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Feminising hormone therapy reduces testicular ACE-2 receptor expression: Implications for treatment or prevention of COVID-19 infection in men

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Abstract

It has been proposed that men hospitalised with COVID-19 be treated with oestrogen or progesterone to improve COVID-19 outcomes. Transgender women (male-to-female) are routinely treated with oestrogen or oestrogen +progesterone for feminisation which provides a model for the effect of feminising hormones on testicular tissue. Our goal was to analyse differences in ACE-2 expression in testicles of trans-women taking oestrogen or oestrogen +progesterone. Orchiectomy specimens were collected from trans-women undergoing gender-affirming surgery, who were taking oestrogen or oestrogen+progesterone preoperatively. For controls, we used benign orchiectomy specimens from cis-gender men. All specimens were stained with H&E, Trichrome (fibrosis), insulin-like 3 antibody (Leydig cell) and ACE-2 IHC. Cells per high-powered field were counted by cell type (Leydig, Sertoli and Germ). Stain intensity was rated on a 0–2 scale. On immunohistochemistry staining for Leydig cells and ACE-2 staining, the oestrogen+progesterone cohort had fewer Leydig cells compared with controls. The oestrogen+progesterone cohort also had greater degree of tissue fibrosis compared with controls and the oestrogen cohort. This work supports the hopeful possibility that a short course of progesterone (or oestrogen+progesterone) could downregulate ACE-2 to protect men from COVID-19 infection.

KEYWORDS
ACE-2, COVID-19, fertility, oestrogen, progesterone, testicle, transgender

1 | INTRODUCTION

Male sex is an independent risk factor for severe COVID-19 infection and death from COVID-19 infection; however, the precise mechanism of the apparent predilection of COVID-19 for men remains unknown (Docherty et al., 2020; Guan et al., 2020; Richardson et al., 2020; Zhou et al., 2020). Experts speculate that men may simply be less likely to comply with social distancing practices and primary prevention than women. A Gallup Poll conducted in March 2020, amid social distancing and stay-at-home mandates across the United States, showed that a lower percentage of men were 'very/somewhat worried' that they or someone in their family would be exposed to SARS-CoV-2 when compared to women (McCarthy, 2020). However, innate anatomical and sex hormone differences between the two genders should be also considered as possible mechanisms.

The angiotensin-converting enzyme 2 (ACE-2) receptor has been identified as the means by which the SARS-CoV-2 virus is able to enter host cells. Among human tissues, the testes have the second highest known expression of ACE-2 receptor, second only to the small intestine (Li et al., 2020b). ACE-2 is expressed predominantly in Leydig cells—the testosterone-producing cells—but also in Sertoli cells and spermatogonia (Verma et al., 2020; Wang et al., 2020). ACE-2 is also expressed in ovarian tissue but in lower concentrations when compared to testis tissue (FM et al., 2011). This differential expression of ACE-2 is at least in part due to sex hormone differences as ACE-2 is regulated by sex hormones (J et al., 2010; Brosnihan et al., 2008). Male and female sex hormones also have immunoregulatory properties: broadly, oestrogen is immunostimulatory while progesterone and testosterone are immunosuppressive (SL & KL, 2016; Moulton 2018). The combination of high ACE-2 concentration in the testes compared with ovaries and the presence of immunosuppressive testosterone make the testes a potential target for SARS-CoV-2 infection. Several institutions, including our own, have demonstrated the ability of SARS-CoV-2 to directly infect testicular tissue; what remains unclear is whether sex hormone modulation—specifically feminising hormone therapy for cis-gender males—can alter outcomes of COVID-19 infection (Achua et al., 2020; Bian et al., 2020; Li et al., 2020; Yang et al., 2020). Administration of progesterone (P) to men with severe COVID-19 infection is currently an active area of investigation (Ghandehari et al., 2021).

Our institution is uniquely positioned to study the effects of feminising hormones on male testicular tissue. Given our busy tertiary care genital gender-affirming surgery (gGAS) practice, we have access to a large repository of orchiectomy specimens from transgender women (TW) undergoing feminising (i.e. male-to-female, MTF) gGAS. TW are typically started on oestrogen (E)-only or combination E + P therapy prior to gGAS. We assessed the effect of feminising hormone therapy on testicular ACE-2 expression compared with cis-gender controls. We hypothesised that feminising hormones will decrease testicular ACE-2 expression, which could be a contributing mechanism by which females tend to have better COVID-19 outcomes compared with males.

2 | MATERIALS & METHODS

This study was conducted with approval by the Institutional review board at Cedar-Sinai Medical Center under IRB# 00000739.

2.1 | Methods—Patient Selection, cohort data, etc

Orchiectomy specimens were collected from TW undergoing gGAS from January 2018 to December 2020, who were taking E-only or E + P preoperatively. For controls, benign (non-testis cancer) orchiectomy specimens from cis-gender men were used. Demographic data were collected for all study subjects including age, BMI and indication for orchiectomy for cis-male controls.

Findings within the two TW cohorts (E-only versus E + P supplementation) were stratified by age: TW over age 40 compared with TW under age 40.

2.2 | Methods: Morphologic analysis

Representative sections of the orchiectomy specimens were submitted in 10% buffered formalin for routine histologic processing and tissue sectioning. Specimens were stained with haematoxylin and eosin (H&E) and subjected to routine light microscopic analysis. Trichrome stain was utilised to assist in the assessment of the presence and degree of interstitial and tubular fibrosis.

Seminiferous tubules were analysed in a semiquantitative manner for degree of spermatogenesis and graded 0–2 (0 = none, 1 = diminished spermatogenesis and 2 = normal levels of spermatogenesis). The numbers of intraluminal mature spermatids served as the reference baseline for comparisons (with 2 being what one would see in the lumens of seminiferous tubules of a normal adult testicle). Interstitial fibrosis of testicular parenchyma was assessed and scored as a percentage of cross-sectional surface area involved. The percentage of seminiferous tubules showing basement membrane fibrosis was recorded. The presence and quantity of interstitial Leydig cells and Sertoli cells were annotated and graded 0–2 (0 = no cells identified, 1 = diminished numbers and 2 = normal numbers). The numbers of Leydig cells and Sertoli cells served as the reference baseline for comparisons (with 2 being what one would see in a normal adult testicle).

Immunohistochemical (IHC) techniques were employed to supplement the morphologic evaluation to better quantify the Leydig cell population; antibody against insulin-like 3 antibody (INSM-3) was utilised.

Immunohistochemical analysis with ACE-2 antibody was utilised to assess for the presence and levels of ACE-2 receptors in specific testicular cell compartments (Leydig cells, Sertoli cells and germinal epithelial cells).

2.3 | Methods: INSL3 and ACE-2 receptor immunohistochemistry (IHC) technique

Samples were Sectioned 4um on Superfrost Plus slides (12-550-15, Fisher Scientific). IHC staining was automatically performed on Ventana Discovery Ultra Instrument (Roche).

Slides were deparaffinised at 72°C with EZ solution (Cat# 950-100, Roche), through CC1 (Tris, pH8.0) (950-500, Roche) antigen retrieval and endogenic peroxidase block (760-4840, Roche), and antibody INSL3 (1:5000, Rabbit polyclonal, HPA028615, Atlas Antibodies AB) or antibody ACE-2 (aa140-172) (1:800, Rabbit polyclonal, LS-B6672, Life Span Bioscience) was manually applied and incubated 32' at 37°C. The following steps were automatically carried out with the detection system of DISC anti-Rabbit HQ (760-4815, Roche Ventana) for 12 min and DISC anti-HQ HRP (760-4820,

Roche Ventana) for 12 min. DISC ChromoMap DAB Kit was finally applied (760-159, Roche Ventana).

After antigen retrieval CC1 (Tris, pH8.0) (950-124, Roche Ventana), primary antibody was manually applied. Antibodies were diluted with Antibody Dilution Buffer (ADB250, Roche Ventana).

Once stained with INSL3, Leydig cells within the testicular tissue sections were semiquantitatively analysed as follows: overall cell numbers graded 0–2 (0 = no cells identified, 1 = few cells and 2 = normal cell numbers) and overall cell staining intensity graded 0–2 (0 = no staining, 1 = weak staining and 2 = strong staining). As Leydig cells are known to express INSL3 (staining intensity 2 is considered strong and normal), this cell population served as the positive control and reference cell population to which other cell populations were semiquantitatively compared.

For ACE-2 receptor immunostained sections, the numbers of cells and staining intensity of staining of ACE-2 were semiquantitatively assessed for each of the cell components Leydig cells, Sertoli cells and germinal cells. The numbers of cells stained were quantified as 0–2 (0 = no stained cells, 1 = few stained cells and 2 = all cells stained). The overall cell staining intensity was graded 0–2 (0 = no staining, 1 = weak staining and 2 = strong staining).

Immunohistochemical scoring was performed in a blinded fashion by a senior genitourinary pathologist.

Mean cell type/high-powered field (HPF) and stain intensity were compared across cohorts using Student's t test ($p < .05$).

3 | RESULTS

Orchiectomy specimens were collected from 58 total TW subjects and ten cis-gender male controls. Among TW subjects, 34 were taking E-only and 24 were taking E + P. Hormone delivery methods and doses varied among TW subjects: 34 used oral E (doses ranged from 2 mg/day to 10 mg/day), 23 used intramuscular E (doses and frequency of injection varied widely) and one used topical E (1.5 mg every three days). All TW subjects using E + P used oral progesterone 100 mg/day or 200 mg/day. The mean age of TW subjects and cis-male subjects was 39 (±14) and 46 (±17) years old respectively; the mean age of TW taking E-only and combination E + P was 38 and 40 years old respectively (Table 1). Mean BMI of TW subjects on E-only, E + P was 27.7 (±5.3), 28.1 (±9.7) respectively; average BMI of cis-male subjects was 23.8 (±5.4). All TW subjects underwent orchiectomy for feminisation as part of gGAS. Indications for orchiectomy for control subjects are described in Table 2.

3.1 | Testicular morphology

Among TW, E + P supplementation was associated with lower overall Leydig cell concentration versus controls ($p = .0001$; Figure 1), but no significant differences were observed between TW over age 40 versus under 40 (Table 1). Sertoli cells showed no significant differences observed versus TW and controls, but within E and E + P

TW groups, older patients had significantly lower concentrations of Sertoli cells as compared to patients under age 40 ($p = .01$ and $p = .003$ respectively; Table 1). No differences were observed in germinal maturation among E and E + P versus controls, nor, across TW age groups.

The degree of fibrosis within testicle tissue was significantly greater among TW on E + P compared with controls ($p = .0003$; Figure 1), and, there was significantly more fibrosis among TW on E + P over age 40 than under age 40 ($p = .0002$; Table 1).

Transgender women on E + P (but not E-only) had significantly fewer Leydig cells as compared to controls ($p = .0035$), though we detected no age-related differences.

Transgender women on E + P had significantly less intense Leydig cell ACE-2 staining than controls and E-only TW ($p = .01$ and $p = .0035$ respectively); older TW on E + P had less intense Leydig cell ACE-2 staining versus TW under age 40 ($p = .024$). Among E-only TW, older subjects had less intense Leydig cell ACE-2 staining than younger subjects ($p = .046$; Figure 1). ACE-2 IHC staining for Sertoli cells showed no differences among TW subjects versus controls, but within the E and E + P groups, TW over age 40 had less ACE-2 staining in their Sertoli cells than younger TW ($p = .0032$ and $p = .0004$ respectively). ACE-2 IHC for germinal cells showed no significant differences between controls and TW, but within TW groups, subjects over age 40 had less ACE-2 staining of germinal cells compared with TW under age 40 ($p = .03$ for E-only and $p = .01$ for E + P respectively).

4 | DISCUSSION

Worldwide, male sex has been shown to be associated with worse outcomes in COVID-19 infection. The exact mechanism of the differential outcomes between the sexes has not been elucidated, but sex hormones likely play a role. P has been proposed as a potential therapeutic agent for men with severe COVID-19 infection. We found that feminising hormone therapy, especially combination E + P, significantly reduced ACE-2 expression in the testicular tissue of TW. This finding is highly relevant to both COVID treatment and prevention in that it offers a possible mechanism explaining the benefit afforded by the combination of E + P in COVID-19-positive patients. We also found that E + P supplementation was associated with a greater degree of testicular fibrosis than cis-male controls, asserting that fertility potential in TW on E + P may be permanently compromised. Within the TW population, there was a greater degree of tissue fibrosis within older TW compared with younger TW. These findings are highly relevant to older transgender women managed with E + P combination therapy who may wish to preserve fertility.

Approximately 60% of patients requiring hospitalisation are male (Docherty et al., 2020; Guan et al., 2020; Richardson et al., 2020; Zhou et al., 2020). In an analysis of 20,133 patients admitted with COVID-19 across 208 hospitals in the United Kingdom, Docherty et al. found male sex to be an independent risk factor for in-hospital mortality (Docherty et al., 2020). Based on epidemiological findings

TABLE 1 Patient demographics and IHC staining findings; results were compared across EP and E-only cohorts and stratified by age within cohorts

	N	Avg age (years)	H/E			Trichrome	
			Leydig	Sertoli	Germ Mat	Int Fib	Tub Fib
			Cells/HPF:			Intensity:	
			0,1,2	0,1,2	0,1,2	0,1,2	% Tub Fib
Controls avg	10	45.7	1.90	1.90	1.70	0.10	45.00
E-only avg	34	37.8	1.24	1.71	0.50	1.44	37.35
EP avg	24	40.1	0.67	1. 58	0.17	1.00	43.75
*TT p-value (EP versus E)			0.01	0.34	0.06	0.53	0.59
TT p-value (EP versus ctrl)			0.0001	0.08	0.00	0.0003	0.94
TT p-value (E versus ctrl)			0.02	0.22	0.00	0.22	0.65
STRATIFIED BY AGE							
Controls age <40 years	4	27.5					
Controls age ≥40 years	6	57.8					
TT p-value			0.45	0.45	0.86	0.45	0.81
E-only age <40 years	21	29.5					
E-only age ≥40 years	13	51.2					
TT p-value			0.21	0.01	0.83	0.11	0.12
EP age <40	13	27.80					
EP age ≥40	11	54.50					
TT p-value			0.87	0.003	0.86	0.55	0.0002

Note: Student's *t* test was performed to compare average cell type counts and average staining intensity; differences were significant at $p < .05$.

*$p < .05$ (highlighted in Green).

TABLE 2 Indications for orchiectomy among control subjects

Subject	Age	Orchiectomy indication
1	35	Gun shot
2	31	Testicular torsion
3	64	Prostate cancer
4	53	Stricture of urethral meatus
5	17	Testicular torsion
6	57	Cryptorchidism
7	64	Prostate cancer
8	27	Acute trauma
9	47	Acute trauma
10	62	Prostate cancer

such as these, there is a growing body of literature investigating the differential effects of sex hormones on COVID-19 outcomes. In a SARS-CoV mouse model, investigators found male mice to be more susceptible to infection than age-matched female controls; these findings held true even after gonadectomy, raising the possibility of a protective effect of oestrogens, not just a detrimental effect of androgens (Liva & Voskuhl, 2001). When considering sex hormones in the context of viral infection, it is important to appreciate that after initial infection via the ACE-2 receptor, death from COVID-19 comes by way of an uncontrolled hyperimmune response known as a 'cytokine storm', in which macrophages produce proinflammatory cytokines (interleukin-6 and tumour necrosis factor-α) via activation of the nuclear factor κB (NF-κB) (Jia, 2016). Understandably, initial cases of severe COVID-19 infection treated with anti-inflammatory corticosteroids demonstrated success (van Paassen et al., 2020).

Conversely, androgens have been shown to promote severe COVID-19 infection at both high and low levels. An Italian study released in August 2020 supports the hypothesis that androgen-deprivation therapy (ADT) may protect men from infection (Montopoli et al., 2020). In this study, prostate cancer patients who were not receiving ADT showed an increased risk of SARS-CoV-2 infection compared with those who were receiving ADT. Of the 5,273 patients with prostate cancer receiving ADT, only four were found to be positive for SARS-CoV-2, compared with the 114 positives of the 37,161 patients with prostate cancer who were not receiving ADT (Cattrini et al., 2020). There are several proposed mechanisms by which testosterone impacts COVID-19 infection. Transmembrane

| INS IHC | | ACE-2 IHC | | ACE-2 IHC | | ACE-2 IHC | |
| Leydig cells | | Leydig cells | | Sertoli | | Germinal cells | |
Cells/HPF:	Intensity:	Cells/HPF:	Intensity:	Cells/HPF:	Intensity:	Cells/HPF:	Intensity:
0,1,2	0,1,2	0,1,2	0,1,2	0,1,2	0,1,2	0,1,2	0,1,2
2.00	2.00	2.00	2.00	2.00	1.00	2.00	1.00
1.24	1.92	1.24	1.84	1.68	1.00	1.59	1.03
0.67	1.91	0.67	1.73	1.54	0.96	1.42	1.13
0.01	0.94	0.01	0.45	0.31	0.24	0.29	0.17
0.0035	0.57	0.0035	0.27	0.09	0.69	0.06	0.46
0.09	0.70	0.09	0.41	0.19	n/a	0.19	0.73
n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
0.21	0.15	0.21	0.046	0.0032	n/a	0.03	0.48
0.87	0.30	0.87	0.024	0.0004	0.37	0.01	0.11

protease, serine 2 (TMPRSS2) has been implicated in host cell invasion by SARS-CoV-2 viral particles both independently and via a synergistic relationship with ACE-2 (Liva & Voskuhl, 2001). TMPRSS2 is upregulated by androgens while simultaneously, ACE-2 has been shown to be downregulated by androgens (Hoffmann et al., 2020; Pozzilli & Lenzi, 2020). Additionally, androgens are known to modulate both innate and adaptive immunity. Androgens generally suppress the inflammatory responses by decreasing the activity of the peripheral blood mononuclear cells and the release of cytokines; they can also promote the synthesis of suppressive cytokines via androgen receptor signalling (Agostino et al., 1999; Cattrini et al., 2020; Liva & Voskuhl, 2001; McKay & Cidlowski, 1999; Musabak et al., 2003). These findings present a dichotomy regarding the relationship between androgens and COVID-19: androgenic immunosuppression appears to portend worse COVID-19 prognosis but should, in theory, also help suppress the cytokine storm associated with the most severe COVID-19 cases.

Oestrogens, specifically oestradiol and progesterone, have immunomodulatory properties as well. The immunologic effect of E2 is concentration dependent: at low levels, E2 has proinflammatory properties which potentiate the immune response while at high levels, E2 has anti-inflammatory properties (Straub, 2007; Villa et al., 2015). Progesterone has exclusively anti-inflammatory properties and has partial glucocorticoid and mineralocorticoid activity (Liu et al., 2020). There is clinical evidence to suggest that pre-menopausal women have better COVID-19 infection outcomes compared with post-menopausal women, who have greatly diminished circulating oestrogens (Ding et al., 2020). Oestrogens have also been shown to downregulate ACE-2 expression in bronchial epithelial cells (Cattrini et al., 2020; Stelzig et al., 2020). Progesterone specifically, given its corticosteroid activity and the early success corticosteroids in the treatment of severe COVID-19 infection, is of particular interest as a treatment adjunct for men with severe COVID-19 infection. Progesterone has been shown to promote T regulatory cell differentiation and enhance interferon-alpha pathways which control viral replication without perpetuating inflammatory pathways (Hall & Klein, 2017; Jakovac, 2020). Results from a pilot study in which hypoxemic men hospitalised with moderate to severe COVID-19 were given progesterone 100mg twice daily by subcutaneous injection in addition to standard of care showed clinical status improvement in just seven days (Ghandehari et al., 2021).
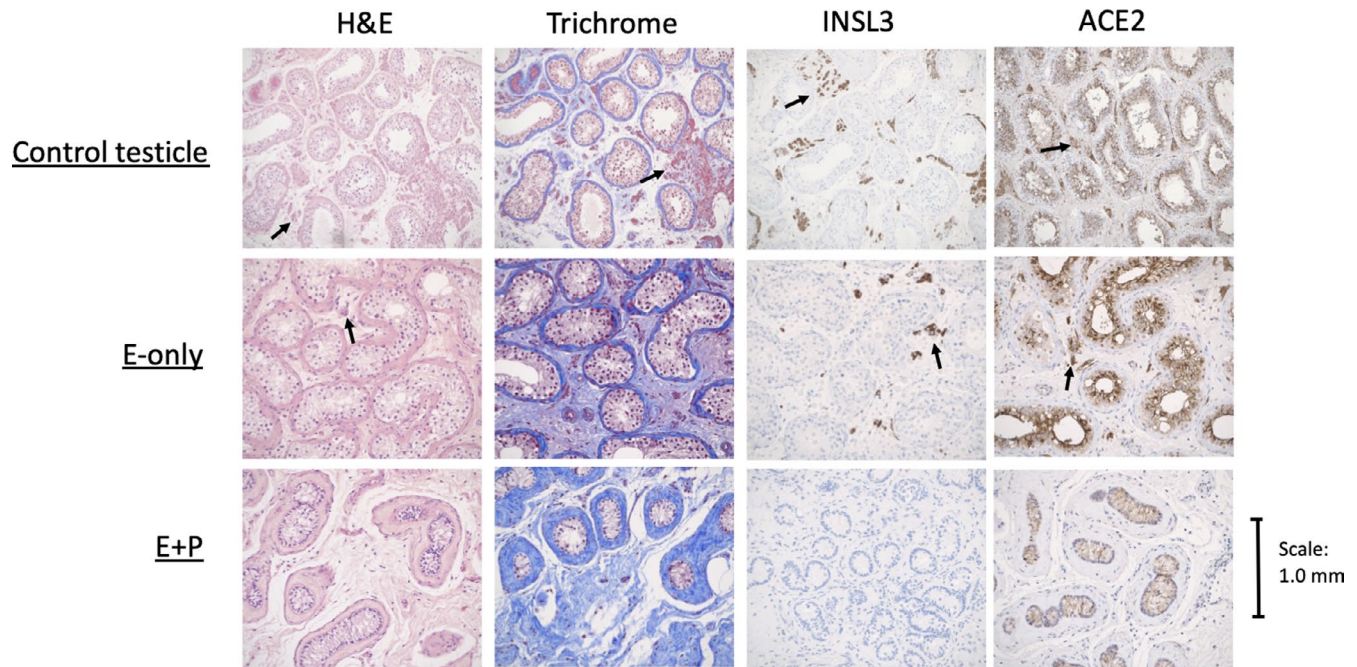
FIGURE 1 Photomicrograph legend (all 200× original magnification). Top row: H & E stain and trichrome stains show control testicular tissue with seminiferous tubules having normal levels of spermatogenesis, no fibrosis, many interstitial Leydig cells (arrows). INSL3 and ACE-2 highlight Leydig cells (arrows), germinal epithelium and Sertoli cells (weakly). Middle row: H&E and trichrome stains in 'oestrogen only' treatment (Case 6) show slightly diminished spermatogenesis and decreased interstitial Leydig cells (arrows). INSL3 shows few Leydig cells (arrow); ACE-2 shows highlights decreased interstitial Leydig cells (arrow), highlight residual germinal cells and Sertoli cells. Bottom row: H&E and trichrome stains in 'oestrogen and progesterone' treatment (Case 28) show interstitial fibrosis, fibrosis and atrophy of seminiferous tubules, complete loss of interstitial Leydig cells, diminished germinal epithelium, no active spermatogenesis. INSL3 shows no Leydig cells and ACE-2 shows complete loss of interstitial Leydig cells, highlight residual germinal cells and Sertoli cells

While it is clear that sex hormones modulate the immune response, another possible mechanism explaining the difference in COVID-19 outcomes between men and women is the effect of sex hormones on ACE-2 expression. It is generally accepted that increased expression of ACE-2 is associated with greater risk of COVID-19 infection, as it is the predominant entry point of SARS-CoV-2 into host cells (Oudit & Pfeffer, 2020). There is evidence to suggest that androgens promote TMPRSS2 expression, which promotes host cell infection along with ACE-2, while oestrogens suppress ACE-2 expression (Gargaglioni & Marques, 2020; J. Liu et al., 2010; Liva & Voskuhl, 2001; Montopoli et al., 2020). Our findings further support this protective mechanism by demonstrating decreased ACE-2 expression in testicular tissue in multiple testicular cell types of TW who had been treated with feminising hormones. Our findings also lend support to the specific use of progesterone as a therapeutic adjunct, given the more dramatic decrease in ACE-2 expression in the E + P cohort compared with the E-only cohort. Further, the effect of ACE-2 downregulation by feminising hormones in our population is age-related: ACE-2 is even more dramatically downregulated in TW over age 40 compared with those under 40.

Separate from the implications of our findings in COVD-19 outcomes, our study highlights valuable information for TW. Not infrequently, TW express desire for fertility preservation before gGAS, and temporarily cease feminising hormone therapy for ≥3 months to allow for procurement of a semen sample for sperm cryopreservation (Sterling & Garcia, 2020). The long-term effects of feminising hormone therapy on testicular tissue and fertility potential are poorly studied (Hembree et al., 2009). To our knowledge, ours is the first study to examine testicular histology following different feminising hormone therapies. Whether the deleterious histologic changes we observed are reversible with EP therapy cessation is unknown; prospective studies to confirm the long-term effects of EP on fertility are warranted. Additionally, the significantly greater suppression of Leydig cells by EP, as compared to E-alone, would suggest decreased intrinsic testosterone production among patients on EP. Decreased testosterone production would be beneficial to TW by decreasing the masculinising effects of testosterone on secondary sex characteristics. However, it would also be harmful to fertility preservation because testosterone production on the seminiferous tubule side of the testis–blood barrier is suppressed. These fertility-related effects on TW should be relayed to TW considering EP.

Our study has several limitations. First, our small sample size and unique patient population limit the wide applicability of our findings to all other cis-gender men. Second, our methods are semiquantitative in nature in that we relied on visual assessment staining techniques rather than quantitative assays; it should be

appreciated however that all staining assessment was performed by a senior genitourinary pathologist. Additionally, the role of ACE-2 expression and COVID-19 infection is somewhat controversial. There is emerging evidence to suggest that increased ACE-2 expression may actually have a protective role in COVID-19 infection in that excess ACE-2 is available for normal function beyond that which is bound by SARS-CoV-2 (Banu et al., 2020). There is also no evidence to suggest that overall ACE-2 expression is different between males and females despite the difference of expression in male versus female gonads (Asselta et al., 2020). Finally, because our case population consisted of only TW, it is unclear whether our results are universally applicable to the cis-male population at large.

5 | CONCLUSIONS

E + P, but not E-alone, is associated with fewer Leydig cells, less ACE-2 receptor expression and greater testicle interstitial fibrosis, as compared to cis-gender controls, and even as compared to TW on E-alone. There appears to be an age-related effect wherein older TW on E + P and E-alone have decreased ACE-2 receptor expression on Leydig cells, Sertoli cells and germinal cells compared with younger TW. These findings have implications both for use of feminising hormones to treat COVID-19 in men and for TW seeking to preserve fertility after prolonged hormone therapy. Regarding COVID-19, whereas E-only does not appear to significantly diminish ACE-2 expression in the testes, P appears to significantly diminish its expression. This has implications for use of feminising hormones to protect from or to treat COVID infection and warrants possibly protective effects of P on other tissues. Regarding TW, use of E + P more significantly diminishes endogenous testosterone production via Leydig cell destruction and induces seminiferous tubule fibrosis, possibly resulting in potentially irreversible fertility effects. Since no group received P only, this study cannot rule out a possible synergistic effect between E + P to decrease ACE-2 expression.

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