UNIVERSITAT DE BARCELONA

Aspectos endocrinológicos y caracterización del tejido ovárico en personas transgénero masculinas. Implicaciones en la preservación de la fertilidad

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TESIS DOCTORAL

ASPECTOS ENDOCRINOLÓGICOS Y CARACTERIZACIÓN DEL TEJIDO OVÁRICO EN PERSONAS TRANSGÉNERO MASCULINAS. IMPLICACIONES EN LA PRESERVACIÓN DE LA FERTILIDAD.

Memoria de Tesis Doctoral presentada por

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Declaramos que **Aina Borrás Capó** ha realizado bajo nuestra supervisión los estudios presentados en la tesis **"Aspectos endocrinológicos y caracterización del tejido ovárico en personas transgénero masculinas. Implicaciones en la preservación de la fertilidad"**.

Esta tesis se estructura siguiendo la normativa para tesis doctorales en formato de compendio de artículos, para la obtención del grado de **Doctor en Medicina**. Los Trabajos abajo mencionados están preparados para ser presentados hoy al Tribunal.

M Dolors Manau Trullàs	**Francesc Fàbregues Gasol**	**Aina Borrás Capó**
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ÍNDICE

GLOSARIO

AFAB *Assigned Female At Birth*
AMH *Anti-Müllerian Hormone*
APA *American Psychiatric Association*
ASRM *American Society for Reproductive Medicine*
DMSO Dimetil Sulfóxido
EOC Estimulación Ovárica Controlada
FIV Fecundación in Vitro
FSH *Follicle Stimulating Hormone*
GAHT *Gender Affirming Hormone Therapy*
GAS *Gender-Affirming Surgery*
GC *Granulosa Cells*
IVG *In Vitro Growth*
IVM *In Vitro Maturation*
LH *Luteinizing Hormone*
MO Microscopia Óptica
MII Metafase II (ovocitos maduros)
OTC *Ovarian Tissue Cryopreservation*
PCOS *PolyCystic Ovary Syndrome*
PF Preservación de la Fertilidad
PrOH 1,2 Propanediol
RHA Reproducción Humana Asistida
SF *Slow-Freezing technique*
TAYAS *Transgender Adolescents and Young Adults*
TEM *Transmission Electron Microscopy*
TRA Técnicas de Reproducción Asistida
VT *Vitrification method*
WPATH *World Professional Association for Transgender Health*

PRESENTACIÓN

La presente tesis se estructura siguiendo la normativa para tesis doctorales en formato de compendio de artículos, para la obtención del grado de **Doctor en Medicina**.

La tesis consta de dos objetivos y dos artículos. Los proyectos incluidos en esta tesis pertenecen a una misma línea de investigación, que ha conducido a la publicación de dos artículos en revistas internacionales:

1. **Borrás A**, Manau MD, Fabregues F, Casals G, Saco A, Halperin I, Mora M, Goday A, Barral Y, Carmona F.
   Endocrinological and ovarian histological investigations in assigned female at birth transgender people undergoing testosterone therapy. Reprod Biomed Online. 2021 Aug;43(2):289-297. doi: 10.1016/j.rbmo.2021.05.010.

   Estado: publicado. Factor de impacto de la revista: 3.82
   1er cuartil en Medicina, Obstetricia y Ginecología.

2. **Borrás A**, Manau MD, Fabregues F, Peralta S, Calafell JM, Casals G, Saco A, Agustí I, Carmona F.
   Comparison between slow freezing and vitrification of ovarian tissue cryopreservation in assigned female at birth transgender people receiving testosterone therapy: data on histological and viability parameters.
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1. INTRODUCCIÓN

1. INTRODUCCIÓN

Estos últimos años se ha observado un interés creciente y una mejora de la asistencia tanto social como médica de las personas trans y la aparición de un nuevo modelo de atención en la salud de este colectivo. Uno de los aspectos que mayor interés suscita, desde el punto de vista de la medicina de la reproducción, es el efecto de la terapia hormonal sobre el ovario y la recuperación de la fertilidad posterior, especialmente si se prolonga en el tiempo o se inicia precozmente, incluso en prepúberes. Además, la mejora en el asesoramiento y la información recibida respecto a las diversas opciones de preservación de la fertilidad, tanto antes como durante la transición, ha aumentado la demanda de estos tratamientos. Todo ello, asimismo, se ha visto apoyado por unos cambios legislativos recientes, concretados en la publicación de una orden ministerial (SND/1215/2021) hace escasos meses (noviembre 2021).

Sin embargo, a pesar de estas mejoras sociales y legislativas antes mencionadas se debe tener presente la escasa evidencia científica de la que disponemos en la actualidad sobre el efecto de la terapia hormonal o de las diferentes técnicas de preservación de la fertilidad en este colectivo y que, por tanto, debería reflejarse en la información aportada antes de iniciarse. En este contexto, la línea argumental de la presente Tesis Doctoral aporta información de gran interés en el abordaje terapéutico de las personas trans en el escenario de la preservación de su fertilidad.

1.1 La persona trans

- **Disforia de género y glosario**

La disforia de género es una condición en la que una persona experimenta conflicto psicológico interno debido a la incongruencia de su género asignado al nacer y el género con el que se identifica (1). No se han realizado estudios epidemiológicos formales sobre la incidencia y prevalencia de personas que se identifican como "transgénero" o "no conforme al género" a nivel mundial ni estatal, y los esfuerzos para llegar a estimaciones realistas están llenos de enormes dificultades. En EUA la prevalencia estimada alcanza aproximadamente el 0.3 - 2% de la población, y extrapolando estos datos la cifra alcanzaría aproximadamente los 25 millones de personas en todo el mundo (2,3). Según datos recientes publicados por WPATH (*World Professional Association for Transgender Health,* por sus siglas en inglés) la estimación de personas transgénero masculinas oscilaría entre 1:200.000 y 1:30.400 personas (4).

La autoconciencia de un individuo como hombre o mujer cambia gradualmente durante la vida infantil y la niñez. Este proceso evoluciona con las interacciones con los padres, compañeros y el entorno y parece que existe un calendario bastante preciso que describe los pasos de este proceso (5). Sin embargo, se desconoce cuándo se adquiere la identidad de género y qué factores contribuyen al desarrollo de una identidad que no es congruente con el género al nacer. Actualmente, se considera que la identidad de género y / o la expresión de género probablemente reflejan una interacción compleja de factores biológicos, ambientales y culturales (6,7).

Los doctores Magnus Hirschfeld y Harry Benjamin, entre otros, fueron pioneros en la valoración de las personas que deseaban vivir una vida que se correspondía con su género experimentado frente al género designado al nacer, y que buscaban una solución a su profundo malestar. El término *"transexual"* se hizo

ampliamente conocido tras la publicación por parte del Dr Benjamin de "The transexual phenomenon" (8) aunque, actualmente, el sistema de clasificación actual de la APA (*American Psychiatric Association*, por sus siglas en inglés) usa el término **disforia de género** en su evaluación (9).
Además, la versión actual de la CIE-11 de la Organización Mundial de la Salud usa el término ***"incongruencia de género"*** al valorar a adolescentes y adultos (10), cuya definición es:

ICD-11 for Mortality and Morbidity Statistics (Versión: 02/2022)

" *La incongruencia de género de la adolescencia y la edad adulta se caracteriza por una incongruencia marcada y persistente entre el género experimentado de un individuo y el sexo asignado, lo que a menudo conduce a un deseo de 'transición', para vivir y ser aceptado como una persona del género autopercibido, a través de tratamiento hormonal, cirugía u otros servicios de atención médica para hacer que el cuerpo del individuo se alinee, tanto como se desee y en la medida de lo posible, con el género experimentado*".

Si bien una discusión exhaustiva de la diversidad de terminología relativa a la identidad y la expresión de género está más allá del alcance de la presente Tesis Doctoral, se incluye un glosario de terminología adaptada en la **Tabla 1**.

Tabla 1. Glosario de terminología transgénero.

TÉRMINO	DEFINICIÓN
Identidad de género	La experiencia interna de género de un individuo o el sentido interno de sí mismo desde una perspectiva de género.
Expresión de género	La manera externa en la que un individuo expresa o muestra su género.
Orientación sexual	Cómo un individuo caracteriza su atracción sexual y emocional hacia los demás.
Disforia o incongruencia de género	Estado de angustia secundaria a la discordancia entre la identidad de género y el sexo asignado al nacer. Para cumplir con los criterios del Manual Diagnóstico y Estadístico de Trastornos Mentales (DSM-5), la angustia debe ser clínicamente significativa y causar un deterioro en el funcionamiento de la persona.
Cisgénero	Describe a un individuo cuya identidad de género es consistente con su sexo asignado al nacer.
Transgénero o trans	Describe a un individuo cuya identidad de género no se corresponde con su sexo asignado en nacimiento.
Persona transgénero masculina o hombre trans	Adjetivo para describir a las personas a las que se les asignó género mujer al nacer que están cambiando o que han cambiado de cuerpo y / o rol de género de mujer asignada por nacimiento a un cuerpo o rol más masculino.
Transición	Período de tiempo en el que los individuos cambian del rol de género asociado con su sexo asignado al nacer a un diferente rol de género. Para muchas personas, esto implica aprender a vivir socialmente en otro rol de género; por para otros, esto significa encontrar un rol y expresión de género que les resulte más cómodo. La transición puede o no conllevar la masculinización del cuerpo a través de hormonas u otros medicamentos o procedimientos. La naturaleza y la duración de la transición son variables e individualizadas.

Adaptada de la publicada por **Baram *et al*; 2019** (11) y **Schwartz *et al*; 2021** (12).

- **Tratamiento médico y quirúrgico en hombres trans**

Gran parte de las personas trans optan por someterse a un tratamiento hormonal para aliviar la angustia asociada con la disforia de género, denominada **terapia hormonal reafirmación de género** (gender affirming hormone therapy o **GAHT** por sus siglas en inglés).

Este tratamiento debe ser en todo momento individualizado y multidisciplinario y consiste, inicialmente, en una terapia hormonal dirigida a suprimir los niveles

de hormonas sexuales endógenas y, por lo tanto, reducir las características sexuales secundarias del género designado del individuo al nacer. Además, se desea reemplazar los niveles de hormonas sexuales endógenas por las consistentes con la identidad de género del individuo mediante el uso de los principios del tratamiento de reemplazo hormonal de pacientes con hipogonadismo (7).

Hay múltiples preparaciones de andrógenos diferentes y eficaces de cara a inducir la masculinización en hombres transgénero. Se suelen usar preparaciones parenterales (intramusculares o subcutáneas) o bien transdérmicas (mediante parches o geles) para conseguir niveles de testosterona en el rango de referencia masculino (generalmente entre 275.0–1000.0 ng / dL de testosterona total). La vía de administración más común son las inyecciones intramusculares o subcutáneas con enantato o cipionato de testosterona, pero otra opción incluiría la administración intramuscular de undecanoato de testosterona, de acción más prolongada, o bien la administración transdérmica con parches o geles (13). Al comparar los niveles de andrógenos tras la administración subcutánea versus la intramuscular no se hallan diferencias en cuanto a la eficacia y seguridad, siendo equivalentes, pero las inyecciones subcutáneas muestran una mejor aceptación por parte de los usuarios. Las preparaciones orales de testosterona rara vez se utilizan debido a la necesidad de dosis frecuentes y la imprevisibilidad de los niveles séricos alcanzados (14).

Aunque los resultados físicos varían de una persona a otra, algunos efectos masculinizantes de la GAHT pueden comenzar a verse tan pronto como tras 1 - 2 meses del inicio del tratamiento. Esta terapia da como resultado un aumento del vello facial y acné, profundización de la voz, piel grasa y se ha descrito un aumento de la libido junto con clitoromegalia (15). Otros cambios, como el crecimiento del vello facial / corporal (especialmente en la cara, el pecho y el abdomen), los cambios en la distribución de la grasa y el aumento de la musculatura tardan más en desarrollarse por completo, en algunos casos hasta 5 años.

La terapia hormonal va a inducir amenorrea en la gran mayoría de los individuos a los escasos meses de iniciarse el tratamiento, alcanzando el 82% a los tres meses y prácticamente el total al año de iniciarse la medicación (16). Además, se han descrito diversos cambios a nivel del sistema reproductivo femenino que expondremos más adelante.

Los riesgos potenciales de la GAHT incluyen eritrocitosis, disfunción hepática y riesgo cardiovascular. Los estudios sobre riesgos a largo plazo que se han realizado en hombres trans han sido, en general, tranquilizadores, lo que sugiere que los efectos adversos son poco frecuentes (7).

Por otra parte, la **cirugía de afirmación de género** puede ser el paso necesario en determinadas personas para lograr su objetivo final de vivir de forma completa el rol de género deseado. Este tipo de cirugía incluye tanto la mastectomía bilateral como la histerectomía y doble anexectomía en la mujer. La cirugía de masculinización más importante es la mastectomía y no tiene repercusión alguna en la fertilidad. En cambio, la histerectomía y doble anexectomía (denominada gender-affirming surgery o **GAS** por sus siglas en inglés) genera una esterilidad irreversible y completa; y suele ser retrasada hasta después de algunos años de terapia con andrógenos.

Según diversos estudios, destacando uno publicado por Wierckx *et al*. (15), la salud mental del individuo mejora de forma significativa al participar en un programa de tratamiento que incluya tanto la terapia hormonal como la cirugía de afirmación de género.

1.2 Efectos del tratamiento sobre la fertilidad y técnicas de preservación

- **Efectos de la GAHT/GAS sobre la fertilidad**

Las opciones terapéuticas comentadas anteriormente ayudan a aliviar los síntomas de la disforia de género, disminuyen los síntomas depresivos y aumentan la autoestima de estas personas (17), pero se debe tener presente que tanto la terapia hormonal como la cirugía de afirmación de género pueden alterar, ya sea de forma **reversible** o **definitiva**, la fertilidad de la persona. Diversas asociaciones médicas profesionales recomiendan que el asesoramiento sobre la preservación de la fertilidad (PF) se lleve a cabo antes de la transición, informando con claridad sobre los posibles impactos negativos de la GAHT y la GAS en la fertilidad y las subsiguientes opciones de PF disponibles (1,7,18–20).

El efecto de la terapia hormonal con altas dosis de andrógenos y mantenida en el tiempo es desconocido, pudiendo ser potencialmente gonadotóxica a largo plazo. La mayoría de los datos disponibles apuntan hacia un efecto sobre todo a nivel ovárico; sin embargo el alcance, la reversibilidad de ese efecto y el tiempo necesario hasta conseguir la recuperación funcional aún no se han explorado en profundidad, siendo todo ello incierto en la actualidad (11).

Según los estudios publicados a este respecto, el efecto hormonal sobre el tejido ovárico es muy controvertido. Estudios observacionales clásicos que valoraban el efecto de la testosterona en dosis altas y periodos prolongados de tiempo informaban principalmente del desarrollo de un patrón histológico similar al presente en el síndrome de ovarios poliquísticos (**PCOS-like**, *PolyCystic Ovary Syndrome - like*, por sus siglas en inglés) (21–23). Estudios mucho más recientes muestran resultados más contradictorios; algunos todavía reportan una histología compatible con PCOS-like (24,25), mientras que otros reflejan una distribución folicular normal (26–28).

Por otra parte, la cirugía de afirmación de género (GAS) es irreversible y conduce a la esterilidad definitiva de la persona. En la actualidad en algunos casos se realiza únicamente la ooforectomía bilateral, sin realizar la exéresis del cuerpo uterino. Esta opción permitiría una futura gestación en el caso de realizarse alguna técnica de reproducción asistida (TRA) con gametos propios o donados.

- **Deseo gestacional en la población trans**

Según datos recientes, un elevado porcentaje de personas trans presenta deseo gestacional actual o futuro, alcanzando hasta un 40 % de los hombres trans (29). De esta población, sólo un 11.7 % de éstos han visto su deseo genésico cumplido, siendo este porcentaje aún más bajo que las cifras publicadas con anterioridad, que estimaban que entre un 25 y un 30 % conseguían ser padres, la mayoría de los cuales tenían hijos biológicamente relacionados (29–31).

Si bien el interés por lograr la paternidad es alto entre los adultos transgénero, los estudios centrados en personas más jóvenes (**TAYAS**, *transgender adolescents and young adults*, por sus siglas en inglés) han mostrado claramente un menor interés en la fertilidad futura y la descendencia biológicamente relacionada. Muchas personas jóvenes están ansiosas por iniciar la transición médica, y algunos incluso optan por comenzar con la terapia hormonal durante su adolescencia o incluso prepuberalmente. Debido a la temprana edad de este colectivo, su futuro de tener hijos biológicos no suele ser motivo de preocupación; sin embargo, es probable que algunos lleguen a desarrollar el deseo de tener hijos biológicos a lo largo de su vida (32–34).

- **Tratamientos de preservación de la fertilidad**

Aunque el deseo gestacional actual o futuro es elevado en este colectivo, múltiples estudios demuestran tasas muy bajas de utilización de los tratamientos de preservación de la fertilidad, que reflejan las barreras significativas a las que enfrentan estas personas (35,36).

La población trans, a pesar de las mejoras acontecidas en la atención médica en los últimos años, sigue siendo una población muy desatendida en estos términos, y únicamente el 30 – 40 % refiere seguir una asistencia sanitaria de rutina (37). La salud reproductiva no es una excepción, por ello aunque destacadas sociedades médicas científicas como la *ASRM* (American Society for Reproductive Medicine, por sus siglas en inglés), la *Endocrine Society* y la *WPATH* han establecido recomendaciones claras a este respecto, el escaso asesoramiento sobre técnicas de PF reflejado en estudios recientes demuestra que no se está cumpliendo con este estándar de atención (7,38–40).

Por otra parte, existen barreras importantes en el momento en que se plantean tratamientos de PF, entre las que destacan, según diversos estudios: el coste económico del tratamiento o la falta de cobertura sanitaria del proceso (36 %), la necesidad de interrumpir o retrasar la GAHT (19 %) y la disforia de género que provoca la estimulación ovárica controlada (EOC) (11 %) (32,33).

Actualmente las opciones de preservación de la fertilidad en hombres transgénero se pueden clasificar en dos grupos diferentes:

I) Opciones disponibles antes y tras del inicio de la terapia hormonal (GAHT).
II) Opciones experimentales, que se pueden realizar al mismo tiempo que la cirugía de afirmación de género (GAS).

Las dos técnicas principales de PF en hombres trans son la **vitrificación de ovocitos** y la **criopreservación de cortex ovárico**, siendo la primera la más establecida y estudiada (41).

I) Opciones disponibles antes y tras el inicio de GAHT

Un hombre trans puede realizar técnicas de PF mediante la criopreservación de ovocitos o embriones tanto antes como tras el inicio de la GAHT (42).
Actualmente no hay estudios claros sobre si el tratamiento con testosterona debe interrumpirse antes del tratamiento y durante cuánto tiempo. En uno de los escasos estudios publicados, los autores optaron por suspender el tratamiento con testosterona durante un período de 3 meses, basando su decisión en datos pertenecientes a otros medicamentos con efecto teratogénico, como el metotrexato (43). Otros grupos abogan por suspender la GAHT durante 6 meses o hasta la restauración de los ciclos ováricos (con menstruación) y conseguir unos niveles basales de testosterona como los de la mujer cisgénero. El pequeño tamaño de la muestra de los estudios y la duración variada de la GAHT antes de la interrupción hacen que este hallazgo sea difícil de generalizar.

La criopreservación de ovocitos y embriones es un método de PF clínicamente establecido en mujeres cisgénero, pero tiene varias consideraciones adicionales para los hombres trans. Además de suspender la GAHT por un período de tiempo indeterminado, deberán someterse a una estimulación ovárica con la administración exógena de hormonas, realizar diversos controles ecográficos de crecimiento folicular y finalmente llevar a cabo un procedimiento transvaginal invasivo para obtener los ovocitos y criopreservarlos posteriormente.

La **tabla 2** muestra las características específicas de este método de PF en la población trans masculina.

Tabla 2. Preservación de la fertilidad mediante criopreservación de ovocitos

Descripción de la TÉCNICA
Estimulación ovárica controlada con gonadotropinas y controles ecográficos seriados. Reclutamiento y maduración de una cohorte de folículos. Punción folicular y obtención de ovocitos maduros, vitrificación de los mismos para uso posterior.
Uso futuro
Los ovocitos pueden ser descongelados y fecundados con semen de la pareja o mediante donación de semen. Los embriones resultantes podrán ser transferidos si la persona trans mantiene útero y desea gestación.
Consideraciones específicas
VENTAJAS Método establecido en mujeres cisgénero con hallazgos iniciales prometedores en series de casos en hombres trans. **INCONVENIENTES** - Suspensión de la GAHT durante un periodo indeterminado de tiempo (no definido). - Puede requerir coste económico elevado según región / país de origen. - Potencial aparición de disforia de género durante la estimulación ovárica (ecografías vaginales seriadas, incremento de los niveles de estradiol, etc). ***Estrategias para reducir la disforia de género*:** - Se puede considerar iniciar la estimulación en cualquier momento del ciclo, sin esperar a iniciarla en fase folicular precoz (evitando así la menstruación). - Se podría considerar minimizar la duración del cese de la testosterona. No está establecido el período mínimo de cese de la medicación, e incluso alguna serie presenta casos sin suspender la GAHT. - Se puede considerar el uso concomitante de letrozol durante el protocolo de estimulación para reducir los niveles de estradiol. - Se puede considerar el uso de ecografías abdominales durante el control de estimulación cuando sea factible.

Adaptada de la publicada por **Schwartz *et al*; 2021** (12).

Escasos estudios han sido publicados sobre la preservación de ovocitos en hombres trans. Muy recientemente han aparecido datos sobre los resultados de vitrificación de ovocitos en personas con GAHT previa respecto a hombres trans antes de iniciar la terapia hormonal, o que comparan los resultados con mujeres cisgénero. Cabe destacar que en todos estos estudios la GAHT había sido suspendida antes de iniciar la estimulación ovárica en todos los individuos, durante un período de tiempo que abarcaba un mínimo de 3 meses con recuperación de ciclos menstruales hasta una media de 6 meses y un rango de entre 1 - 13 meses según las diversas publicaciones.

Adeleye et al. en 2019 publicaron datos retrospectivos de la EOC en 13 hombres trans, 7 de los cuales habían estado previamente con GAHT. No hallaron diferencias en el total de ovocitos recuperados o en el número de ovocitos maduros entre hombres trans y mujeres cisgénero de igual edad; sin embargo, sí informaron de un número reducido de ovocitos recuperados en las personas con GAHT previa frente a los que nunca habían estado expuestos a testosterona (25.5 ovocitos frente a 12, $p = 0.038$). Esta diferencia dejó de ser significativa al eliminar del análisis 2 hombres trans atípicos con marcadores de reserva ovárica muy alterados de inicio (44). Posteriormente, *Amir et al*. mostraron resultados similares en cuanto al número de ovocitos recuperados (no diferencias entre los dos grupos), pero destacó la necesidad de una dosis menor de FSH utilizada para la estimulación en los hombres trans con GAHT previa y unos niveles máximos de estradiol significativamente mayores entre las personas transgénero en comparación con las mujeres cisgénero (45). Finalmente, *Leung et al*. reportó en otro estudio retrospectivo en un total de 26 hombres trans buenos resultados, según el cual se obtuvieron una media de 20 ovocitos por individuo. La mayoría de la cohorte tuvo exposición previa a testosterona (aunque no se comparó esta población con aquella que no había iniciado la GAHT) y entre los individuos con GAHT previa no se hallaron diferencias en el número de ovocitos o la madurez de los mismos en comparación con una cohorte de mujeres cisgénero (46).

Por otra parte, pequeñas series de casos han informado de recién nacidos vivos tras la criopreservación de ovocitos en hombres trans con GAHT previa, así como en aquellos que se sometieron a PF antes del inicio de la terapia hormonal (41,43,47).

II) Opciones experimentales de PF en hombres trans

En aquellos casos en que el aumento de las hormonas no congruentes con el género autopercibido hace que la estimulación ovárica sea inaceptable, o bien en aquellas personas que no se habían planteado la opción de PF antes del inicio de la GAHT (como en el caso de los TAYAS), la **criopreservación de tejido ovárico** (ovarian tissue cryopreservation u **OTC** por sus siglas en inglés) puede ser una opción viable para los hombres trans en el futuro.
La **tabla 3** muestra las características específicas de este método de PF en esta población.

Tabla 3. Preservación de la fertilidad mediante OTC en población trans.

Descripción de la TÉCNICA
Extracción de un fragmento de tejido ovárico quirúrgicamente mediante laparoscopia. Criopreservación posterior de la cortical ovárica fragmentada.
Uso futuro
- El tejido ovárico criopreservado se puede **autotrasplantar** para concebir y restaurar la pérdida de la función ovárica. Tras el autotrasplante, el individuo puede realizar una fecundación in vitro, con la obtención de ovocitos y embriones que podrán transferirse si la persona trans mantiene el útero y desea gestación. - Si **no se desea el autotransplante** del tejido se pueden plantear técnicas *experimentales* como el crecimiento y la maduración in vitro de los ovocitos (IVG y IVM); la fecundación posterior y la transferencia embrionaria si la persona trans mantiene útero y desea gestación.
Consideraciones específicas
VENTAJAS - Evita la estimulación ovárica y la suspensión de la GAHT - Es la única opción de PF en trans prepúberes **INCONVENIENTES** - Se requiere una cirugía (laparoscópica). - El proceso de crecimiento y maduración in vitro es actualmente de naturaleza experimental. ***Estrategias para reducir la disforia de género*:** El tejido ovárico puede recuperarse en el momento de la GAS, en un único procedimiento quirúrgico.

Adaptada de la publicada por **de Roo *et al*; 2016** (48) y **Schwartz *et al*; 2021** (12).

El procedimiento implica un acto quirúrgico mediante una laparoscopia en la cual se extrae un fragmento de corteza ovárica que se criopreserva tras su preparación en el laboratorio. Posteriormente, se llevará a cabo la descongelación del tejido, el autotransplante (en caso de que así se desee) y la maduración de los folículos inmaduros para su uso mediante técnicas de reproducción asistida. En hombres trans, la OTC evitaría los efectos no deseados de la estimulación ovárica, la interrupción de la terapia hormonal y no requeriría procedimientos adicionales, ya que se puede realizar al mismo tiempo que la GAS.
La activación del tejido cortical ovárico que se consigue en las mujeres cisgénero tras su reimplante da como resultado la reanudación del ciclo hormonal femenino y el crecimiento y maduración natural de los ovocitos inmaduros.

Hasta la fecha, se han notificado más de 180 gestaciones y niños nacidos vivos tras el autotrasplante de tejido ovárico descongelado en mujeres cisgénero con enfermedades oncológicas o benignas. El reimplante ortotópico en la pelvis es la localización más común y con mejores resultados reportados, y todas las gestaciones publicadas excepto dos se han logrado mediante la técnica de congelación lenta (**SF**, *slow freezing*, por sus siglas en inglés), que es el método estándar de criopreservación del tejido ovárico (49–51). La técnica de vitrificación (**VT**) se ha desarrollado en los últimos años, siendo más novedosa, rápida y con resultados muy prometedores. Diversos estudios en mujeres cisgénero han comparado ambos protocolos de criopreservación, obteniendo resultados contradictorios especialmente en aspectos como la mejora en la tasa de supervivencia del folículo o la integridad del estroma ovárico.

Sin embargo, las personas trans que **no** desean la restauración del ciclo hormonal femenino, el reimplante del tejido ovárico no se presentaría como opción viable. El planteamiento alternativo sería el crecimiento y maduración de los folículos in vitro (**IVG**, *In Vitro Growth* e **IVM**, *In Vitro Maturation*, por

sus siglas en inglés), técnicas de naturaleza experimental que se hallan en fases muy iniciales de desarrollo a día de hoy (27,52).
En relación a este punto, un estudio de reciente publicación llevó a cabo el cultivo y maduración de ovocitos inmaduros obtenidos durante la GAS en hombres trans que no habían suspendido la terapia hormonal previamente, consiguiendo ovocitos maduros (MII) y hasta en un 87% de los mismos se halló un patrón cromosómico normal (27).

A pesar de ello, el estudio del desarrollo ovocitario tanto en población trans como cis se halla en fases muy iniciales de experimentación, lejos de su uso en la práctica clínica habitual. Destacamos la necesidad de investigar ampliamente en diversos puntos como:

- La valoración del crecimiento y maduración de folículos desde estadios más precoces (incluso folículos primordiales) hasta alcanzar la madurez (MII).

- La capacidad de fecundación de estos ovocitos procedentes de personas con GAHT.

- La valoración del desarrollo embrionario posterior (53–55).

1.3 Relevancia y justificación del estudio

Tal y como hemos comentado con anterioridad, en estos últimos años se ha observado un cambio drástico en la asistencia tanto social como médica en las personas trans, incrementándose el interés tanto en el efecto de la GAHT sobre el ovario y el aparato reproductivo como en la posibilidad de la recuperación de los ciclos ováricos y la fertilidad subsiguiente. Además, se ha mejorado el asesoramiento y la información recibida respecto a las diversas opciones de PF tanto antes de empezar la terapia hormonal como tras su inicio, aumentando así la demanda de estos tratamientos.
Por otra parte, las diversas técnicas de preservación han evolucionado de forma considerable estos últimos años, principalmente a nivel del laboratorio con la aparición, entre otros, de la vitrificación y el incremento de las tasas de éxito en la población.

Nuestro grupo ha sido pionero en la línea de investigación sobre androgenización ovárica en la mujer definida como "baja respondedora" en un ciclo de fecundación in vitro (FIV). Se han publicado diversos artículos sobre el pretratamiento con andrógenos transdérmicos (imprimación ovárica) sobre la base de que la exposición temporal de los folículos a niveles elevados de andrógenos podrían aumentar su capacidad de respuesta en una estimulación ovárica para la posterior fecundación in vitro (56–58). Siguiendo esta línea de investigación consideramos de gran interés valorar las modificaciones observadas en el tejido ovárico tras la iniciarse la terapia con testosterona en dosis elevadas y durante períodos prolongados de tiempo en la persona adulta, debido a la limitada y contradictoria información existente, teniendo como referente las personas transgénero masculinas.

Por otra parte, el Hospital Clínic es centro de referencia en preservación de la fertilidad tanto a nivel autonómico como estatal, altamente especializado en realizar técnicas de preservación desde el año 2007, como es la vitrificación

ovocitaria, la criopreservación corteza ovárica, la cirugía conservadora en cáncer ginecológico, entre otras. Son múltiples las líneas de investigación en este campo, y dado que estos últimos años la técnica de criopreservación de tejido ovárico ha mostrado una gran evolución y desarrollo, hemos considerado estudiar dicha técnica en la población específica de personas trans, en los que no hay datos publicados hasta la fecha.

En esta tesis se realiza el estudio de forma integral de la histología ovárica, el perfil y los cambios hormonales de las personas transgénero masculinas junto con una comparación de diferentes métodos de preservación de la fertilidad. Para conseguirlo se presentan los siguientes estudios:

- Estudio 1: se investiga el efecto a largo plazo de la GAHT en hombres trans a nivel hormonal e histológico, describiendo la población folicular y su comparación con los criterios de Stein-Leventhal que describen la morfología de ovario poliquístico.

- Estudio 2: se investiga si la vitrificación de córtex ovárico es mejor que la congelación lenta preservando el tejido, en términos de morfología folicular y viabilidad tisular, de cara a mantener su potencial futuro reproductivo.

2. HIPÓTESIS

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La administración de testosterona a dosis altas en periodos prolongados de tiempo en personas transgénero masculinas adultas no produce un efecto a nivel histológico y hormonal compatible con PCOS-like.

Además, la vitrificación del tejido ovárico, como técnica de preservación en la persona transgénero masculina, puede mejorar la supervivencia folicular y la funcionalidad de este material en comparación con el método tradicional de congelación lenta.

3. OBJETIVOS

3. OBJETIVOS

1. Investigar el efecto de la terapia hormonal de afirmación de género en las personas trans masculinas mediante la valoración del perfil hormonal y el estudio histológico de las piezas ováricas tras la cirugía de afirmación de género.

2. Comparar los efectos de dos técnicas de criopreservación de tejido ovárico en personas transgénero masculinas en relación con aspectos morfológicos y de viabilidad folicular.

4. MATERIAL, MÉTODOS Y RESULTADOS

La descripción de la población de estudio, la metodología utilizada en las investigaciones llevadas a cabo para el logro de los objetivos antes planteados, así como la presentación de los resultados se encuentran detalladamente expuestos en las secciones de “Materiales y Métodos” y “Resultados” de cada uno de los dos artículos que constituyen el cuerpo doctrinal de la presente Tesis Doctoral.

Dichos artículos se incluyen a continuación tal y como han sido publicados en la literatura científica (páginas 33 a 57)

ESTUDIO 1

Endocrinological and ovarian histological investigations in assigned female at birth transgender people undergoing testosterone therapy.

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Resumen

Estudio de los efectos histológicos, hormonales y ováricos de la terapia hormonal de afirmación de género en personas transgénero masculinas (AFAB, *assigned female at birth* por sus siglas en inglés).

Diseño: estudio observacional prospectivo de 70 personas transgénero previa a la cirugía de afirmación de género (histeroo-oforectomía). Se realiza una ecografía ginecológica y se miden las concentraciones de hormonas séricas, incluida la hormona antimülleriana (AMH) y el perfil androgénico. La evaluación ovárica histológica incluye el estudio de las diferentes etapas de desarrollo folicular y se realiza en ambos ovarios.

Resultados: La edad media de la población es de 27.7+/-5.14 años. Los principales parámetros bioquímicos son: niveles de testosterona total de 781.5 ± 325.9 ng/dl; niveles de AMH de 3.2 ± 1.4 ng/ml; Niveles de FSH y LH de 4.9 ± 2.5 UI/l y 3.9 ± 2.9 UI/l, respectivamente; y valores de estradiol de 47.6 ± 13.7 pg/ml. Se realiza un estudio ecográfico en un total de 55 hombres trans y se han hallado folículos antrales en el 91,5% de los casos (sin presencia de folículo dominante ni cuerpo lúteo). Histológicamente los folículos se hallaron en su mayoría en estadio primordial (88,0%), mientras que el 3,3% eran atrésicos. El grosor de la túnica albugínea ha mostrado elevada heterogeneidad (rango 0.15-1.45 mm) y se ha observado la luteinización de las células del estroma en el 68.6% de las muestras. Se halló una correlación negativa entre los niveles de testosterona y los folículos antrales totales (Rs= -0,306, P = 0,029).

Conclusiones: Las personas transgénero masculinas en terapia hormonal muestran una distribución de folículos corticales en el rango informado previamente en mujeres cisgénero fértiles en edad reproductiva. La población folicular puede no verse alterada como resultado de la terapia, aunque se han observado algunos cambios corticales y estromales.

RBMO

ELSEVIER

ARTICLE

Endocrinological and ovarian histological investigations in assigned female at birth transgender people undergoing testosterone therapy

BIOGRAPHY

Aina Borrás, MD, from Universitat de Barcelona, completed her specialization in Hospital Clinic of Barcelona. In 2011 and 2013, Dr Borrás received two grants for her research into ovarian cryopreservation in the context of fertility preservation. She continues her clinical practice at the Reproductive Unit, Hospital Clinic of Barcelona, Spain.

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KEY MESSAGE

Testosterone therapy in assigned female at birth (AFAB) transgender people does not seem to alter the growing and non-growing follicle population, although some ovarian cortical and stromal changes were observed. Further studies are required to investigate fertility preservation treatment in AFAB transgender people.

ABSTRACT

Research question: What are the hormonal and ovarian histological effects of a gender affirming hormonal therapy in assigned female at birth (AFAB) transgender people?

Design: Prospective observational study of 70 AFAB transgender people taking testosterone therapy before gender-affirming surgery (hystero-oophorectomy). A gynaecological ultrasonographic scan was undertaken and serum hormone concentrations measured, including anti-Müllerian hormone (AMH) and androgenic profile. Histological ovarian evaluation was assessed in both ovaries, including the developmental stages of the follicles.

Results: The mean age of the population was 27.7+/–5.14 years. The main biochemical parameters were total testosterone levels 781.5 ± 325.9 ng/dl; AMH levels 3.2 ± 1.4 ng/ml; FSH and LH levels 4.9 ± 2.5 IU/l and 3.9 ± 2.9 IU/l, respectively; and oestradiol values 47.6 ± 13.7 pg/ml. Fifty-five AFAB underwent gynaecological ultrasound before surgery and antral follicles were found in 43 out of 47 ultrasounds (91.5%) (without the presence of a dominant follicle or corpus luteum). Histological follicles were mostly in the primordial stage (88.0) and 3.3% were atretic. The thickness of the tunica albuginea was widely heterogeneous (range 0.15–1.45 mm) and luteinization of the stromal cells was observed in 68.6% of the samples. A negative correlation between testosterone levels and total antral follicles was found (R_s= –0.306, P = 0.029).

Conclusions: AFAB transgender people taking testosterone therapy show cortical follicle distribution in the range previously reported in fertile cisgender women of reproductive age. The follicular population may not be altered as a result of the gender-affirming hormonal therapy, although some cortical and stromal changes have been observed.

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KEYWORDS

Anti-Müllerian hormone
Assigned female at birth
Gender transition
Ovarian androgenization
Testosterone therapy

INTRODUCTION

Transgender people are individuals whose gender identity differs from their birth-assigned sex. Assigned female at birth (AFAB) transgender people may request gender-affirming hormone therapy (*Hembree et al., 2017*), aimed at suppressing the secondary sex characteristics of the birth-assigned sex and inducing the secondary sex characteristics of the experienced gender. Hormone therapy in AFAB transgender people who desire masculinizing consists of testosterone therapy.

Testosterone therapy causes hyperandrogenism, and classically many of the pathophysiological and clinical alterations related to polycystic ovary syndrome (PCOS) have been attributed to this. Experimental studies in animals have shown this relationship, with many reproductive and metabolic features in rodent models being similar to those of cisgender women with PCOS (*Houten et al., 2012*; *Chen et al., 2015*). In humans, however, the interaction of hyperandrogenism with morphological, endocrinological and clinical aspects of PCOS are still not clearly demonstrated and remain controversial (*Lebbe and Woodruff, 2013*; *Rosenfield, Ehrmann, and Biochemical, 2016*).

Previous studies of AFAB transgender people have shown that the relationship between testosterone therapy and PCOS have not been completely and clearly established, and the expected endocrinological aspects have not been confirmed (*Spinder et al., 1989*). Data on the gonadotrophin profile in AFAB is conflicting, and an increase in anti-Müllerian hormone (AMH) levels or other aspects related to the release of gonadotrophins typical of cisgender women with PCOS have not been demonstrated (*Caanen et al., 2015*).

In relation to ovarian changes in AFAB transgender people, some investigators have reported evident PCOS-like histological changes (*Futterweit and Deligdisch, 1986*; *Grynberg et al., 2010*), whereas others have not confirmed these findings (*Ikeda et al., 2013*; *De Roo et al., 2017*). Recent studies have focused on the cortical and stromal relationship and ovarian aspects with normal and pathological ovarian functionalism, such as in women with PCOS (*Maas et al., 2018*). It has been shown that adequate folliculogenesis depends on known endocrinological aspects and also on elements related to the structure of the ovarian stroma (*De Roo et al., 2020*). In addition, recent studies have described different physical characteristics in the ovarian cortex of AFAB transgender people (*Woodruff et al., 2011*; *De Roo et al., 2019*).

As no consensus on the endocrinological and ovarian changes in AFAB transgender people has been reached, the aim of this study was to evaluate the extensive endocrinological changes observed in the AFAB cohort with testosterone therapy and to evaluate the ovarian histological aspects to determine the follicular population and the stromal and cortical changes and their implications in a future fertility-preservation process.

MATERIALS AND METHODS

Study design, size, duration and settings

This was a prospective observational study of 70 AFAB transgender people after more than 24 months of testosterone therapy recruited between May 2011 and July 2019 before gender-affirming surgery (GAS) (laparoscopic hystero-oophorectomy).

Participants and materials

The hormonal status and histological features of the ovaries of a group of 70 AFAB transgender people were evaluated. The individuals were enrolled when the GAS was scheduled, which consisted of laparoscopic hysterectomy and bilateral adnexectomy. Clinical follow-up took place in the Endocrinology Department at the Hospital Clinic of Barcelona, Spain, and were recruited in two time periods: from May 2011 to March 2013 and from January 2016 to July 2019. None of AFAB were using any contraceptives at baseline or during the study. All had regular menstrual cycles according to their medical history before receiving hormone therapy except for five patients, who presented with oligomenorrhoea, hirsutism and hormone determinations compatible with PCOS. The following exclusion criteria were applied: different sexual development and endocrine pathology other than PCOS, i.e. type 1 diabetes; uncontrolled thyroid disease, i.e. hypothyroidism or hyperthyroidism; and congenital adrenal hyperplasia.

The AFAB transgender people received testosterone therapy with intramuscular testosterone undecanoate (Reandron® 1000 mg) every 2–3 months or cypionate (Testex prolongatum® 250 mg/2 ml) (every 2–4 weeks) for a minimum period of 24 months.

Physical and biochemical studies

Anthropometric measurements (weight, height and body mass index) were carried out in all participants at recruitment and a blood sample was taken to determine serum hormone levels. The body mass index was within the normal range of 20.00–25.00 kg/m^2.

The main biochemical parameters were measured in serum using standard methods in the Core Laboratory of our hospital, and the results were expressed according to male reference ranges, apart from AMH. Sex hormone binding globulin was measured using a chemiluminometric immunoassay run on the Atellica analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY) (limit of quantification [LOQ] 1.11 nmol/l, interassay coefficient of variation <10%, with a normal range of 25.0–96.0 nmol/l). Total testosterone was measured by a chemiluminometric immunoassay (Testosteron II, Elecsys) (Roche, Mannheim, Germany) (LOQ 12 ng/dl, interassay coefficient of variation <5%, with a normal range of 275.0–850.0 ng/dl). Androstendione was measured by immunoradiometric assay (Cisbio assays, Codolet, France) (LOQ 10 ng/dl, interassay coefficient of variation <10%, and with a normal range of 50.0–250.0 ng/dl). Oestradiol, LH and FSH were measured using chemiluminometric immunoassays run on the ATELLICA Immunochemistry analyzer (Siemens Healthineers, Tarrytown, NY, EUA) (interassay coefficient of variation of <5% and a LOQ of 18.8 pg/ml for oestradiol; an intersassay coefficient of variation of less than 10% and an LOQ of 0.55 mUI/ml for FSH; and an interassay coefficient of variation of less than 5% and an LOQ of 1.44 mUI/ml for LH). Normal oestradiol values were between 10.0 and 41.0 pg/ml, whereas normal LH and FSH levels were between 1.5 and 7.5 U/l and 1.7 and 8.0 U/l, respectively. Anti-Müllerian hormone (AMH) was determined in serum by chemoluminescence immunoassay with paramagnetic particles for quantitative determination (AMH B13127 Beckman Coulter kit and in the ACCES2 device) (LOQ 0.02 ng/ml, interassay coefficient of variation <5%; results expressed in ng/

ml; normal female values between 1.0 and 4.5). Anti-Müllerian hormone was only determined in 45 AFAB, owing to difficulties in availability.

A gynaecological ultrasonographic scan (transvaginal or transabdominal) was carried out using a Voluson S6 Unit, General Electric Medical Systems (Austria) equipped with a 5–7 MHz probe or a 9-2 MHz convex curve probe in 55 AFAB transgender people before GAS, in a period time that ranged from 60 days to the day before GAS.

Polycystic ovary syndrome criteria

The diagnosis of PCOS was based on the presence of oligomenorrhoea, amenorrhoea, or both, and clinical, biochemical signs of hyperandrogenism, or both. In some cases, polycystic ovarian morphology was assessed by ultrasonography.

Histopathological study

Ovarian tissue processing

After GAS, the surgical specimens from the hystero-oophorectomy were transported to the laboratory and examined macroscopically. The surgical piece was weighed, and both ovaries were measured (A) longitudinally, (B) transversely and (C) antero-posteriorly, and ovarian volume was calculated according to the formula ½ x A x B x C. Immediately the ovaries were processed by the gynaecological pathologist, and a section comprising the largest diameter of the ovary was removed for histological study and fixed in 10% formalin. A section was taken from both ovaries for each person. All samples were routinely embedded in paraffin and 3-µm sections were made. The sections were stained with haematoxylin and eosin using standard techniques and were examined by a gynaecological pathologist. The morphology of Stein–Leventhal ovary was used as reference (hyperandrogenic PCOS with polycystic ovary morphology), with the following characteristics indicating Stein–Leventhal ovary (*Hughesdon, 1982*): collagenization and thickening of the tunica albuginea; stromal hyperplasia; luteinization of the stromal cells; and ovaries with multiple follicles at various stages of growth and atresia. The thickness of the ovarian cortex along the entire surface of the longitudinal section of the ovary was studied and measured at the thickest and thinnest point of the cortex section in both ovaries. The presence of structures composed of masses of lucent cells within the stroma similar to the corpora albicantia and distinguishable from the surrounding stroma were also evaluated, according to the description from *Ikeda et al. (2013)*. The remaining ovarian tissue was processed and cut in small cortical pieces for other research purposes not reported in this study.

Follicle classification

In the haematoxylin and eosin longitudinal sections, the developmental stages of the follicles were evaluated as defined by Gougeon and Chainy (1997). Primordial follicles were defined as those measuring 30–60 µm in diameter and containing a single layer of flat granulose cells around the oocyte. Primary follicles were those presenting a well-defined layer of cuboidal cells and wider than 60 µm in diameter. Secondary follicles were defined as those presenting two or more layers of cuboidal cells measuring 0.12–0.2 mm in diameter, and early antral follicles had more than two layers of cubical granulosa cells surrounding the oocyte in the presence of a follicular antrum measuring 0.12–2 mm in diameter. The number of antral follicles wider than 2 mm in diameter and atretic follicles were also studied. Follicles were counted in each category separately and were classified on a section containing the nucleus to avoid double counting.

Statistical analysis

IMB SPSS Statistics 23.0 (IBM Corp., New York, USA) was used for statistical analyses. The hormone parameters were evaluated and, for statistical analysis, serum LH levels less than 0.1 U/l were coded as the lower limit, being 0.1 (n = 3). The Kolmogorov–Smirnov test was used to test normality. Correlations analysis between age, hormone levels and the follicular population of the samples were made using Spearman's tests.

The presence or absence of histological characteristics (stromal hyperplasia, stromal luteinization and thickness of the tunica albuginea) was assessed, and the ratio of cases with these histological characteristics calculated. The number and percentage of follicles at different stages of development were also calculated.

Ethical approval

This clinical study was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects (*World Medical Association 2013*), the study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona (registry number 2011/6272) in February 2011, and all participants provided written, informed consent.

RESULTS

An overview of the characteristics of the study population, including the hormonal status and histological parameters observed, are presented in TABLE 1.

Characteristics of the study population, hormonal and ultrasonographic results

The mean age of the AFAB cohort at the time of surgery was 27.7 ± 5.14 years (range 18–40 years). Five individuals presented PCOS criteria at the start of androgenic treatment, with clinical criteria of oligomenorrhoea or amenorrhoea and clinical hyperandrogenism. None of the AFAB people had started androgens on their own before starting testosterone therapy. Of the 70 AFAB people, 21 started Reandrom® 1000 mg every 2–3 months, whereas the remaining 49 received Testex prolongatum® 250 mg/2ml every 2–4 weeks. No significant differences were observed in serum testosterone levels when comparing testosterone undecanoate (Reandron®) (775.1 ± 315.5 ng / dl) versus cypionate (Testex®) (813.7 ± 277.8 ng / dl) (P = 0.46).

All the study population became amenorrhoeic months after starting hormone therapy. Most of the AFAB people (68/70) did not have vaginal bleeding or spotting after the first 6–8 months of testosterone therapy, although two of them required the addition of gonadotrophin-releasing hormone (GnRH) analogues to achieve amenorrhoeic status. Monthly depot intramuscular Decapeptyl® was administered for 2 months in one case and for 6 months in another case. Testex prolongatum® was the androgen preparation used in both AFAB people who required GnRH analogues. At the time of recruitment, after 28.3 ± 3.4 months of hormone therapy, all the participants showed serum testosterone levels within the male physiological range (mean 781.5 ± 325.9 ng/dl) and complete virilization. Forty-eight AFAB people had serum FSH levels within

TABLE 1 CLINICAL, HORMONAL AND HISTOLOGICAL CHARACTERISTICS IN ASSIGNED FEMALE AT BIRTH TRANSGENDER PEOPLE (BEFORE GENDER-AFFIRMING SURGERY)

Clinical features		
Parameter	**Mean ± SD (range)**	**Reference values (male)**
Age, years	27.7 ± 5.1 (18–40)	NA
Weight, kg	60.5 ± 6.0 (45–72)	NA
Height, m	1.6 ± 0.9 (1.5–1.9)	NA
BMI, kg/m^2	22.3 ± 2.5 (20.0–25.0)	20.0–25.0
FSH, IU/l	4.9 ± 2.5 (0.3–9.4)	1.7–8.0
LH, IU/l	3.9 ± 2.9 (0.1–9.5)	1.5–7.5
Oestradiol, pg/ml	47.6 ± 13.7 (10.0–76.0)	10.0–41.0
Total testosterone, ng/dl	781.5 ± 325.9 (309.0–1451.0)	275.0– 850.0
SHBG, nmol/l	38.8 ± 18.3 (13.2–86.8)	25.0–96.0
Androstendione, ng/dl	243.1 ± 62.1 (154–310)	50.0–250.0
AMH, ng/ml	3.2 ± 1.4 (1.3–5.4)	1.0–4.5[a]

Histological features			
Follicle stage	**Mean ± SD**	**Median (range)**	**Sum (%)**
Primordial	61.4 ± 82.6	27 (0–443)	5957 (88.0)
Primary	2.1 ± 3.1	1 (0–15)	209 (3.1)
Secondary	0.5 ± 1.0	0 (0–6)	48 (0.7)
Total antral	3.4 ± 2.3	3 (0–9)	333 (4.9)
Early antral	1.4 ± 1.4	1 (0–6)	132 (1.9)
Antral	2.1 ± 1.9	2 (0–8)	201 (3.0
Atretic	2.3 ± 3.5	1 (0–21)	226 (3.3)

n = 70; anti-Müllerian hormone (AMH) was available for 45 people.

[a] Female pre-menopausal normal range shown for AMH.

BMI, body mass index; NA, not applicable; SHBG, sex hormone-binding globulin.

female pre-menopausal reference range (4.5–10.0 IU/l), corresponding to 68.6% of the population compared with 22 AFAB people presenting suppressed serum levels (minimum value 0.3 IU/l). No differences were found in FSH levels when comparing testosterone undecanoate (Reandron®) (4.8 ± 2.4 U/l) versus cypionate (Testex®) (4.9 ± 1.9 U/l) (P = 0.18). Thirty AFAB people showed suppressed serum LH levels, corresponding to 42.9% of the population, with a minimum value of 0.1 IU/l in three individuals. The AMH was determined in 45 AFAB transgender people, with a mean of 3.2 ± 1.4 ng / ml (range 1.3–5.4). Only two of them showed AMH levels above the female pre-menopausal reference ranges (4.4% of the population), of which one person was diagnosed with PCOS at the start of androgen treatment.

A gynaecological ultrasound was carried out in 55 of the 70 AFAB transgender people before surgery. Thirty-two examinations involved transvaginal ultrasonography, whereas the remaining 23 were transabdominal ultrasonography. In eight cases, the ovaries could not be visualized (all cases were in transabdominal ultrasounds carried out with a full bladder and visualizing the uterus but not the ovaries), whereas the ovaries were found in all the remaining participants. Normal ovaries were found on transvaginal ultrasound in four people initially diagnosed with PCOS and only one 24-year-old person, also previously diagnosed with PCOS, showed ultrasound polycystic ovary morphology criteria and AMH level above the female pre-menopausal reference range (5.4 ng / ml). Antral follicles were found in 43 out of 47 ultrasounds (91.5%) of the AFAB cohort but without the presence of a dominant follicle or corpus luteum. Endometrial thickness was collected in 31 out of 32 transvaginal ultrasound scans, and a thin linear endometrium with a thickness between 1 and 3 mm was described in all cases. Endometrial thickness, however, was not determined in the 23 transabdominal ultrasound studies.

Histological features

The surgical pieces (uterus, ovaries and fallopian tubes) weighed a mean of 80.6 ± 19.4 g, and macroscopically the ovaries did not appear enlarged and showed a smooth outer surface with a light brown parenchyma. The mean ovarian dimensions were 2.5 × 2 × 1 cm (maximum ovarian dimensions 3.5 × 2.5 × 2 and minimum 2 × 1.5 × 1 cm), and the ovarian volume was 3.1 ± 1.3 ml (range 1.3–6.2 ml). The largest diameter of both ovaries was sectioned for histological study in the 70 AFAB cohort (FIGURE 1), and the distribution of histological follicles is presented in TABLE 1.

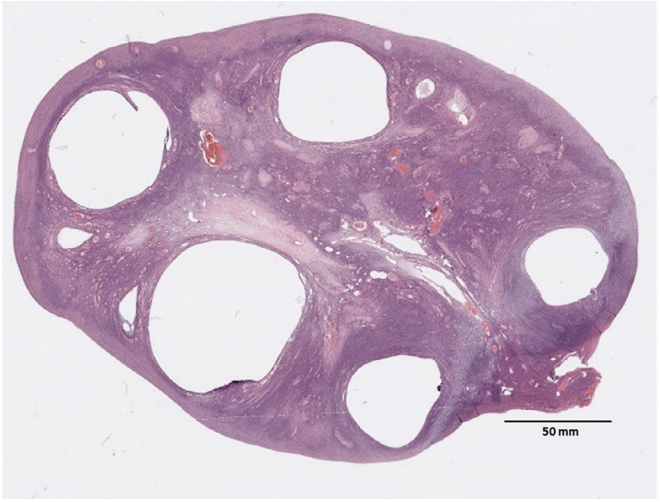

FIGURE 1 An ovarian hemisection showing follicles in different states of maturation (HE, 10x)

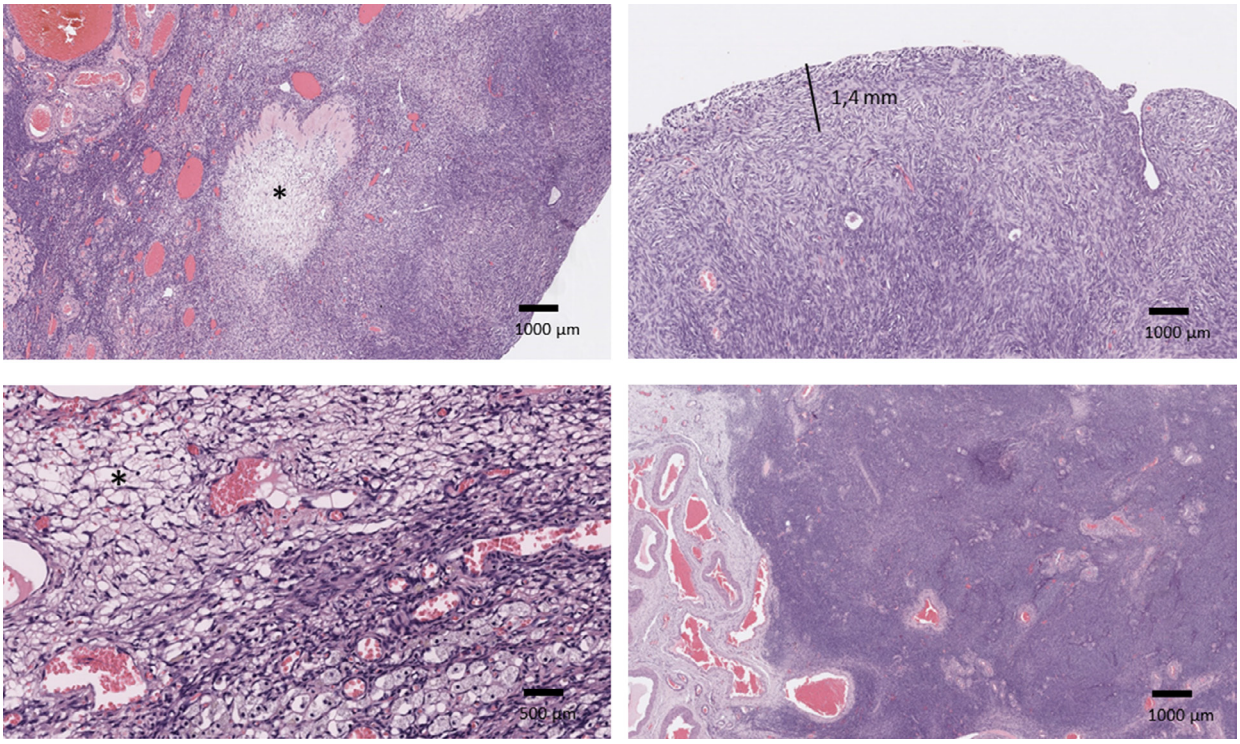

FIGURE 2 Hematoxylin and eosin stained ovarian tissue sections showing (A) a single atretic follicle, marked with an asterisk (100x); (B) tunica albuginea with 1.4 mm of thickness (100x); (C) luteinized stromal cells, marked with an asterisk (200x); (D) ovarian stromal hyperplasia (100x).

The mean count included a total of 6773 follicles, with a mean of 69.1 ± 84.9 follicles per individual (with minimum one and maximum 448 follicles per person). Most of the follicles were primordial (5957 of total follicle count [88.0%]) compared with 209 primary follicles (3.1%). Only 48 secondary follicles were found (0.7%), whereas a greater number of follicles in early antral and antral stages were found (132 and 201 follicles, corresponding to 1.9 and 3.0%). Finally, a total of 226 atretic follicles were found (3.3%) (FIGURE 2A). The mean number of antral and atretic follicles were 2.1 ± 1.9 (range: 0–8) and 2.3 ± 3.5 (range: 0–21), respectively.

The mean thickness of the tunica albuginea was 0.55 ± 0.22 mm, with a notable wide heterogeneity in thickness (range 0.15–1.45) (FIGURE 2B). Luteinization of the stromal cells was frequently observed in 96 ovaries (68.6% of the total samples) (FIGURE 2C); however, diffuse stromal hyperplasia was rare, and only found two cases (1.4% of the samples) (FIGURE 2D). A total of 24 lucent cell clusters, distinguishable from the surrounding stroma, were found in 17 ovaries from a total of 14 AFAB people.

Correlations between serum hormone levels and histological observations

A correlation analysis was carried out between the individual hormonal measurements and the number of follicles found and the corresponding developmental stage so that correlations between hormone levels assessed before surgery and the follicular population in the ovarian tissue in transgender men could be assessed. None of the serum hormone levels correlated with the number of follicles, except for a negative correlation between total testosterone levels and total antral follicles (R_s = –0.306, P = 0.029). No correlation was found between the different hormonal parameters or the follicular population and the age of the patients. The Spearman correlation coefficients are presented in TABLE 2.

DISCUSSION

The present study describes the endocrinological status of AFAB transgender people, including AMH determination, as well as a comprehensive histological study of ovaries resected during GAS.

Gender-affirming hormonal treatment in AFAB transgender people leads to an alteration of FSH, LH and oestradiol levels. Several investigators (*Spinder et al., 1989*; *Caanen et al., 2017*) have also described serum LH and FSH inhibition resulting from elevated testosterone levels, even showing excessive suppression of serum LH (<1.0 U/l). Testosterone treatment in supraphysiological doses has been reported to exert suppressive effect on gonadotrophin secretion, according to different studies. Initially this effect was assessed in rats and monkeys, describing a modification of FSH secretion (*Dubey et al., 1987*), and subsequently studied in agonadal AFAB transgender people and in hypogonadotropic hypogonadism men treated with physiological GnRH doses (*Sheckter et al., 1989*; *Spinder et al., 1989*). According to their results, normal to low serum gonadotrophin levels were found in their population and a decrease in mean FSH and LH serum levels were observed. Other recent studies in AFAB transgender people (*Ikeda et al., 2013*; *De Roo et al., 2017*) showed similar gonadotropin levels as described in the other studies. The previously published studies support the hypothesis that testosterone modifies the gonadotrophin secretion by testosterone itself or by their metabolites directly at central level (pituitary level), causing a functional

TABLE 2 CORRELATIONS BETWEEN PRE-SURGERY HORMONE LEVELS AND FOLLICULAR FINDINGS

	Follicles						
	Primordial	**Primary**	**Secondary**	**Early antral**	**Antral**	**Total Antral**	**Atresic**
FSH	−0.01	−0.06	−0.08	−0.01	−0.04	−0.05	−0.06
LH	−0.04	−0.18	−0.14	−0.14	−0.12	−0.2	−0.08
Oestradiol	−0.18	−0.21	−0.29	0.03	0.08	−0.06	−0.03
Total testosterone	−0.04	−0.10	−0.14	−0.17	−0.22	−0.30[a]	−0.07
AMH	−0.03	0.05	−0.37	0.08	0.01	0.17	0.01
Age	−0.05	−0.04	−0.02	0.05	−0.09	−0.06	0.10

Spearman's correlation coefficients reported.

[a] $P = 0.04$.

AMH, anti-Müllerian hormone.

hypogonadotropic hypogonadism. Hypogonadotropic hypogonadism is typically manifested as amenorrhoea, low or normal gonadotrophin levels, and hypoestrogenaemia without organic abnormalities (*Ferreira et al., 2013*).

One of the major findings of our study was the determination of AMH levels in a total of 45 AFAB people before surgery. Serum AMH levels were found to be within the female pre-menopausal reference ranges, although some AFAB presented higher AMH levels, results consistent to the histological data provided. Only one previous study assessed AMH levels in AFAB transgender people to date, finding even higher levels without exposing a possible pathogenic explanation of these values (*De Roo et al., 2017*). On the other hand, *Caanen et al. (2015)* described a significant decrease in serum AMH levels after 16 weeks of testosterone treatment. The period of testosterone therapy in this latter study was only 16 weeks compared with more than 2 years in the present study. Furthermore, in all cases, hormone therapy was combined with a GnRH agonist and an aromatase inhibitors, which could modify AMH secretion (*Su et al., 2013*; *Marschalek et al., 2015*) and, therefore, these methodological differences make it impossible to compare the data obtained with those of the present study.

Anti-Müllerian hormone is a glycoprotein secreted by the granulosa cells as early as in the primary follicle stage, and its secretion is maximal in the antral stage. It regulates the initial follicular recruitment by an inhibitory effect on the transition from primordial follicle to primary follicle, and it seems to play a role in the follicle growth (*Dewailly et al., 2016*). Some data suggest the existence of a relationship between androgens and AMH, proposing different pathways. On the one hand it has been postulated that androgens directly stimulate AMH secretion to maintain a predominantly androgenic intrafollicular environment (*Lebbe and Woodruff, 2013*) and, on the other hand, it has been suggested indirectly a regulation of AMH via amplification of granulosa cells sensitivity to FSH (*Dewailly et al., 2016*). Additionally, conflicting data have been published on FSH stimulation in AMH secretion in growing follicles; one study supported a positive effect (*Chan and Liu, 2014*) whereas another indicated a negative effect (*La Marca et al 2004*). Altogether, exogenous testosterone administration seems to modify AMH levels directly or indirectly by unknown endocrine pathways; it also seems to alter the AMH secretion by the central inhibition (functional hypogonadotropic hypogonadism) exerted on FSH levels. These complex effects on the hypothalamic–pituitary–ovarian axis might modify the AMH levels but, according to our results, as the follicular cortical population remain similar compared with fertile cisgender women of reproductive age (detailed below), the mean AMH levels also keep within the female pre-menopausal reference ranges.

A moderate, albeit significantly negative, correlation was observed between total testosterone levels and the total number of antral follicles in the histological samples. This may be the result of the testosterone effect at different levels: first due to pituitary inhibition, decreasing the mean FSH and LH levels and the follicular activation (*Dunaif et al., 1984*; *Mueller et al., 2007*; *Defreyne et al., 2020*). On the other hand, it could be induced by a direct local effect on the ovary, either to the previously mentioned alteration in AMH levels, regulating the initial follicular recruitment by exerting an inhibitory effect on the follicular transition, or through a mechanical effect mediated by an increase in the production of type I collagen by fibroblasts (*Jenkins et al., 2007*). The increased collagen layer propitiated by testosterone could alter follicular development, thus leading to a decrease in the growing follicles.

In the present study, five AFAB transgender people (7.1%) presented PCOS criteria before beginning testosterone therapy. This percentage is similar to that observed in the general population, which includes 5–10% of cisgender women of reproductive age (*Asunción et al., 2000*; *Wolf et al., 2018*). The prevalence of PCOS in the AFAB population has been assessed in different studies showing contradictory results. Some investigators found a high percentage of AFAB with PCOS of up to 58% of cases, but none of the papers included a control group (*Baba et al., 2007*; *Becerra-fernández et al., 2014*). According to data from a European study that included a control group of cisgender people, the prevalence of PCOS in AFAB cohort was similar to that of the control group (14.8%, versus 12.8%) and was in line with our results (*Mueller et al., 2008*).

Gynaecological ultrasound was carried out before gender-affirming surgery in a high number of AFAB transgender (almost 80%), with only one of them presenting polycystic ovarian morphology (PCOM). The other 54 ultrasound studies showed an antral follicle count per ovary within the female pre-menopausal reference range (*La Marca and Sunkara, 2013*) or non-

visualized ovaries, ruling out the presence of PCOM. The presence of PCOS was ruled out before testosterone therapy in all people by the assessment of the menstrual cycles and the presence of clinical, biochemical signs of hyperandrogenism, or both, although a baseline ultrasound could not be carried out. Only one person of the five initially diagnosed with PCOS presented ultrasound PCOM criteria before GAS. *Ikeda et al. (2013)* carried out a baseline ultrasound in 11 AFAB, finding two patients with ultrasound PCOS criteria at the start of treatment. In fact, after a histopathological study, they concluded that androgen administration may have converted their PCOM into non-PCOM ovaries (*Ikeda et al., 2013*). In contrast, another study reported that high androgen exposure in adulthood did not seem to induce PCOM (*Caanen et al., 2017*).

One of the strengths of the present study is the histological assessment of ovarian morphology by the evaluation of a complete section of the largest diameter of the ovary, providing an accurate image of the actual state of the organ and allowing all the follicle stages to be included. Macroscopic and histological features of the AFAB cohort ovaries showed similar ovarian measurements as well as a comparable ovarian volume compared with control populations from previous studies. The ovarian size was measured in sections from 18 control patients collected in autopsies (14 out of 18) or surgeries (four out of 18) in the study conducted by *Hughesdon (1982)*. Only two of the three dimensions were reported; however, the dimensions were similar (mean dimensions in control patients ranged from 3.5 × 1.2 to 1.4 × 0.8 cm compared with the AFAB cohort that ranged from 3.5 × 2.5 × 2 to 2 × 1.5 × 1 cm). *Pache et al. (1991)* compared ovarian histolopathological findings from 17 AFAB transgender people with the ovaries from 13 cisgender female of reproductive age (range 27–39 years) in longitudinal ovarian sections. The mean ovarian volume in the control group was 2.7 ± 1.4 ml (range 0.8 – 6.0 ml), comparable with 3.1 ± 1.3 ml (range 1.3–6.2 ml) in the present AFAB population. Most of the follicles studied in the present population were primordial, with fewer being primary, secondary, antral or atretic follicles. The follicular population of the ovaries was also described in fertile cisgender females aged between 19 and 30 years, revealing a similar cortical distribution (*Gougeon and Chainy, 1987*). Their work showed a cortical distribution of 87.59% primordial follicles (compared with 88.0% in our study), 6.16% primary follicles (compared with 3.1%) and 1.26% secondary follicles (compared with 0.7%). The percentage of follicular population was also described in ovarian biopsies from 60 cisgender infertile females, showing 88% of primordial follicles, and 8–4% of primary and secondary follicles respectively (*Lass et al., 1997*). In addition, the whole ovarian cortex was analysed from a 29-year-old cisgender woman with oestrogen receptor-positive breast cancer, and showed a follicular distribution of 94% primordial follicles, 5.3% primary follicles and 0.7% secondary follicles (*Schmidt et al., 2003*). On the basis of these comparisons, the cortical early stage follicle distribution apparently does not shift in ovaries exposed to testosterone therapy in the context of gender affirming hormonal therapy.

It has previously been reported that exogenous androgen increases the number of antral and atretic follicles (*Amirikia et al., 1986*; *Pache et al., 1991*), causing the ovaries of AFAB people to become similar to those found in people with PCOS. *Hughesdon (1982)* studied the antral follicle population (follicles >2 mm) in ovarian sections from 18 cisgender females and reported a mean of 2.37 follicles at this stage, comparable to data described in the present study (a mean of 2.1 ± 1.9 antral follicles). On the other hand, *Amirikia et al. (1986)* studied ovarian sections from three cisgender fertile females aged 31.3 ± 3.2 years as a control group, and reported few atretic follicles (range 2–7) compared with a mean of 2.3 ± 3.5 atretic follicles observed in our cohort (range 0–21). *Ikeda et al. (2013)* also studied these follicular populations in 10 AFAB individuals compared with control participants and reported that the number of primary, secondary, early antral and antral (>2 mm) follicles did not differ between both groups, suggesting that testosterone does not affect the number of these follicles. An increased number of atretic follicles in the AFAB cohort was also found, mainly in patients who had taken testosterone on their own, before the study. *Pache et al., (1991)* showed that control ovaries (n = 14) had neither stromal hyperplasia nor stromal luteinization, whereas 68.6% of the ovarian samples in the present study showed luteinization of the stromal cells, but rarely diffuse stromal hyperplasia. The ovaries from the present AFAB cohort had a wider cortex, with a mean thickness of 0.55 ± 0.22 mm (range 0.15–1.45) compared with 0.24 ± 0.8 mm observed in the control population from *Pache et al., 1991*. Other works also reported similar data in cisgender control females, *Hughesdon (1982)* revealed an average thickness of 0.28 mm and *Amirikia et al. (1986)* a thickness of 0.17 ± 0.29 mm, contrasting with our results.

Androgens stimulate follicle transition because they potentiate FSH-stimulated granulosa cell growth, with follicles becoming more receptive as follicular maturation progresses (*Lebbe and Woodruff, 2013*). Both our results and those of other previous studies did not demonstrate the presence of a greater number of follicles in secondary, early antral and antral stages in AFAB transgender people contrary to PCOS histology. *Vendola et al. (1998)* found that rhesus monkeys treated with testosterone or dihydrotestosterone stimulated early stages of primate ovarian follicular growth. Different and specific testosterone dosages, however, were assessed, and the administration period was short (10 days), in contrast to the present study population. Antral follicles are abundant in patients with PCOS, but *Ikeda et al. (2013)* did not find differences in the number of antral follicles between the AFAB people and the control group. Other studies did not provide clear evidence of increased antral follicle with androgen treatment (*Spinder et al., 1989*; *Grynberg et al., 2010*), owing to lack of control group or follicle count.

The collagenous layer of ovaries in AFAB people on testosterone therapy was heterogenous, with a mean of 0.55 mm, ranging from 0.1 to 1.45 mm and luteinization of the stromal cell was one of the most common findings in our samples. As mentioned previously, exogenous testosterone seems to have a direct effect on ovarian tissue, specifically on stromal and connective tissue. Nuclear staining for androgen receptors in stromal cells surrounding follicles has been described, suggesting that androgens might have some effects on these cells (*Horie and Takakura, 1992*). Moreover, an increase in the production of type I collagen by fibroblasts mediated by testosterone has been reported *in vitro* and could explain the increase and heterogeneity of the collagenous layer (*Jenkins et al., 2007*). In relation to this point, some investigators have studied the

importance of the surrounding ovarian stromal cells and extracellular matrix in the development and maturation of follicles. According to these publications a more rigid ovarian cortex is relatively non-permissive for follicle growth, and AFAB transgender people seem to present a significantly stiffer ovarian cortex compared with that derived from people with a menstrual cycle (*Woodruff et al., 2011*; *De Roo et al., 2019*).

The histological ovarian changes observed in the present study leads to questions about the fertility potential of AFAB transgender people after having initiated hormone therapy. According to our results, testosterone does not seem to alter the number and the distribution of follicles in the ovary but some changes in the cortical and stromal tissue have been reported. Limited data are currently available on ovarian stimulation outcomes among this selective group, with different studies including small sample sizes, different types of testosterone therapy and mean time of discontinuation before stimulation. Conflicting results have been reported about the number of oocytes retrieved or the oestradiol levels achieved at the end of ovarian stimulation compared with a control group (*Adeleye et al., 2019*; *Amir et al., 2020a*; *Amir et al., 2020b*). The length of time AFAB transgender should discontinue testosterone before ovarian stimulation, however, is currently unknown, and also if the stromal changes are transient or may have an effect on subsequent folliculogenesis. In the absence of complete information in AFAB people who have already started testosterone therapy, we consider it at present more appropriate to carry out oocyte–embryo cryopreservation before the start of gender affirming hormonal therapy. Larger studies are required to clarify the effect that testosterone therapy may have on the fertility potential of the ovary.

An important strength of the present study is the large number of AFAB people evaluated, including complete hormonal and histological assessment, and the largest number of AMH determinations in AFAB on hormone therapy to date. The histological study of a longitudinal section of the ovary is the most adequate to describe the total follicle population. In addition, we postulate about the role of testosterone therapy in follicular populations and also in stromal and cortical tissue, seems to modify the stiffness that might play a role in the folliculogenesis. According to our results, the follicular distribution and population might not be altered, but more studies are needed to confirm these results.

The present study has several limitations. A gynaecological ultrasound was not carried out in all AFAB cohort, but most of them considered internal examination unbearable. On the other hand, we did not perform a baseline ultrasound before starting testosterone treatment, although the presence of PCOS was ruled out in all cases, and it was not possible to determine a baseline AMH serum level to compare possible changes. A particular weakness of this study is the absence of an age-matched control group in the histological assessment. In our hospital, only a few pieces of ovarian tissue can be harvested from oncological patients and some samples can be obtained from cisgender fertile women with benign or malignant ovarian mass. Nevertheless, ovarian samples from non-oncological cisgender females with unilateral or bilateral oophorectomy for uterine fibroids or other benign gynaecological diseases was not possible to obtain. *Pavone et al. (2014)*, however, showed that the tissue surrounding an ovarian malignancy is not an appropriate control group as follicle density in the cortex is decreased. Moreover, the ovarian pieces obtained after preserving fertility in a patient with cancer differs greatly from the complete hemisection of the ovary obtained in our study and, therefore, we do not consider this group appropriate as a control.

In conclusion, the present study shows that hormone therapy undertaken by AFAB transgender people alters hormone levels, although ovaries with multiple follicles at various stages of growth were not observed. We found a similar cortical follicle distribution, however, compared with fertile cisgender women from other studies, except in relation to the presence of a higher number of atretic follicles. The ovarian cortex and stroma showed some evidence of PCO morphology, such as thickening of the tunica albuginea and luteinization of the stromal cells but not stromal hyperplasia to indicate a complete PCOM.

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ESTUDIO 2

Comparison between slow freezing and vitrification of ovarian tissue cryopreservation in assigned female at birth transgender people receiving testosterone therapy: data on histological and viability parameters.

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Resumen

El uso de técnicas de preservación de la fertilidad (PF) ha aumentado significativamente en los últimos años en la población transgénero masculina. La criopreservación de ovocitos es el método establecido para la PF, pero la criopreservación de tejido ovárico puede considerarse una opción alternativa, especialmente durante la cirugía de afirmación de género (GAS, *gender-affirming surgery* por sus siglas en inglés). La técnica de criopreservación mediante congelación lenta (SF, *slow freezing* por sus siglas en inglés) es el método estándar en el tejido ovárico humano, pero recientemente diversos estudios han mostrado buenos resultados con la técnica de vitrificación (VT). El objetivo de este estudio ha sido comparar la efectividad de ambas técnicas en tejido ovárico de personas transgénero.

Métodos: estudio prospectivo que incluye 18 hombres trans tras la GAS. Los fragmentos de tejido ovárico se criopreservaron mediante SF y VT y se compararon con tejido fresco del mismo sujeto. Se realizó un estudio mediante microscopía óptica (MO); el porcentaje de folículos viables se estudió mediante la prueba de viabilidad tisular y las diferentes células ováricas se analizaron mediante microscopía electrónica.

Resultados: La técnica de vitrificación conserva el tejido folicular y estromal de forma similar a la congelación lenta, pero con algunas diferencias. La evaluación mediante MO mostró una mejor conservación del folículo tras la VT, pero el estudio ultraestructural mostró la presencia de daños menores con ambas técnicas.

Conclusión: ambas técnicas de criopreservación son correctas para el mantenimiento la población folicular y el tejido estromal. Son necesarios más estudios para determinar el impacto de la VT en el tejido ovárico y los mecanismos de activación folicular subsiguientes en el tejido de las personas transgénero masculinas.

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FERTILITY PRESERVATION

Comparison between slow freezing and vitrification of ovarian tissue cryopreservation in assigned female at birth transgender people receiving testosterone therapy: data on histological and viability parameters

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Abstract

Purpose The use of fertility preservation (FP) techniques has significantly increased in recent years in the assigned female at birth (AFAB) transgender population. Oocyte cryopreservation is the established method for FP, but ovarian tissue cryopreservation may be considered an alternative option, especially during gender-affirming surgery (GAS). The slow freezing (SF) cryopreservation technique is the standard method for human ovarian tissue, but recently, several studies have shown good results with the vitrification (VT) technique. The objective of this study was to compare the effectiveness of VT and SF techniques in ovarian tissue from AFAB transgender people.

Methods This was a prospective study including 18 AFAB transgender people after GAS. Ovarian tissue pieces from each ovary were cryopreserved by SF and VT and compared with fresh tissue. Study by light microscopy (LM) assessed follicular morphology and density. The percentage of surviving and degenerated follicles was studied with the tissue viability test. Oocytes, granulosa cells and stroma were analysed separately by transmission electron microscopy.

Results The VT technique preserves follicle and stromal tissue as well as the SF method, but with some differences. Evaluation by LM showed better follicle preservation with VT, but the ultrastructural study showed the presence of minor damage with both techniques compared to fresh tissue.

Conclusion Both cryopreservation techniques are accurate for maintaining the follicular population and stromal tissue. Further studies are needed to determine the impact of VT on ovarian tissue and the subsequent follicular activation mechanisms in AFAB ovarian tissue.

Keywords Transgender people · Fertility preservation · Ovarian tissue cryopreservation · Vitrification

Introduction

Gender-incongruent persons require the initiation of safe and effective hormone treatment to develop the physical characteristics of their affirmed gender [1]. Assigned female at birth (AFAB) transgender people initiate testosterone therapy to achieve the secondary characteristics of the affirmed gender and maintain sex hormone levels within the normal male range.

The use of fertility preservation (FP) techniques has significantly increased in recent years in this population, because the reproductive wishes of transgender people have intensified, reaching up to 58 % at present [2]. Oocyte and embryo cryopreservations are the established methods for FP, but

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these treatments have certain drawbacks, especially if gender-affirming hormone treatment (GAHT) has already been started. There is a need to temporarily discontinue hormonal treatment, and an ovarian stimulation process increases oestrogen levels which can lead to unwanted physical changes [3]. In cases in which the cessation of hormonal treatment is not desired, ovarian tissue cryopreservation (OTC) may be considered an alternative option to preserve fertility, especially during gender-affirming surgery (GAS) in which the ovaries are removed [4].

OTC is an acceptable fertility preservation technique and is no longer considered experimental in the USA since 2019, according to the American Society for Reproductive Medicine [5]. More than 130 children have been born from this procedure worldwide although it is still considered an experimental FP technique by other European societies [6]. All the pregnancies published to date except two have been achieved in cisgender women with oncological or benign diseases through the slow freezing (SF) cryopreservation technique, which has been the standard cryopreservation method for human ovarian tissue since the end of the 1990s [4, 7, 8]. The vitrification (VT) method began in animal model studies and has been performed in different species (rabbits, monkeys, dogs, cows, rodents and sheeps) [9, 10]. Several studies in humans have compared SF protocols with different VT procedures, with conflicting results especially regarding aspects such as improvement in the survival follicle rate or the integrity of the ovarian stroma. At present, the best method of OTC remains to be determined.

In contrast to the tissue used in studies on ovarian cryopreservation in cisgender women with oncological disease or in the context of a benign gynaecological surgery, several changes mainly of the stromal and connective tissue have been described due to exposure to testosterone therapy in ovarian tissue removed during GAS (laparoscopic hysterooophorectomy) from AFAB transgender people [11–13].

OTC has shown robust results for SF procedures, but various centres worldwide have started to test VT protocols [14]. Neither of these methods has been assessed in ovarian tissue from AFAB transgender people to determine which best preserves their fertility options. Therefore, the aim of this study was to compare the effectiveness of VT and SF cryopreservation techniques in ovarian tissue from AFAB transgender people, analysing morphological and viability parameters by histological means using light and electron microscopy and fluorescent viability markers.

Materials and methods

Study design, size, duration and settings

This was a prospective study including 18 AFAB transgender people aged between 20 and 40 years, receiving testosterone therapy and who were recruited from May 2011 to January 2016 before GAS.

Participants and materials

The study population was enrolled when GAS surgery consisting of hysterectomy and bilateral adnexectomy by laparoscopy was scheduled. Clinical follow-up was carried out in the Endocrinology Department at the Hospital Clinic of Barcelona, Spain. None of the AFAB transgender people used any contraceptives before testosterone therapy or during follow-up. All had had regular menstrual cycles before GAHT. The exclusion criteria were as follows: endocrine pathology, including type 1 diabetes, uncontrolled thyroid disease (hypothyroidism or hyperthyroidism), congenital adrenal hyperplasia and different sexual development. AFAB transgender people started testosterone therapy with intramuscular testosterone undecanoate (Reandron® 1000 mg every 2 to 3 months) which was maintained for a period of 27.3 ± 4.5 months.

Physical and biochemical studies

Weight and height measurements were performed in all AFAB transgender people at recruitment, and a blood sample was taken to determine serum hormone levels. The body mass index (BMI) was calculated, and the main biochemical parameters were measured in serum in the Core Laboratory of Hospital Clínic using standard methods, and the results were expressed according to male reference ranges. Total testosterone (TT) was measured by a chemiluminometric immunoassay (Testosteron II, Elecsys; Roche, Mannheim, Germany, limit of quantification (LOQ) 12 ng/dl, interassay coefficient of variation (CV) < 5%) and was within the normal range of 275.0–850.0 ng/dl. Sex hormone binding globulin was measured using a chemiluminometric immunoassay run on the Atellica analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, LOQ 1.11 nmol/l, interassay CV < 10%), being within the normal range of 25.0–96.0 nmol/l. Androstenedione was measured by immunoradiometric assay (Cisbio assays, Codolet, France, LOQ 10 ng/dl, interassay CV < 10%) showing a normal range of 50.0–250.0 ng/dl. Follicle-stimulating hormone (FSH), luteinizing hormone (LH) and oestradiol (E_2) were measured using chemiluminometric immunoassays in an Atellica Immunochemistry analyzer (Siemens Healthineers, Tarrytown, NY, USA) with an interassay CV of < 10% and an LOQ of 0.55 mUI/ml for FSH, an interassay CV of < 5% and an LOQ of 1.44 mUI/ml for LH and, finally, an interassay CV of < 5% and an LOQ of 18.8 pg/ml for E_2. Normal FSH and LH levels were between 1.7–8.0 U/l and 1.5–7.5 U/l, respectively, while normal E_2 values were

between 10.0 and 41.0 pg/ml. Anti-Müllerian hormone (AMH) was determined in serum by chemoluminescence immunoassay with paramagnetic particles for quantitative determination (AMH B13127 Beckman Coulter kit and in the ACCES2 device, LOQ 0.02 ng/ml, interassay CV <5%), and the results were expressed in ng/ml, with normal female values being between 1.0 and 4.5. AMH determination was only performed in 7 AFAB transgender people, due to difficulties in availability.

Histopathological study

Ovarian tissue processing

After hystero-oophorectomy surgery, both ovaries were transported to the laboratory. Part of the tissue was prepared for histological study, and the remaining tissue was immediately processed. A scalpel was used for dissection of the medulla since the cortex was 1–2 mm thick, and it was cut into squares of approximately 5 × 5 × 10 mm.

A minimum of seven pieces of ovarian tissue from each ovary were studied. In all cases, three pieces were cryopreserved by the SF method and three by VT. Between two and six fresh pieces were analysed as a control. The remaining cortical pieces were cryopreserved for other research purposes, according to the patients' choice. All the samples from any of the patients were processed for each cryopreservation procedure at the same time and were warmed/thawed and analysed in parallel (Fig. 1).

The mean area studied in the ovarian samples by light microscopy was very similar in both ovaries: 103.1 ± 44.1 mm^2 in the right ovary (RO) versus 93.27 ± 38.1 mm^2 in the left ovary (LO). A single ovarian tissue piece (25–30 mm^2 approximately) was assessed by transmission electron microscopy and the tissue viability study (LIVE/DEAD Cell Viability Assays®) per ovary and per patient.

Slow freezing technique/thawing procedure

SF was performed according to a protocol described elsewhere with modifications [15]. The ovarian tissue slices were frozen using a 1.5M PrOH (Fluka Chemika, Switzerland) + 0.2M sucrose (Merck, Darmstadt, Germany) solution in 1.8 ml cryovials (Nunc, Denmark). The cryovials were kept rolling in an ice bath for 30 min at 4°C. Then the samples were placed into a computerized programmable freezer (Planner K10, Planner Ltd., UK).

The cooling programme started from 0 to −9°C at a rate of 2°C/min, and ice nucleation (seeding) was manually induced. After a 10-min holding period, the temperature was lowered to −40°C at 0.3°C/min and from −40 to −140°C at a −10°C/min. Finally, the cryovials were placed into liquid nitrogen for storage.

For the thawing procedure, the cryovials were immersed in a 37°C water bath for 2 min, and the ovarian slices were then equilibrated sequentially in 1.0M PrOH + 0.2M sucrose + 20% serum substitute (5 min); 0.5M PrOH + 0.2M sucrose + 20% serum substitute (5 min); 0.2M sucrose + 20% serum substitute (10 min); and PBS + 20% serum substitute (10 min) at room temperature.

Vitrification/warming procedure

The VT of ovarian tissue pieces was performed according to the protocol described by Kagawa with some modifications [16].

Cortex fragments were incubated in an equilibration solution composed of 7.5% ethylene glycol (Merck, Darmstadt) and 7.5% dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) in a buffer media with 20% human serum albumin (Grifols, Spain), for 25 min at room temperature followed by a 15-min incubation in a vitrification solution composed of 20% ethylene glycol, 20% DMSO and 0.5M sucrose. The samples were transferred to precooled 1.8 ml cryogenic vials and placed directly into liquid nitrogen.

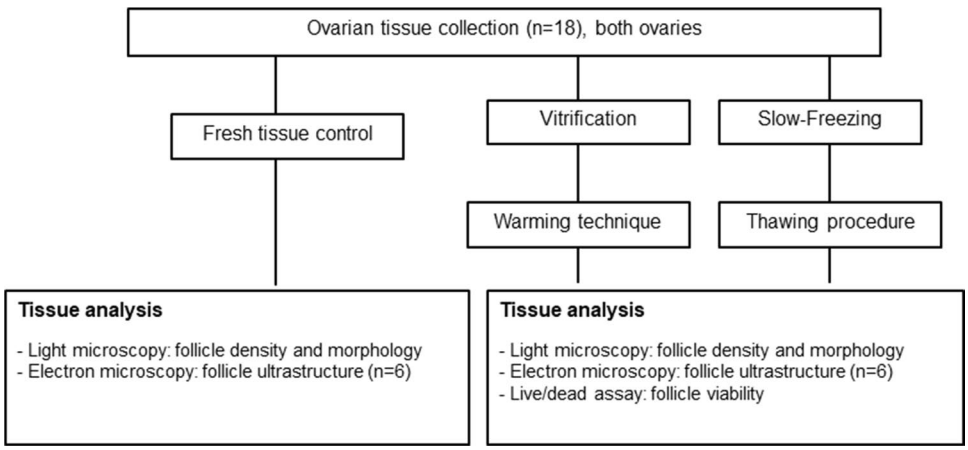

Fig 1 Study flow chart. Follicle morphology, staging, ultrastructure and viability measurements from warmed and thawed ovarian tissue were assessed (n = 18)

For warming, the cryovials were immersed in a 37°C water bath for 2 min, and the ovarian fragments were transferred to a 1M sucrose solution for 5 min and to a solution of 0.5M sucrose for 5 min. Finally, the tissue pieces were washed in buffer media.

Light microscopy evaluation

After the thawing and warming procedures had been performed, the tissue pieces were fixed in 10% formalin. All samples were routinely paraffin embedded, and 3-µm sections were made. The sections were stained with haematoxylin–eosin using standard techniques and were examined by a gynaecological pathologist to perform the ovarian follicle count of each sample.

The developmental stages of the follicles were evaluated as defined by Gougeon in 1996. We defined primordial follicles as those of 30–60 µm in diameter and containing a single layer of flat granulosa cells (GC) around the oocyte. Primary follicles were those presenting a well-defined layer of cuboidal cells and > 60 µm in diameter. Secondary follicles were defined as those presenting two or more layers of cuboidal cells and 0.12–0.2 mm in diameter, and early-antral follicles had more than two layers of cubical GC surrounding the oocyte in the presence of a follicular antrum and 0.12–2 mm in diameter.

The number of primordial follicles per millimetre square of tissue surface was assessed in the samples of both ovaries (left and right), and the follicular density of the processed pieces was also evaluated. The follicular morphology was also assessed considering the parameters previously described by Isachenko [17]: (1) normal follicle being a spherical follicle surrounded by GCs, homogeneous oocyte cytoplasm and slightly granulated nucleus, the centre of which showed objective and spherical dense chromatin and (2) degenerated follicle showing partially or totally disorganized GCs and/or an altered oocyte cytoplasm with pyknotic nuclei.

Follicles were classified on a section containing the nucleus to avoid double counting. The follicular density in fresh tissue and after VT and SF procedures was measured, and the quality of primordial follicles (expressed as percentage of normal follicles) was also investigated.

Tissue viability study (LIVE/DEAD Cell Viability Assays®)

As previously described by Hreinsson et al. 2003, one tissue piece from each ovary and each cryopreservation technique was digested with 1.5 mg/ml collagenase type II (Invitrogen, NY) in pre-equilibrated a-MEM (Invitrogen Inc.) with 10% human serum at 37°C and 5% CO_2 in a humidified incubator. After 1 h, the presence of isolated follicles was checked. If there were follicles floating in the medium, all the medium and tissue were filtered through a 100-µm filter (Gelman, Pall Life Sciences). The pieces on the filter were collected for further digestion, and the flow through medium was centrifuged at 100 g for 5 min. The pellet with a small amount of medium was checked under an inverted microscope for follicle identification. Follicles were stored in culture medium in the incubator, and this procedure was repeated. After 2±3 h digestion, a clear view of the follicles in the stroma was possible, and they were stained along with fully isolated follicles. At this point, the medium containing the tissue was centrifuged at 100 g for 5 min, and the pellet together with the individual follicles was resuspended in live/dead working solution (LIVE/DEAD Viability/Cytotoxicity Kit®) (L-3224), Molecular Probes, Eugene, OR). The working solution was PBS containing 2 mmol/l calcein AM which is converted to calcein by intracellular esterases and produces an intense green fluorescence in live cells and 4 mmol/l ethidium homodimer-1 (EthD-1), which enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, producing bright red fluorescence in dead cells. EthD-1 is excluded by the intact plasma membrane of live cells. After 30-min incubation at room temperature, the cell suspension was centrifuged again (100 g for 1 min), and most of the supernatant was removed. Partly and fully isolated follicles were mounted onto glass slides. After the cover slides were mounted, the viability of the follicles was assessed under a fluorescence microscope. The follicles were classified depending on the staining: viable follicles (green colour) and degenerated follicles (bright red colour). The percentage of surviving and degenerated follicles was studied depending on the cryopreservation technique used.

Transmission electron microscopy analysis

Ovarian tissue samples were collected, placed and fixed (glutaraldehyde 2.5% in 0.1M phosphate buffer). The samples were cut into 1 mm^3 fragments and kept for 2–4 h at 4 °C. Washings were carried out with 0.1 M phosphate buffer (3 × 10 min at 4 °C) and post fixation with 1% osmium tetraoxide (with 0.8 % potassium ferricyanide) in 0.1M phosphate buffer for 1–2 h at 4 °C. New washes were carried out with distilled water (3 × 10 min at 4 ×C), and afterwards, the samples were dehydrated with ketones of different duration depending on the percentage of solvent used (with 50 % solvent, 1 wash was carried out for 10 min at 4 °C). After these steps, the material was included, usually with Spurr and later the infiltration was carried out, varying the time according to the type of tissue to be infiltrated and the size of the sample. Subsequently, the tissue blocks were made and polymerized at 60 °C for 48 h. The sample obtained was then prepared for the study by transmission electron microscopy (TEM).

Oocytes, GC and stroma were analysed separately. In the first two structures (oocytes and GC), the nuclear content, the integrity of the membrane, the density of the cytoplasm, the cytoplasmic organelles and the intracellular contact were evaluated. The nuclear content and the integrity of the extracellular matrix in the stromal cells were also assessed.

The structural changes were evaluated using a score system, with which the value 2 is assigned to the normality of the assessed aspects, the value 1 is given when the observed changes are minimal (slight alterations of the assessed structure) and the value 0 is assigned when the changes shown are severe (according to the criteria established by Hreinsson et al.) [18].

Statistical analysis

The statistical analyses were performed with IMB SPSS Statistics 23.0 (IBM Corp., New York, USA). Normality was tested using the Kolmogorov-Smirnov test. The ANOVA test was used to compare follicular density between samples, and the Pearson chi-square test was used to assess differences in the proportions of viable follicles in light microscopy and after the tissue viability study. The non-parametric Kruskal-Wallis test was used to analyse differences in the scores between groups after assessment by TEM. Correlation analysis between clinical features, hormone levels and the follicular population of the samples was performed using Spearman's test. The level of statistical significance was set at $p < 0.05$.

Ethical approval

This clinical study was conducted according to the Declaration of Helsinki for Medical Research Involving Human Subjects [19]. The study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona (registry number 2011/6272) in 2011. All subjects provided written, informed consent to participate in the study.

Results

Clinical data

An overview of the characteristics of the AFAB transgender cohort including hormonal status is shown in Table 1.

The mean age of the study population at the time of surgery was 26.6 ± 5.5 years (range 20–40). None of the AFAB transgender people started androgens on their own before testosterone therapy, and all received Reandron® 1000 mg every 2 to 3 months.

All the AFAB transgender people became amenorrheic months after starting hormone therapy. None presented vaginal bleeding or spotting after the first 6–8 months of testosterone therapy. At the time of recruitment, when GAS was scheduled, the mean GAHT time was 27.3 ± 4.5 months, and all the participants showed serum testosterone levels within the male physiological range (mean 698.5 ± 309.3 ng/dl) and complete virilization. Fourteen AFAB transgender people (77.8 %) showed serum FSH levels within female pre-menopausal reference ranges (4.5–10.0 U/l) compared to 4 AFAB transgender people presenting suppressed serum levels (minimum value 1.2 U/l). Nine AFAB transgender people (50%) showed suppressed serum LH levels, with a minimum value of 0.1 U/l in one person. AMH determination was performed in a total of 7 AFAB transgender people, with a mean of 2.2 ± 0.5 ng/ml (range 1.4–3.1).

Table 1 Clinical and hormonal characteristics of assigned female at birth transgender people ($N = 18$) before gender affirmation surgery.

Clinical features		
Parameter	Mean (range)	Reference values
Age, years	26.6 ± 5.5 (20–40)	NA
Weight, kg	59.6 ± 5.2 (45–65)	NA
Height, m	1.6 ± 0.1 (1.5–1.9)	NA
BMI, kg/m^2	22.5 ± 2.6 (16.8–25.0)	20.0–25.0
FSH, U/l	5.6 ± 2.4 (1.2–9.2)	1.7–8.0
LH, U/l	3.5 ± 2.6 (0.1–9.5)	1.5–7.5
Oestradiol, pg/ml	45.5 ± 7.8 (34.0–59.0)	10.0–41.0
Total testosterone, ng/ml	698.5 ± 309.3 (309.0–1224.0)	275.0–850.0
SHBG, nmol/l	32.5 ± 15.3 (15.7–64.2)	25.0–96.0
Androstenedione, ng/dl	260.8 ± 58.1 (171.0–310.0)	50.0–250.0
AMH, ng/ml*	2.2 ± 0.5 (1.4–3.1)	1.0–4.5

Acronyms: *BMI* body mass index, *FSH* follicle-stimulating hormone, *LH*, luteinizing hormone, *TT* total testosterone, *SHBG* sex hormone binding globulin, *NA* not applicable

*$n = 18$; anti-Müllerian hormone (AMH) was available for 7 people

Histological results

Light microscopy

Primordial follicles in fresh tissue samples from the left (LO) and right ovaries (RO) of each person were assessed. More than 2000 follicles were studied, and significant variability was found in the different tissue samples. The mean value was 254.62 primordial follicles per sample (range between 8 and 1508). An average of 137.54 follicles in the RO (range 5–970) was found versus 120.15 follicles in the LO (range 1–538 follicles per fragment). Therefore, a heterogeneous distribution of the follicles within the ovarian cortex was observed, but the mean number of primordial follicles remained similar in each ovary ($p > 0, 05$). The follicular density in fresh samples was obtained, with a mean of 1.07 fols/mm^2 (range 0.16–2.71). This value was similar both in the RO (mean of 1.0 fols/mm^2) and in the LO (1.3 fols/mm^2) ($p = 0.09$).

No correlation was found between the different hormonal parameters or patient age and the primordial follicular count.

One tissue sample from each ovary and patient was assessed after SF-thawing and VT-warming (Table 2). The overall evaluation of the thawed/warmed samples showed a greater proportion of grade 1 follicles in the VT-warmed (51.9 %) versus the SF-thawed pieces (43.2 %), but the difference was not statistically significant ($p = 0.7$). No differences were found between the follicular morphology in both cryopreserved tissue samples compared to the control ones (fresh tissue) that showed 59.2 % of grade 1 follicles and 40.8% of grade 2 ones ($p = 0.08$). The follicular density remained stable in both cryopreserved tissue samples, and comparison between the two techniques showed no statistically significant differences ($p = 0.22$). Fig. 2 shows follicular population data from ovaries in fresh pieces, SF-thawed tissue and VT-warmed tissue.

No statistically significant differences were found according to the ovary analysed in the processed tissue samples (thawed and warmed) according to the proportion of grade 1 or 2 follicles or follicular density.

Tissue viability study

The viability study was carried out using the LIVE/DEAD Cell Viability Assays® with fluorescent markers. One tissue piece from each patient and cryopreservation technique was assessed, and all the samples were processed using the aforementioned substances carrying out the subsequent incubation and centrifugation, after which a supernatant was obtained in which the follicles were assessed and identified under a fluorescence microscope. Follicles that exclusively exhibited esterase activity (green colour) were considered viable in the test, while those that stained red were considered non-viable (Fig. 3). The comparison between the two cryopreservation techniques showed no differences in relation to the viability parameters ($p = 0.10$), although there was a trend towards better results with the VT-warmed procedure compared to SF-thawed technique.

Transmission electron microscopy study

Ovarian tissue samples from a total of 6 AFAB transgender people were evaluated, including fresh ovarian and VT and SF cryopreservation samples. A total of 22 follicles were assessed, comprising 22 oocytes and 132 GC and their surrounding stroma.

Seven oocytes and 46 GC from fresh tissue were studied, while eight oocytes and 51 GC from VT-warmed samples and seven oocytes and 35 GC from SF-thawed samples were evaluated. Stromal cells and extracellular matrix were also studied in all the processed samples.

The following items were assessed in each of the structures mentioned above: (1) nuclear content (oocytes, GC and stromal cells); (2) membrane integrity (oocytes and GC); (3) density of the cytoplasm (oocytes and GC); (4) cytoplasmic organelles (oocytes and GC); (5) intracellular contact (oocytes and GC); and (6) integrity of the extracellular

Table 2 Description of different histological parameters in fresh tissue and after slow freezing and vitrification in the study population ($N = 18$).

	SF-thawed tissue	VT-warmed tissue	*P* value
LM study*			
Grade 1 follicles	48.64 ± 45.03 (43.2%)	49.0 ± 41.9 (51.9%)	*P* > 005
Grade 2 follicles	72.36 ± 62.4 (56.8%)	45.29 ± 28.22 (48.1%)	*P* > 005
Follicular density	1.29 ±1.08	1.2 ± 0.8	*P* > 005
Tissue viability study^			
Viable follicles	230 (66.9%)	248 (71.3%)	*P* > 005
Non-viable follicles	114 (33.1%)	100 (28.7%)	*P* > 005

Acronyms: *LM* light microscopy, *SF* slow-freezing, *VT* vitrification

*Data in LM study is shown as mean follicles ± standard deviation (percentage)

^Data in tissue viability study is shown as follicle number (percentage)

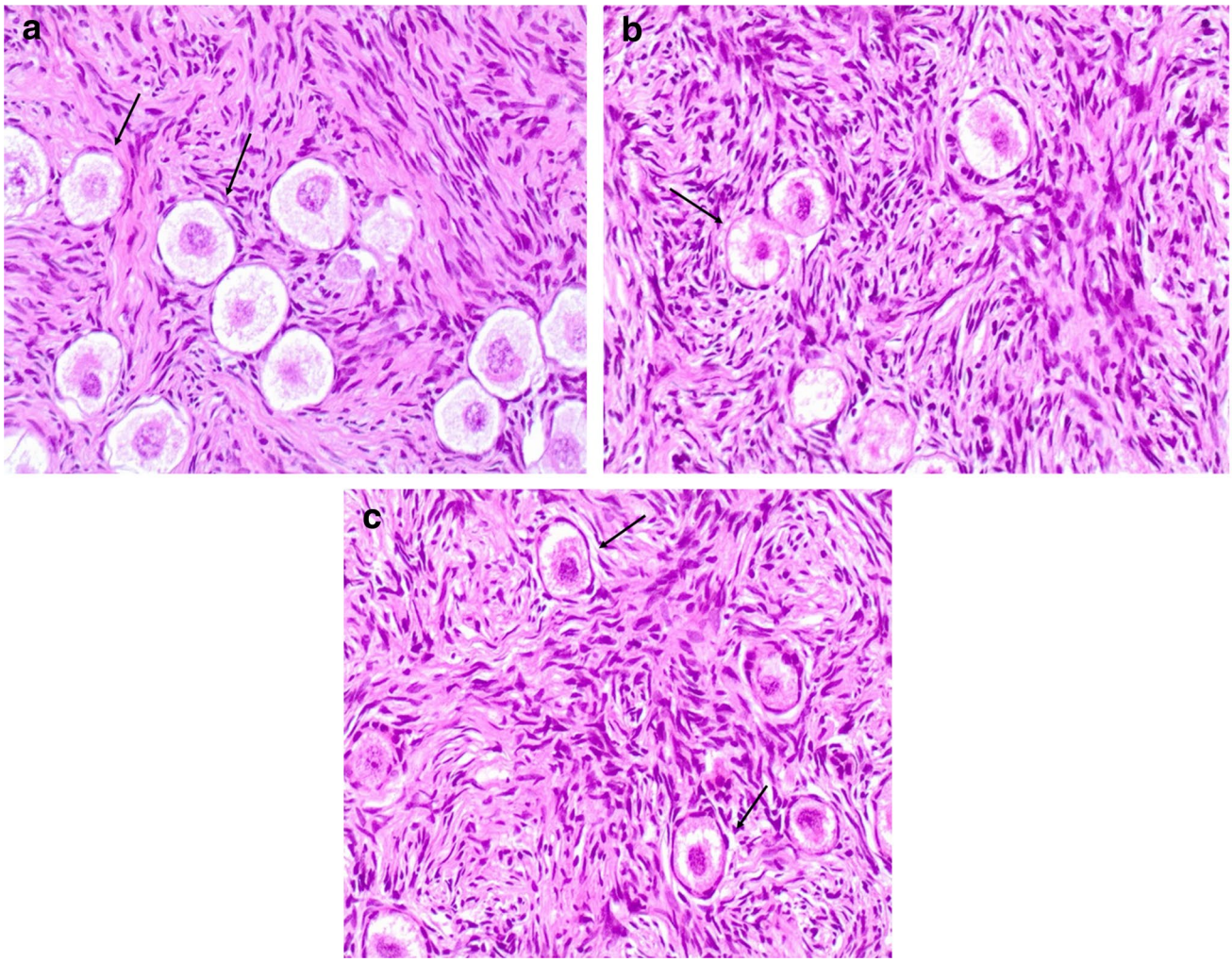

Fig 2 Haematoxylin–eosin stained ovarian tissue sections showing primordial follicles (marked with black arrows) in **a** fresh sample (200×), **b** thawed ovarian piece (200×), and **c** warmed tissue (200×).

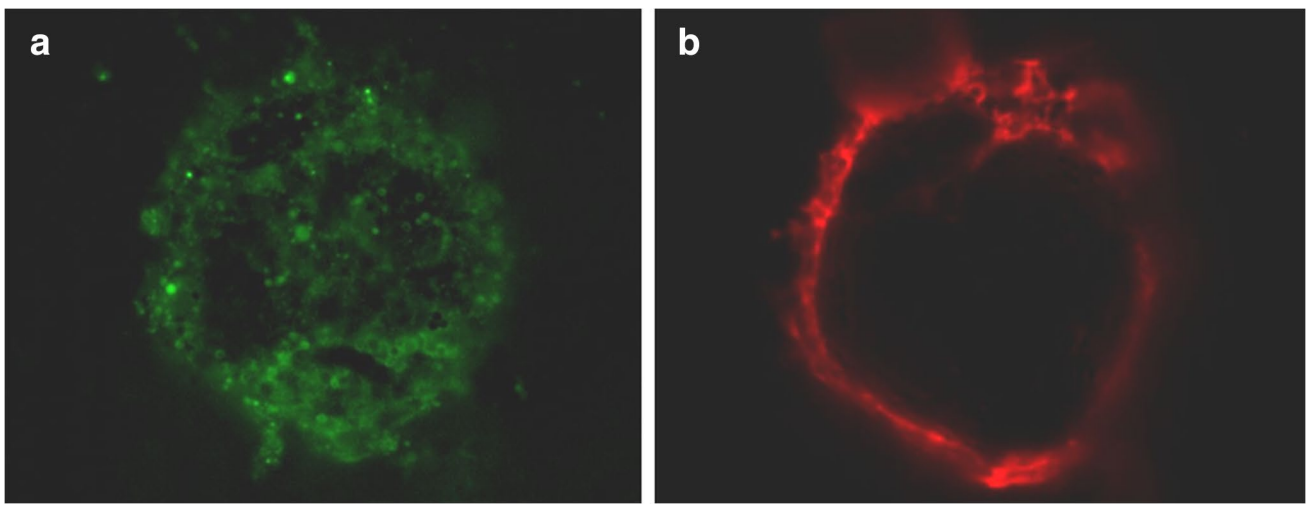

Fig 3 Follicles after LIVE/DEAD assay kit staining: viable follicle (**a**, green) and atretic follicle (**b**, red)

matrix (stromal cells). Structural changes were evaluated using a scoring system (0–2), as mentioned previously.

The ultrastructure of the oocytes did not vary among groups ($p = 0.19$). The number of oocytes containing pyknotic nuclei in VT-warmed or SF-thawed follicles did not differ ($p = 0.9$), but it was of note that some follicles in both techniques showed the presence of a non-rounded nucleus, with a wavy irregular shape compared to control ones (Fig. 4 a, b and c). The cytoplasm was well organized in all cases, containing easily visible microtubules, with no differences on comparing the two techniques ($p = 0.8$). The presence of vacuoles in some oocytes was observed, both in VT-warmed and in SF-thawed oocytes.

Abundant mitochondria with uniform crests were observed in all the oocytes studied, although a few dilated or even oedematous mitochondria were observed in various oocytes, both in VT and SF compared to fresh oocytes (Fig. 5a, b). Organelles such as the Golgi apparatus and lysosomes were correctly preserved in most oocytes. The oocyte cell membrane was well preserved in VT-warmed as well as in SF-thawed follicles compared to fresh follicles, but it should be noted that in some VT-warmed follicles, the oocyte membrane and intercellular space were slightly increased (Fig. 6), without reaching statistical significance ($p = 0.08$).

Granulosa cells surrounding the oocyte were evaluated carefully. Most were well preserved, and close adhesion between oocyte and GC was observed. Junction areas were clearly visible, and in most cases, the nucleus appeared as a homogeneous structure with partial densities and a dense aggregation of heterochromatin adhered to the nuclear membrane. Few atretic GC were observed in SF-thawed follicles, with pyknotic nuclei and granular cytoplasm (Fig. 7).

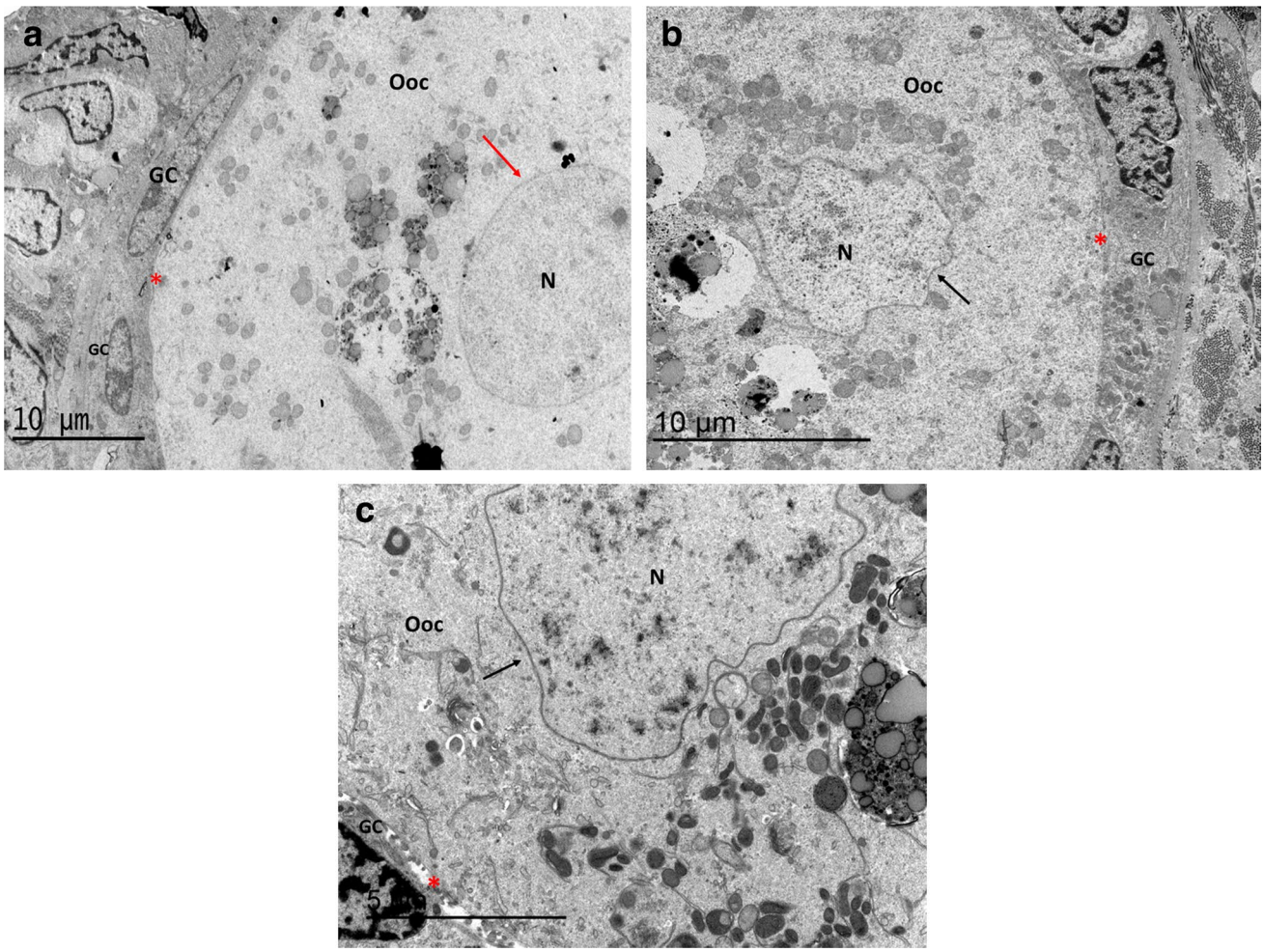

Fig 4. Transmission electron microscopy images of follicles within pieces of non-frozen (control) AFAB transgender ovarian tissue (**a**) (bar 10 µm), SF-thawed tissue (**b**) (bar 10 µm) and tissue cryopreserved using VT-warming protocol (**c**) (bar 5 µm). The oocyte (Ooc) is surrounded by one layer of flattened granulosa cells (GC) that are attached to the basement membrane (red asterisk). The magnification of the images shows the oocyte nuclei with euchromatin (N). SF-thawed and VT-warmed follicles show an irregular shaped nucleus membrane (black arrows) compared to the nucleus membrane from fresh follicle that appear regular and intact (red arrow).

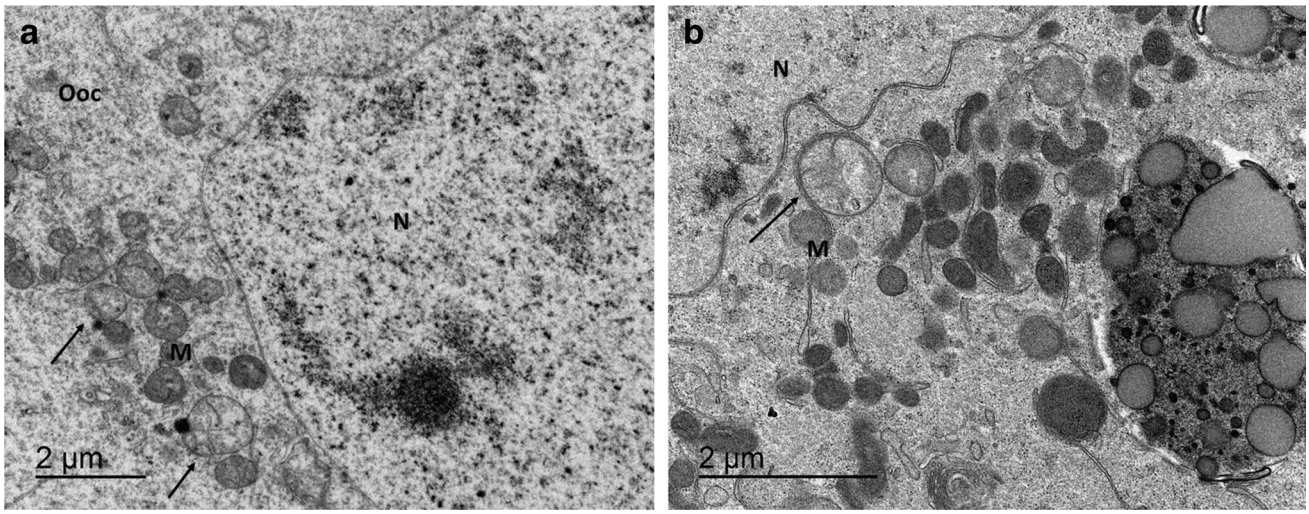

Fig 5 A high magnification of the transmission electron microscopy images of the oocytes (Ooc) of SF-thawed follicle (**a**) (bar 2 µm) and VT-warmed one (**b**) (bar 2 µm) shows abundant mitochondria (M) with uniform cristae grouped around the nucleus (N). The presence of some oedematous mitochondria can be distinguished, marked by black arrows

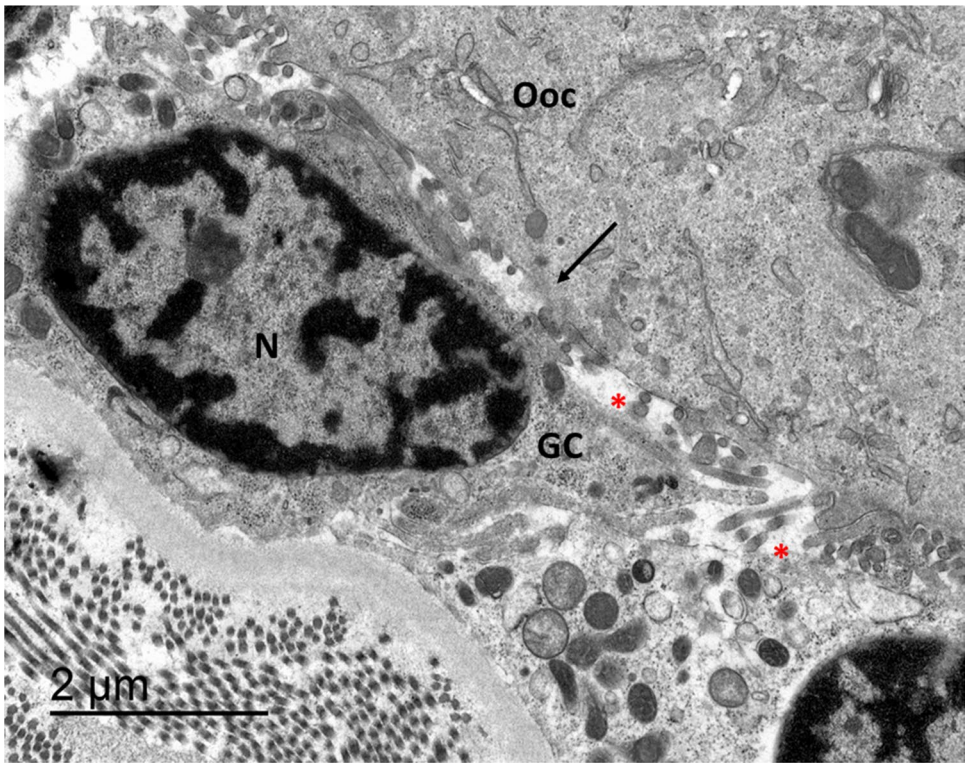

Fig 6 Detail of VT-warmed follicle with a granulosa cell (GC) and an oocyte (Ooc) (bar 2 µm). The granulosa cell shows a well-preserved nucleus (N) and is attached to thin basement membrane of the oocyte (black arrow) but shows a partially detached and expanded intercellular space, marked with red asterisks.

On the other hand, alterations in the cell cytoplasm were observed in some VT-warmed GC, highlighting vacuolization in the endoplasmic reticulum, an early sign of necrosis. In addition, lipid vacuoles were also observed in these GC, which were not observed in SF or fresh follicles (Fig. 8).

The morphology of the stroma, composed of collagen fibres and spindle-shaped fibroblast-like, was well-preserved and very similar among the three processed samples ($p= 0.4$). A higher magnification evaluation showed that the stromal cell nuclei exhibited a normal morphology, and the presence of some mitochondria was observed. Stromal collagen fibre disruption was not observed in either transverse or longitudinal sections in any of the samples studied.

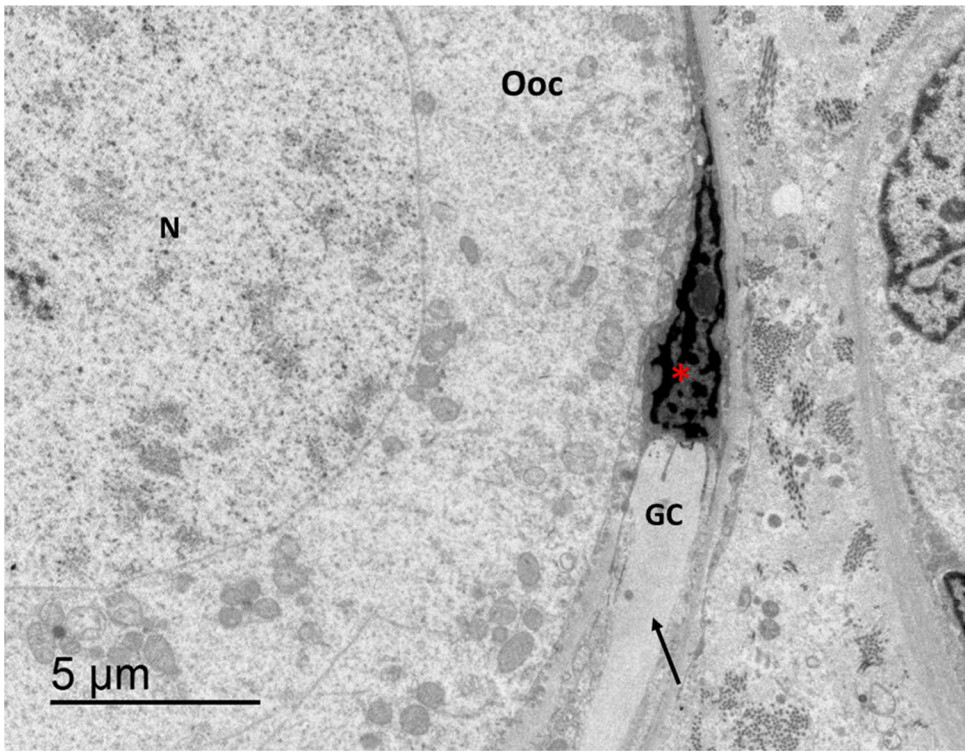

Fig 7 Detail of SF-thawed follicle with a granulosa cell (GC) and an oocyte (Ooc), with a well-preserved nucleus with homogenous euchromatin (N) (bar 5 µm). The granulosa cell shows clear signs of apoptosis: pyknotic nucleus, marked with a red asterisk, and granular and condensed cytoplasm, marked with a black arrow.

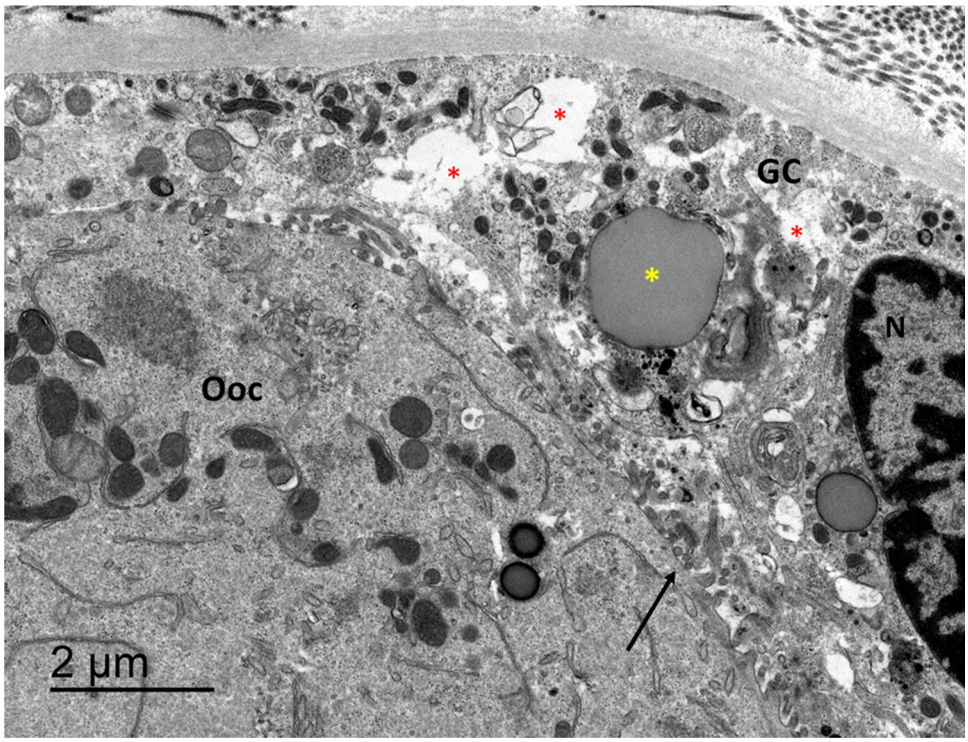

Fig 8 High magnification of the transmission electron microscopy image of a VT-warmed follicle that shows an oocyte (Ooc) and a granulosa cell (GC) attached to the thin basement membrane (black arrow) (bar 2 µm). A detailed observation of the granulosa cell shows a well-preserved nucleus (N), but a vacuolization in the endoplasmic reticulum can be observed forming empty spaces (red asterisks) and also a big lipid vacuole (yellow asterisk).

Discussion

The present study is the first to evaluate and compare two cryopreservation techniques in ovarian tissue from AFAB transgender people. The results indicate that VT preserves follicle and stroma morphology as well as the SF method, but with some differences. While evaluation by light microscopy showed better follicle preservation with VT and similar follicular density, the ultrastructural

study exhibited the presence of minor changes with both techniques, mainly in the preservation of GC.

OTC is a FP technique recommended in cisgender female patients undergoing gonadotoxic treatment in whom oocyte/embryo cryopreservation is not feasible, or for patient preference. According to the European Society of Human Reproduction and Embryology guideline on FP, OTC can be proposed as an experimental option when ovaries are removed during GAS in transgender men [4] and is no longer considered experimental and can be used in prepubertal patients or when there is not time for ovarian stimulation according to the American Society for Reproductive Medicine [5]. At present, GAHT is one of the indications for FP according to various organizations and recent literature since testosterone therapy may adversely affect fertility and GAS may involve the removal of both ovaries [1, 20, 21]. Different studies have evaluated the wishes of up to 47% of transgender individuals to have children with whom they are genetically related [22]. In a previous study, Wierckx et al. reported that up to 54% of transgender men manifested a desire for children, and 37.5% would have considered freezing germ cells had it been offered [23].

Several studies have reported that many transgender individuals are eager to initiate GAHT, with the number of AFAB transgender young adults and adolescents referred to clinics increasing, and some even choose to start GAHT during adolescence [2, 24]. Due to the young age of these AFAB transgender people, they may not yet be concerned about a future with biological children; however, it is likely that some may develop a desire to have biological children later in life [25, 26].

Given that oocyte cryopreservation requires discontinuation of testosterone therapy for varying lengths of time before starting ovarian stimulation (OS) (ranging from 1 to 12 months according to previous studies), almost all transgender men will experience menses, usually within 3 months of stopping testosterone, and this can cause gender dysphoria [27–32]. Moreover, in these cases, the increase of oestrogen levels during OS can cause mental distress and adversely affected the view of their bodies [33, 34]. In addition, this process can be an expensive medical procedure in some countries, and cost could be a major barrier preventing AFAB transgender people from pursuing FP. OTC, however, can be performed at the same time as GAS without the need for OS or gynaecologic examination, but no publication has evaluated OTC in the transgender population [24].

Previous reports performing systematic comparisons of the two cryopreservation procedures have been carried out in human ovarian tissue from cisgender females with different and contradictory results [35–38]. Only two studies have been performed with ovarian tissue from AFAB transgender people after GAS (n total = 20) to test VT protocols [39, 40]. In these two studies, comparison between the two available cryopreservation techniques was not made, but only the use of VT as a technique for the preservation of ovarian tissue was validated.

All other studies have compared the two techniques with ovarian tissue from cisgender women of reproductive age with an oncological disease or benign gynaecological pathology. According to our results, the study of follicular morphology by light microscopy showed that 51.9 % of the VT-warmed follicles were considered normal follicles, compared to 43.2 % of the thawed follicles. No statistical differences were found, although a clear trend towards better preservation was observed with the VT method. Li et al. [41] also described similar results with both cryopreservation techniques, although a higher percentage of follicles in both groups had a normal appearance (80.3 % in VT group after warming vs. 72.6 % in SF group after thawing). Other publications comparing the follicular morphology between cryopreservation techniques reported similar follicular normality rate [–, 17, 42, 43], while some groups reported clearly worse results after the VT procedure [35, 44–47]. On the other hand, histomorphometric analysis in our study showed that follicular densities were comparable between fresh, warmed and thawed tissues, in agreement with previous studies [48], while other authors reported a significant decrease in the follicular population after cryopreservation techniques and mainly after the VT procedure [44, 45]. In addition, two recent studies have reported in vitro and xenotransplantation data confirming worse results of follicular density after the VT procedure [47, 49]. Of note is the great heterogeneity in the preservation protocols applied and/or the devices used to cryopreserve the tissue, to which the ovarian components may be particularly sensitive to [43, 50, 51]. Regarding this issue, Fabbri et al. [50] studied ovarian tissue from six cisgender women with oncological diseases and compared 4 cryopreservation techniques (2 SF procedures and 2 VT protocol with 5- or 10-min incubation in each vitrification solution) and concluded that samples showed cryodamage of small entity with both techniques and different protocols compared to fresh samples, but no differences were found among the four techniques. In our study, the protocols used were previously published by Fabbri and Kagawa, who established groups with experience in ovarian tissue preservation [40, 50, 52, 53], with slight differences being found in our samples.

Little data is available regarding the ultrastructural study of the oocytes, GC and stroma. The same study described above showed no differences in TEM between protocols, although warmed samples showed oocyte nuclei with slightly thickened chromatin and irregular shapes [50]. In agreement with this study, our results showed good preservation of the follicles with both cryopreservation techniques, although GC showed poor preservation with VT and SF compared to fresh cells. In some VT-warmed follicles,

the oocyte membrane and intercellular space were slightly increased, but important and significant alterations were not observed in the ultrastructural assessment, and no follicle destruction by ice formation was noted, contrary to what was described by Lee et al. [47]. This latter study assessed primordial follicles in three ovarian samples from cisgender women who underwent benign ovarian surgery, and according to their results, many of the VT-warmed follicles and stroma were deformed and destroyed by ice crystal formation. Follicles from the SF and control group were intact and showed no morphological deformation. On the contrary, Keros et al. [36] compared 4 cryopreservation techniques (2 SF procedures with different cryoprotectants and 2 VT protocols with 5 or 10 min of incubation in each vitrification solution) and concluded that the ovarian stroma was better preserved by VT than SF, whereas more necrotic cells and empty areas were found in the stromal tissue after the SF technique. Slightly different results regarding preservation of the GC and stromal tissue were observed in our study compared to this last study. These differences may be due to the ovarian tissue samples, since in our study the pieces were obtained from AFAB transgender people who present differential characteristics with respect to the ovarian tissue of cisgender females.

Initial publications in the AFAB cohort reported that ovaries removed during GAS showed polycystic ovary morphology (PCOM) features [54–57] according to Stein-Leventhal criteria [58]. In the last decade, five groups have studied ovarian morphology in an AFAB cohort after initiation of GAHT. According to the studies by Grynberg et al. and Loverro et al. [59, 60], the morphology and histology of the majority of ovarian specimens (79–82%) resembled that of PCOS. However, other recent studies revealed a normal follicular distribution compared with controls [11–13]. Two of the studies found a higher proportion of atretic follicles within the AFAB group compared with age and BMI-matched controls or with published literature [11, 13]. Among the histological changes described, a thicker ovarian cortex, more hyperplastic collagen, ovarian stromal hyperplasia and stromal luteinization were of note.

Based on the data currently available and given that a normal follicular distribution has been observed but stromal changes have been described in AFAB people with testosterone therapy, both VT and SF seem to be good techniques to preserve oocytes and stromal tissue, with similar results in ovarian tissue samples from cisgender women and AFAB transgender people. On the other hand, both procedures showed minor changes in the preservation of GC compared to fresh samples, perhaps due to the differential characteristics of ovarian tissue from the AFAB cohort with GAHT.

Otherwise, OTC as an option for FP remains in the developmental phase, while the method to mature the immature follicles is being perfected [61]. Some authors [62, 63] have reported that during the OTC, cumulus-oocyte complexes could be found, and in vitro maturation (IVM) could be possible in AFAB transgender people who had not stopped GAHT prior to oophorectomy. Spindle analysis performed in these studies found a normal chromosomal pattern in 87% of the metaphase II oocytes obtained. However, achievement of metaphase II does not equate the ability for successful fertilization, and, to date, successful fertilization and embryo implantation following IVM of an immature oocyte from a AFAB population has not been reported [61, 64]. It is necessary to increase our knowledge regarding OTC and the subsequent in vitro culture of ovarian tissue and IVM of oocytes in order to activate, mature and use primordial follicle pools in patients who are not eligible for transplantation, as the transgender people [63]. Nowadays, efficient use of the cryopreserved cortical follicular reserve remains a challenge in this population, and techniques of in vitro growth combined with IVM are currently being developed in non-human and human primates [65].

An important strength of this study is that it is the first to compare SF and VT cryopreservation techniques in AFAB transgender people on GAHT, including hormonal assessment and histomorphometric study by light microscopy and TEM and also with viability investigation of the follicles. Histological study describing normal follicular population and density along with the ultrastructural description of the ovarian cells is the most adequate to assess the cryopreservation techniques. In addition, we discuss the differences found with both procedures in AFAB transgender people. According to our results, both cryopreservation techniques preserve follicles and stroma correctly, but with some differences. While VT showed better follicle preservation according to light microscopy study, the ultrastructural study exhibited minor cryoinjury levels with both techniques compared to the fresh samples. More studies are needed to confirm these results.

Our study also has several limitations. Only ovarian tissue samples from six AFAB transgender people were evaluated by TEM, but the assessment of the follicles and surrounding stroma was comprehensive and from fresh and processed samples by both techniques. Moreover, the absence of a control group is another limitation. The same ovarian tissue from AFAB transgender people has been considered as a control group, and some pieces were evaluated in fresh, as a control. In our hospital, only a few pieces of ovarian tissue can be harvested from oncological patients, and some samples can be obtained from cisgender fertile women with ovarian masses. However, Pavone et al. [66] showed that the tissue surrounding an ovarian malignancy is not an appropriate control group as follicle density in the cortex is decreased, and therefore, we do not consider this group suitable as a control. On the other hand, the lack of functional tests that would allow determining whether the damage to

follicles and stroma was reversible or not is another limitation. Further investigations such as xeno-transplantation might give information about viability of the follicles within the tissue.

In conclusion, this study shows that OTC by VT and SF cryopreservation techniques is effective to maintain the follicular population and the stromal tissue. Further studies are needed to determine the impact of VT on ovarian tissue and evaluate the subsequent follicular activation mechanisms with a view to the use of this tissue for FP in the AFAB transgender population.

Author contribution All authors contributed to the study conception and design. Material preparation was performed by Josep Maria Calafell, Adela Saco and Aina Borrás. Data collection and analysis were performed by Aina Borrás, Inés Agustí, Sara Peralta and Gemms Casals. The original draft preparation was performed by Aina Borrás. The study supervision was performed by Dolors Manau, Francesc Fabregues and Francisco Carmona. All authors commented on previous versions of the manuscript and approved the final version of article submitted.

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Declarations

It was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects [19]. The study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona (registry number 2011/6272) in 2011. All subjects provided written, informed consent to participate in the study.

Competing interests The authors declare no competing interests.

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5. DISCUSIÓN

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La presente tesis describe el estado endocrinológico de las personas transgénero masculinas, así como realiza un estudio histológico completo de los ovarios extirpados durante las GAS. Además, expone los resultados de la comparación de dos técnicas de criopreservación de tejido ovárico, no descritas previamente en hombres trans.

Diversos estudios, publicados a lo largo de los años, han expuesto que la terapia con testosterona a dosis suprafisiológicas ejerce un efecto supresor en sobre la secreción de gonadotropinas, tanto en modelo animal como humano (59). Los los estudios de la presente Tesis corroboran estos datos, mostrando que la terapia hormonal de afirmación de género (GAHT) altera los niveles hormonales ejerciendo un efecto directo a nivel central (hipofisario). Tanto estudios previos en hombres con hipogonadismo hipogonadotrópico tratados con dosis fisiológicas de GnRH como en hombres trans corroboran estos resultados, apoyando la hipótesis de que la testosterona modifica la secreción de gonadotropinas al afectar el eje hipotálamo-hipofisario-ovárico, provocando un hipogonadismo hipogonadotrópico funcional (22,28,60–63).

La determinación de los niveles de hormona antimülleriana (AMH, por sus siglas en inglés) es uno de los principales hallazgos de la presente Tesis, hallándose ésta en la mayoría de los casos dentro del rango de referencia de la mujer premenopáusica. Escasos estudios han valorado esta hormona en hombres trans, sobre todo tras el inicio de la GAHT (27,63–65). En tres de éstos, la población se administraba concomitantemente otros tratamientos hormonales (análogos de la GnRH, inhibidores de la aromatasa o gestágenos) y / o la duración del tratamiento era escasa (12 y 16 semanas), por lo que estas diferencias metodológicas hacen imposible comparar los datos obtenidos con los de nuestro estudio. Únicamente la publicación realizada por *De Roo et al.* (27) halla valores similares a los de nuestro estudio, e incluso más elevados en su población de 35 hombres trans.

Algunos datos han sugerido la existencia de una relación entre los andrógenos y la AMH, aunque muy compleja y poco conocida (66,67). Según estos datos, la administración de testosterona exógena podría modificar los niveles de AMH de forma directa o indirecta por vías endocrinas desconocidas. A pesar de ello, dado que la población cortical folicular sigue siendo similar a la de las mujeres cisgénero en edad reproductiva según nuestros resultados, ello explicaría que los niveles medios de AMH también se mantengan dentro los rangos de referencia femeninos.

Por otra parte, hemos observado una correlación moderada, aunque significativamente negativa, entre los niveles de testosterona total y el número de folículos antrales en las muestras histológicas. Esta relación inversa podría ser el resultado del efecto de la testosterona a diferentes niveles: por una parte debido a la inhibición hipofisaria ya comentada, que conllevaría una inactivación folicular y un menor reclutamiento de folículos (68–70); además de un efecto local directo sobre el ovario, mediante la alteración de las propiedades físicas de la corteza ovárica, como un aumento de la colagenización subepitelial y alteraciones estromales (26,27,71). Diversas investigaciones han destacado el papel de las células estromales ováricas circundantes y la matriz extracelular en el desarrollo y maduración de los folículos, postulando que una corteza ovárica más rígida sería relativamente menos permisiva para su crecimiento, como sucedería en las personas trans masculinas en comparación con la presente en las mujeres cisgénero con ciclos ováricos regulares (72,73).

La valoración anatomo-patológica de las muestras ováricas del primer estudio de la actual Tesis no halló cambios en el tamaño o el volumen global al comparar dichos resultados con los reportados en mujeres cisgénero, aunque una publicación reciente halló una correlación inversa significativa entre la duración del uso de testosterona en la población trans y el volumen ovárico. Los autores postulaban que a medida que la exposición a testosterona se prolonga en el tiempo se observa una disminución progresiva del volumen ovárico, sobre todo por encima de los dos años de tratamiento, como en el caso

de nuestra población (74). En la misma línea, otro estudio que comparaba el volumen ovárico ecográfico de una cohorte de hombres trans versus mujeres cisgénero de la misma edad halló incluso un volumen medio mayor en el grupo control, aunque no de forma estadísticamente significativa (28).

La población folicular en estadios precoces (folículos primordiales, primarios y secundarios) ha sido descrita en diversos estudios en mujeres cisgénero en edad reproductiva, aunque cabe destacar la gran heterogeneidad de muestras procesadas (fragmentos de corteza ovárica, cuñas ováricas o incluso un ovario en su totalidad) y la edad de las mujeres estudiadas. Entre las diversas publicaciones destaca la de *Gougeon y Chainy* en 1987, referente en la descripción de la población folicular temprana en la mujer cisgénero en edad reproductiva (75). Sobre la base de estas comparaciones, la distribución de folículos en la corteza ovárica en estos estadios precoces no se ve modificada por la GAHT, en consonancia con lo expuesto por otras publicaciones similares a la nuestra (27).

Por el contrario, es preciso señalar que según diversos estudios la administración de andrógenos en hombres trans (en edad adulta) aumenta el número de folículos antrales y atrésicos (22,23). *Hughesdon* observó en su población control de mujeres cisgénero un número similar de folículos antrales a los reportados en nuestro estudio, mientras que el grupo de *Ikeda et al.*, al comparar ambas cohortes (trans y mujeres cisgénero) tampoco confirmó la presencia de un número mayor de folículos antrales en el grupo trans (26,76). Respecto a los folículos atrésicos también hallamos resultados algo contradictorios, mientras que algunos grupos exponen un número mayor de estos folículos en la cohorte trans respecto al control, otros grupos hallan un número similar e incluso menor respecto a la mujer cisgénero, con cifras similares a las obtenidas en nuestra población (23,26,77). Escasos estudios han valorado de forma adecuada el número de folículos atrésicos como para extraer conclusiones firmes respecto a este punto, pero la mayoría abogan por la presencia de un número incrementado éstos en las muestras de cohorte trans estudiadas.

Adicionalmente, los cambios estromales descritos en los estudios de esta Tesis muestran la presencia de luteinización estromal casi en el 70 % de las muestras estudiadas y un grosor de la túnica albugínea aumentado de forma heterogénea, aunque prácticamente no se halló la presencia de hiperplasia estromal como para considerar el *ovario de Stein-Leventhal* (76). Nuestros hallazgos con concordantes con observados en otras series (22,26,27) y difieren de forma clara respecto a los ovarios control de mujeres cisgénero estudiados en diversas publicaciones, que revelan la ausencia de luteinización estromal y un grosor de la corteza ovárica significativamente menor (23,76,77). Según las observaciones realizadas, la testosterona exógena tendría un efecto directo sobre el estroma y el tejido conectivo, provocando una colagenización subepitelial. Se han descrito receptores de andrógenos en las células estromales perifoliculares que podrían mediar en la producción de colágeno tipo I por los fibroblastos del estroma, lo que podría explicar el engrosamiento y la heterogeneidad de la túnica albugínea de la corteza ovárica (71,78).

Por otra parte, la presente Tesis profundiza en las técnicas de preservación de la fertilidad en personas transgénero masculinas, concretamente en la criopreservación de tejido ovárico (OTC), comparando dos métodos de congelación de tejido ovárico en hombres trans por primera vez.

Se han publicado múltiples estudios comparando la técnica estándar de congelación lenta (SF, por sus siglas en inglés) versus la vitrificación (VT) en tejido ovárico humano de mujeres cisgénero con resultados contradictorios (79–82). Todos los estudios hasta la fecha han comparado ambos métodos en esta población específica al presentarse una enfermedad oncológica o patología ginecológica benigna; y solamente dos han valorado alguna técnica en tejido ovárico de hombres trans tras la GAS, únicamente de cara a validar los protocolos de vitrificación descritos por ambos grupos (83,84).

No se han hallado diferencias estadísticamente significativas entre las dos técnicas al estudiar la morfología folicular mediante microscopia óptica según los resultados observados en el segundo estudio de la Tesis, aunque se ha observado una clara tendencia hacia una mejor conservación con el método de

vitrificación (51.9 % de folículos normales tras la VT versus el 43.2 % tras la SF). *Li et al* (85) también describió resultados similares con ambas técnicas de criopreservación en mujeres cisgénero, aunque cabe destacar que observó un porcentaje mayor de folículos morfológicamente normales en ambos grupos (80.3 % en el grupo VT versus el 72.6 % en el grupo SF). Estos datos irían en concordancia con los expuestos recientemente por *Bailie et al* (86) según los cuales se hallaría un porcentaje menor de folículos morfológicamente normales en hombres trans respecto a la población control de mujeres cisgénero, debido a un posible efecto de la testosterona sobre la salud folicular. Nuestro estudio no ha comparado la normalidad folicular con tejido ovárico de mujeres cis, sino que la misma muestra en fresco al ser procesada ha sido el grupo control para comparar ambas técnicas de PF, objetivo del presente estudio.

Otras publicaciones que han comparado la morfología folicular entre ambas técnicas han reportado una tasa similar de normalidad folicular en algunos estudios (87–92), mientras que otros grupos han informado de resultados claramente peores tras la vitrificación del tejido (80,93–96). La densidad folicular en nuestra serie se mantuvo comparable en los tres grupos de estudio (incluidas las muestras de tejido en fresco), aunque algunos autores habían reportado una disminución significativa en la población folicular tras el uso de las técnicas de criopreservación y principalmente tras la VT (89,90). Además, dos estudios de reciente publicación reportaron menor densidad folicular tras la VT y el xenotrasplante posterior del tejido (92,94).

De todas maneras, es imperativo destacar la gran heterogeneidad en los protocolos de preservación utilizados por los diferentes grupos, en los que la modificación en la concentración de las diversas soluciones utilizadas [etilenglicol, dimetil sulfóxido (DMSO),1,2-propanediol (PrOH) entre otros] podrían alterar de forma significativa los diferentes tipos celulares y su supervivencia posterior (88,95-97). Nuestro estudio se ha realizado siguiendo los protocolos de grupos con amplia experiencia, como son los descritos por *Fabbri* en congelación lenta y *Kagawa* para la vitrificación del tejido, con ligeras modificaciones respecto a sus protocolos originales (83,95,98,99).

Disponemos de escasos datos sobre la ultraestructura de los ovocitos, células de la granulosa (GC, *granulosa cells*, por sus siglas en inglés) y del estroma tras el uso de técnicas de PF, y ninguno descrito en la población transgénero masculina. En mujeres cisgénero destaca el estudio de *Fabbri et al*, que no mostró diferencias en el estudio mediante microscopia electrónica de transmisión (TEM, por sus siglas en inglés) entre los diferentes protocolos, aunque las muestras tras VT mostraron alteraciones en el núcleo del ovocito (95). De acuerdo con este estudio, nuestros resultados mostraron una buena conservación de los folículos con ambas técnicas, aunque las GC mostraron una preservación claramente deficiente tras aplicar ambos métodos de congelación en comparación con las células en fresco. En algunos folículos tras la VT la membrana ovocitaria y el espacio intercelular estaban ligeramente aumentados, pero no se observaron alteraciones importantes y significativas en la evaluación ultraestructural, y no se observó la destrucción del folículo, al contrario de lo descrito por otros autores (92). El grupo de *Keros et al* (80), al comparar 4 técnicas de criopreservación (2 procedimientos de SF y 2 protocolos de VT) halló que tras la congelación lenta se observaban más células necróticas y vacías en el tejido estromal, concluyendo que el estroma ovárico se conservaba mejor con la vitrificación. La preservación de las GC y el tejido estromal en nuestros resultados han mostrado diferencias claras respecto a los observados en el estudio comentado anteriormente de *Keros et al*, al no hallarse diferencias significativas al aplicar ambas técnicas de criopreservación. Ello podría deberse a las mismas muestras del tejido ovárico, que en nuestra investigación proceden de personas trans con características tisulares diferenciales respecto al de mujeres cisgénero, ya descritos con anterioridad (engrosamiento de la túnica albugínea y luteinización de células del estroma, principalmente). Así pues, de forma global, los resultados de esta Tesis muestran que tanto la vitrificación como la congelación lenta parecen ser buenas técnicas para preservar el tejido ovárico en personas trans, con resultados similares a los reportados en mujeres cisgénero.

Una fortaleza importante del compendio de estudios de la presente Tesis es la gran cantidad de personas transgénero incluidas, mediante una valoración histológica y hormonal completas. El estudio histológico de un corte longitudinal del ovario es el más adecuado para describir la población total de folículos, la corteza ovárica y el estroma.
Además, comparamos ambas técnicas de criopreservación de tejido ovárico en hombres trans, mediante la evaluación por microscopia óptica y TEM, junto con la valoración de la viabilidad folicular tras una tinción celular. El estudio histológico que describe la población folicular normal y la densidad junto con la descripción ultraestructural de las células ováricas es el más adecuado para examinar ambas técnicas de criopreservación. Asimismo, se realiza una comparación y discusión exhaustiva de las diferencias encontradas en el tejido ovárico de hombres trans y los hallazgos observados tras el uso de ambas técnicas en población de mujeres cisgénero en edad reproductiva.

Los estudios que abarcan la presente Tesis también presentan diversas limitaciones. Una de las principales es la ausencia de un grupo de control de la misma edad en la evaluación histológica de las muestras. En nuestro centro únicamente podemos obtener piezas de tejido ovárico de pacientes oncológicas durante la PF o bien algunas muestras de mujeres cisgénero en edad fértil que presenten alguna tumoración ovárica benigna o maligna. Sin embargo, no ha sido posible obtener muestras de tejido ovárico de mujeres cisgénero no oncológicas con ovariectomía unilateral o bilateral por miomas uterinos u otras enfermedades ginecológicas benignas (en edad reproductiva). Determinados autores, en relación con este punto, mostraron que el tejido ovárico que rodea una tumoración no es un grupo de control apropiado ya que la densidad de folículos en la corteza disminuye significativamente (100). Además, las piezas de tejido ovárico obtenidas tras la PF en una paciente oncológica difieren mucho de la hemisección completa del ovario obtenida en nuestro estudio y, por tanto, no consideramos adecuado incluir este grupo como control.
Al comparar ambas técnicas de criopreservación utilizamos el mismo tejido ovárico de hombres trans como grupo control, evaluándose así diversas piezas

en fresco a la vez que las procesadas. No consideramos adecuado comparar los resultados obtenidos con muestras de tejido ovárico de otro tipo de pacientes (p.e oncológicas tras PF) dado el escaso número de muestras preservadas y que todas ellas se criopreservan mediante congelación lenta para su uso propio en un futuro. Además, el objetivo de nuestro estudio era valorar la eficacia de ambas técnicas en población trans, por lo que consideramos que la muestra control debía ser de la misma población.

Otras limitaciones de nuestros estudios incluirían la ausencia de control ecográfico previo a la cirugía de afirmación de género en todos los hombres trans, pero algunos consideraron totalmente indeseable realizar el examen de sus genitales internos. Por otro lado, no realizamos una ecografía basal antes de iniciar el tratamiento con testosterona, aunque en todos los casos se descartó la presencia de PCOS; y no fue posible determinar el nivel sérico de AMH basal para comparar los posibles cambios tras el inicio de la terapia hormonal. Finalmente, la falta de pruebas funcionales que permitan determinar si el daño folicular y estromal es reversible o no sería otra limitación.

En relación con este punto, consideramos de interés remarcar la publicación reciente del desarrollo de un modelo murino que imita la terapia hormonal del hombre trans y confirmaría los resultados histológicos observados en las diferentes series en modelo humano. Según los datos publicados la administración de testosterona de forma continuada en etapa postpuberal resultaría en un aumento de los folículos atrésicos sin reducción alguna en el recuento de folículos antrales, primordiales, primarios, secundarios o totales en los ratones androgenizados en comparación con los controles, junto con una ausencia completa de cuerpos lúteos (101). El desarrollo de este modelo permitirá mejorar la comprensión de los cambios reproductivos producidos durante la terapia hormonal y la reversibilidad de cualquier cambio ovárico observado tras el cese de la testosterona, de forma controlada y con una posible extrapolación de los resultados a la población trans que considere el uso de técnicas de PF o una paternidad futura.

En base a los resultados de los estudios expuestos en la presente Tesis nos planteamos el potencial fértil en los hombres trans tras haber iniciado la terapia hormonal. Según nuestros datos, la testosterona no parece alterar el número y la distribución de los folículos en el ovario, es decir, no tiene un efecto gonadotóxico directo sobre la población folicular, pero, sin embargo, hemos observado algunos cambios en el tejido cortical y estromal.

Con respecto a las implicaciones que esto conlleva sobre la preservación de la fertilidad mediante la *vitrificación de ovocitos*, disponemos actualmente de datos limitados sobre los resultados de una estimulación ovárica en este colectivo (44–46,102). De forma global los resultados son satisfactorios en cuanto a número de ovocitos obtenidos, aunque se debe tener presente la elevada heterogeneidad en la edad de la población estudiada (rango de edad 16.4 - 30.3 años), el número escaso de pacientes valorados (N máxima 26) y con periodos de suspensión de la GAHT diferentes según la serie.

Por lo tanto, a la luz de nuestros resultados, aunque los cambios estromales son leves, desconocemos si son transitorios o pueden tener un efecto prolongado en la foliculogénesis posterior. En consecuencia, no sabemos el tiempo necesario de suspensión de la terapia hormonal antes de iniciar la estimulación ovárica y, por todo ello, nuestra recomendación es la de intentar realizar una criopreservación de ovocitos antes de iniciar la GAHT, aunque los diversos estudios apuntan a una recuperación funcional correcta.

Paralelamente, y con respecto a la otra técnica disponible para preservar la fertilidad en las personas trans, la *OTC* es aún una técnica considerada experimental en Europa y no existe experiencia publicada en cuanto a su aplicación en este colectivo (51). Los datos histológicos aportados en la presente tesis corroboran la opción de criopreservar tejido ovárico en el momento de la cirugía de afirmación de género en esta población (periodo de terapia de 24 meses aproximadamente). Sin embargo, en estos momentos, esta opción sería recomendable en aquellos individuos que se planteen como opción un futuro autotransplante.

Ahora bien, se debe tener presente también en qué situación se realizaría el autotransplante posterior, es decir, si se plantearía la suspensión la terapia hormonal previamente a la cirugía y durante cuánto tiempo; siendo además interesante la valoración posterior de ese tejido tras el reimplante. La pérdida folicular por isquemia acontecida los 4 - 5 primeros días tras el autotransplante condiciona la funcionalidad del tejido posterior, y se ha descrito que puede alcanzar hasta el 50% de la población folicular (103–105). El tejido ovárico de la persona trans, al presentar unas características diferenciales (principalmente modificaciones estromales) podría ser más resistente a esta pérdida folicular o bien más sensible, sin que dispongamos actualmente de ninguna información al respecto. La realización de estudios mediante el xenotransplante de este tejido ovárico en modelo murino, por ejemplo, podría aportar información fundamental en relación con este aspecto.

En aquellos individuos que no se planteen o no deseen el autotransplante posterior, se requerirán técnicas experimentales que estos momentos se encuentran en fases muy iniciales de su desarrollo, como son el crecimiento y la maduración in vitro de los folículos (42). Hoy en día, el uso eficiente de la reserva folicular cortical criopreservada sigue siendo un desafío en esta población, así como en otros grupos de pacientes en los que no es posible / deseable trasplantar el tejido ovárico preservado, como las pacientes afectas de neoplasias hematológicas altamente infiltrativas y con posibilidad de mestástasis ováricas como la leucemia. Actualmente se están desarrollando técnicas de crecimiento in vitro combinadas con IVM en modelo humano, y se ha conseguido una *"proof of concept"* según la cual es posible el crecimiento y la maduración completa del ovocito. Aun así, queda un largo camino por recorrer y años de experimentación antes de iniciar su uso en la práctica clínica (53,55).

Por otro lado, no debemos olvidar el potencial efecto directo de la testosterona sobre el ovocito. Algunos autores han reportado el cultivo y la maduración de ovocitos inmaduros obtenidos durante la cirugía de afirmación de género (y la preservación del tejido obtenido) en hombres trans que no habían suspendido la terapia hormonal previamente (54,106). Prosiguiendo con esta línea, un

estudio reciente del mismo grupo ha conseguido la fecundación de estos ovocitos madurados, aunque han mostrado un desarrollo embrionario deficiente y ninguno de éstos consiguió evolucionar a fase de blastocisto (107), objetivando así el posible efecto de la testosterona sobre la salud ovocitaria.

Finalmente, es necesario mencionar que los estudios de la presente Tesis se han realizado en población adulta, postpuberal, y con ciclos ováricos establecidos. La tendencia actual de iniciar la transición en edades más precoces, incluso en prepúberes, hace que estos resultados no sean aplicables, debido al posible efecto que puede tener la testosterona sobre un tejido ovárico claramente inmaduro. En estos casos consideramos imprescindible dar a la persona y su familia toda la información de la que disponemos antes plantear esta técnica de preservación de la fertilidad en este subgrupo de personas.

Recientemente, en noviembre de 2021, se ha publicado un cambio legislativo de gran interés como es la **Orden SND/1215/2021** por la que se modifica el RD (Real Decreto 1030/2006) que regula las técnicas de reproducción humana asistida (RHA).
Este documento contempla que podrán optar a tratamientos de RHA también "*mujeres sin pareja, personas transexuales que conservan la capacidad de gestar y mujeres lesbianas*". Además, respecto a la preservación de gametos para uso propio diferido en el contexto de PF en situaciones asociadas a procesos especiales, la orden indica que se realizará "*exclusivamente por indicación médica y en pacientes con posible riesgo de pérdida de su capacidad reproductiva asociada a exposición a tratamientos gametotóxicos o a procesos patológicos con riesgo acreditado de fallo ovárico prematuro*", sin que se aplique en este supuesto el límite mínimo de edad de 18 años.
La publicación de esta normativa abre la puerta a realizar PF y técnicas de RHA en este colectivo y plantea la necesidad de tener el máximo conocimiento posible para poder aplicarlas con la máxima formalidad.

Es importante, pues, destacar los resultados de la presente Tesis Doctoral, que aporta datos concretos que podrían ayudar en la elaboración de protocolos asistenciales, optimizando así este recurso disponible y, sobre todo, permitiendo ofrecer opciones reproductivas cimentadas en bases científicas en la población trans.

6. CONCLUSIONES

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1. La terapia con testosterona a dosis elevadas, durante dos años en personas transgénero masculinas produce un estado endocrinológico de anovulación por inhibición hipofisaria, manteniendo valores de AMH dentro del rango de referencia en mujeres cisgénero.

2. La terapia hormonal de afirmación de género mantenida en dichas condiciones no altera la densidad ni la distribución folicular a nivel de la corteza ovárica, aunque se observan algunas alteraciones estromales acompañantes. Por tanto, no parece tener un efecto gonadotóxico directo sobre la gónada.

3. La vitrificación y la congelación lenta son técnicas equivalentes en la conservación de la morfología y la viabilidad folicular del tejido ovárico; por lo tanto, ambas son opciones válidas a considerar para preservar la fertilidad de las personas trans.

7. REFERENCIAS

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