Ovarian tissue cryopreservation in female-to-male transgender people: insights into ovarian histology and physiology after prolonged androgen treatment

Chloë De Roo a,*,1, Sylvie Lierman a,1, Kelly Tilleman a, Karen Peynshaert b,c, Kevin Braeckmans b, Mirte Caanen d, Cornelius B Lambalk d, Steven Weyers a, Guy T'Sjoen e, Ria Cornelissen f, Petra De Sutter a

a Division of Reproductive Medicine, Department of Gynaecology, Ghent University Hospital, 9000 Ghent, Belgium
b Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, Ghent University, 9000 Ghent, Belgium
c Centre for Nano- and Bio Photonics (NB-Photonics), Ghent University, 9000 Ghent, Belgium
d Division of Reproductive Medicine, Department of Obstetrics and Gynaecology, VU medical centre (VUmc), 1007 MB Amsterdam, The Netherlands
e Department of Endocrinology and Centre for Sexology and Gender, Ghent University Hospital, 9000 Ghent, Belgium
f Department of Basic Medical Science, Faculty of Medicine and Health Science, Ghent University, 9000 Ghent, Belgium

Chloë De Roo concluded her medical studies at the University of Ghent in 2012. She is combining her specialization in gynaecology and obstetrics with fundamental and applied scientific research at the Centre for Reproductive Medicine at the Ghent University Hospital in order to obtain a PhD degree in fertility preservation.

**KEY MESSAGE**
This study describes a surprisingly normal distribution of cortical follicles in the ovaries of trans men after more than a year of testosterone treatment. This work confirms the presence and in-vitro maturation potential of cumulus-oocyte complexes obtained during the tissue processing of ovaries procured in trans men.

**ABSTRACT**
Female-to-male transgender people (trans men) are faced with the risk of losing their reproductive potential owing to gender-affirming hormone treatment and genital reconstructive surgery. This observational, prospective cohort study investigates the effect of prolonged androgen therapy on their ovarian histology and fertility preservation perspectives. Hormone serum levels, ovarian histology and cumulus-oocyte complexes (COC) of 40 trans men were analysed at the moment of hysterectomy with bilateral oophorectomy in the context of genital reconstructive surgery after testosterone treatment (58.18 ± 26.57 weeks). In the cortex, most follicles were primordial (68.52% total follicle count) compared with 20.26% intermediate and 10.74% primary follicles. Few secondary follicles (0.46%) and a single antral follicle were found in the sections analysed. In total, 1313 COC were retrieved from the medulla of 35 patients (37.51 ± 33.58 COC per patient). Anti-Müllerian hormone serum levels were significantly correlated with number of

* Corresponding author.
E-mail address: chloe.deroo@ugent.be (C De Roo).
1 Contributed equally to this work.
Introduction

Female-to-male transgender people (trans men), are faced with the risk of losing their reproductive potential owing to gender-affirming hormone treatment and genital reconstructive surgery. Transgender people also tend to start sex assignment treatment at a young age, when reproductive wishes are not yet clearly defined nor fulfilled (De Sutter, 2001). About one-half of trans men, however, express the desire to have children (Wierckx et al., 2012). It is, therefore, recommended by the most recent Standards of Care of the World Professional Association for Transgender Health to clearly discuss fertility and fertility preservation before any treatment (Coleman et al., 2012). Hence, transgender people represent a new group of patients consulting fertility centres. Their specific needs certainly require more insight into the effects of gender-affirming hormone therapy on fertility and an adjusted approach in fertility centres.

If taking steps towards preservation of fertility, cryopreservation of ovarian tissue at the time of hysterectomy and oophorectomy is a possibility (De Roo et al., 2016; De Sutter, 2001). No additional surgical procedure is then needed, nor ovarian stimulation in combination with frequent vaginal ultrasound monitoring as would be the case if oocyte cryopreservation is chosen. The latter is perceived as both a physical and a psychological burden by trans men (De Roo et al., 2016). Future use of the frozen tissue could either be transplantation of thawed cortex or in-vitro activation, growth and maturation of the cortical immature follicles. Although surgical challenges can be overcome in time, making future use of banked ovarian cortex of men a reality, transplantation of ovarian cortical strips in men can include unwanted side-effects by restoring female hormone activity. In this perspective, in-vitro activation, growth and maturation of these immature follicles would broaden reproductive perspectives for trans men. The developmental potential of the residing follicles in the ovary, having been exposed for a prolonged period to supraphysiological doses of testosterone, remains to be determined.

Primordial follicles are not depleted from the ovarian cortex of trans men as a result of the gender-affirming hormone treatment (Van den Broecke et al., 2001a). Furthermore, it has been shown that the cortical-residing follicles actually can resume growth and maturation after xenotransplantation (Van den Broecke et al., 2001b).

The standard procedure for banking of ovarian tissue, as described elsewhere (Donnez et al., 2004; Segers et al., 2015; Wilken-Jensen et al., 2014) still implies in many centres, that the cortical region composed of primordial follicles is cryopreserved and the medulla discarded. This could lead to discarding potentially important gametes as growing antral follicles have been found in the medulla during the manipulation of the ovarian cortex for cryopreservation in oncolgic fertility preservation programmes (Fasano et al., 2011; Huang et al., 2008). It has been shown, in an oncolgic patient cohort, that these cumulus-oocyte complexes (COC) can be recovered from the antral follicles and subsequently matured and cryopreserved in vitro (Fasano et al., 2011; Huang et al., 2008; Segers et al., 2015; Wilken-Jensen et al., 2014). The presence of these COC, nor their in-vitro maturation potential, has so far been confirmed during the processing of ovaries originating from trans men.

The primary aims of this study were to investigate ovarian histology in trans men and the possibility of ex-vivo harvesting of COC in the medulla during the processing of ovarian tissue cryopreservation after a prolonged period of testosterone use. These biological observations will be plotted against the hormonal status of the trans men at the time of ovarian tissue cryopreservation, aiming to find associations between ovarian cortical follicle count or medulla derived COC content and clinical or biochemical markers.

Materials and methods

Study design

This study was approved by the Ethical Committee of Ghent University Hospital (UZ Ghent Reference: 2012/780, Belgian registration number B670201 21 5468) on 13 November, 2012. A total of 40 people were included between April 2013 and May 2015. Of these 40 people, cortical histology and anti-Müllerian hormone (AMH) serum levels were analysed. Additional hormone serum level sampling (FSH, LH, oestrogen, progesterone, testosterone, total and free fraction and sex hormone binding globulin (SHBG)) was introduced in the study protocol after inclusion of the first 10 people (n = 30). Information on the number of COC was included in 35 people. After a period of optimization (Lierman et al., 2014), COC of 27 individuals could be in-vitro matured following our established research protocol (as described below). In 16 individuals, 124 of the matured second metaphase (MII) oocytes were subsequently used for detailed spindle analysis. The remaining matured MII oocytes (two MII oocytes were lost during fixation/staining procedure) were used for other research projects.

Hormone serum levels

A preoperative blood sample, at the time of hysterectomy with bilateral oophorectomy, was taken to define the hormone serum levels. An overview of the patient characteristics, including hormonal status and reference values, is given in Table 1. Hormone levels were determined at the Department of Clinical Biology of Ghent University Hospital as part of the standard patient follow-up using a E170 Modular® (Roche Diagnostics, Mannheim, Germany), except for AMH serum levels (A73818, Elisa Immunotech, Beckman Coulter, Woerden, Nederland). Because of changes in the Department of Clinical Biology of Ghent University Hospital, AMH serum levels of five patients were determined using the AMH Roche E170 (Roche Diagnostics, Mannheim, Germany), and were therefore excluded from our analysis as it is not recommended to compare absolute AMH values from different assays (Dewailly et al., 2014). Serum-free testosterone was calculated from the total testosterone fraction and SHBG according to Vermeulen et al. (1999).
Ovarian tissue collection and processing

Immediately after hysterectomy with bilateral oophorectomy, the entire ovaries were transported to the laboratory on ice, in Leibovitz L-15* medium (Life Technologies, Merelbeke, Belgium), supplemented with 0.45% human serum albumin (Red Cross, Belgium) (further referred as manipulation medium). The ovaries were bisected and the medulla was carefully removed by scraping with a scalpel to prepare the cortical tissue to the required thickness of 1 mm, as described elsewhere (Donnez et al., 2004; Segers et al., 2015; Wilken-Jensen et al., 2014). This cortex was subsequently fragmented in pieces of 5 × 5 mm². Cortical strips of both ovaries were pooled and one piece was randomly collected, fixed in 10% buffered formalin (Sigma-Aldrich, Bornem, Belgium) for a maximum of 24 h and embedded in paraffin (Thermo Scientific, Erembodegem, Belgium). The remaining cortical pieces were cryopreserved for either fertility preservation or research purposes, according to the patients’ choice.

During ovarian tissue processing, the medulla was minced into small pieces in a petri dish with manipulation medium. These dishes were examined under a stereomicroscope for the presence of COC. All COC were transferred to gamete buffer medium (Cook Ireland Ltd, Limerick, Ireland) at 37°C, which served as a holding medium during the process of the COC collection. Finally, each COC was transferred to a modified pre-equilibrated in-vitro maturation (IVM) medium as described further.

For two patients, whole-ovary fixation was carried out. During this procedure, the entire ovary was fixed in 10% buffered formalin for the purpose of whole-organ paraffin embedding.

Follicle classification

The paraffin-embedded ovarian cortical tissue piece was serially sectioned at 5 μm, resulting in 10 slices of 5 mm × 5 mm (surface) × 5 μm (depth), and stained with (Mayer) haematoxylin (Merck, Overijse, Belgium) and eosin (Thermo Scientific, Erembodegem, Belgium). Follicles were analysed using an inverted microscope with a 40x magnification. Follicles were classified according to the Gougeon (1986) classification for human follicles (Figure 1): primordial follicle (an oocyte surrounded by a single layer of flattened granulosa cells (Figure 1a); intermediate follicle (a single layer of flattened and cubical...
granulosa cells surrounds the oocyte ([Figure 1b]); primary follicle (a single layer of exclusively cubical granulosa cells surrounds the oocyte) ([Figure 1c]); secondary follicle (an intact second layer of cubical granulosa cells surrounds the oocyte) ([Figure 1d]); antral follicle (more than two layers of cubical granulosa cells surrounds the oocyte in presence of an antrum) (not shown) (Gougeon, 1986). Follicles were classified on the section containing the nucleus to avoid double counting. Two independent observers analysed the follicles and the mean of the two observations was used for further analysis. Hence, the follicle count sometimes resulted in decimal numbers.

In-vitro maturation

The IVM medium consisted of tissue culture medium 199 (Sigma-Aldrich, Bornem, Belgium) supplemented with 10 ng/ml epidermal growth factor (Sigma-Aldrich, Bornem, Belgium), 1 μg/ml oestradiol (Sigma-Aldrich, Bornem, Belgium), 10 μIU/ml recombinant FSH (Puregon, Organon, The Netherlands), 0.50 μIU/ml HCG (Pregnyl, Organon, The Netherlands), 1 mM L-glutamine (Sigma-Aldrich, Bornem, Belgium), 0.30 mM sodium pyruvate (Sigma-Aldrich, Bornem, Belgium), 0.80% human serum albumin, 100 IU/ml penicillin G (Sigma-Aldrich, Bornem, Belgium) and 100 μg/ml streptomycin sulphate (Sigma-Aldrich, Bornem, Belgium). All medullar COC were cultured individually in 25 μl drops of IVM medium overlaid with pre-incubated embryo-tested light mineral oil in a humidified atmosphere at 5% CO₂ and 6% O₂ at 37°C for 48 h. In-vitro maturation time was studied in a one-step 24-h, one step 48-h, and a consecutive 24-48-h protocol, where the percentage of MII oocytes was highest when the one-step 48-h in-vitro maturation time was carried out (data not shown) (Lierman et al., 2014).

Spindle analysis: fixation, fluorescence labelling and confocal microscopy

As described by Mattson and Albertini (1990), MII oocytes were simultaneously fixed and extracted in a microtubule-stabilizing buffer. To visualize microtubules, MII oocytes were incubated in the presence of a mixture of mouse monoclonal anti-α, β-tubulin (1:200); Sigma, Bornem, Belgium) overnight at 4°C, followed by Alexa Fluor 488 conjugated goat-anti-mouse immunoglobulin G (1:200); Molecular Probes, Life Technologies, Merelbeke, Belgium) for 2 h at 37°C. Chromatin was stained with ethidium homodimer-2 (1:500); (Molecular Probes, Life Technologies, Merelbeke, Belgium) for 1 h at 37°C. Labelled MII oocytes were washed and mounted in Mowiol 4-88 (Sigma-Aldrich, Bornem, Belgium) containing 0.01% phenylenediamine (Sigma-Aldrich, Bornem, Belgium) as an anti-fading reagent. Slides were stored in the dark at 4°C until analysis. Images of labelled MII oocytes were observed using a Nikon scanning laser confocal microscope (SLCM) with a 60x oil immersion objective (Nikon, NA = 1.40). A three-dimensional image of the micro tubular structure and chromosomes was rendered from Z-axis stacks (0.75 μm/step) by using ImageJ software (rsweb.nih.gov/j/). Meiotic spindle configuration of the oocytes was classified based on the classification of Combelles et al. (2011): normal if microtubules were barrel shaped and formed two opposite pointed poles, also called bipolar; or slightly aberrant, if shapes were bipolar with no pointed poles. Abnormal if shapes were bipolar with irregularities at the equator or at the poles; or non-bipolar, including mono-, or multi-polar instances. On the basis of chromosomal distribution in the metaphase plate, oocytes were classified as followed: normal when chromosomes were aligned at equatorial plate, or if six chromosomes or less were separated from the equatorial region. Abnormal chromosome distribution if dispersed, or more than six chromosomes away from equatorial region or all chromosomes located throughout the spindle. If both meiotic spindle configuration as well as chromosomal distribution were evaluated as normal, the spindle analysis was classified as normal.

Statistical analysis

Statistical analysis was conducted with IBM SPSS Statistics 23 (IBM Corp., New York, USA). For statistical analysis, hormone serum levels of LH less than 0.10 U/L were coded as the lower limit, being 0.10 (n = 3) and an immeasurable high testosterone level was defined as missing (n = 1). To correct for the variation in the number of sections per sectioned paraffin embedded ovarian cortical tissue piece, the results were recalculated per section unit. The inter-observer variation in follicle classification was verified according to Bland-Altman’s limits of agreement. If the difference between the two observations exceeded a single standard deviation, the section was counted again. Normality was tested using the Shapiro–Wilk test. Correlation analysis was conducted through inspection of scatter plots to rule out other correlations than linear and by using Spearman’s rank-order correlation tests. A linear regression test was calculated to predict the number of COC based on AMH serum levels. Regression diagnostics did not reveal any substantial discrepancies for the assumption of the regression analysis (visual inspection of a residual plot, a normal probability plot of the residuals and statistically non-significant test for heteroscedasticity). P < 0.05 was considered to be statistically significant.

Results

Patient characteristics

Forty patients with a mean age of 24.30 ± 6.15 years, after excluding one person with known polycystic ovary syndrome were finally included in this study. Of these 40 people, 24 (60%) did not smoke, four (10%) were active smokers and 12 (30%) stopped smoking. All of them underwent a hysterectomy with bilateral oophorectomy in the context of genital reconstructive surgery after a period of testosterone treatment (mean duration 58.18 ± 26.57 weeks). Transvaginal ultrasound was not carried out during the treatment, because of the additional psychological distress for the patients. Testosterone treatment consisted of intramuscular testosterone undecanoate 1000 mg every 12 weeks [Nebido®] (n = 26 [65%]), oral testosterone undecanoate 160 mg daily [Pantostone®] (n = 1 [2.5%]), transdermal testosterone gel 50 mg daily [Androge®] (n = 3 [7.5%]) or an intramuscular blend of four oesterized testosterone compounds 250 mg every 2 weeks [Sustanon®] (n = 10; 25%). Some of the patients (n = 4) using an intramuscular blend of four oesterized testosterone compounds [Sustanon®] were still increasing their dose (25 to 50 to 75 mg regimen), with a median dose of 50 mg at the moment of surgery. Statistical sub-analysis (kruskal–wallis test) did not show a significant difference in serum testosterone levels (free and total testosterone) when comparing the different testosterone treatment types and dosages. An overview of patient characteristics, including hormonal status and reference values, can be found in Table 1.
**Count and percentage distribution of cortical follicles**

The ovarian cortical tissue slices per patient were counted by two observers. The mean count of the two observers showed a total of 3613.50 follicles, with a mean of $90.34 \pm 118.02$ follicles per patient (with minimum 2.00 and maximum 614.50 follicles per patient). The detailed follicle distribution is presented in Table 2. Most of the follicles were primordial (2476.00 primordial follicles in total, being 68.52% of the total follicle count) compared with 732.00 intermediate follicles (20.26% of total) and 388.00 primary follicles (10.74% of total). Very few secondary follicles (16.50 secondary follicles, 0.46% of total) and only a single antral follicle (0.03% of total) were found in all sections analysed in the study.

**Modelling of hormone serum levels and ovarian cortical observations**

In order to determine correlations between the ovarian cortical observations and the hormone serum levels in trans men, a correlation analysis between the individual hormone serum levels and the total number of follicles as well as the different follicle stages was carried out. None of the hormone serum levels correlated with the follicle numbers, nor did the duration of the testosterone therapy or the patients’ age. An overview of the Spearman correlation coefficients and corresponding P-values of the correlation analyses is presented in Table 3. The correlation with the number of antral follicles could not be studied, as only one antral follicle was found in the randomly selected ovarian cortical tissue strips. Additional inter-hormonal correlation analysis was conducted to study possible interactions. This analysis showed a significant positive and strong correlation between FSH and LH ($R_s = 0.762, P < 0.001$), between the total and the free fraction of testosterone ($R_s = 0.933, P < 0.001$) and a significantly moderate correlation between SHBG and the total testosterone fraction ($R_s = 0.429, P = 0.018$). The duration of testosterone treatment did not correlate with any hormone serum level.

**Cumulus-oocyte complexes and in-vitro maturation**

In total, 1313 COC were collected from the medulla suspensions of 35 patients. A median of 27 COC per patient were retrieved (minimum four COC and maximum 174 COC). These COC are most probably released from antral follicles by scraping the cortex on the dissection plane as described further.

After 48 h, IVM (34.30% [334/974]) of MII oocytes were observed ($n = 27$ patients) (Figure 2). MII oocytes ($n = 124$) obtained from 16 patients were fixed and stained for detailed spindle analysis. A normal spindle pattern was observed in 94.35% (117/124); being 71 (57.26%) bipolar spindles with pointed poles and 46 (37.10%) bipolar spindles with flattened poles in spindles. Abnormal shapes included three (2.42%) bipolar spindles with irregularities at poles or equatorial plate and four (3.23%) non-bipolar, mono- or multipolar spindles. A normal chromosome pattern was seen in 87.10% (108/124) of the spindle configurations, of which 82 (66.13%) were perfectly aligned and 26 (20.97%) chromosome patterns were mostly aligned with six chromosomes or less away of the equatorial plate. An abnormal (dispersed) chromosome pattern could be observed in 16 (12.90%) of the spindles. Considering a normal spindle pattern as being bipolar spindle structures with or without pointed poles and a chromosome alignment with

| Table 2 – Cortical count and percentage distribution of follicles. |
|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Follicle stage | Total | Primordial | Intermediate | Primary | Secondary | Antral |
| Mean | 90.34 | 61.90 | 18.30 | 9.70 | 0.41 | 0.02 |
| SD | 118.02 | 90.66 | 23.56 | 11.50 | 0.76 | 0.16 |
| Median | 59.25 | 39.50 | 9.75 | 5.25 | 0.00 | 0.00 |
| Minimum | 2.00 | 0.00 | 0.50 | 0.00 | 0.00 | 0.00 |
| Maximum | 614.50 | 501.50 | 101.50 | 45.00 | 4.00 | 1.00 |
| Sum | 3613.50 | 2476.00 | 732.00 | 388.00 | 16.50 | 1.00 |
| (68.52%) | (20.26%) | (10.74%) | (0.46%) | (0.03%) |

a The follicle classification is the mean of calculations performed by two independent observers after correction for the number of slices. Percentage (%) of total follicle count is included between brackets.

| Table 3 – Correlation between hormone serum levels and follicle classification. |
|-------------|-----------------|-----------------|-----------------|-----------------|
| | Total | Primordial | Intermediate | Primary | Secondary | Antral |
| AMH | −0.130 | 0.027 | −0.019 | 0.100 | 0.180 | NA |
| FSH | 0.140 | 0.112 | 0.062 | 0.138 | 0.148 | NA |
| LH | 0.088 | 0.117 | 0.030 | 0.095 | −0.087 | NA |
| Oestradiol | −0.071 | −0.084 | −0.053 | −0.054 | −0.044 | NA |
| Progesterone | −0.048 | −0.039 | −0.045 | 0.035 | −0.177 | NA |
| Testosterone | −0.085 | −0.065 | −0.049 | 0.103 | −0.256 | NA |
| Free testosterone | −0.067 | −0.081 | −0.014 | −0.067 | −0.163 | NA |
| SHBG | 0.160 | 0.214 | 0.105 | −0.010 | −0.193 | NA |
| Age | 0.028 | 0.004 | 0.091 | 0.089 | −0.097 | NA |
| Duration of testosterone treatment | 0.229 | 0.255 | 0.209 | 0.254 | 0.053 | NA |

a Spearman correlation coefficients. All investigated correlations were non-significant. $P < 0.05$ was considered to be statistically significant. AMH, anti-Müllerian Hormone; NA, the correlation with the number of antral follicles could not be studied as only one antral follicle was found; SHBG, sex hormone binding globulin.
none, or six chromosomes or less out of the equatorial plate, we calculated 87.10% (108/124) normal spindles (Figure 3).

Modelling of hormone serum levels and ovarian medulla observations

To determine correlations between the ovarian medulla observations and the hormone serum levels in trans men, a correlation analysis between the individual hormone serum levels and the total number of recovered COC and in-vitro matured MI was conducted. AMH serum levels correlated strongly with the number of COC ($R_s$ 0.787, $P < 0.001$), as well as with the number of in-vitro matured MI oocytes ($R_s$ 0.760, $P < 0.001$), MI oocytes ($R_s$ 0.768, $P < 0.001$) and germinal vesicle oocytes ($R_s$ 0.787, $P < 0.001$). Next, linear regression analysis showed an equation $y = 0.847 + 7.48 \times (R^2 0.714)$ with a non-significant intercept ($b_0 = 0.847; 95\%$ CI $-10.44$ to $12.19$) but a significant increase of the estimated mean number of COC with factor 7.48 per unit AMH ($\mu g/L$) ($P < 0.001, 95\%$ CI $5.73$ to $9.23$), as shown in Figure 4. The number of COC did not correlate with other hormone serum levels nor with duration of testosterone treatment. An overview is given in Table 4.

Histological analysis of the entire ovary

To locate the AMH-producing follicles, giving rise to the COC found in the medulla, whole ovary fixation was carried out. The organ was embedded in paraffin and stained with haematoxylin and eosin. Antral follicles were clearly located on the transition plane between cortex and medulla tissue (Figure 5). This borderline is actually the exact dissection plane during ovarian tissue cryopreservation procedures. Therefore, the more mature follicle stages are cut out of their location and found in the residual manipulation medium during processing. This observation clarifies the reason why only one antral follicle could be found in the cortical tissue slices.

Discussion

In the present study, ovarian histology in trans men at the moment of ovarian tissue cryopreservation was investigated after more than a year of testosterone treatment. The first part addressed the cortical follicle count. Most of the follicles were primordial, fewer were intermediate and primary follicles and very few were secondary follicles. A particular and specific weakness of this study is the inability to include an age-matched control group. In the described age group ($24.3 \pm 6.15$ years), oophorectomy in healthy normal fertile women rarely occurs, in some exceptions for a benign or malignant ovarian mass. On the basis of the study by Pavone et al. (2014), it is shown that this is not an appropriate control group as follicle density in the cortex surrounding an ovarian malignancy is decreased. In case of benign ovarian lesions, ovarian stroma may proliferate, resulting in the same number of follicles but spread over a larger surface area (Pavone et al., 2014). Also, preventive oophorectomy in patients carrying a BRCA mutation did not seem an appropriate control group as the mutation might be associated with reduced follicular pools (Pavone et al., 2014). To put our results in perspective, we compared our findings to ovarian histology of age-matched normal fertile
women in other published studies. Gougeon and Chainy (1987) described the ovaries of normal fertile women age 19–30 years. Their work showed a cortical distribution of 65.20% primordial follicles (compared with 68.52% in our study), 27.39% intermediate follicles (compared with 20.26%), 6.16% primary follicles (compared with 10.74%) and 1.26% secondary follicles (compared with 0.46%). On the basis of this careful comparison, it is noteworthy that the cortical follicle distribution apparently does not shift in ovaries exposed to supraphysiological doses of testosterone for about a year. Plotting these histological findings against clinical and biochemical markers showed no correlations with the cortical follicle numbers at the moment of fertility preservation. The heterogeneous cortical follicle distribution undoubtedly affects the interpretation of the correlation analyses described in this study and thus, additionally, our results cannot entirely address all potential effects of supraphysiological doses of testosterone. In ovarian physiology, the two-cells-two-hormones model describes the cooperation of theca cells and granulosa cells and the role of gonadotrophins in steroidogenesis. In theca cells, androstenedione and testosterone are produced in response to the LH stimulus. Following passive transport, the androgens are converted in oestrogens (oestrone and oestradiol respectively) by aromatase enzyme under the stimulus of FSH (Gervásio et al., 2014; Kim, 2013). Androgens are known to be enhancers of FSH-dependent follicular growth and development by inducing follicular FSH receptor expression (Caanen et al., 2015; Dumesic et al., 2015; Gervásio et al., 2014) and lower levels of androgens are likely to have anti-atretic effects (Dumesic et al., 2015). Importantly, the effects of androgens are mediated through the androgen receptor and not by conversion to oestrogens, as the administration of dihydrotestosterone (not convertible to oestrogens) results in the same ovarian effects (Gervásio et al., 2014). Apart from possible effects on the follicular unit, supraphysiological testosterone doses can also have a potential influence on the ovarian stroma (Ikeda et al., 2013). Additionally, high testosterone serum levels do not necessarily correlate with high levels of testosterone in the ovary. Therefore, our results cannot unravel the entire effect of testosterone on the ovary, however, it might contribute to fertility counselling concerning follicle presence at the moment of fertility preservation for trans men after a prolonged period of gender-affirming testosterone treatment.

One of the major findings of our work is the high number of COC (median of 27 per patient) recovered during the preparation of the tissue. As previously mentioned, the applied procedure is the current standard procedure of tissue preparation for ovarian cortex cryopreservation. This research indicates that, similar to the findings
in an oncologic patient cohort (Segers et al., 2015), the current procedure leads to discarding potentially important gametes. These high numbers of COC also explain the relatively high AMH serum levels (mean 4.66 ± 3.84 µg/L) in relation to the rather scarce AMH-producing follicle yield in the cortex. As known thus far, AMH is secreted by the granulosa cell, starting at the primary follicle stage. The granulosa cells of the primordial follicles, the larger antral and pre-ovulatory follicles do not contribute to the AMH production (Dewailly et al., 2014; Grynnerup et al., 2012; Weenen et al., 2004). The described follicle pool in the cortex could, therefore, not entirely explain the observed AMH-production. A limitation in our study is, however, the lack of baseline AMH serum levels in order to be able to study possible changes. Caanen et al. (2015) described a significant decrease in AMH serum levels after 8 weeks of daily transdermal testosterone gel, which is significantly shorter and lower dosed than the administration of more than a year of intramuscular testosterone (Caanen et al., 2015). Furthermore, in their population, the testosterone treatment was combined with a GnRH agonist and an aromatase inhibitor (Caanen et al., 2015). Although interesting, these methodological differences make it impossible to correctly compare results.

We have calculated an average estimated increase in the number of COC with about 7.5 per unit AMH in trans men. This is important information for patient counselling as it is possible to estimate an average yield based on AMH levels before the surgery. The number of collected COC in trans men, a mean of 37.51 ± 33.58 in our study, compared with a recovery rate of 8.1 COC in cancer patients (as reviewed by Segers et al., 2015) is remarkable. The high number of COC recovered in trans men could also be caused by the androgen-induced FSH and LH down regulation resulting in anovulation and subsequently the accumulation of antral follicles. It is important to state that the oocyte yield in our study population is the result of processing two ovaries in trans men, in contrast to one ovary or even biopsies in cancer patients. A maturation rate of 36% (Segers et al., 2015) is comparable to the in-vitro maturation rate of COC retrieved from the ovaries of trans men (34.30%). Apart from the normal spindle structure in 87.10% of the in-vitro matured MII oocytes, it is necessary to determine the developmental capacity of

**Figure 4** – Correlation between anti-Müllerian serum levels and cumulus-oocyte complexes. Linear regression analysis $y = 7.48x$ ($b_0 = 0.847$; non-significant; 95% CI $-10.44$ to $12.19$; $b_1 = 7.48$; $P < 0.001$; 95% CI $5.73$ to $9.23$; $R^2 = 0.714$). AMH, anti-Müllerian hormone.

**Figure 5** – Hematoxylin/eosin staining of an entire ovary (scale bar 2000 µm). Antral follicles are located on the boundary between cortex (*) and medulla (**).

<table>
<thead>
<tr>
<th>P-value</th>
<th>COC</th>
<th>MII</th>
<th>MI</th>
<th>GV</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>0.787*</td>
<td>0.760*</td>
<td>0.768*</td>
<td>0.734*</td>
<td>0.612*</td>
</tr>
<tr>
<td>FSH</td>
<td>$-0.271$</td>
<td>$-0.213$</td>
<td>$-0.256$</td>
<td>$-0.224$</td>
<td>$-0.213$</td>
</tr>
<tr>
<td>LH</td>
<td>0.088</td>
<td>$-0.103$</td>
<td>0.058</td>
<td>0.064</td>
<td>$-0.015$</td>
</tr>
<tr>
<td>Ostradiol</td>
<td>0.128</td>
<td>0.176</td>
<td>0.197</td>
<td>0.081</td>
<td>0.034</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.249</td>
<td>$-0.362$</td>
<td>0.210</td>
<td>0.126</td>
<td>0.173</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.263</td>
<td>$-0.342$</td>
<td>0.300</td>
<td>0.198</td>
<td>0.190</td>
</tr>
<tr>
<td>Free testosterone</td>
<td>0.331</td>
<td>$-0.131$</td>
<td>0.323</td>
<td>0.316</td>
<td>0.165</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.056</td>
<td>0.182</td>
<td>0.253</td>
<td>$-0.029$</td>
<td>0.017</td>
</tr>
<tr>
<td>Age</td>
<td>$-0.195$</td>
<td>$-0.05$</td>
<td>$-0.256$</td>
<td>$-0.160$</td>
<td>$-0.072$</td>
</tr>
<tr>
<td>Duration of testosterone treatment</td>
<td>$-0.215$</td>
<td>$-0.307$</td>
<td>$-0.239$</td>
<td>$-0.183$</td>
<td>$-0.369$</td>
</tr>
</tbody>
</table>

Spearman correlation coefficients.

* AMH serum levels correlated strongly with the number of COC, MII, MI, and GV oocytes ($P < 0.001$); statistically significant. $P < 0.05$ was considered to be statistically significant.

AMH, anti-Müllerian hormone; SHBG, sex hormone binding globulin; COC, cumulus-oocyte complex; MII, metaphase II; MI, metaphase I; GV, germinal vesicle; others, fragmented or degenerated complexes.
these in-vitro matured oocytes. Wissing et al. (2014) showed that embryos from hyper-androgenic women with polycystic ovary syndrome developed slower from fertilization to the seven-cell stage compared with embryos from healthy controls, so impaired embryo development of in-vitro matured oocytes derived from ovaries of trans men should be taken into account.

In conclusion, this study shows that cortical follicle distribution in trans men seems to be surprisingly normal after more than a year of testosterone treatment. This study confirms the presence and in-vitro maturation potential of COC obtained during the tissue processing of ovaries procured in trans men. An estimated increase of 7.5 COC in relation to a single unit AMH enables the prediction of COC yield in clinical practice based on AMH serum levels. The recovered COC show a maturation percentage of 34.30% and a normal spindle structure and chromosome alignment.

Acknowledgements

We are grateful to L Pieters for her help in the histological preparation of the samples, and to T Fiers for the additional information on the hormone serum level measurements in our Department of Clinical Biology. We would like to express our gratitude to the patients who donated their ovarian tissue for this scientific purpose. This research has been conducted through collaboration with the Bimera biobank, a high quality bio-repository for Ghent University Hospital and Ghent University.

REFERENCES


