



FACULTEIT GENEESKUNDE EN  
GEZONDHEIDSWETENSCHAPPEN

Academiejaar 2018 – 2019

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**Gertjan Vereecke**

Promotor : Prof. dr. T'Sjoen G.

Masterproef voorgedragen in de master in de specialistische geneeskunde  
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## Abstract

**Introduction:** Treatment of gender dysphoria may include hormone therapy to induce the development of desired secondary sex characteristics conform the gender identity and to diminish those of the birth-assigned sex. However, the effects on fertility and testicular function are less known. Several studies have been performed with various results regarding germ cell maturation and testicular function in this population. In order to give correct information, we find it important, as a referral center for transgender care, to compare results of earlier studies with our population.

**Material & Methods:** Testicular tissue retrieved during gonadectomy from 97 participants was processed and stained for four different markers. Immunohistochemical staining was first performed for Melanoma-Associated antigen A4 (MAGE-A4), which is expressed by spermatogonia. The second marker used was Boll, expressed in secondary spermatocytes and round spermatids. The third marker, cAMP Responsive Element Modulator (CREM) , is a marker for round spermatids. Finally Acrosin, marker for acrosome visualization in round elongating and elongated spermatids, was the last marker used. After the MAGE-A4 staining, the number of spermatogonia and primary spermatocytes were counted per square millimeter. Controls with normal spermatogenesis were used for comparison. Hormone levels were measured prior to surgery, using immunoassays.

**Results:** 92 % (89/97) of the participants had an adequately suppressed testosterone level (< 50ng/dl) prior to surgery and the mean time on hormone therapy prior to surgery was 685 days. In 88% (85/97) of the sections the staining for spermatogonia (MAGE-A4) was positive. Further immunohistochemical staining could not reveal complete spermatogenesis in any of the participants. There was a positive correlation between serum testosterone value and number of spermatogonia counted per mm<sup>2</sup>. Participants with higher serum testosterone values were more likely to have a later arrest.

**Conclusion:** In conclusion, we showed that adequate hormonal treatment leads to complete suppression of spermatogenesis in transgender women.. We believe that this different outcome can be explained by the adequately suppressed testosterone levels in our population, which lacked in the earlier studies. Because of the induced infertility, it is very important to discuss sperm preservation before the start of hormone therapy.

## Introduction

Treatment of gender dysphoria may include hormone therapy and a positive effect on the quality of life of those desiring medical treatment has been reported (Fisher et al.,2016). The goals of hormonal treatment are to induce the development of the secondary sex characteristics of the identified sex and to diminish those of the natal sex (Gorin-Lazard et al.,2012). Gender affirming surgery may be another step in the transition and has been shown to be beneficial for psychological, social and sexual health (Da Silva et al.,2016).

The prevalence of transgender women attending clinical services was estimated at 6.8/100 000 and 2.6/100 000 for transgender males in a meta-analysis (Arcelus et al.,2015). Over the last decade increasing attention has been given to transgender care and rights. The effects of hormonal therapy on physical, psychological and mental health are being studied (T'Sjoen et al.,2018) and recent guidelines from the Endocrine Society have been published (Hembree et al.,2017).

However, the effects on fertility and testicular function in trans women are less known. In the past a number of papers have been published in which testicular morphology was studied in a limited number of patients, who received mainly estrogen therapy. The largest study included 11 patients and the microscopic appearance of testicular tissue was quite uniform. Narrowed seminiferous tubules, which contained Sertoli cells and spermatogonia (type A) exclusively have been reported. There were no typical Leydig cells (Schulz et al., 1988). In 2018 these histological findings were confirmed in 50 patients undergoing gender affirming surgery, although little was known about previous hormonal therapy. In contrast, in this study it was reported that in 50 % of these patients Leydig cells were seen and spermatozoa in up to 20% of participants (Matoso et al.,2018) Recently semen parameters among seven transgender women who were under hormone therapy were analysed, showing heterogeneous results varying from normal sperm quality to azoospermia. Hormone levels were lacking and hormone regimens were again not standardized (Adeleye et al., 2018).

A much larger recent multicenter study from 108 transgender women, treated with different hormone regimens from three different centers is available. The authors reported that 24% of these patients presented with qualitatively normal spermatogenesis and variable hormonal status on the day of gender affirming surgery (Schneider et al., 2015). To our knowledge no

other major studies have been performed, investigating the influence of cross-sex hormone therapy on germ cell characterisation in transgender women.

According to the Endocrine Society Clinical Practice Guidelines it is recommended that clinicians inform and counsel all individuals seeking gender-affirming medical treatment regarding options for fertility preservation prior to treating with hormonal therapy of the affirmed gender in both adolescents and adults (Hembree et al.,2017). In order to give correct information we found it important as a referral center for people with gender dysphoria, to evaluate the findings mentioned above with our data from the ENIGI cohort (Dekker et al., 2016).

## Materials and Methods

This research is part of the endocrine part of “European Network for the Investigation of Gender Incongruence” (ENIGI) protocol, a collaboration of four major West European gender identity clinics (Amsterdam, Ghent, Florence and Oslo), a study group created to obtain more transparency in diagnostics and treatment of gender dysphoria. This substudy on testicular tissue was restricted to the Ghent cohort.

From February 15, 2010 to September 30, 2015, 162 trans women were included in the ENIGI study at the Ghent University Hospital in Belgium of which 124 underwent genital surgery before June 2018, of which 107 in the Ghent University Hospital. All patients included in the ENIGI study were at least 16 years old and underwent a standardized diagnostic procedure before initiating treatment (Dekker et al., 2016). Patients were included in the ENIGI endocrine protocol when they started hormonal treatment for gender incongruence. Every patient was treated in accordance with the WPATH Standards of Care, Version 7 (Davies et al., 2015). Exclusion criteria were previous use of gender affirming hormones and insufficient knowledge of the native languages (Dutch or French). At the start of the study, patients received oral and written information about the ENIGI endocrine protocol by the physician. Written informed consent was obtained according to the Ethics Committee of the Ghent University Hospital, which approved all aspects of the study.

To compare hormone levels with histologic findings we chose to include only those of whom we could present hormone levels prior to bilateral orchiectomy and who were in follow up until sex reassignment surgery. This resulted in 97 participants (Table 1). The detailed study protocol of the endocrine part of the ENIGI study can be found in Dekker et al., 2016. The short-term follow-up currently consists of a visit at baseline and after 3, 6, 9, 12, 18, 24, and 36 months of gender affirming hormone therapy.

In trans women, gender-affirming hormonal treatment consists of cyproterone acetate (CPA) (Androcur®; Bayer, Diegem, Belgium) 50 mg once daily, combined with an oral estradiol agent, estradiol valerate (Progynova®; Bayer) 2 mg twice daily. In patients older than 45 years, estradiol is administered transdermally in the form of estradiol patches (Dermestril®; Besins, Brussels, Belgium) in a dose of 50–100 µg/24 h, to avoid increased thrombotic risk associated with the administration of oral estrogens caused by the hepatic first-pass effect (Dekker et al., 2016). In the case of non-tolerance, 2 mg of transdermal 17-β E2 gel twice daily (Oestrogel®;

Besins) is given. Two weeks prior to surgery hormonal treatment (estrogens and CPA) is put on hold to reduce the perioperative thrombotic risk. After orchiectomy, estrogens only are continued in an unchanged dose.

Three controls having normal spermatogenesis were used for comparison. These are patients who underwent a re-anastomose after vasectomy. Testicular tissue was obtained at the time of re-anastomose.

## Immunohistochemistry

All participants (table 1) underwent orchiectomy in our center. Biopsies were taken by the surgeon. The testicular specimens underwent fixation in formalin and were embedded in paraffin. The samples were sectioned at 1-1.5 $\mu$ m thickness and transported to the research group Biology of The Testes (BITE) from the department Reproduction Genetics and Regenerative medicine at the Vrije Universiteit Brussel (VUB).

Immunohistochemical staining of the sections was performed using four different markers to identify the level of differentiation within the seminiferous tubules. The first marker, Melanoma-Associated antigen A4 (MAGE-A4), is expressed by spermatogonia and by the primary spermatocytes (Aubry et al.,2001). The second marker which was used is Boll, an RNA-binding protein, expressed in secondary spermatocytes and round spermatid. The third marker, cAMP Responsive Element Modulator (CREM) , is a marker for round spermatids. Finally Acrosin (ACR), marker for acrosome visualization in round, elongating and elongated spermatids was the last marker used (Human Protein Atlas, 2018 and Cell Signaling Technology, 2018).

If the sections of a participant were positive for MAGE-A4, staining with Boll was performed on the next section of this participant, and this was continued with markers for CREM and ACR until full characterisation of differentiation was achieved. If the staining was negative for a marker, no subsequent staining on sections of these patients was executed.

The sections were first deparaffinized in xylene and were subsequently rehydrated in decreasing ethanol. After washing with Phosphate Buffered Saline (PBS) for 5 min, endogenous peroxidase was blocked with a 0.3% peroxide solution in methanol for 30 minutes. After washing in PBS for 5 min, a heat induced antigen retrieval was performed by incubating the sections (slides) in a retrieval solution (citric acid buffer pH 6,0). A water-bath (95°C) for 75



min was used for MAGE-A4 and Boll, a water-bath (95°C) for 30 min was used for ACR and a pressure boiler (121°C) for 15 min was used for CREM. After cooling down and washing, a blocking step was performed by using 4% Normal Goat Serum (NGS; b104; Rockland) for MAGE-A4/Boll/ACR or CAS block reagent for CREM. After the blocking step, primary antibodies were applied to the sections (MAGE-A4 (1/200; Gift from Dr. Spagnoli), Boll (1/400; H0006637; Novusbio), CREM (1/2000; hpa001818; Merck), or ACR antibody (1/500; sc 67151; Santa Cruz biotechnology). The sections were incubated in a humidified chamber overnight at 4°C. No primary antibody was applied to the negative control (PBS instead of primary antibody).

The next day after three washing steps, sections were incubated with a peroxidase labeled secondary antibody (Dako Real Envision Detection System; k5007; Dako) at room temperature for 1 hour. After washing, visualization was performed by using 3,3'-diaminobenzidine 'DAB' (Dako Real envision Detection System; k5007; Dako). After counterstaining with haematoxylin, sections were subsequently dehydrated in increasing ethanol series and mounted for microscopic evaluation.

## Microscopic analysis

The stained sections (transgender and controls) were analyzed by using an inverted light microscope (Olympus IX80). Pictures of the sections were taken with a computer program 'CELL F' (Imaging software from Life Science Technology). Five representative pictures of the MAGE-A4 stained sections were taken and used to perform counting. Other representative pictures of the sections (random numbers) for the controls, Boll, CREM and ACROSIN were taken as evidence. The expression pattern of the samples (n=97) were compared with the expression pattern of the control group. A scoring system from zero to five for MAGE-A4 was used as mentioned above. MAGE-A4 counting was performed by using the program Fiji. All positive cells within 5 microscopic fields at 10x magnification were considered for analysis. The final result per section was expressed in the number of MAGE-A4 positive cells per square millimeter. Sections were scored as (0) if expression was absent, as (1) if less than 50 cells per mm<sup>2</sup> were expressed, as (2) between 50 and 200 cells per mm<sup>2</sup>, as (3) between 200 and 350 cells per mm<sup>2</sup>, as (4) between 350 and 500 cells per mm<sup>2</sup> and as (5) if more than 500 cells per mm<sup>2</sup> were counted. A scoring system from zero to one for Boll, CREM and ACROSIN was used. Sections were scored as (0) if expression was absent and as (1) if expression was present.

## Laboratory analysis

Competitive chemiluminescent immunoassays were run for oestradiol (E170 Modular, Roche, Gen III, LOQ 25 pg/mL, interassay CV 3.2%), serum testosterone (E170 Modular, Roche, Gen II, LOQ 10 ng/dL (0.4 nmol/L), interassay CV 2.6%), LH (E170 Modular, Roche, Gen III, interassay CV 3.48%, LOQ 0.1 mIU/mL), and FSH (E170 Modular, Roche, Gen III, interassay CV 3.3%, LOQ 0.1 mIU/mL), and for SHBG, a sandwich-type chemiluminescent immunoassay was employed (E170 Modular, Roche, Gen III, interassay CV 4.06%, LOQ 0.35 mIU/mL). Before March 19, 2015, estradiol was measured using an E170 Modular (Gen II; Roche Diagnostics, Mannheim, Germany). For conversion of oestradiol values measured before March 19, 2015, the formula  $\text{Gen III} = 6.687940 + 0.834495 * \text{Gen II}$  was used (E170 Modular; Roche Diagnostics, Mannheim, Germany).

## Statistical analysis

Data were analyzed using IBM SPSS 25.0 (SPSS, Chicago, IL, USA). Data were verified for normal distribution using the Shapiro–Wilk test. Normal data are shown as mean  $\pm$  standard deviation (SD), not normally distributed values as median [percentile 25 – percentile 75]. For normally distributed values, correlations were tested using Pearson's R, not normally distributed values using Spearman's Rho. Not normally distributed variables were compared among groups using Mann-Whitney U test. Differences in frequencies between groups were compared using Chi Square statistic. In case of low cell count, differences in frequencies were compared using Fisher's Exact Test.

## Results

The average age of our population at the time of surgery was 31.2 years. 71% of the participants were treated with an oral estrogen while 29% were treated with a transdermal estrogen. All participants used cyproterone acetate, as an anti-androgen. The mean time from start with hormonal treatment to surgery was 644 days and in 92% (89/97) of the participants the serum testosterone level prior to surgery was adequately suppressed ( $< 50\text{ng/dl}$ ) with median value of 17,92 [10,40-25,35] ng/dl. The median serum testosterone level was 17,92 [10,40-25,35] ng/dl. Only 8 % ( 8/97) of the participants in our study had a testosterone value above 50ng/dl and only one participant's testosterone level was within normal male range (328ng/dl). The median LH and FSH were respectively 0,1 [0,1-0,1] U/L and 0,19 [0,12-0,43] U/L. Oestradiol, prolactin and SHBG values were to be found in the normal female range. The median serum testosterone level prior to initiation of hormonal therapy was 513.8 [413.7 – 640.9] ng/dl (Table 1).

## Immunostaining

As reported above MAGE-A4 staining and cell counting were performed on specimens of all the participants. Only on the positive samples further staining with Boll was performed and this was continued with markers for CREM and Acrosin until full characterisation of differentiation was achieved. If the staining was negative for a marker, no subsequent staining on sections of these patients was executed (Fig 1).

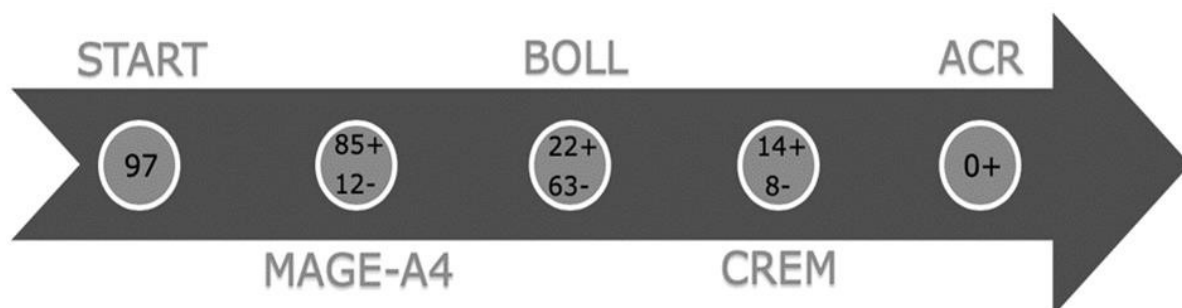


Figure 1: Schematic overview of the results from the immunostaining.

In 77,3% (75/97) of the participants no spermatogenesis was observed. In 12,4% (12/97) Sertoli cell only image was seen. In the other 63 transgender women where no spermatogenesis was seen, spermatogonia were observed but in much smaller numbers than the control group. The mean MAGE-A4 count in the control group was 595 positive cells per mm<sup>2</sup> whereas in our cohort of transgender women the mean count is 122 cells/mm<sup>2</sup>.

<b>Table 1: Patient characteristics at time of surgery</b>		<b>n</b>
Age at surgery (years)	31,19 [23,25 - 45,78]	97
Weight (kg)	74,00 [68-81,50 ]	97
Height (cm)	177,93 ± 6,88	97
BMI (kg/m <sup>2</sup> )	23,39 [21,69-25,91]	97
Current smoking	16,5% (16/97)	
Mean pack year	10,1	
Time last visit to surgery (days)	90 [57-153]	
Time to surgery (days)	644 [452-853]	97
Serum testosterone (ng/dl) prior to hormonal therapy	513.8 [413.7 - 640.9]	95
Serum prolactin( µg/L)	18,70 [13,70-27,75]	93
Serum LH (U/L)	0,1 [0,1-0,1]	97
Serum FSH (U/L)	0,19 [0,12-0,43]	97
Serum oestradiol (ng/dl)	65,50 [45,64-94,92]	97
Serum testosterone ng/d)	17,92 [10,40-25,35]	97
SHBG (nmol/L)	47,00 [30,70-68,70]	95
Serum free testosterone (ng/dl)	0,33 [0,21-0,50]	65
Type of estrogen therapy		
Oral	71% (69/97)	
Transdermal	29% (28/97)	
Type of anti-androgen therapy		
CPA 50mg daily	100% (97/97)	

Tests for normality were performed by Shapiro-Wilk. For normally distributed values, mean values ± standard deviations are shown. For values that are not normally distributed, median values and IQR [ P25 and P75].

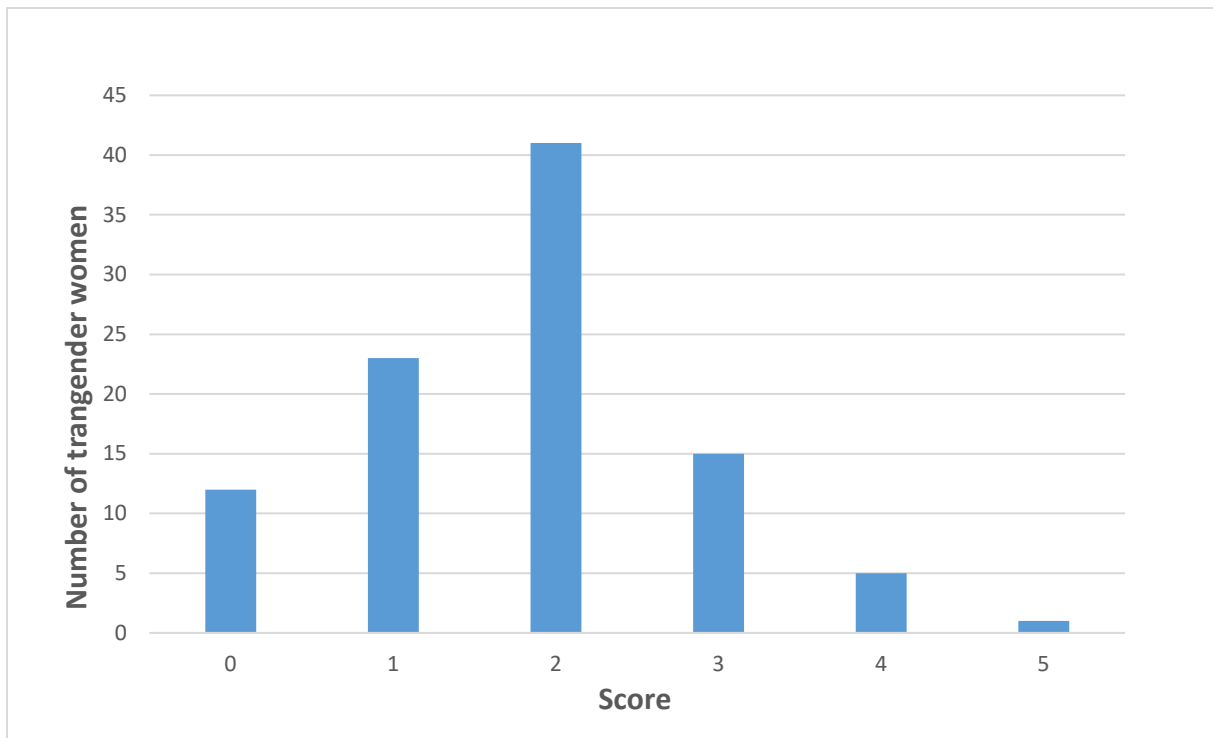
In 22,7% (22/97) immunostaining reveals partial spermatogenesis. 8,2 % (8/97) show early maturation arrest at the stage of secondary spermatocytes. In 14,4% (14/97) there is a late maturation arrest at the level of the round spermatids. No further maturation beyond round spermatids was seen in any of our specimens (Table 2)

**Table 2:** Summary of characterisation of spermatogenesis

	n	%
No spermatogenesis	75 /97	77,3%
Sertoli cell only	12/97	12,4%
Spermatogonia only	63/97	65,0%
Partial spermatogenesis	22/97	22,7%
Early maturation arrest	8/97	8,2%
Late maturation arrest	14/97	14,4%
Complete spermatogenesis	0	0%

## MAGE-A4

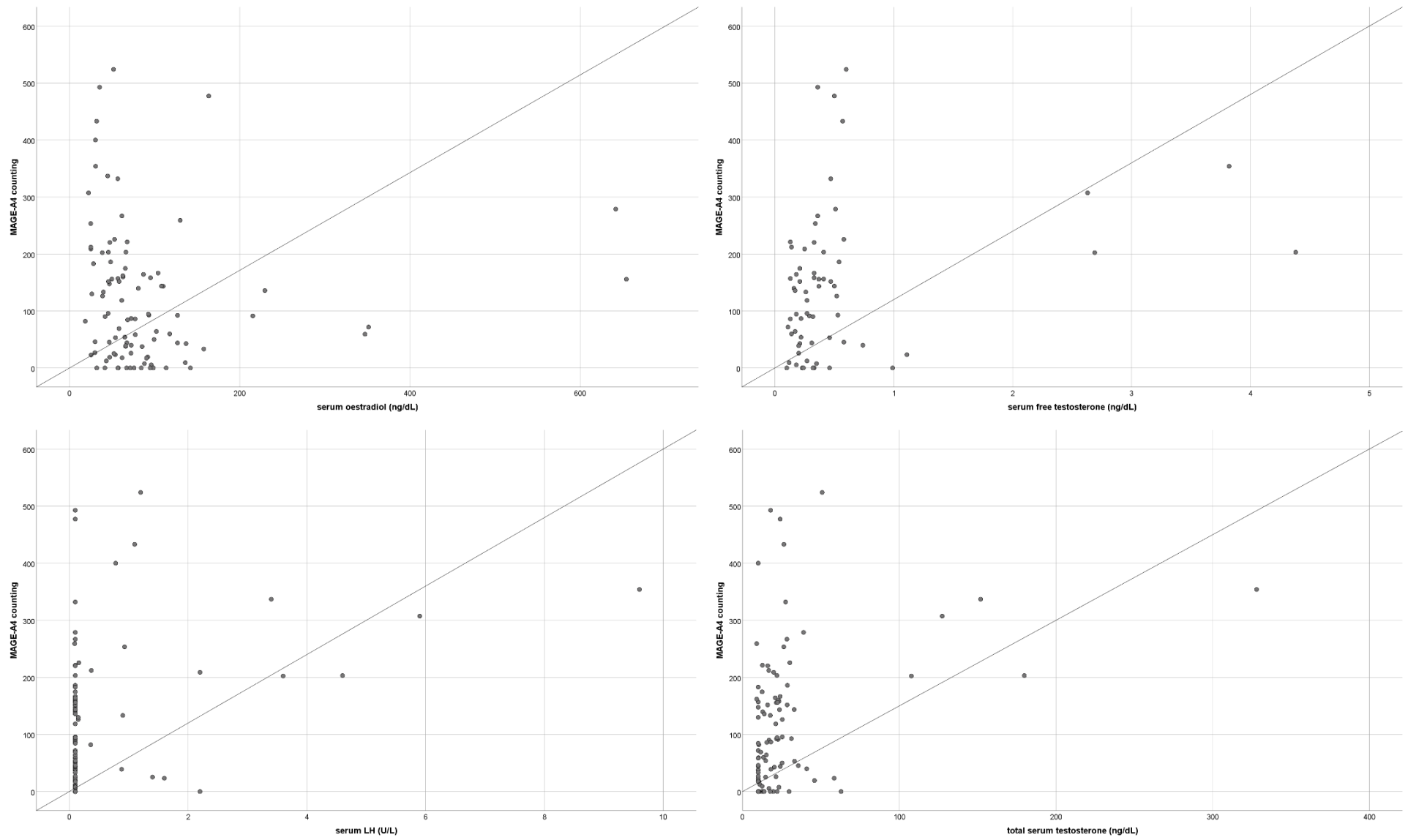
In 87,6% (85/97) of the sections the staining for spermatogonia with MAGE-A4 was positive. In 27.1% (23/85) scored (1), in 48.2% (41/85) scored (2), in 17.6% (15/85) scored (3), in 5.9% (5/85) scored (4) and 1.2% (1/85) sections scored (5). In 12,4% (12/97) no spermatogonia were visualized in the specimens. ( Score (0) is absent expression, score (1) less than 50 cells per mm<sup>2</sup>, score (2) between 50 and 200 cells per mm<sup>2</sup>, score (3) between 200 and 350 cells per mm<sup>2</sup>, score (4) between 350 and 500 cells per mm<sup>2</sup> and score (5) more than 500 cells per mm<sup>2</sup>.) (Figure 2 & 4). In the control group staining was strongly positive with a mean counting of 595 positive cells per mm<sup>2</sup> of score (5).



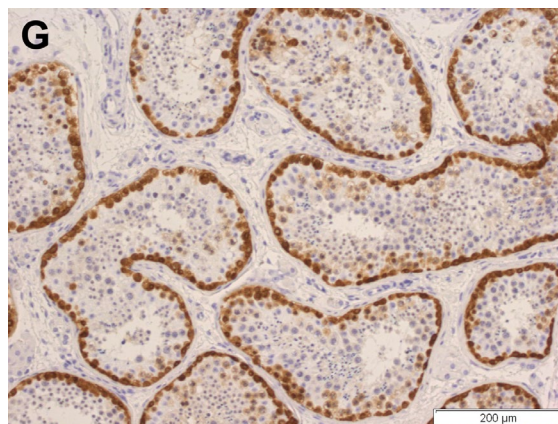
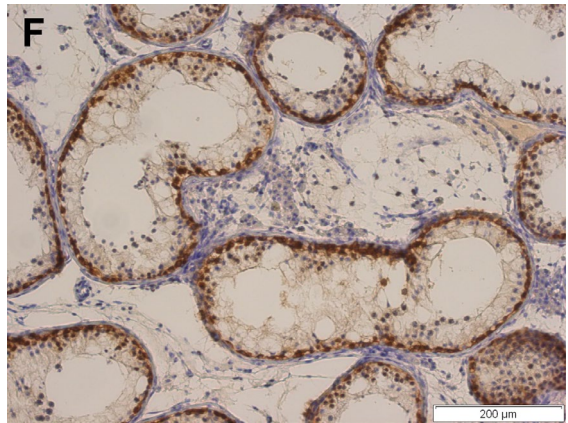
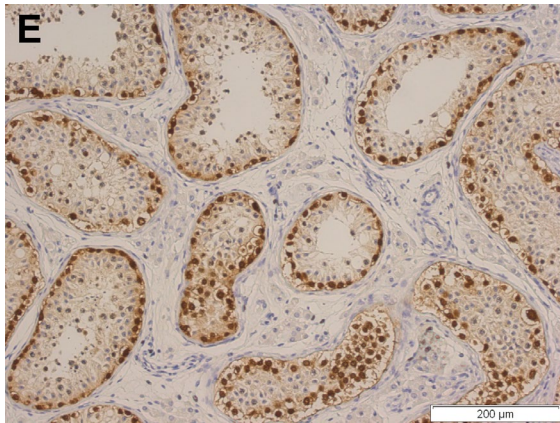
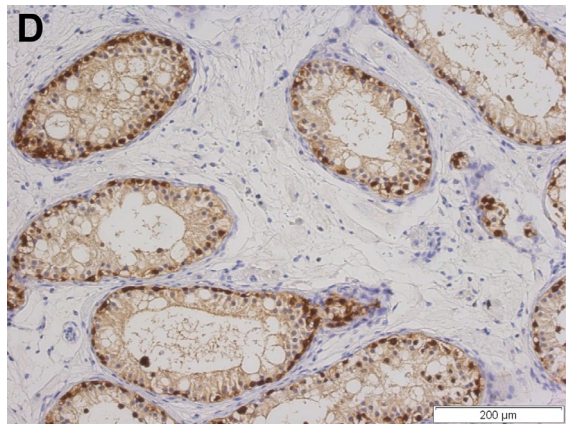
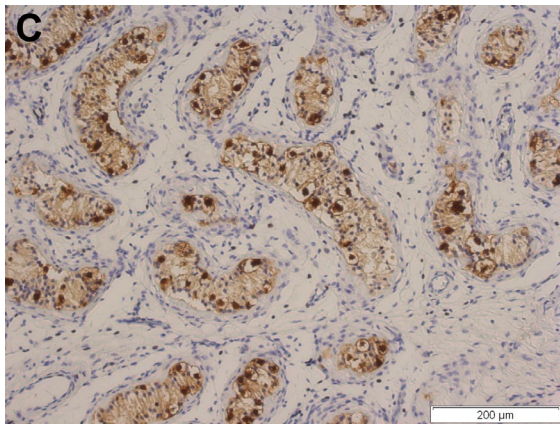
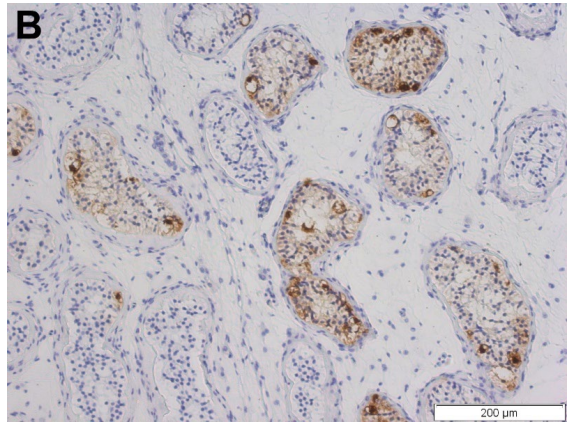
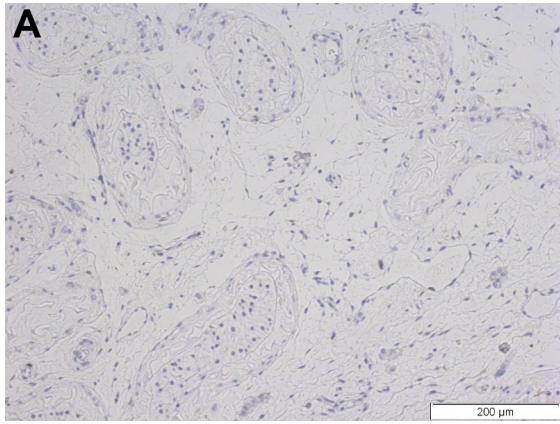
**Figure 2: Scoring of MAGE-A4 positive cell counting in transgender women.** Number of transgender women of whom the specimens were scored from 0 to 5.

There was a positive correlation between MAGE-A4 count and serum total testosterone ( $\rho = 0.320$ ,  $P = 0.001$ ), serum free testosterone ( $\rho = 0.314$ ,  $P = 0.007$ ) and LH ( $\rho = 0.296$ ,  $P = 0.003$ ). There was a negative correlation between MAGE-A4 count and serum oestradiol ( $\rho = -0.252$ ,  $P = 0.013$ ) and also between MAGE-A4 count and age at the time of surgery ( $\rho = -0.314$ ,  $P = 0.002$ ) (Figure 3).

When correlating MAGE-A4 count with patient characteristics, we found no correlation for BMI ( $P = 0.885$ ), serum prolactin ( $P = 0.582$ ), serum FSH ( $P = 0.99$ ), serum SHBG ( $P = 0.143$ ) and pack years ( $P = 0.445$ ).



**Figure 4 :** A-C show correlations between MAGE-A4 counting and serum oestradiol (ng/L), serum free testosterone (ng/dl), serum LH (U/L) and total serum testosterone (ng/dl), respectively.

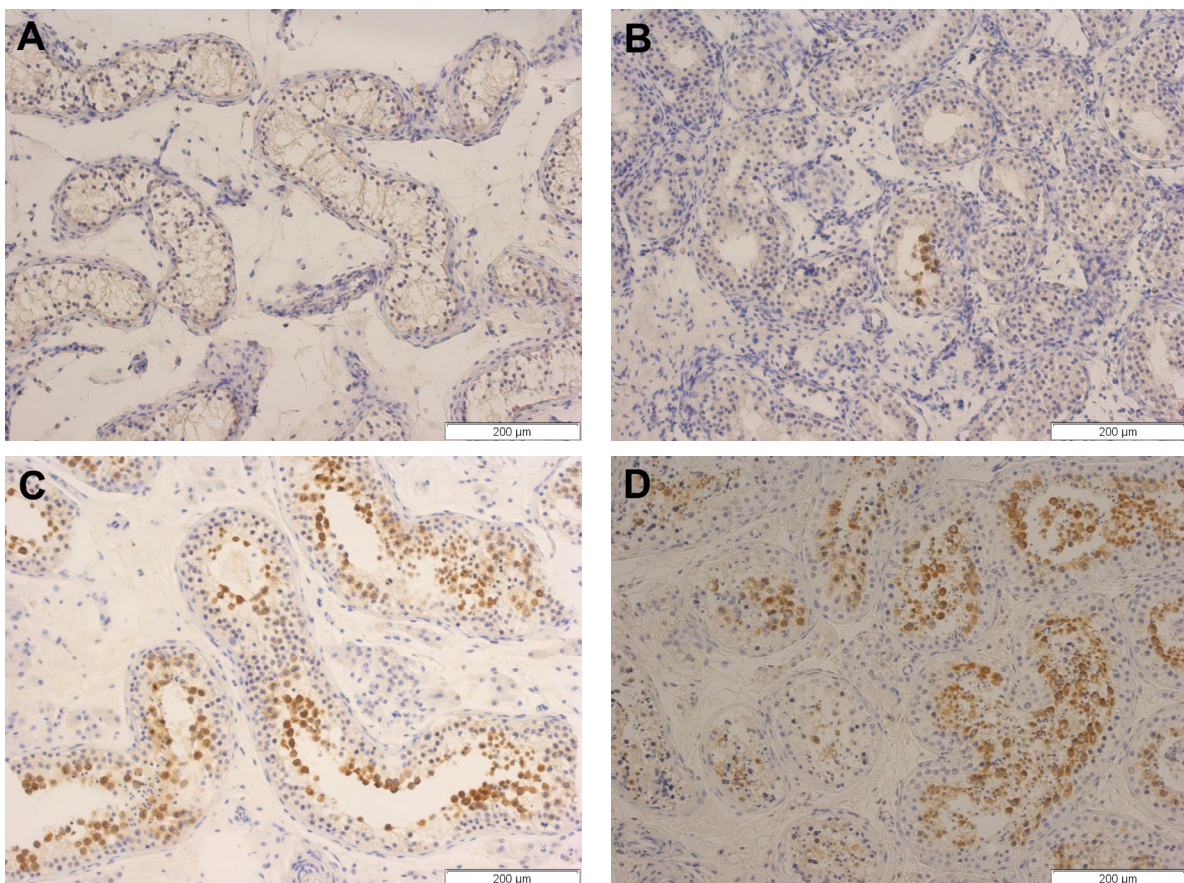




**Figure 5: Expression of the MAGE-A4 marker in testicular tissue from 6 different transgender women (A-F) and one control man in normal tissue, obtained at 10x magnification (light microscope). Nuclear staining highlights spermatogonia. Note the narrowed seminiferous tubules and increased intertubular space in A-F. (A) Negative staining (score 0), (B) positive staining counting 23 positive cells/mm<sup>2</sup> (score 1), (C) positive staining counting 140 positive cells/mm<sup>2</sup> (score 2), (D) positive staining counting 307 positive cells/mm<sup>2</sup> (score 3), (E) positive staining counting 433 positive cells/mm<sup>2</sup> (score 4), (F) positive staining counting 524 positive cells/mm<sup>2</sup> (score 5), (G) positive control counting 651 positive cells/mm<sup>2</sup>.**

## Boll

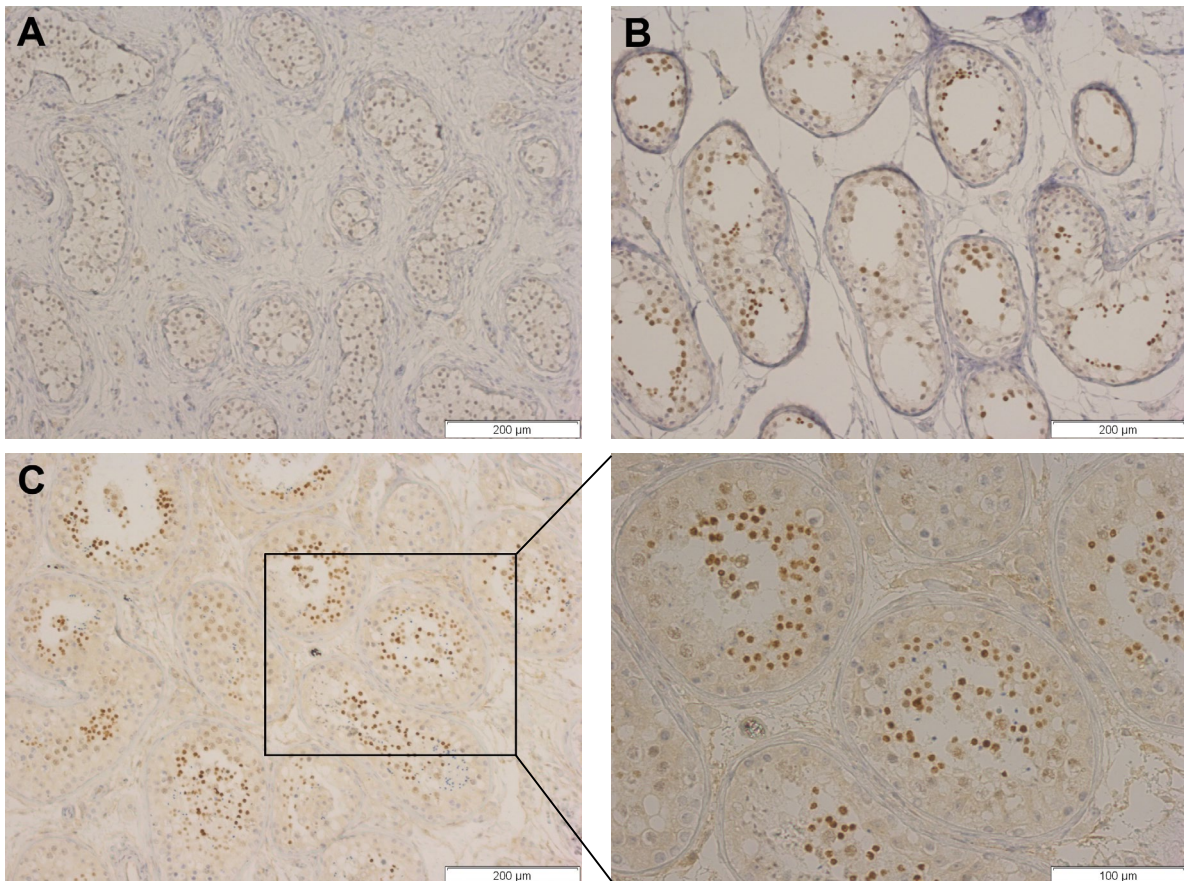
When stained for Boll, a marker for secondary spermatocytes (meiotic cells), 25,9% (22/85) of the 85 MAGE-A4 positive sections were positive. 54.5% (12/22) were scored as (1) and 45.5% (10/22) as (2) (Figure 6).



**Figure 6: Expression of the Boll marker in testicular tissue from 3 different transgender women (A-C) and one control man in normal tissue, obtained at 10x magnification (light microscope). Staining highlights secondary spermatocytes. (A) Negative staining, (B) slight positive staining (score 1), (C) positive staining (score 2), (D) positive control.**

## CREM

When stained for CREM, a marker for round spermatids, 63,6 % (14/22) of the Boll positive sections were positive. All Boll positive sections which were scored as (2) were positive when stained for CREM (Figure 7).



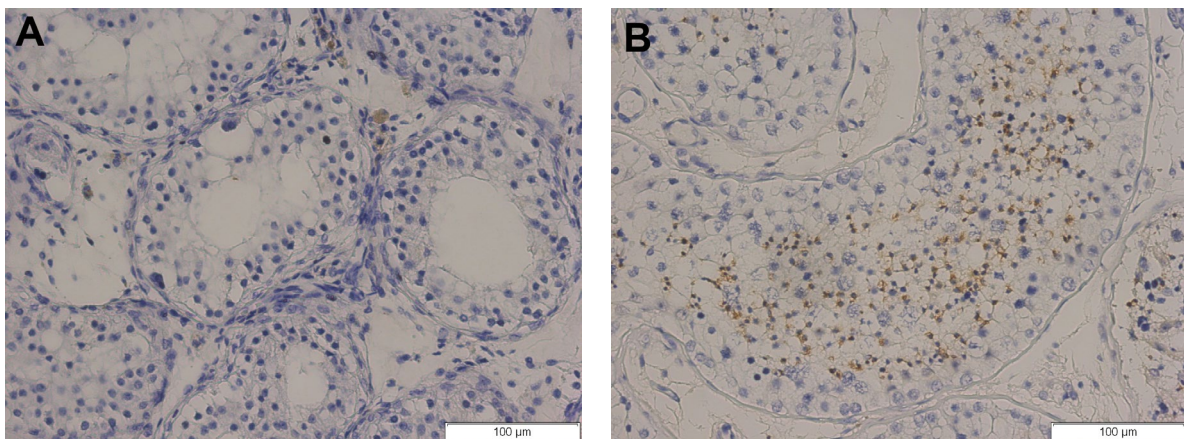
**Figure 7: Expression of the Boll marker in testicular tissue from 2 different transgender women (A-B) and one control man in normal tissue (C), obtained at 10x and 20x (light microscope). (A) Negative staining, (B) positive staining, (C) positive control with 20X magnification.**

We noticed that participants with a serum testosterone above 50 ng/dl are more likely to reach maturation up to round spermatids ( $P < 0.001$ ). The same observation was made for specimens of participants who were younger than 30 years at the time of gender affirming surgery ( $P = 0.022$ ).

There is a significant difference in serum testosterone ( $P= 0.007$ ) when comparing the CREM positive group (27.43 [15.06-133.53] ng/dl) with the CREM negative group (16.89 [10.36-23.32] ng/dl). This difference is also present for serum free testosterone ( $P = 0.001$ ) (0.59 [0.44-2.97] ng/dl vs. 0.29 [0.20-0.41] ng/dl). In addition we observed a difference in age at the time of surgery ( $P = 0,042$ ) when comparing the CREM positive group (24.59 [20.37-30.80] years ) with the CREM negative group (32,56 [23.32-47.37] years).

## ACROSIN

None of the CREM positive sections stained positive for Acrosin, marker of acrosome visualization in elongating and elongated spermatids (Figure 8).



**Figure 8: Expression of the Acrosin marker in testicular tissue from one transgender women and one control man in normal tissue, obtained at 20x magnification (light microscope). (A) Negative staining, (B) positive control.**

## Discussion

We found that long term hormonal treatment leads to absence of complete spermatogenesis in all transgender women with documented low testosterone values.

The results presented here are in conflict with earlier observations. In recent data (Schneider et al., 2015) up to 24% of transgender women presented with normal spermatogenesis on the day of gender affirming surgery. In this German cohort of 108 transgender women another 24% presented with meiotic arrest. As mentioned above, in our study we observed no complete spermatogenesis in any of the participants and only 8,2% presented with meiotic arrest. Few other studies can be found in which the influence of hormone therapy on testicular morphology in transgender women has been examined. These studies show heterogeneous results, are performed with limited number of participants, lack hormonal values and patients are mainly treated with estrogens only as reported in a recent review (Schneider et al.,2017).

In a study with more participants (Matoso et al.,2018) histologic findings in orchiectomy specimens of 50 transgender women were evaluated, but neither hormone therapy regimens nor serum hormone levels were available. Regarding spermatogenesis the authors in this study describe in 80% a maturation arrest at the level of the spermatogonia but in 20% focal mature spermatozoa were present.

We hypothesise that the large difference in serum testosterone levels between our study and the German are the explanation of these differences. In this German cohort the mean testosterone value was 195ng/dl (6,76nmol/L which is above the upper target value of 50ng/dl, stated by the Endocrine Society in their recent guidelines (Humbree et al.,2017). In our study 92% of the participants had a serum testosterone level below this threshold.

The mean estradiol level in the German study was 56,7 ng/L (208,1 pmol/L) which is comparable to the estradiol levels in the current study and within normal premenopausal female range (table 1). It has to be stressed that in the German cohort 22 of the 108 participants had already interrupted their hormonal treatment for 6 weeks, and 51 of them for 2 weeks before the hormonal analysis. Only 35 were with active hormonal treatment at the time of the hormonal analysis. It is suggested that because of early cessation there is a virilised hormonal status on the day of surgery. Although, the results of the hormonal analysis are not completely comparable, it cannot be the explanation for complete spermatogenesis in so many participants

since the duration of spermatogenesis is estimated between 68 and 74 days (Amann, 2008). Likewise male contraception studies demonstrate that hormone therapy, by down-regulating the release of gonadotropins, can adequately induce sterility but it can take months to recover after cessation of these hormonal regimens (Liu et al. 2006).

Another possible explanation is the difference in type and dosage of the anti-androgen used. In our center all patients are treated equally with 50mg cyproterone acetate once daily, which is an androgen receptor antagonist. CPA reduces serum testosterone levels by acting as a direct antagonist on the peripheral androgen receptor and by its progestational and weak glucocorticoid activity that inhibits luteinizing hormone (LH) release (Defreyne et al., 2017). In the study of Schneider et al. not everyone was on anti-androgen treatment. Referring to this study (Schneider et al., 2015) only 59 out of 108 subjects took CPA but in varying doses (10-100mg daily) combined with different doses of estrogens. Thirteen were on estrogens only and one participant took spironolactone in combination with estrogens. In 32% (35/108) there seems to be no information whatsoever about the hormonal treatment.

Altogether it seems that a lack of adequate hormonal treatment with inadequately suppressed serum testosterone levels is the main reason why, in other studies, spermatogenesis is not suppressed. Therefore it is important for clinicians to evaluate serum estradiol and serum testosterone and maintain them at the level for premenopausal females (Hembree et al., 2017).

In our study we see no maturation beyond meiosis and this is not surprising. From studies concerning testosterone signaling and regulation of spermatogenesis we learn that withdrawal of testosterone or knock out of the androgen receptor (AR) in Sertoli cells results in three major impairments to fertility. First, the integrity of the blood testis barrier (BTB) is compromised, which exposes post meiotic germ cells to autoimmune attack and cytotoxic factors. Second, there is a block in conversion of round spermatids to elongated spermatids due to a defect in cell adhesion that causes the premature detachment of round spermatids from Sertoli cells. Third, fully mature spermatozoa cannot be released from Sertoli cells and the germ cells are phagocytized by the Sertoli cells (Walker., 2011).

Nevertheless in our study serum testosterone and serum LH are not completely suppressed and this is probably the reason why there is still initiation of spermatogenesis. In our study population we noticed that with higher serum testosterone levels there are a significantly higher number of spermatogonia, measured by MAGE-A4 positive cell count. It seems that these hormonal levels are not high enough to overcome meiotic arrest.

Another observation is the higher the oestradiol levels are the lower the number of spermatogonia are. It is indeed known that oestradiol impairs spermatogenesis in transgender women ( Sapino et al., 1987 ) and we demonstrate in our study that this effect is significantly correlated with serum oestradiol levels.

The participants with the highest serum testosterone level do not only have the highest number of spermatogonia, expressed by MAGE-A4 positive cell count, but are more likely to have a later arrest in spermatogenesis compared with participants with a lower serum testosterone. In 75% (6/8) of the participants who have a testosterone value above 50ng/dl we see maturation until round spermatids and in only 9% (8/89) of the participants with a testosterone value beneath 50ng/dl round spermatids were visualized (Figure 9).

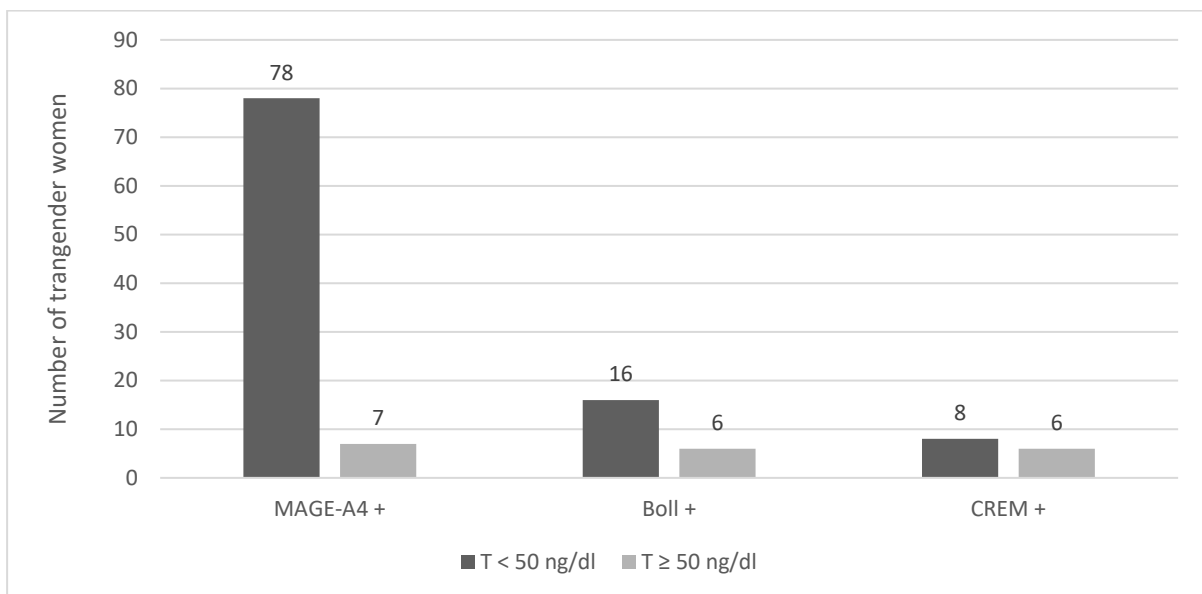


Fig 9: Positive staining for MAGE-A4, Boll and CREM in testicular tissue from transgender women in relation to the 50ng/dl threshold. T = Testosterone

Another finding was that age is as also negatively correlated with the MAGE-A4 count. We know indeed there are age-related changes in spermatogenesis with, among other, decreased number of germinal cells. This reflects in a consistent age-dependent decline in semen quality. Some studies have reported age thresholds for the onset of decline in sperm quality, with the earliest decline starting from the age of 30 to 35 year. (Kaufman et al.,2019).

In the current study we found no correlation between MAGE-A4 count and smoking, although tobacco smoking may influence the level of reproductive hormones and negatively influence

the number of germ cells (Dai et al., 2015). Neither was there a correlation between BMI and MAGE-A4 count. In literature there is emerging evidence that male obesity negatively impacts fertility through changes to hormone levels (Palmer et al., 2012) but in our study there were no obese transgender women.

For the participants without suppressed testosterone value in our study several reasons can be postulated. Most likely non-adherence is the reason, but also interaction with other medication (substrate of CYP3A4) ) and alcohol are known possibilities.

As for the strengths of this study, this is the first prospective analysis, that show that there is absence of complete spermatogenesis in all transgender women who adequately undergo long term hormonal treatment, documented with serum hormone values. Another strength of this study is the size of our cohort with 97 participants, the largest study for this purpose to date. In all participants immunohistochemical staining was performed for different stages of spermatogenesis.

Some limitations of this study should be considered. We have no information about testicular function of our participants prior to initiation of hormonal treatment, but all patients have normal serum testosterone value before initiation of hormonal treatment. Another limitation is that the hormone levels mentioned in this study are not from blood analysis on the day of gender affirming surgery but from the day of the last visit in our outpatient clinic prior to the surgery. Equally this may be a strength since this resembles more the hormonal status throughout treatment.

In conclusion, we showed that adequate hormonal treatment leads to complete suppression of spermatogenesis in transgender women. We agree to the recommendations of the World Professional Association of Transgender Health (WPATH) and Endocrine Society Clinical Practice Guidelines to monitor sex hormones during hormonal treatment and maintain premenopausal female target levels throughout the treatment. Therefore it is important to counsel transgender women regarding options for fertility preservation prior to treating with hormonal therapy. It is equally important to inform transgender women that in case these hormonal premenopausal levels are not maintained there is a risk for continuing or reinitiating spermatogenesis which may lead to unwanted pregnancies. Gender affirming hormone treatment cannot be advised as reliable contraception.

## **Acknowledgments**

I would like to thank all endocrinology residents who made a valuable contribution to the ENIGI study: Greet Roef, Mirra Boer, Marijn Carpentier, Liesbeth Van Huffel, Sara Vandewalle, Loes Moernaut, Sabine Vermeersch, Xavier-Philippe Aers, Charlotte Verroken, Emmanuelle Versele, Eva Van Caenegem, Katrien Wierckx, Justine Defreyne and Bram Vantomme for their outpatient care, Charlotte Bultynck and Charlotte Pas for their help with the dataset, our study nurse Kaatje Toye for handling the extensive administration to the study, and all participants in the ENIGI study protocol.

I would also like to thank Prof. Goossens Ellen, Van Saen Dorien and Adams Clement from the department ‘Biology of the Testis, Research cluster Reproduction, Genetics and Regenerative medicine’ at the Vrije Universiteit Brussel for their help on the immunostaining. I would like to thank Lynn Supply of the department ‘Pathology’ of the University Hospital Ghent for her help in processing the testicular specimens. I would like to give special thanks to my promotor Prof. Dr. T’Sjoen Guy for his guidance throughout this project.



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## Nederlands abstract

Bij de behandeling van genderdysforie kan onder andere gebruik gemaakt worden van hormonale behandeling met als doel het ontwikkelen van de secundaire geslachtskenmerken van het gewenste geslacht en het onderdrukken van de aangeboren geslachtskenmerken. De effecten van hormonale behandeling op testiculaire functie bij transgender vrouwen is echter weinig gekend. We vonden het als referentiecentrum belangrijk om dit te bestuderen om correcte informatie te kunnen geven aan onze patiënten. Dit onderzoek maakt deel uit van de ‘‘European Network for the Investigation of Gender Incongruence’’ (ENIGI).

Testiculair weefsel werd verkregen door biopsies tijdens orchiëctomie van 97 deelnemers aan de studie. Immunohistochemische kleuring tegen vier verschillende markers gebeurde met als doel de complete spermatogenese in kaart te brengen. Een eerste kleuring gebeurde voor ‘Melanoma-Associated antigen A4’ (MAGE-A4), dewelke tot expressie komt in spermatogonia en primaire spermatocyten. De tweede merker die werd gebruikt is ‘Boll’, die vooral tot expressie komt in secundaire spermatocyten. De derde merker ‘cAMP Responsive Element Modulator’ (CREM), is een merker voor ronde spermatiden. Finaal werd gekleurd tegen Acrosin, een merker voor acrosoom visualisatie. Als een coupe van een deelnemer positief was voor de MAGE-A4 kleuring gebeurde een verdere kleuring voor Boll op een andere coupe, enzovoort voor CREM en Acrosin. Na de MAGE-A4 kleuring gebeurde ook een telling van het aantal primaire spermatocyten per vierkante millimeter. Testisweefsel met normale spermatogenese werd gebruikt ter vergelijking. Hormonale analyses gebeurden via immunoassays op de laatste consultatie alvorens heekunde.

92 % (89/97) van de deelnemers had een adequaat onderdrukte testosteronemie ( $< 50\text{ng/dl}$ ) alvorens heekunde. De gemiddelde tijd van start hormonale behandeling tot heekunde bedroeg 685 dagen. In 88% van de deelnemers konden spermatogonia aangetoond worden middels de MAGE-A4 kleuring. Hoe hoger de serum testosteroone spiegel hoe meer spermatogonia werden gevisualiseerd alsook was er bij een hogere testosteronemie een verdere uitrijping van de spermatogonia. Doch na verdere kleuring kon bij geen enkele patiënt volledige spermatogenese aangetoond worden.

In conclusie kunnen we stellen dat adequate hormonale behandeling tot complete onderdrukking van de spermatogenese leidt. Deze verschillende uitkomst in vergelijking met andere studies is volgens ons omwille van een adequaat onderdrukt serum testosteroone spiegel

in onze studie, iets wat niet het geval is in andere studies. Daar door de behandeling er een complete onderdrukking is van de spermatogenese is het zeer belangrijk om transgender vrouwen goed in te lichten hierover en de mogelijkheden tot cryopreservatie van sperma te bespreken, alvorens het opstarten van hormonale behandeling.