012).

6.2.2. Conazoles

Conazole fungicides are triazole-based compounds used both in agriculture and pharmaceuticals, and which exert their fungicidal effects via broad inhibition of CYP enzymes (reviewed in Sheehan *et al.* 1999). CYP26 inhibition has been suggested to be specifically involved in the teratogenic effects of triazoles (reviewed in Menegola *et al.* 2006). Triazole-containing conazoles have been shown to cause decreased hepatic RA levels in mice (Chen *et al.* 2009), and the ability of the triazole compound flusilazole to modulate RA homeostasis has been hypothesized to be an important

mechanism underlying its developmental toxicity (Tonk *et al.* 2015). Reprotoxic effects have been observed following *in vivo* exposure (Vickery *et al.* 1985, Taxvig *et al.* 2007, Schwartz *et al.* 2019).

In the pharmaceutical industry, structurally related compounds such as liarozole and talarozole have been considered for treatment of some cancers and dermatological diseases (Stevison *et al.* 2017). One mechanism of action of these compounds is the blocking of RA catabolism *via* inhibition of CYP26. Transient increased testicular RA levels have been observed in mice given talarozole (Stevison *et al.* 2017). In VAD mice administered liarozole after a dose of RA, the RA-induced proliferative effects on A spermatogonia were less than in VAD mice given RA but not liarozole (Gaemers *et al.* 1997). Ketoconazole was used to suppress Cyp26 activity in fetal mouse testis and thereby preventing degradation of RA; the result was induction of Stra8 expression which was followed by a premature meitotic entry in male germ cells (Bowles et al

10. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/021951Orig1s000PharmR.pdf, downloaded 4 September 2018.
11. International Council for Harmonization of technical requirements for pharmaceuticals for Human use; https://www.ich.org/

2006). Although ketoconazole blocks CYP enzymes without specificity for CYP26B1, the observation that ketoconazole, if used in combination with the RAR antagonist BMS-204493, has no effect on Stra8 expression in *ex vivo* cultured mouse fetal testis further proves the role of RA for Stra8 induction (Koubouva *et al.* 2006). This indicates that the ketoconazole effect was due to Cyp26b1-inhibition.

6.2.3. Bisphenol A

In mice, neonatal exposure to bisphenol A has been shown to cause a decrease in sperm number and damage to sperm motility and morphology in adult mice.. The effect was, to some extent, ameliorated by vitamin A supplementation and aggravated under vitamin A-deficient conditions (Nakahashi *et al.* 2001, Aikawa *et al.* 2004). The authors suggested that the development and functional differentiation of the reproductive tract and the gonads may be controlled by a balance between levels of estrogen and retinoids.

In mouse embryonic stem cells, bisphenol A was found to upregulate the expression of *Stra8* (amongst other genes) in a manner that appeared to be consistent with a feminizing effect, but bisphenol A seemed to act *via* a non-RA and non-RAR mediated mechanism (Aoki *et al.* 2012).

6.2.4. Organotin compounds

Triorganotins are considered endocrine-disrupting compounds and have been shown to bind RXR. Macejova and co-workers reviewed effects of triorganotin compounds on male reproductive organs in rats, but it was unclear if these effects were the result of a disrupted retinoid pathway (reviewed in Macejova *et al.* 2016). Several studies have shown that organotins act via PPARγ/RXR heterodimers (see *e.g.* Kanyama *et al.* 2005, reviewed in Grün and Blumberg 2006). However, since RXR is a silent partner in the PPARγ/RXR heterodimer (Mangelsdorf and Evans 1995), involvement of the retinoid pathway is uncertain.

6.2.5. Phthalates

Some phthalate esters are anti-androgenic, and thus considered potential causing agents in the "testicular dysgenesis syndrome" (Skakkebæck *et al.* 2001). These compounds have now been shown to also interfere with RA synthesis, both *in vitro* (Chen and Reese 2016) and *ex vivo* (Spade *et al.* 2019b). It has been suggested that a dual mechanism of both anti-androgen and retinoid disruption may lead to developmental effects in humans and rodents (Chen and Reese 2016).

6.2.6. Hexachlorocyclohexane (the gamma isomer is known under the name of lindane)

Lindane administration caused atrophy of the epididymidis and seminal vesicles, along with decreased sperm count in the epididymis and reduced activities of steroidogenic enzymes, in VAD rats but not in rats given a diet with a sufficient amount of retinoids (Pius *et al.* 1990).

6.2.7. BMS-189453

BMS-189453 is a synthetic RARα-, β- and γ-antagonist. Following oral administration to rats and rabbits for up to one month, BMS-189453 caused testicular degeneration and atrophy (Schulze *et al.* 2001). In later studies using lower doses, this compound was shown to reversibly inhibit spermatogenesis in rats, without other adverse testicular effects (Chung *et al.* 2016).

6.2.8. WIN 18,446

While the BMS synthetic retinoid above inhibits RA signaling, WIN 18,466 (a bisdichloroacetyl diamine) acts by lowering local RA concentration *via* inhibition of RALDH2 (Kogan *et al.* 2014). WIN 18,466 administration to neonatal mice caused meiotic defects in spermatocytes (Kent *et al.* 2016). In WIN 18,466-treated rodents, the progression of progenitor cells from A spermatogonia into A_1 spermatogonia is blocked (Griswold and Hogarth 2018).

6.2.9. R115866

In an *in vitro* dog testis model, treatment with the triazole CYP26 inhibitor R115866, caused an upregulation of *e.g. Stra8* both at the mRNA and protein level (Kasimanickam and Kasimanickam 2014). Increased *Stra8* expression has also been observed in *ex vivo* cultured mouse fetal testes treated with R115866 (Bowles *et al.* 2006).

6.2.10. Ro 23-2895

High doses of the synthetic retinoid Ro 23-2895, presumably a RAR agonist, administered to rats caused testicular degeneration, decreased testicular weight, delayed sperm release and retention, and disorganization/desquamination of the tubular epithelium accompanied by reduced numbers of mature elongated spermatids (Bosakowski *et al.* 1991). These effects resemble those caused by vitamin A deficiency. In a parallel study, plasma and testis ROH levels were lower compared to controls, suggesting that Ro 23-2895 caused testicular degeneration by interfering with normal retinoid homeostasis (Bosakowski *et al.* 1991).

6.2.11. Isotretinoin/13-cis-RA

Results from a pilot study suggest that treatment with 13-*cis*-RA (used for *e.g.* acne treatment) can increase sperm production (Amory *et al.* 2017). The mechanism behind this possible effect is not known, but it can be noted that reduced testicular concentrations of 13-*cis*-RA have been observed in men with abnormal sperm production (Nya-Ngatchou *et al.* 2013).

7. Potential adverse outcome pathways in female and male reproduction

Adverse Outcome Pathways (AOPs) form a framework for organizing data on the relationships between a molecular initiating event (MIE) induced by the interaction of a stressor (*i.e.* a chemical) with a molecular target and the resulting sequence of key events (KE), leading to an adverse outcome (AO). AOPs can establish a rationale for the use of particular assays or *in vivo* endpoints, and/or highlight the need for developing assays or exploring existing test guidelines to cover one or more components of an AOP.

Currently, there are no AOPs focusing on the disruption of the retinoid pathway published in the OECD series on Adverse Outcome Pathways[12]. In the AOP Wiki database[13] one AOP (Id 297) is under development, linking RALDH inhibition with visual impairment in fish (*not open for citing*). Additional AOPs (AOP Id 37, 107, and 149) mention either "retinoic" or "retinoid". Retinoid-relevant KE in these AOPs include "retinaldehyde dehydrogenase inhibition", "retinoic acid (RA) synthesis decreased", "plasma RA levels decreased". In addition, the Belgian SPF Santé Publique Sécurité de la Chaîne Alimentaire et Environnement recently published a call for tender to develop an AOP for inhibition of retinol dehydrogenase leading to urogenital and cardiovascular malformations.[14] In the scientific literature, several attempts to build AOPs and other frameworks to understand RA-dependent effects on embryogenesis have been published (Tonk *et al.* 2015, Baker *et al.* 2018, Battistoni *et al.* 2019, Di Renzo *et al.* 2019, Piersma *et al.* 2019[15]), with disruption of CYP26 enzymes and RALDH2 suggested to be important KEs. Previous efforts have not focused specifically on the reproductive system *per se*, even though embryo development of several other organ systems is discussed.

Since there is substantial cross-talk between the retinoid system and other nuclear hormone systems (see section 4), there are additional AOPs that may be involved with retinoid pathway effects on reproduction. For example, activation of PPARα may impair steroidogenesis, and could lead to impaired fertility in males (AOP18[16]). RXR is a PPAR heterodimer partner (see section on cross-talk), and it is possible that RXR may be involved in this AOP.

The following illustrations are not complete AOPs, neither in terms of how they are constructed, whether or not KEs are measurable, nor in terms of the amount or type

12. https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x
13. https://aopwiki.org/aops (accessed in September 2019).
14. https://enot.publicprocurement.be/enot-war/preViewNotice.do?noticeId=356068&saveSearchParams=true&useWorkingOrganisationId=%66%61%6C%73%65&allLanguages=%66%61%6C%73%65&selectAllChildren=%74%72%75%65&isPopup=&advancedSearch=&publicationDateBDATo=&publicationNumberBDA=&versionReferenceNumber=&tenderSubmissionDeadline=&title=&marketPlaceType=%65%4D%61%72%6B%65%74&publicationDateBDAFrom=%31%35%2F%31%30%2F%32%30%31%39¬iceStatus=%31&purchaseAuthority=&
15. Abstract SOT 2019: https://cfpub.epa.gov/si/si_public_record_Report.cfm?dirEntryId=345956&Lab=NCCT
16. https://aopwiki.org/aops/18

of data supporting the AOPs. Rather, they are attempts to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity, as covered in this report. Hopefully, these illustrations can be a starting point for future efforts to develop AOPs in this area.

7.1. Proposed AOP for meiosis initiation, oogenesis, folliculogenesis and female fertility, focusing on Stra8

The role for RA for meiosis initiation, oogenesis and folliculogenesis in the female fetus is discussed in Chapter 6 and 7. In female fetal offspring of VAD rats, a delay or failure of meiosis initiation was observed in oogonia (Li and Clagett-Dame 2009). Although RA levels were not measured, the low Stra8 expression suggest lower than normal RA levels in the ovaries. The critical role of Stra8 in meiosis, oogenesis and follicular development is also evident in Stra8$^{-/-}$ female mice, which are infertile and have smaller ovaries with no oocytes or follicles, while heterozygotes are fertile (Baltus *et al.* 2006). Since Stra8 expression is regulated by RA, it is plausible that lack of Stra8 expression could be caused by insufficient fetal ovarian RA levels, which in turn could be the result of a decrease in the synthesis of RA (see Figure 13 below); alternatively, RAR antagonists could lead to the same effect without affecting RA levels. Animal experiments with bisphenol A and DEHP have connected lack of increased Stra8 expression with impaired female fertility; however, the effects of bisphenol A (Zhang *et al.* 2012) and DEHP (Zhang *et al.* 2015) appear to by-pass the retinoid system and instead affect Stra8 expression via epigenetic mechanisms.

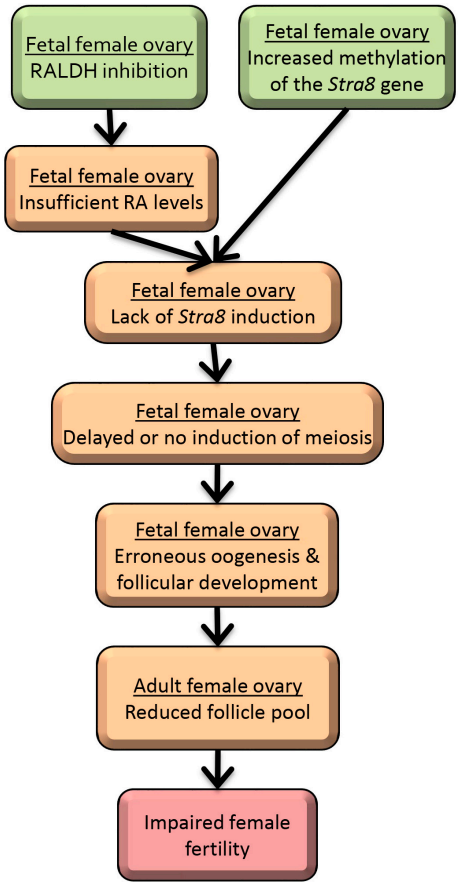

Figure 13: Proposed AOP for absence of ovarian Stra8 induction in utero possibly leading to impaired female fertility

Note:
The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity.

7.2. Proposed AOP for endometriosis in the adult female, focusing on CYP26A1

In endometrial tissue, RA appears to inhibit the decidualization of stromal cells, which is a prerequisite for blastocyst implantation after fertilization; thus, reduced concentration of RA in the endometrial tissue seems to be necessary for successful implantation (Deng *et al.* 2003). As discussed in Chapter 7, CYP26A1 catabolizes endometrial RA, and its expression normally increases during the endometrial secretory phase (lowering the RA levels) when compared to the proliferative phase, during which RA-synthesizing enzymes such as RALDHs are increased (Deng *et al.* 2003).

When compared to healthy women, expression of CYP26A1 is down-regulated in both the secretory and proliferative phases in endometrial biopsies from women with moderate or severe endometriosis (Burney *et al.* 2007). The availability of RA may therefore be increased in endometrial tissue of women with endometriosis (see Figure 14).

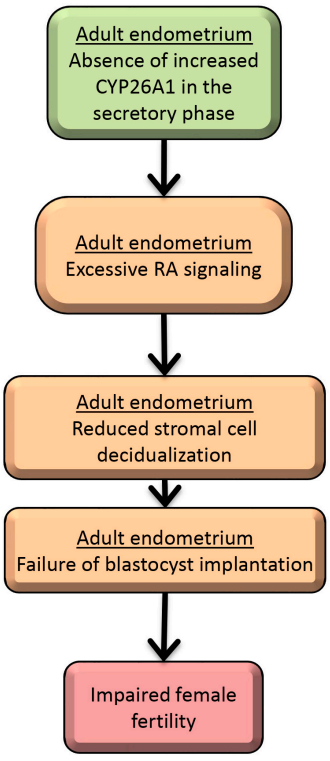

Figure 14: Proposed AOP for lack of CYP26A1 increase in the adult human endometrium possibly leading to impaired female fertility

Note:
The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity.

7.3. Proposed AOP for *in utero* CYP26B1 inhibition effects on male fertility

As described in Chapters 5 and 6, temporally and spatially regulated RA concentrations in the fetal gonads appear to play a role both in the proper development of the testis and in the differentiation of gonocytes into either oogonia or spermatogonia. In the fetal testis, perturbed RA catabolism caused by inhibition of Cyp26b1 (putative MIE) could disrupt the normal RA signaling pathway, which would lead to the AO impaired male fertility (See Figure 15). Disturbed RA-signalling has been observed after exposure of adult mice to chemicals acting as RAR antagonists (Schulze *et al.* 2001) and it is reasonable to assume that RAR agonists would also disturb RA signaling.

Following inhibition of the Cyp26b1 enzyme or complete knockout of the *cyp26b1* gene in the male mouse fetus, aberrant meiotic and apoptotic germ cells are observed in the testis (Bowles *et al.* 2006, MacLean *et al.* 2007, Teletin *et al.* 2017), and McLean and co-workers observed that virtually no germ cells are present in testes from neonatal pups (McLean *et al.* 2007). It has also been shown that ketoconazole can act, at least in an *ex vivo* mouse model, to inhibit Cyp26b1 activity and thereby induce abberant meiotic entry in fetal testes (Bowles *et al.* 2006). *In vitro*, Cyp26 inhibition prevents the normal mitotic arrest of the male germ cells and induces apoptosis (Teletin *et al.* 2017).

Male *cyp26b1*$^{-/-}$ homozygote 13.5 dpc mouse embryos display a mild ovotestis phenotype, with an "ovarian component" at the anterior end of the gonad which is where RA levels are expected to be higher due to the connection to the mesonephric tubules at this end (Bowles *et al.* 2018). In addition, abnormal development of the Leydig cells and of the Müllerian and Wolffian ducts was observed in the same male mouse embryos (Bowles *et al.* 2018). Thus, it is possible that the observed effects on the germ cells and on testis development may potentially lead to adverse effects on spermatogenesis and malfunctioning testes, which would eventually lead to impaired male fertility (see Figure 15 below).

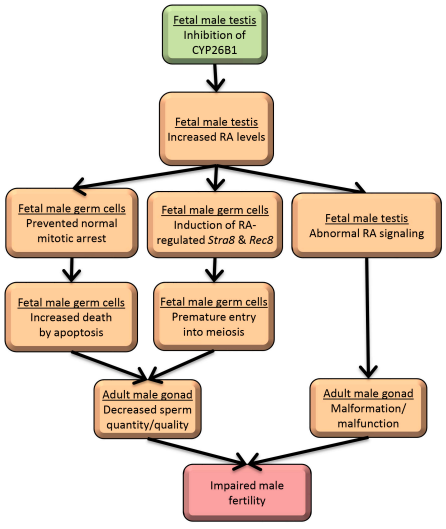

Figure 15: Proposed AOP for how Cyp26b1 inhibition in the fetal mouse testis may lead to impaired male infertility

Note:
Studies looking at Cyp26b1 inhibition were based on gene deletion or chemical inhibition of the enzyme. The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity.

7.4. Proposed AOP for effects of RALDH inhibition on male fertility

As described in Chapter 8, a tight regulation by RA-synthesizing (Raldh) and RA-catabolizing (Cyp26) (putative MIEs) is required for maintaining spermatogenesis, implying that chemicals that affect these enzymes may cause adverse effects on the spermatogenic process, leading to the AO impaired male fertility (see Figure 16 below). It is conceivable that exposure to chemicals acting as RAR antagonists could have the same effect; in such cases, altering of endogenous RA levels would not be needed.

At least in the mouse, RA is also required to disengage spermatozoa from the Sertoli cell cytoplasm during spermiation (Spiller and Bowles 2015, Teletin *et al.* 2017). It seems possible that increased RA testis levels *via* chemically-induced CYP26B1 inhibition (putative MIE) could lead to similar adverse effects on spermatogenesis.

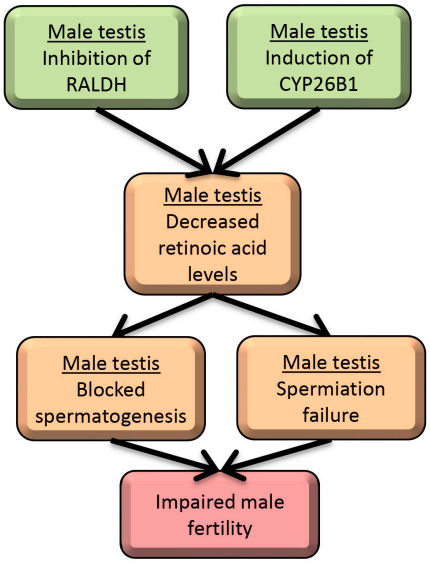

Figure 16: Proposed AOP for how Raldh inhibition in the adult mouse testis may lead to impaired male infertility

Note:
Studies looking at Raldh-inhibition were based on gene deletion, rather than chemical inhibition of the enzymes. The proposed AOP is an attempt to visualize possible pathways between effects on retinoid homeostasis and reproductive adversity.

8. Initial scoping effort: assays and endpoints for effects of chemicals on female and male reproduction *via* the retinoid system

Several existing OECD test guidelines can provide information on endpoints relevant to the estrogen, androgen, steroidogenesis, and thyroid hormone pathways, but currently, no OECD test guidelines include endpoints specifically indicative of retinoid system modulation. Some reproductive parameters already included in existing test guidelines, would also provide information on adverse effects stemming from retinoid disruption. However, due to extensive cross-talk with other pathways, there is no endpoint identified that will give information specifically on disruption of retinoid signaling. The information assembled in this report regarding the role of retinoids in female and male reproduction indicates possible *in vitro*/*ex vivo* assays that could associate adverse reproductive outcomes to disruption of retinoid signaling.

Some of the proposed assays are suitable for screening larger numbers of chemicals (*e.g. in silico*/*in vitro*, OECD Conceptual Framework [CF] levels 1, 2), while *in vivo* test methods address more complex mechanistic and organ system effects (CF level 3, 4, 5). One other option could be to include *ex vivo* models.

8.1. *In silico* methods

In silico methods, such as molecular docking models and quantitative structure-activity relationship (QSAR) models for binding to *e.g.* CYP26, RALDH, RAR and RXR, could, if available, be integrated into CF Level 1. Also, homology modeling and computational docking simulations for CYP26A1 and CYP26B1 have been performed to identify inhibitors for these enzymes (Foti *et al.* 2016). Several studies describe QSAR models for binding to and activating PXR (an RXRα-heterodimerizing partner) and CYP3A4 (Rosenberg *et al.* 2017b), thyroperoxidase (TPO; Rosenberg *et al.* 2017a) and AhR (Klimenko *et al.* 2019). Similar models for RARs and RXRs would be valuable, and should be developed.

8.2. *In vitro* assays and *ex vivo* methods

Temporal and spatial regulation of local availability of RA is critical for normal reproductive development. Thus, in addition to nuclear receptor activation assays, assays measuring expression and/or activity of the enzymes involved in the metabolism of RA can be considered as candidate *in vitro* assays (Piersma *et al.*

2017; see also section 10).

Considering the complex RA modulation of both female and male reproduction, a panel of *in vitro* assays with the following endpoints is suggested:

- CYP26A1, CYP26B1 (induction, inhibition);
- RALDH (induction, inhibition);
- STRA8 (induction);
- RAR/RXR transactivation.

The proposed top candidate enzyme for fetal exposure to chemicals is the RA-catalyzing **CYP26B1** enzyme. Inhibition of CYP26B1 in the fetal testis (leading to increased RA levels) would cause serious adverse effects on normal development of testicular somatic and germ cells, while induction of the CYP26B1 enzyme in the female fetus could potentially lead to decreased RA tissue levels and subsequent adverse effects on initiation of germ cell meiosis. For evaluating adult females, **CYP26A1** might be the more relevant enzyme, considering its role in the endometrium.

Inhibition of the RA-synthesizing **RALDH** enzymes could decrease RA levels, and assays measuring RALDH inhibition may therefore be considered for screening purposes. In the postnatal male, RALDH inhibition and the subsequent decrease in RA levels would interfere with spermatogenesis.

Stra8 is regulated by RA, and is expressed only in germ cells (Oulad-Abdelghani *et al.* 1996, Mark *et al.* 2008). *Stra8* is required for germ cells to enter meiosis (Baltus *et al.* 2006). Therefore, an *in vitro* screening assay for *Stra8* expression could be used to identify substances that, through disturbed RA signaling, affect meiosis initiation.

The different **RAR** and **RXR** transcription factors mediate the retinoid signal when activated by RA or 9-*cis*-RA RXRs can also act as a silent partner, *i.e.* no RXR ligand is necessary for RXR to bind and activate genes. Activation or inactivation of these transcription factors by chemicals could lead to different effects on the cellular level, which may be translated to effects on tissue/organ level.

For any *in vitro* assay, the choice of cellular system is important in terms of both relevance and technical considerations. Ovarian stem cell test systems have been described, in which pre-meiotic oogonia in the post-natal ovary are used (Bhartiya and Patel 2018). However, the existence of such cells is highly controversial (Frydman *et al.* 2017, Wagner *et al.* 2019). For screening large numbers of chemicals, gonadal cell or tissue models (preferably of human origin) would probably be necessary. Given the sex-specific effects of RA pathways, using two model systems in parallel (ovarian/follicular and testicular) is recommended. Any *in vitro* model should be characterized in terms of expression and function of *e.g.* CYP26, RoDH2, and RALDH.

Ex vivo model systems consisting of rodent follicular cells or whole rodent neonatal ovaries (Hannon and Flaws 2015) could generate valuable information on chemical disruption of the possible association between RA and the formation and maturation of follicles (Minkina *et al.* 2017, Damdimopoulou *et al.* 2019). Pre-meoitic germ cells, which can be obtained from fetal rodent ovaries and cultured *in vitro* (Sun *et al.* 2010, Paczkowski *et al.* 2012) can possibly provide information on chemical effects on RA-mediated meiotic induction. Both human and rat fetal testis

cultures have been used to study the effects of hormone disrupting chemicals on the development of human fetal testis (Lambrot *et al.* 2006, Spade *et al.* 2019b).

8.3. *In vivo* endpoints

Detection of of *e.g.* CYP26, RoDH2, RALDH, and Stra8 expression using immunohistochemistry or *in situ* hybridization could be performed on tissues from *in vivo* studies, for obtaining information on the mechanistic level.

Retinoid pathway signaling is highly variable by life stage, tissue type, and sex. Additionally, in animal studies, measuring retinoids in different tissues (including serum) is problematic in many aspects since the polyene chain of retinoids make them vulnerable to light, oxidation, acids and heat (reviewed in Gundersen 2006). In addition, the existence of more relevant endpoints such as the histopathological endpoints mentioned below, and methods that indirectly informs of retinoid levels (*e.g.*, mRNA expression of RARE-controlled genes such as *Stra8*) makes measurements of tissue retinoid levels a lower priority.

The detailed histopathological examination of reproductive organs/organ structures that are part of animal studies performed according to OECD reproductive development guideline studies could add valuable information when evaluating a possible retinoid-disrupting impact of the test chemical on these organs (Table 3 and 4; CF Level 3 or 4). In TGs 421 and 422, evaluating the value of addition of ovary histopathology of pups, could be valuable. Although it is not an endpoint specific for retinoids, retinoids are important for female meiosis, and could thus consequently also affect the size of the ovarian reserve. This is currently a data gap in these screening studies. Serum levels of AMH in the adult female as a proxy for follicle counting, could potentially be evaluated, although it currently appears unclear if serum AMH levels reflect fertility status in women (see *e.g.* Kahn *et al.* 2019). In addition, measurements of male and female steroid serum levels could potentially add value considering the extensive RXR cross-talk (Jacobs 2005). In females, examined organs should include ovaries, as well as uterus and vagina. In males, parameters such as sperm number and sperm quality, cryptorchidism and the presence of PGC in the adult testis is relevant.

Table 3: Current OECD TG endpoints which might capture possible retinoid effects on mammalian female reproductive health, at CF level 4 and 5

Retinoid effects	OECD Test Guidelines (TG)*	Comments
Reproductive development (female rodents)		
Oogenesis, follicular count	Extended one-generation reproductive toxicity study (TG 443); 2-Generation reproduction toxicity study (TG 416).	TG 443: Quantitative (most sensitive). Histopathological examination should be aimed at detecting a quantitative evaluation of primordial and small growing follicles, as well as corpora lutea, in F1 females. TG 416: only qualitative (limited sensitivity). A quantitative evaluation of primordial follicles should be conducted for F1 females.
Oestrus cycles	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Vaginal smears should be examined daily for all F1 females in cohort 1A, after the onset of vaginal patency, until the first cornified smear is recorded, in order to determine the time interval between these two events. Oestrous cycles for all F1 females in cohort 1A should also be monitored for a period of two weeks, commencing around PND 75. TG 421/422: Not included in the offspring and also not possible due to, termination of the offspring on PND 14, i.e. before puberty. TG 416: Oestrous cycle length and normality are evaluated in F1 females by vaginal smears prior to mating, and optionally during mating, until evidence of mating is found.
Vagina, uterus with cervix, and ovaries	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Uterus (with oviducts and cervix), ovaries will be weighed (F1). Full histopathology is performed for all high-dose and control F1 animals. All litters should be represented by at least 1 pup per sex. Organs and tissues demonstrating treatment-related changes and all gross lesions should also be examined in all animals in the lower dose groups to aid in determining a NOAEL. TG 421/422: Not included and not possible due to termination on PND 14 (F1).

		TG 416: Vagina, uterus with cervix, and ovaries (preserved for histopathology (parental F1 animals) determining a NOAEL. TG 421/422: Not included and not possible due to termination on PND 14, although follicular counts can be made at this age (F1). TG 416: Vagina, uterus with cervix, and ovaries (preserved for histopathology (parental F1 animals).
Adult exposure (female rodents)		
Vagina, uterus with cervix and ovaries (histopathology)	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Uterus (with oviducts and cervix), ovaries will be weighed. Full histopathology is performed for all high- dose and control P animals. Organs demonstrating treatment-related changes should also be examined in all animals at the lower dose groups to aid in determining a NOAEL. Additionally, reproductive organs of all animals suspected of reduced fertility should be subjected to histopathological evaluation. TG 421/422: optional: paired ovaries (wet weight) and uterus (including cervix) in females (P). TG 416: Examination of the ovaries of the P animals is optional.
Oestrus cycles	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG 443: Normally the assessment of oestrous cyclicity (by vaginal cytology) will start at the beginning of the treatment period and continue until confirmation of mating or the end of the 2-week mating period. TGs 421/422: Oestrous cycles should be monitored before treatment to select study females with regular cyclicity. Vaginal smears should also be monitored daily from the beginning of the treatment period until evidence of mating. TG 416: Oestrous cycle length and normality are evaluated in P females by vaginal smears prior to mating, and optionally during mating, until evidence of mating

is found.

Note:
Also indicated are limitations and data gaps (adapted from the draft DRP)
** All OECD test guidelines are available via https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788*

Table 4: Current OECD TG endpoints which might capture possible retinoid effects on mammalian male reproductive health, at CF level 4 and 5

Retinoid effects	OECD Test Guidelines (TG) *	Comments
Reproductive development (male rodents)		
Hypospadias, dysgenesis of external reproductive organs, cryptorchidism	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update); Prenatal Developmental Toxicity Study (TG 414).	TG443: Most sensitive design due to 20 litters per group, 2 or more males per litter and termination after puberty. TG 421/422: Limited sensitivity due to limited group size (n~8-10) and termination before puberty (hypospadia should be detectable at birth). TG416: Sensitive design due to 20 litters per group and termination after puberty, but only 1 male per litter decreases the sensitivity compared to TG443. TG 414: Sensitive design due to 20 litters per group, but limited/ unknown sensitivity as the offspring is terminated before birth (although e.g. hypospadia should still be detectable).
Testes development (weight and histopathology)	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG443: Most sensitive design due to 20 litters per group and 2 adult male offspring per litter. TG 421/422: Not included but could be assessed at termination on PND 14. Limited sensitivity due to limited group size (n~8-10) and termination well before puberty. TG416: Sensitive design due to 20 litters per group and termination after puberty, but only 1 male per litter decreases the sensitivity compared to TG443.
Spermatogenesis (sperm quality and testicular histology)	Extended one-generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update).	TG443: sensitive design due to 20 litters per group, 1 adult male offspring per litter examined. TG 421/422: Not included and not possible due to termination on PND 14. TG416: Sensitive design due to 20 litters per group, 1 adult male offspring per litter examined (only included in the most recent update in 2001).
Adult exposure (male rodents)		

Spermatogenesis (sperm quality, testis weight and testicular histopathology)	Extended one- generation reproductive toxicity study (TG 443); Reproductive screening test (TG 421); Combined 28- day/ reproductive screening assay (TG 422); 2-Generation reproduction toxicity study (TG 416 most recent update);, Repeated Dose 90-day Oral Toxicity Study in Rodents (TG 408); Repeated Dose 28-Day Oral Toxicity Study in Rodents (TG 407).	TG 443: Sperm parameters assessed in around 20 parental males unless there is existing data to show that sperm parameters are unaffected in a 90-day study. TG 421/422: Limited sensitivity due to limited group size (n~8-10) and assessment of sperm quality is not required; TG416: Sperm parameters assessed in around 20 parental males (sperm quality only included in the most recent update in 2001). TG 408, 90-day study: Limited sensitivity due to limited group size (n~10) and assessment of sperm quality is not required. TG407, 28 day study: Very limited sensitivity due to limited group size (n~5), assessment of epididymal sperm parameters are optional.

Note:
Also indicated are limitations and data gaps (adapted from the draft DRP).
** All OECD test guidelines are available via https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788*

9. Concluding remarks

Potential endpoints for studying effects on female and male reproduction caused by chemical exposure, possibly affecting the retinoid pathway, have been identified and are described in this report.

In silico methods, such as QSAR and molecular docking models for *e.g.* CYP26, RALDH, RAR, RXR could be integrated into OECD CF Level 1. A small number of *in silico* tools for retinoid signaling pathways (all focusing on CYP26) have been developed (Battistoni *et al.* 2019, Foti *et al.* 2016). Such tools exist for other signaling pathways (Rosenberg *et al.* 2017a, Rosenberg *et al.* 2017b, Klimenko *et al.* 2019), which suggests that they could be developed further also for the retinoid pathway. It should be recognized that large amounts of data are needed to build QSAR models.

Some *in vitro* assays are available (See Chapter 14) that measure retinoid-relevant endpoints such as RAR/RXR activation. Other assays (*e.g.* CYP26, Stra8, RALDH) that might be suitable for integration into OECD CF Level 2 remains to be developed (see section 14 for more information). The CYP26 and RALDH isomers appear to be the best candidates at present, since they play a critical role in regulating RA concentrations in several reproductive tissues in both males and females. RAR antagonists and agonists could lead to the same effects as altered RA concentrations. CYP26 isomer expression and activity appears to be well studied in many laboratories, suggesting that these assays might be mature enough for further development. Assays focusing on steroidogenesis should also be considered. However, a more comprehensive analysis has to be performed in order to identify the most suitable candidate assay. Recent development of models such as the human female reproductive tract-on-a-chip (Xiao *et al.* 2017) needs to be evaluated for possible regulatory use in the future.

Regarding current mammalian *in vivo* OECD TG studies, no endpoint specifically relevant for retinoid-disruption have been identified that could be added to already existing test methods (such as TG 443 which has the most sensitive design). One exception could be evaluating the value of addition of ovary histopathology of pups in OECD TGs 421/422, although not an endpoint specific for retinoids, retinoids are important for meiosis, and this could consequently also affect the size of the ovarian reserve. This is currently a datagap in these screening test guidelines. Serum levels of AMH in adult females, as a proxy for follicle counting, could potentially also be evaluated, although it is currently unclear if serum AMH levels reflect the fertility status in women. Certain already existing endpoints (in studies covering sensitive windows; see Table 1) could be informative regarding retinoid disruption. For females, ovaries uterus and vagina are of particular interest, and for males, testes and sperm parameters. Serum level measuments of steroids could add value considering the extensive RXR cross-talk. However, no endpoints that would provide information specifically on retinoid disruption have been identified, since the control of male and female reproduction involves many other endocrine pathways.

The initial scoping effort in this report highlights that there are several challenges before *in silico* or *in vitro* screening assays, for identifying retinoid disruption, could

be added to the test guidelines programme, or *in vivo* endpoints added to already existing test guidelines:

- Due to extensive cross-talk, it is difficult to distinguish between adverse fertility effects caused specifically by retinoid disruption, from effects caused by disruption of other endocrine signaling pathways important for proper reproduction;

- More research is needed to not only to clarify the role of RAR- or RXR-mediated influence on cross-talk in retinoid homeostasis and its role in normal reproduction, but also to further clarify the spatial and temporal control of RA availability in reproductive tissues;

- The scientific approach used in the reviewed publications did not evaluate retinoid homeostasis (in relevant organs) concomitantly with recording adverse effects on reproduction. This is a major data gap. Consequently, no suitable reference chemical, known to affect reproduction specifically *via* the retinoid pathway, has been identified (except a very small number of specific RA-disrupting compounds originating from development of pharmaceuticals). Thus, validation of potential endpoints and assays described herein will be challenging.

Finally, the pathway visualizations in the proposed AOPs of this report point to similar early events, *i.e.*, dysregulated RA levels in target reproductive tissues. RA levels are controlled by a set of enzymes under physiological conditions, but it should be recognized that other enzymes capable of affecting RA levels could become relevant after exposure to chemicals. While it is currently unclear which degree of RA dysregulation that is necessary for adverse effects to occur, the suggestion of these KEs can be useful for guiding future work. Currently, the level of knowledge on cross-talk in RA-signaling is limited, and thus RAR/RXR activation was not included in the proposed AOPs in the present report. When the understanding of cross-talk in RA-signaling has matured, RAR/RXR activation should be taken into account when AOPs are developed.

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Appendices

A1. Assay methods and availability

RA levels

Can be measured in tissues (organs, blood) or cells with *e.g.* liquid chromatography methods coupled to ultraviolet or mass spectrometric detection (a multitude of publications reviewed in the D-DRP).

Can also be measured with ELISA kits (available for several species).

https://www.mybiosource.com

CYP 26

The different isomers can be studied on mRNA and protein level using commercially available probes and antibodies (see *e.g.* Topletz *et al.* 2012).

ELISA kits for CYP26A1, B1 and C1 for several species are available.

https://www.mybiosource.com

Enzyme activity can be measured in microsomes as conversion of RA to its metabolites (Thatcher *et al.* 2010 analyzed human liver microsomes).

RDH 10

ELISA kits for several species are commercially available.

https://www.mybiosource.com

Stra8

mRNA and protein levels can be measured (Mark *et al.* 2008).

cDNA and antibodies for multiple species are commercially available, as are ELISA kits.

https://www.mybiosource.com

https://www.labome.com

https://www.biocompare.com

RARs, RXRs

Several RAR/RXR activation assays are commercially available, many use HepG2 cells:

http://alttox.org/application-of-medium-and-high-throughput-screening-for-in-vitro-toxicology-in-the-pharmaceutical-industry/

http://www.attagene.com (used in ToxCast™).

https://ncats.nih.gov/tox21/projects/assays

AhR

AhR mRNA induction or activation can be measured in *e.g.* human HepG2 cells; commercially available.

http://www.attagene.com (used in ToxCast™).

https://ncats.nih.gov/tox21/projects/assays (Tox21 used in ToxCast™).

CYP1A1

CYP1A1 activity can be measured in microsomes.

A multitude of assays (mRNA, protein, activity) are commercially available.

A description of a cell-based method (using mouse pluripotent P19 cells) for identifying chemicals that disrupt RA signaling pathways was published by Chen and Reese in 2013. It has been used to test phthalate esters (Chen and Reese 2016).

A2. PubMed search strategy – retinoid parameters, chemical names, female/male reproduction parameters

Retinoid parameters were combined with chemical names (see Table A, below) and with either female or male reproduction search terms (see Tables B and C, below). No cut-offs (for *e.g.* year of publication) were used. The searches yielded several hundred hits, however, only a small percentage was deemed relevant. In fact, a larger number of relevant articles was found via non-structured searches (using *e.g.* references in articles, citing articles, etc).

Retinoid parameters: (retinoic OR retinoid OR retinol OR retinyl OR retinal). Inclusion of the term "vitamin A" rendered the exact same number of hits as when this term was excluded.

The selected chemicals are a combination of a) chemicals handpicked by Nancy Baker and co-workers at EPA, from ToxCast™ and other sources; b) ToxCast™ chemicals with activity on RARs/RXRs and CYP1A1 (information found in tables in the draft-DRP); c) CYP26 inhibitors found via text mining (information found in table in the draft-DRP); and finally, d) chemicals classified as Persistent Organic Pollutants by the Stockholm Convention.

Table A. Search terms for chemicals
"ser 2–7" (methyl 3-(1H-imidazol-1-yl)-2,2-dimethyl-3-(4-(naphthalen-2-ylamino)phenyl)propanoate
1-(6-tert-Butyl-1,1-dimethyl-2,3-dihydro-1H-inden-4-yl)ethanone
1,2,3-Trichlorobenzene
1,3,5-Triisopropylbenzene
2,2-dimethyl-3-(4-(naphthalen-2- ylamino)phenyl)-3-(1,2,4)triazol- 1-yl-propionic acid methyl ester
2,4,5-Trichlorophenol

2,4,6-Tris(tert-butyl)phenol

2,4-Bis(1-methyl-1-phenylethyl)phenol

2,6-Di-tert-butyl-4-ethylphenol

2,6-Di-tert-butyl-4-methoxyphenol

2-Mercaptobenzothiazole

2-Naphthylamine

3-(6-(2-dimethylamino-1- imidazol-1-yl-butyl)naphthalen-2- yloxy)-2,2-dimethyl-propionic acid

4,4'-Sulfonylbis[2-(prop-2-en-1-yl)phenol]

4-Nonylphenol

4-Nonylphenol, branched

4'-octyl-4-biphenylcarboxylic acid

AC-41848 hydrate

Acitretin

Adapalene

Aldrin

all/trans-Retinoic acid

Alpha-hexachlorocyclohexane

AM580

AR7

Azinphos-methyl

Bensulide

Benzyl alcohol

Beta-hexachlorocyclohexane

Bifenazate

BMS 493

BMS-189453

BMS-195614

Butylated hydroxytoluene

CD437

Chlordane

Chlordecone

Chlorthal-dimethyl

Citric acid

Clorophene

Coumaphos

CP-532623

DDT OR Dichlorodiphenyltrichloroethane

Dieldrin

Diethylaminobenzaldehyde

Difenoconazole

Diniconazole

Dioxin OR dibenzofuran

Dodecyl gallate

Dysprosium(III) chloride

Econazole nitrate

Endosulfan

Endrin

EPN OR ethyl phenylphosphonothioate

Esfenvalerate

Fludioxonil

Gentian Violet

Heptachlor

Hexabromobiphenyl

Hexabromocyclododecane

Hexabromodiphenyl ether and heptabromodiphenyl ether

Hexabromodiphenyl ether OR heptabromodiphenyl ether

Hexachlorbenzene

Hexachlorobutadiene

Imazalil

Imidazole

Isoniazid

Isopentyl benzoate

LE 135

Liarozole

Lindane OR gamma-hexachlorocyclohexane

Mepanipyrim

Methoprene acid

methyl 3-(4-(6-bromopyridin-3- ylamino)phenyl)-3-(1H-imidazol- 1-yl)-2,2-dimethylpropanoate

Mirex

N,N-Dimethylformamide

Nicotine

N-Phenyl-1,4-benzenediamine

Organotin

Pentachlorobenzene

Pentachlorphenol
Perfluorooctanesulfonic acid OR perfluorooctanesulfonyl fluoride
Phosalone
Polychlorinated AND (naphtalene OR naphtalenes OR napthalene OR napthalenes)
Polychlorinated biphenyls OR PCBs
Polychlorinated AND (dibenzo-p-dioxins OR dibenzofurans)
Prochloraz
Pyraclostrobin
R115866 OR rambazole
R116010
SR271425
Symclosene
Talarozole
Tetrabromodiphenyl ether and pentabromodiphenyl ether
Tetrabromodiphenyl ether OR pentabromodiphenyl ether
Tetrabutyltin
Toxaphene
Tributyltin benzoate
Tributyltin chloride
Tributyltin methacrylate
Triflumizole
Triphenyltin hydroxide
Triticonazole
TTNPB

B. Search terms for female reproduction	C. Search terms for male reproduction
“female genitalia”	Aspermia
“female gonad”	Asthenozoospermia
“Female reproduction”	Azoospermia
“Granulosa cells”	Blood-Testis Barrier
“Polycystic ovarian syndrome”	Cryptorchidism
“Theca cells”	Ejaculation
Abortion	Epididymis
Cervix OR cervical	Epididymitis
Corpus Luteum	Erectile
Decidua OR decidualization	Fertilization

Eclampsia

Embryo OR embryonic

Endometriosis

Endometrium OR endometrial

Estrous

Fallopian

Fertility OR infertility

Fetus OR fetal

Folliculogenesis

Gestational

Lactation

Litter

Luteal

Maternal

Menopause

Menstrual OR menstruation

Oocyte

Oogenesis

Oogonia

Ovary OR Ovarian

Ovulation

Placenta OR placental

Pregnancy OR pregnant

Prenatal OR postnatal

Puberty

Trimester

Uterus OR uterine

Vagina OR vaginal

Hemospermia

Hypospadia

Impotence

Leydig

male infertility

Oligospermia

Orchitis

Penile

Prostate

Prostatic

Prostatitis

“Rete Testis”

Scrotum

Seminiferous

Sertoli

Sperm

Spermatid

Spermatocele

Spermatocyte

Spermatogenesis

Spermatogonia

Spermatozoa

Testes

Testicular

Testis

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