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ARTICLE

Steroid profiles by liquid chromatography-mass spectrometry of matched serum and single dominant ovarian follicular fluid from women undergoing IVF

BIOGRAPHY

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KEY MESSAGE

The steroid profile, measured by liquid chromatography-mass spectrometry, of follicular fluid collected from the single dominant follicle in women undergoing ovarian stimulation for IVF does not predict pregnancy outcome. Instead, the most consistent predictor of IVF outcomes is baseline serum anti-Müllerian hormone.

ABSTRACT

Research question: Can IVF outcomes be predicted from the steroid profile generated by liquid chromatography-mass spectrometry (LC-MS/MS) from follicular fluid collected from a single dominant follicle and serum after ovarian stimulation.

Design: Prospective observational cohort study in which serum and follicular fluid were collected from women and used to generate steroid profiles by LC-MS/MS. A total of 93 consecutive women enrolled for IVF treatment were recruited at the Fertility Unit, Royal Prince Alfred Women and Babies Hospital, Sydney between September 2014 and July 2015. Baseline and serum levels at oocyte retrieval, as well as follicular fluid samples from the largest single antral follicle, were collected. All samples underwent steroid analysis within a single batch to measure progesterone (P4), oestradiol (E2), oestrone (E1), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone (T), dihydrotestosterone (DHT), and 3 α, 5α androstanediol (3α-diol) and 3β, 5α androstanediol (3β-diol).

Results: P4, E2, E1, A4, T, DHEA and A4 were detectable in all baseline serum levels, at oocyte retrieval and in follicular fluid samples, whereas DHT, 3α-diol and 3β-diol were only detectable in a minority of samples. The most consistent predictor of pre-transfer (number of follicles >14mm in diameter, oocytes retrieved or fertilized, day-5 blastocysts) outcomes was baseline serum anti-Müllerian hormone. In follicular fluid, E2 was a negative predictor of the number of oocytes retrieved and the number of day-5 blastocysts but no follicular fluid steroids predicted pregnancy outcome.

Conclusions: None of the nine steroids measured in follicular fluid predicted pregnancy outcome in women undergoing IVF.

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KEYWORDS
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INTRODUCTION

Follicular development in the ovary depends on numerous extra- and intra-ovarian factors. Bioactive steroids, in particular androgens (testosterone, dihydrotestosterone), oestrogens (oestradiol, oestrone) and progesterone play important roles in regulating ovarian folliculogenesis and maturation. These steroids and their precursors are synthesized in the follicle and secreted by granulosa and theca cells, with follicular fluid representing a milieu in which the oocyte develops and matures to achieve fertilizing capacity. Yet, the steroid milieu required for optimal oocyte development and maturation remains to be defined.

Variable success rates are achieved with IVF, such that even an average of 40% for a first IVF cycle resulting in a live birth is considered successful (*Wade et al., 2015*). Therefore, improving the IVF per cycle live birth rate remains an important priority. Consequently, numerous studies have aimed to determine predictive factors, either positive or negative prognostic markers, for IVF success (*Revelli et al., 2009; van Loendersloot et al., 2010; Kushnir et al., 2012*). The ability to assess human oocyte quality more definitively in IVF could optimize oocyte selection, allowing for optimal choice of oocytes for transfer or cryostorage, which would improve IVF success per cycle and reduce excessive exposure of women to ovarian stimulation as well as wasted embryo overproduction (*Revelli et al., 2009*). As the oocyte microenvironment is likely to be crucial for healthy development and maturation, analysis of follicular fluid biochemistry may provide informative biomarkers of oocyte health and maturation. The maturing oocyte is surrounded by follicular fluid, a complex fluid comprising a plasma transudate and follicular secretions, including bioactive steroids secreted from granulosa and theca cells, namely androgens, oestrogens and progesterone (*Revelli et al., 2009*). Recent in-vitro evidence suggests the existence of an intrafollicular feedback circuit regulating steroidogenesis in mice (*Lebbe et al., 2017*), which may have a role in regulating an optimal steroidal milieu for oocyte maturation. As follicular fluid is available for collection at oocyte retrieval, it provides a valuable resource for biochemical analysis of the oocyte milieu.

Several studies have analysed follicular fluid steroid concentrations to determine whether these can predict IVF outcomes (*De Sutter et al., 1991; Andersen,1993; Costa et al., 2004; Smitz et al., 2007; Kushnir et al., 2009; Rosen et al., 2009; Lamb et al., 2010; Naessen et al., 2010; Wen et al., 2010; Kushnir et al., 2012; Kushnir et al., 2016*). Most, however, have used steroid immunoassays, which are suboptimal when applied to biological fluids other than human serum (*Handelsman, 2017*). Because of the requirement for a different immunoassay for each steroid and their low sensitivity, these studies have often used pooled fluid from multiple follicles to obtain sufficient sample for multiple steroid measurement. Furthermore, the limited specificity of steroid immunoassays dictated by their antibody epitopes can lead to inaccuracy owing to cross-reactivity with structurally related steroids.

Recently, only one group has used steroid liquid chromatography-mass spectrometry (LC-MS/MS) to measure follicular fluid steroids in a series of studies (*Kushnir et al., 2009; 2012; 2016; Naessen et al., 2010*). We, therefore, sought to investigate follicular fluid steroid profiles using multi-analyte LC-MS/MS profiling (*Harwood and Handelsman, 2009*). This is the reference method for steroid specificity and, together with its high sensitivity, is now the gold standard for clinical research in endocrinology (*Handelsman and Wartofsky, 2013*) and reproductive medicine (*Handelsman, 2017*). We aimed to use LC-MS/MS to examine the potential of follicular fluid steroid profiles to predict IVF outcomes. The aim of this study was to use an LC-MS/MS method to profile nine steroids: progesterone (P4), oestradiol (E2), oestrone (E1), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone (T), dihydrotestosterone (DHT), 3α, 5α androstanediol (3α-diol) and 3β, 5α androstanediol (3β-diol) in serum measured at baseline and at oocyte retrieval (34–36 h after the ovulatory trigger) together with fluid from the largest single antral follicle in women undergoing IVF stimulation, and to determine whether such highly specific steroid measurements improved prediction of IVF outcomes.

MATERIALS AND METHODS

Study participants, ovarian stimulation, embryo assessment and transfer

Between September 2014 and July 2015, 93 eligible, consenting patients enrolled to undergo IVF treatment at the Royal Prince Alfred Hospital, Sydney, were recruited. After exclusion of 15 patients whose cycles did not proceed and one patient whose follicular fluid samples was unusable owing to accidental dilution with an unknown volume of buffer during oocyte retrieval, 77 women participated in the study (FIGURE 1). Demographics, infertility causes, treatments and outcomes are presented in TABLE 1. The study was approved by the Sydney Local Health District Human Ethics Committee (RPA Hospital), Sydney, Australia (HREC/14/RPAH/181), on 24 July 2014, and all participants provided written, informed consent.

Protocols used for ovarian stimulation were either gonadotrophin releasing hormone agonist 'long' down-regulation or gonadotrophin releasing hormone antagonist 'short' cycles, as previously described (*Marren et al., 2016*). Follicular growth was promoted by recombinant FSH (Puregon, MSD or Gonal F; Merck Serono, Darmstadt, Germany). Recombinant HCG 250 μg (Ovidrel; Merck Serono) or urinary HCG 5000–10,000 IU (Pregnyl; MSD, Kenilworth, NJ, USA) were used to trigger ovulation when two or more follicles had a diameter greater than 18mm in a long down-regulation cycle or three or more leading follicles had a diameter greater than 17mm in a short cycle. Follicles with a diameter more than 14mm in diameter were quantified by transvaginal ultrasonography. Oocytes were retrieved under transvaginal ultrasound guidance 34–36 h after the ovulatory trigger.

Semen preparation, IVF, intracytoplasmic sperm injection (ICSI) and physiological intra-cytoplasmic sperm injection (PICSI) were carried out according to the unit's routine protocols. Between 18 and 20 h after insemination, oocytes obtained from ICSI or PICSI were assessed for signs of fertilization. Oocytes with two pro-nuclei and two polar bodies were cultured and embryonic development evaluated on the morning of day 3 and 5 (day of embryo transfer) as previously described (*Marren et al., 2016*). One blastocyst was selected for embryo transfer 5 days after fertilization. If the schedule did not permit, a four to 10-cell embryo or a morula was transferred 3 or 4 days after fertilization.

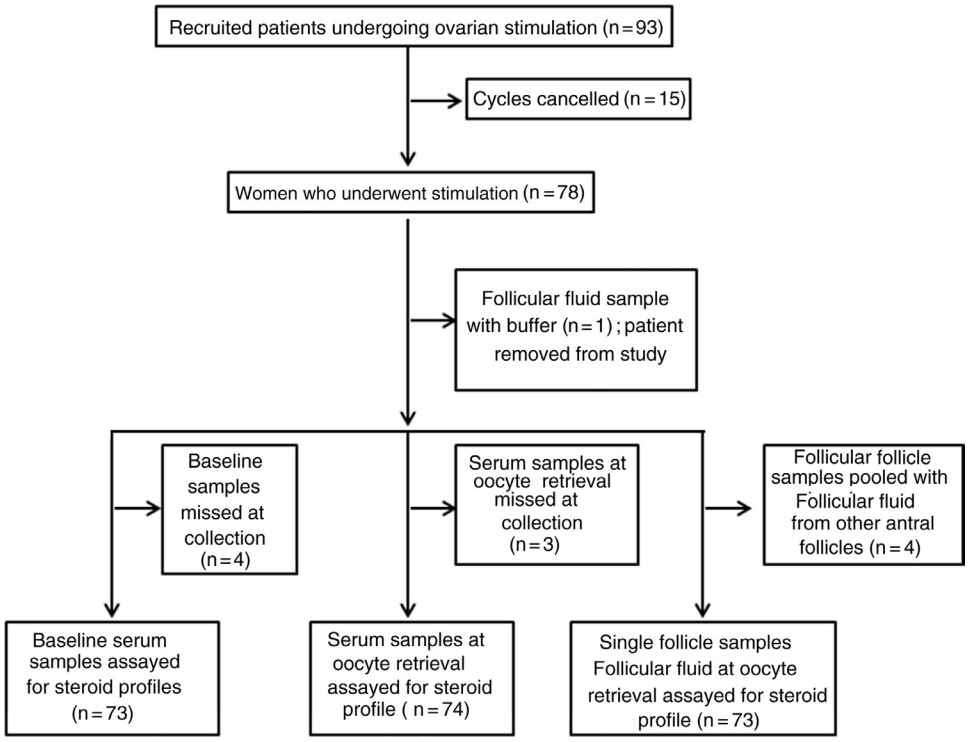

FIGURE 1 Sample distribution and numbers in the study analysis.

Collection and handling of follicular fluid samples

Single follicle follicular fluid was aspirated from the largest follicle during the oocyte retrieval process by ultrasound-guided aspiration. Care was taken to prevent any buffer contamination of follicular fluid. Follicular fluid was collected into a Cryo tube (Nunc), frozen in liquid nitrogen, transferred to the Anzac Research Institute Andrology laboratory on dry ice and then stored at –30 C until LC-MS/MS analysis.

LC-MS/MS methods, reagents and calibrators

Steroids were measured in organic solvent extracts of 200 µl of serum or follicular fluid samples using LC-MS/MS by a stable-isotope dilution method with atmospheric pressure photoionization (*Harwood and Handelsman, 2009; Hsu et al., 2016*). Details of the LC-MS/MS methods are presented in the **Appendix** (see Supplementary Materials). Briefly, samples, standards and quality controls underwent liquid–liquid extraction with methyl tert- butyl ether to quantify P4, E2, E1, DHEA, A4, T, DHT, 3α-diol and 3β-diol. Because of the high concentrations of P4, E2 and E1, follicular fluid samples were run a second time diluted 300-fold with phosphate buffered saline (pH 7.4) to quantify those three steroids. Accuracy was assessed by spiked recovery of serum pools, and imprecision by quality control samples run at three levels (low, medium and high) prepared by spiking charcoal-stripped serum with appropriate volumes of steroid stock solutions. The limits of detection (LOD), limit of quantification (LOQ) and reproducibility (coefficient of variation %) of the steroids were T (10 pg/ml, 25 pg/ml, 5%), DHT (50 pg/ml, 100 pg/ml, 10%), E2 (2.5 pg/ml, 5 pg/ml, 10%), E1 (2.5 pg/ml, 5 pg/ml, 11%), 3α-diol (50 pg/ml, 200 pg/ml, 7%), 3β-diol (50 pg/ml, 200 pg/ml, 5%), DHEA (50 pg/ml, 100 pg/ml, 5%), P4 (50 pg/ml, 100 pg/ml, 7%) and A4 (25 pg/ml, 50 pg/ml, 7%).

Serum AMH

Serum AMH was measured in baseline serum samples by a commercial enzyme-linked immunosorbent assay (ELISA) immunoassay (ultrasensitive AMH ELISA, ANSH Labs) with a detection limit of 0.05 ng/ml according to the manufacturer's direction.

Data analysis

SPSS and NCSS software (NCSS Statistical Software version 11, Kaysville, UT) were used for statistical analysis. Steroid concentrations were not normally distributed so were transformed before analysis using a Box–Cox analysis to determine the optimal normalizing power transformation, which was in all cases a log transform. Serum AMH was cube-root transformed to normalize distribution, which was determined as optimal under Box–Cox transformation. For analysis, undetectable steroid concentrations were set at the limit of detection. This was based on the most appropriate simple substitution methods for quantifying left censored data using a value between zero and the quantification limit (*Hewett and Ganser, 2007; Huynh et al., 2014*), for which the limit of detection serves well as an independently defined threshold between quantification and detection limits. The predictive relationships of serum and follicular steroids for IVF outcomes were investigated by negative binomial regression for count data, logistic regression for binary analysis of pregnancy (clinical pregnancy, confirmed by ultrasound showing an intrauterine sac at 7 weeks' gestation) and multiple linear regression for the proportion of oocytes fertilized (after arcsin transformation). The regression analyses were run separately for each of the three sets (baseline serum, pick-up serum and follicular fluid) of steroid concentrations. In addition to the steroid concentrations, the regression models included age, total FSH dose, number of days of FSH stimulation, baseline serum AMH and body mass index. In further

TABLE 1 ANTHROPOMETRIC AND MEDICAL FEATURES OF PARTICIPANTS, PATIENT TREATMENT AND OUTCOME MEASUREMENTS

Variable	
Patients characteristics	
Age (years)	38 ± 1 (25–43)
Height (cm)	164 ± 1 (145–186)
Weight (kg)	66 ± 2 (42–100)
Body mass index (kg/m^2)	25 ± 1 (16–34)
Nulliparity, n (%)	52/76 (68)
Current smokers, n (%)	3/76 (4)
Cause of infertility, n (%)[a]	
Unexplained	32/77 (42)
Male factor	26/77 (34)
Endometriosis	13/77 (17)
Tubal factor	10/77(13)
Ovulation disorder	9/77 (12)
Polycystic ovary syndrome	7/77 (9)
Chemotherapy	1/77 (1)
Baseline AMH	
AMH (ng/ml)	3.3 ± 2.6 (0.1–11.1)
Treatment	
Total dose FSH (IU)	222 ± 8 (75–350)
Number of days FSH	11 ± 0.2 (8–16)
Outcomes	
Number of antral follicles ≥14 mm	6 ± 0.4 (1–17)
Number of oocytes retrieved	7 ± 0.5 (0–23)
% of oocytes fertilized	65% ± 2.9 (0–100)
Number of blastocysts on day 5	2 ± 0.2 (0–8)
Confirmed pregnancy, n (%)[b]	13/64 (20)

Data are mean ± SEM (range) or numbers (%).

[a] Numbers include multiple causes for some patients.

[b] Does not include patients who had no embryos available for transfer, or pregnancies from embryos frozen in this cycle for transfer in a future cycle.

AMG, anti-Müllerian hormone.

analyses the ratio of E2 to T and E_1 to A4, both indirect markers of aromatization, as well as E_2 to E_1 and total oestrogens (E_2 + E_1), and the percentage increase from baseline to pick-up in serum T, A4 or DHEA concentrations, in all three biological fluids were analysed as potential predictors of IVF outcomes. $P < 0.05$ was considered statistically significant.

RESULTS

Steroid profiles of serum and follicular fluid from a single follicle

Detectable levels of P4, E2, E1, DHEA, A4 and T were observed in all serum and follicular fluid aspirated from the single largest antral follicle samples at oocyte retrieval, whereas E1, DHEA, A4 and T were detected in all baseline serum samples (TABLE 2). However, DHT, 3α-diol and 3β-diol were only detectable in a minority of serum and follicular fluid samples, though concentrations were higher, and a higher proportion were detectable in serum at oocyte retrieval. Ovarian stimulation increased median serum concentrations of P4 (150-fold), E2 (79-fold) and E1 (31-fold), with much smaller increases in T (four-fold) and A4 (three-fold), but DHEA was not increased (TABLE 2 and FIGURE 2). Compared with levels at oocyte retrieval, median follicular fluid steroid concentrations were further increased for P4 (1880-fold), E2 (570-fold), E1 (49 fold) with smaller increases in A4 (6-fold) and T (2-fold), whereas DHEA was not increased (TABLE 2 and FIGURE 2). Further analysis was undertaken to determine if baseline (B) serum and oocyte retrieval/pick-up (P) serum and follicular fluid (F) steroid concentrations at oocyte retrieval were correlated (TABLE 3). A statistical relationship was identified between B T and P T, but no relationship was found between any baseline steroid concentrations and follicular fluid steroid concentrations (TABLE 3). Similarly, no relationship was found between any steroid concentrations at oocyte retrieval and follicular fluid steroid concentrations (TABLE 3).

Correlations with IVF outcomes

Steroid concentrations in baseline serum, and serum and follicular fluid collected at oocyte retrieval were analysed in separate models as predictors of IVF outcomes together with age, BMI, total FSH dose and days of FSH stimulation and baseline serum AMH (TABLE 4).

Analysis of baseline serum steroid profiles revealed that for pre-transfer IVF outcomes (number of follicles >14 mm, oocytes retrieved and fertilized, day-5 blastocysts), only baseline serum AMH was a consistent positive predictor. No significant steroid predictors were identified other than E1–A4 ratio being a negative predictor of blastocysts on day 5 (TABLE 4).

AMH serum at oocyte retrieval was a significant (positive) predictor of some (oocytes retrieved and fertilized and day-5 blastocysts) but not all (follicles >14 mm) pre-transfer IVF outcomes. Steroid levels at oocyte retrieval were not found to be strong predictors of pre-transfer IVF outcomes with only E2–T ratio being a negative predictor of oocytes retrieved and fertilized, (TABLE 4). In addition, the percentage increase from baseline to serum T, A4 or DHEA at oocyte retrieval did not predict any IVF outcome (data not shown). Follicular fluid AMH was also found to be a significant (positive) predictor of the pre-transfer IVF outcomes of number of follicles with a diameter greater than 14mm and oocytes retrieved and fertilized, but not day-5 blastocysts. Apart from E2 being a negative predictor of oocytes retrieved and blastocysts on day 5, and E1 a positive predictor of oocyte retrieved, no other steroids significantly predicted pre-transfer IVF outcomes (TABLE 4).

For clinical pregnancy outcomes, only serum DHEA (positive) at oocyte retrieval was found to be predictive. No other baseline, follicular fluid steroids or AMH levels at oocyte retrieval were predictive.

TABLE 2 STEROID PROFILES MEASURED BY LC-MS/MS IN SERUM AND FOLLICULAR FLUID COLLECTED FROM WOMEN UNDERGOING OVARIAN STIMULATION. DATA ARE PER CENT DETECTABLE SAMPLES, MEDIAN AND RANGE. NON-DETECTABLE STEROID VALUES WERE TREATED AS THE VALUE SET FOR THE LIMIT OF DETECTION

	Baseline serum			Serum at oocyte retrieval			Follicular fluid		
Steroids	**Per cent detectable samples**	**Median (Q1, Q3)**	**Min–max**	**Per cent detectable samples**	**Median (Q1, Q3)**	**Min–max**	**Per cent detectable samples**	**Median (Q1, Q3)**	**Min–max**
P4 (ng/ml)	36	0.05 (0.05, 0.19)	0.05–7.8	100	7.55 (4.63, 11.28)	0.65–52.1	100	14190 (11250, 19860)	2166–47400
E2 (pg/ml)	81	10.20 (3.86, 22.80)	2.5–116	100	802 (471, 1029)	45.8–2030.0	100	459000 (312000, 705000)	86700–1929000
E1 (pg/ml)	100	21.90 (14.90, 33.60)	6.5–77.7	100	680 (343, 1105)	54.60–3650.0	100	33600 (24300, 51000)	5910–149100
DHEA (ng/ml)	100	3.59 (2.39, 5.82)	0.1–12.9	100	2.83 (2.19, 4.15)	0.66–17.5	100	2.15 (1.59, 2.95)	0.17–12.6
A4 (ng/ml)	100	0.60 (0.45, 0.75)	0.1–1.6	100	1.68 (1.11, 2.17)	0.34–4.68	100	10.20 (6.73, 15.10)	2.23–73.7
T (ng/ml)	100	0.13 (0.08, 0.20)	0.05–0.4	100	0.49 (0.31, 0.67)	0.06–1.3	100	1.11 (0.79, 1.35)	0.27–3.73
DHT (ng/ml)	12	0.05 (0.05, 0.05)	0.05–0.1	24	0.05 (0.05, 0.05)	0.05–0.6	20	0.05 (0.05, 0.05)	0.05–0.7
3α-diol (ng/ml)	7	0.05 (0.05, 0.05)	0.05–0.1	40	0.05 (0.05, 0.06)	0.05–0.16	12	0.05 (0.05, 0.05)	0.05–0.14
3β-diol (ng/ml)	11	0.05 (0.05, 0.05)	0.05–0.1	57	0.06 (0.05, 0.10)	0.05–0.32	18	0.05 (0.05, 0.05)	0.05–0.12

A4, androstenedione; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E1, oestrone; E2, oestradiol; LC-MS/MS, liquid chromatography-mass spectrometry; P4, progesterone; T, testosterone; 3α-diol, 3 α, 5α androstanediol; 3β-diol, 3β, 5α androstanediol.

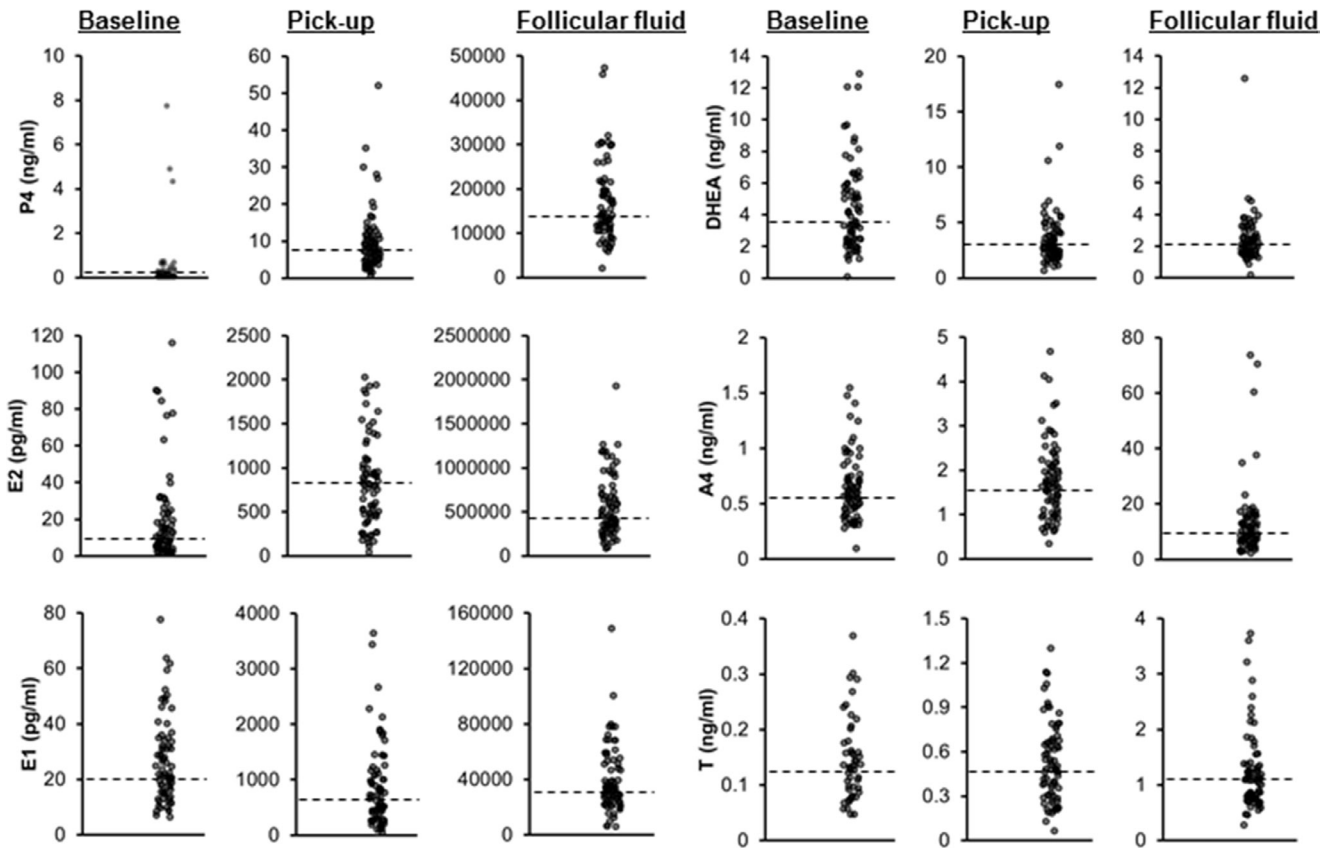

FIGURE 2 Distribution of steroid concentrations in baseline serum and serum at oocyte retrieval (pick-up), as well as follicular fluid from the single dominant follicle collected from women undergoing ovarian stimulation. Dashed line indicates median. E1, oestrone; E2, oestradiol; P4, progesterone; DHEA, dehydroepiandrosterone; A4, androstenedione; and T, testosterone.

TABLE 3 CORRELATIONS BETWEEN SERUM BASELINE, SERUM AT OOCYTE RETRIEVAL AND FOLLICULAR FLUID STEROID CONCENTRATIONS

	B_P4	B_E2	B_E1	B_A4	B_T	P_P4	P_E2	P_E1	P_A4	P_T	F_P4	F_E2	F_E1	F_A4	F_T
B_P4	1.000	0.363	0.291	0.040	0.098	0.202	0.161	0.093	0.164	0.137	−0.014	0.015	−0.051	0.120	0.042
B_E2		1.000	0.662	0.252	0.320	0.193	0.290	0.300	0.361	0.343	0.067	0.192	0.246	0.049	−0.074
B_E1			1.000	**0.448**	**0.481**	0.083	0.236	0.288	0.289	0.335	−0.130	−0.065	−0.051	0.138	0.099
B_A4				1.000	**0.754**	−0.153	−0.011	−0.116	0.118	0.207	−0.276	−0.234	−0.212	0.223	0.139
B_T					1.000	0.056	0.288	0.153	0.355	**0.501**	−0.363	−0.409	−0.251	0.243	0.110
P_P4						1.000	**0.627**	**0.634**	**0.530**	**0.442**	0.023	−0.080	−0.053	−0.130	−0.147
P_E2							1.000	**0.854**	**0.843**	**0.850**	−0.012	−0.185	−0.097	0.200	0.125
P_E1								1.000	**0.680**	**0.653**	−0.015	−0.123	−0.029	0.026	0.039
P_A4									1.000	**0.906**	−0.033	−0.123	−0.103	0.179	0.092
P_T										1.000	−0.089	−0.227	−0.138	0.297	0.167
F_P4											1.000	**0.644**	**0.486**	−0.068	0.011
F_E2												1.000	**0.802**	−0.025	0.052
F_E1													1.000	0.004	0.070
F_A4														1.000	**0.794**
F_T															1.000

Spearman rank correlation coefficients (n = 66) between serum and follicular fluid. steroid concentrations with bold values indicating statistically significant relationships after a Bonferroni adjustment for multiple comparisons (r = 0.44 for P = 0.0002).
B, baseline; F, follicular fluid steroid concentrations; P, pick-up (retrieval).

DISCUSSION

The present study aimed to measure, by sensitive and specific multi-analyte LC-MS/MS (*Harwood and Handelsman, 2009*), concentrations of nine steroids in matched samples of serum collected at baseline and oocyte retrieval and follicular fluid collected at oocyte retrieval from the largest antral follicle of women undergoing IVF stimulation. In addition, we investigated whether using the steroid LC-MS/MS profile of serum or follicular fluid predicted IVF outcomes.

Follicular fluid provides the microenvironment in which an oocyte develops and matures. This fluid is routinely available during oocyte retrieval in an IVF cycle so that it has always been available for biochemical measurements to gain insight into oocyte development, fertilization capacity and for predicting IVF outcomes. Consequently, many studies have analysed the follicular fluid steroid profile as potential predictors of IVF reproductive outcomes. Most of these studies measured one or more steroids using a different steroid immunoassay for each steroid as required by the inherently mono-analyte steroid immunoassay methodology. Steroid immunoassays, however, have

TABLE 4 PREDICTORS OF IVF OUTCOMES

IVF Outcome	Baseline serum			Serum at oocyte retrieval			Follicular fluid		
	Variable	Coefficient ± SE	*P*-value	Variable	Coefficient ± SE	P-value	Variable	Coefficient ± SE	*P*-value
Follicles >14 mm, n	AMH	0.754 ± 0.280	**0.007**				AMH	0.676 ± 0.296	**0.023**
Oocytes retrieved, n	AMH	1.076 ± 0.291	**0.0002**	AMH	0.774 ± 0.306	**0.011**	AMH E2 E1	0.808 ± 0.299 −3.37 ± 1.52 3.17 ± 1.52	**0.007** **0.027** **0.037**
Oocytes fertilized, n	AMH	0.967 ± 0.323	**0.003**	AMH E2–T ratio	1.233 ± 0.339 −0.003 0.001	**0.0003** **0.027**	AMH	0.798 ± 0.336	**0.018**
Oocytes fertilized (proportion)				E2–T ratio Total E	−0.001 ± 0.0003 0.0002 ± 0.0001	**0.007** 0.054	T	−1.26 ± 0.66	0.063
Blastocysts day 5 (number)	AMH E1–A4 ratio	0.938 ± 0.443 −0.095 ± 0.033	**0.034** **0.004**	AMH	1.23 ± 0.51	**0.016**	Total FSH E2	−0.007 ± 0.003 −4.73 ± 2.28	**0.028** **0.038**
Clinical pregnancy	A4 T	−28.1 ± 14.4 8.54 ± 4.43	0.051 0.054	DHEA	6.41 ± 2.87	**0.026**			

Predictive models were developed separately for baseline and pick-up serum and for follicular fluid. In each model, steroid measurements in that fluid together with age, BMI, total FSH used, days of FSH treatment and baseline serum AMH were run in negative binomial regression models for pre-transfer counts data (numbers of follicles >14 mm, numbers oocytes retrieved or fertilized, day 5 blastocysts), in multiple regression for percentage of oocytes fertilized (arcsin transformed) and logistic regression for clinical pregnancy. Bold values indicate statistically significant differences.
A4, androstenedione; AMH, anti-Müllerian hormone; E1, oestrone; E2, oestradiol; DHEA, dehydroepiandrosterone; T, testosterone.

substantial limitations in specificity when applied to biological fluids other than serum testosterone for men and serum oestradiol for pre-menopausal women (*Rosner et al., 2007; Handelsman and Wartofsky, 2013; Rosner et al., 2013*), the type of samples for which those commercial immunoassays were developed and optimized. As a result, discrepancies between studies of follicular fluid steroid content and their relationships with IVF outcomes may arise, among other reasons, from the non-specificity of steroid immunoassays arising from antibody-based cross-reactivity with structurally related steroids, including precursors and metabolites (*Handelsman, 2017*). Furthermore, the need for separate samples for each immunoassay dictated a need for larger sample volume, which was often achieved by pooling follicular fluid; however, this made it impossible to provide comprehensive analysis of steroid profile in individual follicles. Other studies using different analytical methods such as capillary gas (*Vanluchene et al., 1990*), high pressure liquid chromatography (*Vanluchene et al., 1991*) or steroid LC-MS/MS (*Kushnir et al., 2009; Naessen et al., 2010; Kushnir et al., 2012; Kushnir et al., 2016*) have reported measurement of steroids in follicular fluid. Hence, in this study, we aimed to expand on these studies by using multi-analyte steroid LC-MS/MS for nine bioactive steroids in 200 µl matched samples of follicular fluid (and <1 µl for the major ovarian steroids P4, E2 and E1) as well as baseline and oocyte retrieval serum samples from individual women undergoing IVF ovarian stimulation.

The follicular fluid steroid concentration measurements in this study show that dominant ovarian follicular steroid (P4, E2 and E1) levels were greatly increased (30-150 fold) in serum collected at oocyte retrieval after ovarian stimulation compared with baseline serum levels, and were even further increased in follicular fluid (50-1800 fold versus contemporaneous oocyte retrieval serum). In contrast, other sex steroids, T and A4, increased only more modestly in serum and follicular fluid, whereas DHEA remained similar at baseline, at oocyte retrieval and in follicular fluid during IVF stimulation. These findings are consistent with previously reported studies of follicular fluid after ovarian stimulation with steroids measured by gas chromatography-mass spectrometry (*Dehennin et al., 1987*) or LC-MS/MS (*Kushnir et al., 2016*). The first exploratory analysis using mass spectrometry-based steroid measurements of steroids in follicular fluid of 44 women undergoing ovarian stimulation as well as a pool of 50 follicular fluids (*Dehennin et al.,1987; 1990*) did not report any predictive biomarkers for IVF outcome or pregnancy. More recent studies have reported LC-MS/MS steroid profiles in follicular fluid of women with regular menstruation or undergoing IVF stimulation (*Kushnir et al., 2009; Naessen et al., 2010*) but only a single previous study has investigated follicular fluid steroid concentrations using multi-analyte LC-MS/MS as a predictor of IVF outcomes (*Kushnir et al., 2016*). In the study by *Kushnir et al. (2016)*, steroid profiles were generated from follicular fluid collected from 22 follicles of 14 women undergoing IVF, and after analysis of 14 steroids and 28 derivatives of these measurements, the only significant predictor of pregnancy outcome was the ratio of A4 to DHEA. The findings from our present study are generally consistent with the lack of predictive power for IVF outcomes from steroid LC-MS/MS profiles and their derivatives, but confirms the predictive power of baseline serum AMH for most pre-transfer IVF outcomes.

The highly sensitive and specific multi-analyte LC-MS/MS method allows simultaneous measurement of nine bioactive steroids from small sample volumes. A limitation of the present study is that by focusing on the dominant follicle but without tracking of fate of individual oocytes, it was not possible to determine whether the oocyte from the dominant follicle was the one that led to pregnancy. This may explain why the follicle steroid concentrations from the dominant follicle may not correlate with pregnancy or other IVF outcomes. Further this does not allow testing of the hypothesis that follicular androgens may influence oocyte health, including fertilizing capacity. In the future, evaluation of the predictive properties of follicular fluid steroid profiles for IVF outcomes would be rendered more specific if steroid profiles were matched with data from individually tracked oocytes through fertilization and subsequent steps. An additional potential limitation is that women undergoing ovarian stimulation had diverse individualized stimulation regimens as determined by treating clinicians. Although we included FSH treatment as a covariable, future studies should control for additional details of individual stimulation regimens.

In conclusion, use of multi-analyte LC-MS/MS steroid profiling of serum at baseline, at oocyte retrieval or of follicular fluid, from the single dominant follicle in women undergoing IVF stimulation does not predict IVF outcomes. Nevertheless, the highly sensitive and specific LC-MS/MS methods are suitable for studies aiming to identify the relationship between the follicular fluid microenvironment and oocyte quality and viability. Future studies more specifically focused on the follicular fluid microenvironment of individual oocytes will provide further insight, and may ultimately be used to optimize individual IVF treatment regimens, as well as facilitating selection of the 'best' oocyte(s) for fertilization and producing live births.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2018.10.006.

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