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The impact of testosterone on the non-growing follicles and stroma in the transgender ovary

Dr Emily Bailie

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Declaration

April 2024

I declare that all the work presented within this thesis was composed by myself and that it has not been submitted in any other degree or professional qualification. Except where stated otherwise in the text, the work presented is entirely my own.

Emily Bailie

2024

Abstract

There is an increasing awareness of a need for fertility preservation measures for the transgender population prior to commencing gender affirming endocrine therapy. Current options for transgender men include embryo and oocyte cryopreservation but ovarian tissue cryopreservation may be a viable fertility preservation option for transgender men if immature follicles from removed ovarian cortex could be developed *in vitro* to maturity. However, there are few data on the effect of long-term exposure to high levels of testosterone as found in transgender men taking testosterone therapy on the non-growing follicles of the ovary.

Androgens are essential in normal ovarian function and follicle health, with androgen receptor expression found on the granulosa and theca cells as well as the oocyte. Androgens promote follicle growth, improve the responsiveness of granulosa cells to FSH and are an essential substrate for oestradiol production. However, an excess of androgen, as seen in polycystic ovary syndrome (PCOS), is associated with increased follicular recruitment with subsequent development arrest and anovulation, poor ovarian follicle health and subfertility. Hyperandrogenism as seen in PCOS and transgender men on testosterone therapy also drives changes within the ovarian stroma including thickening of the tunica albuginea, stromal cell hyperplasia and stromal cell luteinisation, contributing to reduced follicle maturation.

In this study, the effect of testosterone on the development, morphological health and DNA damage and repair capacity of human ovarian follicles *in vivo* and their survival *in vitro* was investigated. Potential alterations in the composition of the ovarian stroma as a result of

testosterone exposure and how this can impact follicle growth and activation were also investigated.

Whole ovaries were obtained from transgender men taking pre-operative testosterone therapy (mean age: 27.6 ± 1.7 years; range 20-34 years, $n = 8$) at oophorectomy. This was compared to cortical biopsies from age-matched healthy women obtained at caesarean section (mean age: 31.8 ± 1.5 years; range= 25-35 years, $n=8$). Cortical tissues were dissected into fragments and either fixed immediately for histological analysis or cultured for 6 days and subsequently fixed. Follicle classification and morphological health were evaluated from histological sections stained with haematoxylin and eosin (H&E) and expression of γ H2AX as a marker of DNA damage by immunohistochemistry. Alteration of the composition of the ovarian stroma compared to control ovarian cortex was assessed by quantifying collagen deposition using histological stains picrosirius red and Masson's Trichrome, and elastin deposition using immunofluorescence. Stromal cell density was quantified from H&E stained sections.

In uncultured tissue, testosterone exposure was associated with reduced follicle growth activation, poor follicle health and increased DNA damage. After 6 days of culture, there was enhanced follicle activation compared to control, but with further deterioration in morphological health and increased DNA damage. Transgender ovarian tissue had more collagen and less elastin compared to control with a subsequently higher collagen/elastin ratio, indicating tissue fibrosis. The stromal cell density was significantly higher in transgender tissue, in keeping with stromal cell hyperplasia.

The findings from this study indicate that high circulating concentrations of testosterone have effects on the primordial and small-growing follicles of the ovary. In addition, it was found

that the ovaries of transgender men are more collagenous and less elastic, indicating tissue fibrosis. This enhanced tissue rigidity may contribute to reduced follicle activation *in vivo* and have implications on reproductive outcomes for transgender men considering pregnancy or fertility preservation measures.

Lay Summary

As part of transitioning from female to male, transgender men take testosterone therapy. Prior to starting testosterone therapy, they are encouraged to consider freezing eggs or embryos as the impact of testosterone on the function of the ovary or future reproductive potential is unclear. Ovarian tissue cryopreservation, where a small portion of the ovary is removed via keyhole surgery, may be a way to preserve fertility for transgender men if immature eggs (follicles) could be grown to maturity *in a laboratory dish*.

Androgens are important for normal ovarian function and follicle health however, if there are excessive androgen levels, as seen in women with polycystic ovary syndrome, this can result in ovulation problems, difficulties getting pregnant and miscarriage.

In this study, transgender men who were on long-term testosterone treatment and having a hysterectomy (removal of their uterus) and removal of their ovaries and fallopian tubes as part of their transitioning process were recruited and donated their ovaries. The ovaries were prepared by removing the outer most layer, the ovarian cortex, from the rest of the ovary. Some tissue was analysed without further treatment, other ovarian tissue was used in culture experiments to assess how well ovarian follicles grow. The results were compared to women of the same age who donated ovarian tissue at the time of caesarean section.

This study aimed to see whether testosterone affects how ovarian follicles grow and their overall health, and whether it causes DNA damage. It also assessed the effect of testosterone on the composition of the ovary stroma, which is the supportive connective tissue which surrounds and supports the ovarian follicles, after testosterone exposure. The results showed there was a higher proportion of non-growing ovarian follicles in the ovaries of transgender men, and that these follicles appeared less healthy with higher levels of DNA damage. After 6 days of tissue culture, there were more growing follicles in transgender ovaries compared to

control, but follicle health further deteriorated and there were increased levels of DNA damage. In the ovarian stroma, there was more collagen and less elastin found, resulting in the stroma being very stiff and rigid. As a more rigid stroma reduces the ability of the follicle to be able to expand and develop, this could explain the finding of more non-growing follicles in transgender tissue compared to control.

The results of this study have identified new effects of testosterone on the ovary. It emphasises the importance of discussing fertility preservation options with transgender men prior to starting testosterone therapy.

Publications and Presentations Relating to This Thesis

Publications

Bailie E, Maidarti M, Hawthorn R, Jack S, Watson N, Telfer EE, Anderson RA. 2023. The ovaries of transgender men indicate effects of high dose testosterone on the primordial and early growing follicle pool. *Reproduction and Fertility*, 4, e220102.

Oral presentations

Dr E Bailie, Dr M Maidarti, Dr S Jack, Dr R Hawthorn, Dr N Watson, Prof EE Telfer, Prof RA Anderson (2020). The ovaries of trans-men indicate effects of testosterone on the primordial follicle pool. Oral presentation at Fertility Meeting January 2020 Edinburgh, UK.

Dr E Bailie, Dr M Maidarti, Dr S Jack, Dr R Hawthorn, Dr N Watson, Prof EE Telfer, Prof RA Anderson (2021). Testosterone treatment affects non-growing and early growing ovarian follicles. Oral presentation at Fertility Meeting January 2021, online.

Dr E Bailie, Dr J Grosbois, Dr S Jack, Dr R Hawthorn, Dr N Watson, Prof RA Anderson, Prof EE Telfer. High dose testosterone induces changes in stromal collagen and elastin content of ovaries of transgender men. Oral presentation at Fertility Meeting January 2022, online *

Dr E Bailie, Dr J Grosbois, Dr S Jack, Dr R Hawthorn, Dr N Watson, Prof RA Anderson, Prof EE Telfer). Testosterone treatment induces ovarian fibrosis in the ovaries of transgender men. Oral presentation at Fertility Meeting January 2024 Edinburgh, UK

*Awarded the Iwan Lewis Jones Prize for best oral presentation in a category

Poster presentations

Dr E Bailie, Dr M Maidarti, Dr S Jack, Dr R Hawthorn, Dr N Watson, Prof EE Telfer, Prof RA Anderson (2021). The ovaries of transgender men indicate effects of high dose testosterone on the primordial and early growing follicle pool. E-Poster presentation at the 38th Annual Meeting of the European Society of Human reproduction and Embryology (ESHRE); June 2021, online.

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Abbreviations

Abbreviations

Description

ADSCs	Adipose-derived stem cells
Akt	Protein kinase B
APLF	Aprataxin-and-PNK-like factor
ATM	Ataxia telangiectasia mutated
AMH	Anti-mullerian hormone
AR	Androgen receptor
ART	Assisted reproductive techniques
A4	androstenedione
bFGF	Basic fibroblast growth factor
Bcl-2	B-cell lymphoma/leukemia-2
Bad	Bcl-2-associated death promoter
Bax	Bcl- associated X protein
BMP	Bone morphogenetic protein
BMP4	Bone morphogenetic protein 4
BMP7	Bone morphogenetic protein 7
BMP15	Bone morphogenetic protein 15
bpv(HOpic)	Dipotassium bisperoxo (5-hydroxypyridinecarboxyl)oxovanadate (V)
BRCA1	Breast cancer susceptibility gene1
BRCA2	Breast cancer susceptibility gene2
Chk1	Checkpoint kinase 1

Chk2	Checkpoint kinase 2
COC	cumulus oocyte complex
CTGF	Connective tissue growth factor
CYR61	cysteine-rich angiogenic protein 61
DAB 3,	3'-diaminobenzidine
DDR	DNA damage response
dH ₂ O	Distilled water
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DHT	dihydrotestosterone
DPX	Dibutylphthalate Polystyrene Xylene
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DMC1	Disturbed meiotic cDNA1
DSBs	Double-strand breaks
D0	Day 0
D6	Day 6
ECM	extracellular matrix
EGF	epidermal growth factor
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
FOXO3	forkhead box transcription factor 2
FOXO3	Forkhead box O3
FP	Fertility preservation
GC	Granulosa cells
GDF9	Growth differentiation factor 9

GnRH	Gonadotrophin releasing hormone
H & E	Haematoxylin and eosin
HGF	hepatocyte growth factor
HR	Homologous recombination
HSA	Human serum albumin
IGF	Insulin-like growth factor
IVA	In vitro activation
IVF	In vitro fertilization
IVG	In vitro follicle growth
IVM	In vitro maturation
KGF	keratinocyte growth factor
KL	KIT ligand
LATS1/2	Large tumour suppressor homolog 1/2
LIF	Leukaemia inhibitory factor
LH	Luteinising hormone
MDC1	Mediator DNA damage checkpoint protein
M1	Metaphase I
M2	Metaphase II
Mob	Mps one binder
mTORC	Mammalian target of rapamycin complex
mTORC1	Mammalian target of rapamycin complex I
mTORC2	Mammalian target of rapamycin complex 2
MRN	MRE11, Rad50, NBS1
MRE11	Meiotic recombination 11
NBF	Normal buffered formalin

NBS1	Nijmegen breakage syndrome 1
NGFD	Non growing follicle density
NHEJ	Non-homologous end joining
NOV	nephroblastoma overexpressed
NT4	neurotrophic factor 4
OTC	Ovarian tissue cryopreservation
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline-triton
PCOS	Polycystic ovary syndrome
PFA	primordial follicle activation
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PKD1	Phosphatidylinositol-dependent kinase 1
PGCs	Primordial germ cells
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-bisphosphate
POF	Premature ovarian failure
POI	Premature ovarian insufficiency
PSR	Picrosirius red
PUMA p53	upregulated modulator of apoptosis
ROI	Region of interest
rpS6	Ribosomal protein S6 kinase
ROS	Reactive oxygen species
SAV	the adaptor Salvador

SPSS	Statistical Package for the Social Sciences
SRY	sex determining region Y
SSB	single stranded break
Streptavidin HRP	Streptavidin Horseradish Peroxidase
STSW	Scott's tap water substitute
S6K1	Ribosomal protein S6 kinase 1
TBS	Tris-buffered saline
TBST	Tris-buffered saline-tween
TAp63	Trans-activating p63
TAZ	Transcriptional coactivator with PDZ-binding Motif
TEAD	transcriptional enhanced associate domain
TGF β	Transforming Growth Factors- β
TID	Transcriptional inhibitory domain
TSC1/2	Tuberous sclerosis complex 1/2
TZPs	Transzonal projections
VEGF	vascular endothelial growth factor
WNT4	wingless-related integration site 4
WPATH	World Professional Association for Transgender Health
XRCC4	X- ray cross complementing protein 4
XLF	XRCC4-like factor
YAP	Yes-associated protein
3D	Three dimensional

4EBP

eukaryotic translation initiation factor 4E

γ H2AX

gamma H2A histone family member X

Chapter 1

Introduction

1.1 Introduction

Ovarian tissue cryopreservation (OTC) is becoming increasingly available to patients at risk of premature ovarian insufficiency (POI), in particular oncology patients as a result of gonadotoxic treatments. This involves laparoscopic removal of part of the ovarian cortex or a whole ovary. For patients who survive cancer and subsequent gonadotoxic treatments, tissue can be autografted to an orthotopic or heterotopic site with the aim of restoring fertility and ovarian function (Anderson and Baird, 2019a). To date, more than 200 live births have been reported in the literature (Dolmans et al., 2020). OTC is increasingly becoming utilised by women with benign conditions at high risk of POI including genetic disorders e.g. galactosaemia and Turner's syndrome (Dunlop et al., 2023) and patients requiring gonadotoxic treatments for benign conditions such as alkylating agents for autoimmune conditions e.g. systemic lupus erythematosus (SLE) (Condorelli and Demeestere, 2019).

OTC could potentially be utilised as a fertility preservation (FP) option for transgender men (Bailie et al., 2023, De Roo et al., 2017). Rather than planned auto-transplantation of ovarian cortical tissue, the non-growing follicles within the ovarian cortical tissue could be subjected to a multi-step tissue culture process with the aim of producing mature oocytes that could subsequently be fertilised to create embryos (section 1.16 and 1.18).

1.2 Primordial follicle formation and ovarian reserve

The two key functions of the ovary are to produce hormones and form the female germline (Liu et al., 2010). Primordial follicle formation occurs during fetal life in humans. Primordial germ cells (PGCs) are the precursors to oocytes or spermatogonia and differentiate

depending on the somatic environment (McLaren, 1991). The PGCs migrate to the genital ridge where PGC proliferation occurs prior to entry into meiosis and formation of oogonia and subsequent formation of oocytes (Stringer et al., 2023). Sex differentiation occurs by 6 weeks gestation (Liu et al., 2010). In the absence of sex-determining region Y (SRY) and in the presence of wingless-related integration site 4 (WNT4) and forkhead box transcription factor 2 (FOXL2), ovarian development occurs.

Between 11-12 weeks' gestation, some oogonia begin to enter meiosis and progress to the diplotene stage of prophase 1 to form oocytes. The number of oocytes present in the human ovary reaches a peak number of 6-7 million at 16-20 weeks' gestation (Baker, 1963). Oocytes must form associations with flattened squamous granulosa cells originating from the mesenchyme of the genital ridge to form primordial follicles (De Felici et al., 2005, Picton et al., 1998, Telfer et al., 2023). Not all oocytes form primordial follicles, with the primordial follicle pool being 500,000 to 1 million primordial follicles at term, constituting the female ovarian reserve (Findlay et al., 2015) (figure 1.1). The remaining oocytes undergo degradation via apoptosis and autophagy (Stringer et al., 2023, Albamonte et al., 2008). The reason for this significant loss of oocytes is unknown but it is likely a quality control mechanism (Tilly, 2001a), eliminating low quality or defective oocytes (Findlay et al., 2015). Oocytes are arrested in meiotic prophase 1 and remain in this quiescent state until ovulation (Telfer et al., 2023)

The size of the primordial follicle pool, constituting the ovarian reserve, can widely vary and correlates with the time of onset of menopause, when the ovarian reserve drops to below 1000 remaining oocytes (Depmann et al., 2015, Faddy and Gosden, 1996) (figure 1.1). A study using mathematical modelling, showed that the primordial follicle pool size can range between 34,800 and 2,508,000 primordial follicles at 18–22 weeks post-conception, with

women who go through menopause at the average age having approximately 295,000 non-growing follicles (NGFs) present at birth per ovary, women with earlier menopause having with approximately 35,000 NGFs at birth per ovary and those with late menopause having over 2.5 million NGFs per ovary at birth (Wallace and Kelsey, 2010). Throughout a human reproductive span, only 400-600 follicles complete the process of ovulation (Wallace and Kelsey, 2010), with up to 75% of primordial follicles being lost before puberty (Telfer et al., 2023).

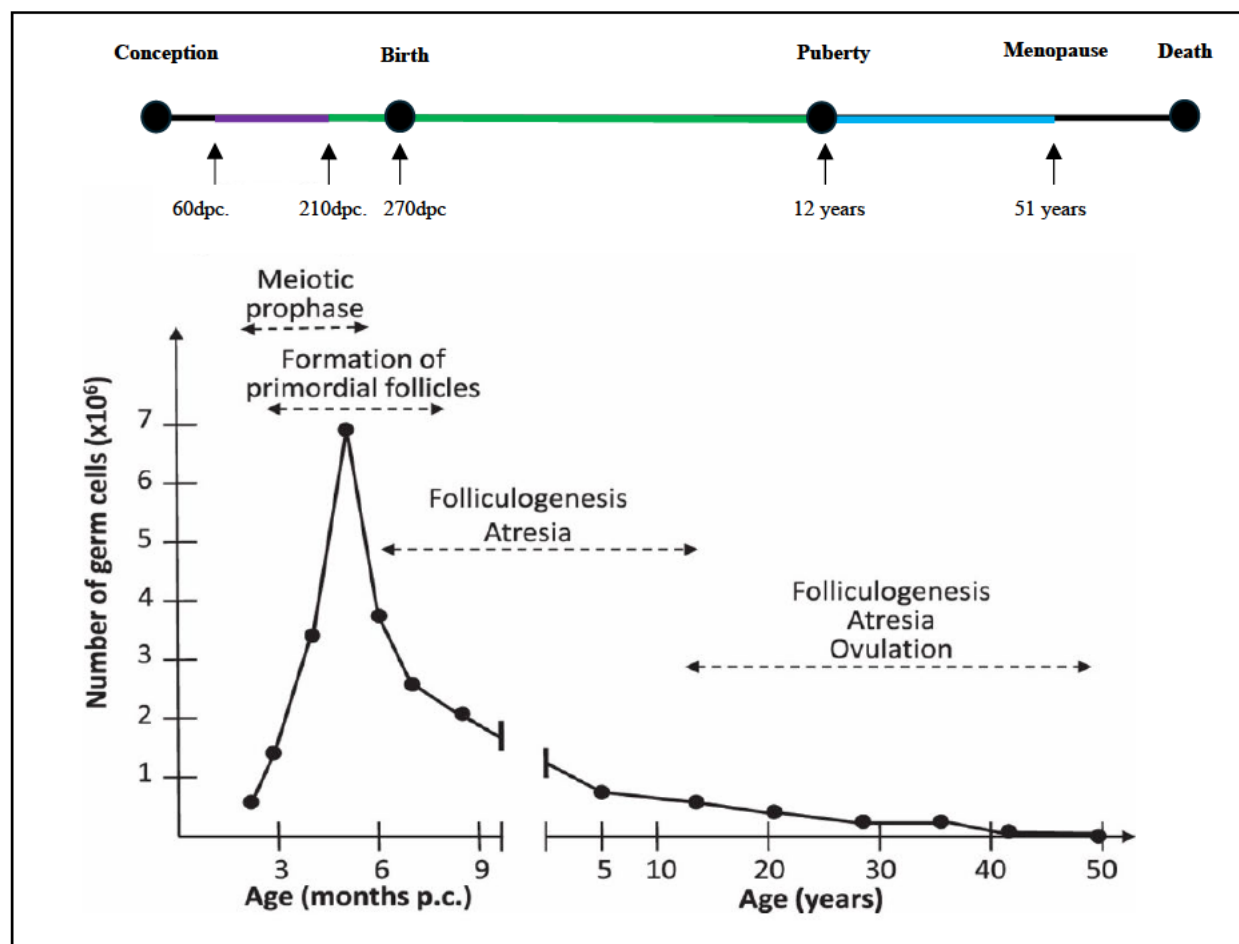


Figure 1.1: Germ cell attrition with age. PGCs migrate to the genital ridge where they start to undergo proliferation. Between 11-12 weeks' gestation, some oogonia begin to enter meiosis and progress to the diplotene stage of prophase 1 to form oocytes. The majority of

oocytes by this stage will have been lost to apoptosis, however those that survive are surrounded by pre-granulosa cells to form the primordial follicle pool. During follicle development, a number of primordial follicles are activated to grow, with the emergence of a dominant follicle that is subsequently ovulated. Ovarian follicle reserve is depleted with age leading to menopause when <1000 follicles remaining. Figure adapted from (Monniaux et al., 2014)- purple bar-meiosis initiation and formation of primordial follicles, green bar-folliculogenesis, purple bar- folliculogenesis and ovulation.

1.3 The process of follicle development

The stages of follicle development include primordial (single layer of flattened granulosa cells), transitional (single layer of granulosa cells with a mixture of flattened and cuboidal in shape) , primary (single layer of cuboidal granulosa cells), secondary (2 or more layers of cuboidal granulosa cells), preantral, multilaminar stage prior to formation of an antral cavity antral and preovulatory (Pedersen and Peters, 1968).

The process of follicle development is split into 2 distinct phases- gonadotrophin independent and gonadotrophin dependent, with the gonadotrophin dependent phase further split into FSH-dependent progression, and LH-responsive maturation (Hillier, 2001). Follicle development begins with the initial recruitment of primordial follicles into the growing follicle pool in a continuous manner when appropriate signals are received (McGee and Hsueh, 2000). Once follicles are activated, subsequent growth to primary and secondary stages occurs independently of endocrine factors and is controlled by paracrine factors, for example oocyte factors such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (Esencan et al., 2022).

FSH, a gonadotrophin produced and released from the anterior pituitary as a response to the pulsatile release of gonadotrophin releasing hormone (GnRH) from the hypothalamus, promotes the growth and differentiation of the granulosa cells by binding to the FSHRs located on the granulosa cells. The FSH receptor (FSHR) is not present at the primordial follicle stage but is expressed by follicles as they enter their growth phase (Oktay et al., 1997). This indicates that follicle stimulating hormone (FSH) does not play a role in primordial follicle activation. The FSHR is present on the primary follicle, however growth at this stage does not require gonadotrophins. This is demonstrated in ovaries of women with Kallmann syndrome (GnRH deficient), where ovarian histology showed small growing follicles with up to 3 layers of granulosa cells (Goldenberg et al., 1976). In hypogonadotrophic mice, pre-antral follicles were identified histologically, subsequently confirming the lack of dependence on gonadotrophins to this stage of development (Halpin et al., 1986). Mutations of FSHR are very rare but can present in women as primary and secondary amenorrhoea, premature ovarian insufficiency (POI) and infertility. Ovarian histology demonstrates impaired follicle maturation with arrest at the small antral follicle stage, again indicating early stages of follicle growth are gonadotrophin independent (Desai et al., 2013, Kuechler et al., 2010, Liu et al., 2017). This has also been demonstrated in ovarian tissue culture, whereby supplementation of culture media with FSH has been shown to have no effect on follicle activation and growth in the early stages of tissue culture (Wright et al., 1999).

As follicles progress to the pre-antral follicle stage, they begin to express the FSHR, but they do not rely on FSH for growth and development (Oktay et al., 1997, Craig et al., 2007). However, during the transition between pre-antral and antral follicle stages, reaching a diameter of 2-5mm, the follicle becomes increasingly responsive to and subsequently

dependent on FSH (Craig et al., 2007), switching the development mechanism from intraovarian factors (Orisaka et al., 2021). Cyclical recruitment commences with puberty and is in response to increased FSH levels in the follicular phase of the menstrual cycle. Every month, approximately 5-15 antral follicles commence FSH dependent growth as a response to cyclical increasing circulating FSH levels during the luteal-follicular phase (Orisaka et al., 2021). In the human, only a single follicle from the cohort is selected to gain dominance and ovulate every cycle. Due to the FSH-dependence at this stage of follicle development, only those antral follicles that are at a more advanced stage of maturation, and therefore more sensitive to FSH at the inter-cycle rise in FSH, will continue to mature when the FSH levels begin to decline, known as the FSH window (Palermo, 2007, van Santbrink et al., 1995). As the FSH levels fall, all remaining follicles below the FSH window threshold arrest in their development and undergo atresia, resulting in a single dominant follicle in preparation for ovulation (van Santbrink et al., 1995). Luteinising hormone (LH) is another gonadotrophin hormone produced and released by anterior pituitary. In the latter stages of follicle development, it promotes follicular growth and steroidogenesis, primarily in the theca cells of the follicle. As a result of FSH, the dominant follicle, at around 10mm diameter, develops LH receptors, increasing its sensitivity to LH in preparation for the subsequent LH surge and ovulation. Subsequently, the dominant follicle becomes more responsive to LH and less dependent on FSH. The FSH levels gradually decline during the follicular phase, until the mid-cycle rapid increase in LH level as a result of increasing oestrogen concentrations with a switch from hypothalamic and pituitary negative feedback to positive feedback. This initiates final oocyte maturation and ovulation with the remaining somatic cells forming the corpus luteum (Kumar and Sait, 2011).

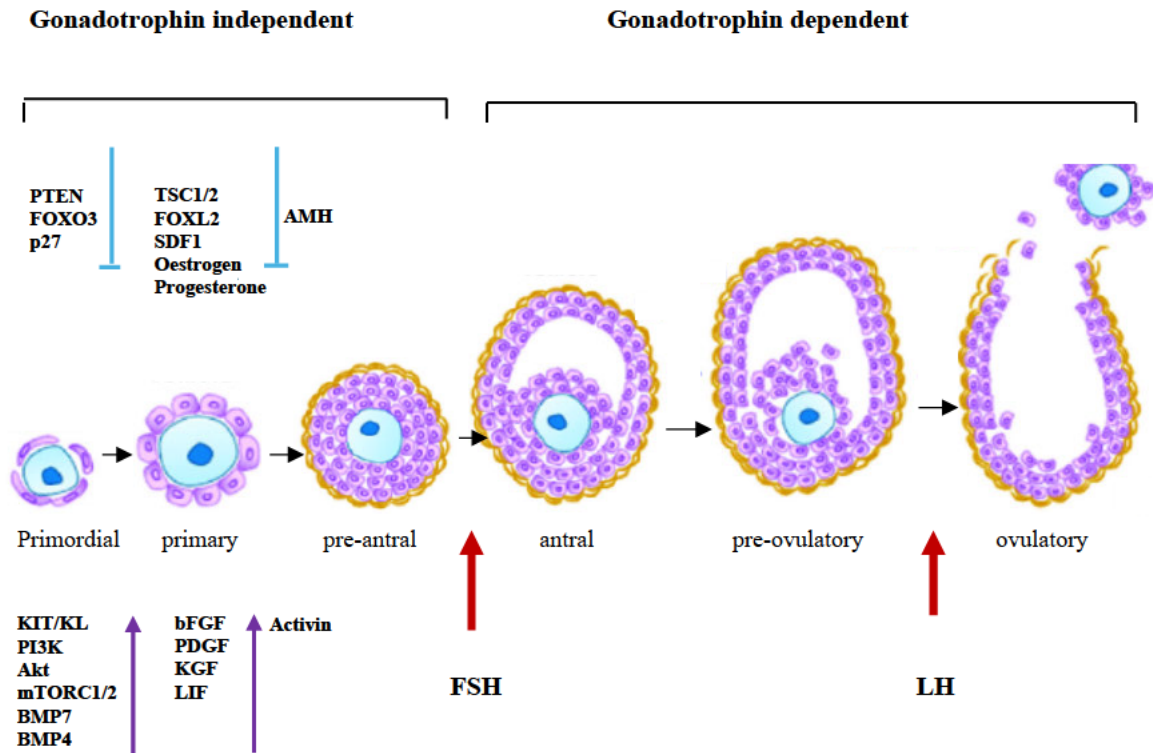


Figure 1.2: Follicle development. Oocytes are surrounded by flattened granulosa cells to form the primordial follicle pool. During primordial follicle activation, granulosa cells take a cuboidal shape to form primary, secondary and antral follicles. Up to this stage of follicle development, the process is considered gonadotrophic independent. The FSHR is present, however, they do not rely on FSH for growth and development. Primordial follicle activation is influenced by follicle activation (purple arrow) and repressor pathways (blue line) and the surrounding extracellular matrix. Created using Biorender.

1.4 Primordial follicle activation

Primordial follicles in human ovaries reside in the ovarian cortex and constitute the ovarian reserve. The process of primordial follicle activation is the initial recruitment of the follicle into the growing follicle pool. It is characterised by the differentiation of flattened pre-granulosa cells to cuboidal granulosa cells (Gougeon and Chainy, 1987b). Granulosa cells

become mitotically active, forming layers and maintaining connections with the oocyte that also undergoes growth (Reddy et al., 2008a).

There are a number of processes that influence follicle activation. These include activation pathways to initiate follicle activation and repressor pathways to maintain follicle dormancy. The surrounding extracellular matrix can also influence follicle activation (Nagamatsu et al., 2019, Woodruff and Shea, 2011, Xu et al., 2006c). It is the fine balance between the activation pathways and repressor pathways which dictates a female's reproductive lifespan, allowing for a steady supply of follicles to support final maturation to ovulation and preventing precocious activation. As discussed above, primordial follicles lack gonadotrophin receptors therefore it is widely accepted that this phase of follicle activation is gonadotrophin independent (Oktay et al., 1997) and given their lack of an immediate blood supply within the ovarian cortex, primordial follicles have to rely on effective paracrine signalling within the local follicle microenvironment.

1.5 PI3K/AKT signalling pathway

There are a number of locally acting growth factors in the human ovary involved in initiating and regulating primordial follicle activation. KIT ligand (KL) (also known as stem cell factor) is a cytokine growth factor derived from granulosa cells (Thomas and Vanderhyden, 2006) whose role includes regulation of cell survival and proliferation, regulating theca cell growth and subsequent production of theca cell factors including keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and transforming growth factor β (TGF β) (Jones and Pepling, 2013) (figure 1.3). Granulosa cell (GC) induction of mammalian target of rapamycin complex (mTOR) leads to the secretion of KL which binds to c-KIT receptor on the surface of the oocyte, activating the phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt)

signalling pathway, one of the major signalling pathways that coordinates the activation, growth and differentiation of primordial follicles (Reddy et al., 2008a). Other growth factors involved in activation of the PI3K pathway include insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF). Upon ligand binding, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), a process that can be reversed by Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN). PIP3 recruits next binds to phosphoinositide dependent-kinase 1 (PDK1) and Akt and subsequently recruits them to the membrane, resulting in PDK1 phosphorylating AKT (Telfer et al., 2023). PDK1 can also activate other protein kinases of the AGC family, such as Ribosomal protein S6 kinase beta-1 (S6K1), the kinase of ribosomal protein S6 (rpS6), via phosphorylation of T229 in S6K1 (Reddy et al., 2009b).

Akt is further phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) at serine 473 (Ser473) for its full activation, which in turn regulates a number of downstream targets. Activated Akt phosphorylates downstream forkhead box O3 transcription factor (FOXO3A). This results in nuclear exclusion of FOXO3A, leading to primordial follicle activation. FOXO3A is an inhibitor of primordial follicle activation, and this is demonstrated in studies with *Foxo3a*^{-/-} female mice where global primordial follicle activation results in oocyte depletion and resultant primary ovarian failure (POF) (Castrillon et al., 2003).

PTEN is an inhibitor of the PI3K pathway, suppressing primordial follicles to maintain them in a dormant state (Reddy et al., 2008b). Numerous studies have shown that a loss or inhibition of PTEN results in ovarian follicle activation with resultant oocyte depletion and premature ovarian insufficiency (POI) (Reddy et al., 2008b, Maidarti et al., 2019). In human ovarian tissue, after treatment with dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate (V) (bpV(HOpic)), a PI3K pathway promoter, there was enhanced

follicle activation with progression to secondary follicle stage, however subsequent growth was poor with significant deterioration in morphological health (McLaughlin et al., 2014a).

PDK1 is an important downstream effector of the PI3K pathway. It is essential for maintaining primordial follicle survival during the prolonged period of dormancy, thus determining the duration of female fertility. Deletion of PDK1 in mouse oocytes resulted in accelerated loss of primordial follicles from their quiescent state due to under activation of Akt/S6K1/rpS6 signalling in oocytes (Reddy et al., 2009b). This differs to PTEN loss which is associated with excessive primordial follicle activation resulting in loss of the resting follicle pool due to atresia. Both processes of primordial follicle loss are likely to be implicated in ovarian aging (Reddy et al., 2009a). However, PTEN deletion in growing follicles has not been shown to have a significant effect on follicle growth (Reddy et al., 2008a).

mTORC is another downstream substrate of Akt (Zhao et al., 2021). mTORC is a serine/threonine kinase which comprises of 2 distinct protein complexes, mTORC1 and mTORC2. Following phosphorylation of Akt by mTORC2, a number of downstream targets are activated. Akt activates the mTOR1 pathway through destabilising tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2), resulting in the loss of inhibitory effect on mTOR and subsequently upregulating mTOR activity. The newly activated mTORC1 proceeds to phosphorylate and subsequently activate its downstream effectors S6 kinase 1 (S6K1), ribosomal protein S6 (rpS6), and eukaryotic translation initiation factor 4E (4EBP1), promoting cell growth and proliferation (Huang and Manning, 2008). Deletion of TSC1 or TSC2 from the oocytes of mice leads to mass activation of primordial follicles at the time of puberty, resulting in depletion of the entire resting follicle

pool and POF (Adhikari and Liu, 2010). This was found to be due to elevated mTORC1 activity, suggesting that suppression of mTORC1 activity by TSC1-TSC2 complex is necessary to maintain primordial follicle quiescence. Equally, when ovarian tissue from *Pten*-mutant mice (*OoPten*^{-/-} is treated with the mTORC1 inhibitor rapamycin, the ovarian reserve was largely reserved, thus preventing POF (Adhikari et al., 2013).

Work using knockout mouse models has highlighted the importance of the phosphatidylinositol-3'-kinase (PI3K-AKT) signalling pathway within the oocyte in regulating follicle activation (Reddy *et al.*, 2008). Using pharmacological inhibitors of PTEN *in vitro* leads to increased activation of human primordial follicles (Li *et al.*, 2010; McLaughlin *et al.*, 2014), whereas treatment with rapamycin (an inhibitor of mammalian target of rapamycin complex 1: mTORC) results in decreased activation (McLaughlin *et al.*, 2011).

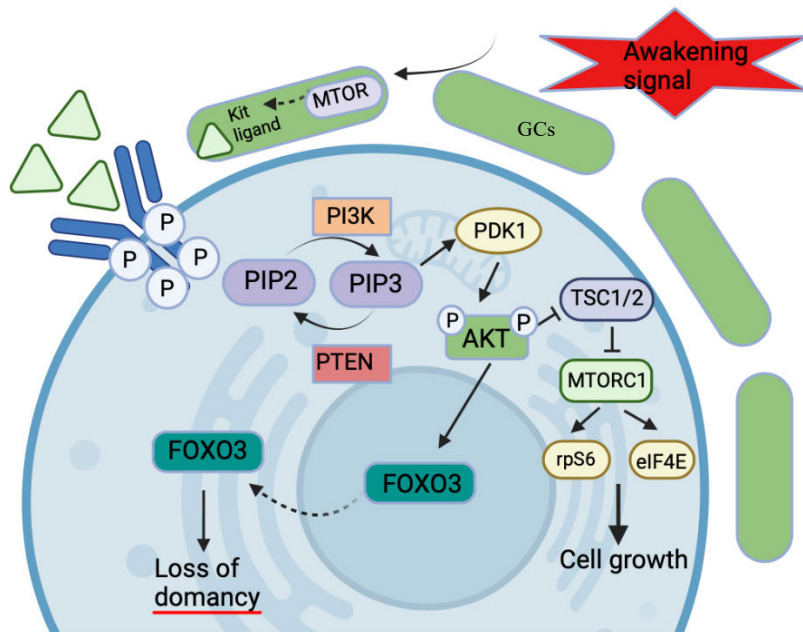


Figure 1.3: PI3K pathway for primordial follicle activation. Granulosa cell (GC)

induction of mTOR leads to the secretion of KL which binds to c-KIT receptor on the oocyte surface that triggers the activation of the PI3K pathway. Phosphorylation of AKT results in the nuclear exclusion of FOXO3A which results in loss of follicle dormancy and subsequent follicle activation and activates downstream mTOR pathway, promoting cell growth.

Adapted from (Telfer et al., 2023). Created with BioRender.com

1.6 Hippo signalling pathway

Another important pathway implicated in primordial follicle activation is the Hippo signalling pathway, an inhibitory pathway which maintains organ size in mammals by controlling the balance between cell proliferation and apoptosis (Tumaneng et al., 2012). It consists of a kinase cascade pathway which involves large tumour suppressor kinase (LATS1/2), mammalian Ste-20 like kinase (MST1/2), the adaptor Salvador (Sav) and Mps one binder (Mob) proteins which prevent nuclear access to Hippo signalling effectors Yes-

associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) via phosphorylation (figure 4). When Hippo signalling is disrupted, non-phosphorylated YAP enters the nucleus and interacts with transcriptional enhanced associate domain (TEAD) transcriptional factors. This increases the expression of downstream biochemical signals for the CCN growth factors (CYR61 (cysteine-rich angiogenic protein 61 or CCN1), CTGF (connective tissue growth factor or CCN2) and NOV (nephroblastoma overexpressed or CCN3)) (CCN) and baculoviral inhibitors of apoptosis repeat containing (BIRC) apoptosis inhibitors resulting in the stimulation of cell growth (Pan, 2007). The Hippo signalling pathway has no specific receptors or ligands but rather responds to intracellular and extracellular stimuli including changes within the cytoskeleton and the changes to cell-cell contact (Tsoi et al., 2019).

The role of the Hippo signalling pathway in follicle activation in the ovary is increasingly apparent. Multiple studies have used mouse models with knock out mice for different Hippo pathway effectors. LATS1-null female mice demonstrate widespread primordial follicle activation with subsequent POF (St John et al., 1999). Knockout of Yap1 in ovarian granulosa cells resulted in disruption of ovarian follicle development with subsequent subfertility due to anovulation (Lv et al., 2019).

Disruption of the Hippo signalling pathway also results from non-physiological conditions including physical disruption of the integrity of the ovarian cortex during fertility preservation procedures, ovarian tissue drilling and with chemotherapy exposure (Devos et al., 2023). Ovarian cortical fragmentation triggers actin polymerization (G-actin to F-actin) leading to decreased YAP phosphorylation with subsequent enhancement of follicle growth activation (Hsueh and Kawamura, 2020, Kawamura et al., 2013a, De Roo et al., 2020a, Devos et al., 2019, Grosbois and Demeestere, 2018a).

Inhibition of the Hippo signalling pathway is via the mechanical forces created by the ECM within the ovarian tissue (Kawamura et al., 2013a). Cytoskeleton stiffness and cell-cell contact, indicating increased cell density, are both implicated in the upstream regulation of the Hippo signalling pathway, resulting in increased phosphorylation of YAP and subsequent inactivation (Halder et al., 2012a, Fu et al., 2022). Several proteins can act as sensors for the Hippo signalling pathway, for example the Crumbs complex. In high density cell cultures, the Crumbs complex senses cell density via the Hippo signalling pathway, and subsequently interacts with YAP/TAZ and facilitates its phosphorylation and subsequent suppression of the TGF- β -SMAD signalling pathway (Varelas et al., 2010).

As early as the 1930s, ovary damaging procedures have been used to encourage ovulation in PCOS patients firstly through ovarian wedge resection (Stein and Leventhal, 1935) and more recently ovarian drilling with diathermy or laser (Farquhar et al., 2012). Similarly, in patients undergoing ovarian tissue cryopreservation as a measure of fertility preservation for oncological conditions, when the tissue was auto-transplanted back with the aim of restoring ovarian function and reproductive potential, there was evidence of follicle growth in the tissue fragments (Donnez et al., 2011). A study looking at culturing ovarian cortical tissue confirmed that fragmenting the ovarian cortex results in disturbance of the Hippo signalling pathway with subsequent increased primordial follicle activation (Grosbois and Demeestere, 2018b). They also identified a decrease in TSC1 and LATS1 expression on day 1 of tissue culture, plus a 2nd wave of Hippo disturbance on day 6 of culture, resulting in increased BIRC1 and CCN2 expression, concomitant with high expression levels of KL and Akt, indicating that there is a synergistic relationship between the PI3K/Akt and Hippo signalling pathways during ovarian follicle development. Both follicle activation and quiescence are

influenced by mechanical signalling within the ovary, and this occurs through both the Hippo and PI3K/Akt pathways involving YAP, TAZ, PTEN, mTOR, and Foxo3A (Shah et al., 2018).

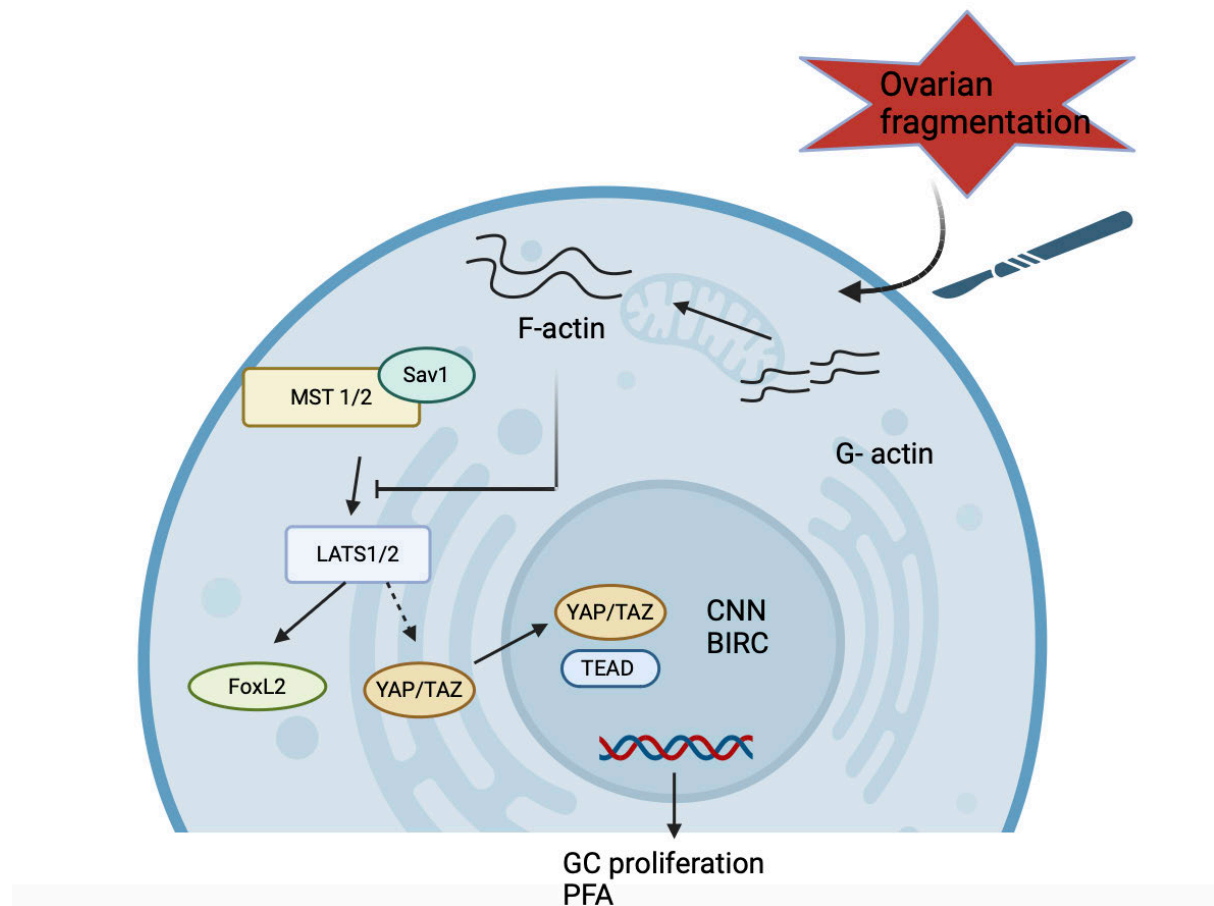


Figure 1.4: Hippo signalling pathway. Disruption of the Hippo signalling pathway through ovarian cortex fragmentation results in a switch in the G-actin/F-actin ratio in the GCs, resulting in the inhibition of LATS1/2 activity and YAP1 dephosphorylation and translocation into the nucleus. YAP/TAZ interact with the TEAD transcription factors which results in the expression of target genes important for primordial follicle activation (PFA) and

GC proliferation. Adapted from (Hsueh et al., 2015b) and (Telfer et al., 2023), Created with BioRender.com.

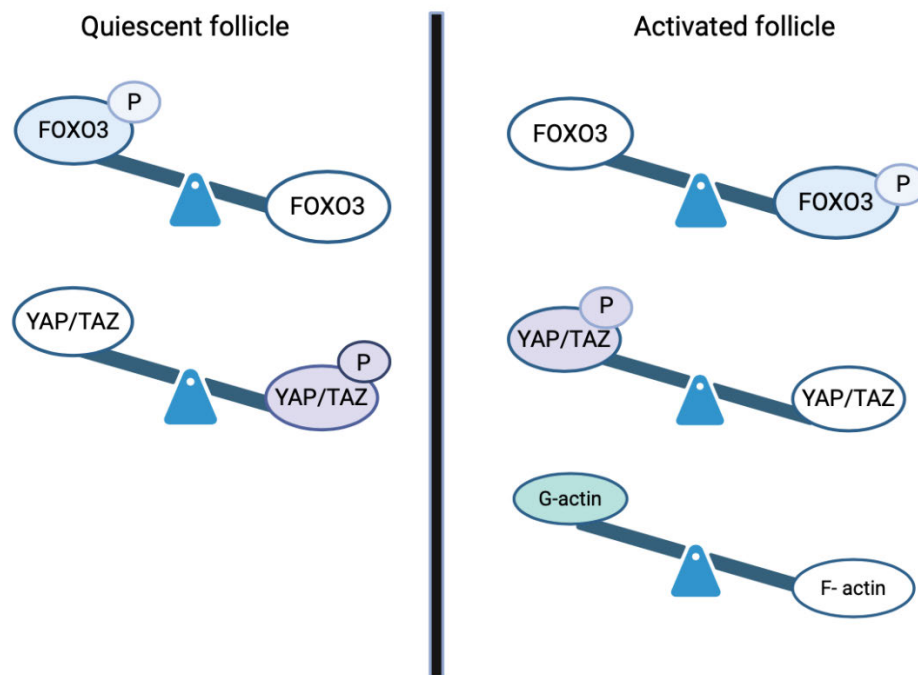


Figure 1.5. Follicle quiescence and activation. Illustrations represents a weighing scale; scale down indicates a higher proportion of substrate present compared to the scale in the up position. There is a delicate balance between the activator and repressor pathways. In quiescence, the PI3K Akt pathway will keep FOXO3A unphosphorylated to prevent follicle activation, the Hippo signalling pathway YAP/TAZ remains phosphorylated to prevent nuclear inclusion. In an activated follicle, phosphorylation of FOXO3A results in nuclear exclusion, enabling follicle activation. In the Hippo signalling pathway, when a follicle senses mechanical disruption, G-actin is polymerised to F-actin in the GCs, resulting in increased phosphorylation of YAP/TAZ, leading to follicle activation. Adapted from (Shah et al., 2018). Created with BioRender.com.

1.7 Other regulators of primordial follicle activation

There are a number of factors that are involved in promoting PFA. Basic fibroblast growth factor (bFGF) is involved in a wide range of ovarian functions including steroidogenesis and granulosa cell mitosis. It has also been found to induce PFA in a rat model similar to KL (Nilsson et al., 2001). Leukaemia inhibitory factor (LIF), a granulosa cell-secreted cytokine, acts within another activation pathway, the JAK-STAT pathway and like bFGF, has been shown to encourage primordial to primary follicle transition (Nilsson et al., 2001). Bone morphogenetic proteins 4 and 7 (BMP4 and BMP7) are growth factors within the TGF β superfamily (Skinner, 2005) and have been shown to promote primordial follicle activation, with BMP-4 having an important role in follicle survival (Skinner, 2005).

Anti-Mullerian hormone (AMH), like the BMPs, is a member of the TGF β superfamily, but conversely has an inhibitory effect on primordial follicle activation. AMH is produced in the granulosa cells of growing follicles and is used in clinical application to predict ovarian response to exogenous gonadotrophins during the controlled ovarian stimulation in IVF.

Studies confirming AMH's inhibitory effect on primordial follicle activation have shown that AMH null mice show early depletion of primordial follicle pool (Durlinger et al., 1999) and that by adding AMH to ovarian tissue culture, fewer growing follicles were found (Durlinger et al., 2002).

1.8 The ovarian stroma and its effect on primordial follicle activation

The ovarian stroma is the supportive connective tissue framework that encompasses the ovarian follicles, providing structural support and facilitating bidirectional paracrine

signalling necessary for follicle development (Kinnear et al., 2020, Shea et al., 2014). It consists of connective tissue containing stromal cells, neurons, blood vessels and the extracellular matrix (ECM) (Shea et al., 2014, Kinnear et al., 2020). The ECM is an intricate network of macromolecules, arranged in a particular orientation to facilitate several processes including adhesion, migration and proliferation through cell receptor interactions and the effect of mechano-transduction (Taipale and Keski-Oja, 1997). Recent proteomic studies have identified and spatially mapped over 1500 proteins in the human ovary (Ouni et al., 2022) including collagen subtypes, essential in providing structural support (Kinnear et al., 2020), glycoproteins including elastin for tissue flexibility (Vindin et al., 2019), and fibronectin and laminin which contain integrin binding sequences essential for initiating proliferation and differentiation of follicular somatic cells (Kinnear et al., 2020, Monniaux et al., 2006).

Collagen is the most abundant of the ECM proteins, accounting for 30% of protein mass in humans (Tang, 2020) and 49% of the total ovarian cortical matrisome (Ouni et al., 2022). They belong to a large family of proteins which are made up of combinations of 3 different polypeptide chains assembled in a triple helix structure (Lind et al., 2006). There are a number of different types of collagen present in the human ovary. Within the human ovarian cortex, collagen I and III have been found in distinct concentric layers in the outer ovarian stroma between the tunica albuginea and the stroma layer surrounding the primordial follicles, and collagen IV most abundantly in the basal lamina (Lind et al., 2006). However, a recent proteomic study found collagen VI to be the most abundantly found, particularly in the ECM (Ouni et al., 2019). Collagen VI has been found to have multiple roles within the ovarian stroma, firstly providing structural support and tensile strength and secondly providing a protective effect in counteracting oxidative damage and apoptosis (Cescon et al., 2015).

The composition of the ovarian stroma, in particular the distribution of stromal collagen, changes throughout the process of follicle development to be able to facilitate the demands of the growing follicle (Grosbois et al., 2023). Matrix metalloproteinases (MMP) 2 and 9 are responsible for degrading basement membrane collagens in preparation for ovulation. Lysyl oxidase (LOX) stabilises collagen by catalysing collagen cross linking formation within the ECM (Endo et al., 2006).

Elastin is an important component of the ECM, inferring flexibility and resiliency and allows for reversible extensibility. It is essential for maintaining function in several different organ systems including skin, lungs and vasculature. Elastin fibres are approximately 1 μm in diameter (Trębacz and Barzycka, 2023) and consist of 2 discrete components, elastin, the main component and microfibrils, mainly fibrillin-1 (Ouni et al., 2021). Tissue elasticity is not only based on the actual quantity of elastin within the tissue but also from the type of elastin fibre network and its spatial arrangement within the tissue. Elastin synthesis in human begins during late fetal stages, peaks the early post-natal period with gradual production decline and eventual cessation in adulthood (Swee et al., 1995). The elasticity of the ECM varies with age. The ECM of the prepubertal ovary, along with increased collagen content, is found to contain less elastin, contributing to increasing tissue rigidity, to maintain primordial follicle quiescence. Moving towards puberty, the elasticity of the ECM significantly increases in order to facilitate follicle development, likely as a response to increasing oestrogen levels (Ouni et al., 2020) before elastin content declines with age, contributing to age related ovarian fibrosis (Ouni et al., 2021).

The role of different pathways in promoting and inhibiting primordial follicle activation are well studied, however there is significant interest in the role of the ovarian stroma in follicle development, from as early as primordial follicle activation.

The ovary has two very distinct compartments, which differ greatly due to the composition, organisation and density of the ovarian stroma. The stiffer outer ovarian cortex is the residing place of the resting primordial follicle pool. This rigid environment, namely due to the collagen composition in the ovarian cortex, through mechano-transduction, prevents precocious activation of the resting follicle pool thus ensuring a reproductive lifespan of several decades (Silber et al., 2018). The majority of primordial follicles are found in the outer most aspect of the ovarian cortex, with fewer found towards the ovarian medulla.

A recent study evaluated the role of the ECM maintaining dormancy in mouse primordial follicles in culture (Nagamatsu et al., 2019). After incubating the ovaries in a solution containing collagenase, thus digesting the key component of the ECM, collagen, they found significantly more cytoplasmic FOXO3A compared to control, indicating the oocytes had exited their dormant state. However, after applying exogenous pressure, the percentage of oocytes with cytoplasmic FOXO3A significantly decreased, cancelling out the effect of the collagenase solution. This demonstrates the role of mechano-transduction in maintaining primordial follicle dormancy.

The ovarian medulla differs significantly to the cortex, as it consists of loose connective tissue and is abundant in blood and lymphatic vessels and nerve fibres.

A recent study demonstrated that in the human ovarian tissue, there was uniform distribution of growing follicles across the ovarian cortex *in vivo*, however after 6 days of culture, there was a notable migration of growing follicles towards the cortical-medullary border (Grosbois et al., 2023). This study also demonstrated throughout the culture period, there was a graduation

degradation of collagen with a concomitant rise in elastin deposition, demonstrating ECM remodelling which is necessary to facilitate the demands of growing follicles.

More information on the impact of the ECM on follicle activation is notable in studies that have focused on engineering an artificial ovary where the major challenge is replicating a dynamic ovarian environment (Buckenmeyer et al., 2023). Requirements of a suitable bioengineered matrix include sufficient support to maintain a growing follicle's structure, pervasive to maintain cell to cell connectivity, deliver essential endocrine and paracrine support to promote further follicle development and the follicle being easily retrievable (Shea et al., 2014). A number of different hydrogel matrices have been reported, including collagen (Telfer et al., 1990), plasma clots (Gosden, 1990), fibrin (Kniazeva et al., 2015, Smith et al., 2014, Luyckx et al., 2014, Paulini et al., 2016), and alginate (Jamalzaei et al., 2020, Xu et al., 2006c, Hornick et al., 2013) with promising results. Using these matrices allows for investigating different mechanical properties of an ECM and assess how it impacts follicle growth and development. A study using different concentrations of alginate matrices, demonstrated that primordial follicle survival and normal morphology was higher in the stiffer matrix (Hornick et al., 2012). Using varying concentrations of alginate in a 3-dimensional culture system, a permissive, low density (low alginate concentration) matrix demonstrated enhanced oocyte growth with increased granulosa and theca cell proliferation with the high subsequent fertilisation and blastocyst formation compared to matrices of increasing stiffness (Xu et al., 2006c). This was further demonstrated in a study where more follicles in a less dense matrix achieved meiotic competence compared to those in a stiffer matrix (Jiao and Woodruff, 2013b) highlighting the influence a follicle's microenvironment can have on its developmental capacity.

1.9 Alterations in the composition of the ovarian stroma

There are a number of both physiological and pathophysiological conditions where alteration in the composition of the ECM has resulted in disordered follicle development (changes to the ovarian stroma as a result of hyperandrogenism is covered in section 1.23). Ovarian aging is characterised by progressive tissue fibrosis and is one of the first human organs to demonstrate fibrosis as a result of aging. This results in a more rigid ovarian stroma, limiting follicle expansion and maturation, and subsequent follicle quiescence. A study of ageing mouse ovaries showed increased tissue rigidity demonstrated by Young's modulus, as a result of increased stromal collagen content (Armagent et al., 2020). Young's modulus is a measure of stiffness within a material when pressure is being applied to the subject in one direction. A higher value for Young's modulus indicates increased stiffness.

When the older mouse ovaries were incubated in collagenase, the Young's modulus was similar to that found in the ovaries of younger mice. In a porcine model, significantly higher concentrations of collagen fibres were found in older ovaries with fewer elastin fibres compared to the young ovaries (Pennarossa et al., 2022). In human ovaries, there was increased collagen and reduced elastin deposition within the ovarian stroma with advancing age (Ouni et al., 2020).

POI is a condition that affects up to 1% of the female population and is characterised by menopause prior to the age of 40. However, women with POI can have sporadic ovarian activity as there are still some follicles within the ovary, with the chance of natural conception between 5-10% (Ben-Nagi and Panay, 2014). Studies have been performed involving laparoscopic ovarian *in vitro* activation, a technique whereby 10-12 small pieces of ovarian cortex are removed from one ovary and auto-transplanted in the contralateral ovary

and peritoneal pocket near to the ovary (Kawamura et al., 2020b) (Méndez et al., 2022). Results showed patients had evidence of resumption of ovarian function, spontaneous conception and conception through assisted reproductive techniques (ART). These studies would indicate that increased ovarian stiffness in POI patients can be counteracted by ovary damaging procedures, likely through activation of the Hippo signalling pathway and reducing the intrinsic tissue rigidity with the potential for recovering reproductive potential.

1.10 DNA damage in ovarian follicles

The current consensus is that human females are born with their entire supply of oocytes and new oocytes cannot be produced under normal physiological conditions. Oocytes can remain dormant for up to 5 decades and because of this, oocytes are susceptible to DNA damage. To be able to protect the genome stability, there needs to be a robust system within oocytes to firstly identify DNA damage and secondly to either repair or destroy the oocyte. This allows for a pool of high quality oocytes to remain available for development and subsequent ovulation, fertilisation and embryological development.

There are several different types of DNA damage but the most severe form being DNA double stranded breaks (DSBs). This type of DNA damage can result in structural changes to the DNA, genomic rearrangements and lead to the loss of functionality of the oocyte (Winship et al., 2018a). These can occur in the presence of a number of different factors including maternal aging as a result of oxidative stress (Titus et al., 2013), chemotherapy including cyclophosphamide, cisplatin and doxorubicin (Kujjo et al., 2010, Spears et al., 2019) radiotherapy (Kerr et al., 2012) and environmental toxins including bisphenol A (Susiarjo et al., 2007). After these exposures, oocytes can undergo repair but often will

undergo cell death and apoptosis, if sufficiently widespread potentially resulting in premature primordial follicle pool depletion and subsequent infertility and POI.

1.11 DNA repair pathways in oocytes and granulosa cells

DNA damage can accumulate over time, particularly in non-dividing or slowing dividing cells. If the DNA damage does not trigger cell cycle arrest, the DNA damage is subsequently left unrepaired, resulting in loss of function and mutations. It is essential that primordial follicles have a robust system of DNA damage recognition and repair mechanisms to ensure genetic integrity. Given that oocytes within primordial follicles can remain in an arrested stage at the diplotene stage of meiosis prophase 1 for up to several decades, they are more prone to accumulate DNA damage. Granulosa cells, as actively dividing cells, are also sensitive to DNA damage. Genetic integrity in granulosa cells is achieved by activating DNA damage repair pathways which lead to the subsequent arrest of the cell cycle.

Unrepaired or incorrectly repaired DNA DSBs result in infertility, miscarriage and genetic defects in offspring (Stringer et al., 2018). When recognised, all cells have the capacity to repair DNA damage, which typically occurs at the G1/S and G2/M-phase transition of the cell cycle (Oktay et al., 2015). Primordial follicles have a particular propensity to opt for apoptosis rather than DNA repair and this is likely a mechanism to best preserve the genomic integrity of the remaining follicle pool overall (Suh et al., 2006). Similarly, if it has not been possible to repair any DNA damage and the cell remains in an arrested state, the oocyte will undergo apoptosis (Nowsheen and Yang, 2012). However, studies have demonstrated that primordial follicles are capable of efficient and effective DNA damage repair (Nguyen et al., 2021, Stringer et al., 2020, Kerr et al., 2012). This could lend itself to the development of

treatments targeting inhibiting apoptosis of the primordial follicles to preserve the ovarian reserve in patients undergoing gonadotoxic treatment.

There are two principal repair mechanisms that are available to repair DNA DSBs. These are homologous recombination (HR) and non-homologous end-joining (NHEJ) (Winship et al., 2018a, San Filippo et al., 2008).

The preferred mechanism of DNA repair in primordial follicles is homologous recombination (HR), a process of repair which uses the sister chromatid as a template for error-free repair (Winship et al., 2018b) (figure 1.6). The process of HR is initiated by the MRN complex consisting of Meiotic Recombination 11 (MRE11), Rad50 and Nijmegen breakage syndrome 1 (NBS1), recognising the DNA DSB (Maidarti et al., 2020). The binding of the MRN complex to the DNA DSBs allows for the interaction of the NBS1 protein with Ataxia telangiectasia mutated (ATM), resulting in autophosphorylation of ATM at a serine residue (Rein and Stracker, 2014). Following this, ATM phosphorylates gamma H2A histone family member X (H2AX) at the C-terminal serine 139 (γ H2AX) (Nguyen et al., 2021). γ H2AX is a DNA repair protein that binds to the location of DNA damage and controls recruitment of DNA repair proteins (Winship et al., 2018b). γ H2AX binds to the DNA DSB, resulting in the initiation of the downstream pathway whereby DNA is either repaired or apoptosis occurs (Oktay et al., 2015). It binds to the DNA DSBs in a ratio of 1:1, making it a useful protein for accurate quantification and location of DNA damage (Nazarov et al., 2003). When proceeding with DNA repair, mediator DNA damage checkpoint protein (MDC1) is next activated and binds to γ H2AX, facilitated by breast cancer susceptibility gene 1 (BRCA1). MDC1 form foci with γ H2AX which then drives further recruitment of more MRN complexes and subsequent resources for DNA repair. In oocytes, the DNA strand resection is activated and leads to HR.

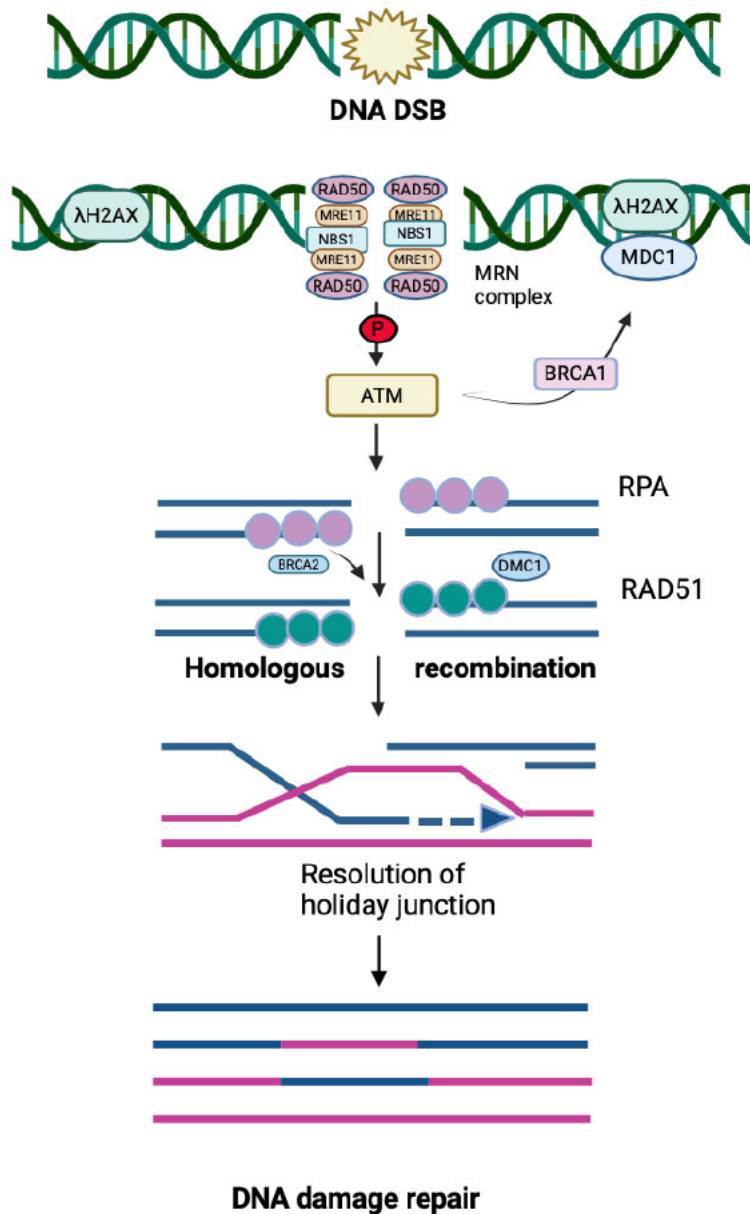


Figure 1.6. Homologous recombination for repair of DNA DSBs. The process of HR is initiated by the MRN complex recognising and binding to the DNA DSB. This allows for the interaction of the NBS1 protein with ATM, resulting in autophosphorylation of ATM at a serine residue. This in turn results in the phosphorylation of H2AX, producing foci of γ H2AX. γ H2AX binds to the DNA DSB, resulting in the initiation of the downstream pathway whereby DNA is either repaired or apoptosis occurs. Adapted from (Maidarti et al., 2020). Created using Biorender.

NHEJ repair involves the ligation of the two ends of the DNA DSB after the removal of any DNA single stranded break (SSB) overhang (Winship et al., 2018a) (figure 1.7). The first step of NHEJ repair involves Ku protein, a heterodimer of KU70 and KU80, detecting and subsequently binding to the ends of the DNA DSB. This prevents any further DNA damage and interaction with other DNA which could be detrimental to the repair process. Once bound to the DNA, the Ku protein recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs), X-ray cross-complementing protein 4 (XRCC4), DNA Ligase IV, XRCC4-like factor (XLF), and Aprataxin-and-PNK-like factor (APLF) to the sites of the DNA DSBs (Winship et al., 2018a). The Ku protein and DNA-PKcs recruit processing enzymes to the DSB and XRCC4/ Ligase IV complex join the two blunt ends of DNA together, resulting in the generation of an intact double helix. If end processing results in an overhang of single stranded DNA, this can be rectified by nucleus enzyme Artemis or alternatively the MRN complex (Ahmad et al., 2008).

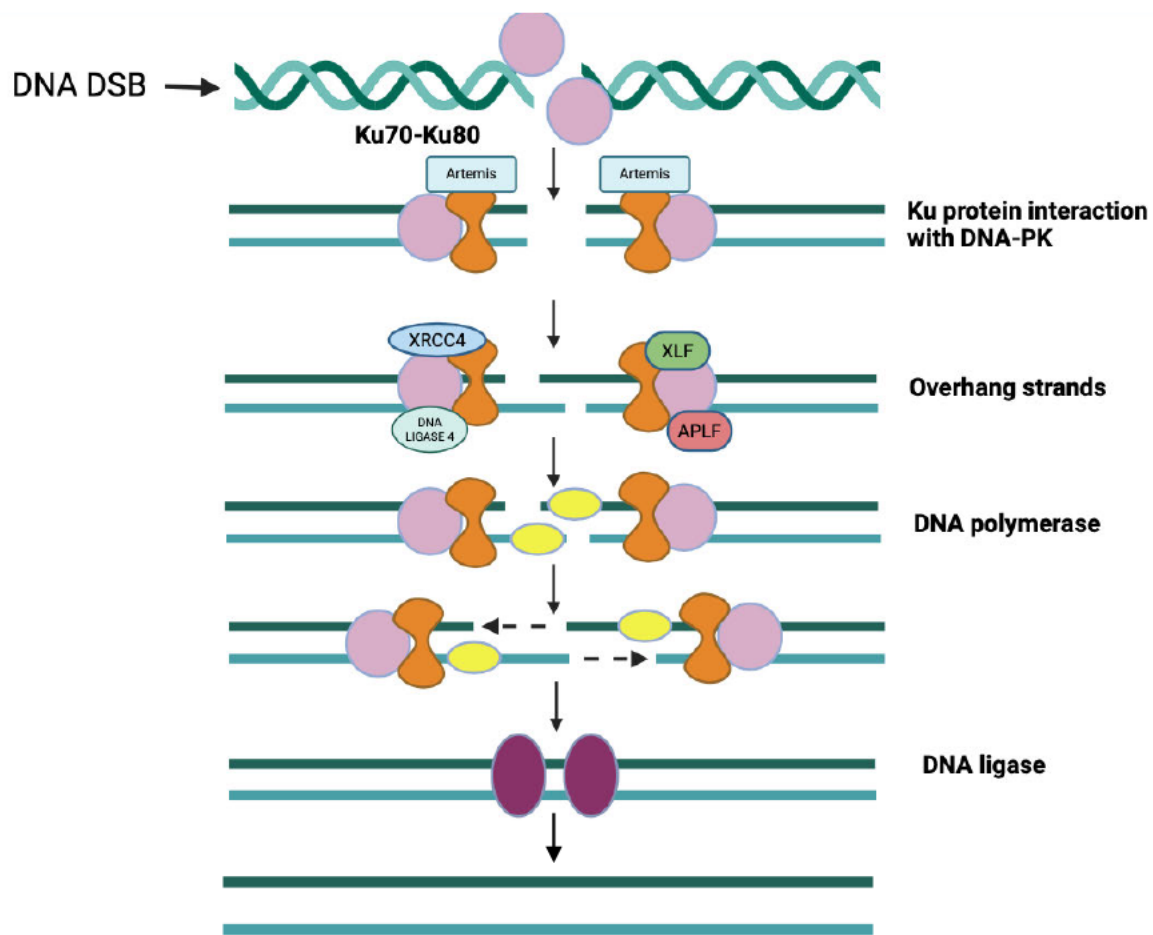


Figure 1.7. DNA DSBs NHEJ repair pathway. When the DNA DSB is detected, protein Ku70-Ku80 is activated and binds to the ends of the DNA break. This results in the recruitment of DNA-PKcs, XRCC4, DNA Ligase IV, XLF, and APLF which interact with the Ku protein and form a complex. This results in the end processing which may result in overhang strands which is rectified by DNA polymerase. DNA ligase joins the ends of DNA together, completing the process. Created using Biorender.com.

There are differences between these two processes. HR appears to be the preferred process of DNA repair for primordial follicles (Winship et al., 2018a). HR is an error free process of DNA repair as it uses a sister chromatid as a template for repair. However, it is restricted to the S (DNA synthesis) and G₂ (growth and preparation for mitosis) phases of the

cell cycle due to the necessity of the presence of the sister chromatid. NHEJ is more error prone compared to HR as it does not require sequence homology. This could result in inappropriate ends of DNA being ligated and subsequent chromosomal translocations which are associated with cancer (Helleday et al., 2007). It can however take place in any stage of the cell cycle and is not restricted to proliferating cells. The selection process resulting in the activation of one DNA repair pathway over the other is not fully understood. Components of both HR and NHEJ are expressed and functional in mammalian oocytes therefore both pathways are likely utilised (Zheng et al., 2005). NHEJ is the most commonly deployed DNA repair process in mitotic cells and in particular, oocytes in the later stages of development (Martin et al., 2018). HR predominantly repairs DNA DSBs in meiotic cells however it can be used in all cell types (Hong et al., 2013). The type of DNA DSB will also dictate which pathway of DNA repair is utilised, for example, if there is an overhang of DNA, NHEJ will be preferentially deployed (Frank-Vaillant and Marcand, 2002).

1.12 Meiotic DSBs

Meiosis is the process which involves the production of haploid gametes from diploid germ cells with one single round of DNA replication followed by two rounds of division. During fetal life, the female's entire pool of germ cells will enter meiosis and following HR, they will arrest in the diplotene/diactyate stage of prophase1 of meiosis (Pailas et al., 2022) and only resume meiosis when fully grown in response to an LH surge. Meiosis commences at the G1 (growth) stage of the cell cycle, whereby there is 1 pair of homologous chromosomes, one inherited from each parent. During the S stage, DNA replication occurs, and a pair of sister chromatids are created for each chromosome, held together by chromatid cohesion. Next, DNA DSBs are deliberately induced by endonuclease SPO11 at the start of meiotic

prophase to start meiotic HR, recruiting DNA repair proteins and forming recombinant foci. During this process, genetic material from the maternal and paternal copies of each chromosome are exchanged, producing new combinations of code on each chromosome, thus promoting genetic diversity (Murakami and Keeney, 2008, MacLennan et al., 2015). Importantly for meiotic DSBs, there need to be checkpoints to detect DNA damage. The process of repair must be tightly controlled to firstly prevent apoptosis occurring (Mahadevaiah et al., 2001) and secondly to prevent the transmission of genetic abnormalities (MacLennan et al., 2015). There are studies which have created DSB repair deficient mouse models and have shown complete oocyte elimination (Lutzmann et al., 2012) as lack of DNA repair by HR as part of meiosis resulted in loss by apoptosis (Di Giacomo et al., 2005, Inagaki et al., 2016)

1.13 Apoptosis in oocytes and granulosa cells

Over 99% of the cells in the female germline are eliminated via apoptosis and follicle atresia at varying times of follicle development (Tilly, 2001b). DNA damage checkpoints (DDC) exist from the time of meiotic arrest. The 2 points in the cell cycle where DNA damage repair (DDR) mechanisms are in place are G1/S and the G2/M (mitosis)-phase transitions. At G1, if DNA DSBs are identified, the cell cycle is arrested and the first stage initiated is the activation of ATM, leading to Checkpoint kinase 2 (Chk2) activation, resulting in cell cycle arrest. The main downstream effector of phosphorylated ATM and Chk2 is transcription factor 53 (p53) (Carroll and Marangos, 2013) (figure 1.8).

Transcription factor Trans-activating p63 (TAp63), an isoform of p63 and a member of the p53 family, is a key protein involved in oocyte apoptosis (Suh et al., 2006). TAp63 responds to DNA damage identified after prophase 1 of meiosis and only becomes active when DNA damage has occurred. During embryonic development and the process of meiosis, the oocyte

can tolerate hundreds of DSBs whereas postnatally, if a very small number of postnatal DSBs are induced, the oocyte is at risk of apoptosis. This may be due to lack of expression of TAp63 in prenatal meiotic oocytes (Kim and Suh, 2014).

There are 5 different variants of TAp63, α , β , γ , δ , and ϵ (Luan et al., 2021). TAp63 α variant is highly expressed in the oocytes of primordial follicles (Livera et al., 2008) and when DNA DSBs occur, this results in the activation of TAp63 α and subsequently apoptosis ensues (Luan et al., 2021). Mutations or inactivation in TAp63 α results in poor quality oocytes within the resting follicle pool, leading to increasing miscarriage rate and infertility. When TAp63 null mice were subjected to γ -radiation, ovarian histology showed that the primordial follicle pool was preserved, unlike in wild mice where the primordial follicle pool was largely lost indicating TAp63's essential role in apoptosis (Suh et al., 2006).

TAp63 is held in a closed dimeric form by transcriptional inhibitory domain (TID), which blocks the tetramerization interface and keeps TAp63 in an inhibited state (Deutsch et al., 2011, Straub et al., 2010). Further stabilisation of TAp63 occurs with the interaction of N-terminal transactivation (TAD) with TID and with the oligomerization domain, subsequently making the amino acids that are essential for transactivation even less accessible. The active form of TAp63 is the tetrameric formation and this is induced in the presence of genotoxins including chemotherapy and radiotherapy (Deutsch et al., 2011). In its active form, TAp63 enables the transcription of Puma and Noxa, 2 proteins that are members of the pro-apoptotic arm of the B-cell lymphoma/leukemia-2 (Bcl-2) family. These proteins have been shown to inhibit pro-survival Bcl-2 proteins and promote the function of BAX and BAK, two major pro-apoptotic Bcl-2 family members resulting in cell apoptosis. A study involving *Puma*^{-/-} and *Puma*^{-/-}*Noxa*^{-/-} mice demonstrated that primordial follicles were not lost to apoptosis after exposure to gamma radiation and interestingly the γ H2AX DSB foci that had formed as a result of the radiation exposure had resolved within 5 days. Subsequently healthy

offspring were produced, indicating that even after exposure to potentially genotoxic treatment, the DNA repair capacity is preserved and able to maintain genomic integrity (Kerr et al., 2012).

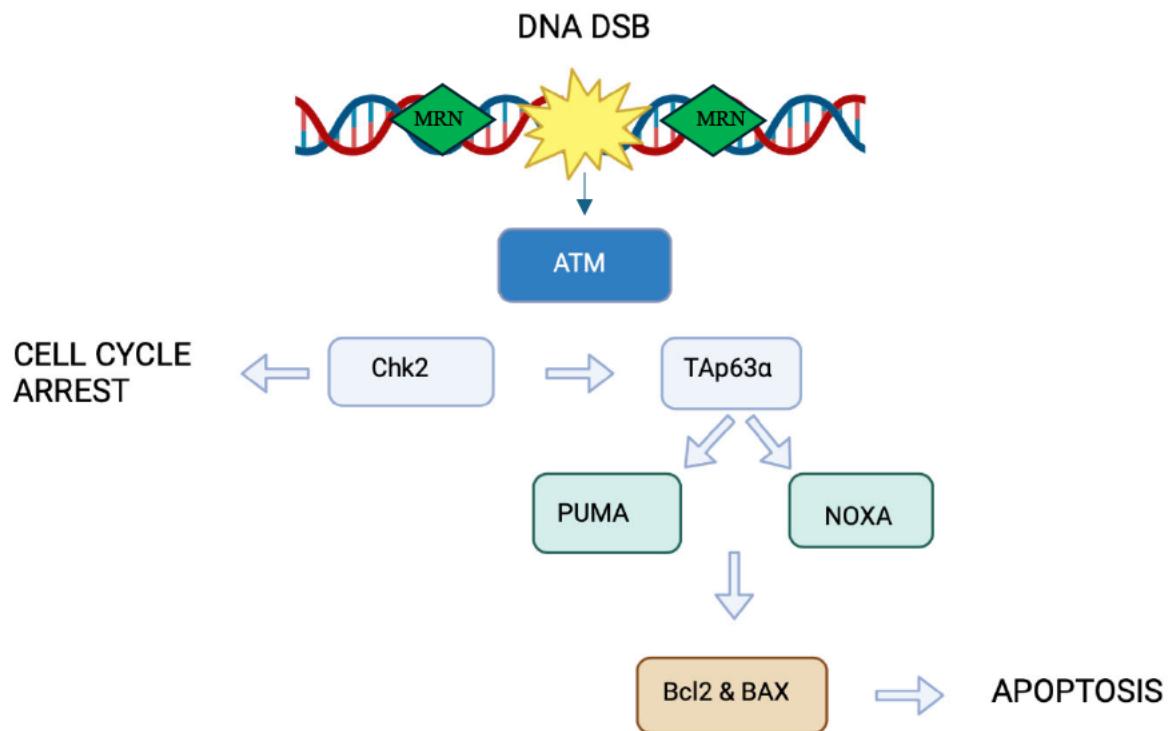


Figure 1.8: Apoptosis pathway in primordial follicles. On recognising the presence of DNA DSBs, the MRN complex phosphorylates ATM which in turn phosphorylates Chk2 leading to cell cycle arrest. TAp63 α is the downstream effector of Chk2 and ATM. Phosphorylation of TAp63 results in the active tetrameric formation results in the transcription of PUMA and NOXA, proapoptotic proteins, promoting the function of pro-apoptotic Bcl-2 family members BAX and Bcl2, leading to cell apoptosis. Created using Biorender.com.

1.14 Development of an *in vitro* culture system

Tissue culture systems have been developed over decades with the goal of producing developmentally competent oocytes (Bertoldo et al., 2018, Telfer and Andersen, 2021). Complete *in vitro* growth of primordial follicles with subsequent *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and production of live offspring was first achieved in the mouse (Eppig et al., 1996, O'Brien et al., 2003a). This ground-breaking work was not without flaws, with only a single mouse offspring being born who subsequently developed abnormalities in adulthood. It did however prove that the concept of complete oocyte development *in vitro* was potentially achievable (Eppig and O'Brien, 1996). Subsequent work to optimise culture conditions with the development of a 2 step culture system (O'Brien et al., 2003b) saw an improvement of mouse oocyte developmental competence with dozens of live offspring produced. This tissue culture model was subsequently trialled in other species (Hovatta et al., 1997, Telfer and McLaughlin, 2012, Picton and Gosden, 2000). The initial development of culture systems in human tissue which supported the activation and growth of primordial follicles was achieved, however development did not progress past secondary follicle stage, indicating these follicles would need to be isolated from the surrounding tissue in order to proceed with *in vitro* growth and maturation (Hovatta et al., 1997, Hovatta et al., 1999).

In the Telfer lab, much has been achieved in developing a multi-step serum free tissue culture system, able to demonstrate significant follicle growth and survival in both human and domestic animal models (Telfer et al., 2008a, McLaughlin et al., 2018, Walters et al., 2006). In 2008, the Telfer lab achieved primordial follicle activation and oocyte growth to the pre-antral follicle stage using human ovarian cortical tissue that had been mechanically loosened and did not require any artificial supportive matrices (Telfer et al., 2008a). Following on from this, they further developed their culture system and successfully produced human MII

oocytes from primordial follicles (McLaughlin et al., 2018). A multi-step process is required to achieve complete *in vitro* growth (IVG) and maturation of primordial follicles to metaphase 2 (MII) (figure 1.9). A most recent development has seen the development of human embryos with the potential for transfer, *in vitro* grown and matured from pre-antral follicles with the addition of neutrotrophic factor 4 (NT4) in culture (Guo et al., 2024).

The first step is primordial follicle activation and development, the second step is to optimise the growth of follicles from preantral to antral stage, the third step is ongoing oocyte growth in preparation for IVM in the fourth step (Telfer, 2019). Similarly to the ovarian stroma's role in follicle development, the culture system needs to be able to adapt to the changing demands of the growing oocyte and surrounding granulosa cells, whilst maintaining oocyte-somatic cell interactions as it progresses through the steps of ovarian tissue culture (Telfer, 2019).

1.15 Primordial follicle activation in *in vitro* culture

The majority of follicles within the ovarian cortex will be primordial follicles. Some of the earliest work into primordial follicle culture involved isolating primordial follicles prior to culture and encapsulating on to an artificial matrix, however the follicles did not grow past 24 hours of culture (Abir et al., 1999). This indicated that there is a role of the surrounding stromal tissue in ovarian follicle development (Hovatta et al., 1997, Hovatta et al., 1999). The step of PFA usually takes between 6-8 days to produce multi-laminar pre-antral (secondary) follicles (section 2.2). The ovarian cortical tissue is cut into strips with excessive stromal tissue removed and mechanically loosened (Telfer and McLaughlin, 2012). As a result of tissue preparation, the rate of PFA *in vitro* is enhanced compared to *in vivo*. This is likely due to the disruption of the Hippo signalling pathway (section 1.6). Tissue shape and stromal density can affect follicle activation rate. Culturing ovarian cortical tissue in strips appears to

promote more effective oocyte development compared to cubes (Wright et al., 1999), further highlighting the role of the ovarian stroma and mechano-transduction in maintaining follicle quiescence.

At the end of 6 days of tissue culture, any secondary follicles are isolated from the cortical stromal environment and grown individually. The process of isolating growing follicles from the surrounding ovarian cortical tissue can either be carried out enzymatically or through microdissection techniques or a combination of the two. During step 2 of tissue culture, the isolated preantral follicles are cultured in individual wells in culture plates for 6-8 days where antral cavities are formed. Studies have reported the use of different matrices to support preantral follicle growth including alginate hydrogels (Xu et al., 2006a), decellularized ovarian tissue and 3D microporous scaffolds (Laronda et al., 2015) (Laronda et al., 2017). At the end of this culture period, oocyte granulosa cells complexes can be retrieved from the follicle by applying gentle pressure.

For step 3 of the multi-step culture process, only complexes with complete cumulus and adherent granulosa cells are selected and cultured on membranes with activin-A and rFSH. The final stage of the multi-step culture process that has to occur before IVF can be carried out is IVM. This enables the oocyte to resume meiosis to the point of producing an MII oocyte capable of fertilisation.

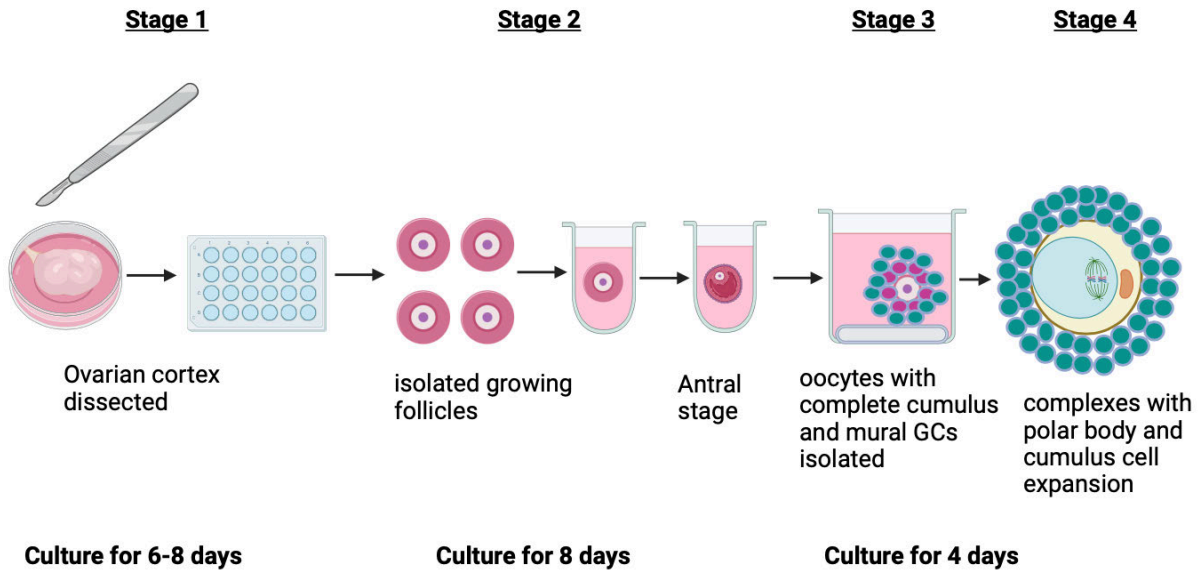


Figure 1.9. Multi-step tissue culture system for human follicle development. Step one- dissecting ovarian cortex from the medullary tissue, preparing primordial follicles for culture. Step two- after 6-8 days of culture, the growing follicles need to be isolated from the stromal tissue to be cultured from pre-antral to antral follicle stage. Step three- cumulus oocyte complexes (COCs) are isolated from antral follicles and are cultured for a further 4 days to 100µm diameter. Step four- COCs are prepared for IVM by placing in new culture medium and are examined for cumulus cell expansion (green), MII spindle formation and the presence of a polar body. Adapted from (Telfer, 2019). Image created using biorender.com.

1.16 Clinical application

The use of ovarian tissue culture systems to grow and mature oocytes *in vitro* from the primordial follicle stage has a wide range of clinical applications.

OTC as a form of fertility preservation (FP), has primarily been offered to girls and young women diagnosed with malignancy who plan to undergo gonadotoxic chemotherapy

treatment which would irreversibly affect their fertility (Anderson and Baird, 2019b). With improvement in cancer treatment and subsequent higher childhood cancer survival rates, more patients are considering pre-treatment OTC as an option.

A major concern with OTC for patients with a history of malignancy is reintroduction of malignant cells from the ovarian cortical strips. Bioengineering the artificial ovary provides a potential solution for this issue. Follicles are isolated and reconstituted with somatic cells and implanted within an artificial matrix prior to reimplantation to prevent malignant cells being reintroduced (Dolmans and Amorim, 2019a). The development of an ovarian tissue culture system capable of producing MII oocytes for IVF with successful live birth in humans from primordial follicle stage would avoid the need for OTC. It provides an exciting opportunity for women to have their own biological child, particularly those who are unsuitable for conventional artificial reproductive techniques (ART) e.g. women with POI, aggressive or hormone responsive malignancy and prepubertal females (Ghezelayagh et al., 2022).

In vitro tissue culture systems could be manipulated pharmacologically with the aim of maximising oocyte yield. PFA is primarily through the PI3K pathway (section 1.5). Multiple studies in animal models have shown that excessive PI3K activation results in enhanced PFA which leads ultimately to primordial follicle depletion and POF (Sobinoff et al., 2012, Reddy et al., 2008a). In studies where PTEN, a major negative regulator of the PI3K pathway, is inhibited, there is enhanced PFA with increased proportions of primary and secondary follicles. However, the morphological health and subsequent growth and survival of these growing follicles is compromised (McLaughlin et al., 2014a, Grosbois and Demeestere, 2018a). This is due to the accumulation of DNA damage and impaired DNA repair capacity as demonstrated in bovine oocytes when ovarian cortex was exposed to a PTEN inhibitor bpv(HOpic) (Maidarti et al., 2019). These findings are significant as bpv(HOpic) and the subsequent *in vitro* activation (IVA) has been utilised as a treatment option in women with

POI.(Kawamura et al., 2013b, Suzuki et al., 2015). These studies involved laparoscopic removal of ovaries from women with POI, with the ovarian cortex fragmented and treated with bpv(HOpic) and reimplanted after a period of vitrification. After ovarian stimulation, IVF and embryo transfer, a small proportion of women in both studies did achieve a pregnancy.

Ovary damaging procedures have been utilised to promote ovulation (Hsueh et al., 2015b). Ovarian drilling is a laparoscopic procedure that involves applying diathermy to the ovarian cortex and has been used for several decades in anovulatory females diagnosed with polycystic ovary syndrome (PCOS). Spontaneous ovulation and pregnancy rates vary with different techniques, ranging within one year of the procedure from 30-90% to 13-88% respectively (Mitra et al., 2015). The exact mechanism of action has yet to be elucidated but one theory is that the process of thermal destruction to the ovarian stroma results in a reduction in the local androgen levels, thus creating an intrafollicular environment less rigid and more permissive to follicle maturation resulting in a secondary rise in FSH levels, allowing for the emergence of a dominant follicle with subsequent mono-ovulation (Mercorio et al., 2022, Woodruff and Shea, 2011).

Another mechanism of the promotion of follicle growth as a result of ovarian drilling is through the activation of the Hippo signalling pathway (Hsueh and Kawamura, 2020, Kawamura et al., 2013a) (section 1.6). Studies involving ovarian cortex dissection in preparation for both tissue culture and fertility preservation have observed the effects of disrupting the Hippo signalling pathway (section 1.6). In patients with POI, drug free IVA has been used to promote follicle growth through the disruption of the Hippo signalling pathway. This involved removal of ovarian cortex laparoscopically and auto-transplanting the tissue into the pelvic sidewall near the contralateral ovary during the same surgical procedure

(Kawamura et al., 2020a). This approach shows promise of being able to promote fertility in women with diminished ovarian reserve, however given the degree of variation in success, a standardised approach needs to be established.

1.17 Transgender population: epidemiology

Gender identity describes the gender that individual recognises as their own (Korpaisarn and Safer, 2019), which may differ from their biological sex. For transgender people, their gender identity is incongruent with the gender assigned at birth, often leading to gender dysphoria and mental health issues. The incidence within the general population identifying as transgender has increased over the last few decades. In 2021 in the UK, the question of gender identity was added to the Census, allowing for the first time for the collection of data on the official size of the transgender population in England and Wales. 262,000 people, equating to 0.5% of the population, answered no when asked whether their gender identity was the same as their birth assigned gender (Biggs, 2023).

1.18 Transgender men- gender transitioning and fertility preservation

For transgender men, gender transitioning in a step wise approach includes social, medical and surgical transitioning. To gain the desired masculinising effects and suppress feminizing characteristics, transgender men may consider commencing gender affirming endocrine therapy, in the form of exogenous testosterone (Unger, 2016). Prior to commencing testosterone therapy, it is recommended by the most recent Standards of Care of the World Professional Association for Transgender Health (WPATH) to discuss fertility preservation options with patients (Coleman et al., 2012, Hembree et al., 2009). These discussions should occur regardless of age of transition and or interest in having a family (Park et al., 2022,

Feigerlová et al., 2019, De Roo et al., 2016). Fertility preservation should ideally occur prior to commencing testosterone therapy (Chen et al., 2018) due to the lack of definitive information regarding the impact of testosterone on reproductive potential and ovarian function and the degree of reversibility (Kolbuck et al., 2020).

As a growing population group, the transgender community are a pressing challenge for fertility services, with inconsistent NHS funding across UK and Ireland (Reckhow et al., 2023). In the UK, current fertility preservation options available to transgender men include oocyte and embryo cryopreservation. The patient undergoes ovarian stimulation with gonadotrophin injections, followed by oocyte retrieval, +/- fertilisation and subsequent freezing. These oocytes or embryos can be stored for up to 10 years and could subsequently be used in artificial reproduction by either the patient or surrogate. Fertility preservation should ideally occur prior to commencing gender affirming endocrine therapy, however if this has already been commenced, cessation from testosterone of 1-3 months is advised (Reckhow et al., 2023) although there are case reports of successful ovarian stimulation without discontinuing testosterone treatment. Despite 40% of transgender men expressing a desire to have their own biological children (Defreyne et al., 2020), the uptake for fertility preservation is as low as 3% (Auer et al., 2018, Nahata et al., 2017). Reasons include evoking gender dysphoria from invasive nature of transvaginal scanning and oocyte retrieval and the feminising hormonal effects from supra-ovulation (Cheng et al., 2019) (Reckhow et al., 2023).

There have been a number of studies that have reassuringly concluded that there appears to be a degree of reversibility of ovarian function and the preservation of reproductive potential in transgender men after cessation of testosterone therapy. Transgender men have been able

to conceive both naturally and through ART after cessation of testosterone therapy with no significant impact on pregnancy, delivery and birth outcomes (Light et al., 2014a, Thornton and Mattatall, 2021). A study of 12 transgender men, 6 of which had prior testosterone use and 6 without any testosterone use, compared ovarian stimulation outcomes with cisgender women who were oocyte donors (Amir et al., 2020). Results showed no difference in peak oestradiol levels, in the number of oocytes retrieved, the number of MII oocytes or oocyte maturity rates between groups. Another study of 13 transgender men, 7 with prior testosterone use and 6 without testosterone use, compared to cisgender females, showed no difference in peak oestradiol level number of oocytes retrieved or oocyte maturity rates between groups (Adeleye et al., 2019a).

A study compared ART outcomes between transgender men and age matched cisgender control women with male or tubal factor infertility (Leung et al., 2019). In the transgender male group, 61% of participants had previously been taking testosterone therapy and all had stopped it for their IVF cycle. Results showed more oocytes were collected from transgender male ovaries compared to control however, sub analyses showed that there was no significant difference between number of oocytes or percentage of mature oocytes retrieved between patients who had and had not been taking testosterone therapy. This study offered reassurance that even after testosterone therapy, that the ovarian stimulation outcomes were favourable for transgender men. All studies looking at ART outcomes for transgender men have reported successful live births with ART (Adeleye et al., 2019b, Amir et al., 2020, Leung et al., 2019). A recent study looked at the effect of testosterone treatment on oocyte fertilization and preimplantation embryo development in embryos from transgender men who had been previous taken testosterone therapy and compared to cisgender women. They found no difference in the number of suitable embryos to cryopreserve or in the morphokinetic parameters ascertained from time-lapse imaging between groups (Israeli et al., 2022)

indicating previous testosterone exposure did not appear to impact the number or quality of embryos produced. Collectively, this provides a degree of reassurance that transgender men who have a history of testosterone therapy use do retain reproductive capacity. However, to date, there are no studies that have demonstrated the rate of reversibility or whether it is dose or treatment duration dependent.

1.19 Ovarian tissue cryopreservation for transgender men

There is an interest in OTC for the transgender male population and how this could be carried out concomitantly at the time of their gender reassignment surgery which involves bilateral oophorectomy. Instead of future autografting the ovarian tissue, for transgender men, lab-based tissue culture techniques could be employed to activate, grow and mature non-growing ovarian follicles found in the ovarian cortex.

There are no studies that have successfully produced mature oocytes or embryos from transgender testosterone exposed ovaries from the primordial follicle stage of development (Bailie et al., 2023). There have been studies that have looked at this process from more mature follicle stages. One study took ovaries from transgender men on testosterone therapy and extracted COCs from ovarian medulla. They underwent *in vitro* maturation with 34.3% of collected COCs reaching MII oocyte stage, with 94.4% exhibiting a normal spindle pattern (De Roo et al., 2017). Another study conducted from the same lab group whereby all transgender men had a history of testosterone treatment, compared the oocyte survival rate and rate of normal spindle pattern between fresh transgender oocytes and those that had been cryopreserved at the COC stage and subsequently thawed. They found the rate of oocyte survival post thaw was 67.7% and there was comparable proportion of normal spindle structure and chromosomes alignment, 85.7% vs. 92.2% (Lierman et al., 2017). This is

significant as if OTC is to be a viable option for transgender men, oocytes must be able to survive the freeze thaw process.

The next important step is to ascertain whether testosterone exposed oocytes, matured *in vitro* can successfully be fertilised and demonstrate normal embryo development. Interestingly, from this same lab group, when MII oocytes were fertilised by intracytoplasmic sperm injection, fertilisation was achieved in 48 oocytes (34.5%), 25 (52.1%) embryos developed up to day 3, of which 11 (44%) had ≥ 6 cells with only one blastocyst being obtained on day 5 out of 1903 COCs. With significantly lower than expected maturation and fertility rates, aberrant cleavage patterns, and low rate of development to the blastocyst stage, this study concluded that testosterone treatment is likely to have a detrimental effect on the developmental competence of ovarian follicles (Lierman et al., 2021). This highlights that if OTC is to be considered a fertility preservation option for transgender men who have taken testosterone therapy, further studies are required to optimise this maturation process.

A subsequent study looked at the feasibility of improving the suboptimal embryo development rate through the use of spindle transfer (Christodoulaki et al., 2023). They collected ovaries from transgender men, one ovary from each pair was placed in cold medium, the other ovary in warm medium. Isolated COCs were *in vitro* matured for 48 hours, and the quality of the oocytes was assessed by calcium pattern releasing ability, embryo development after ICSI and staining for mitochondrial membrane potential. Control oocytes were from women undergoing ovarian stimulation as part of IVF treatment. They found similar oocyte maturation rate between the cold and warm medium (54% vs 57%). Control oocytes showed a significantly higher calcium releasing ability compared to transgender oocytes, where there was no difference in rate between cold and warm media (2.3AU vs 1.47AU vs 1.43AU, $p=0.046$). There was no difference in mitochondrial membrane potential across groups. After ICSI, fertilisation rate in the control group (84.2%) was significantly

higher than in the cold (40.4%, $p<0.01$) and warm (50%, $p<0.01$) groups, with significantly higher D5 blastocyst rate (control 25.4% vs cold 0%, $p=0.014$, vs warm 4.1%, $p=0.026$). When spindle transfer was applied to the cold and warm groups in an attempt to rescue poor embryo development, the day 5 blastocyst rate was improved in the cold group from 0% to 20.6% ($p=0.034$). This study concluded that oocytes from testosterone exposed were of an inferior quality and likely due to cytoplasmic issues which is why blastocyst numbers improved with spindle transfer, calling into question their suitability for fertility preservation.

All of the above studies were performed using COCs. A hurdle to OTC being a viable fertility preservation option for transgender men is that there are no studies that have demonstrated competence in *in vitro* activation, growth and maturation of oocytes to MII stage from primordial follicle.

The most recent ESHRE guideline on fertility preservation states that currently, OTC should not be used first line for fertility preservation for transgender men, however it can be proposed as an experimental option during oophorectomy as part of surgical gender transitioning (Anderson et al., 2020).

1.20 Androgen production and the androgen receptor in the ovary

In females, the circulating androgen precursors and active androgens are dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone and dihydrotestosterone (DHT) (Walters and Handelsman, 2018). In the presence of steroidogenic enzymes, DHEA can be converted to androstenedione and testosterone, which can both subsequently be aromatised into oestrogen.

Theca cells are endocrine cells in the ovary that arise from the ovarian stroma. After primordial follicle activation, granulosa cells change from a flattened to a cuboidal shape and in conjunction with the ovary, begin to secrete paracrine signals for theca cell formation from ovarian stromal cells. Once formed, the theca develops into 2 distinct layers with differing roles within follicle development; the theca interna and theca externa. The theca interna comprises of steroidogenic cells which under LH stimulation produce androgen, namely androstenedione, which is subsequently aromatised to oestrogen in the granulosa cells. The theca externa layer provides structural support to the growing follicle through production of collagen, vimentin, vascular cell adhesion molecule, laminin, fibronectin, proteoglycans, tenascin C, and fibrillins. Like the ovarian stroma, the ovarian theca undergoes remodelling during the process of follicle development. Theca cells are visible around a follicle once the follicle has developed 2 or more layers of granulosa cells as it is at this time that the theca cells become responsive to LH.

Androgens play a key role in maintaining oocyte health and in ovarian follicle development. They exert their actions on the ovary in both a direct and indirect manner. The direct mode of action is through the androgen receptor, which is found in the ovarian follicles and corpora lutea, although most abundantly in the granulosa cells (Sen and Hammes, 2010). In the human ovary, the androgen receptor is also localised to the ovarian stroma (Horie et al., 1992). The androgen receptor is found in the cell cytoplasm in an inactive complex bound to chaperone heat shock proteins (Walters, 2015). DHT and testosterone are the only natural androgens that can bind to and act on the androgen receptor, whereas pro-androgens DHEA, DHEAS and A₄ require conversion to testosterone or DHT to exert their effects on the androgen receptor (Burger, 2002). When testosterone or DHT bind to the receptor, the heat shock proteins are dissociated, thus activating the receptor and allowing for androgen mediated gene transcription (Walters, 2015).

Androgens promote granulosa cell proliferation resulting in pre-antral follicle growth. However they also play a role in ensuring only one dominant follicle proceeds to ovulation by suppressing later stages of follicular development in the non-dominant follicles through induction of granulosa cell apoptosis (Lim et al., 2017). The importance of androgens in follicle development is demonstrated through the use of androgen receptor knock out (ARKO) mice where subfertility is observed in the form of reduced litter size and dysfunctional ovarian follicle development (Walters et al., 2007, Shiina et al., 2006, Hu et al., 2004).

In the human ovary, androgen receptor gene expression is first expressed in primary follicles and as follicles develop, the intensity of androgen receptor expression increases to a maximal point at the small antral follicle stage (approx. 6mm in diameter) and gradually declines as follicle development progresses towards ovulation (Rice et al., 2007) (figure 1.10). The change in androgen receptor expression throughout the process of follicle development implies that the role of androgen receptor mediated actions differs throughout the process of follicle development. Androgen receptor signalling is regulated by various posttranslational modifications, including ubiquitination, phosphorylation, acetylation, methylation and sumoylation (Coffey and Robson, 2012). Studies looking at AR signalling in the prostate have indicated that SMAD3 and Akt play both stimulatory and inhibitory roles in AR expression (Kollara et al., 2020). Testosterone has been shown to increase AR mRNA in granulosa cells in the primate ovary (Weil et al., 1998a).

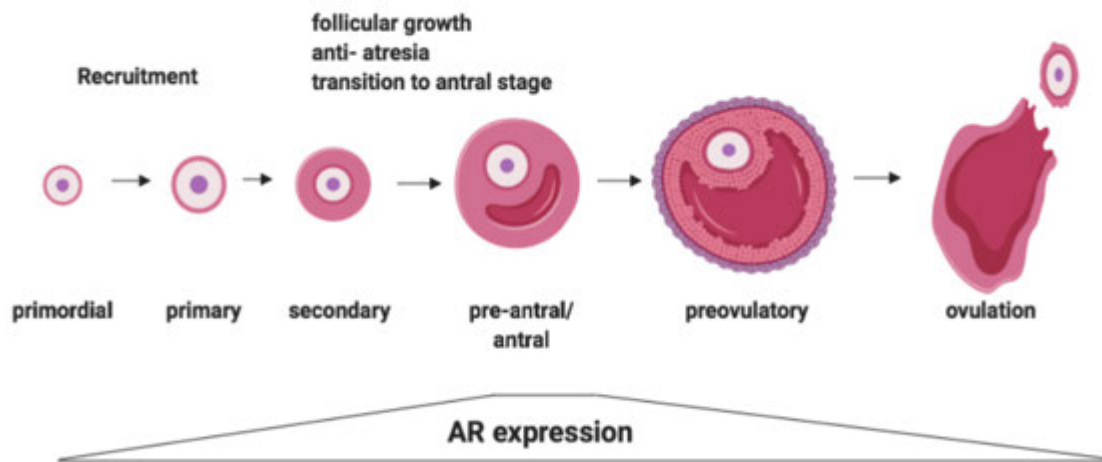


Figure 1.10- The pattern of androgen receptor expression throughout follicle

development. The androgen receptor is first expressed from primary follicle stage and remains detectable throughout follicle development. The intensity of AR expression in the granulosa cells progressively increases to maximal expression at the small antral follicle stage before a decline in expression as the follicle progresses towards ovulation. Overall, androgens have a role in stimulating follicle development and in optimising follicle growth and health and ovulation. Created using Biorender.com.

Androgens also exert their action indirectly on the ovary through aromatisation to oestrogen with subsequent action through the oestrogen receptor, and via production of factors of androgen regulated genes, such as FSH or IGF1 (Walters, 2015). Androgens have a stimulatory effect on genes involved in granulosa cell differentiation. Even in the absence of gonadotrophins and in the presence of Cyp19 and 5 α -reductase, testosterone has been shown to increase granulosa cell expression of steroidogenic enzymes Cyp19 and P450scc (Wu et al., 2011).

1.21 The effect of androgens on ovarian follicle growth

Androgens receptors are not expressed in primordial follicles therefore are not directly involved in primordial follicle activation (section 1.4 and 1.5). They may have an indirect role in primordial follicle activation via the PI3K pathway. Administration of testosterone to mouse primordial follicles was found to increase the ratio of primary to primordial follicles to more than 2-fold through nuclear exclusion of FOXO3A (Yang et al., 2010)(section 1.5).

Apparent primordial follicle activation in sheep with treatment with DHEA (Narkwichean et al., 2014) and in primates with DHT and testosterone (Vendola et al., 1999) would indicate that androgens can promote primordial follicle activation, however given the lack of AR expression in primordial follicles, this must be via a different pathway for example via the upregulation of insulin-like growth factor 1 (IGF1) and/or its receptor (Vendola et al., 1999).

As a follicle progresses from a primary to secondary follicle, it gains a second layer of granulosa cells. Establishing cell-cell contact between the oocyte and the granulosa cells is of the utmost importance to the health of the follicle. Androgens are thought to improve intercellular communication within the ovarian follicle. Expression levels of connexin 43, a gap junction protein, are upregulated in granulosa cells from rats after *in vitro* treatment with DHT (Talhouk et al., 2012). Androgens have been found to have a stimulatory effect of pre-antral follicle development in multiple species including mouse (Murray et al., 1998, Laird et al., 2017), primates (Vendola et al., 1998), rat (Mannerås et al., 2007) and sheep (Forsdike et al., 2007). Androgens also have an impact the rate of follicle atresia. In primate ovaries, after systemic treatment with testosterone or DHT, the levels of apoptotic granulosa cells and atretic follicles were significantly decreased (Weil et al., 1998b).

As the follicle progresses to antral follicle size, they become gonadotrophin responsive (section 1.3). Androgens continue to exert their action through the androgen receptor however, androgens have a synergistic relationship with FSH which in turn has a profound benefit to ovarian follicle development. In primates, treatment with testosterone increased FSH receptor mRNA levels in follicles at all stages of development (Weil et al., 1999). Androgens also enhance a follicle's responsiveness to FSH as demonstrated in a mouse model where DHT enhanced FSH-mediated preantral to antral follicle development (Sen et al., 2014). A positive correlation was found between AR receptor mRNA, A4 and testosterone levels in the follicular fluid of small antral follicles from human ovaries and FSHR expression in granulosa cells, indicating that a follicle's sensitivity to FSH stimulation may be enhanced by androgens via the AR (Nielsen et al., 2011). From early reproductive age, androgen levels begin to decline which may contribute to the reduced ability of the aging ovary to respond to FSH in the setting of IVF. In human studies, pre-treatment with transdermal testosterone in patients who have previously had a poor response to ovarian stimulation, has been shown to significantly improve clinical pregnancy rates and live birth rates, increase the number of oocytes retrieved per cycle and reduce the dose of exogenous FSH required for adequate ovarian stimulation (Vollenhoven et al., 2008, Luo et al., 2014).

1.22 The implications of androgen excess on the ovary- animal studies

An optimal level of androgens is essential to maintain ovarian function. The impact of androgen excess on ovulation and follicle health has been demonstrated in females with polycystic ovary syndrome (PCOS), women with congenital adrenal hyperplasia and transgender men taking testosterone therapy, although in all these situations there are also changes in gonadotropin concentrations.

To gain further understanding of the pathophysiology of PCOS, multiple animal species models have evolved. After *in utero* exposure to androgens, rodents exhibit features in keeping with PCOS including irregular and prolonged oestrus cycles and increased LH pulsatility (Walters et al., 2012). In sheep following prenatal testosterone exposure, there was increased frequency of LH pulses with impaired oestrogen and progesterone feedback resulting in ovulation dysfunction. On histological examination, there was evidence of increased follicle recruitment with a lower proportion of primordial follicles and a higher proportion of primary follicles with evidence of follicle persistence and reduced apoptosis (Padmanabhan and Veiga-Lopez, 2013). Prenatally androgenised female monkeys exhibit multiple findings similar to PCOS including multi-follicular ovaries of increased stromal volume with keeping with stromal hyperplasia and reduced blastocyst development with IVF (Abbott et al., 2005). Pharmacological studies creating hyperandrogenic models using mice and primates have shown that prolonged exposure to androgen excess results in initial enhanced follicle growth, but the oocytes produced exhibited higher levels of oxidative stress, poorer morphological health and subsequent impaired developmental competence in the form of a significant decrease in fertilisation rate (Liu et al., 2015, Yang et al., 2010, Bertoldo et al., 2019, Walters et al., 2018).

1.23 The implications of androgen excess on the ovary - human studies

PCOS is a state of endogenous hyperandrogenism, which affects 5-15% of the female population and accounts for 75% of anovulatory infertility (Balen et al., 2003). Clinical manifestations include hirsutism, hair loss, acne and oligo-anovulation. Metabolic abnormalities associated with PCOS include obesity, insulin resistance, type 2 diabetes mellitus, hyperlipidaemia and cardiovascular disease. The underlying aetiology of PCOS is poorly understood, however both genetic and environmental factors are implicated. Women

with PCOS have reduced ovarian follicle health, ovulatory dysfunction and subfertility.

The ovulation dysfunction in PCOS, resulting from hyper-secretion of androgens from theca cells, is characterised by an initial increased growth of small follicles with an accumulation of primary and pre-antral follicles with an arrest in growth before maturation. Women with PCOS will often have a high or upper limit LH. In comparison to their LH levels, they will often have low circulating FSH levels, which may explain the failure to select a dominant follicle with a subsequent arrest in growth at the antral follicle stage (Dumesic et al., 2015, Franks et al., 2008). High levels of LH can negatively impact follicle growth through premature luteinisation of the granulosa cells resulting in reduced sensitivity to FSH. Increased levels of LH will increase theca cell androgen production, further driving ovulation dysfunction.

Hyperactivity of the theca cells and thus excessive androgen production is typically seen in the ovaries of women with PCOS. This is likely due to altered hypothalamic LH production and increased insulin levels which has been shown to increase LH induced androgen synthesis in the theca cells of women with PCOS.

Effects of exogenous hyperandrogenism have been demonstrated in the ovaries of transgender men taking testosterone therapy. Whilst the results of animal studies have uniformly demonstrated that the administration of high doses of testosterone does induce ovarian morphological features in keeping with PCOS (Murray et al., 1998, Vendola et al., 1998, Abbott et al., 2009), the results have been more variable in human studies. Some studies have found the ovaries from transgender men have increased numbers of antral and atretic follicles, similar to PCOS (Amirikia et al., 1986, Futterweit and Deligdisch, 1986b, Spinder et al., 1989a, Pache et al., 1991, Grynberg et al., 2010) whilst other studies have found a normal follicle distribution (De Roo et al., 2017, Ikeda et al., 2013a).

It is well documented that hyperandrogenism drives changes within the ovarian stroma. This has been demonstrated in the ovaries of women with PCOS and in transgender men taking testosterone therapy. Histological changes noted in both groups include thickening of the tunica albuginea, stromal cell hyperplasia and stromal cell luteinisation (Ikeda et al., 2013b, Chadha et al., 1994, Grynberg et al., 2010, Futterweit and Deligdisch, 1986b, Spinder et al., 1989b, Pache et al., 1991, Hughesdon, 1982). In women with PCOS, on ultrasound, ovarian cortical thickness has been found to be increased by 1/3 compared to normal. Women with PCOS have been found to have increased levels of basic fibroblast growth factor (bFGF) in serum and follicular fluid (Artini et al., 2006). This leads to aberrant collagen synthesis (Papachroni et al., 2010) resulting in increased rigidity of the ovarian stroma. A rigid micro-environment has been shown to be detrimental to follicle growth (section 1.8). The stromal hyper-rigidity found in PCOS may contribute to the known impaired follicle development. This is evident in the use of ovarian drilling a laparoscopic procedure involving electrocautery to the ovarian cortex, which increases the rate of spontaneous ovulation through disruption of the Hippo signalling pathway (Hsueh et al., 2015a) (section 1.6) and through the reduction of ovarian androstenedione and testosterone secretion (Hendriks et al., 2007).

The ovarian cortex from transgender men has also demonstrated increased stiffness. A study carried out texture profile analysis on cortical tissue from the ovaries of transgender men taking testosterone therapy and compared it to an oncological control group (De Roo et al., 2019). They found increased stiffness in the transgender tissue compared to control as a result of testosterone exposure in keeping with increased subepithelial collagenization and stromal hyperplasia previously described (Ikeda et al., 2013a). Increased tissue stiffness has also been demonstrated in physiological ovarian aging as a result of increased collagen deposition within the extra-cellular matrix (section 1.9).

The androgen receptor has been shown to be expressed in the ovarian stroma (section 1.20). A study looking at stromal AR expression found significantly increased AR expression in the ovaries of transgender men on testosterone therapy compared to control postmenopausal ovaries (Chadha et al., 1994). Similar AR receptor staining was also seen in ovaries from women with PCOS and appeared similar to those from transgender men, however this was not quantified in this study. The correlation of AR expression in the ovarian stroma in the presence of hyperandrogenism would indicate it may play a role in the development of the androgen induced stromal change.

Hypothesis

The studies in this thesis aim to address the hypothesis that testosterone affects the follicle distribution in ovarian cortical tissue, causes DNA damage and may impair the DNA repair capacity of ovarian follicles.

Secondly, that excessive testosterone exposure is associated with increased ovarian tissue rigidity, precipitated by changes in ovarian stromal composition.

Aims

To test the hypothesis, the following specific aims were defined:

1. To assess the distribution of the stages of follicle development and morphological health in follicles from testosterone exposed ovaries *in vivo* compared to control
2. Quantify DNA damage and DNA damage repair capacity of oocytes and granulosa cells *in vivo* in testosterone exposed ovaries compared to control
3. To determine the effect of culture conditions on the growth and survival of follicles from testosterone exposed ovaries
4. To investigate changes within the composition of the ovarian stroma as a result of testosterone exposure through measuring stromal cell density and quantifying collagen and elastin content compared to control.

Chapter 2

General materials and methods

2.1. Ovarian Cortical Fragment Preparation and Dissection

2.1.1. Collection of human Ovaries

Ovarian tissue was obtained with informed consent from patients undergoing hysterectomy and bilateral oophorectomy as part of gender transitioning (mean age: 27.6 ± 1.7 years; range 20-34 years, $n = 8$). All patients had received 1000mg of testosterone undecanoate treatment every three months prior to tissue collection (range 18 months-10 years). Centres taking part in this study include the Royal Infirmary Edinburgh, the Queen Elizabeth University hospital, Glasgow and Spire Thames Valley hospital, Slough. Ethical approval of this study was given (ref LREC 10/S1101/2). When the tissue was acquired in theatre, a full thickness section of both ovaries was sent for hospital histopathology assessment. Next, a small section of ovarian cortex was immediately dissected and fixed in 10% normal buffered formalin (NBF). The remaining ovarian tissue was transported in pre-warmed dissection medium (2.1.2.). On arrival to the laboratory, the ovaries were weighed. The ovarian cortex was next dissected from the medullary tissue using a scalpel blade no. 24 in a laminar flow hood. The ovarian cortical tissue was transferred into pre-heated, fresh dissection medium comprising of supplemented Leibovitz medium (Gibco BRL) (section 2.1.3).

2.1.2. Media

Holding medium for transporting the ovaries to the laboratory comprised of M199 (HEPES buffered) (Gibco BRL, Life Technologies Ltd., Paisley, Renfrewshire, UK) supplemented with sodium pyruvate (2 mM), glutamine (2 mM), human serum albumin (HSA) (3 mg/ml), penicillin G (75 µg/ml), streptomycin (50 µg/ml) and amphotericin B (2.5 µg/ml) (all chemicals from Sigma-Aldrich Chemicals, Poole, Dorset, UK). pH was adjusted to 7.2-7.4. Dissection medium was used during preparation of ovarian cortical strips for tissue culture.

Dissection medium was prepared from Leibovitz medium (GIBCO BRL) supplemented with sodium pyruvate (2mM), glutamine (2mM), HSA (Fraction V. 3mg/ml), penicillin G (75µg/ml) and streptomycin (50µg/ml) and was preheated to 37 °C in an incubator prior to use.

2.1.3. Ovarian Cortical Tissue Dissection

After gross dissection of the cortex from the underlying medulla, ovarian fragments were placed in a petri dish with dissection medium and examined carefully using light microscopy. This allowed for identification of medullary and haemorrhagic tissue which was trimmed away using forceps and a scalpel blade. The tissue was gently stretched using the blunt edge of a scalpel blade with the cortex uppermost. Using an angled incision to maximise the volume of ovarian cortex per fragment, the tissue was cut into small strips approximately 4×2×1 mm in size. Individual ovarian cortical fragments were next placed in flat-bottomed 24 well culture plates (Corning Costar Europe, Badhoevedorp, The Netherlands) for tissue culture.

2.2. Tissue Culture

2.2.1. Culture Medium

Basic culture medium comprised of McCoys 5a medium with bicarbonate and HEPES (20mM) (GIBCO BRL, Life Technologies Ltd. Paisley, Renfrewshire, UK) supplemented with HSA (1 mg/ml), glutamine (3 mM), penicillin G (0.1 mg/ml), streptomycin (0.1 mg/ml), transferrin (2.5 µg /ml), selenium (4 ng/ml), insulin (10 ng/ml), FSH (1 ng/ml), and ascorbic acid (50 µg/ml) (all obtained from Sigma-Aldrich Chemicals, Dorset, UK, unless stated otherwise). Prior to use, the culture medium was filter sterilised (Corning Costar Europe, Badhoevedorp, The Netherlands) and equilibrated in a humidified incubator at 37°C and an

air atmosphere with 5% CO₂. The same basic culture medium was used for all experiments including initiation of tissue culture and subsequent alternate days medium changes throughout the culture process.

2.2.2. Ovarian Cortical Fragments Culture

Human ovarian cortical strips were cultured in flat-bottomed 24-well culture plates (Corning Costar Europe, Badhoevedorp, The Netherlands). One cortical fragment was placed in each well containing 300µl of culture medium at 37°C in a humidified air atmosphere with 5% CO₂ for 24 h. During the culture process, 150µl of culture medium per well was removed and replaced with fresh culture medium on alternate days (days 2 and 4). Every experiment was performed under the same conditions (Fig 2.1). After a total of 6 days of culture, the cortical strips were removed from the wells and placed into NBF for fixation.

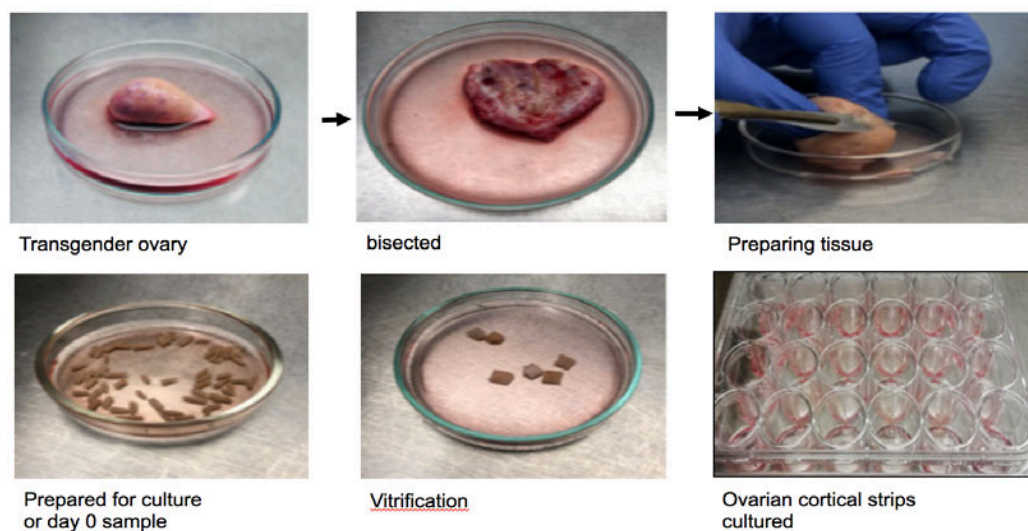


Figure 2.1- ovarian tissue dissection and culture. On arrival to the lab, the transgender ovary was bisected, and ovarian cortex was removed using a scalpel. Ovarian cortical tissue was prepared into small strips and either fixed for day 0 analysis, vitrified for future use or placed in tissue culture of 6 days in flat-bottomed 24-well culture plates.

2.2.3. Control ovarian tissue

Control ovarian cortical tissue was obtained after informed consent was given by healthy women undergoing an elective caesarean section at the Royal Infirmary Edinburgh. The tissue was age matched, thus the upper age limit for tissue used as a control for this study was 35 years old. A small piece of ovarian cortical tissue was dissected intra-operatively using a scalpel by obstetric colleagues and immediately placed in pre-warmed holding medium as described in 2.1.2. and transferred to the lab. The process of tissue dissection and preparation for culture was as described above in 2.1.4. and 2.2.2. Two ovarian cortical fragments per patient were fixed in NBF for day 0 analysis. The remaining tissue fragments underwent tissue culture for a total of 6 days and then was subsequently fixed in NBF for analysis.

2.3. Histological Techniques

2.3.1. Tissue Fixation, Embedding, and Sectioning

After the cortical tissue was removed from culture, it was placed in NBF solution for 24 hours at room temperature. The tissue was then removed from the fixative and dehydrated through a series of increasing concentrations of ethanol (70%, 90% and 100%) for one hour each to displace the water. Absolute alcohol was cleared and replaced with cedarwood oil (BDH Laboratory Supplies, Poole, UK) for a minimum of 24 hours. Following dehydration, the tissue was then subsequently placed in toluene in a fume hood (Fisher Scientific UK Ltd, Loughborough, UK) for 30 minutes to achieve complete oil clearance. Individual ovarian cortical fragments were placed in moulds (Peel-a-way disposable embedding moulds, Polysciences Inc, USA) filled with liquid paraffin at 60°C for a total of 4 hours, with 3 changes of wax at hourly intervals to ensure complete clearance of toluene. The tissue was finally manually embedded and cooled on a cool plate.

2.3.2. Sectioning and Mounting

Paraffin mounted ovarian cortical fragments were serially sectioned at 6µm using a microtome (Leica, model Jung RM2035, Nussloch, Germany). The sections were floated on a water bath at 42°C and mounted on SuperFrost Plus slides (VWR International Ltd., Leicestershire, UK). The slides were left to dry overnight at 37°C in a slide oven.

2.3.3. Dewaxing and Tissue Rehydration

Sections were dewaxed by placing the slides in xylene (VWR International) for 20 minutes (15 minute immersion followed by 5 minute immersion) and dehydrated by subsequent immersions in decreasing concentrations of alcohol for 2 minutes each (100%, 90% and 70%). Slides were then rinsed in running tap water and were then ready for staining or antigen retrieval.

2.3.4. Haematoxylin and Eosin (H&E) Staining

Following dewaxing and rehydration and rinsing in tap water (2.3.3), the sections were placed in Harris' Haematoxylin (BDH Laboratory Supplies, Poole, UK) for 3 minutes, then washed in tap water, followed by immersion in Scotts tap water substitute (STWS; CellPath) for 2 minutes and then placed back in tap water for further 2 minutes. Slides were then placed in 1% eosin (Sigma Chemicals, Poole, Dorset UK) solution for 2 minutes, immersed briefly in tap water before being placed in potassium aluminium solution for 2 minutes. Next, the slides were rinsed under running tap water and completed dehydration through increasing concentrations of ethanol (70%, 90% and two washes of 100%) for 30 seconds each. Slides were then immersed in xylene for 15 minutes and lastly mounted using Dibutylphthalate Polystyrene Xylene (DPX) mounting medium (BDH Laboratory Supplies, Poole, UK) and

glass coverslips (BDH Laboratory Supplies, Poole, UK) and left to dry for 24 hours in a fume hood at room temperature.

2.4. Immunohistochemistry

2.4.1. Immunohistochemistry Protocol

Ovarian cortical strips were processed and embedded as described in Section 2.3.

Immunohistochemistry was performed to determine the localisation of antibodies used in this study. Details of the primary and secondary antibodies used, incubation time, and the concentration of primary and secondary antibodies, antigen retrieval method, endogenous peroxidase quenching and visualisation techniques for specific antibody are detailed in the Methods and Materials sections of each chapter.

In general, following dewaxing and rehydration in decreasing concentration of ethanol, slides were immersed in water for 5 minutes and then washed once in phosphate-buffered saline with 0.1% Triton (PBST) (pH 7.2–7.4) for 5 minutes. Slides were then equilibrated for 5 minutes in 10 mM sodium citrate. Two methods of antigen retrieval were used- using microwave and autoclave. Antigen retrieval by microwave was performed by heating the slides in the sodium citrate (pH 6.0) buffer on a low setting for 20 minutes. The slides were allowed to cool in the buffer for approximately 20 minutes. Antigen retrieval by autoclave was performed by placing the slides in a slide holder filled with sodium citrate buffer and heating in the autoclave for 2 hours, then allowing to cool for 30 minutes before continuing the remainder of the experiment.

Following antigen retrieval, slides were washed in PBST (2x5 minutes) and then placed in 3% hydrogen peroxide (H₂O₂ Sigma-Aldrich) in methanol solution (VWR International) for

10 minutes to block endogenous peroxidase activity and then washed again in 2x5 minutes PBST. Sections were then incubated with the most appropriate blocking solution for the antibody in use (Vectastain® ABC Kit, Vector Laboratories- rabbit and mouse Vectastain kits required) diluted in PBST (3 drops of serum in 10 ml PBST) for 1 hour. The slides were then incubated overnight in a solution containing the blocking solution (5% Goat serum in PBST) with primary antibodies in the concentration specific for the particular antibody being used at 4°C. Blocking solution without antibody acted as a negative control. Following incubation with the primary antibody, slides were washed in PBST (2 x 5 minute), and sections were incubated for 30 minutes with biotinylated secondary antibody at room temperature (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK) and then washed in PBST (2x5 minutes). The secondary antibodies were selected based on the primary antibody of choice. Slides were then incubated with Streptavidin Horseradish Peroxidase (Streptavidin HRP) (made up as per the manufacturer's instructions; Vectastain® ABC Kit) for 30 minutes at room temperature. Following the PBST wash, DAB (3, 3'-diaminobenzidine) peroxidase substrate kit (Vector Laboratories, made up as per the manufacturer's instructions) solution was added to the sections for between 1 and 5 minutes. Prior to applying DAB solution to the tissue sections, the slides were placed on white tissue to enable visualising the colour change within the sections as timely as possible. The slides were placed in tap water to stop the DAB reaction when the tissue sections began to change to a pale brown colour, counterstained with haematoxylin and finally dehydrated in graded alcohol and mounted with DPX.

2.4.2. Immunofluorescence Protocol

As previously described, mounted tissue sections were deparaffinised and rehydrated and then washed in PBST for 5 minutes. Slides were then equilibrated for 5 minutes in 10 mM

sodium citrate prior to antigen retrieval. Antigen retrieval was performed as previously described. Following this, the slides were incubated for 1h at room temperature (RT) with blocking solution (5% goat serum in PBST). Primary antibody was applied to tissue sections and incubated overnight at 4 °C. Blocking solution without primary antibody acted as a negative control. After washing with PBST (4 washes x5 minutes), sections were incubated with an appropriate secondary antibody for 1 hour and then washed for a further 4x5 minutes. The slides were then mounted in Vectashield hardset with 4',6-diamidino-2-phenylindole (DAPI) (H-1500, Vector Laboratories) and stored at 4°C.

Optimisation of IHC methods was required for one antibody- Ki67. This was carried out by changing different steps of the IHC process including altering the method of antigen retrieval, different concentrations of primary antibody, using PBS instead of PBST and incubating the sample for longer with primary antibody. Using autoclave for antigen retrieval allowed for successful identification of the primary antibody within the sample.

In this study, the control used was a negative control i.e. one where the primary antibody was omitted. In retrospect, the omission of the primary antibody is not evidence for the specificity of staining with the primary antibody but more so a control for nonspecific binding of the secondary antibody. Another type of negative control that could have been used is an isotype control where a non-immune antibody of the same isotype at the same concentration as the primary antibody is used and checks that the observed staining is not caused by non-specific interactions of the antibody with the tissue. This type of control is considered to be the most robust. An absorption control was another option; this is the use of a pre-absorbed antibody instead of the primary antibody. Other options that could have been used include wider use of a positive control. This was utilised in the experiments with Ki67 where granulosa cells in growing follicles were positive for Ki67, confirming within the tissue section being analysed that the IHC method was working.

2.4.3. Image Acquisition and Processing

Images were captured in the IMPACT imaging facility (Centre for Discovery Brain Sciences, The University of Edinburgh) and Queens Medical Research Institute (University of Edinburgh). Details of imaging are provided as appropriate in subsequent chapters. Identical laser settings were applied for all samples imaged within each experiment, for each of confocal, immunofluorescence and polarised light microscopy.

2.4.4. Massons Trichrome

Following dewaxing and rehydration and rinsing in tap water, slides were incubated in Bouin's fluid (Abcam) for 24 hours at room temperature in a fume hood. Next, the slides were immersed in running tap water until the tissue sections were completely clear from yellow staining. Following this, the slides were rinsed for one minute in distilled water and then immersed in working Weigert's iron Haematoxylin (Abcam) solution for 5 minutes. The slides were then transferred into running tap water for 2 minutes to rinse. Biebrich Scarlet/acid fuchsin solution (Abcam) was applied to the slides for 15 minutes and subsequently rinsed in distilled water for 5 minutes. Next, the slides were differentiated in Phosphomolybdic/phosphotungstic acid solution (Abcam) for 10 minutes or until the collagen was no longer red and then without rinsing, placed in Aniline Blue solution for 10 minutes. Next, the slides were further rinsed in distilled water and immersed in acetic acid solution 1% (Abcam) for 5 minutes. The slides were quickly dehydrated in 2 changes of 95% ethanol followed by 2 changes of 100% ethanol, cleared in xylene for 15 minutes and mounted with DPX and glass covered slips.

2.4.5. Picrosirius Red staining

Slides were dewaxed and rehydrated as per 2.3.3 and placed in Harris' haematoxylin for 8 minutes. Slides were rinsed under running tap water for 10 minutes then stained with Picrosirius red (Abcam) for 1 hour. Following this, the slides were rinsed in 1% acetic acid x2 washes and then most of the water was removed from the slides through vigorous shaking. Slides were then dehydrated in 3 separate changes in 100% alcohol, cleared in xylene and mounted in DPX.

2.5. Collection and Analysis of results

2.5.1. Collection and analysis of Histological Results

Analysis of follicles within cortical pieces from H&E-stained sections and sections that underwent immunohistochemistry was carried out using light microscopy with a crossed micrometre under x40 magnification. Every 10th section was analysed for follicle counts and classification, therefore any follicle with a nucleus present within the section was included in analysis. Follicle classification and morphological health was recorded. The mean diameter of two perpendicular measurements of follicles (a+b) and oocytes (x+y) was recorded and true follicle/oocyte diameter was calculated using the algorithm $a+b$ or $x+y/2 \times 2.651$ (microscope correction factor).

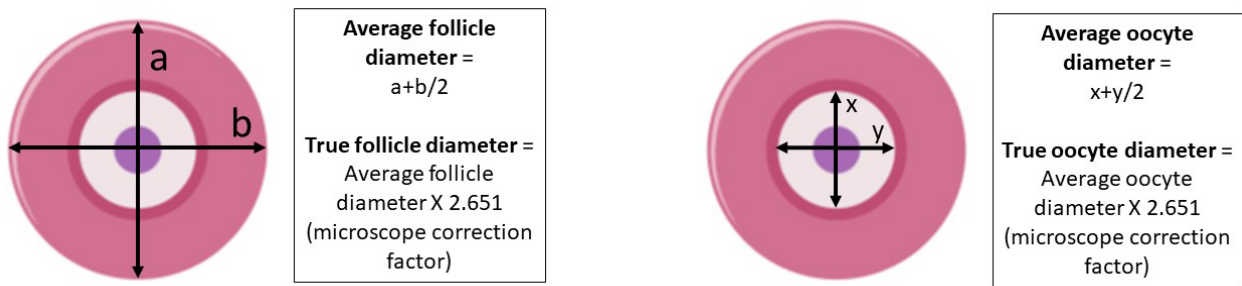


Figure 2.2 method of measuring follicle and oocyte diameter. The mean diameter of two perpendicular measurements of follicles ($a+b$) and oocytes ($x+y$) was recorded and true follicle/oocyte diameter was calculated using the algorithm $a+b$ or $x+y/2 \times 2.651$ (microscope correction factor). The theca cell layer was not included in the follicle diameter.

The Pederson classification (Pedersen and Peters, 1968) to identify follicle type was used as follows:

- (1) Primordial follicles: oocyte surrounded by a complete or incomplete single layer of a flattened granulosa cells.
- (2) Transitional follicles: oocyte surrounded by a mixed layer of flattened and cuboidal granulosa cells.
- (3) Primary follicles: oocyte surrounded by a single layer of cuboidal granulosa cells.
- (4) Secondary stage: oocyte surrounded by two or more complete layers of cuboidal granulosa cells.
- (5) Antral follicles: oocyte surrounded by two or more complete layers of cuboidal granulosa cells and the presence of an antral cavity.

The number of follicles at each stage of growth was recorded, for day 0 and day 6 for both the control and the transgender groups. With regards to human ovarian follicles, there is debate as to whether transitional ovarian follicles are part of the non-growing follicle pool

(McLaughlin et al., 2015, Schmidt et al., 2003b, Gaytan et al., 2015). For this analysis, primordial and transitional follicles have been combined and analysed as non-growing follicles.

The morphological health of a follicle was determined using the criteria set out by Telfer *et al.* (2008) with modifications (Telfer et al., 2008b, McLaughlin and Telfer, 2010). To classify a follicle as morphologically normal, the oocyte must be grossly circular, surrounded by a zona pellucida, have a visible germinal vesicle and defined nucleolus and have less than 10% of pyknotic granulosa cells present (pyknotic granulosa cells were identified as misshapen and had much darker staining than others). Results for both the follicle classification and morphological health are presented as mean $\pm$ SEM.

2.5.2. Collection and Analysis of immunohistochemical Results

Data regarding follicle classification, morphological health and follicle and oocyte diameter was collected the same way as described in 2.5.4. Immunohistochemistry analysis regarding antibody protein expression was analysed separately in oocytes and granulosa cells. The proportion of oocytes with positive expression of a primary antibody was calculated per patient and analysed as the mean $\pm$ SEM. For the granulosa cells, the proportion of follicles with any positive expression of primary antibody within the granulosa cells was calculated per patient followed by calculating the proportion of positive granulosa cells per total number of granulosa cells per follicle. Both results were analysed as the mean $\pm$ SEM.

2.5.3. Collection and Analysis of immunofluorescence results

The expression of γ H2AX within ovarian follicles was analysed separately in the oocytes and granulosa cells. Images were taken via confocal light microscopy of every 10th tissue section

and the entire section analysed. DAPI nuclear staining identified ovarian follicles with a present nucleus and these follicles were included in analysis. γ H2AX expression in oocytes and granulosa cells was collected in the same fashion as described above in 2.5.2.

To quantify expression of KI67, images of every 10th tissue section was taken using Leica upright Brightfield and epifluorescence microscope x20 magnification in IMPACT imaging facility. Three regions of interests (ROIs) were selected across each tissue section analysed. Expression of KI67 in the ovarian stroma was analysed as a proportion of positive expression of the antibody in stromal cells per total number of stromal cells which are identified with DAPI staining.

2.5.4. Collection and analysis of follicle density

H&E-stained sections for quantification of follicle classification and morphological health were imaged using Leica upright Brightfield and epifluorescence microscope x2.5 magnification in IMPACT imaging facility. A scale bar measuring 1cm was imaged at the same magnification and the image was opened on Microsoft PowerPoint. The length of the imaged scale bar 1cm was measured at 4.42cm. The images taken for follicle density were opened in Microsoft PowerPoint and the length and breadth measurements taken. These measurements were subsequently divided by 4.42 to give the adjusted dimensions.

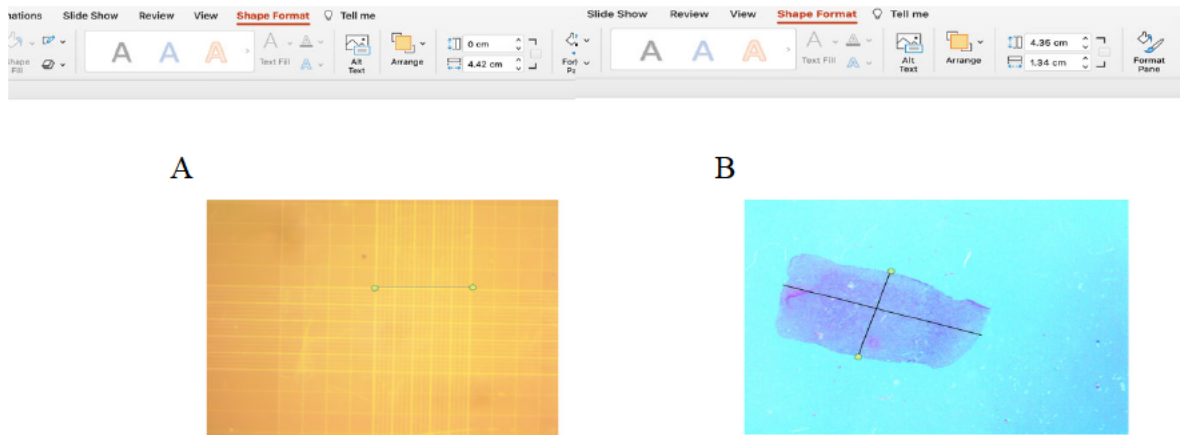


Figure 2.3: Calculation of area of tissue segments using Microsoft Excel ((McLaughlin et al., 2015). (A) A scale bar measuring 1cm was imaged at the same magnification and the image was opened on Microsoft PowerPoint. The length of the imaged 1cm scale bar was measured at 4.42 cm (B) image of tissue section x 2.5 magnification-The images of sections taken for follicle density were opened in Microsoft PowerPoint and the length and breadth measurements taken. These measurements were subsequently divided by 4.42 to give the adjusted dimensions measurement.

Next, the area of each tissue section was calculated (McLaughlin et al., 2015). For each patient, the total tissue area was calculated by adding the area of every tissue section analysed for each patient in mm^2 . Next, the total tissue area per patient was next multiplied by 0.006 to calculate the total tissue volume in mm^3 (each tissue section being of $6\mu\text{m}$ thickness). Given that every tenth tissue section has been analysed rather than every section, the tissue volume was multiplied by 10 to take into account the distance between sections (Lopes et al., 2020). The mean non-growing follicle density (NGFD) was calculated by dividing the total number of non-growing follicles per patient by the volume of tissue analysed in mm^3 . Predicted NGFDs were taken from table 1 of (McLaughlin et al., 2015), an externally validated model of age related mean NGFD derived from human ovarian cortex. Correlation between observed NGFD from transgender and control tissue and predicted NGFD was measured by

calculating the Pearson product-moment correlation coefficient.

2.5.5. Analysis of stromal cell density

Five tissue sections previously analysed for follicle classification, morphological health and follicle density were selected per patient for analysis of stromal density. Rather than imaging the entire tissue section under a low magnification, three ROI were selected across each tissue section and imaged using Leica Upright Brightfield and epifluorescences microscope x 20 magnification. The area of tissue was automatically calculated using Image J software in mm^2 with the same area used for each ROI ($315 \times 235 \times 5 \mu\text{m} = 370125 \mu\text{m}^3$). Within each individual ROI, the number of stromal cells was manually counted using Image J software. The tissue volume in mm^3 was calculated by multiplying the tissue area by 0.006 to account for tissue thickness ($6 \mu\text{m}$). The stromal cell density per ROI was calculated using the following equation: total number of stromal cells/ total tissue volume mm^3 . The stromal cell density of individual tissue sections was calculated by taking the average stromal cell density of the 3 ROIs per tissue section. Next, the stromal cell density was calculated per patient and results were presented as the mean $\pm$ SEM $\times 10^6$ cells/ mm^3 .

2.5.6. Collection and analysis of Picrosirius red (PSR) results

These slides were subjected firstly to bright field microscopy. Images were taken of every 10th section using Leica upright Brightfield and epifluorescences microscope x2.5 magnification in IMPACT imaging facility. In addition to brightfield imaging, the same PSR stained sections were imaged using a Zeiss Axiovert 200 manual microscope (Queens Medical Research Institute) to expose the natural birefringence of collagen under polarised light. Each section was imaged under x10 magnification under the same conditions

(exposure, gain, saturation and gamma). Depending on the thickness and packing density of the collagen fibres, the colour ranges from red (thickest collagen fibres) to green (thinnest collagen fibres). To establish the proportion of different colours of collagen fibres, a macro program on Image J was used to automate quantification. This macro program determines the total area of polarized pixels using the colour threshold tool on Image J and classifies them into three categories based on the hue histogram. We used a modification of hue definitions applied by MacKenna *et al.* (1994): red 2–9, yellow/orange 10–38, green 24–135. The percentage of each colour was calculated by dividing the pixel count of each colour by the total pixel count for each image.

2.5.7. Analysis of collagen by Massons Trichrome staining

To quantify ovarian fibrosis in the stromal tissue, new collagen deposition was quantified using Massons Trichrome staining of ovarian tissue sections (Pampanini et al., 2019). Images of every 10th section were taken using Leica upright Brightfield and epifluorescences microscope x2.5 magnification in IMPACT imaging facility. Using Image J software, the area of the collagen staining was firstly measured, followed by the total area of each tissue section. The percentage area of positive staining for collagen per tissue section was calculated (area of collagen-stained tissue/total area of ovarian tissue section x100) and the average percentage area of positive staining for new collagen deposition per patient was calculated by taking an average area of positive staining of all sections analysed. The results for area of positive staining of new collagen deposition are presented as mean $\pm$ SEM.

2.5.8. Hormone profile collection and results

Prior to hysterectomy and oophorectomy, the patients gave consent for a blood sample to be taken for follicle stimulating hormone (FSH), luteinising hormone (LH), oestradiol and

testosterone levels. The blood sample was drawn the day of the operation whilst the patients were still taking testosterone therapy and sent to the biochemistry lab for analysis. The samples are processed in the Biochemistry lab in the hospital the patient's operation is taken place and results made available. A correlation analysis was performed (5 transgender patients consented to having serum hormone levels preoperatively) to investigate potential relationships between serum hormone levels and ovarian follicle distribution. Serum hormone levels were compared to the percentage proportion of ovarian follicles within each classification (non-growing, primary, secondary) for each individual patient. Pearson's correlation co-efficient were calculated and statistical significance was assigned $p < 0.05$. In addition, an inter-hormonal correlation analysis was performed, between FSH/LH, FSH/testosterone and LH/testosterone.

2.6. Commonly Used Solutions

2.6.1. Sodium Citrate

The stock solution of 0.1 M sodium citrate was made by dissolving 29.4 g dihydrate tri-sodium citrate in 1000 ml of distilled water. The pH was altered to 6.0 with sodium hydroxide (all Sigma-Aldrich). The stock solution was sterilised and stored at room temperature. When used, 900 ml of distilled water was added to 100 ml stock solution to reach a final concentration of 10 mM.

2.6.2. Phosphate-Buffered Saline (PBS) and PBST (Triton)

PBS used was prepared by dissolving 5 PBS tablets (Sigma-Aldrich) into 1000 ml of distilled water. PBST was made by adding 1 ml of Triton X-100 to 1000 ml of sterilised PBS.

2.7. Statistical Analysis

All data was analysed using SPSS statistical software version 24 (SPSS, Inc., Chicago, USA) and graphs were produced using PRISM Graph Pad version 8 (GraphPad Inc., San Diego, California, USA). Quantitative data was expressed as mean $\pm$ SEM. Given the potential for large interpatient variability, non-parametric Kruskal–Wallis test with Dunn post hoc test or Mann Whitney test were used for statistical analyses. Statistical significance was assigned as $P < 0.05$.

Chapter 3

The effects of testosterone therapy on follicle growth, DNA damage and DNA repair capacity of oocytes and granulosa cells in the human ovary

3.1. Introduction

Tissue culture systems have been continually modified over decades with the goal of producing developmentally competent oocytes (Bertoldo et al., 2019). Complete growth in culture from primordial follicle stage with subsequent IVF and production of live offspring from immature oocytes has been achieved in the mouse model (Eppig and O'Brien, 1996, O'Brien et al., 2003a) however this has not yet been achieved in human (Telfer et al., 2008a) or larger animal models (McLaughlin et al., 2018). More recently in the human model, the *in vitro* culture system has been optimised to support oocytes through follicle activation, *in vitro* growth and maturation to produce oocytes that survive culture, successfully emitting a polar body and displaying metaphase II stage of meiosis (McLaughlin et al., 2018).

In vivo, primordial follicles are gradually recruited over a female's reproductive lifespan. As they enter the growing follicle pool, the flattened granulosa cells differentiate to form cuboidal cells, providing nutrients to support further oocyte metabolic activity. Not all follicles that are recruited from the primordial follicle pool complete the process of follicle development, but rather many are lost through apoptosis (McGee and Hsueh, 2000).

It is recognised that by removing follicles from their physiological environment, the process of tissue culture can accelerate growth and result in mass activation of primordial follicles (Hovatta et al., 1999, Telfer et al., 2008b) which can have a detrimental impact on the oocyte's competence (McLaughlin et al., 2014b). In order to obtain a population of good quality oocytes, limiting and controlling such precocious primordial follicle activation is essential to an efficient and effective culture system. The PI3K pathway is recognised as having a central role in activation of primordial follicles). Testosterone is known to be able to influence primordial follicle activation through the PI3K pathway by promoting nuclear exclusion of FOXO3a (Yang et al., 2010).

There have been multiple studies that have looked at the *in vitro* maturation potential of testosterone exposed oocytes (section 1.19). Medullar cumulus-oocyte complexes (COCs) have been extracted from ovaries from transgender men who were taking testosterone preparations prior to their hysterectomy and oophorectomy, *in vitro* matured for 48 hours with 34.1% reaching metaphase II stage with 87.1% displaying normal spindle structure and chromosome alignment (De Roo et al., 2017, Lierman et al., 2017). However, more recently published work has shown that when these testosterone exposed oocytes were fertilised, there was significantly impaired fertilisation rates and embryo development, indicating that post-exposure to long term testosterone treatment, oocytes have a low developmental capacity. This highlights the need for more clarity regarding the long-term sequelae of testosterone on ovarian follicles to guide prescribing endocrine therapies for transgender men and aid discussion regarding fertility preservation options. It also indicates a need for further optimisation of the culture system and the potential need for customisation depending on the ovarian tissue's *in vivo* environment. This study is the first to look at the first step of tissue culture- follicle activation - in ovaries from transgender men. For ovarian tissue cryopreservation to be a viable fertility preservation option for transgender men, complete *in vitro* growth from primordial follicle stage needs to be achieved.

The aims of the experiments in this chapter are to identify whether exogenous high dose testosterone exposure impacts the rate of follicle activation and survival in culture, causes DNA damage (by identifying expression of H2AX, a marker for DNA double stranded breaks (DNA DSBs)) and has any impact on the DNA repair capacity of ovarian follicles(by identifying expression of DNA repair proteins MRE11, RAD51 and ATM, key proteins involved in DNA repair by homologous recombination).

3.2. Materials and methods

3.2.1. Hormone profile collection and results

Pre-operatively, transgender patients gave consent for a blood sample to be taken to be tested for FSH, LH, oestradiol and testosterone levels whilst still taking testosterone therapy as described in section 2.5.8.

A correlation analysis was performed ($n=5$) to investigate potential relationships between serum hormone levels and ovarian follicle distribution. Serum hormone levels were compared to the percentage proportion of ovarian follicles within each classification (non-growing, primary, secondary) for each individual patient. Pearson's correlation co-efficient were calculated and statistical significance was assigned $p<0.05$. In addition, an inter-hormonal correlation analysis was performed, between FSH/LH, FSH/testosterone and LH/testosterone.

3.2.2. Ovarian Cortical Tissue Collection and Preparation

Human ovaries were collected and transported to the lab as described in chapter 2 section 2.1.1. On arrival to the lab, the ovaries were bisected and weighed. The tissue was prepared for culture as described in section 2.1.3.

3.2.3. Ovarian Tissue Fragments Culture

Basic culture medium was prepared as described in section 2.1. Each experiment was from a single set of human ovaries. Multiple pieces of tissue 4x4mm were prepared for vitrification as described in 2.2 and stored in -80°C . For each experiment, 12-15 pieces of tissue were randomly selected for day 0 control and placed in 10% NBF as described in 2.2. The remaining tissue fragments were cultured in flat-bottomed 24-well culture plates containing 300 μl basic culture medium as described in 2.2. On days 2 and 4, half of the culture medium was exchanged with fresh medium. On day 6, on completion of the culture period, all

remaining tissue fragments were fixed in 10% NBF for future histological and immunohistochemical analysis.

3.2.4. Histological Methods and Tissue Analysis

Details of histological analysis are in chapter 2 section 2.4. In brief, after fixation in NBF for 24 hours, tissue fragments were further processed and embedded individually into paraffin wax blocks and serially sectioned at 6µm thickness. From each wax block that was serially sectioned, slides were prepared for both H&E staining, immunohistochemistry and immunofluorescence. 20-30 sections were placed on each slide for H&E staining and 10 sections per slide for immunohistochemistry and immunofluorescences. Follicles were analysed on every 10th section under the light microscope with a crossed micrometre under x40 magnification. Follicles with a nucleus were included in analysis. Follicle developmental stage was categorised using the classification as described in 2.5.4 (Pedersen and Peters, 1968). Primordial and transitional follicles were classified as non-growing follicles. The number of follicles within each stage of development was recorded, both at day 0 and after 6 days of culture. The proportion of follicles at different developmental stages was defined as a percentage of the total follicle count per patient and the results were given as mean ± SEM. Morphological health of follicles within each follicle classification was defined as the proportion of morphologically healthy follicles of the total number of follicles per patient and the results were displayed as mean ± SEM. The mean diameter of follicles and oocytes was measured as described in 2.5.1. Briefly, the mean diameter of two perpendicular measurements of follicles (a+b) and oocytes (x+y) was recorded and true follicle/oocyte diameter was calculated using the algorithm $a+b$ or $x+y/2 \times 2.651$ (microscope correction factor).

3.2.4.1. Immunohistochemistry

Quantitative analysis for DNA repair protein response was performed using immunohistochemistry. Antibodies against RAD51, MRE11 and ATM were used to identify DNA repair response within the oocytes and granulosa cells (table 3.1).

Immunohistochemistry protocol used is described in 2.4.1. In brief, slides were dewaxed, dehydrated in decreasing concentrations of alcohol, rinsed in tap water and 0.1% PBST and equilibrated in 10 mM sodium citrate (pH 6.0). Antigen retrieval was performed by placing the slides immersed in 10 mM sodium citrate in the microwave on a simmer setting for 20 minutes and then allowed to cool for 20 minutes. The slides were next placed in hydrogen peroxide in methanol solution to quench peroxide activity and incubated with blocking solution for 1 hour at room temperature. Next, primary antibody was applied to all slides, omitting the negative control. The next day, primary antibody was washed off and the slides were incubated for 30 minutes with biotinylated secondary antibody (Table 3.2). Next, slides were incubated in Streptavidin Horseradish Peroxidase (Streptavidin HRP) for 30 minutes at room temperature. Following this, the slides were washed in PBST. DAB peroxidase substrate kit solution was applied, observing for reaction then timely plunged in tap water and counterstained with haematoxylin. Sections were analysed using a light microscope at x20 magnification.

Expression of DNA repair proteins was analysed in granulosa cells and oocytes separately. The proportion of oocytes with positive expression of a DNA repair protein was calculated per patient and was analysed as the mean $\pm$ SEM. For the granulosa cells, the proportion of follicles with any positive expression of primary antibody within the granulosa cells was calculated per patient followed by calculating the proportion of positive granulosa cells per

total number of granulosa cells per positively stained follicle. All results were analysed as the mean $\pm$ SEM.

3.2.4.2. Immunofluorescence

Immunofluorescence was used to identify and quantify the presence of γ H2AX in the oocytes and granulosa cells of ovarian follicles from both transgender day 0 and day 6 tissue and control day 0 and day 6 tissue. γ H2AX is a marker of DNA damage. It binds to double stranded breaks, the most potent form of DNA damage, in a ratio of 1:1, making it a useful tool to quantify DNA damage (Redon et al., 2009).

As previously described, mounted tissue sections were deparaffinised, dehydrated in decreasing concentrations of alcohol and washed in tap water and 0.1% PBST. Next, slides underwent antigen retrieval for 20 minutes using a microwave on a simmer setting. Following this, the slides were allowed to cool for at least 20 minutes in room temperature. After 2x5min washes in PBST, slides were incubated for 1 hour at room temperature with blocking solution (5% goat serum in PBST). Tissue sections were then probed with primary antibody γ H2AX (table 3.1) overnight at 4°C. Blocking solution without primary antibody acted as a negative control. The following day, the slides were washed in PBST 4x5mins and then incubated with secondary antibody (Table 3.2) for 1 hour. Next, following further PBST washes 4x5mins and 1x5min PBS wash, the sections were mounted in Vectashield hardset with DAPI.

Images were captured using a Zeiss LSM 800 confocal microscope with x20 magnification in the IMPACT imaging facility. Images were taken of every follicle with an oocyte nucleus present as demonstrated by DAPI staining for analysis. Images were analysed using ImageJ. The proportion of oocytes with positive expression of γ H2AX was calculated per patient and

analysed as mean $\pm$ SEM. For the granulosa cells, the proportion of follicles with any positive expression of primary antibody within the granulosa cells was calculated per patient followed by calculating the proportion of positive granulosa cells per total number of granulosa cells per positively stained follicle. Both results were analysed as the mean $\pm$ SEM.

ImageJ was used to quantify the intensity of γ H2AX immunofluorescence staining in both oocytes and granulosa cells. This was carried out by measuring the mean gray value. To calculate the intensity of γ H2AX staining in the oocyte, the nucleus of the positively stained oocyte was manually highlighted, and the mean gray value was calculated. For granulosa cells, the area of granulosa cells with positive antibody staining was calculated by setting a threshold value on the fluorescence intensity. This is achieved by manually adjusting the intensity value to cover the positively stained area. This was next divided by the total area of the granulosa cells compartment and expressed as percentage. The results were analysed per patient and presented as the mean $\pm$ SEM.

Table 3.1. primary antibodies used immunohistochemistry/immunofluorescence

Blocking serum	Primary antibody	Product number	Species raised	Final concentration	Source
Goat serum (Sigma Aldrich)	γ H2AX	NB100-384	Rabbit polyclonal	1 μ g/ml	Novusbio
Goat Serum Vectastain Elite Kit PK-6101, Vector Laboratories, California, USA. 150 μ l horse serum+ 10 ml PBST	Rad51	137323	Rabbit monoclonal	2.22 μ g/ml	Abcam
	MRE-11	NB100-142	Rabbit polyclonal	1.89 μ g/ml	Novusbio
Horse Serum Vectastain Elite Kit PK-6102, Vector Laboratories, California, USA. 150 μ l horse serum+ 10 ml PBST	ATM	ab78	Mouse monoclonal	3.70 μ g/ml	Abcam

3.2 Secondary antibodies used in immunohistochemistry/immunofluorescence

Secondary antibody	Product number	Final concentration	source
Vectastain Elite ABC HRP Kit (peroxidase, mouse IgG)	PK 1601	50 μ l biotinylated secondary antibody+ 150 μ l goat serum+ 10 ml PBST	Vector Laboratories
Vectastain Elite ABC HRP Kit (peroxidase, rabbit IgG)	PK1602	50 μ l biotinylated secondary antibody+ 150 μ l horse serum+ 10 ml PBST	Vector Laboratories
Cy3 AffiniPure Donkey anti-rabbit IgG	711-165-152	5.98 μ g/ml	Jackson Laboratories

3.5. Statistical analysis

All data was analysed using SPSS statistical software version 24 (SPSS, Inc., Chicago, USA).

Graphs were generated using GraphPad software version 7 (Inc., San Diego, California,

USA). All results were presented as mean $\pm$ SEM. Given the potential for large interpatient variability, analysis was performed using non-parametric Kruskal Wallis test with post-hoc Bonferroni test (details of test used found in each figure). Statistical significance was assigned $p<0.05$.

3.6. Results

3.6.1. Patient characteristics

In total, eight transgender patients who underwent a hysterectomy and bilateral salpingo-oophorectomy as part of gender transitioning (mean age: 27.6 ± 1.7 years; range 20-34 years) were included in this study. All patients had received 1000mg of testosterone undecanoate treatment every three months prior to tissue collection (range of treatment 18 months- 10 years). All patients were non or ex-smokers and none had any significant past medical history, in particular, polycystic ovary syndrome. An overview of blood results taken from the transgender patients who had been taking pre-operative testosterone and reference ranges can be found in table 3.6.1. There are no blood results available from the control patients. The blood results from the transgender patients ($n=5$ - the number of participants who consented to pre-operative blood tests) showed hormone levels FSH, LH, oestradiol and testosterone within expected reference range as set by NHS Lothian, indicating that testosterone levels were in the normal adult male range, without marked gonadotropin suppression.

variable	mean	SEM	range	reference
age	27.6	2.3	20-34	
FSH	4.1	0.4	2.9-4.9	1-10IU/l
LH	3.7	1.7	0.5-9.6	1-9IU/l
Oestradiol	122.8	16.3	63-156	0-160pmol/l
Testosterone	15.5	0.8	12.3-17.2	10-38nmol/l

Table 3.3. Overview of age of transgender patients (n=8), blood results (5 patients consented for blood tests pre-operatively) and reference ranges (normal male reference ranges as set by laboratory NHS Lothian). Results presented as mean and SEM.

3.6.2. Comparing serum hormone levels and ovarian follicle distribution

A correlation analysis was performed (5 patients) to determine if there was any link between serum hormone levels and ovarian follicle distribution. Serum hormone levels were compared to the percentage proportion of ovarian follicles within each classification (non-growing, primary, secondary, table 3.6.2) for each individual patient. There were no significant correlations identified between serum hormone levels and percentage follicle classification for any follicle type. Pearsons' correlation co-efficients are presented in table 3.6, none of the p values were less than 0.05 and therefore were not significant.

	Non-growing	Primary	secondary
FSH	-0.17	0.15	0.15
LH	0.42	-0.59	0.37
oestradiol	0.52	-0.44	-0.5
testosterone	-0.65	0.52	0.69

Table 3.4. Pearson's correlation co-efficients comparing hormone levels (FSH, LH, oestradiol, testosterone) to follicle distribution (non-growing, primary, secondary). Statistical significance assigned $p < 0.05$. All p levels > 0.05 therefore non-significant.

3.6.3. Analysis of follicle distribution and morphological health

A total of 32 cortical fragments were obtained on day 0 from 8 transgender and 8 control patients to determine follicle classification and morphological health, with a total of 4526 follicles analysed (see table 3.4).

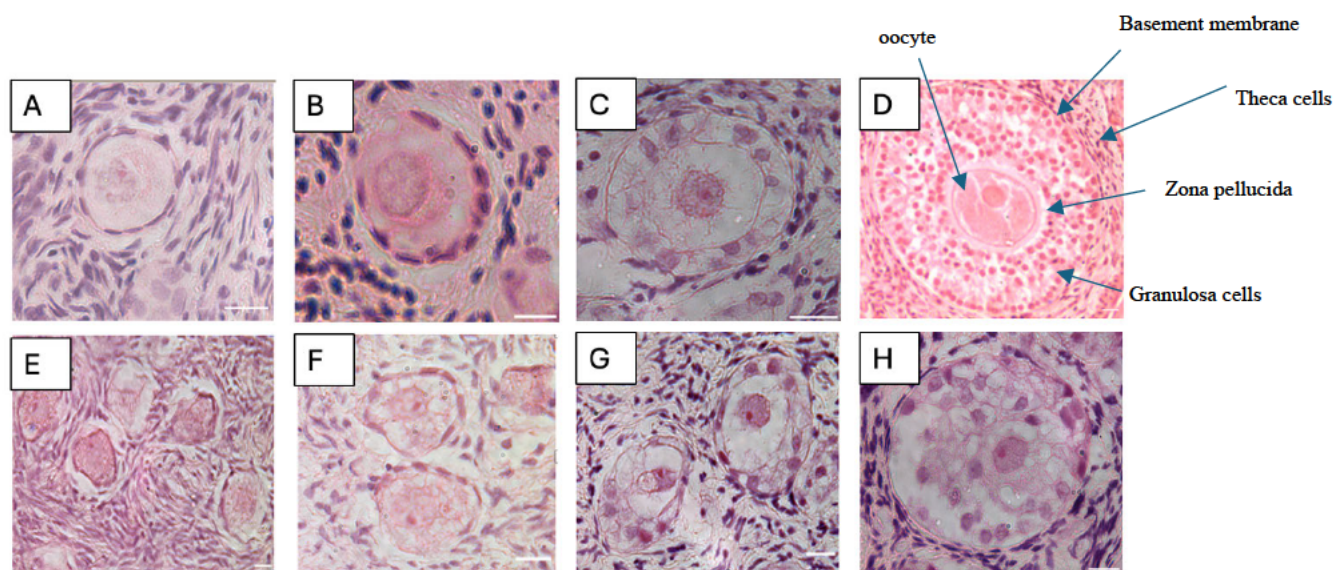


Figure 3.1- Representative images of haematoxylin and eosin (H&E) stained sections showing ovarian follicle development. (A) primordial follicle with a single layer of flattened granulosa cells from control day 0 tissue, (B) transitional follicles from control day 0 tissue,

(C) single layer of cuboidal granulosa cells in primary follicles from cultured control tissue (day 6), (D) multilayer cuboidal granulosa cells in a secondary follicle from cultured control tissue, ((E) primordial follicles and (F) transitional follicles from transgender day 0 tissue. (G) primary follicles from transgender cultured tissue, (H) secondary follicle from cultured transgender tissue. Scale bar 20µm.

Patient	Non-growing (% total)	Primary (% total)	Secondary (% total)	total
transgender	3619 (93.9)	192 (4.7)	60 (1.4)	3871
1	550 (88.3)	51 (8.2)	22 (3.5)	623
2	780 (90.1)	68 (7.9)	17 (2)	865
3	522 (96.1)	13 (2.4)	8 (1.5)	543
4	123 (94.6)	6 (4.6)	1 (0.8)	130
5	371 (98.1)	4 (1.1)	3 (0.8)	378
6	695 (97.3)	16 (2.2)	3 (0.5)	714
7	238 (93)	13 (5.0)	5 (2)	256
8	340 (93.9)	21 (5.8)	1 (0.3)	362
Mean±SEM	93.9%±1.2	4.7%±0.9	1.4%±0.4	
Control	578 (88.4)	64 (7.7)	13 (1.9)	655
1	92 (87.6)	10 (9.5)	3 (2.9)	208
2	164 (78.8)	38 (18.3)	6 (2.9)	208
3	35 (97.2)	1 (2.8)	0 (0)	36
4	40 (90.9)	4 (9.1)	0 (0)	44
5	63 (92.6)	3 (4.5)	2 (2.9)	68
6	56 (88.9)	5 (7.9)	2 (3.2)	63
7	101 (81.5)	3 (2.5)	0 (0)	124
8	25 (89.3)	2 (7.1)	1 (3.6)	28
Mean±SEM	88.4%±2.1	7.7±1.8	1.9%±0.6	
Total	4197	256	73	4526

Table 3.5 - table of total follicle counts and percentage distribution per patient for both control (n=8) and transgender groups (n=8) at day 0. Note all data have subsequently been analysed as the mean and SEM.

At day 0, the most prevalent follicle type was non growing, and there were significantly more non growing follicles found in transgender tissue compared to the age matched control (93.9%±1.2 vs 88.4%±2.1 p<0.05). In addition to this, the proportion of primary (4.7%±0.9 vs 7.7%±1.8) and secondary (1.4%±0.4 vs 1.9%±0.6) follicles was lower in transgender tissue with no pre-antral or antral follicles identified (figure 3.2A).

At day 0, the proportion of morphologically healthy non-growing follicles was significantly lower in the transgender tissue compared to control (57.5%±2.8 vs 79%±4.6, p≤0.001). The morphological health of primary and secondary follicles between both groups at day 0 was comparable (primary 66.8%±2.8 vs 59.7%±4.6, secondary 76.2%±10.8 vs 91.7%±8.3) (figure 3.2B).

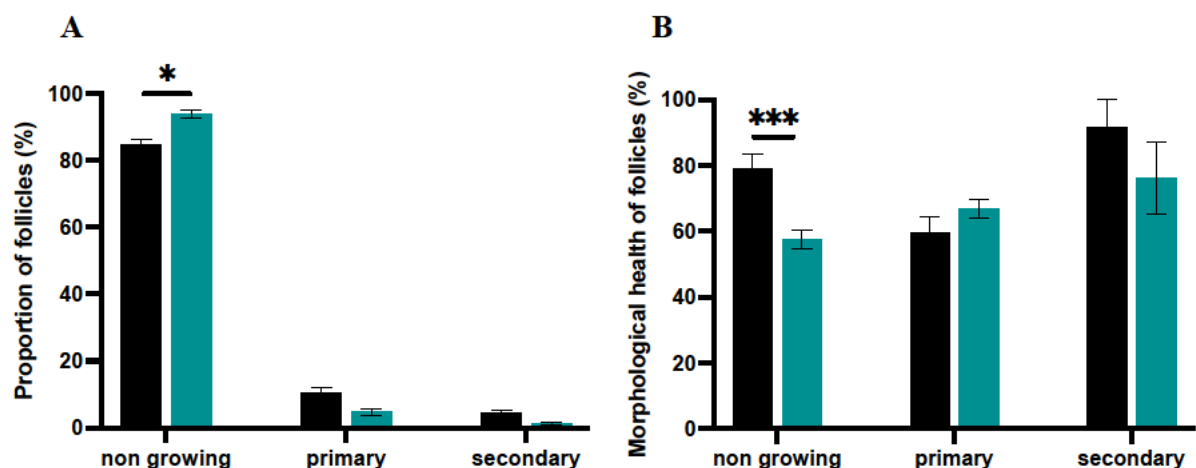


Figure 3.2. (A) Follicle classification- proportion of follicles in each follicle classification stage in transgender and control tissue at day 0. (B) Morphological health- proportion of morphologically healthy follicles at each follicle stage in control and transgender tissue day

0. Both charts- Black bar- control day 0, green bar- transgender day 0. Analysis was performed for both sets of data using Kruskal Wallis test with post- hoc Bonferroni test, p-value < 0.05 was considered significant, asterisk (***) ≤ 0.001 , (**) ≤ 0.01 , (*) < 0.05 .

At day 0, there was no significance difference in follicle diameter between control and transgender non-growing (transgender $43\mu\text{m}\pm 0.9$ vs control $37.7\mu\text{m}\pm 2.0$) and secondary follicles ($112.1\mu\text{m}\pm 14.6$ vs $76.8\mu\text{m}\pm 10.7$) (figure 3.3A). The diameter of transgender primary follicles was significantly greater than in control ($51.5\mu\text{m}\pm 1.2$ vs $43.3\mu\text{m}\pm 3.5$, $p < 0.05$). For oocyte diameter, non-growing follicles in transgender tissue had a significantly larger oocyte diameter (transgender $38.7\mu\text{m}\pm 1$ vs control $33.6\mu\text{m}\pm 1.7$, $p < 0.05$) than control oocytes. There was no significant difference in oocyte diameter in primary and secondary follicles between groups (figure 3.3B).

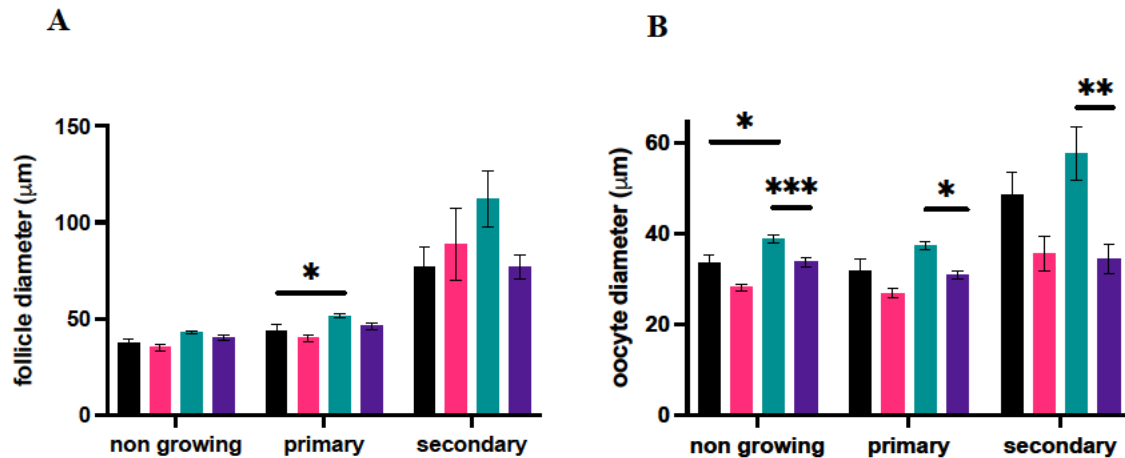


Figure 3.3- follicle and oocyte diameter in control and transgender tissue at day 0 and day 6. (A) follicle diameter analysed with Kruskal Wallis test with post- hoc Bonferroni test. (B) oocyte diameter analysed with Kruskal Wallis test with post- hoc Bonferroni test. Black bar- control day 0, pink bar- control day 6, green bar- transgender day 0, purple bar- transgender

day 6. All data displayed as mean $\pm$ SEM, statistical significance assigned $p < 0.05$, Asterisk (***) ≤ 0.001 , (**) ≤ 0.01 , (*) < 0.05 .

3.6.4. The impact of testosterone on follicle activation and survival

A total of 32 cortical strips were obtained to determine the follicle classification and morphological health of follicles before and after 6 days of culture and a total of 3367 follicles were analysed (see table 3.5). In both the transgender and control groups, after 6 days of culture, the proportion of non-growing follicles significantly declined compared to day 0 (transgender $93.9\% \pm 1.2$ to $56.0\% \pm 3.3$, control $88.4\% \pm 2.1$ to $65.5\% \pm 3.2$, both $p \leq 0.001$). The proportion of non-growing follicles in the transgender group was significantly lower than that found in control tissue ($56\% \pm 3.3$ vs control $65.5\% \pm 3.2$, $p < 0.005$).

In both groups, this decline was balanced with a reciprocal increase in the proportion of growing follicles, in particular primary follicles (transgender: primary $4.7 \pm 0.9\%$ to $35.5 \pm 1.9\%$, $P < 0.001$, secondary $1.4 \pm 0.4\%$ to $8.5 \pm 2.3\%$, $P < 0.005$, control: primary $7.7 \pm 1.8\%$ to $30.6 \pm 2.4\%$, $P < 0.001$, secondary $1.9 \pm 0.6\%$ to $4.2 \pm 1.1\%$, $P = 0.99$) (figure 3.4A).

Patient	Non-growing (% total)	Primary (% total)	Secondary (% total)	total
transgender	1402 (56.0)	934 (35.5)	281 (8.5)	2617
1	136 (42.8)	125 (39.3)	57 (17.9)	318
2	401 (48.8)	275 (33.5)	145 (17.7)	821
3	179 (50.6)	148 (41.8)	27 (7.6)	354
4	70 (55.1)	43 (33.9)	14 (11)	127
5	170 (72.3)	58 (24.7)	7 (3)	235
6	73 (65.2)	37 (33)	2 (1.8)	112
7	192 (57.3)	127 (37.9)	16 (4.8)	335
8	164 (56)	116 (39.6)	13 (4.4)	293
Mean±SEM	56.0±3.3	35.5±1.9	8.5±2.3	
Control	483 (65.5)	233 (30.6)	34 (4.2)	750
1	36 (54.5)	26 (39.4)	4 (6.1)	66
2	197 (67.7)	86 (29.6)	8 (2.7)	291
3	37 (66.1)	16 (28.5)	3 (5.4)	56
4	48 (61.5)	26 (33.4)	4 (5.1)	78
5	92 (60.1)	48 (31.4)	13 (8.5)	153
6	20 (57.2)	13 (37.1)	2 (5.7)	35
7	39 (72.2)	15 (27.8)	0 (0)	54
8	14 (82.4)	3 (17.6)	0 (0)	17
Mean±SEM	65.5±3.2	30.6±2.4	4.2±1.1	
Total	1885	1167	315	3367

Table 3.6- table of total follicle counts and percentage distribution per patient for both control (n=8) and transgender groups (n=8) at day 6. Note all data has subsequently been analysed as the mean and SEM.

On day 6, in the transgender tissue, there was a reduction in the proportion of morphologically healthy follicles at all stages compared to day 0 (non-growing $57.5\% \pm 2.8$ to $32\% \pm 4.4$, primary $66.8\% \pm 6.5$ to $25.1\% \pm 4.6$, secondary $76.2\% \pm 10.8$ to $36.3\% \pm 4.2$, $p < 0.05$ for all follicle types) (figure 3.4B). A decline in morphological health was seen in control non-growing follicles ($79\% \pm 4.6$ to $54.4\% \pm 3.3$, $p < 0.005$) with the non-significant decline in morphological health of primary and secondary follicles. The decline in the proportion of morphologically healthy follicles in the transgender ovarian follicles was greater than control (non-growing $44.8\% \pm 6.9$ vs $35.9\% \pm 4.6$ $p < 0.01$, primary $62\% \pm 6.8$ vs $5.3\% \pm 30.1$, $p < 0.05$, secondary $39.4\% \pm 23$ vs $43.2\% \pm 12.9$) after 6 days of culture.

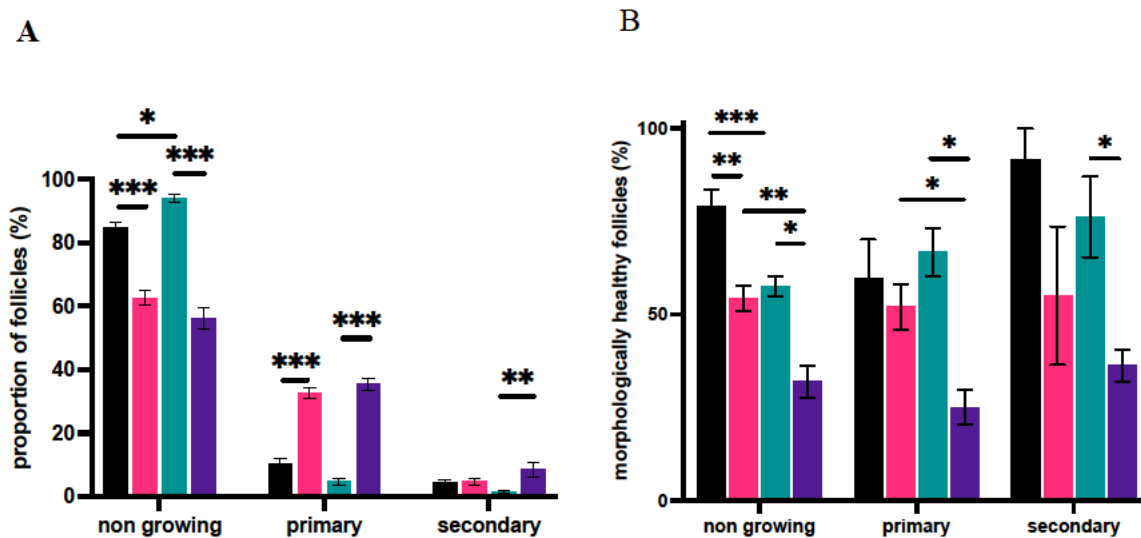


Figure 3.4. (A) Follicle classification- proportion of follicles in each follicle classification stage in transgender and control tissue at day 0 and day 6. (B) Morphological health- proportion of morphologically healthy follicles at each follicle stage in control and transgender tissue day 0 and day 6. Both charts- Black bar- control day 0, pink bar- control

day 6, green bar- transgender day 0, purple bar- transgender day 6. Analysis performed for both sets of data using Kruskal Wallis test with post- hoc Bonferroni test, p-value < 0.05 was considered significant, asterisk (***) ≤ 0.001 , (**) ≤ 0.01 , (*) < 0.05.

There was no significant difference in follicle diameter after 6 days of culture both within and between groups (transgender non-growing $43\mu\text{m}\pm 0.9$ to 40.1 ± 1.2 , primary $51.5\mu\text{m}\pm 1.2$ to $46\mu\text{m}\pm 1.8$, secondary $112.1\mu\text{m}\pm 14.6$ to $77\mu\text{m}\pm 6.1$, vs control non-growing $37.7\mu\text{m}\pm 2.0$ to $35\mu\text{m}\pm 1.6$, primary $43.3\mu\text{m}\pm 3.5$ to $39.9\mu\text{m}\pm 1.6$, secondary $76.8\mu\text{m}\pm 10.7$ to $88.5\mu\text{m}\pm 13.5$) (figure 3.3A). Although not statistically significant, in the transgender group, a reduction in follicle size was seen across all follicle types, whereas in the control group, there was a reduction in size in non-growing and primary follicles but an increase in size in secondary follicles.

In transgender follicles, the oocyte diameter decreased in non-growing, primary and secondary follicles after 6 days of culture (non-growing $38.7\mu\text{m}\pm 1$ to $33.7\mu\text{m}\pm 1$, primary $37.3\mu\text{m}\pm 1$ to $30.9\mu\text{m}\pm 1$, secondary $57.5\mu\text{m}\pm 5.9$ to $34.4\mu\text{m}\pm 3.2$, $p < 0.05$ for all follicle types) (figure 3.3B). This reflects poor follicle health with retraction of the oocyte from the basement membrane, resulting in oocyte shrinkage. A similar but non statistically significant decline in the oocyte diameter was identified in control follicles (non-growing $33.6\mu\text{m}\pm 1.7$ to $28.1\mu\text{m}\pm 0.8$, primary $31.8\mu\text{m}\pm 2.6$ to $26.9\mu\text{m}\pm 1.1$, secondary $48.5\mu\text{m}\pm 5.1$ to $35.5\mu\text{m}\pm 3.8$). There was no significant difference in oocyte diameter between groups on day 6 across all follicle types.

3.6.5. The effects of testosterone on DNA damage

A total of 16 cortical strips were obtained to identify and quantify expression of γ H2AX in the oocytes and granulosa cells of ovarian follicles. Both uncultured day 0 and day 6 control and transgender tissue was analysed. The analysis included 840 follicles.

At day 0, the proportion of oocytes from non-growing and primary follicles expressing γ H2AX was higher in transgender tissue compared to control (non-growing $49.1\% \pm 8.5$ vs $28.6\% \pm 8$, primary $40\% \pm 12.7$ vs $3.3\% \pm 3.3$, both $p < 0.05$) (figure 3.6A). No secondary follicles were identified for analysis in the control group or on day 0 in the transgender group.

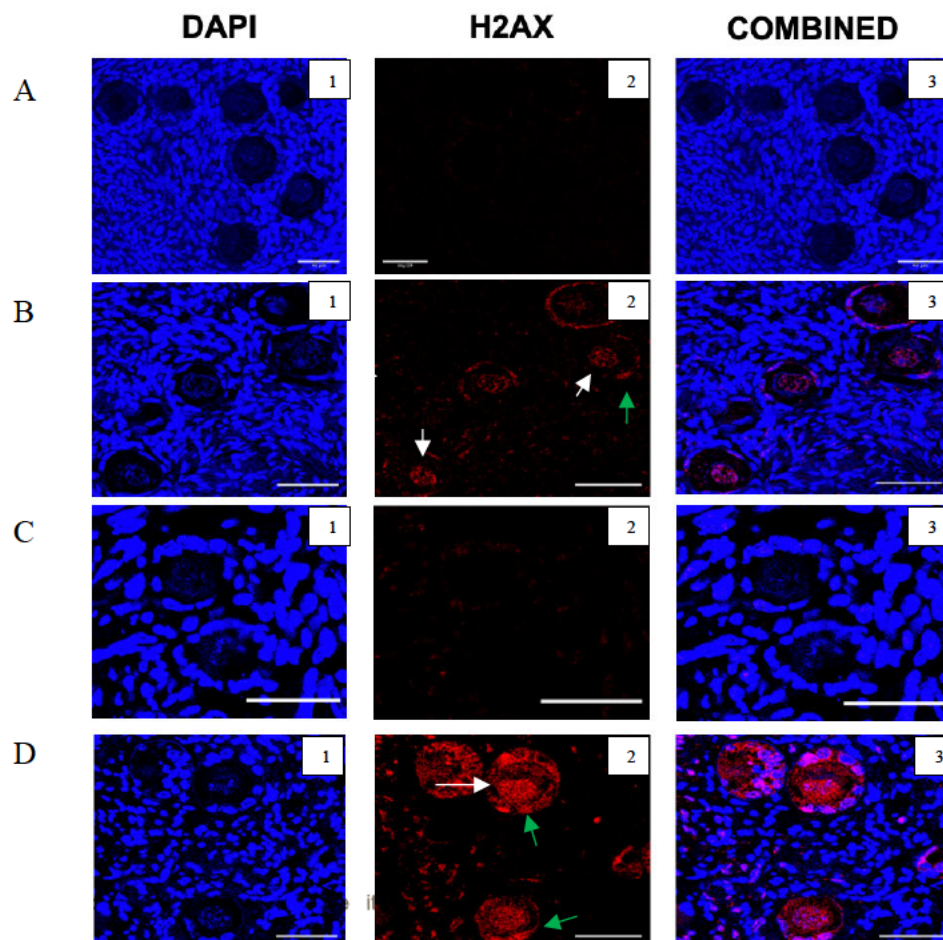


Figure 3.5. Representative images showing localisation of γ H2AX by immunofluorescence in human ovarian tissue. γ H2AX (red) and DAPI (blue) staining in

oocyte and granulosa cells. γ H2AX staining is identified as red foci within nuclei (white arrows) in oocytes and granulosa cells (green arrows). (A) control day 0- non-growing follicles, oocytes and granulosa cells negative for γ H2AX, (B) transgender day 0- foci of γ H2AX identified within the oocytes and granulosa cells of non-growing follicles, (C) control day 6- growing follicles, negative staining for γ H2AX, (D) transgender day 6- growing follicles, positive foci identified within oocytes and granulosa cells. Scale bar = 40 μ m.

The proportion of non-growing follicles expressing γ H2AX in the granulosa cells at day 0 did not significantly differ between groups ($7.3 \pm 2.5\%$ vs $4.7 \pm 2.4\%$), with comparable proportions of granulosa cells per follicle affected ($5.3 \pm 1.7\%$ vs $4.2 \pm 1.4\%$) (figure 3.6B). In primary follicles, there was an increase in the proportion of follicles expressing γ H2AX in the granulosa cells in the transgender tissue compared to control ($19 \pm 9.2\%$ vs $6.6 \pm 6.7\%$) but with comparable proportions of granulosa cells per follicle affected ($20.7\% \pm 14.7$ vs 26.3%) (figure 3.6C).

After 6 days of culture, expression of γ H2AX in oocytes of non-growing follicles remained high in transgender tissue ($49.1\% \pm 8.5$ to $49.5 \pm 13\%$), whereas in control non-growing oocytes the expression of γ H2AX declined ($28.6\% \pm 8$ to $19.4\% \pm 6.9$ $p=0.1$). Expression of γ H2AX increased in primary oocytes in both groups (transgender $40\% \pm 12.7$ to $48.9 \pm 12.5\%$, $p=0.5$ vs control $3.3\% \pm 3.3$ to $22.6 \pm 4.8\%$ $p=0.5$) and was identified in $36.7 \pm 18.6\%$ of oocytes from secondary follicles in the transgender group (figure 3.6A).

After 6 days of culture, the proportion of follicles expressing γ H2AX in the granulosa cells in control non-growing follicles was comparable to day 0 ($4.7\% \pm 2.4$ to $5.6\% \pm 3.2$), whereas in the transgender tissue, the expression was increased ($7.3\% \pm 2.5$ to $18.1\% \pm 9.8$) (figure 3.6B). The percentage of granulosa cells positive for γ H2AX per follicle in the control group after 6

days of culture was static in non-growing follicles ($4.2\% \pm 1.4$ to $4.2\% \pm 0.5$) but increased in the transgender non-growing follicles ($5.4\% \pm 1.7$ to $20.4\% \pm 8.7$, $p < 0.05$) (figure 3.6C).

In primary follicles, the proportion of follicles expressing γ H2AX in the granulosa cells increased non-significantly in both the control ($6.7\% \pm 6.7$ to $17\% \pm 5.9$) and transgender tissue ($19\% \pm 9.2$ to $47.3\% \pm 11.8$). In control primary follicles, the percentage of granulosa cells positive for γ H2AX per follicle declined after 6 days of culture (26.3% to $11.8\% \pm 2.4$) whereas it increased in transgender primary follicles ($20.8\% \pm 14.7$ to $27.1\% \pm 8.7$).

At day 6, γ H2AX was identified in $57.5\% \pm 9.2$ of secondary follicles, with $23.3\% \pm 11.3$ of granulosa cells per positively stained follicle affected (not included in graphs in figure).

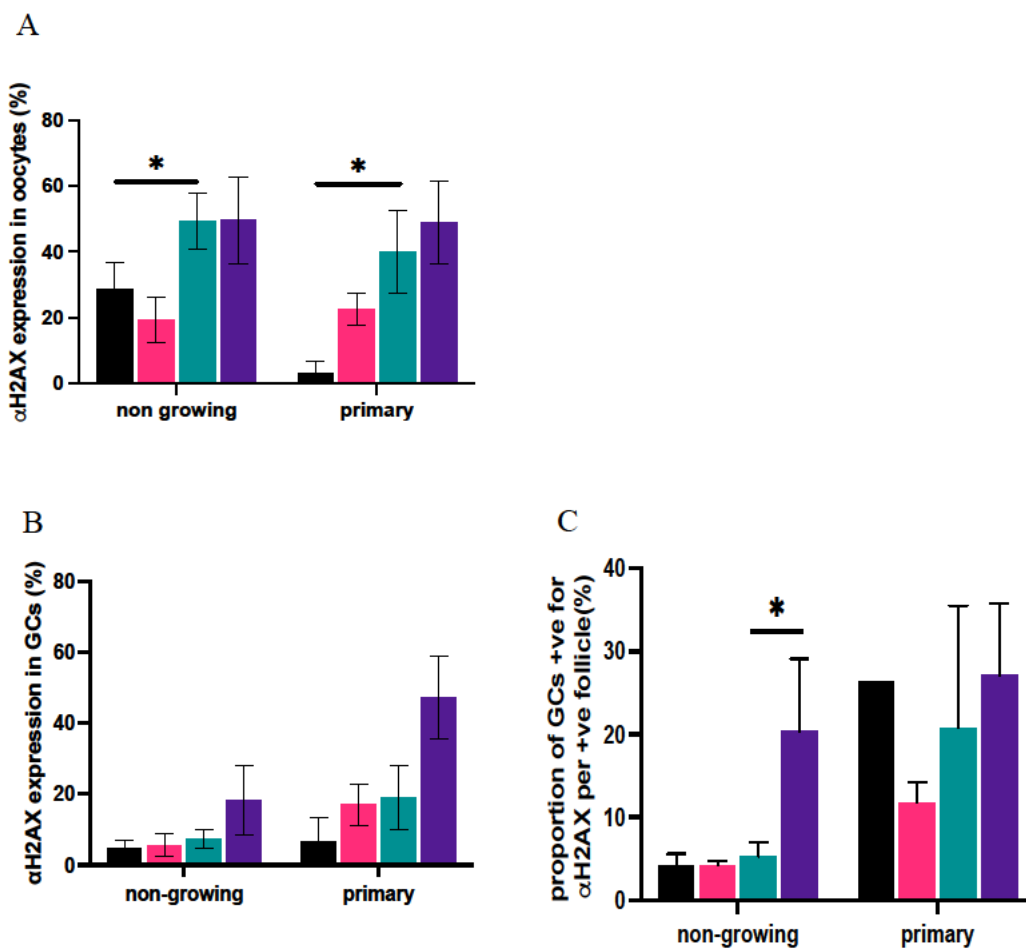


Figure 3.6. Localisation of γ H2AX in ovarian follicles. Positive expression of γ H2AX in oocytes (A) and granulosa cells (B and C) (total number of follicles n=840, control day 0 n=122, transgender day 0 n=384, control day 6 n=145, transgender day 6 n=189). In all charts- Black bar- control day 0, pink bar- control day 6, green bar- transgender day 0, purple bar- transgender day 6. Results were displayed as the mean $\pm$ SEM. Expression of γ H2AX in oocytes and granulosa cells and proportion of granulosa cells in affected follicles were analysed using Kruskal Wallis test followed by post-hoc Bonferroni test. Statistical significance assigned $p < 0.05$. Asterisk (***) $p \leq 0.001$, (**) $p \leq 0.01$, (*) $p < 0.05$.

In ovarian follicles, where there was positivity for γ H2AX in the oocytes and/or granulosa cells, the intensity of the immunofluorescent staining was measured by calculating the mean gray value. In both the oocytes and the granulosa cells, there was no significant difference in the intensity of the staining between control and transgender tissue at day 0 or after 6 days of culture across all follicle types (figure 3.7).

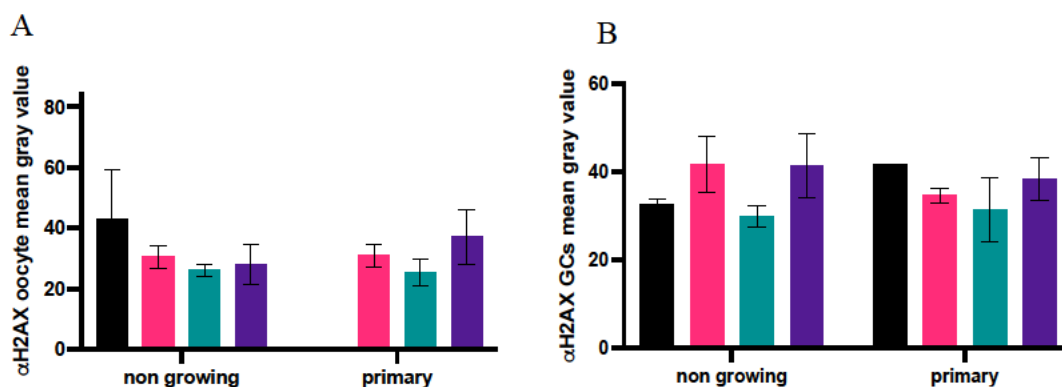


Figure 3.7. Intensity of γ H2AX immunofluorescence staining in oocytes (A) and granulosa cells (B) in control (n=8) and transgender groups (n=8). Both charts- Black bar- control day 0, pink bar- control day 6, green bar- transgender day 0, purple bar- transgender day 6.

Analysis was performed using Kruskal Wallis test with post-hoc Bonferroni test for both oocytes and granulosa cells analysis. Statistical significance was assigned $p < 0.05$.

3.6.6. The effects of testosterone on DNA repair capacity of ovarian follicles

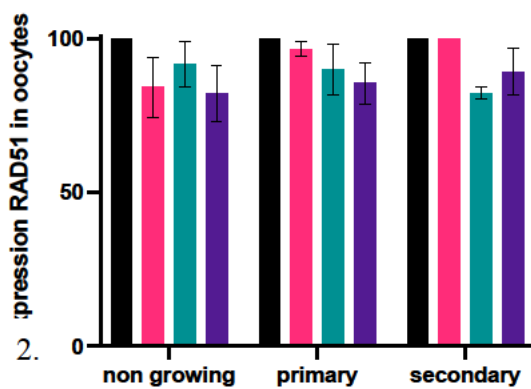
To identify whether testosterone affects the DNA repair capacity of ovarian follicles, expression of ATM, RAD51 and MRE11 in oocytes and granulosa cells was analysed in follicles from transgender and control tissue at day 0 and after 6 days of culture.

A total of 16 cortical strips were obtained to identify and quantify expression of DNA repair proteins in oocytes and granulosa cells across 8 control and 8 transgender tissue samples. For expression of RAD51, 834 follicles were analysed. At day 0, there was no significant difference in expression of RAD51 in the oocytes or granulosa cells across all follicle types between control and transgender groups (figure 3.8). Very high levels of expression of RAD51 were found in oocytes at day 0 in both groups (transgender: non-growing $91.7\% \pm 7.4$, primary $90\% \pm 8.2$, secondary $82.3\% \pm 2$, control: 100% expression of RAD51 in the oocytes of all follicle types). The expression of RAD51 in granulosa cells is very low at day 0 across all follicle types in both groups (transgender: non-growing $2.5\% \pm 1.3$, primary $8.5\% \pm 4.7$, secondary $12.5\% \pm 12.5$, control: non-growing $6\% \pm 4.2$, primary $4.8\% \pm 4.8$). Of the ovarian follicles with positive foci of RAD51 in their granulosa cells, no significant difference was found in the proportions of granulosa cells that were positive per affected follicle between transgender (non-growing $27.4\% \pm 12.4$, primary $30\% \pm 25$, secondary 2%) and control groups (non-growing $56.7\% \pm 43.3$, primary 14.3%).

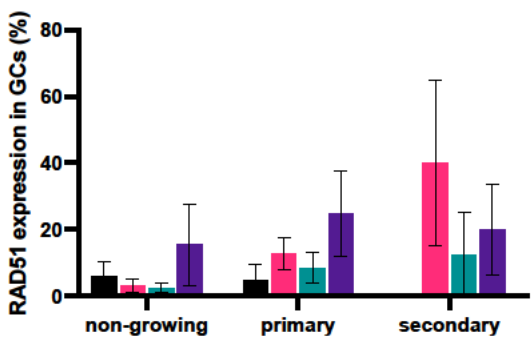
After 6 days of culture, there was no significant difference in expression of RAD51 in the oocytes or granulosa cells across all follicle types between control and transgender groups (figure 3.8). In both groups, there was a very similar slight decline in expression of RAD51 in

oocytes across all follicle types. In both groups, the expression of RAD51 in granulosa cells remained low in non-growing and primary follicles, with increased expression in secondary follicles. Of the ovarian follicles with positive foci of RAD51 in their granulosa cells, a similar trend was seen within both groups with an increase in the proportion of granulosa cells positive for RAD51.

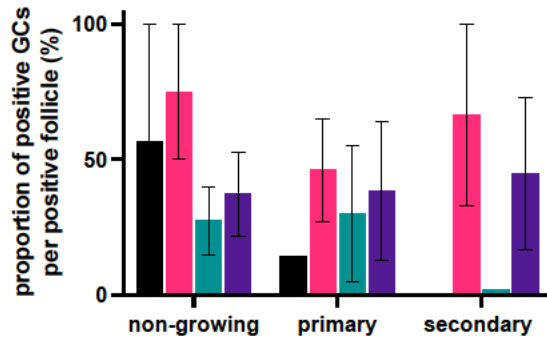
A



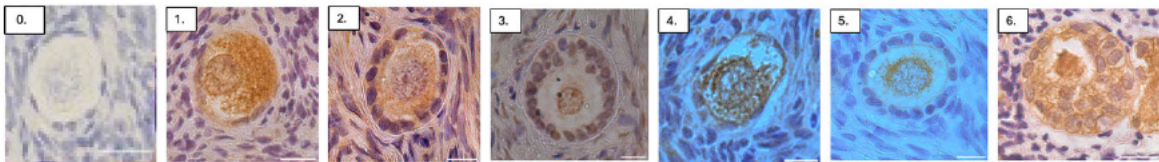
B



C



D



E

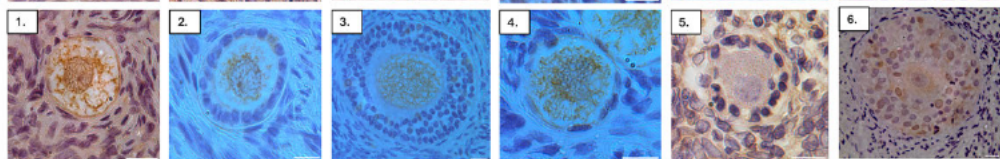


Figure 3.8. Positive expression of RAD51 in oocytes (A) and granulosa cells (B) and (C).

Results were displayed as the mean $\pm$ SEM. All charts- Black bar- control day 0, pink bar- control day 6, green bar- transgender day 0, purple bar- transgender day 6. Expression in oocytes and granulosa cells was performed using Kruskal Wallis test with post-hoc Bonferroni test. Statistical significance assigned $p < 0.05$. (D/E) Representative images of detection of RAD51. Photomicrographs of ovarian follicles for RAD51 from negative control (D0), transgender day 0 (D1–3), transgender day 6 (D4–6), control day 0 (E1–3) and control day 6 (E4–6). Positive staining (brown) in oocytes in D4 and D6, AND E1–5. Positive staining in granulosa cells in D5, E2 and E6. Scale bar = 20 μ m.

For expression of ATM, 800 follicles were analysed. At day 0, in oocytes, there was reduced expression of ATM in transgender non-growing and primary follicles compared to control (transgender: non-growing 60.3% $\pm$ 13.4, primary 68.3% $\pm$ 30.9, control: non-growing 93.9% $\pm$ 4.9, primary 85.7%) (figure 3.9A). 61.1% $\pm$ 30.9 of transgender secondary follicles expressed ATM in their oocytes and there were no secondary follicles present in the control tissue for comparison. At day 0, in both the transgender and control group, no follicles expressed ATM in the granulosa cells (figure 3.9B/C).

After 6 days of culture, a non-significant rise in expression of ATM in transgender non-growing and primary oocytes was seen whereas there was decreased expression in control non-growing and primary oocytes (figure 3.9A). There was expression of ATM in 100% of oocytes of control secondary follicles whereas the expression remained similar after 6 days of culture in the oocytes of transgender secondary follicles (61.1% $\pm$ 30.9 to 60% $\pm$ 24.5).

After 6 days of culture, in both transgender and control ovarian follicles, there was a non-significant increase in expression of ATM in granulosa cells across all follicle types (transgender: non-growing 19% $\pm$ 10.1, primary 36% $\pm$ 13.4, secondary 25.3% $\pm$ 12.6, control:

non-growing $15.8\% \pm 10.8$, primary $8.3\% \pm 7$, secondary $25\% \pm 25$) with a similar proportion of positive granulosa cells per positively affected follicle (figure 3.9B/C).

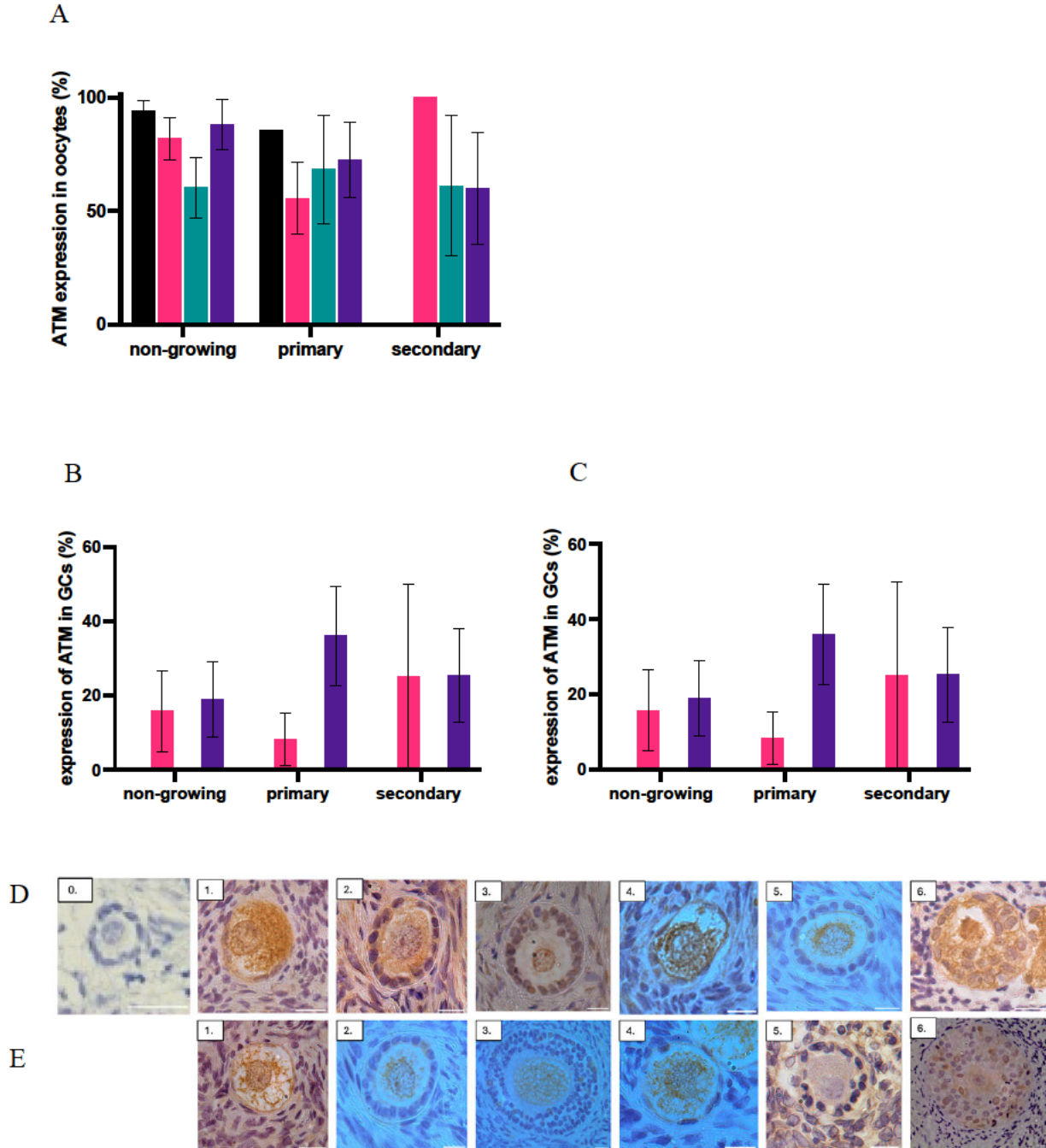


Figure 3.9. Positive expression of ATM in oocytes (A) and granulosa cells (B) and (C).

Results were displayed as the mean $\pm$ SEM. All charts- Black bar- control day 0, pink bar- control day 6, green bar- transgender day 0, purple bar- transgender day 6. Expression in

oocytes and granulosa cells was analysed using Kruskal Wallis test with post-hoc Bonferroni test. Statistical significance assigned $p < 0.05$. (D/E) Representative images of detection of ATM. Photomicrographs of ovarian follicles for ATM from negative control (D0), transgener day 0 (D1–3), transgener day 6 (D4–6), control day 0 (E1–3) and control day 6 (E4–6). Positive staining (brown) in oocytes in D1 and D3–6 and E1–4. Positive staining in granulosa cells in D1, D3–4 and D6, E2–3 and E6. Scale bar = 20 μm .

For expression of MRE11, 550 follicles were analysed. At day 0, there was no significant difference in expression of MRE11 in the oocytes or granulosa cells across all follicle types between control and transgener groups. Very high levels of expression of MRE11 were found in oocytes at day 0 in both groups (transgener: non-growing $87.5\% \pm 6.9$, primary $80\% \pm 20$, secondary 100%, control: non-growing $83.3\% \pm 16.7\%$, primary 100%, secondary 100%) (figure 3.10A). At day 0, the proportion of non-growing follicles expressing MRE11 in the granulosa cells is very low in both groups (transgener $6.9\% \pm 5.4$, control $4.2\% \pm 2.8$) and higher in primary and secondary follicles (transgener: primary 100%, secondary 100%, control: $50\% \pm 50$, secondary 100%) (figure 3.10B). In follicles where MRE11 was expressed, the proportion of positive granulosa cells was very high in both groups (transgener: non-growing $87.5\% \pm 12.5$, primary 100%, secondary 100%, control: 100% expression across all follicle types) (figure 3.10C).

After 6 days of culture, in both the control and transgener groups, there was a non-significant decline in expression of MRE11 in oocytes across all follicle types (figure 3.10A). A similar decline was seen in the proportion of follicles expressing MRE11 in the granulosa cells across all follicle types, whilst in follicles where MRE11 was expressed, the proportion of positive granulosa cells per follicle remained high (figure 3.10B/C).

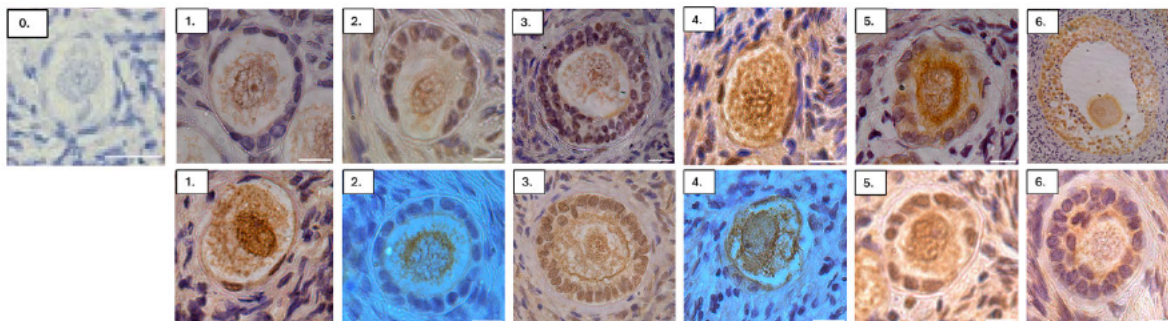
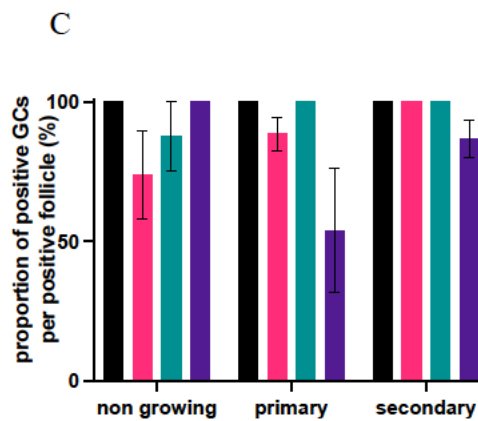
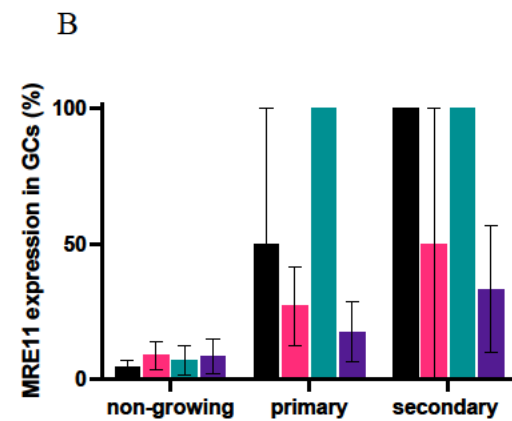
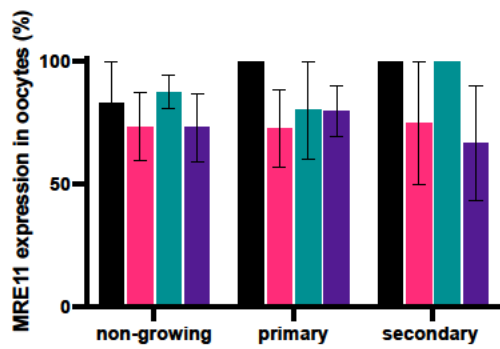


Figure 3.10- Positive expression of MRE11 in oocytes (A) and granulosa cells (B) and (C). Results were displayed as the mean $\pm$ SEM. All charts- Black bar- control day 0, pink bar- control day 6, green bar- transgender day 0, purple bar- transgender day 6. Expression of MRE11 in oocytes and granulosa cells was analysed using Kruskal Wallis test with post-hoc Bonferroni test. Statistical significance assigned $p < 0.05$. (D/E) Representative images of detection of MRE11. Photomicrographs of ovarian follicles for MRE11 from negative control (D0), transgender day 0 (D1–3), transgender day 6 (D4–6), control day 0 (E1–3) and control

day 6 (E4-6). Positive staining (brown) in oocytes in D1-6 and E1-6. Positive staining in granulosa cells in D2, D4 and D6, E1, E3-5. Scale bar = 20µm.

3.7. Discussion

In this study, a significant difference in the distribution of follicle classification by developmental stage in the ovaries of transmen compared to age matched controls was identified. The proportion of non-growing follicles in transgender tissue was significantly higher, suggesting that high dose testosterone therapy suppresses *in vivo* follicle development. This finding differs from previous studies (De Roo et al., 2017) (Boras et al 2021), where results indicate that testosterone exposed ovaries have a similar cortical follicle distribution to cisgender females. However, those studies used historic controls from the literature (Gougeon and Chainy, 1987a), where the age range was 19-49 and where results were based on findings from cisgender females who were known to be infertile (Lass, 1999). This study has used aged-matched females who donated ovarian cortical tissue at the time of caesarean section, and the analyses were performed at the same time and using the same methodology. In pregnancy, GnRH, FSH and LH levels are low as a result of negative feedback loop from high levels of oestrogen and progesterone. A similar negative feedback loop is active in transgender men as a result of taking gender affirming endocrine therapy as supraphysiological levels of testosterone will inhibit the production and release of gonadotrophins, although measured gonadotropin levels were in fact within the normal range. The non-growing follicle pool is of primary interest in this study and given that there is no evidence of any effect of high levels of hCG and oestradiol on these follicles with expected similar levels of gonadotrophins in both groups, our chosen age matched control is appropriate. However, the effect of pregnancy on follicle DNA damage and capacity for repair is unknown. Accessing tissue from healthy, aged matched non-pregnant women is very

difficult but possible examples may include age matched women having a hysterectomy for benign gynaecological conditions such as heavy menstrual bleeding. Using tissue from patients with ovarian pathologies has its own limitations: a study of follicle distribution and ovarian reserve in patients with a range of both benign and malignant ovarian pathologies concluded that the cortex surrounding an ovarian malignancy has reduced follicle density, and in women with benign ovarian lesions, stromal proliferation was observed (Pavone et al., 2014). Similarly, the ovarian cortex from women with an endometrioma had a reduced follicle number, poorer morphological health and fibrotic change within the stroma (Maneschi et al., 1993).

One possible explanation for reduced follicle activation in testosterone exposed tissue is related to intrinsic tissue pressure. Mechanical stress has been shown to maintain dormant state of primordial follicles (Nagamatsu et al., 2019). The dense, fibrous nature of the tissue of the ovarian cortex contributes to suppression of primordial follicles to prevent mass and premature activation, resulting in early depletion. Given that the ovarian cortex in transgender tissue has been shown to be stiffer in comparison to oncological tissue (De Roo et al., 2019), this increased tissue rigidity could be a possible explanation for reduced follicle activation *in vivo* (this is further explored in chapter 4). Another explanation could be due to the significantly higher proportion of morphologically abnormal follicles in the transgender tissue compared to control found in this study, indicating that the follicles from testosterone exposed tissue may have a limited potential for development.

Unlike other studies (De Roo et al., 2017, Ikeda et al., 2013a) there were no pre-antral/antral follicles identified for analysis in either the control or transgender groups. This may be due to the technique of tissue preparation, whereby ovarian medulla and areas of ovarian cortex containing haemorrhagic or cystic follicles were avoided.

In this study, after six days of culture, the rate of activation of non-growing follicles in transgender tissue was enhanced compared to control. The process of tissue dissection mechanically loosens the ovarian cortex and reduces intrinsic tissue pressure. In the transgender tissue where the follicles have been subjected to supraphysiological levels of testosterone, this results in enhanced follicle activation compared to control. This enhanced activation may be mediated through via the PTEN pathway where testosterone is known to mediate primordial follicle activation through nuclear exclusion of FOXO3A (Yang et al., 2010). In studies where primordial follicle activation was enhanced through suppression of PTEN, using hpV(HOpic), there was an increase in the proportion of growing follicles but a significant reduction in morphological health (McLaughlin et al., 2014a, Maidarti et al., 2019). In this study, the morphological health of the ovarian follicles after 6 days of culture declined to a greater extent compared to control. These findings are consistent with these studies, whereby enhanced rate of follicle activation was associated with poorer morphological health and could be considered detrimental to follicle health.

A limitation of this study is that there is a discrepancy in the volume of tissue available between groups, with whole ovaries collected from transgender patients and small fragments of ovarian cortex from control subjects. Unlike in rodent studies where absolute follicle counts can be achieved, as often in human studies where biopsies of the ovarian cortex are obtained, the proportion of follicle classification is calculated. Within human cortical tissue, the distribution of follicles can vary significantly between ovarian cortical fragments from the same ovary (Schmidt et al., 2003a). This presents a difficulty in assessing primordial follicle activation through classifying and counting follicles in uncultured and cultured tissue (Telfer and Zelinski, 2013). It is not possible to follow follicle activation in real time, therefore, to comment on follicle activation through assessing different ovarian cortical fragments pre and post culture is difficult.

Another consideration in this study is regarding how the ovarian cortical samples were obtained. The transgender ovarian cortical samples were from whole ovaries compared to the control samples which were taken by obstetric colleagues at the time of caesarean section. Given that the smaller control fragments were more likely to be subjected to increased tissue handling and dissection, this may increase the rate of follicle activation in culture. Despite this, there was significantly more follicle activation in the transgender tissue compared to control after 6 days of tissue culture, further confirming enhanced follicle activation as a result of testosterone exposure.

γ H2AX is a DNA repair protein that binds to the location of DNA damage and controls recruitment of DNA repair proteins. In this study, we found that expression of γ H2AX in oocytes of uncultured D0 non-growing follicles was significantly higher in the transgender group compared to control, in keeping with histological findings of poorer morphological health. This may indicate that high dose testosterone therapy induces DNA damage in non-growing ovarian follicles *in vivo*. After 6 days of culture, the expression of γ H2AX in the oocytes of non-growing follicles in the control group declined. This could indicate that DNA damage occurred in transit to the lab and in tissue preparation but was repaired during culture, as previously demonstrated using bovine ovary (Maidarti et al 2018). After 6 days of culture, levels of γ H2AX in the non-growing follicles of the transgender group remained stable and indeed increased in primary oocytes. The cause of sustained DNA damage in these testosterone exposed follicles is not clear but studies that have used an experimental hyperandrogenic model in culture (Bertoldo et al., 2019) found increased levels of reactive oxidative species (ROS). Oxidative stress is a state whereby ROS outbalances antioxidant levels, resulting ultimately in DNA damage and/or cell apoptosis. Polycystic ovary syndrome (PCOS) is a state of endogenous hyperandrogenism affecting 5-10% of females of reproductive age (Balen et al., 2003). There is an increasing body of literature looking at the

role of ROS and its contribution in the pathogenesis of PCOS (Zhang et al., 2008, Escobar-Morreale et al., 2011). Multiple studies have demonstrated increased levels of biochemical markers of oxidative stress in PCOS patients compared to control (Murri et al., 2013, Sabuncu et al., 2001, Palacio et al., 2006), whilst others have variable findings when looking for markers for anti-oxidative stress levels (Saleem et al., 2014, Zhang et al., 2008, Sabuncu et al., 2001). Given the known similarities between testosterone exposed tissue from transgender men and patients with PCOS, it would be reasonable to hypothesise that ROS are responsible for the DNA damage seen in testosterone exposed ovarian follicles.

It is essential that primordial follicles have a robust system of DNA damage recognition and repair mechanisms to ensure genetic integrity. Unrepaired or incorrectly repaired DNA DSBs result in infertility, miscarriage and genetic defects in offspring (Stringer et al., 2020). The preferred mechanism of DNA repair in primordial follicles is homologous recombination (HR), whereby a sister chromatid is used as a template to aid accurate, error free repair (Winship et al., 2018a).

The DNA repair proteins selected for this study are key players within the process of HR. Meiotic recombination 11 (MRE11)-Rad50, Nijmegen breakage syndrome 1 (NBS1) form the MRN complex which identifies the DNA DSB and initiates the DNA repair process (Rein and Stracker, 2014). Next, NBS1 protein interacts with ATM resulting in autophosphorylation of ATM at a serine residue. Following this, ATM next phosphorylates H2AX at the c-terminal serine 139 (γ H2AX). γ H2AX binds to the DNA DSB, resulting in the initiation of the downstream pathway whereby DNA is either repaired or apoptosis occurs (Oktay et al., 2015).

In this study, results showed that in cultured control tissue, a decline in expression of γ H2AX in non-growing oocytes was mirrored in a decline in expression of RAD51, ATM and

MRE11, and increasing levels of γ H2AX in primary follicles was found with high levels of expression of DNA repair proteins in the oocyte. No secondary follicles were identified for analysis in the control D0 group for γ H2AX to allow for identification of trend of DNA repair protein expression. In the cultured transgender non-growing oocytes, despite the level of expression of γ H2AX remaining stable, the levels of DNA repair protein expression had a non-significant decline. Similarly in growing oocytes, increasing levels of γ H2AX expression, indicating increased DNA damage were found, however levels of RAD51, MRE11 and ATM either had a minimal increase or declined after 6 days of culture, indicating a suboptimal DNA repair response. Small numbers of subjects and follicles being included in analysis is likely to be the reason for lack of statistical significance in these experiments. However, it was felt most appropriate to analyse the data per patient i.e. n=8 per group rather than analysing per follicle type. It prevented inter-patient variation skewing the data as different numbers of follicles were analysed per patient, however it results in small numbers which can make it more difficult to detect statistical significance. However, the trend in DNA repair protein expression would indicate that the DNA repair protein recruitment in transgender tissue is suboptimal. With RAD51, whose function is to find and invade homologous DNA sequences to enable accurate and timely DNA repair, in both groups there was high expression in oocytes in D0 and a similar slight decline in expression at D6. With ATM, whose role in HR includes DSB end resection, expression in oocytes, was reduced in transgender non-growing and primary follicles compared to control whereas on D6 a non-significant rise in expression of ATM in transgender non-growing and primary oocytes was seen whereas there was decreased expression in control non-growing and primary oocytes. With MRE11, the DNA binding and processing core of MRN complex, very high levels of expression were found in oocytes at D0 in both groups whereas at D6 there was a non-significant decline in expression of MRE11 in oocytes across all follicle types. This would

indicate that in transgender tissue, the expression of DNA repair proteins did not reflect an appropriate response to the levels of DNA damage identified. Ineffective DNA repair is pathognomonic in reproductive aging, resulting in accumulating DNA double stranded breaks (DSBs) (Gorbunova et al., 2007, Lieber and Karanjawala, 2004, Winship et al., 2018a, Ruth et al., 2021). DSBs have been found to accumulate in ovarian follicles of aging mice, with downregulation of key DNA repair proteins. Given the similarity of results, it would be reasonable to suggest testosterone impairs DNA repair capacity of ovarian follicles, resulting in compromised genetic integrity and reduced oocyte quality. However, this study only looks at the first step of this process- follicle activation therefore the results of these experiments cannot take into consideration the potential for DNA damage and DNA repair capacity in later stages of follicle growth.

To note, the analysis of the experiments in this chapter was carried out by 1 person and the slides were not masked for group (control vs transgender). This could allow for bias to be introduced. When measuring follicle diameter, particularly in secondary follicles, the theca layer was not included in the measurement. In retrospect, this would been useful as in transgender men taking testosterone therapy, there might be a degree of theca cell hyperplasia as a result of high circulating levels of testosterone. In addition, it also would have been of benefit to collect data on patient sex hormone binding globulin (SHBG) and AMH levels. SHBG is a protein synthesised in the liver which binds to steroid hormones testosterone, oestradiol and DHT and transports them in the blood in an inactive form. In PCOS, SHBG levels tend to be low due to excessive androgens, it would be reasonable to expect a similar finding in transgender patients taking exogenous testosterone. Whether AMH levels would have been raised is more debatable. Our study showed the highest proportion of non-growing follicles compared to control and no pre-antral/antral follicles were identified.

Some studies have concluded that testosterone does not affect the ovarian cortical follicle distribution (De Roo et al., 2017, Ikeda et al., 2013a) whilst other studies have found increased numbers of antral and atretic follicles, similar to PCOS (Amirikia et al., 1986, Futterweit and Deligdisch, 1986b, Spinder et al., 1989a, Pache et al., 1991, Grynberg et al., 2010). Small antral and preantral follicles produce AMH. AMH is raised in PCOS due to an accumulation of pre-antral and antral follicles, arrested in follicle development and is considered a biochemical marker for PCOS. A similar finding was not identified in this study so it cannot be assumed that transgender men on testosterone therapy would have a raised AMH.

The blood results from the transgender patients in this study are given with a male testosterone level reference range. In women, the local hospital normal reference range for serum testosterone is 0.7-2.8 nmol/L. Typically accepted values for a biochemical indicator of PCOS is testosterone levels 2.8-5.2nmol/l. In women, with a testosterone level >6.9nmol/l, it would be reasonable to further investigate for an ovarian or adrenal tumour. In this study, the mean serum testosterone levels were 15.5 ± 0.8 . This indicates that the testosterone levels in transgender men in this study are very substantially higher than in women with PCOS.

In this chapter, there was a wide range of duration of testosterone treatment (range 18 months–10 years). We found no relationship between the markers of DNA damage and duration of treatment, but it is possible that such a relationship exists and would be identified in a larger study focusing on that question.

The impact of exogenous testosterone on the ovary and the degree of reversibility is largely unknown. To date, there have not been any human studies that have addressed this but it is reassuring that on discontinuing testosterone therapy, transgender men have been able to readily conceive both naturally and through ART (Light et al., 2014b, Ghofranian et al.,

2023, Moravek et al., 2020). Animal models have been developed to try and mimic the effect of gender affirming endocrine therapy to assess for reversibility. A study was conducted whereby female mice received a subcutaneously implant testosterone pellet for 6 weeks. Results showed that when the testosterone pellet was removed, 100% of mice resumed cycle within 1 week, testosterone levels fell to control levels and there was no difference to follicle counts or corpora luteal formation 4 cycles later (Kinnear et al 2021). The difficulty in extrapolating findings from small animal studies is that it is not necessarily reflective of more long-term gender affirming endocrine therapy in transgender men.

In summary, the experiments in this chapter have demonstrated that testosterone appears to suppress *in vivo* follicle development, with poorer morphological follicle health and increased levels of DNA damage. However, once the ovarian cortical tissue is dissected and mechanically loosened, there is enhanced activation in culture. This enhanced level of activation is accompanied by a marked deterioration in morphological health, static or increased levels of DNA damage and compromised DNA repair capacity. This data suggests that testosterone has a detrimental effect on the non-growing ovarian follicle pool. This information is of relevance when considering commencing patients on testosterone therapy prior to considering fertility preservation options.

Chapter 4

The impact of testosterone on the composition of the stroma of the human ovary

4.1 Introduction

Within the ovarian cortex, primordial follicles comprising of non-growing oocytes surrounded by flattened squamous granulosa cells and enclosed within a basement membrane, form the resting pool of ovarian follicles where many can lie dormant for decades. Primordial follicles are continuously recruited into the growing follicle pool, characterised by growth of the oocyte and the surrounding granulosa cells becoming cuboidal and proliferating to form multiple layers of granulosa cells. Next, the theca cell layer differentiates from the ovarian stroma to form preantral follicles, with these early stages of growth being mainly regulated by paracrine and autocrine factors. Once follicles reach the preantral stage, they become dependent upon follicle stimulating hormone (FSH) to stimulate the progressive growth and development of a dominant antral follicle, with subsequent ovulation facilitated by a surge of luteinising hormone (Woodruff and Shea, 2007).

The ovarian stroma is the supportive connective tissue framework that encompasses the ovarian follicles, providing structural support and facilitating bidirectional paracrine signalling necessary for follicle development (Kinnear et al., 2020, Shea et al., 2014). It consists of connective tissue containing stromal cells, neurons, blood vessels and the extracellular matrix (ECM) (Shea et al., 2014, Kinnear et al., 2020). Recent proteomic studies have identified and spatially mapped over 1500 proteins within the human ovary (Henning et al., 2019a, Ouni et al., 2022). Key components of the ECM include collagen subtypes, essential in providing structural support (Kinnear et al., 2020), glycoproteins including elastin for tissue flexibility (Vindin et al., 2019), and fibronectin and laminin which contain integrin binding sequences essential for initiating proliferation and differentiation of follicular somatic cells (Kinnear et al., 2020, Monniaux et al., 2006). Several studies have concluded that in order to support the complex and dynamic process of follicle development, the composition

of the ovarian stroma must be able to undergo extensive remodelling to facilitate the demands of the growing follicle (Grosbois et al., 2023, Curry and Smith, 2006, Berkholtz et al., 2006). This has led to the biological function of the ECM and process of tissue remodelling becoming of particular interest in both the pathophysiology of conditions including polycystic ovary syndrome (PCOS), premature ovarian insufficiency (POI) and in physiological ovarian aging. It may also be the key to optimising ovarian tissue culture systems for fertility preservation and in tissue engineering developing the artificial ovary (Telfer et al., 2008b, Dolmans et al., 2008, Soares et al., 2015, Luyckx et al., 2014).

The impact that an alteration in the composition of the ovarian stroma has on reproductive outcomes is not well understood in humans, however more understanding has been gained from *in vitro* culture and in animal models. Altering the microenvironment of follicles with different 3D alginate hydrogels allows for the impact of changing the stiffness of the follicular microenvironment to be analysed. Results showed that a stiffer, less permissive environment impaired follicular growth *in vitro*, reduced the conversion of androgen and progesterone to oestrogen and produced oocytes with limited ability to resume meiosis (West et al., 2007, Jiao and Woodruff, 2013a). This indicates that the ovarian follicles' microenvironment has a significant impact not just on the ability of the ovarian follicles to grow and function but can influence their developmental capacity to produce high quality oocytes (West-Farrell et al., 2009, Xu et al., 2006b).

Hyperandrogenism drives changes within the ovarian stroma, and this has been demonstrated in the ovaries of women with PCOS and in transgender men taking testosterone therapy. Changes noted in both groups include thickening of the tunica albuginea, stromal cell hyperplasia and stromal cell luteinisation (Ikeda et al., 2013b, Chadha et al., 1994, Grynberg

et al., 2010, Futterweit and Deligdisch, 1986b, Spinder et al., 1989b, Pache et al., 1991, Hughesdon, 1982). Results from experiments in chapter 3 revealed that *in vivo*, testosterone exposed ovaries had a higher proportion of non-growing ovarian follicles compared to age matched control ovarian tissue from healthy pregnant women donated at the time of caesarean section, indicating that testosterone had a suppressive effect on activation of follicle development. This was an unexpected finding as testosterone has been reported to enhance primordial follicle activation in the mouse ovary through nuclear exclusion of Forkhead box O3A (FOXO3A) (Yang et al., 2010), an important transcription factor for maintaining primordial follicle dormancy within the PI3K/AKT pathway (John et al., 2008, Yang et al., 2010).

The experiments in this chapter aim to identify whether testosterone exposure alters the composition of the ovarian stroma as demonstrated in ovaries of transgender men taking exogenous testosterone therapy, namely by quantifying collagen and elastin content. The experiments also aim to quantify stromal cell density (SCD) and identify any relationship between SCD and follicle density in testosterone exposed tissue compared to age matched healthy controls.

4.2 Materials and Methods

4.2.1. Collection and analysis of follicle density

The H&E-stained slides that had been previously prepared and analysed for follicle classification, morphological health, follicle and oocyte diameter (Chapter 3) were used to quantify follicle density. Tissue sections from transgender and control D0 tissue that were

used to analyse the number and type of follicles, were used to calculate non growing follicle density (NGFD). Details of the method for analysing follicle density can be found in section 2.5.4. Briefly, sections were imaged using Leica upright Brightfield microscope x2.5 magnification in the IMPACT imaging facility. A scale bar measuring 1cm was imaged at the same magnification and the image was opened on Microsoft PowerPoint. The length of the imaged scale bar 1cm was measured at 4.42cm. The images taken for follicle density were opened in Microsoft PowerPoint and the length and breadth measurements taken. These measurements were subsequently divided by 4.42 to give the adjusted dimensions. Next, the area of each tissue section was calculated. For each patient, the total tissue area was calculated by adding the area of every tissue section analysed for each patient in mm^2 . Next, the total tissue area per patient was multiplied by 0.006 to calculate the total tissue volume in mm^3 (each tissue section thickness being $6\mu\text{m}$). Given that every tenth tissue section has been analysed, the tissue volume was multiplied by 10 to take into account the distance between sections (Lopes et al., 2020). The mean NGFD was calculated by dividing the total number of non-growing follicles per patient by the volume of tissue analysed in mm^3 . To assess if testosterone has an impact on NGFD, the NGFDs were compared between the transgender and control groups using the non-parametric Mann-Whitney test. To assess whether age had an impact on the NGFD, the ages of the patients of the patients in both groups were also compared using the Mann Whitney test, followed by comparison of NGFDs between groups. Predicted NGFDs were taken from table 1 of (McLaughlin et al., 2015), an externally validated model of age related mean NGFD derived from human ovarian cortex. Correlation between observed NGFD from transgender and control tissue and predicted NGFD was measured by calculating the Pearson product-moment correlation coefficient.

4.2.2. Analysis of stromal density

Details of analysis of stromal density (SCD) are in section 2.5.5. In brief, tissue sections from transgender D0 and control D0 groups (that were used for follicle classification) were analysed, with 5 tissue sections selected per patient, with three regions of interest (ROIs) selected across each section. The area of tissue was calculated using Image J software (0.02mm^2) and the tissue volume in mm^3 was calculated by multiplying the tissue area by 0.006 to account for tissue thickness $6\text{ }\mu\text{m}$ ($1.2 \times 10^4 \text{mm}^3$). The SCD per ROI was calculated using the following equation: total number of stromal cells/ total tissue volume mm^3 . The mean SCD of individual tissue sections was calculated by taking the average SCD of the 3 ROIs per tissue section. Next, the SCD was calculated per patient and data were presented in a box plot (Grosbois et al., 2023). A relationship between NGFD and SCD was tested using Spearman's correlation.

4.2.3. Analysis of collagen content by Picrosirius Red staining (PSR)

Tissue from transgender D0 and control D0 were analysed for stromal collagen content, with 5 sections selected per patient. The histological technique PSR is detailed in section 2.4.5. In brief, slides were dewaxed and rehydrated as per 2.3.3 and placed in Harris' haematoxylin for 8 minutes. The slides were next rinsed under running tap water for 10 minutes. Next, the slides were stained with PSR (Abcam) for 1 hour. Following this, the slides were rinsed in 1% acetic acid x2 washes and then most of the water was removed from the slides through vigorous shaking. The slides were next dehydrated in 3 separate changes in 100% alcohol, cleared in xylene and mounted in DPX.

Details of analysis of PSR staining are detailed in section 2.4.5. Briefly, the slides were first analysed using brightfield microscopy and imaged using Leica upright Brightfield

microscope x2.5 magnification. In addition to brightfield imaging, tiled images of the same PSR stained sections were taken using a Leica THUNDER polarised light microscope at x10 magnification in the IMPACT Imaging Facility to detect the collagen birefringence. Collagen fibres stained with PSR and viewed under polarised light, will display different colours depending on their fibre thickness and packing density; red (thickest collagen fibres), orange (medium thickness fibres) and green (thinnest collagen fibres). The hue (colour) of each pixel was quantified using a customised automated macro package and analysed using FIJI software. A colour threshold was used as previously described in the literature (Ouni et al., 2020, Grosbois et al., 2023): red 2–9, yellow/orange 10–38, green 24–135. The relative percentage of each colour was calculated by dividing the pixel count of each colour by the total pixel count for each image. Both brightfield and polarised light results are presented as mean and SEM.

4.2.4. Analysis of newly formed collagen in the ovary: Massons' Trichrome staining

Massons' Trichrome staining was used to identify newly formed collagen within the ovarian stroma (Pampanini et al., 2019). The histological technique is detailed in section 2.4.4. In brief, following dewaxing and dehydration and rinsing in tap water, slides were incubated in Bouins' fluid (Abcam) for 24 hours at room temperature. Next, the slides were immersed in running tap water until the tissue sections were completely clear from yellow staining. Following this, the slides were rinsed for one minute in distilled water and then immersed in working Wiegert's iron Haematoxylin (Abcam) solution for 5 minutes. The slides were then transferred into running tap water for 2 minutes to rinse. Biebrich Scarlet/acid fuchsin solution (Abcam) was applied to the slides for 15 minutes and subsequently rinsed in distilled water for 5 minutes. Next, the slides were differentiated in

Phosphomolybdic/phosphotungstic acid solution (Abcam) for 10 minutes or until the collagen was no longer red and then without rinsing, placed in Aniline Blue solution for 10 minutes. Next, the slides were further rinsed in distilled water and immersed in acetic acid solution 1% (Abcam) for 5 minutes. The slides were next quickly dehydrated in 2 changes of 95% ethanol followed by 2 changes of 100% ethanol, cleared in xylene for 15 minutes and mounted with DPX and glass covered slips.

Analysis of Massons' Trichrome is detailed in section 2.4.4. In brief, images of sections were taken using Leica upright Brightfield microscope x2.5 magnification in IMPACT imaging facility. A macro package was developed to identify the blue staining of new collagen formation using hue pixels 157-180 (blue colour). The percentage area of positive staining for collagen per tissue section was calculated (area of collagen-stained tissue/total area of ovarian tissue section x100) and the average percentage area of positive staining for new collagen deposition per patient was calculated by taking an average area of positive staining of all sections analysed. The results were presented as mean $\pm$ SEM.

4.2.5 Immunofluorescence

Immunofluorescence was carried out to identify and quantify elastin and Ki67 in the ovarian stroma. The immunofluorescence protocol is detailed in section 2.4.2. Briefly, mounted tissue sections were deparaffinised and rehydrated and then washed in phosphate-buffered saline (PBS) with 0.1% Triton (PBST) (pH 7.2–7.4) for 5 minutes. Slides were then equilibrated for 5 minutes in 10 mM sodium citrate prior to antigen retrieval. For elastin, antigen retrieval was performed as previously described in section 2.4.2. For Ki67, antigen retrieval required optimisation and was ultimately carried out using an autoclave at 125°C for 25 minutes and allowing 30mins to cool. Following this, the slides were incubated for 1h at RT with blocking

solution (5% goat serum in PBST). Primary antibody was applied to tissue sections and incubated overnight at 4 °C. Blocking solution without primary antibody acted as a negative control. After washing with PBST (4 washes x5 minutes), sections were incubated with an appropriate secondary antibody for 1 hour and then washed for a further 4x5 minutes. The slides were then mounted in Vectashield hardset with 4',6-diamidino-2-phenylindole (DAPI) (H-1500, Vector Laboratories) and stored at 4°C. Details of primary and secondary antibodies used in tables 4.1 and 4.2.

Blocking serum	Primary antibody	Product number	Species raised	Final concentration (dilution)	Source
Goat serum (Sigma Aldrich)	Elastin	Ab213720	Rabbit monoclonal	2.2µg/ml (1:250)	Abcam
Goat serum (Sigma Aldrich)	Ki67	M7240	Mouse	1:100	Dako

Table 4.1: Primary antibodies used in Immunofluorescence.

Secondary antibody	Product number	Final concentration	source
Cy3 AffiniPure Donkey anti-rabbit IgG	711-165-152	5.98 ug/ml (1.5 mg/ml)	Jackson Laboratories
Cy3 AffiniPure goat anti-mouse IgG	115-165-146	1.5mg/ml	Jackson Laboratories

Table 4.2: Secondary antibodies used in Immunofluorescence.

To quantify elastin within the ovarian stroma, five sections were analysed per patient from transgender D0 and control D0 groups. Images of the whole section were taken using the

Leica upright Brightfield and epifluorescence microscope x5 magnification and analysis of images was carried out using Image J software. The percentage area of positive staining for elastin per tissue section was calculated (area of elastin-stained tissue/total area of ovarian tissue section x100) and the average percentage area of positive staining for elastin content per patient was calculated by taking an average area of positive staining of all sections analysed. The percentage signal intensity was calculated per tissue section by quantifying the mean gray value of staining (positive elastin staining intensity/total tissue section intensity x100) and the average signal intensity was calculated per patient by taking an average of signal intensity of elastin of all sections. Both the results for area of positive staining and staining signal intensity are presented as mean $\pm$ SEM.

To quantify expression of Ki67, five sections were analysed per patient from both transgender and control D0 groups. Images of tissue sections were taken using the Leica upright Brightfield and epifluorescence microscope x20 magnification. Three ROIs were selected across each tissue section for analysis. Expression of KI67 in the ovarian stroma was analysed as a proportion of positive expression of the antibody in stromal cells per total number of stromal cells identified with DAPI staining.

4.3 Statistics

All data were analysed using SPSS statistical software version 24 (SPSS, Inc., Chicago, USA) and graphs were produced using PRISM Graph Pad version 8 (GraphPad Inc., San Diego, California, USA). Normality of data was tested using Shapiro-Wilk due to the sample size <35. Where 2 groups of data were compared, for normally distributed data, Student's t test was used. When data were not normally distributed, non-parametric Mann Whitney U test was used. Where more than 2 groups were compared, the parametric one-way analysis of variance (ANOVA) test with Tukey post hoc test was used on normally distributed data and

the non-parametric Kruskal Wallis test with the Dunne post hoc test was used for data that were not normally distributed. Data are presented as either box plots or mean $\pm$ SEM, with statistical significance assigned as $p \leq 0.05$. Pearsons' correlation efficient was used to correlate SCD and age and Spearman's correlation efficient was used to correlate SCD and NGFD.

4.4 Results

4.4.1. The impact of testosterone on NGFD

The number of follicles and their developmental stage were determined from uncultured tissue from 8 control and 8 transgender patients (D0). NGFD was calculated by dividing the total number of NGFs by the volume of tissue (McLaughlin et al., 2015).

Group	Age	Total number of non-growing follicles (NGFs)	Observed non-growing follicle density (NGFD) NGF/mm ³
transgender	34	67	12.9
	27	342	45.6
	32	229	26.3
	25	330	33
	30	187	25.3
	20	117	50.9
	30	278	30.5
	23	582	109.8
control	36	40	12.5
	33	42	19.1
	35	24	12
	32	35	21.9
	25	65	40.6
	26	32	40
	32	50	19.2
	31	43	22.6

Table 4.3- Observed NGFD for each patient in both transgender and control groups.

The follicle density in the transgender tissue was comparable to that in control tissue 31.8/mm³ (25.6-49.6) vs 20.6/mm³ (14.2-35.7) p=0.065 (figure 4.1A). However, this data does not reflect patient age (transgender: mean age: 27.6 ± 1.7 years; range 20-34 years, control: mean age: 31.8±1.5 years; range= 25-35 years). Observed mean follicle densities from both control and transgender groups were then compared to predicted mean follicle

densities using an age-related model of mean follicle densities in the cortex of the human ovary (McLaughlin et al., 2015). This analysis showed that the NGFD in testosterone exposed tissue closely correlated to both age matched controls and those of age matched model predicted densities (Pearson's correlation coefficient- control expected vs observed $r=0.993$, CI 95% 0.948-0.999 $p=0.00$, transgender expected vs observed $r=0.87$, 95%CI 0.57-0.99, $p=0.01$, expected vs combined observed $r=0.92$, 95% CI 0.854-0.981, $p=0.00$) indicating that testosterone does not accelerate or delay depletion of the non-growing follicle pool (figure 4.1B).

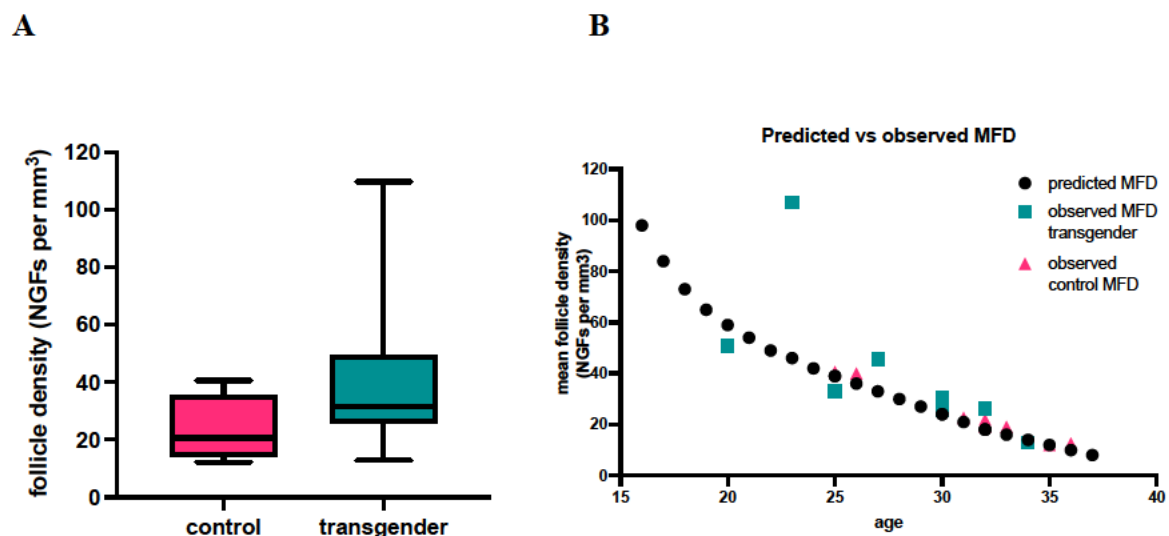


Figure 4.1- NGFD in transgender and control ovarian tissue. (A) Box plot of NGFD (n=8 in each group). Analysis performed using the Mann Whitney test, data displayed as a box plot, pink box for control, green box for transgender. (B) observed and predicted NGFD compared to age, black circle depicts data of predicted NGFD taken from table 1 in (McLaughlin et al., 2015), pink triangle observed control NGFD, green square observed transgender NGFD.

4.4.2 The impact of testosterone on stromal cell density

Sections that were used for follicle density were also used to analyse the SCD from 8 control and 8 transgender patients (D0 tissue). Five sections were analysed per patient with three ROIs selected across each section (figure 4.2B). In the transgender tissue, the SCD was significantly higher than control tissue ($2.5 \times 10^6 \pm 0.1 \text{ cells/mm}^3$ vs $1.7 \times 10^6 \pm 0.1 \text{ cells/mm}^3$ $p < 0.01$) (figure 4.2A). This is in keeping with stromal cell hyperplasia that has been described previously in the ovaries of transgender men who have taken high dose testosterone therapy (Futterweit and Deligdisch, 1986a, Grynberg et al., 2010, Pache et al., 1991, Spinder et al., 1989a).

In this study, no correlation was found between SCD and age in each group or with both groups combined (transgender: Pearson's correlation $r=0.5$, $p=0.2$, control: Pearson's correlation $r=0.7$, $p=0.5$, combined: Pearson's correlation $r=-0.37$, $p=0.27$). There was also no correlation found between SCD and duration of testosterone treatment (Pearson's correlation $r=0.52$, $p=0.23$).

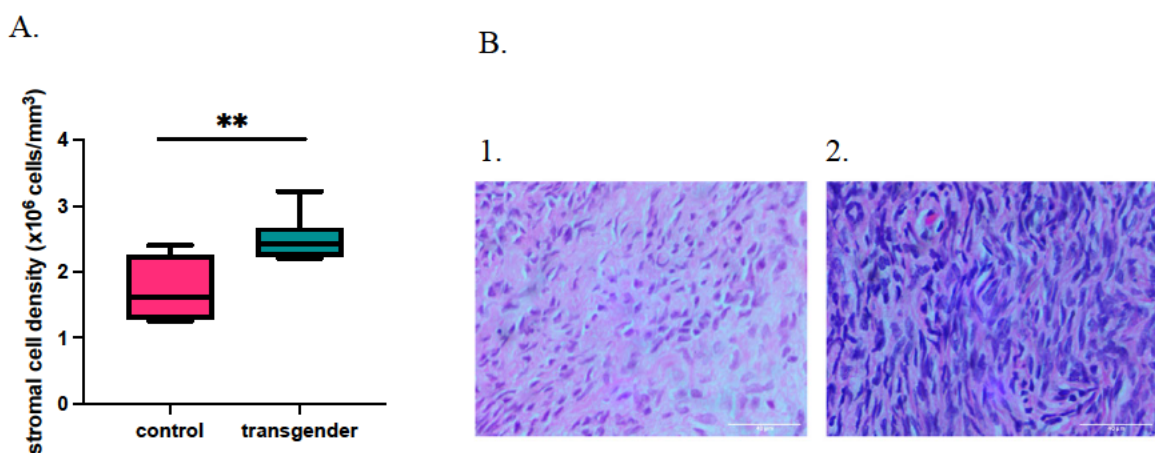


Figure 4.2- (A) Stromal cell density in control (n=8) and transgender tissue (n=8). Analysis performed using Student's t test. Data displayed as box plot. (**) $p \leq 0.01$ (B) Representative

images showing stromal cells in human ovarian tissue x20 magnification. (1.) control ovarian tissue (2.) transgender ovarian tissue. Scale bar = 40 μ m.

4.4.3 The relationship between stromal cell density and follicle density

To identify if there was an association between NGFD and SCD, data from the experiments in 4.4.1 and 4.4.2 were tested by correlation analysis. When the NGFD and SCD were compared within each individual group, there was no significant correlation (transgender: Spearman's correlation $r=-0.24$, $p=0.9$, control: $r=0.49$, $p=0.33$) (figure 4.3A). Comparison with both the transgender and control data combined resulted in a stronger correlation between SCD and NGFD ($r=0.53$), however the result was not significant ($p=0.052$) (figure 4.3B). To identify outliers within the data sets, a Z score was performed for identification of data that is a certain number of standard deviations away from the mean. A data point with a Z score >3 or <-3 is considered an outlier. Z score analysis was performed on Microsoft Excel using the formula: **follicle density-average follicle density/standard deviation**. For one patient's follicle density result, the Z score was >3 therefore this data point was excluded from the next analysis. Repeat correlation analysis with groups combined, Spearman's correlation increased $r=0.69$, $p=0.03$ (figure 4.3C) therefore with the excluded outlier data point, a significant correlation was found in that with increasing SCD, there was an increase in NGFD.

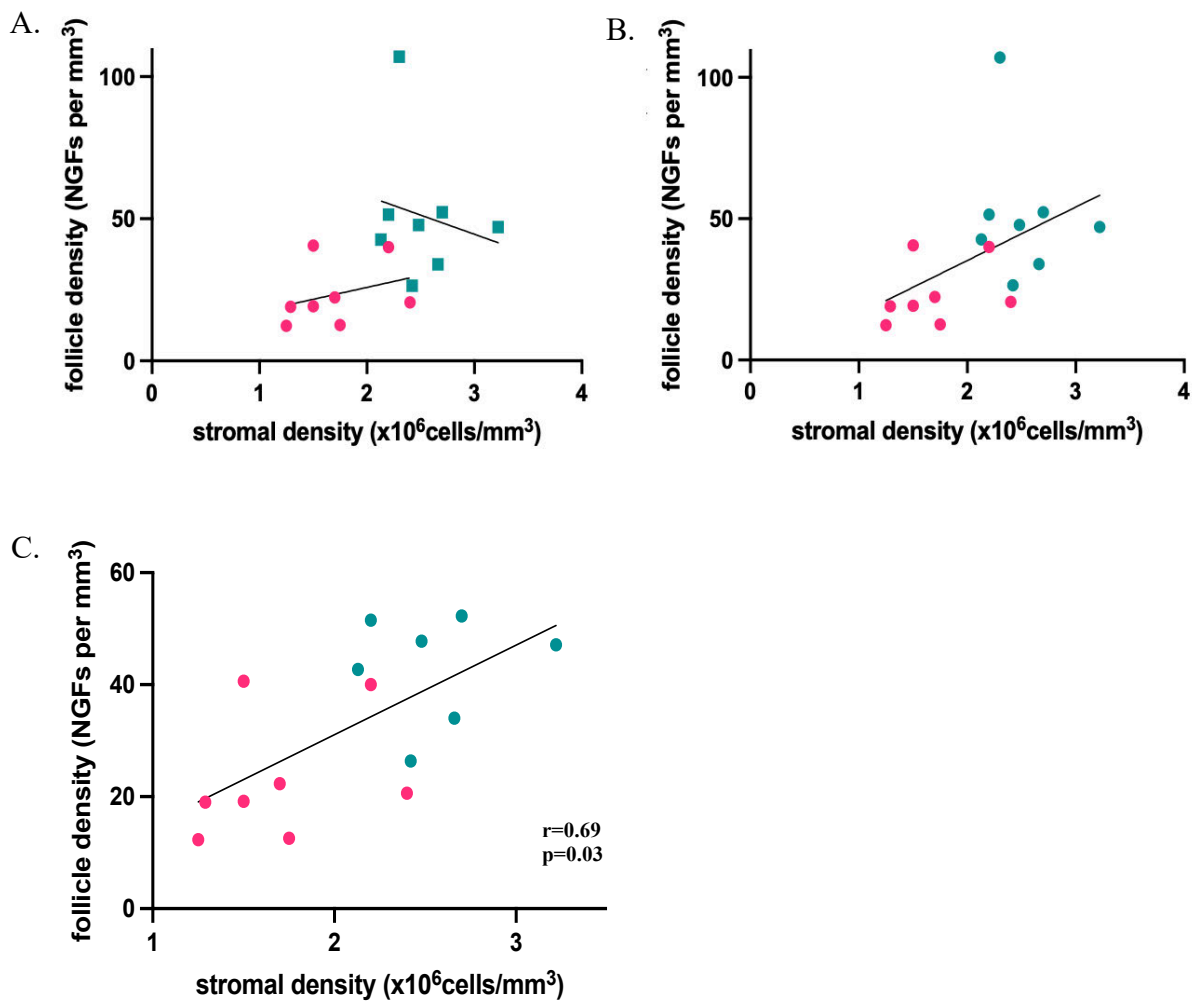


Figure 4.3- SCD vs NGFD, correlation analysis using Spearman's correlation efficient. (A) shows correlation within groups, transgender (n=8) $r=-0.19$, $p>0.05$, control (n=8) $r=0.31$, $p>0.05$. Green dots represent transgender data points, pink dots represent control data points. (B) shows correlation within data when groups combined (n=16), $r=0.53$, $p=0.052$. (C) data point removed as Z score >3 (n=15), $r=0.69$ $p=0.03$.

4.4.4 The impact of testosterone on ovarian stromal collagen content- brightfield analysis

Quantitative analysis of collagen content in the ovarian stroma was carried out using PSR

stained sections from 8 transgender and 8 control patients (D0 tissue). (figure 4.4B). The testosterone exposed tissue from transgender patients had a greater area of collagen staining and thus a higher collagen content ($77.2\% \pm 1.2$) compared to control ($31.3\% \pm 3.3$) ($p < 0.005$) (figure 4.4A).

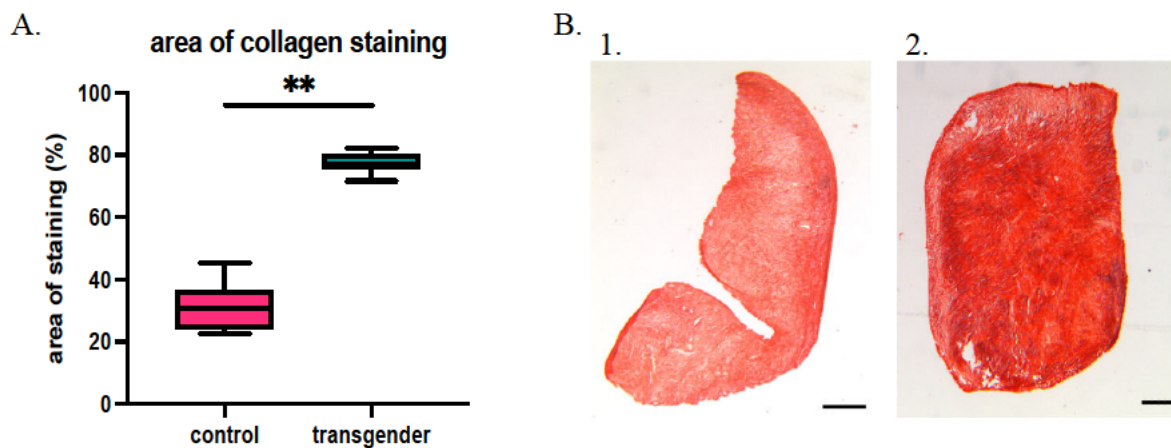
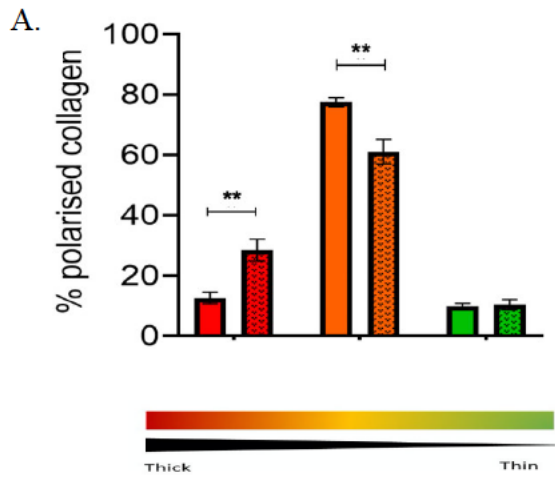


Figure 4.4- analysis of collagen staining using PSR in transgender (n=8) and control tissue (n=8)- brightfield analysis. (A) area of total collagen in transgender and control tissue-data are displayed as box plots, with analysis performed using Mann Whitney U test, (**) $p \leq 0.01$. (B) Representative images of PSR stained sections from (1) control and (2) transgender tissue at x2.5 magnification. Scale bar=40 μ m.

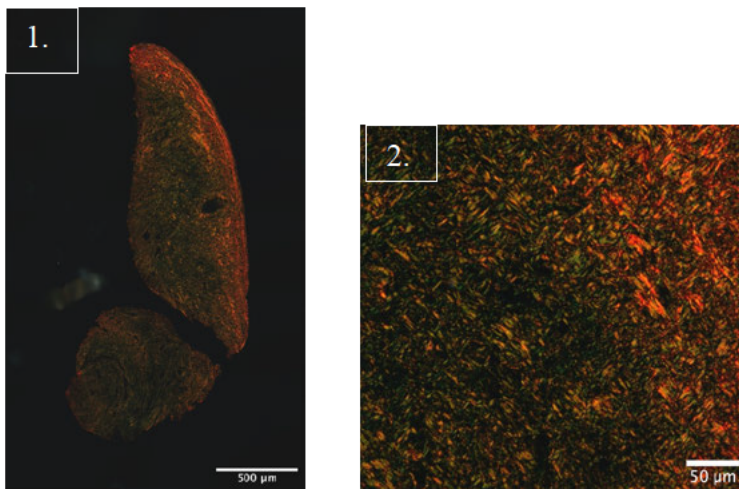
4.4.5. The impact of testosterone on ovarian stromal collagen content- polarised light microscopy analysis

Analysis of brightfield images indicated there is increased collagen fibre deposition associated with testosterone exposure in ovarian cortical tissue. Therefore, to identify the characteristics of the stromal collagen, the PSR sections were subjected to polarised light microscopy to identify collagen birefringence under x10 magnification (figure 4.5B/C).

The distribution of fibre size was found to be significantly different between the transgender and control tissue (figure 4.5A). In the transgender tissue, there were significantly more of the thickest red fibres compared to control tissue ($28.5\% \pm 3.6$ vs $12.6\% \pm 1.9$, $p < 0.005$). Significantly fewer medium thickness collagen fibres were found in the testosterone exposed tissue compared to control ($61.1\% \pm 4.1$ vs 77.5 ± 1.5 , $p < 0.005$) The least abundant thickness of fibre in both the transgender and control tissue was the thin green collagen fibres with comparable proportions found within both groups ($10.4\% \pm 1.7$ vs $9.9\% \pm 0.9$, $p = 0.6$).



B.



C.

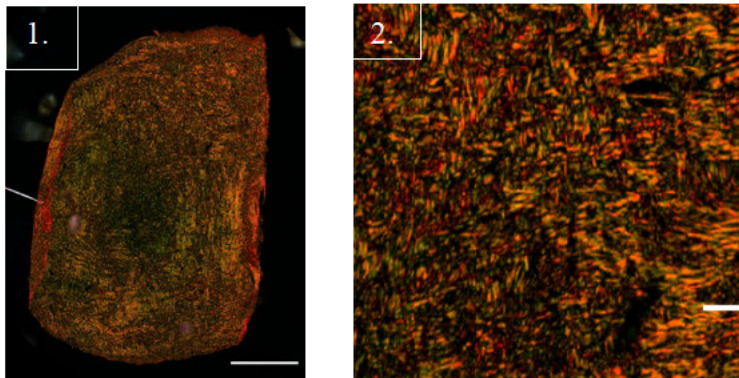


Figure 4.5- Collagen deposition in the human ovarian cortical stroma visualised under polarised light. PSR stained sections from control (n=8) and testosterone exposed tissue (n=8) imaged under polarised light. Whole sections were analysed using a custom FIJI macro package to threshold the main three colours seen under polarised light in: red- thickest fibres

(hue pixels 2-9), orange- medium thickness collagen fibres (hue pixels 10-38), and green- thinnest collagen fibres (hue pixels 24-135). (A)

Quantification of the relative percentage of each collagen colour type. All results displayed as mean $\pm$ SEM. Control, non-hatched bars, transgender samples, hatched bars. Analysis was performed using non-parametric test Kruskal Wallis with post hoc Bonferroni test. (**)
 $p < 0.01$. Representative images of PSR stained sections from (B) control and (C) transgender tissue under polarised light microscopy. Images (B1) and (C1) represent full sections, images (B2) and (C2) are ROIs. Scale bars on images.

4.4.6. The impact of testosterone on new collagen deposition- analysis using Masson's Trichrome stain

Five sections per patient from transgender D0 and control D0 groups were stained with Massons' trichrome to identify new collagen deposition as a measure of ongoing ovarian tissue fibrosis (Pampanini et al., 2019) (figure 4.6B). All results were expressed as the mean $\pm$ SEM. There was no significant difference in area of new collagen staining found between testosterone exposed and control tissue (figure 4.6A) ($38.6\% \pm 6.1$ vs $33.7\% \pm 2.0$). It is worth noting that the range in results of the area of the new collagen staining (transgender: 13.4%-59.5%, control 26.5%-40.5%) is much wider in the testosterone exposed tissue which may reflect the wide range of duration of testosterone treatment pre-operatively (range 18 months-10 years), however there was no significant correlation between treatment duration and new collagen deposition ($r=0.48$, $p=0.22$).

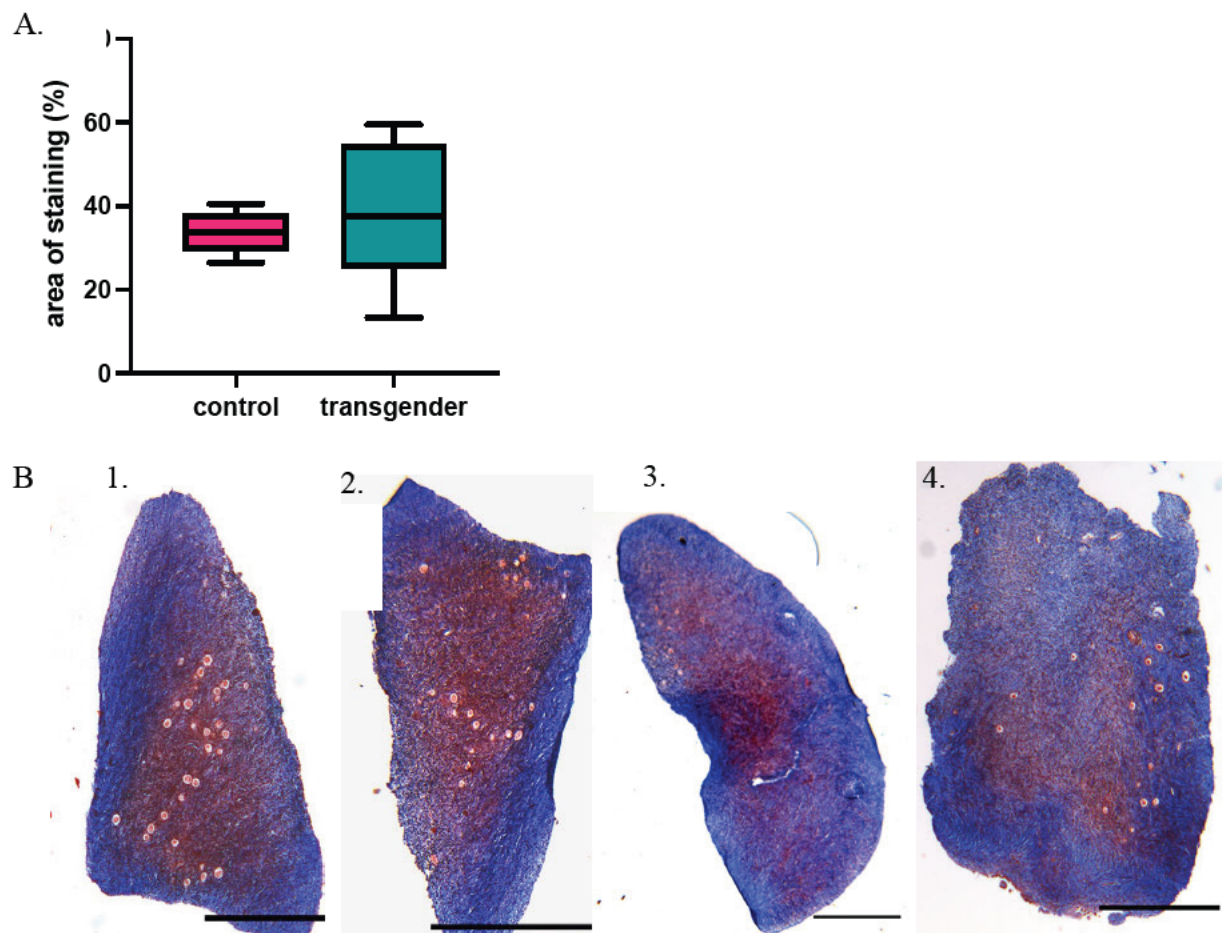


Figure 4.6- (A) Analysis of Massons Trichrome stained sections for expression of new collagen deposition in the ovarian stroma in control (n=8) and testosterone exposed tissue (n=8). Data are presented as box plots and analysis was performed using non-parametric test Mann Whitney U test. (B) Representative images of Massons' Trichrome stained sections of ovarian cortical tissue from (1-2) control and (3-4) transgender subjects, scale bar=1mm. With Massons' trichrome, collagen is stained blue, and cytoplasm is stained pink.

4.4.7. The impact of testosterone on ovarian stromal elastin content

Elastin was localised within the ovarian stroma of control and testosterone exposed tissue using immunofluorescence. Five sections were imaged per patient using Zeiss LSM 800

confocal microscope with x10 magnification and analysed using Image J software (figure 3.7B). The area of elastin staining was quantified per section and results were expressed as the mean $\pm$ SEM.

The area of elastin staining and thus the elastin content was significantly higher in the control tissue compared to the testosterone exposed tissue ($3.6\% \pm 0.6$ vs $1.3\% \pm 0.1$, $p < 0.005$) (figure 4.7A).

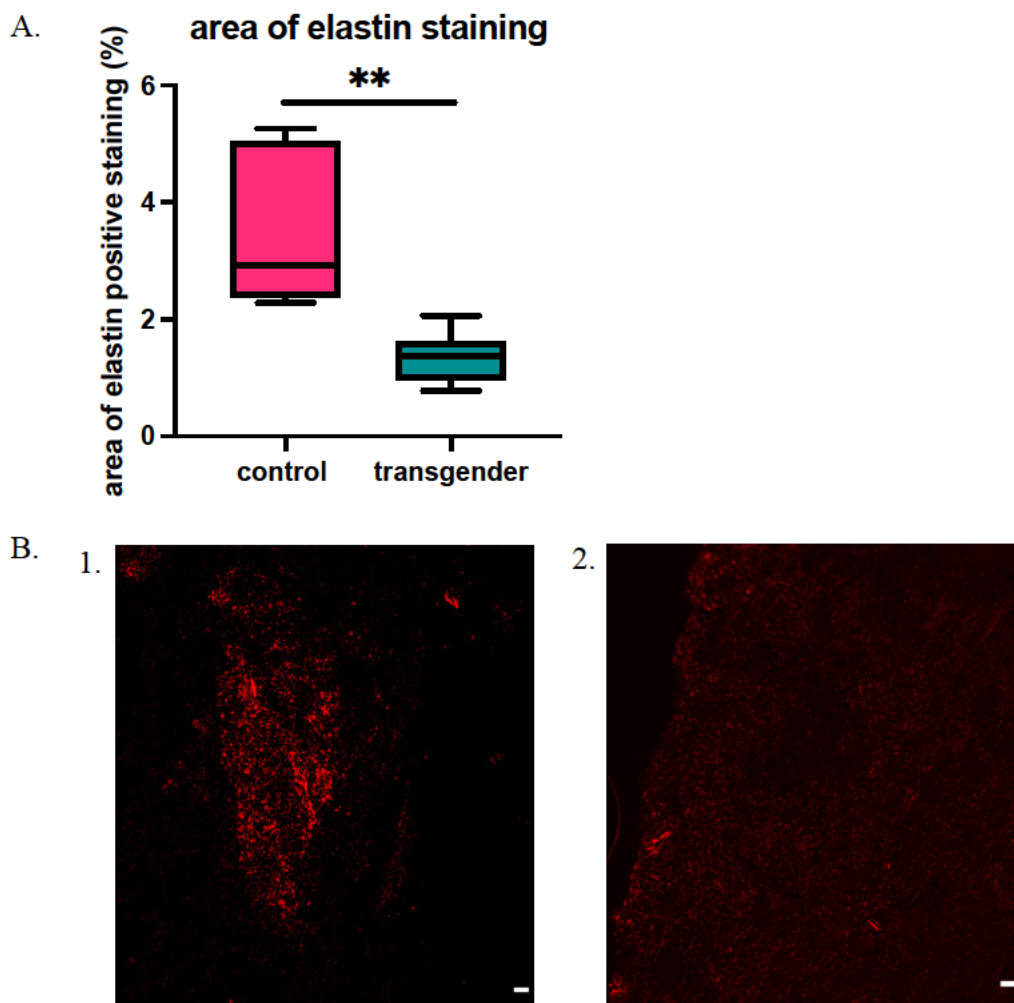


Figure 4.7- (A) analysis of immunofluorescent stained sections for expression of elastin in the ovarian stroma in control (n=8) and testosterone exposed tissue (n=8). Data are presented as box plots. Analysis was performed using non-parametric test Mann Whitney test and statistical significance assigned $p < 0.05$ (**) $p < 0.01$ (B) Representative images of

immunofluorescence-stained sections from (1) control and (2) transgender tissue. Scale bar=100µm.

4.4.8. The impact of testosterone on collagen elastin ratio- a measure of ovarian fibrosis

The results from the experiments in sections 4.4.3 and 4.4.6 have demonstrated that the transgender tissue had a higher collagen content ($77.2\% \pm 1.2$) compared to control ($31.3\% \pm 3.3$) ($p < 0.005$) (figure 4.4A) but a lower elastin content ($1.3\% \pm 0.1$ vs $3.6\% \pm 0.6$, $p < 0.005$) (figure 4.7A). Subsequently, the collagen/elastin ratio can be calculated and used as a measure of demonstrating intrinsic tissue rigidity and ovarian fibrosis (Dodson et al., 2013, Zhang et al., 2016). The collagen/elastin ratio was significantly higher in the transgender tissue compared to control (63.1 ± 7.9 vs 10 ± 1.3 , $p < 0.005$) (figure 4.8). This indicates that the ovarian cortical tissue from transgender patients that has been exposed to supraphysiological levels of testosterone has increased ovarian fibrosis, with likely consequent intrinsic tissue rigidity.

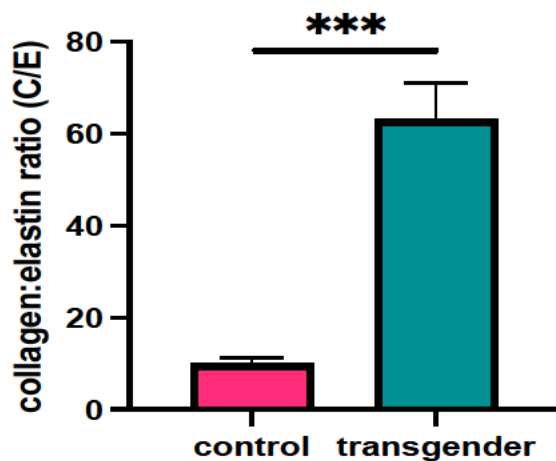


Figure 4.8- Analysis of collagen elastin ratio, a measure of intrinsic tissue rigidity and fibrosis (area of total collagen (%)/area of total elastin (%)) for control (n=8) and transgender tissue (n=8). All results displayed as mean $\pm$ SEM. Analysis was performed using non-parametric test Mann Whitney test. Statistical significance assigned $p < 0.05$, asterisk (***) $\leq$

0.001.

4.4.9. The impact of testosterone on stromal mitotic activity using proliferation marker Ki67

Ki67 is a widely used marker for cell proliferation. It is found to highly expressed in cells that are actively moving through the cell cycle and downregulated in non-replicating cells (Gerdes et al., 1983, Sun and Kaufman, 2018). In both groups, there was minimal positivity for Ki67 in the stromal cells despite positive expression of Ki67 in the granulosa cells of growing ovarian follicles (figure 4.9). The results of this experiment showed that despite increase in SCD, there was no detectable increase in ovarian stromal mitotic activity compared to control.

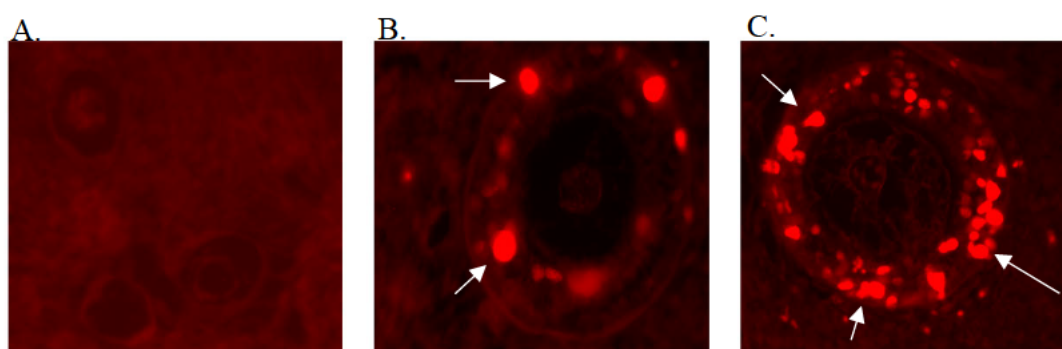


Figure 4.9- Localisation of Ki67 in human cortical ovarian stroma. Representative images showing localisation of Ki67 in human ovarian tissue. Ki67 is identified in the granulosa cells of growing follicles (white arrows) (positive control). (A) Control D0- non-growing follicles: oocytes, granulosa cells and surrounding stromal cells negative for Ki67 (magnification x40), (B) transgender D0 secondary follicle, some granulosa cells positive for Ki67 (magnification x60), (C) control D0 secondary follicle, some granulosa cells positive for Ki67 (magnification x60). Scale bar= 40µm.

4.5 Discussion

In this chapter, we have evaluated the composition of the ovarian stroma, namely collagen and elastin content, in ovaries exposed to high dose testosterone from transgender men and compared to control tissue. The effect of the composition of the ovarian stroma on follicle activation *in vivo* is discussed, together with analysis of SCD in comparison to NGFD.

Follicle development is a complex and dynamic process, tightly regulated by endocrine and paracrine factors (Thomas et al., 2003). However, the role and subsequent influence the ovarian stroma and extracellular matrix has on the ovarian follicle's ability to progress effectively through stages of follicle development is becoming increasingly evident (Grosbois et al., 2023, Curry and Smith, 2006, Berkholtz et al., 2006). The optimum follicle microenvironment is one that provides support and rigidity to maintain a follicle's 3D structure, but permissive to allow for follicle activation and subsequent growth and expansion.

The results of the experiments in this chapter have revealed that the composition of the ovarian stroma in testosterone exposed tissue is significantly different to age matched control tissue from healthy women. The first significant finding was that in testosterone exposed ovarian cortical tissue, there was a significant higher collagen content ($77.2\% \pm 1.2$) compared to control ($31.3\% \pm 3.3$) ($p < 0.005$) (figure 4.4A). Collagen is a major component of the ovarian extracellular matrix and ovarian stroma, accounting for 30% of protein mass in humans (Tang, 2020) and 49% of the total ovarian cortical matrisome (Ouni et al., 2022). It infers tensile strength and rigidity to tissue. Excessive extracellular matrix deposition in the ovarian stroma has been implicated in both physiological and disease processes. Ovarian fibrosis, as part of the physiological aging process, is largely attributed to excess stromal

collagen deposition (Briley et al., 2016, Amargant et al., 2020). In a study comparing the ovaries of older mice and younger mice, the ovaries of older mice were stiffer with a significantly higher collagen content (Amargant et al., 2020). Ovaries of older mice incubated with collagenase resulted in the collagen content similar to that found in the younger mice and reverted their biomechanical properties to those of their younger controls (Amargant et al., 2020).

Several studies have reported an increase in the thickness of the tunica albuginea layer due to excess collagen deposition in the ovaries of transgender men taking testosterone therapy (Chadha et al., 1994, Futterweit and Deligdisch, 1986b, Ikeda et al., 2013b, Pache et al., 1991, Spinder et al., 1989b) which is consistent with the findings in this chapter. Collagen is produced by fibroblasts found in the supportive stromal tissue (Lind et al 2006). The mechanism for excessive collagen deposition in the ovary as a consequence of exposure to supraphysiological levels of testosterone is currently unknown. In a study studying rat coronary artery adventitial fibroblasts, exogenous testosterone increased the production of collagen 1 by 20% *in vitro*, with resulting coronary vessel stiffening and hypertension (Jenkins et al., 2007).

Transforming growth factor - β (TGF- β) family members are growth factors which play an integral role in cell proliferation, differentiation and apoptosis (Kubiczkova et al., 2012) and are the most intensively studied regulators of the ECM (Wynn, 2008). However, they are also implicated in many disease processes including inflammation, fibrosis and malignancy (MacFarlane et al., 2017, Kubiczkova et al., 2012). There are 3 known isoforms of TGF- β , 1, 2 and 3, with isoform 1 widely expressed in mammalian tissue and the most implicated in tissue fibrosis (Frangogiannis, 2020, Biernacka et al., 2011). TGF- β 1 is stored within the cell

in an inactive form, however, once activated, TGF- β 1 signals for phosphorylation and nuclear translocation for SMAD 2/3 proteins, which modulate the transcription of target genes including procollagen 1 and 3 (Roberts et al., 2003). TGF- β 1 also upregulates collagen producing fibroblasts through the activation of mesenchymal cells which differentiate into myofibroblasts (Wynn, 2008).

In PCOS, ovarian cortical thickening and increased stromal volume due to pathological collagen synthesis is a sequela of TGF- β dysregulation (Raja-Khan et al., 2014). Given that there are many similarities with the transgender ovary subjected to high dose testosterone and PCOS, it would be reasonable to consider that the excessive extracellular matrix deposition, namely collagen in the ovarian stroma of transgender men taking testosterone therapy is due to overactivity of TGF β . This has been demonstrated in a mouse model in a recent study where primary granulosa cells were treated with testosterone to evaluate the effect of testosterone on the expression of fibrosis markers (Han et al., 2023). They found testosterone promoted the expression of TGF β 1 and other fibrotic markers including protein inhibitor TIMP1, whilst inhibiting the expression of MMP2, a proteolytic enzyme involved in tissue ECM remodelling.

There is an ever-growing body of evidence indicating that mechanical forces created by the extracellular matrix within the ovarian stroma maintain dormancy of the resting ovarian follicle pool (Curry and Smith, 2006, Woodruff and Shea, 2011, Shea et al., 2014, Xu et al., 2006c). The ovary's dynamic architecture has two distinct compartments- the cortex and the medulla. The dense, fibrous nature of normal human ovarian cortex contributes to suppression of primordial follicle growth, thus preventing mass and precocious activation, which would result in early depletion (Silber et al., 2018). However, during the process of follicle activation, follicles migrate towards the ovarian medulla whose environment contains

loose connective tissue and ovarian vasculature which is more conducive to continuing growth and development (Kinnear et al., 2020, Grosbois et al., 2023).

Ovarian stiffness has been shown to correlate with ovarian fibrosis in both senescent ovaries (Briley et al., 2016, Amargant et al., 2020) and ovaries from women with PCOS (Sumbul et al., 2022) due to aberrant collagen deposition within the stromal ECM. The ovarian cortex in transgender tissue has been found to be stiffer in comparison to tissue from oncology patients (De Roo et al., 2019). The results of the experiments in this chapter suggests that this is due to excessive collagen deposition in the ovarian stroma compared to control. This would explain the perceived reduced follicle developmental capacity of ovarian follicles in testosterone exposed tissue *in vivo*- the increased tissue turgor prevents follicle expansion, resulting in enhanced primordial follicle suppression. This is also seen in the pre-pubertal ovary where there is a significantly higher collagen content compared to reproductive age tissue in humans (Ouni et al., 2020), inferring increased tissue rigidity which functions to preserve the primordial pool in a quiescent state.

The distribution of collagen fibre thickness is best defined in PSR stained sections under polarised light to demonstrate its birefringence. The different colours demonstrated under polarised light indicate collagen thickness with red fibres being the thickest and green fibres being thinnest. Differing collagen fibre thickness has been implicated in both the normal and aging ovary and in disease processes. A study assessing collagen distribution showed significantly different collagen fibre thickness distribution across the human ovarian cortex, with predominantly thick and mid-size collagen fibres found in the outer cortex where the resting primordial follicle pool resides and more thin fibres at the cortico-medullary junction where follicles migrate during follicle development (Grosbois et al., 2023). The thickness of

collagen fibres in a human ovary have been shown to evolve with age, with pre-pubertal ovarian tissue being characterised with thin fibres with a gradual increased fibre thickness through reproductive age and menopause (Ouni et al., 2020). In the aging human heart, increased deposition of thick collagen fibres and loss of thinner fibres has been demonstrated over time with associated impaired cardiac function (Mendes et al., 2012). In malignancy, degradation of the normal ECM is an important part of the disease process and precedes the deposition of a stiff extracellular matrix, facilitating local tumour invasion and spread (Schedin and Keely, 2011, Wozniak et al., 2003, Paszek et al., 2005). In ovarian cancer, a strong correlation has been found between increased density and thickness of collagen I fibres, the thickest collagen fibre, and an increasingly poor prognosis (Kuczek et al., 2019, Sarwar et al., 2022). In human tissue, during normal wound healing, the ECM remodels with the loss of the typical weave basket collagen fibre formation with replacement scar tissue where the collagen is crosslinked to maintain thicker collagen fibres in a single direction (Ehrlich and Krummel, 1996).

The mechanism of increased collagen fibre thickness in the testosterone exposed ovarian cortical tissue is not clear. It has been hypothesised that the increasing collagen thickness occurring in puberty may be due to increased oestrogen production, thus increasing the activity of lysyl oxidase (LOX) (Ouni et al., 2021). LOX is a copper-dependent amine oxidase which catalyses the cross linking of collagen, resulting in increased tensile strength and resistance to degradation by MMPs (Erler et al., 2009, Vater et al., 1979).

In human PCOS ovaries, there is increased LOX mRNA expression in ovarian granulosa cells and increased LOX activity in follicular fluid (Zhang et al., 2018). In the same study, using a rat PCOS model, they found that by administering a LOX inhibitor, β -aminopropionitrile (BAPN), there were more oestrous cycles, reduced LH levels and reduced LH/FSH ratio, more oocytes retrieved during ovarian stimulation and less stroma collagen deposition.

Another study (T. Endo et al., 2001) demonstrated a three-fold increase in LOX mRNA expression with DHEA treatment compared to control in a rat PCOS model, with a reciprocal decline in MMP-2 mRNA expression. It is reasonable to hypothesise that the increased collagen fibre thickness seen in transgender ovaries may be a consequence of enhanced LOX activity secondary to testosterone treatment.

Androgens exert their actions on the ovary via the androgen receptor (Franks and Hardy, 2018). However, the androgen receptor is not expressed in primordial follicles (Rice et al., 2007) therefore primordial follicle activation is not directly regulated by androgens. The PI3K/Akt signalling pathway is one of the major signalling pathways that coordinates the activation, growth and differentiation of primordial follicles (Maidarti et al., 2020, Grosbois and Demeestere, 2018b). FOXO3A is an important transcription factor for maintaining primordial follicle dormancy within the PI3K/AKT pathway (John et al., 2008, Yang et al., 2010) with overexpression making follicles refractory to growth (Liu et al., 2007) and defects in the FOXO 3A gene resulting in premature depletion of the follicle pool due to mass activation (Liu et al., 2007, Castrillon et al., 2003). Testosterone has been found to influence the PI3K pathway and promote primordial follicle activation through rapid phosphorylation and nuclear exclusion of Foxo3a (Yang et al., 2010). Therefore, the results of the experiments in chapter three showing significant follicle growth suppression in testosterone exposed tissue was unexpected. A study using mice ovaries evaluated whether the ECM has an impact on maintaining follicle dormancy (Nagamatsu et al., 2019). After incubating neonatal ovaries in a collagenase containing solution, there was a significant rise in cytoplasmic FOXO3A in primordial follicles with subsequent enhanced follicle activation after 14 days of ovarian tissue culture compared to control. Interestingly, with exogenous compression, nuclear FOXO3A localisation was restored, almost completely cancelling the effect of the

collagenase containing solution. This indicates that intrinsic pressure provided by the ECM, namely collagen, maintains follicle dormancy and suppresses development and is in keeping with the present findings of reduced follicle development in testosterone exposed tissue where excessive collagen deposition has resulted in increased the intrinsic tissue pressure.

The results of chapter 3 showed that testosterone exposed tissue showed a higher proportion of primordial follicles than in control tissue, indicating a lower rate of *in vivo* activation. This is consistent with the findings in this chapter, of a higher ovarian collagen content. However, after 6 days of culture, the testosterone exposed tissue, compared to control, demonstrated enhanced follicle activation. We hypothesise that this enhanced follicle activation is due to the release of increased intrinsic tissue pressure created by increased collagen deposition which occurs when the tissue is dissected in preparation for ovarian tissue culture, allowing increased follicle activation. This theory would be in keeping with patients with PCOS whereby a treatment option includes the use of ovarian drilling, a laparoscopic procedure involving electrocautery to the ovarian cortex. The exact mechanism of action has yet to be elucidated but one theory is that the process of thermal destruction to the ovarian stroma results in a reduction in the local androgen levels, thus creating an intrafollicular environment less rigid and more permissive to follicle maturation resulting in a secondary rise in FSH levels, allowing for the emergence of a dominant follicle with subsequent mono-ovulation (Mercorio et al., 2022, Woodruff and Shea, 2011).

Another mechanism of enhanced follicle activation in the testosterone exposed tissue in this study may involve the Hippo signalling pathway. Disruption of the Hippo pathway during ovary-damaging procedures has been shown to promote follicle growth (Hsueh and Kawamura, 2020, Kawamura et al., 2013a). A study followed Hippo signalling pathway driven primordial follicle activation after ovarian cortical tissue from testosterone exposed

transgender tissue and oncological tissue (De Roo et al., 2020b). Interestingly, there was significantly less YAP expression in the granulosa cells of all follicle types over all culture days in the oncological tissue compared to transgender tissue. They concluded that this may be due to increased ECM stiffening in the transgender tissue secondary to testosterone therapy which has been shown to increase YAP activity, resulting in a more pronounced effect when the tissue was dissected (Hsueh et al., 2015b, Halder et al., 2012b). This is in keeping with the findings of this chapter where we have found enhanced follicle activation in testosterone exposed tissue after dissection in *in vitro* culture.

Elastin fibres are an essential element of the extracellular matrix within the ovarian stroma. Its ability to stretch and retract is integral in ECM remodelling to facilitate follicle development. A recent study mapped the presence of elastin in the ovarian stroma throughout follicle culture, with a progressive increase in elastin deposition found throughout the duration of the culture period (Grosbois et al., 2023). This was paired with a gradual degradation of collagen, reiterating that the ECM needs to be able to create a progressively permissive environment to accommodate the changing demands of the follicle. The results of the experiments in this chapter found that the testosterone exposed tissue had a reduced elastin content compared to control ($1.3\% \pm 0.1$ vs $3.6\% \pm 0.6$, $p < 0.005$) (figure 3.7A) with a significantly higher collagen/elastin ratio (63.1 ± 7.9 vs 10 ± 1.3 , $p < 0.005$) (figure 3.8). This further supports our hypothesis that testosterone exposure creates a rigid microenvironment, thus inhibiting follicle development *in vivo*. Along with increased collagen deposition, reduced elastin content has been demonstrated in both physiological aging (Pennarossa et al., 2022) and in the pre-pubertal ovary (Ouni et al., 2020) and can be considered as another marker of tissue stiffness (Dodson et al., 2013, Zhang et al., 2016).

Stromal cell density is an important indicator of tissue integrity (Alves et al., 2016). In this chapter, an increase in stromal density was found in the testosterone exposed tissue compared to control ($2.5 \times 10^6 \pm 0.1 \text{ cells/mm}^3$ vs $1.7 \times 10^6 \pm 0.1 \text{ cells/mm}^3$) (figure 4.2). This is in keeping with previous studies of the ovarian stroma of transgender men where stromal hyperplasia had been documented (Ikeda et al., 2013a, Amirikia et al., 1986, Futterweit and Deligdisch, 1986b, Pache et al., 1991) and correlates with increased androgen receptor expression in ovarian stroma in response to high dose exogenous testosterone therapy (Chadha et al., 1994). This is the first study to quantify stromal cell density in testosterone exposed ovarian cortical tissue. Interestingly, when data from both testosterone exposed and control tissue were amalgamated, we found that with increasing SCD, there was a significant increase in NGFD. This supports our hypothesis that with a denser and more compact ovarian stroma, there is a decreased rate of follicle activation. In this chapter, we found that the NGFD in testosterone-treated ovaries correlated closely to both our age matched controls and with those of age-matched model-predicted densities. This is in keeping with the finding that testosterone therapy does not accelerate primordial follicle depletion (Van Den Broecke et al., 2001).

The results from these experiments further adds to the body of evidence that suggests that the ovarian microenvironment influences follicles' developmental potential (Amargant et al., 2020, Briley et al., 2016, Castrillon et al., 2003, Curry and Smith, 2006, De Roo et al., 2017, Dolmans et al., 2008, Henning et al., 2019b, Grosbois et al., 2023, Hovatta et al., 1997, Luyckx et al., 2014, Ouni et al., 2020, Shea et al., 2014, Woodruff and Shea, 2011). Gaining invaluable insight into the dynamics of the ECM is essential in continuing to optimise *in vitro* culture systems, with the goal of maximising the quality and quantity of oocytes produced. Creating culture conditions to support follicles of every stage of follicle development is particularly challenging given the continuous tissue remodelling that occurs *in vivo* in

response to the demands of the growing follicle. What we do know is that when follicles are isolated, their rate of maturation is impaired, indicating that the ovarian stroma has a more complex role in follicle development than just a supportive capacity (Aharoni et al., 1997). Equally, when cubes of ovarian cortex are cultured (Hovatta et al., 1997), follicle growth and activation is significantly less than in experiments where strips of ovarian cortex are cultured, indicating that the tissue shape and the stromal density are influential (Telfer et al., 2008b, McLaughlin et al., 2018).

The experiments in this chapter only focus on the composition of the ovarian stroma *in vivo*. In retrospect, it would be of value to perform the same experiments on ovarian tissue that had completed 6 days of tissue culture so to evaluate how well the testosterone exposed ovarian stroma could remodel compared to control in response to follicle development.

Another area where gaining more insight into the importance of the role of the ovarian stroma and ECM is pertinent, is fertility preservation. OTC, involving removal of ovarian cortex which is subsequently frozen and can be thawed for reimplantation, remains the only fertility preservation option for pre-pubertal girls (Wallace et al., 2016). However, there is a real concern of the risk of reimplanting malignant cells with the graft tissue (Dolmans and Amorim, 2019b). The advent of a bioengineered graft mitigates this risk. Requirements of a suitable bioengineered matrix include sufficient support to maintain a growing follicle's structure, pervasive to maintain cell to cell connectivity, deliver essential endocrine and paracrine support to promote further follicle development and the follicle being easily retrievable (Shea et al., 2014). Proposed matrices include collagen (Telfer et al., 1990), plasma clots (Gosden, 1990), fibrin (Kniazeva et al., 2015, Smith et al., 2014, Luyckx et al.,

2014, Paulini et al., 2016), and alginate (Jamalzai et al., 2020, Xu et al., 2006c, Hornick et al., 2013) with promising results.

Tissue fibrosis, as a result of excessive ECM deposition, forms the basis of a wide plethora of health conditions, with subsequent progressive, incurable organ dysfunction (Ley and Collard, 2013, Conrad et al., 2018, Furst et al., 2012, Fleming et al., 2008). Specifically, to the ovary, fibrotic conditions include physiological aging, endometriomas, PCOS and POI. Targeting ECM remodeling enzymes to prevent and even reverse pathophysiological changes within the ECM has created much interest. More recently, it has emerged that metformin, an antidiabetic drug, has anti-fibrotic effects mainly through inhibiting TGF- β 1 production (Xiao et al., 2010, Fan et al., 2017). Specifically to the ovary, the ovaries of women with type 2 diabetes mellitus taking metformin exhibit normal ovarian collagen organization and reduced M2 macrophage polarization compared to the ovaries of non-diabetic women whose ovaries showed age related fibrotic change (McCloskey et al., 2020). Following this, a study using a mouse model, demonstrated that whilst metformin did not reverse ovarian fibrosis, it very effectively prevented age associated ovarian fibrosis, with collagen proportion and thickness similar to young mice (Landry et al., 2022). Further studies and clinical trials are warranted to evaluate if similar findings are indeed found in human ovaries as this may have significant clinical applications for patients with POI, PCOS and for transgender men taking testosterone therapy. When commencing gender affirming testosterone therapy, prophylactic metformin therapy could be considered to prevent testosterone driven ovarian fibrosis, as demonstrated in the results of the experiments in this chapter, particularly if they are considering pregnancy in the future after commencing testosterone therapy.

In summary, the results of the experiments in this chapter have demonstrated that ovaries from transgender men taking testosterone therapy have a higher collagen content and lower elastin content compared to age matched controls. This change to the composition of the ECM has resulted in a more rigid, less permissive microenvironment and ultimately reduced the functionality of the ovarian stroma to support follicle development. This work provides insight for transgender patients considering commencing hormone therapy into irreversible fibrotic changes to the ovary because of testosterone treatment and how this could negatively influence the ability to conceive in the future if they so wished. Much more work is needed in the form of clinical trials to ascertain the potential of metformin in preventing ovarian fibrosis and its use as a treatment adjunct for transgender men on testosterone therapy.

Chapter 5

General discussion

5.1 General discussion

Androgens play a key role in maintaining oocyte health and in ovarian follicle development. However, an optimal level of androgens is likely to be essential to maintain normal ovarian function. The impact of high dose testosterone on ovarian follicle health and reproductive potential remains largely unknown. Similarities can be drawn between the exogenous hyperandrogenism of transgender men on gender affirming endocrine therapy and the endogenous hyperandrogenism seen in women with PCOS, in particular, changes within the ovarian stroma including stromal cell hyperplasia, cortical thickening and stromal cell luteinisation.

The experiments in this thesis were designed firstly to investigate the effect of testosterone on non-growing and small growing ovarian follicles *in vivo* and evaluate the effect of culture conditions on the growth and survival of follicles compared to control ovarian tissue. In addition, this study assessed the effect of exogenous testosterone on the composition of the ovarian stroma and how this may impact follicle growth activation.

5.2 Testosterone exposure alters stromal composition which affects follicle distribution *in vivo* and growth activation *in vitro*

There have been a number of studies that have assessed the distribution of ovarian follicles in the ovaries of transgender men taking testosterone therapy. Some studies have concluded that testosterone does not affect the ovarian cortical follicle distribution (De Roo et al., 2017, Ikeda et al., 2013a) whilst other studies have found increased numbers of antral and atretic follicles, similar to PCOS (Amirikia et al., 1986, Futterweit and Deligdisch, 1986b, Spinder et al., 1989a, Pache et al., 1991, Grynberg et al., 2010). The work reported in this thesis has shown that in ovaries from transgender men taking testosterone therapy, there is an increased

proportion of non-growing follicles compared to control. This would indicate that there is reduced follicle growth activation as a consequence of testosterone exposure. This contrasts with the major finding in women with PCOS where there is an accumulation of pre-antral and antral follicles arrested in development *in vivo*.

Hyperandrogenism has also been shown to alter the ovarian stroma with ovaries from transgender men being shown to be stiffer compared to an oncological control (De Roo et al., 2019). Results from experiments in chapter 4 revealed the ovarian stroma from transgender men consisted of more collagen and less elastin, inferring increased tissue rigidity. It is well documented in the literature that increasing tissue rigidity limits follicle growth and development (Nagamatsu et al., 2019, Xu et al., 2006c, Jiao and Woodruff, 2013b) therefore the tissue fibrosis found in the transgender tissue as a result of testosterone exposure may contribute to the reduced follicle growth activation *in vivo* seen in the transgender tissue compared to control.

The role of the Hippo signalling pathway has not been directly measured in this study, however it may contribute to the follicle quiescence *in vivo* in transgender tissue. Hippo signalling is regulated within a tissue's microenvironment with increased mechanical forces created within the ECM resulting in enhanced inhibition of the Hippo signalling pathway (Kawamura et al., 2013a). Cytoskeleton stiffness and cell-cell contact, indicating increased cell density, are both implicated in the upstream regulation of the Hippo signalling pathway, resulting in increased phosphorylation of YAP and subsequent inactivation (Halder et al., 2012a, Fu et al., 2022).

Increased stromal cell density was also found in the transgender tissue compared to control, in keeping with previous findings of stromal hyperplasia (Ikeda et al., 2013b, Chadha et al., 1994, Grynberg et al., 2010, Futterweit and Deligdisch, 1986b, Spinder et al., 1989b, Pache et al., 1991, Hughesdon, 1982). Interestingly, results in chapter 4 found a positive correlation between stromal density and non-growing follicle density, again indicating that a more compact, dense stroma confers follicle quiescence.

The aim of any ovarian tissue culture system is to produce competent oocytes, capable of fertilisation and embryological development. Major advances have been made in human tissue with complete oocyte growth being achieved from primordial follicle to the MII stage (McLaughlin et al., 2018, Xu et al., 2021). Most recently, pre-antral follicles have been matured *in vitro* with the addition of neutrotrophic factor 4 (NT4) to produce MII oocytes that have been fertilised and have developed into morphologically normal blastocysts with transfer potential (Guo et al., 2024). The experiments in chapter 3 focused on the first stage of tissue culture, primordial follicle activation, and analysed the influence testosterone can have on follicle *in vitro* growth and development. In this study, the transgender tissue showed enhanced follicle activation after 6 days of tissue culture compared to control. Testosterone has been shown to be able to influence primordial activation, not through the androgen receptor which is notably absent from primordial follicles (Vendola et al., 1998) but through the PI3K/Akt with testosterone increasing nuclear exclusion of FOXO3A which in turns results in follicle activation (Yang et al., 2010). Increased levels of testosterone in the transgender ovarian tissue may have contributed to the enhanced follicle activation seen in the transgender tissue compared to control.

Ovary damaging procedures have been found to increase primordial follicle activation as a result of disruption to the Hippo signalling pathway (Donnez et al., 2011). A recent study analysing key players of the Hippo signalling pathway during *in vitro* culture compared transgender tissue to oncological control (De Roo et al., 2020b). They found a difference in YAP expression between groups. At day 0, there was no YAP expression in the granulosa cells of the oncological control whereas YAP was found in primary and secondary follicles granulosa cells. During the culture period, YAP was found in the transgender follicle granulosa cells in all stages of follicle development whereas YAP was absent in the oncological granulosa cells. This study concluded that these findings could be attributed to the malignant disease process but more likely due to the increased ovarian cortical stiffness found as a result of testosterone exposure (De Roo et al., 2020b, De Roo et al., 2019). This increased stromal rigidity could result in a stiffer cortical ‘niche’, favouring Hippo pathway signalling and resulting in enhanced follicle activation upon disruption (Hsueh et al., 2015b, Halder et al., 2012b, De Roo et al., 2019).

5.3 Testosterone affects follicle health, with increased levels of DNA damage and an impaired DNA repair response

A significant finding from the experiments in chapter 3 was the ovarian follicles from transgender tissue showed reduced morphological health *in vivo* compared to control which may indicate limited potential for survival and development. DNA DSBs, the most lethal form of DNA damage, were quantified using immunostaining of γ H2AX as a marker of DNA damage. The results showed that the expression of γ H2AX in oocytes of uncultured D0 non-growing follicles was significantly higher in the transgender group compared to control, in keeping with histological findings of poorer morphological health, indicating that

testosterone may induce DNA damage *in vivo*. We hypothesise this may be due to increasing levels of ROS, which has been demonstrated in a PCOS mouse model using DHT implants, resulting in DNA damage and/or cell apoptosis (Murri et al., 2013, Bertoldo et al., 2019). Similarly in women with PCOS, increased levels of biochemical markers of oxidative stress have been found (Murri et al., 2013).

There have been multiple studies that have demonstrated that primordial follicles are capable of efficient and effective DNA damage repair (Nguyen et al., 2021, Stringer et al., 2020, Kerr et al., 2012). However, results from the experiments in chapter 3 despite showing difference in the expression of DNA damage proteins RAD51, ATM and MRE11 between control and transgender oocytes, these were not statistically significant and may indicate an impaired DNA damage repair response as a result of testosterone exposure. It is important to note that a small numbers of subjects and follicles being included in analysis may be the reason for lack of statistical significance in these experiments. Unrepaired or incorrectly repaired DNA DSBs result in infertility, miscarriage and genetic defects in offspring (Stringer et al., 2018). Similarly, if it has not been possible to repair any DNA damage and the cell remains in an arrested state, the oocyte will undergo apoptosis (Nowsheen and Yang, 2012). Reassuringly, testosterone exposure does not appear to result in accelerated follicle loss and premature ovarian insufficiency, and this may be due to the ability of testosterone to inhibit apoptosis (Ono et al., 2014, Yoo et al., 2020).

After 6 days of tissue culture, with enhanced follicle activation, there was a further deterioration in morphological health across all follicle types; this was not associated with a change in levels of γ H2AX in the non-growing follicles but was increased in primary oocytes. Enhanced primordial follicle activation via the PI3K/Akt pathway as a result of testosterone exposure may contribute to the deterioration in the morphological health of follicles from

transgender tissue during tissue culture. Excessive PI3K/Akt activity has been shown to lead to low survival of growing follicles, morphological abnormalities, and DNA repair defects (Maidarti et al., 2019, McLaughlin et al., 2014b, Grosbois and Demeestere, 2018b).

These results raise concerns regarding DNA damage in oocytes as a result of testosterone exposure. It is important to note that the experiments in this thesis only look at the first stage of the tissue culture process, primordial follicle activation, therefore repair of DNA damage at a later stage cannot be excluded. It does however fit with findings from another lab of issues with fertilisation and embryological development of *in vitro* matured oocytes from transgender tissue (Christodoulaki et al., 2023, Lierman et al., 2021).

5.4 Study limitations

As previously mentioned in the discussions of chapters 3 and 4, there were some limitations and areas for consideration in the design and methodology of this study. Deciding on the most appropriate control for this study was difficult. The control used was age matched ovarian cortical tissue donated by healthy women at the time of caesarean section. The benefits of using this control are that it is age matched, available from the local obstetrics unit and there is no evidence that HCG has any effect on the non-growing ovarian follicles.

However, the effect on DNA repair capacity and the ovarian stroma is unknown. Accessing tissue from healthy, aged matched non-pregnant women is rarely possible but examples may include age matched women having a hysterectomy for benign gynaecological conditions.

Using tissue from patients with ovarian pathologies equally has its own limitations: a study of follicle distribution and ovarian reserve in patients with a range of both benign and malignant ovarian pathologies concluded that the cortex surrounding an ovarian malignancy has reduced follicle density, and in women with benign ovarian lesions, stromal proliferation was observed (Pavone et al., 2014). Similarly, the ovarian cortex from women with an

endometrioma had a reduced follicle number, poorer morphological health and fibrotic change within the stroma (Maneschi et al., 1993).

It is important to note that in the immunohistochemical experiments, there was only a single observer who was unblinded. This introduces a risk of bias. The negative controls used was omission of the primary antibody and an immunoglobulin fraction from the same species as the primary antibody would have been more robust. Alternatively, use of polymer-based technologies would increase specificity in immunohistochemical experiments. and is a learning point I take forward.

A missed opportunity within chapter 4 was regarding only focussing on the composition of the ovarian stroma *in vivo*. In retrospect, it would be of value to perform the same experiments on ovarian tissue that had completed 6 days of tissue culture so to evaluate how well the testosterone exposed ovarian stroma could remodel compared to control in response to follicle development.

During follicle classification analysis, it would have been of benefit to observe for theca cell hyperplasia in growing follicles. This finding might well have been present and has been overlooked but would have added to documented changes to ovarian follicles and stroma as a result of high dose exogenous testosterone exposure.

Another limitation in this study was duration of testosterone treatment which was patient reported duration, with most of them being reported in years. Ideally, it availability of specific commencement dates would have been useful to be able to analyse in weeks or months of treatment however this was not generally available, particularly due to the several sites where patients were recruited from.

This study focussed on the impact of testosterone on the non-growing follicles and the ovarian stroma in the outer cortex. However, the ovarian medulla was available for analysis as whole ovaries were obtained. It would have been interesting to have performed some experiments on the ovarian stroma, e.g. follicle classification, DNA damage and composition of collagen and elastin. There have been a number of studies published on the transgender ovary which have been performed from tissue from the ovarian medulla ((De Roo et al., 2017, Lierman et al., 2021). It may have allowed me to perform these experiments on more mature oocytes, in particular DNA damage, which would allowed me to analyse whether there appears to be evidence of effective DNA repair in the latter stages of follicle development. Another point to mention in steroid production from the ovarian cortical strips. This was not included in this study but would have been of interest as it may have given insight to levels of sex steroids within the ovarian cortex and compared to serum hormone levels.

5.5 Future directions

There is significant interest in OTC as a form of fertility preservation for transgender men. However, for OTC to be a viable option, complete follicle activation, growth and maturation from primordial follicle stage needs to be achieved *in vitro*. Much work is being carried out to try and improve the developmental competency of oocytes in the *in vitro* growth and maturation stages of tissue culture (Lierman et al., 2021, Christodoulaki et al., 2023), however this is the first study that has looked at the first step of this process; primordial follicle activation.

The ovarian tissue culture protocol used in this study (McLaughlin et al., 2018) is unlikely to effectively support complete follicle development in transgender tissue but instead will

require further optimisation and even personalisation towards the tissue being used. For example, increased follicle activation was seen in transgender tissue *in vitro* therefore the administration of a substrate during tissue culture that could control the rate of follicle activation could be of benefit to prevent excessive activation and subsequent deterioration in follicle health. This may involve manipulation of the PI3K/Akt pathway which has been shown to be influenced by increased testosterone levels (Yang et al., 2010). It is likely that both disruption of the Hippo signalling pathway and a loss of intrinsic tissue rigidity within the transgender tissue are also contributing to excessive follicle activation so altering the tissue preparation technique could be investigated as a way of reducing follicle activation. The tissue shape and stromal density are known to influence follicle growth and activation in culture as follicles in cubes of ovarian cortex in culture grow less than those cultured in strips (Hovatta et al., 1997) (Telfer et al., 2008b, McLaughlin et al., 2018).

An important finding in this thesis was that *in vivo*, testosterone treatment was associated with increased DNA damage in transgender oocytes. This may be contributing to the reduced developmental capacity of more mature oocytes found in other studies (Lierman et al., 2021, Christodoulaki et al., 2023). It would be of benefit to investigate if ROS are contributing to the DNA damage. If this was the case, this could potentially be managed by 2 different approaches, firstly to try and reduce the ROS and secondly by trialling anti-oxidative treatment (Poljsak, 2011). One example, melatonin, has demonstrated significant antioxidant effects both directly through scavenging for free radicals and indirectly by modulating DNA damage response and repair pathway. (Galano et al., 2018). In aged bovine oocytes, the administration of melatonin resulted in a reduction in ROS levels with a subsequent improvement of DNA damage (Liang et al., 2017). In human oocytes, melatonin has been shown to reduce oxidative stress levels, resulting in an improved fertilisation rates in IVF (Li

et al., 2019). If melatonin was found to be beneficial in preventing oocyte DNA damage *in vivo* in testosterone exposed tissue, this could be prescribed and given to transgender men on commencing testosterone therapy, particularly those who may consider having their own biological offspring in the future.

Moreover, another direction could be to optimise DNA repair capacity in transgender oocytes with studies addressing the repair capacity of oocytes and granulosa cells in tissue subjected to chemotherapy, radiation and in the aging ovary. A study using an aging mouse ovary model demonstrated that the supplementation of RAD51 in culture reduced the occurrence of DNA DSBs, reduced apoptosis and improved the developmental potential of embryos (Kujjo et al., 2010). This is another example of how the ovarian tissue culture process could be personalised to the transgender tissue.

Further studies to determine a relationship between treatment duration and dose on ovarian follicle health would be beneficial in particular when counselling patients prior to commencing testosterone therapy for medical transitioning. In this study, no link was found between treatment duration and follicle classification and health, in keeping with other studies (Ikeda et al., 2013b, De Roo et al., 2017). However, this is a small study with 8 patients recruited therefore it would be beneficial to look at a larger cohort of patients.

The results in this thesis indicate that testosterone induces ovarian tissue fibrosis similar to that seen in PCOS and physiological ovarian aging. Metformin, an anti-diabetic drug, has shown to have anti-fibrotic effects mainly through inhibiting TGF- β 1 production as demonstrated in women with type 2 diabetes mellitus (Xiao et al., 2010, Fan et al., 2017).

Transgender men who are due to commence testosterone therapy and are planning bilateral oophorectomy as part of surgical transitioning may make good subjects for testing the

efficacy of metformin in preventing ovarian fibrosis as a result of the testosterone therapy. If it was shown to demonstrate anti-fibrotic tendencies, metformin, could be offered to transgender men starting treatment with the aim of preventing ovarian fibrosis, particularly if they are considering pregnancy in the future after commencing testosterone therapy. It may also benefit women with PCOS to try and reduce androgen induced stromal fibrosis which contributes to ovulation dysfunction.

The experimental methods used in this study were well established. However, there are novel approaches which could be used going forward to gain further insight into the transgender ovary. Ultrasound elastography is a non-invasive approach to assess a tissue's hardness and rigidity (Dewilde et al., 2023). This novel technique is based on tissue deformation when a certain force or stress is applied. The elastic modulus of the tissue is assessed, with an increased modulus indicating increased tissue stiffness. Ultrasound elastography is currently being used to assess several organs including breast, prostate, thyroid and kidney. However, more recently, it is being employed in the myometrium to assess for fibroids and adenomyosis and in the endometrium to assess for polyps and endometrial cancer. Shear wave elastography is an ultrasound imaging tool which uses a push pulse which is produced by the ultrasound probe and generate shear waves to the intended soft tissue target, travelling faster through stiffer tissue (Zaniker et al., 2024). This is subsequently converted into Young's modulus in kPa. This non-invasive, low cost, low risk imaging modality could be used to monitor for fibrosis detection and progression for transgender men who commence testosterone therapy but who wish to potentially retain fertility potential. For the transgender patients who have a hysterectomy and oophorectomy, it would be of interest to perform a pre-operative scan and compare to the results of the ovarian cortical stromal assessment for tissue fibrosis and assess the correlation between findings.

5.6 Concluding remarks.

The work presented in this thesis indicates that high circulating concentrations of testosterone have previously unrecognised effects on the primordial and small-growing follicles of the ovary. It also quantifies the alteration of major components of the ovarian stroma, namely collagen and elastin and that it negatively affects follicle growth activation *in vivo*. These results support the recommendations that fertility preservation measures should ideally be explored prior to commencing gender affirming endocrine therapy. The impact of exogenous testosterone on the ovary and the degree of reversibility is largely unknown. To date, there have not been any human studies that have addressed this but it is inanately reassuring that on discontinuing testosterone therapy, transgender men have been able to readily conceive both naturally and through ART (Light et al., 2014b, Ghofranian et al., 2023, Moravek et al., 2020). Much more work is needed to assess the validity of OTC as a potential fertility preservation option for transgender men who have already commenced testosterone therapy. The results in this thesis also add to the body of literature indicating the influence the ovarian stroma on follicle growth and development, namely increased rigidity of the ECM results in reduced follicle growth and development.

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Appendix 1

Patient	age	Duration of testosterone (months)	Testosterone level
1	32	120	16.4
2	30	36	15.5
3	23	60	12.3
4	30	18	17.2
5	20	36	16.1
6	25	60	
7	34	96	
8	27	84	

Table 1: Duration of testosterone (n=8, patient reported duration in months) and serum testosterone levels (n=5).

Patient group	Patient number	Age
transgender	1	32
	2	30
	3	23
	4	30
	5	20
	6	25
	7	34
	8	27
control	1	36
	2	33
	3	35
	4	32
	5	32
	6	26
	7	25
	8	35

Table 2: Ages of all patients included in the study, transgender mean age: 27.6 ± 1.7 years; range 20-34 years, n=8, control mean age: 31.8 ± 1.5 years, range 25-36, n=8.

Appendix 2

RESEARCH

The ovaries of transgender men indicate effects of high dose testosterone on the primordial and early growing follicle pool

Emily Bailie¹, Mila Maidarti¹, Robert Hawthorn², Stuart Jack³, Neale Watson⁴, Evelyn E Telfer¹ and Richard A Anderson⁵

¹Institute of Cell Biology, Hugh Robson Building, University of Edinburgh, Edinburgh, UK

²Queen Elizabeth University Hospital, Glasgow, UK

³Simpson Centre for Reproductive Health, Royal Infirmary, Edinburgh, UK

⁴Spire Thames Valley Hospital, Wexham St, Slough, UK

⁵MRC Centre for Reproductive Health, Queens Medical Research Institute, University of Edinburgh, Edinburgh, UK

Correspondence should be addressed to E Bailie; Email:

Abstract

Androgens are essential in normal ovarian function and follicle health, but hyperandrogenism, as seen in polycystic ovary syndrome, is associated with disordered follicle development. There are few data on the effect of long-term exposure to high levels of testosterone as found in transgender men receiving gender-affirming endocrine therapy. In this study, we investigate the effect of testosterone on the development, morphological health and DNA damage and repair capacity of human ovarian follicles *in vivo* and their survival *in vitro*. Whole ovaries were obtained from transgender men (mean age: 27.6

± 1.7 years; range: 20–34 years, $n = 8$) at oophorectomy taking pre-operative testosterone therapy. This was compared to cortical biopsies from age-matched healthy women obtained at caesarean section (mean age: 31.8 ± 1.5 years; range: 25–35 years, $n = 8$). Cortical tissues were dissected into fragments and either immediately fixed for histological analysis or cultured for 6 days and subsequently fixed. Follicle classification and morphological health were evaluated from histological sections stained with hematoxylin and eosin and expression of γ H2AX as a marker of DNA damage by immunohistochemistry (IHC). In uncultured tissue, testosterone exposure was associated with reduced follicle growth activation, poor follicle health and increased DNA damage. After 6 days of culture, there was enhanced follicle activation compared to the control with further deterioration in morphological health and increased DNA damage. These data indicate that high circulating concentrations of testosterone have effects on the primordial and small-growing follicles of the ovary. These results may have implications for transgender men receiving gender-affirming therapy prior to considering pregnancy or fertility preservation measures.

Lay summary

As part of gender transitioning, transgender men take testosterone therapy. While androgens like testosterone are essential to maintain ovarian health, the effects of long-term testosterone treatment on the ovary are unclear. This study examines whether testosterone impacts ovarian follicle growth activation, follicle health and whether it causes DNA damage. It also looks at how well these follicles grow in tissue culture. The results showed there was a higher proportion of non-growing ovarian follicles in the ovaries of trans men, they appeared less healthy and there were higher levels of DNA damage. After 6 days of tissue culture, there were more growing follicles in transgender ovaries compared to control, but follicle health further deteriorated and there are increased levels of DNA damage. These results identify new effects of testosterone on the ovary and highlight the importance of discussing fertility preservation options prior to starting testosterone.

Keywords: ▶ transgender ▶ testosterone ▶ non-growing ovarian follicles ▶ primordial follicles ▶ effect
▶ impact

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Introduction

Reproductive choices are important for transgender individuals, as they are for the general population (De Sutter 2001). In the absence of surgery, trans men retain the potential for pregnancy, and international guidelines recommend consideration of fertility preservation options prior to commencing gender-affirming testosterone therapy (Coleman *et al.* 2012, Anderson *et al.* 2020). Current fertility preservation options available are oocyte and embryo cryopreservation (Mattawanon *et al.* 2018, Anderson *et al.* 2020). However, despite 54% of transgender men expressing a desire to have their own biological children (Wierckx *et al.* 2012), the uptake of fertility preservation interventions is as low as 3% (Birenbaum-Carmeli *et al.* 2021). Ovarian tissue cryopreservation is also potentially of value to the transgender population, with the aim of utilising immature oocytes from the preserved ovarian cortex (De Roo *et al.* 2016). Tissue culture systems have been developed over decades with the goal of producing developmentally competent oocytes (Bertoldo *et al.* 2018, Telfer & Andersen 2021). Complete *in vitro* growth of primordial follicles with subsequent *in vitro* maturation (IVM), IVF and production of live offspring has been achieved in the mouse (Eppig *et al.* 1996, O'Brien *et al.* 2003) and systems that support the development of human immature follicles to antral stages (Telfer *et al.* 2008) and to the development of mature oocytes that can reach Metaphase II have been developed (McLaughlin *et al.* 2018). There are, however, limited data on the effects of long-term gender-affirming testosterone treatment on the ovary.

Androgens are essential in maintaining normal ovulatory function and follicle health (Bertoldo *et al.* 2019). Excessive androgen as demonstrated in the ovaries of females with polycystic ovary syndrome (PCOS) results in an increase in follicular recruitment with subsequent arrest in follicle development and accumulation of pre-antral follicles (Nisenblatt & Norman 2009). Along with anovulation, these patients typically have poor ovarian follicle health and subfertility (Dumesic *et al.* 2015). Animal studies have revealed that administering high doses of testosterone results in an ovarian morphology with similarities to PCOS (Murray *et al.* 1998, Vendola *et al.* 1998, Abbott *et al.* 2009). However, the results of studies investigating the effect of exogenous testosterone on human ovaries are variable. The ovaries of transgender men undergoing testosterone therapy have been reported to show increased numbers of antral and atretic follicles, similar to PCOS (Amirikia *et al.* 1986, Futterweit &

Deligdisch 1986, Spinder *et al.* 1989, Pache *et al.* 1991, Grynberg *et al.* 2010), whilst others have found a normal follicle distribution (Ikeda *et al.* 2013, De Roo *et al.* 2017).

This study aimed to investigate the effects of long-term exposure to high levels of testosterone on the human ovary. Specifically, to identify if exogenous testosterone therapy impacts the pool of non-growing and early follicles *in vivo*, and the response to *in vitro* culture. The effects on follicle health and growth initiation, markers of DNA damage and its repair were also investigated.

Materials and methods

Ovarian cortical tissue

Whole ovaries were obtained with informed consent from transgender patients undergoing hysterectomy and bilateral oophorectomy as part of gender transitioning (mean age: 27.6 ± 1.7 years; range: 20–34 years, $n = 8$). Ethical approval of this study was given by the local ethics committee (ref LREC16/SS/0114). Ovaries were collected in theatre and placed in pre-warmed dissection medium (Leibovitz L-15 supplemented with glutamine (2 mM; Gibco), sodium pyruvate (2 mM), human serum albumin (HSA) (3 mg/mL), penicillin G (75 µg/mL) and streptomycin (50 µg/mL) all from Sigma-Aldrich).

Age-matched contemporaneous control ovarian cortical biopsies (mean age: 31.8 ± 1.5 years; range: 25–35 years, $n = 8$) were obtained from women undergoing elective caesarean section (Telfer *et al.* 2008).

Tissue preparation and fragment culture

On arrival in the laboratory, ovaries were transferred into fresh, pre-warmed dissection medium and then placed individually on a shallow glass Petri dish. The ovarian cortex was dissected into fragments using a no. 24 scalpel blade and fine forceps to secure the ovary, ensuring to avoid areas containing haemorrhagic or cystic follicles and then placed in fresh pre-warmed dissection medium. Cortical strips including samples from both ovaries were selected at random, fixed in neutral-buffered formalin (NBF) and processed as uncultured controls for histological

evaluation of follicle distribution and morphology. Tissue stretching was performed as previously described (McLaughlin *et al.* 2018) to promote *in vitro* activation of follicles and to aid follicle visualisation. Using an angled incision, tissue strips were divided into fragments $\sim 4 \times 2 \times 1$

mm thick. Fragments were cultured individually in 24-well flat-bottomed plates at 37°C in 300 µL/well culture medium (McCoys 5a medium supplemented with 25 mM HEPES (Gibco), glutamine (3 mM; Gibco), HSA (0.1%), penicillin G (0.1 mg/mL), streptomycin (0.1 mg/mL), transferrin (2.5 µg/mL), selenium (4 ng/mL), human insulin (10 ng/mL), recombinant human FSH (1 ng/mL) and ascorbic acid (50 µg/mL) all from Sigma-Aldrich) for 6 days in humidified air with 5% CO₂; half the culture medium was removed and replaced with fresh medium on alternate days (days 2 and 4). At the end of the culture period, all fragments were fixed in NBF for histological processing.

Histological methods and tissue analysis

After fixation for 24 h, individual fragments were dehydrated through graded alcohols and cleared in cedar wood oil before being embedded in paraffin wax and serially sectioned at 6 µm thickness. Tissue sections were stained with hematoxylin and eosin and every 10th section was analysed using a light microscope under 40× magnification. Follicles were classified using a modification of an established system: primordial follicle (oocyte surrounded by a complete or incomplete single layer of flattened granulosa cells), transitory follicle (an oocyte surrounded by a mixed layer of flattened and cuboidal granulosa cells), primary follicle (oocyte surrounded by a single layer of cuboidal granulosa cells) and secondary follicle (an oocyte surrounded by two or more complete layers of cuboidal granulosa cells) (Pedersen & Peters 1968). For the main analysis of the effect of culture, primordial and transitory follicles were distinguished and analysed separately. For the DNA damage and repair immunohistochemical analyses, they were combined and analysed as non-growing follicles (Gougeon & Chainy 1987b, Westergaard *et al.* 2007). The number and developmental stage of follicles from D0 and cultured D6 tissue were recorded per patient. To prevent double counting, only follicles with an oocyte nucleus were analysed. The classification of healthy follicles was based on the same criteria as previously described (Telfer *et al.* 2008, McLaughlin *et al.* 2014). In brief, for follicles to be categorised as morphologically normal, the oocyte must be grossly circular, surrounded by a zona pellucida and have <10% of pyknotic granulosa cells present (Fig. 1A).

Immunofluorescence

Immunofluorescence was used to identify and quantify the presence of γH2AX, a marker of DNA damage, in

oocytes and granulosa cells of ovarian follicles from both transgender and control D0 and D6 tissue. Tissue sections were dewaxed, rehydrated and immersed in phosphate-buffered saline (PBS) with 0.1% (v/v) Triton X-100 (PBST) (pH 7.2–7.4). Antigen retrieval in 10 mM sodium citrate (pH 6.0) was performed by microwaving slides followed by cooling at room temperature. After washing, slides were incubated for 1 h at room temperature with blocking solution (5% goat serum in PBST) and then probed with primary antibody γ H2AX (NB100-384, 1:1000; Novusbio, Centennial, CO, USA) overnight at 4°C. Blocking solution without primary antibody acted as a negative control. Next, the slides were washed and incubated with a secondary antibody (Cy3-conjugated affinity pure donkey anti-rabbit IgG (H + L), 1:250; Jackson Laboratories) for 1 h. Following further PBST washes, the sections were mounted in a Vectashield hardset with 4'-6-diamidino-2-phenylindole (DAPI) (H-1500; Vector Laboratories, Peterborough, UK).

Images were captured using a Zeiss LSM 800 confocal microscope with $\times 20$ magnification (Fig. 2A). Five sections were analysed per patient and images were taken of every follicle with an oocyte nucleus present and analysed using ImageJ. The proportion of oocytes with positive expression of γ H2AX was calculated per patient and analysed as mean $\pm$ s.e.m. For the granulosa cells, the proportion of follicles with any positive expression of primary antibody within the granulosa cells was calculated per patient followed by calculating the proportion of positive granulosa cells per total number of granulosa cells per positively stained follicle. Both results were analysed as the mean $\pm$ s.e.m. ImageJ was used to quantify the intensity of γ H2AX immunofluorescence staining in both oocytes and granulosa cells. This was carried out by measuring the mean grey value. The results were analysed per patient and presented as the mean $\pm$ s.e.m.

Immunohistochemistry

DNA repair proteins were localised in tissue sections from both control and transgender ovarian tissue at D0 and D6 using antibodies against Rad51 (137323; 1:500; Abcam), Ataxia-Telangiectasia Mutated (ATM) (ab78; 1:500; Abcam) and meiotic recombination (MRE) 11 (NB100-142; 1:1000; Novusbio). Antigen retrieval was performed as described earlier, then slides were immersed in 3% (v/v) hydrogen peroxide to quench endogenous peroxidase activity. The slides were washed and incubated for 1 h in appropriate blocking solution (150 μ L goat serum in 10 mL PBST) followed by incubation in suitably diluted primary

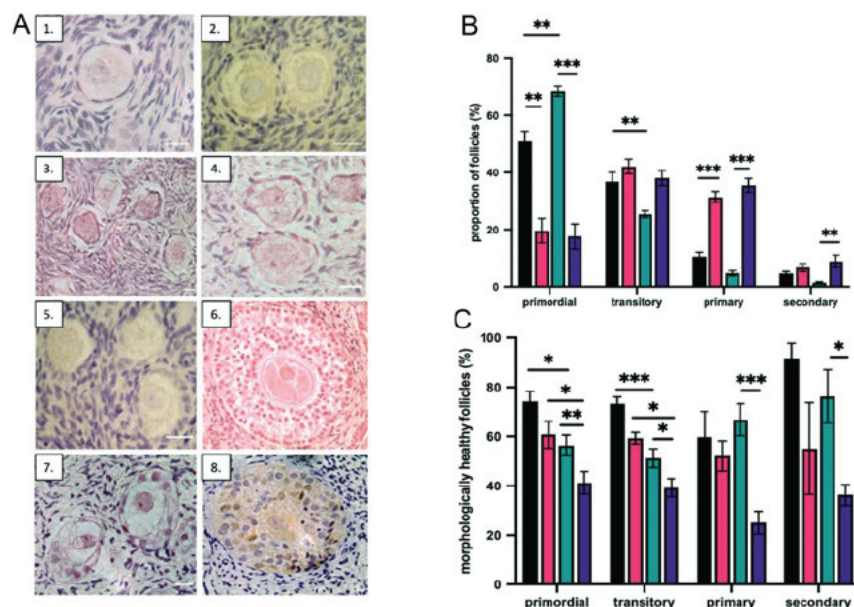


Figure 1 (A) Photomicrographs of non-growing and growing follicles from both groups. (A1) primordial follicle from control tissue, (A2) transitory follicles from control tissue, (A3–4) primordial follicles from transgender tissue, (A5) primary follicles from control tissue, (A6) secondary follicle from control tissue, (A7) primary follicles from transgender tissue and (A8) secondary follicle from transgender tissue. (B) Follicle classification: proportion of follicles in each classification stage in transgender and control tissue at D0 and D6 ($n = 8$ patients per group). In the analysis using Kruskal–Wallis test with *post hoc* Dunn test, results were analysed per patient and expressed as mean $\pm$ s.e.m., P -value < 0.05 was considered significant, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P < 0.05$. Black bars control D0, pink bars control D6, green bars transgender D0 and purple bars transgender D6. (C) Morphological health: proportion of morphologically healthy follicles at each follicle stage in control and transgender tissue D0 and D6 ($n = 8$ patients per group). In the analysis performed using Kruskal–Wallis test with *post hoc* Dunn test, results were analysed per patient and expressed as mean $\pm$ s.e.m., P -value < 0.05 was considered significant, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P < 0.05$. Black bars control D0, pink bars control D6, green bars transgender D0 and purple bars transgender D6.

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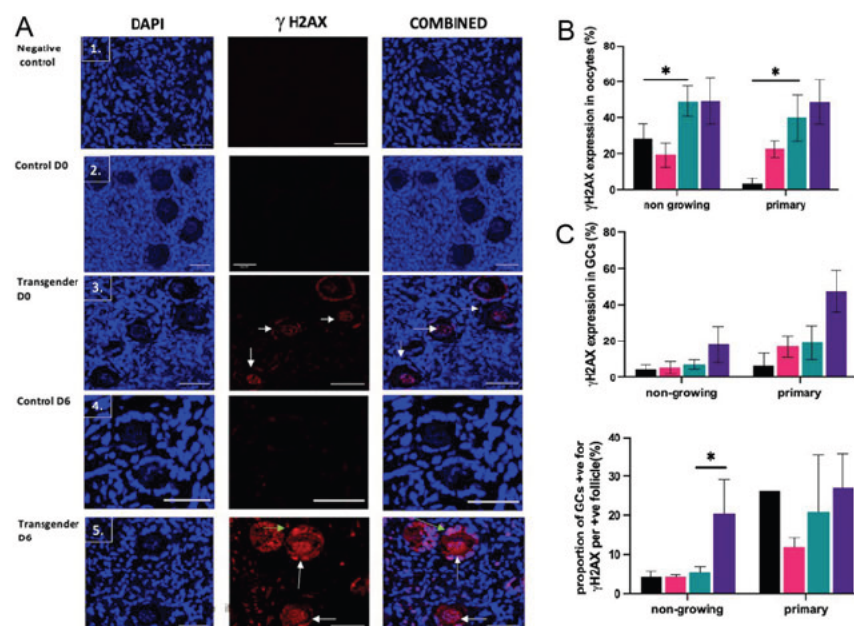


Figure 2 (A) Localisation of γ H2AX by immunofluorescence in human ovarian tissue. γ H2AX (red) and DAPI (blue) staining in oocyte and granulosa cells. γ H2AX staining is identified as red foci within nuclei (white arrows) in oocytes and granulosa cells (green arrows). (1) negative control, (2) control D0 – non-growing follicles, oocytes and granulosa cells negative for γ H2AX, (3) transgender D0 – foci of γ H2AX identified within the oocytes and granulosa cells of non-growing follicles, (4) control D6 – growing follicles negative staining for γ H2AX, (5) transgender D6 – growing follicles positive foci identified within oocytes and granulosa cells. Scale bar = 40 μ m. (B–C) Positive expression of γ H2AX in oocytes (B) and granulosa cells (C) (total number of follicles $n = 840$, control D0 $n = 122$, transgender D0 $n = 384$, control day 6 $n = 145$, transgender day 6 $n = 189$). Expression of γ H2AX in oocytes and granulosa cells was analysed per patient ($n = 8$ patients per group) and results are expressed as the mean $\pm$ s.e.m. Expression in oocytes was analysed using the Kruskal–Wallis test with the *post hoc* Dunn test. Expression of γ H2AX in granulosa cells and proportion of granulosa cells in affected follicles were analysed

using the Kruskal–Wallis test followed by the *post hoc* Dunn test. Statistical significance assigned $P < 0.05$. * $P < 0.05$. Black bars control D0, pink bars control D6, green bars transgender D0 and purple bars transgender D6.

antibodies overnight at 4°C. Blocking solution without primary antibody acted as a negative control.

After washing, sections were incubated for 30 min with biotinylated secondary antibody at room temperature (Vector Laboratories) and then processed using an ABC kit as per manufacturer instructions (Vectastain Elite ABC kit; Vector Laboratories). DAB (3,3'-diaminobenzidine) peroxidase substrate kit (Vector Laboratories) solution was applied to the sections and then counterstained with haematoxylin. Five sections were analysed per patient. The proportion of oocytes with positive expression of a DNA repair protein was calculated per patient and analysed as the mean $\pm$ s.e.m. For the analysis of granulosa cells, the proportion of follicles with any positive expression of primary antibody within the granulosa cells was calculated per patient followed by calculating the proportion of positive granulosa cells per total number of granulosa cells per positively stained follicle.

Statistical analysis

All data were analysed using SPSS statistical software version 24 (SPSS, Inc.). Graphs were generated using GraphPad software version 7 (Graphpad Inc.). All results are presented as mean $\pm$ s.e.m. Given the potential for large interpatient variability, non-parametric Kruskal–Wallis test with Dunn *post hoc* test was used for all statistical analyses. Statistical significance was assigned as $P < 0.05$.

Results

Patient characteristics

In total, tissue from eight trans men who underwent a hysterectomy and bilateral oophorectomy (mean age 27.6 ± 1.7 years; range 20–34 years, BMI 27.5 ± 1.4) was included in this study. Pre-operative measurement of reproductive hormones (Table 1) showed values in the normal male range. All patients had been treated with 1000 mg of testosterone undecanoate at approximately 12-week intervals prior to tissue collection (range of treatment 18 months–10 years) and were non- or ex-smokers with no significant past medical history, in particular, no history of PCOS (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article).

Testosterone exposure and follicle distribution and morphological health

On D0, 32 cortical fragments were obtained, 16 from 8 transgender patients and 16 from 8 control patients, to

Table 1 Overview of patient blood results ($n = 5$, patients who consented to hormone analysis). Samples were assayed as part of normal care in a hospital laboratory.

Variable	Mean	s.e.m.	Range	Reference range
Age, years	27.6	2.32	20–34	
FSH, μL	4.06	0.4	2.9–4.9	1–10
LH, μL	3.7	1.7	0.5–9.6	1–9
Oestradiol, pmol/L	122.8	16.3	63–156	0–160
Testosterone, nmol/L	15.5	0.84	12.3–17.2	10–38

determine follicle classification and morphological health. As more tissue sections were available from the transgender tissue, more follicles were analysed, transgender 3871 follicles, control 655 follicles, totalling 4526 follicles (Fig. 1A). At D0, the most prevalent follicle type in both groups was primordial, with a significant higher proportion in transgender tissue compared to the age-matched control ($67.4 \pm 1.7\%$ vs $50.9 \pm 1.5\%$, $P < 0.005$) (Fig. 1B). There was a smaller proportion of transitory follicles in the transgender tissue ($26.5 \pm 1.2\%$ vs $33.9 \pm 3.2\%$, $P < 0.005$). Differences in the proportion of primary ($4.7 \pm 0.9\%$ vs $10.6 \pm 1.5\%$, $P = 0.054$) and secondary ($1.4 \pm 0.4\%$ vs $4.6 \pm 0.7\%$, $P = 0.2$) follicles did not reach statistical significance, and no pre-antral or antral follicles were identified in either group.

At D0, the proportion of morphologically healthy primordial and transitory follicles was significantly lower in the transgender tissue compared to the control

(primordial: $56.4 \pm 4.2\%$ vs $72.1 \pm 4.2\%$, $P \leq 0.05$,

transitory: $51.2 \pm 3.8\%$ vs $73.3 \pm 2.7\%$, $P \leq 0.001$ (Fig. 1C). There were no differences in morphological health of primary and secondary follicles between groups at D0 (primary: $66.8 \pm 2.8\%$ vs $59.7 \pm 4.6\%$, $P = 0.9$, secondary: $76.2 \pm 10.8\%$ vs $91.7 \pm 8.3\%$, $P = 0.78$).

Testosterone exposure and follicle activation and

survival *in vitro*

On D6, 32 cortical fragments were obtained, 16 from 8 transgender patients and 16 from 8 control patients, to determine the follicle classification and morphological health of follicles after 6 days of culture with a total of 3367 follicles (transgender: 2617 follicles, control: 750 follicles) analysed (Fig. 1A). The proportion of primordial follicles was significantly lower at D6 compared to D0 in both groups (transgender $57.5 \pm 2.8\%$ vs $17.6 \pm 4.4\%$, $P < 0.001$, control $50.9 \pm 3.6\%$ vs $19.6 \pm 4.3\%$, $P < 0.005$ (Fig. 1B) with no change in the proportion of transitory

follicles. Thus, the population of non-growing follicles

after 6 days in culture was lower in the transgender group than in controls ($55.6 \pm 3.3\%$ vs $61.7 \pm 2.4\%$, $P < 0.005$). In both groups, this decline was balanced with a reciprocal increase in the proportion of growing follicles, in particular primary follicles (transgender: primary $4.7 \pm 0.9\%$ to $35.5 \pm 1.9\%$, $P < 0.001$, secondary $1.4 \pm 0.4\%$ to $8.5 \pm 2.3\%$, $P < 0.005$, control: primary $10.6 \pm 1.5\%$ to $32.5 \pm 1.7\%$, $P < 0.001$, secondary $4.6 \pm 0.7\%$ to $8.5 \pm 2.3\%$, $P = 0.99$).

On D6 in the transgender tissue, there was a reduction in the proportion of morphologically healthy follicles across all follicle types from D0 (primordial $56.4 \pm 4.2\%$ to $41.2 \pm 4.6\%$, transitory $51.2 \pm 3.8\%$ to $39.2 \pm 3.7\%$, primary $66.8 \pm 6.5\%$ to $25.1 \pm 4.6\%$, secondary $76.2 \pm 10.8\%$ to $36.3 \pm 4.2\%$, $P < 0.05$ for all follicle types) (Fig. 1C). In contrast, in control tissue, on day 6, the morphological health of follicles remained stable across all follicle types (primordial: $74.2 \pm 4.1\%$ to $60.8 \pm 5.5\%$, $P = 0.71$, transitory: $73.3 \pm 2.7\%$ to $59.3 \pm 3.3\%$, $P = 0.40$, primary: $59.6 \pm 10.4\%$ to $52.1 \pm 2.1\%$, $P = 0.88$, secondary $91.7 \pm 8.3\%$ to $55.1 \pm 18.6\%$, $P = 0.21$).

cells per follicle affected ($20.8 \pm 14.7\%$ vs $26.3 \pm 2.1\%$, $P = 0.67$).

Testosterone exposure and DNA damage and DNA double-strand breaks repair capacity in follicles

To identify whether testosterone exposure was associated with DNA damage, five sections per patient from both transgender and control groups were used to identify expression of γ H2AX as a marker of DNA damage in oocytes and the granulosa cells of ovarian follicles (Fig. 2A–E). Both D0 and D6 tissue was analysed and included 840 follicles (transgender 573 follicles, control 267 follicles).

At D0, the proportion of oocytes from non-growing and primary follicles expressing γ H2AX was significantly higher in transgender compared to control tissue (non-growing: transgender $49.1 \pm 8.5\%$ vs control $28.6 \pm 8\%$, primary $40 \pm 12.7\%$ vs $3.3 \pm 3.3\%$, both $P < 0.05$) (Fig. 2B). Insufficient numbers of secondary follicles were identified for analysis.

The proportion of non-growing follicles expressing γ H2AX in the granulosa cells at D0 did not significantly differ between groups (Fig. 2C), with comparable proportions of granulosa cells per follicle affected (Fig. 2C). In primary follicles, a higher proportion of follicles expressed γ H2AX in the granulosa cells in the transgender tissue compared to control ($19 \pm 9.2\%$ vs $6.6 \pm 6.7\%$, $P < 0.05$) but with comparable proportions of granulosa

On D6, expression of γ H2AX in the oocytes of non-growing follicles remained unchanged in both groups (Fig. 2B). Expression of γ H2AX remained high in primary oocytes in transgender tissue ($40 \pm 12.7\%$ to $48.9 \pm 12.5\%$, $P = 0.5$); while there appeared to be an increase in control, this was variable and not statistically significant $3.3 \pm 3.3\%$ to $22.6 \pm 4.8\%$, $P = 0.5$).

At D6, in the transgender non-growing follicles, there was a significant increase in the percentage of granulosa cells positive for γ H2AX per follicle ($5.4 \pm 1.7\%$ to $20.4 \pm 8.7\%$, $P < 0.05$), whereas γ H2AX expression remained at low levels in control tissue follicles (Fig. 2C). In primary follicles, there was no significant change in the proportion of follicles expressing γ H2AX in the granulosa cells nor the percentage of granulosa cells positive for γ H2AX per follicle in both groups (Fig. 2C).

No significant differences in the intensity of staining in oocytes and granulosa cells were detected between control and transgender tissue across all follicle types at D0 and D6.

To identify whether testosterone affects the DNA repair capacity of ovarian follicles, expression of the DNA repair proteins ATM, RAD51 and MRE11 which are involved in the process of homologous recombination was analysed (Fig. 3A1–3/B1–3/C1–3). Five sections per patient from both control and transgender groups were analysed for expression in both oocytes and granulosa cells.

At D0, there was no significant difference in expression of RAD51, ATM and MRE11 in oocytes and granulosa cells between transgender and control follicles ($P > 0.05$) (Fig. 3). At D0, RAD51, ATM and MRE11 were found in a high proportion of both transgender and control oocytes across all follicle types (Fig. 3 A4/B4/C4). In both groups, RAD51 and MRE11 were expressed in a low proportion of granulosa cells in non-growing and primary follicles, with no expression of ATM identified (Fig. 3 A5/B5/C5).

At D6, there was no significant difference in the expression of RAD51, ATM and MRE11 in oocytes and granulosa cells between groups ($P > 0.05$). At D6, in both groups, expression of RAD51 remained high in oocytes and low in granulosa cells across all follicle types (Fig. 3A4 and Fig. 3A5). Expression of ATM in oocytes in both groups was comparable to D0 across all follicle types (Fig. 3B4). ATM was detected in variable levels in granulosa cells in all follicle types in both groups (Fig. 3A5). In both groups, levels of MRE11 expression remained high in oocytes at D6 (Fig. 3C4) with more variable expression of MRE11 in granulosa cells, particularly in growing follicles (Fig. 3C5).

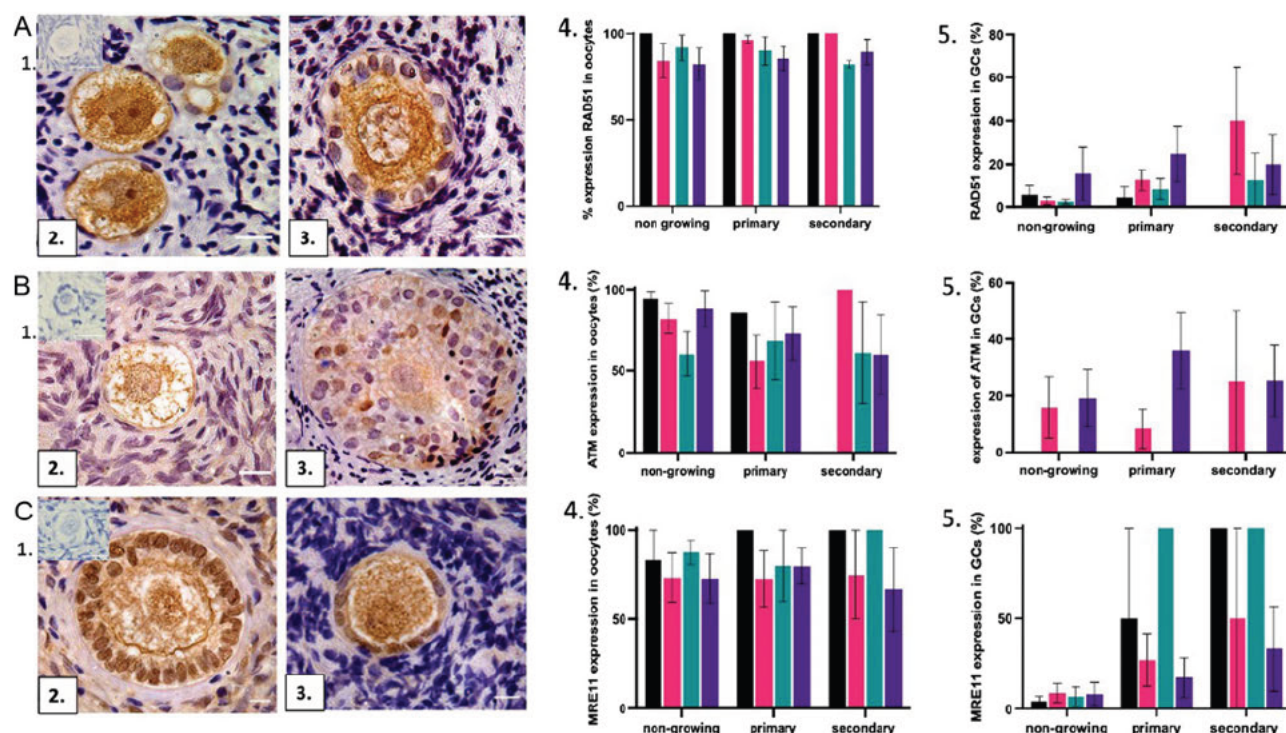


Figure 3 Immunohistochemical detection of RAD51, ATM and MRE11. Photomicrographs of ovarian follicles for RAD51 (A1–3), ATM (B1–3) and MRE11 (C1–3). Negative control (1), control follicles A2/B2/C2, transgender follicles A3/B3/C3. Positive staining (brown) in oocytes in A2–3, B2–3 and C2–3. Positive staining in granulosa cells in B3 and C2–3. Scale bar = 40 μ m. Graphs showing positive expression in oocytes (A4, B4, C4) and granulosa cells (A5, B5, C5) (total number of follicles, RAD51 $n = 834$, ATM $n = 800$, MRE11 $n = 550$, $n = 8$ patients per group). Results were analysed per patient and expressed as the mean $\pm$ s.e.m. Expression in oocytes was analysed using Kruskal–Wallis test with the *post hoc* Dunn test. Expression in granulosa cells was analysed using Kruskal–Wallis test with the *post hoc* Dunn test. Statistical significance assigned $P < 0.05$. Black bars control D0, pink bars control D6, green bars transgender D0 and purple bars transgender D6.

Discussion

In this study, we have compared the distribution of follicle stages and their health in ovaries exposed to high levels of testosterone in trans men compared to controls. The effect of *in vitro* culture on follicle distribution and health was also examined, together with an analysis of markers of DNA damage and its repair. A significantly higher proportion of primordial follicles was found in transgender tissue compared with similarly aged controls, and these follicles had poorer morphological health and increased levels of DNA damage.

An increased proportion of primordial follicles in transgender ovarian tissue compared to control suggests that testosterone therapy has a suppressive effect on follicle activation *in vivo*. Similar proportions of primordial follicles were found in other studies (De Roo et al. 2017, Borrás et al. 2021), but our study found a smaller proportion of primary follicles. These studies concluded that testosterone-exposed ovaries had a similar cortical follicle distribution to cisgender females. However, those studies did not use contemporaneous controls and used historic

data from the literature (Gougeon & Chainy 1987a) where the age range was 19–49 or based on data from cisgender females who were known to be infertile (Lass et al. 1997). This study used closely aged women who donated ovarian cortical tissue at the time of caesarean section with samples analysed at the same time by the same observer. In pregnancy, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels are persistently low for the duration of pregnancy as a result of the inhibitory effects of high levels of circulating oestradiol and progesterone on the hypothalamus and pituitary, suppressing ovulation (Choi & Smits 2014, Stilley & Segaloff 2018). However, the process of follicle recruitment and early growth is gonadotrophin-independent; therefore, pregnancy is likely to have no effect on this process (Hsueh et al. 2015, Zhang et al. 2023). In transgender men taking gender-affirming endocrine therapy, FSH and LH levels are also low or similar to the follicular phase of the menstrual cycle in cisgender women (De Roo et al. 2017, Greene et al. 2021) with low levels of oestradiol (Chan et al. 2018). In this study, testosterone levels were within the male range, without marked gonadotrophin suppression.

The choice of appropriate controls is important for these studies, as accessing tissue from healthy, aged matched non-pregnant women is rarely possible. The use here of samples from pregnant cisgender females has advantages as they are fertile healthy samples, and additionally, the high steroid/low gonadotrophin environment has parallels to gender-affirming treatment but with female testosterone levels. However, the effect of pregnancy on follicle DNA damage and capacity for repair is unknown. Using tissue from patients with ovarian pathologies equally has its own limitations: a study of follicle distribution and ovarian reserve in patients with a range of both benign and malignant ovarian pathologies concluded that the cortex surrounding an ovarian malignancy has reduced follicle density, and in women with benign ovarian lesions, stromal proliferation was observed (Pavone *et al.* 2014). Similarly, the ovarian cortex from women with an endometrioma had a reduced follicle number, poorer morphological health and fibrotic change within the stroma (Maneschi *et al.* 1993).

There is limited information regarding the long-term impact of testosterone on ovarian follicles. Reassuringly, after cessation of testosterone therapy, many transgender men successfully conceive naturally (Light *et al.* 2014) or through artificial reproductive techniques (Adeleye *et al.* 2019, Leung *et al.* 2019). Testosterone has been reported to have marked effects on ovarian morphology with abnormalities including stromal hyperplasia, thickened ovarian cortex and polycystic ovary morphology (Futterweit & Deligdisch 1986, Spinder *et al.* 1989, Grynberg *et al.* 2010, Ikeda *et al.* 2013). Testosterone therapy does not appear to deplete the primordial follicle pool from the ovarian cortex of transgender men (Van Den Broecke *et al.* 2001) or affect the *in vitro* maturation potential of cumulus–oocyte complexes (COCs) harvested from the ovarian medulla (De Roo *et al.* 2017, Lierman *et al.* 2017). It has however been reported to enhance follicle activation in the mouse ovary through the nuclear exclusion of Forkhead box O3X (FOXO3A) (Yang *et al.* 2010) although others have found that androgen receptors are not expressed by primordial follicles (Gervásio *et al.* 2014, Walters *et al.* 2018). The dense, fibrous nature of normal human ovarian cortical tissue contributes to suppression of primordial follicle growth, thus preventing mass and premature activation, which would result in early depletion (Silber *et al.* 2018). The ovarian cortex in transgender tissue has been found to be stiffer in comparison to tissue from cancer patients (De Roo *et al.* 2019). This increased resting tissue rigidity may enhance the suppression of residing primordial follicles

and be an explanation for the finding here of reduced follicle activation *in vivo*.

Following 6 days of culture, the proportion of non-growing follicles in transgender tissue was significantly lower than in controls, despite being higher before culture. This suggests that the rate of activation of non-growing follicles *in vitro* is higher than in controls. The process of tissue dissection mechanically loosens the ovarian cortex and reduces intrinsic tissue pressure, increasing follicle activation (Smitz & Cortvrindt 2002, Telfer *et al.* 2008, McLaughlin *et al.* 2018, Grosbois & Demeestere 2018). This may have had a greater effect on the testosterone-exposed tissue as accelerated activation can have a detrimental impact on follicle quality (Smitz & Cortvrindt 2002, McLaughlin *et al.* 2014). In studies where primordial follicle activation was enhanced by suppressing phosphatase and tensin homolog deleted on chromosome 10 (PTEN) using Dipotassium bisperoxo oxovanadate (V) (bpV(HOpic)), an increase in the proportion of growing follicles was accompanied by a significant reduction in morphological health in both human (McLaughlin *et al.* 2014, Maidarti *et al.* 2019) and bovine ovarian follicles (Maidarti *et al.* 2019). In this study, with increased follicle activation in cultured transgender ovarian tissue, there was a significant decline in the morphological health of growing follicles compared to the control. This highlights that further optimisation of this stage of the culture process is needed to control follicle activation to ultimately produce a population of high-quality oocytes (Telfer & Zelinski 2013).

γ H2AX is a DNA repair protein that binds to the location of DNA damage and controls recruitment of DNA repair proteins (Winship *et al.* 2018). In this study, the proportion of oocytes expressing γ H2AX in uncultured D0 non-growing follicles was significantly higher in the transgender group compared to the control, consistent with the histological findings of reduced follicle health. After 6 days of culture, levels of γ H2AX in the non-growing and primary follicles from transgender tissue remained stable. The cause of increased DNA damage in testosterone-exposed follicles is not clear, but studies that have used an experimental hyperandrogenic model in culture (Bertoldo *et al.* 2019) found increased levels of reactive oxidative species (ROS) resulting in DNA damage. Oxidative stress is a state whereby ROS outbalances antioxidant levels, resulting ultimately in DNA damage and/or cell apoptosis (Lee *et al.* 2022). There is also an increasing body of literature on the role of ROS in the pathogenesis of PCOS (Karadeniz *et al.* 2008, Zhang *et al.* 2008). Increased levels of biochemical markers of oxidative stress have been reported in women with PCOS compared to controls

(Sabuncu *et al.* 2001, Palacio *et al.* 2006, Murri *et al.* 2013) whilst others have found variable findings when looking for markers for anti-oxidative stress levels (Sabuncu *et al.* 2001, Zhang *et al.* 2008, Seleem *et al.* 2014). Given that similar histological findings have been identified in both transgender male ovaries and females with PCOS, for example stromal hyperplasia, tunica albuginea thickening (Baba *et al.* 2007), it is possible that ROS could be responsible for the increased DNA damage seen in testosterone exposed ovarian follicles.

It is essential that primordial follicles have a robust system of DNA damage recognition and repair mechanisms to ensure genetic integrity. Unrepaired or incorrectly repaired DNA double-strand breaks (DSBs) result in infertility, miscarriage and genetic defects in offspring (Stringer *et al.* 2018). The preferred mechanism of DNA repair in primordial follicles is homologous recombination (HR), a process of repair which uses the sister chromatid as a template for error-free repair (Winship *et al.* 2018). The process of HR is initiated by the MRN complex consisting of Meiotic recombination 11 (MRE11)-Rad50, Nijmegen breakage syndrome 1 (NBS1), recognising the DNA DSB (Maidarti *et al.* 2020). The binding of the MRN complex to the DNA DSBs allows for the interaction of the NBS1 protein with ATM, resulting in the autophosphorylation of ATM at a serine residue (Rein & Stracker 2014). Following this, ATM phosphorylates H2AX at the C-terminal serine 139 (γ H2AX) (Nguyen *et al.* 2021). γ H2AX binds to the DNA DSB, resulting in the initiation of the downstream pathway whereby DNA is either repaired or apoptosis occurs (Oktay *et al.* 2015). The DNA repair proteins analysed here are key players within the process of HR.

Similar levels of expression of DNA repair proteins in oocytes were found between the transgender and control groups despite differing levels of expression of γ H2AX and morphological health. Small numbers of subjects and follicles being included in the analysis may explain the lack of statistical significance in these experiments. However, a lack of increased expression of DNA repair proteins with increased levels of DNA damage in transgender tissue could indicate suboptimal DNA repair protein recruitment. Ineffective DNA repair is pathognomonic in reproductive aging, resulting in accumulating DNA DSBs (Karanjawala & Lieber 2004, Gorbunova *et al.* 2007, Winship *et al.* 2018). DSBs have been found to accumulate in ovarian follicles of aging mice, with the downregulation of key DNA repair proteins (Oktay *et al.* 2010, Titus *et al.* 2013). It is possible that testosterone may impair the DNA repair capacity of ovarian follicles, resulting in compromised genetic integrity and reduced oocyte quality. However,

this study only looks at the first step of follicle growth; therefore, the results of these experiments cannot take into consideration the potential for DNA repair at later stages. This would however align with findings that when COCs harvested from the ovarian medulla of transgender men on testosterone therapy were fertilised, there were significantly impaired fertilisation rates and embryo development (Lierman *et al.* 2021).

A limitation of this study is that there is a discrepancy in the volume of tissue available between groups, with whole ovaries collected from transgender patients and small fragments of ovarian cortex from control subjects. Unlike in rodent studies where absolute follicle counts can be achieved, as often in human studies where biopsies of the ovarian cortex are obtained, the proportion of follicle classification is calculated. Within human cortical tissue, the distribution of follicles can vary significantly between ovarian cortical fragments from the same ovary (Schmidt *et al.* 2003). This presents a difficulty in assessing primordial follicle activation through classifying and counting follicles in uncultured and cultured tissue (Telfer & Zelinski 2013). It is not possible to follow follicle activation in real time, therefore, to comment on follicle activation through assessing different ovarian cortical fragments pre and post culture is difficult.

In this study, there was a wide range of duration of testosterone treatment (range 18 months–10 years). We found no relationship between the markers of DNA damage and duration of treatment (not shown), but it is possible that such a relationship exists and would be identified in a larger study focusing on that question.

In summary, the results from this study indicate that testosterone exposure *in vivo* leads to reduced follicle growth activation, and a reduction in morphological health with apparent DNA damage, with further deterioration after 6 days of culture compared to our control population. These results highlight that further studies are needed to evaluate the long-term sequelae of testosterone on ovarian follicles to guide discussion regarding future fertility and the potential value of fertility preservation in trans men before initiating testosterone treatment. They also indicate a need for further optimisation of the culture system and for customisation depending on the source of ovarian tissue being cultured.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/RAF-22-0102>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author	contribution	statement
EB	contributed to the conception and design of study, experimental work and data acquisition, analysis and interpretation, and manuscript preparation.	
MM	contributed to the experimental work and data acquisition, analysis and interpretation, and final approval of manuscript.	
EET	contributed to the conception and design of study, data analysis and editing and final approval of manuscript.	
RAA	contributed to the conception and design of study, data analysis and editing and final approval of manuscript.	
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