REVIEW

Potential ovarian toxicity and infertility risk following targeted anti-cancer therapies

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Abstract

Unlike traditional chemotherapy agents which are generally cytotoxic to all cells, targeted anti-cancer therapies are designed to specifically target proliferation mechanisms in cancer cells but spare normal cells, resulting in high potency and reduced toxicity. There has therefore been a rapid increase in their development and use in clinical settings, including in curative-intent treatment regimens. However, the targets of some of these drugs including kinases, epigenetic regulatory proteins, DNA damage repair enzymes and proteasomes, have fundamental roles in governing normal ovarian physiology. Inhibiting their action could have significant consequences for ovarian function, with potentially long-lasting adverse effects which persist after cessation of treatment, but there is limited evidence of their effects on reproductive function. In this review, we will use literature that examines these pathways to infer the potential toxicity of targeted anti-cancer drugs on the ovary.

Lay summary

Compared to traditional chemotherapy agents, anti-cancer therapies are thought to be highly effective at targeting cancer cells but sparing normal cells, resulting in reduced drug side effects. However, many of processes within the cells that these drugs affect are also important for the ovary to work normally, so suppressing them in this way could have long-lasting implications for female fertility. This review examines the potential toxicity of anti-cancer therapies on the ovary.

Keywords: ▶ fertility preservation ▶ ovary ▶ reproductive toxicology

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Introduction

More than 85% of young people diagnosed with cancer in the UK survive their disease beyond 5 years, thus the treatment of cancer is increasingly turning from focussing on survival to a recognition of the long-term effects of treatment on subsequent quality of life. These issues are extremely important to patients and all involved in their care. In a recent UK research priority setting initiative, research into the consequences of cancer and cancer treatments was rated as a top priority by patients, their families, and healthcare professionals; among late effects, the potential impact on fertility is a very high priority for patients (Peate *et al.* 2009, James Lind Alliance. http://www.jla.nihr.ac.uk/priority-setting-partnerships/teenage-and-young-adult-cancer/the-top-10-priorities.htm 2019).

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Despite this, reproductive toxicity is rarely assessed in contemporary curative-intent breast cancer clinical trials which enrolled premenopausal women (Cui *et al.* 2021), although there are calls for this to be made routine across all cancer diagnoses affecting reproductive age males and females (Anderson *et al.* 2021). Fertility preservation interventions are therefore increasingly widely used with anti-neoplastic treatments with known adverse effects on ovarian function, supported by international guidelines (Loren *et al.* 2013, Anderson *et al.* 2020, Lambertini *et al.* 2020), but are not currently recommended in patients receiving novel classes of anti-cancer treatments such as targeted therapies due to lack of knowledge of their impact on reproductive function.

For many years, the mainstays of cancer treatment have been surgery, radiotherapy, and chemotherapy, with cytotoxic chemotherapy being the main systemic approach to cancer drug therapy. Traditional chemotherapeutic agents generally take advantage of the relatively rapid proliferation of cancer cells and disrupt fundamental cellular processes required for cell division and proliferation. However, the broad cellular processes involved are common with normal cells, resulting in significant systemic toxicity and adverse side effects. In recent decades, advances in our understanding of the molecular pathways that drive the development and progression of human cancers have heralded a new age in cancer treatment, with the advent of targeted anti-cancer therapies. These targeted agents act to exclusively block the growth and survival of cancer cells by specifically targeting molecules and signalling cascades uniquely required for tumourigenesis, and thus, these therapies may have both better efficacy and less off-target adverse side effects when compared with their chemotherapy counterparts. However, this improved selectivity is relative rather than absolute, and anti-cancer agents also disrupt signalling transduction in normal cells. While detailed toxicity data are mandated in cancer clinical trials to identify treatment-emergent and treatment-related adverse effects, there remain little data on the impact of novel classes of anti-cancer therapies on gonadal function, and a recent review discussing the effect of chemotherapy treatment on the ovary highlighted that even within this field, there is a near complete absence of human data (Spears *et al.* 2019). In this review, we will use literature that examines the importance of these targeted pathways in normal ovarian physiology (as summarised in Fig. 1 and Table 1) to infer the potential toxicity of targeted anti-cancer drugs on the ovary and evaluate the few preclinical studies that have been published in this field. This review is not intended to be a comprehensive summary on the signalling pathways themselves, and where appropriate, we have directed the reader to articles that explore the signal transduction aspects more fully.

EGFR/HER inhibitors

The human EGF receptor (HER) family of membrane-bound receptor tyrosine kinases (RTKs) consists of four members: EGF receptor (EGFR) also known as HER1 or ErbB1, EGFR2 more commonly known as HER2 or ErbB2, HER3/ErbB3, and HER4/ErbB4 (Roskoski 2014). After binding of a ligand, receptor subunits dimerise, which leads to autophosphorylation of intracellular tyrosine residues and the creation of docking sites for numerous effector proteins, thereby generating multiple signal transduction cascades. These signalling cascades include the rat sarcoma virus (Ras)-rapidly accelerated fibrosarcoma (Raf)-mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) kinase (MEK) (Ras-Raf-MEK-ERK) pathway, the phosphatidyl-inositol 3 kinase (PI3K)

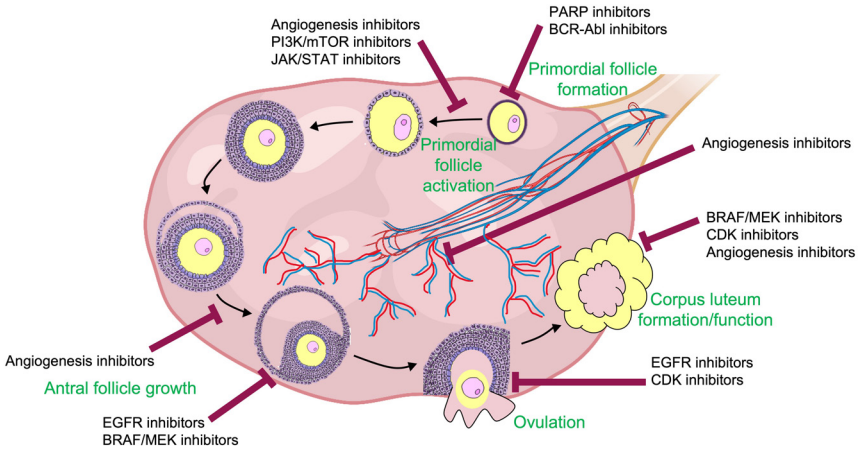

Figure 1 The potential effect of anti-cancer drugs on healthy ovarian function.

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Table 1 Summary of drug classes and the pathways they target, with examples and potential implications for ovarian function.

Class of drug	Examples	Targeted pathway	Relevant ovarian function(s)
EGFR/HER inhibitors	Erlotinib, gefitinib, osimertinib, lapatinib, afatinib, neratinib trastuzumab, pertuzumab, cetuximab	Receptor tyrosine kinases signalling through Ras/Raf/MEK/ERK, PI3K/Akt/mTOR, and JAK/STAT pathways; also direct nuclear translocation	Oocyte maturation, cumulus expansion, and ovulation
BRAF/MEK inhibitors	BRAF: vemurafenib, dabrafenib, encorafenib; MEK: trametinib, binimetinib, cobimetinib	Serine/threonine kinase signalling through RAS/RAF/MEK/ERK	Follicle growth, cumulus cell-oocyte complex expansion, oocyte maturation and luteinisation
PI3K/Akt/mTOR inhibitors	Sirolimus (rapamycin), temsirolimus, everolimus	PI3K/Akt/mTOR pathways regulate cell growth, motility, survival, metabolism, and apoptosis	Primordial follicle dormancy/growth activation, granulosa cell proliferation, follicle survival, possible chemoprotection
JAK/STAT inhibitors	Ruxolitinib, fedratinib, tofacitinib, baricitinib	Cytokine signal transduction through STAT phosphorylation; crosstalk with PI3K pathway	Primordial follicle growth activation, follicle survival
BCR-Abl	Imatinib, asciminib	MAPK, PI3K/Akt/mTOR, and JAK/STAT signalling	Primordial follicle survival/apoptosis, possible chemoprotection
CDK inhibitors	Palbociclib, ribociclib, abemaciclib	Cell cycle: transition from G1 to the S phase of the cell cycle	Ovulation, corpus luteum formation
PARP inhibitors	Olaparib, rucaparib, niraparib, and talazoparib	DNA repair including single-strand breaks, nucleotide excision repair, non-homologous end joining, homologous repair, and DNA mismatch repair	Direct damage and death of primordial follicle oocytes, granulosa cell dysfunction/follicle growth
Anti-angiogenesis	Bevacizumab ramucirumab, nintedanib, pazopanib, sorafenib	VEGF, FGF, PDGF receptor tyrosine kinase signalling	Possibly primordial follicle activation, antral follicle growth, corpus luteum function

pathway, and the signal transducer and activator of transcription (STAT) pathway, ultimately affecting a wide range of cellular processes (Oda *et al.* 2005, Wieduwilt & Moasser 2008). Independent of kinase-dependent signal transduction pathways, EGFR may also be internalised and translocated to the nucleus, where it participates in gene transcription and DNA-repair mechanisms (Lin *et al.* 2001, Dittmann *et al.* 2005).

A number of RTK inhibitors targeting HER are currently in routine clinical use. These include RTK inhibitors targeting EGFR (such as erlotinib, gefitinib, and osimertinib), RTK inhibitors with dual targets (such as lapatinib which targets both EGFR and HER2), and pan-HER RTK inhibitors (such as afatinib and neratinib). These drugs have been licensed for the treatment of non-small cell lung cancer (NSCLC) harbouring *EGFR* activating mutations (erlotinib, gefitinib, afatinib, and osimertinib), advanced breast cancer (lapatinib and neratinib), and advanced pancreatic cancer (erlotinib). HER is also targeted using MAB therapies, with compounds targeting the extracellular domain of EGFR (such as cetuximab) and HER2 (such as trastuzumab and pertuzumab) available for clinical use. Furthermore, the antibody–drug conjugate Ado-trastuzumab emtansine (T-DM1) utilises the targeted action of trastuzumab to selectively deliver cytotoxic DM-1 to HER2-expressing tumour cells and is widely used in both early-stage and advanced breast cancer. As 15–40% of NSCLC harbour an activating *EGFR* mutation (Zhang *et al.* 2016) and nearly 25% of breast tumours overexpress HER2, these compounds are widely used to treat these tumours in both early and metastatic phases (reviewed in Zhong *et al.* 2021).

In the ovary, EGFR activity is essential for oocyte maturation, cumulus expansion, and ovulation. This is because the EGF signalling network is responsible for communicating luteinising hormone (LH) and growth factor signals from the mural granulosa cells across the follicle to the oocyte via paracrine signals from EGF-like peptides AREG, EREG, and BTC (Hsieh *et al.* 2009). Indeed, although an Egfr-null mutation is embryonically lethal in mice (Sibilia & Wagner 1995), decreased Egfr signalling caused by a hypomorphic mutation yields offspring with impaired reproductive potential as LH-induced meiotic resumption and cumulus expansion are reduced in preovulatory follicles, leading to decreased ovulation. Furthermore, *in vitro* culture of bovine (da Rosa *et al.* 2017)

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and porcine (Nagyova 2012) oocyte–cumulus complexes indicated that the EGFR inhibitor AG1478 and lapatinib inhibited oocyte maturation and reduced expression of cumulus expansion-associated transcripts, respectively. Based on these findings, one would anticipate that EGFR inhibitors would have an adverse effect on fertility; however, a recent preclinical study where female mice were treated with either vehicle or lapatinib (100 or 200 mg/kg/day orally) for 4 weeks found no significant differences in ovarian morphology, total follicle numbers, anti-Mullerian hormone (AMH) levels, oestrous cyclicity, or mating outcomes in the three groups of mice (Liao *et al.* 2020). Further Western blotting and immunohistochemical investigation of the EGFR and its main downstream signalling pathways in isolated ovary cultures showed increased phosphorylation of STAT3 in the lapatinib-treated groups compared to the vehicle group, and it was hypothesised the lack of effect of lapatinib on ovarian function may be due to a compensatory effect of STAT3 signalling pathway activation. In fact, STAT3 signalling upregulation has been shown to be one of the mechanisms of anti-EGFR therapy resistance, with patients who are resistant to gefitinib, cetuximab, and lapatinib having increased STAT3 activity in their tumour tissue (Zulkifli *et al.* 2017). It may also be possible that the doses of lapatinib used in the experiments by Liao *et al.* were too low to observe ovarian toxicity. While the dose per kg body weight is high compared to clinical doses (Geyer *et al.* 2006), differences in the rate of metabolism and excretion make comparison difficult without knowledge of blood or tissue concentrations.

Lower rates of severe toxicity effects of EGFR inhibitors have also been observed in clinical settings compared to traditional chemotherapy. Although gefitinib has been shown to reduce testosterone and DHEA to lower than baseline levels in 31 women during 14–18 days of treatment (Nishio *et al.* 2005), there have been no clinical studies describing the impact of other EGFR inhibitors on fertility. Evidence from the adjuvant paclitaxel and trastuzumab (APT) clinical trial, a single-arm phase 2 study of 12 weeks of adjuvant paclitaxel and trastuzumab followed by 9 months of trastuzumab monotherapy, indicated that amenorrhea rates among premenopausal women treated with adjuvant non-alkylating paclitaxel and trastuzumab for early-stage breast cancer appeared lower than those historically seen with standard alkylating-based breast cancer regimens (28% in this study vs approximately 50% in earlier studies) (Ruddy *et al.* 2015). These findings were recapitulated in a separate study examining breast cancer patients treated with chemotherapy with or without trastuzumab, and while AMH levels declined to undetectable levels by 6 months post-treatment, 57.1% of the HER2-positive cohort showed a degree of recovery of AMH to detectable levels by 12 months post-treatment, compared to 36.8% in the negative cohort (Levi *et al.* 2020). Further exploration in a prepubertal mouse model injected with cyclophosphamide or paclitaxel with or without trastuzumab, or saline, and sacrificed 1 week or 3 months later, showed that trastuzumab significantly reduced the vascular damage of both cyclophosphamide and paclitaxel, with these ovaries exhibiting less apoptosis (determined using qualitative TUNEL staining and quantitative CD34+ blood vessel analysis) and a higher ovarian reserve (assessed via ovarian weight, serum AMH, and ovarian follicle stage proportions) following treatment (Levi *et al.* 2020). Collectively, these studies suggest that EGFR inhibitors may be safer than standard chemotherapy treatments, with no lasting effects on ovarian function and fertility; however, additional long-term follow-up studies are required to examine oocyte quality and subsequent offspring health.

BRAF/MEK inhibitors

BRAF belongs to the rapidly accelerated fibrosarcoma (RAF) family of serine/threonine kinases and is one of three distinct RAF proteins (also ARAF and CRAF) that functions as an effector in the MAPK signalling cascade (Dhillon *et al.* 2007). Normally activated following ligand binding to receptor tyrosine kinases, such as EGFR, dimerization of RAF family members and downstream activation of kinases including MEK and ERK lead to direct and indirect transcriptional regulation of genes implicated in cell survival and proliferation (Dankner *et al.* 2018). Additionally, the RAF-MEK-ERK pathway transduces signals from the RAS small GTPase, one of the most frequently mutated oncogenes in human cancers; however, due to its picomolar affinities to GTP, it was an undruggable target for many years until the recent advent of small-molecular covalent KRAS inhibitors. Dysregulation of Ras/Raf/MEK/ERK signalling in cancers is also commonly related to mutations in *BRAF*, with *BRAF* mutations (mostly *BRAF* V600E) resulting in constitutive pathway activation evident in ~40–50% of melanomas and ~6% of all other malignancies, thus making it an attractive therapeutic target (Flaherty & McArthur 2010).

Small molecule BRAF inhibitors can be categorised into two types based upon their interaction with the ATP-binding pocket. Type I inhibitors bind the ATP-binding pocket of BRAF in its active confirmation, particularly

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BRAF V600E, and here three inhibitors have been licensed for clinical use for the treatment of *BRAF* V600E mutant melanomas as single agents or combined with MEK inhibitors, namely vemurafenib, dabrafenib, and encorafenib. Combination BRAF/MEK inhibitors are also approved for use in advanced colorectal cancer and NSCLC. Sorafenib is an example of a type II-BRAF inhibitor, which stabilises the kinase in its inactive form; however, this compound also shows inhibitory activity against VEGFR, PDGFR, FLT1, KIT, and RET and is currently only approved for the treatment of hepatocellular carcinoma, advanced renal cell carcinoma, and some thyroid carcinomas. MEK inhibitors used clinically are trametinib, binimetinib, and cobimetinib, and these are often used in combination with BRAF inhibitors (refer to (Zhong *et al.* 2021) review for more detailed information about their usage). Currently, there are no ERK inhibitors approved for clinical use.

Given that RAF is a key downstream effector of EGFR signalling, one would anticipate that BRAF and MEK inhibitors may have potential effects on fertility, as discussed above. Indeed, the MAPK/ERK pathway transduces FSH signals via G-protein coupled receptors and is important for the transcription of genes associated with the preovulatory phenotype, including upregulation of *CYP19A1*, *LHCGR,* and *EGRF* genes (Donaubauer *et al.* 2016). Furthermore, disruption of ERK1/2 in mouse granulosa cells *in vivo* abolishes ovulation, cumulus cell–oocyte complex expansion, oocyte maturation, and luteinisation (Fan *et al.* 2009). The MAPK/ERK pathway is also active in proliferating cells, and culture of rat granulosa cells isolated from large preantral follicles showed that ERK is activated following stimulation by FSH, leading to granulosa cell differentiation (Das *et al.* 1996). In addition, inhibition of ERK reduced gonadotropin- and IGF-stimulated hormone production by bovine granulosa and theca cells *in vitro*, and experiments in ewes demonstrated that ERK inhibition reduced follicle growth and oestradiol production (Ryan *et al.* 2008). Based on these findings, we hypothesise that the consequences of anticancer drugs on this pathway would have widespread implications for ovarian follicles, especially given that proper differentiation and the acquisition of additional layers of granulosa cells is imperative to support follicle growth and critical to follicle survival, and we would anticipate these effects to be transient, with granulosa cell proliferation resuming in the absence of the inhibitors. However, the potential impact of disrupted granulosa cell function on the primordial follicle pool and at the time of follicle growth initiation is unknown and may also have long-term effects on ovarian function. To the best of our knowledge to date, there have been no published preclinical or clinical studies that have independently investigated the potential effects of this class of inhibitors on female fertility. However, consistent with these likely effects, the European Medicines Agency summary of product characteristics for dabrafenib, trametinib, and cobimetinib states that a reduced number of ovarian corpora lutea was observed in female rats, though no detailed ovarian follicle data were provided (Hassel *et al.* 2021).

PI3K/Akt/mTOR inhibitors

The PI3K/protein kinase B(Akt)/mammalian target of rapamycin (mTOR) pathway is one of the most important intracellular signalling pathways, driving a myriad of cellular processes including cell growth, motility, survival, metabolism, and apoptosis (Liu *et al.* 2009). PI3Ks are a family of lipid kinases that catalyse the phosphorylation of second messengers leading to activation of Akt, the main downstream effector of this pathway, and subsequently, activation of mTOR, whose main substrates include translation initiation factors and ribosomal proteins, ultimately promoting protein synthesis and cell proliferation (see (Vanhaesebroeck *et al.* 2012, Fruman *et al.* 2017) for details on PI3K signal transduction). This signalling pathway is dysregulated in many human cancers, with amplifications and activating mutations found in *PI3K, Akt,* and *mTOR*, and loss of function variants in *PTEN* frequently observed in a variety of tumours, leading to constitutive activation of this pathway. The value of targeting this pathway for therapeutic intervention is clear; however, only a few inhibitors of PI3K and mTOR have been approved for treatment, with the development of Akt-specific inhibitors being slow. We will focus our discussion here on the effects of mTOR inhibitors, as there are numerous studies that have examined the potential implications of these anti-cancer agents on the ovary. mTOR inhibitors can be divided into two categories: first-generation rapamycin analogues, which only inhibit the activity of mTORC1 but not mTORC2, and second-generation ATP-competitive inhibitors which can simultaneously suppress the activity of both mTOR proteins. At present, no second-generation inhibitors have been licensed for clinical use, whereas sirolimus (rapamycin), temsirolimus, and everolimus are rapalogs currently used for the treatment of various cancers including renal cell, breast, and pancreatic carcinomas, among others (Zhong *et al.* 2021).

PI3K/Akt/mTOR signalling is probably the most intensely investigated pathway in the oocyte with regards to

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primordial follicle activation, with additional roles in later follicle development. The phenotypes of mice null for Pten or Foxo3 (an oocyte-specific transcription factor on which this pathway converges) show premature reproductive senescence due to unrestricted, global activation of the primordial follicle pool (John *et al.* 2008, Jagarlamudi *et al.* 2009). Subsequent knock-out studies confirmed that intra-oocyte PI3K activation led to primordial follicle activation and growth initiation (Reddy *et al.* 2008). Independent of this, several studies suggest that mTOR itself is a positive regulator of granulosa cell proliferation, follicular survival, activation, and development, as well as translation of maternal transcripts (Guo & Yu 2019, Correia *et al.* 2020). In mouse oocytes, disruption of the negative mTOR regulators *Tsc1* (Adhikari *et al.* 2010) and *Tsc2* (Adhikari *et al.* 2009) led to overactivation of primordial follicles and subsequent infertility, though interestingly, mTOR itself may not be necessary for primordial follicle activation due to compensatory potential of the PI3K/Akt pathway. Conditional deletion of oocyte *Rptor*, a protein required for mTORC1 assembly, had no reproductive consequences despite the loss of mTORC1 activity, and it is thought that Akt signalling via FOXO3 can support a normal rate of follicle activation (Gorre *et al.* 2014). However, conditional deletion of *Rptor* in granulosa cells prevented the differentiation of flattened granulosa cells to cuboidal cells, with nearly all the primordial follicles in mutant ovaries remaining quiescent and very few growing follicles by juvenile age (Zhang & Liu 2015), implicating mTOR signalling in maintaining follicle dormancy. These data also suggest that crosstalk in mTOR signalling exists between granulosa cells and oocytes, as although *Rptor* deletion was only in granulosa cells, no enlarged and growing oocytes were observed in the cortical region of the adult mouse ovaries. Further evidence of this is observed in oocyte-specific conditional mTOR knockout mice, where granulosa cells of primordial follicles shift to an immature Sertoli-cell phenotype, including the expression of RNAs *Gata1*, *Hsd3b6,* and *Sox9* characteristic of this cell type (Guo *et al.* 2018), though how mTOR signalling regulates cellular identity remains unclear.

Sirolimus was first approved for use as an immunosuppressant for transplant patients. An analysis of the oestrous cycles and ovaries of adult female rats injected daily for 4 weeks with 2 mg/kg/day of sirolimus (this is 4–11 times the clinical dose adjusted for body surface area) (Shivaswamy *et al.* 2011) showed reductions in ovarian weight and size with fewer oestrous cycles when compared to the vehicle group, though the sirolimus-treated mice did not stop cycling altogether. Upon histological analysis, sirolimus-treated mice had both developing and large antral follicles, and the presence of corpora lutea indicating ovulation; however no detailed staging or quantification of follicles were performed in this study, making these data difficult to interpret and extrapolate. Other studies support the effect of sirolimus on oestrous cycles, and here ovarian morphological assessments showed an increased number of primordial follicles and fewer growing follicles and corpora lutea (which may potentially suggest reduced primordial follicle activation), although no sirolimus-treated mice had pregnancies during mating studies (Zhang *et al.* 2013). In a study of the effects of sirolimus on human ovarian tissue *in vitro*, oocyte loss within growing follicles was observed (McLaughlin *et al.* 2011). The mechanism underlying this finding is unclear, but the preferential loss of oocytes rather than of follicles would be expected to impact fertility with relative maintenance of endocrine ovarian function, albeit only during ongoing treatment rather than resulting in a longer-term damage to the ovary. In the clinic, sirolimus or everolimus treatment increased the risk of oligomenorrhea and/or ovarian cysts (Parazzini *et al.* 2021) in patients with tuberous sclerosis complex (Sparagana *et al.* 2017) following transplantation (Alfadhli *et al.* 2009) or with autosomal dominant polycystic kidney disease (Braun *et al.* 2012). Although more than a third of patients in a meta-analysis of 112 patients from 3 separate multi-centre studies experienced at least 1 menstrual irregularity (defined as amenorrhea or irregular menstruation) over the study period, these disturbances did not persist after 2 months from stopping treatment (patients were followed-up for 4–5 years), consistent with effects of these mTOR inhibitors on the growing follicle population or not prolonging beyond cessation of treatment (Sparagana *et al.* 2017).

In addition to these studies, a series of investigations into the ovarian effects of mTOR inhibitors have been undertaken to investigate these anti-cancer agents in limiting the harmful effects of some chemotherapies themselves. Alkylating chemotherapy agents such as cyclophosphamide are highly gonadotoxic and thought to induce ovarian damage in part by upregulation of PI3K/Akt signalling, leading to primordial follicle activation and subsequent premature depletion of the primordial follicle reserve (Kalich-Philosoph *et al.* 2013, Sonigo *et al.* 2019). In an *in vivo* mouse model of cyclophosphamide-induced gonadotoxicity, daily administration of everolimus or the experimental drug INK128 (an mTOR1/2 inhibitor) achieved a two- to four-fold attenuation of mTOR activity (as measured via Western blotting of PI3K signalling effectors and quantification of primordial follicle P-S6K immunostaining), compared with cyclophosphamide

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alone, and this was able to maintain primordial follicles in their resting state during chemotherapy treatment, maintain normal serum AMH levels, and preserve normal fertility (Goldman *et al.* 2017); similar findings have been obtained following experiments undertaken with sirolimus (Zhou *et al.* 2017). While the data presented here suggest that mTOR inhibitors may have low gonadotoxicity as anti-cancer or chemoprotective agents, it is important to highlight that oocyte-specific conditional knockout of mTOR impairs the completion of the first meiotic division, thus these agents may have an impact on oocyte quality and mating studies should be undertaken to ascertain this effect (Guo *et al.* 2018).

JAK/STAT inhibitors

There are four Janus kinase (JAK) isoforms which belong to the family of non-receptor tyrosine kinases that communicate signals from cell-membrane receptors elicited by ligands such as LIF and KIT in the ovary, by recruiting and phosphorylating STAT proteins, and promoting their translocation to the nucleus (Bousoik & Montazeri Aliabadi 2018). STAT then regulates target-gene transcription to coordinate the induction or prevention of apoptosis. Not only is JAK/STAT signalling an attractive target for the treatment of multiple autoimmune diseases, due to its important role in cytokine signal transduction, mutations in genes encoding STAT and JAK proteins are also implicated in the development of malignancy, especially haematopoietic cancers, and JAK has been targeted with inhibitors given it is the main upstream protein of STAT (Thomas *et al.* 2015). To date, four JAK inhibitors have been approved for clinical use, namely ruxolitinib, a JAK1/2 inhibitor and fedratinib, a JAK2-selective inhibitor (both approved for treatment of myelofibrosis); and tofacitinib and baricitinib (used clinically for the treatment of autoimmune diseases), with other JAK inhibitors currently undergoing clinical trials (Zhong *et al.* 2021).

In the ovary, JAK/STAT signalling plays an important role in mammalian folliculogenesis (Sutherland *et al.* 2012, Pastuschek *et al.* 2015, Hall *et al.* 2018). Analysis of JAK expression identified *JAK1* as the predominant transcript of the Jak family, and JAK1 protein expression localised predominantly within granulosa cells of primordial and growing follicles in mouse (Sutherland *et al.* 2018). Ruxolitinib has been used as a potent and selective inhibitor in mouse neonatal ovary cultures to elucidate the functional role of Jak1 in primordial follicle regulation (Sutherland *et al.* 2018). Morphological analysis of ovaries cultured with ruxolitinib for 48 h showed a decreased proportion of primordial follicles, with an increase in the percentage of follicles undergoing activation, though important to note this did not change the percentage of follicles transitioning to the pre-antral stage of development. Furthermore, ruxolitinib-treated ovaries had increased levels of follicle atresia, ascertained through qualitative TUNEL analysis. This suggests that JAK/STAT inhibitors have the potential to be highly gonadotoxic to the ovaries, activating primordial follicles with their subsequent demise; however, given that this study only provided follicle proportions rather than absolute follicle counts and did not quantify TUNEL staining by follicle stage, these data are difficult to conclusively interpret. Increased primordial follicle growth activation with poor health of growing follicles (determined morphologically) was also observed in *in vitro* culture experiments using human (McLaughlin *et al.* 2014) and bovine (Maidarti *et al.* 2019) ovary during treatment with PI3K pathway activators. Crosstalk exists between JAK/STAT and the PI3K signalling pathway in mammary glands (Rädler *et al.* 2017), suggesting perhaps these pathways may also interact in the ovary. There is therefore the potential for JAK/STAT inhibitors to have compounded negative effects mediated via this secondary route, and this should be explored further perhaps using conditional deletion or knockout rodent models.

BCR-Abl

A reciprocal translocation between chromosomes 9 and 22, which cytogenetically results in the Philadelphia chromosome, molecularly gives rise to the chimeric BCR-Abl gene. This is the underlying cause of chronic myeloid leukaemia (CML), which accounts for 15–20% of all cases of adult leukaemia in Western populations (Redaelli *et al.* 2004). This oncoprotein is also observed in ~20% of acute lymphoblastic leukaemias (ALL) (Klein *et al.* 2004). BCR-Abl is a constitutively active kinase that drives tumourigenesis through phosphorylation and activation of a broad range of downstream substrates that play critical roles in cell signal transduction and transformation. Among these downstream substrates are activation of MAPK signalling, PI3K-Akt signalling, and JAK/STAT signalling (Quintás-Cardama *et al.* 2007), thus inhibition of BCR-Abl represents a therapeutic approach with widespread implications, as previously discussed in this review.

Imatinib mesylate, a RTK inhibitor which targets ABL as well as platelet-derived growth factor (PDGF) receptor

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and c-kit, was the first approved BCR-Abl inhibitor (in CML and ALL) and c-kit inhibitor (in gastrointestinal stromal tumours); however, the data regarding its effects on the ovary, and indeed whether it might offer protection against some chemotherapies, are conflicting. Adult mice given daily intraperitoneal injections of imatinib for 4-6 weeks had a shift in follicle distribution, with imatinib-treated females having fewer primordial follicles, but increased numbers of primary and secondary follicles (Salem *et al.* 2020), While there was no observed effect on ovulation or fertilisation rates, blastocysts derived from superovulated imatinib-treated females had fewer total number of cells, with fewer cells in the inner cell mass and increased numbers of trophectoderm cells (Salem *et al.* 2019, 2020). In similar *in vivo* studies, a single dose of imatinib alone increased the numbers of pyknotic bodies (oocytes with nuclear fragmentation, a hallmark of apoptosis) by nearly five-fold, 3 days later (Kerr *et al.* 2012), suggesting that imatinib has pro-apoptotic activity in oocytes most likely through inhibition of c-kit signalling (Hutt *et al.* 2006). Supporting this, studies using the human ovary also suggest a toxic effect of imatinib (Bildik *et al.* 2018), which was similar to that seen with an antagonist against c-kit, but was not seen with another c-Abl inhibitor. However, there are reports that describe no adverse effects of imatinib treatment on folliculogenesis (Maiani *et al.* 2012, Schultheis *et al.* 2012, Morgan *et al.* 2013). Differences in variables that could potentially account for this include mouse age and strain, *in vitro* vs *in vivo* studies, and most importantly, route of imatinib delivery and doses, with the latter having a ten-fold difference between studies. Interestingly, one study reported that imatinib exposure in a newborn mouse ovary culture model led to a higher number of follicles at the end of the 6-day culture period compared to controls (Morgan *et al.* 2013), which the authors suggest is a result of BCR-Abl signal inhibition on promoting primordial germ cell survival, which following nest breakdown during the culture period, could lead to increased primordial follicle formation. Clinical data are very limited, but there are case reports indicating temporary compromise of ovarian response to gonadotropin stimulation during imatinib treatment (Zamah *et al.* 2011), and of premature ovarian insufficiency (Christopoulos *et al.* 2008).

Additionally, imatinib and asciminib have been suggested to offer protection to primordial follicles during treatment with cisplatin and cyclophosphamide, respectively, although this is controversial. An initial report showed that imatinib provided protection against cisplatin-induced oocyte death in mice (Gonfloni *et al.* 2009), supported by work showing that imatinib reduced cisplatin-induced Bax expression and apoptosis in mouse oocytes (Kim *et al.* 2013). Others however found no evidence of such protection in mouse (Kerr *et al.* 2012) or in the human ovary (Bildik *et al.* 2018) and, as discussed above, proposed that imatinib itself had toxic effects on follicles through inhibition of c-kit signalling. Supporting a protective effect, addition of imatinib during cisplatin treatment of *in vitro* newborn mouse ovary cultures reduced the percentage of unhealthy follicles by 21% compared to control ovaries, while imatinib alone only led to a small reduction which was not significant and imatinib did not protect against the effects of doxorubicin (Morgan *et al.* 2013). Similarly, concomitant *in vivo* administration of asciminib with cyclophosphamide in postnatal day 6 mice showed a significant rescue in the number of primordial follicles; however, this finding was dose dependent, with evidence that higher doses of asciminib (four times more than the effective dose) have a minor toxic effect (Mattiello *et al.* 2021). The dose-effect observed by Matiello *et al.* suggests the dose of BCR-Abl inhibitor alone and its ratio to the chemotherapy itself may be critical in delivering its effect.

CDK inhibitors

Tumour-associated cell cycle deregulation, either continued proliferation or unscheduled re-entry into the cell cycle, is often mediated by alterations in cyclin-dependent kinases (CDKs). CDKs are critical enzymes that govern cell cycle progression and require cyclin proteins for activation and downstream phosphorylation (Malumbres 2014). Among the numerous CDKs and cyclins that have been identified in humans, only a certain subset of CDKs are directly involved in driving the cell cycle, notably CDK4 and CDK6, which are activated by D-type cyclins, and drive the transition from G1 to the S phase of the cell cycle via phosphorylation of retinoblastoma protein 1 (RB1). This subsequently promotes the release of E2F transcription factors, driving the transcription of cell cycle progression genes (Chen *et al.* 2016, O'Leary *et al.* 2016). Given the critical role of this pathway in mediating cell cycle progression, inhibition of CDK4 and CDK6 is a promising anti-cancer intervention (Asghar *et al.* 2015, Roskoski 2019). These proteins are targeted together as they have identical biological functions, and dual inhibition is essential to limit compensatory effects. To date, three CDK 4/6 inhibitors are clinically available, palbociclib, ribociclib, and abemaciclib, and these have been approved for the treatment of hormone receptor-positive and

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HER2 negative breast cancers in combination with other endocrine therapies (Zhong *et al.* 2021).

Evidence from knockout mice suggest the potential involvement of CDK4 and CDK6 in fertility and subsequent embryo-fetal development. Mice deficient in either CDK4 or CDK6 are viable, and although these kinases are present in mouse oocytes at varying levels throughout development (Kohoutek *et al.* 2004), an infertility phenotype is only apparent in CDK4 null mice. Female Cdk4 knockout mice have small ovaries and are sterile, with reduced ovulation efficiency and defective corpus luteum formation, leading to implantation failure (Moons *et al.* 2002, Pagano & Jackson 2004). These mice are unable to support embryo implantation because of defective progesterone secretion, and this was revealed to be a consequence of the inability of Cdk4-deficient mice to produce prolactin due to an 80% decrease in the pituitary lactotroph population (Rane *et al.* 1999, Moons *et al.* 2002). Interestingly, the gonadotrophs were unaffected, and transplanted blastocysts derived from Cdk4-null oocytes to the uteri of WT mice developed normally, suggesting that the oocytes themselves were unaffected (Moons *et al.* 2002). However, despite these findings in female Cdk4-deficient mice, preclinical studies carried out in rats who were administered doses up to 300 mg/kg/day of palbociclib once daily for 15 days showed no evidence of any impact on fertility, as determined by no deviations in oestrous cyclicity, functional fertility, and uterine parameters (Catlin *et al.* 2019). The authors propose that this finding may reflect the difference between complete absence of Cdk4 during fetal development in knockout mice compared to the transient pharmacological inhibition achieved in their study, thus bringing into question the potential long-term consequences of prolonged CDK4 inhibitor exposure that may be used in a clinical setting, particularly in the curative treatment setting where 2 years of CDK4/6 inhibitor therapy is recommended.

PARP inhibitors

Poly (ADP-ribose) polymerases (PARPs) are a family of proteins involved in a diverse range of cellular processes, with PARP1 being the most well-characterised family member due to its distinct role in the detection and repair of single-strand DNA breaks. Although homologous recombination is the major pathway by which dsDNA breaks are repaired with high fidelity, the PARP pathway is an important alternative DNA repair mechanism especially in cells with defects in homologous recombination (Morales *et al.* 2014). In response to DNA damage, PARP is involved in base excision repair, by binding tightly to DNA strand breaks and promoting chromatin remodelling through the recruitment of DNA repair effectors. PARP may also have a role in alternative DNA repair pathways including nucleotide excision repair, non-homologous end joining (both classical and alternative), homologous repair, and DNA mismatch repair (Caldecott 2014, Vyas & Chang 2014). As anti-cancer agents, PARP inhibitors suppress the repair of single-strand DNA breaks, and these unrepaired breaks can result in double-strand DNA breaks in cancer cells with impaired DNA repair mechanisms, resulting in apoptosis. In instances where the cell already has defective DNA repair mechanisms, for example, where there are existing mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* that are crucial for homologous recombination and double-strand DNA repair, this can create a 'synergistic lethal' effect, resulting in genomic instability and cancer cell death. Several PARP inhibitors have been licensed for the treatment of germline and/or somatic *BRCA1/2*-mutated breast, ovarian and pancreatic cancer, namely olaparib, rucaparib, niraparib, and talazoparib (Zhong *et al.* 2021).

Despite considerable knowledge gained from completed clinical trials regarding the potential side-effects of PARP inhibitors, specifically olaparib (Pujade-Lauraine *et al.* 2017, Robson *et al.* 2017), assessment of ovarian function or fertility has been overlooked as an assessable endpoint. However, information from a limited number of laboratory studies suggests that this anti-cancer agent (and conceivably other PARP inhibitors) may have a detrimental effect on primordial follicles, thus diminishing the ovarian reserve. In one study, 8-week-old female mice were given intraperitoneal injections of a single dose of either cyclophosphamide, doxorubicin, carboplatin, paclitaxel, or saline vehicle-control, and a daily subcutaneous injection of olaparib or saline vehicle-control for 28 days, at doses known to partially deplete primordial follicles or successfully reduce breast cancer tumour burden in mouse models, respectively (Winship *et al.* 2020). Olaparib alone significantly depleted primordial follicles by 36% compared with controls, and this was independent of primordial follicle loss resulting from premature activation, as olaparib did not alter growing follicle populations (determined both histologically and via serum AMH concentrations). There also appeared to be evidence of increased DNA damage in surviving primordial follicles in olaparib-treated ovaries; however, this was not statistically significant. Interestingly, immunohistochemical analyses of olaparib-exposed ovaries showed the presence of primordial follicle

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remnants that contained intact granulosa cells but no oocyte, suggesting this drug caused direct damage and death to primordial follicle oocytes (Winship *et al.* 2020).

Granulosa cells (and thus normal follicle growth and hormone production) may also be adversely affected by PARP inhibition. In an *in vitro* study where granulosa cells isolated from mouse antral follicles were cultured with olaparib for 6 h, RT-qPCR analyses showed significant downregulation of characteristic granulosa cell transcripts *CYP19A* and *FSHR,* and these findings were supported with analyses demonstrating decreased oestradiol production from olaparib-cultured ovaries (Nakamura *et al.* 2020). The authors suggest that olaparib causes granulosa cell dysfunction, affecting both granulosa cell quality and quantity, in addition to its effects on the ovarian reserve. Although this study was undertaken over a shorter duration (only 14 days) and in much younger mice (aged PND21), the effects observed on granulosa cells here may have been related to the high olaparib dose investigated, which was in fact six times higher than that used by Winship *et al*. Nevertheless, this work also suggests that the ovary can potentially recover from a short duration of olaparib treatment, as following 3-week cessation of olaparib administration, ovarian function in these mice appeared to recover, with there being no difference in the number of retrieved oocytes and fertilisation rates following IVF, when compared to vehicle-control animals. While this may be encouraging for women, the impact of longer duration PARP inhibitor therapy is unknown and the irreversible harmful effects that olaparib has on the ovarian reserve is still likely to be an important factor for women seeking natural and assisted conception. Furthermore, *in utero* toxicity potential is also a concern in the situation of treatment of a woman while pregnant, given the importance of this period in establishing the ovarian reserve.

Anti-angiogenesis

Angiogenesis is the complex process of forming new blood vessels from pre-existing vessels, with vascular endothelial growth factor (VEGF) secreted by hypoxic cells acting on nearby endothelial cells to stimulate proliferation and thus new vessel growth (Shibuya 2011). This is essential to supply oxygen and nutrients to tumour cells and remove metabolic waste, thus facilitating tumour growth and metastasis. Indeed, it has been shown that in the absence of angiogenesis, human solid cancers are unable to grow larger than 1–2 mm (Potente *et al.* 2011), and in some cancers, such as mesothelioma, breast and gastric cancers, high levels of VEGF and other angiogenic factors indicate high-risk disease and poor prognosis. Targeting the tumour vasculature therefore has received considerable interest as an optimal target for anti-cancer strategies. Clinical results have however been mixed across different tumour types, with improvements in both progression-free and overall survival demonstrated in some studies with the addition of anti-angiogenic agents to chemotherapy in patients with advanced colorectal cancer (Baraniskin *et al.* 2019) and NSCLC (Liu *et al.* 2021); however, in other cancer types, such as advanced breast and advanced ovarian cancer, improvements in progression-free survival did not translate into improved overall survival. In addition to VEGF, there are several other angiogenic growth factors including fibroblast growth factor (FGF) and PDGF (Bouïs *et al.* 2006), and anti-angiogenetic treatments have mainly focussed on inhibiting the actions of these factors by targeting the activity of their receptors. Most of the inhibitors that have been approved for use in this category are multi-kinase inhibitors, thus have the potential for widespread implications for ovarian function. Given that there are numerous anti-angiogenic therapies available (El-Kenawi & El-Remessy 2013), we will infer the effects of these agents based on the role of pro-angiogeneic factors in the healthy ovary and limit our discussion here to those agents that have specifically been investigated in a fertility context.

VEGF is one of the most important local regulators of ovarian vascular physiology (McFee *et al.* 2012, Araújo *et al.* 2013). While primordial follicles are not closely vascularised, VEGF peptide expression increases as follicles mature, with expression mainly localised to thecal cells and to granulosa cells nearest the oocyte in antral follicles, and in the very highly vascularised corpus luteum (Gordon *et al.* 1996). VEGF TRAP treatment used to inhibit VEGF activity during the follicular phase in marmosets resulted in a significant decrease in thecal, granulosa, and endothelial cell proliferation, compromising antral follicle development, with an absence of ovulatory follicles (Wulff *et al.* 2002, Taylor *et al.* 2007). VEGF is secreted into the follicular fluid, and its abundance has been correlated with increased perifollicular vascularity, higher oocyte fertilisation rates, better embryo quality, and higher pregnancy rates in women undergoing IVF treatment (Monteleone *et al.* 2008). In addition to its role in these later stages of follicle growth, VEGF may also be important in the activation and development of preantral follicles. Several reports describe the expression of VEGF in the oocytes of

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human primordial and primary follicles (Otani *et al.* 1999, Harata *et al.* 2006) and rat primary follicles (Celik-Ozenci *et al.* 2003). VEGF was also shown to be significantly upregulated during primordial follicle development in a rat ovary gene expression profiling study (Kezele *et al.* 2005). Experimentally, *in vivo* injection of VEGF into the ovarian bursa of mice enhanced neovascularisation and vascular permeability of developing follicles, consequently reducing follicle atresia (Danforth *et al.* 2003, Quintás-Cardama *et al.* 2007). These findings were observed in a dose- and time-dependent manner and suggest a potential role for VEGF in primordial follicle activation. Indeed, systemic and intrabursal injection into adult and prepubertal mice, respectively, with antibodies designed to neutralise VEGF or inhibit its interaction with its receptors, significantly reduced primordial follicle numbers by ~25%, and this was evident within 1–3 days of administration (Roberts *et al.* 2007); there were no effects on primary or secondary follicle number, though these may become apparent after higher doses or prolonged exposure.

Based on the consequences of inhibiting VEGF alone, one might expect systemic delivery of anti-angiogenic inhibitors to women undergoing cancer treatment to not only impact growing follicles but possibly also the ovarian reserve as well. However, given the multi-kinase action of these drugs, there needs to be consideration of the implications of inhibiting other pro-angiogenic factors, such as PDFG, which along with its receptors, has been detected in human oocytes and granulosa cells respectively, leading to the suggestion that binding of PDGF ligands to their receptors on granulosa cells may be involved in primordial follicle activation (Pinkas *et al.* 2008). Although absent PDGF receptor expression is lethal before ovarian follicle development (Soriano 1994, 1997), mutations in selected target genes of PDGF signalling in mice compromised female fertility, with female sterile mutants exhibiting defects in ovarian size, follicle development, ovulation, and theca cell-related oestrogen production (Schmahl *et al.* 2008). Similarly, Pascuali *et al.* demonstrated that inhibition of PDGF signalling via ovarian injection of a selective PDGFR inhibitor decreased the proportion of preantral and early antral follicles, while increasing the percentage of atretic follicles compared to control ovaries, and this was thought to be mediated via alterations in the expression of pro-apoptotic factors Bax and cleaved caspases 3 and 8 (Pascuali *et al.* 2015). Thus, inhibition of this pathway is also likely to be detrimental for ovarian function; however, there are no publications describing controlled investigations into the effects of anti-angiogenic agents on female fertility at present.

One published case report describes transient ovarian insufficiency in a woman receiving pazopanib treatment for 10 months, which the authors propose was caused by the treatment as menses returned regularly following discontinuation of treatment after 2 months (De Sanctis *et al.* 2019), but these findings are not interpretable in relation to potential effects on primordial follicles.

Concluding remarks

Despite the large number of small molecule targeted anti-cancer drugs that have been approved to treat various human cancers, our knowledge and understanding of the implications of these therapies on female fertility and ovarian function remains minimal. The pathways targeted are all of relevance to physiological ovarian function, across the range from *in utero* oocyte and primordial formation, through maintenance and growth initiation of the ovarian reserve, to follicle development and ovulation, thus are therefore expected to have temporary or permanent effects on female reproductive function. The pre-clinical and clinical data are however scarce, and there is a significant need for accurately controlled studies with robust analyses, particularly including absolute follicle counts and quantification of apoptosis and DNA damage where possible, which will allow valid comparisons and conclusions to be drawn from multiple independent studies. Particularly for those drugs likely to have direct impact on primordial follicles themselves, it will be important for oncologists to inform patients about potential treatment-related infertility and discuss fertility preservation options prior to treatment (Loren *et al.* 2013, Anderson *et al.* 2020, Lambertini *et al.* 2020), particularly in patients receiving curative-intent anti-neoplastic treatment where long-term treatment toxicities can have profound implications on survivorship. As with investigations into the effects of chemotherapy in the ovary (Spears *et al.* 2019), the lack of human data strongly suggests the need to develop and for wider use of appropriate models that can be used to examine both the short-term and sustained consequences of treatment.

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