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A multi-centre international study of salivary hormone oestradiol and progesterone measurements in ART monitoring

BIOGRAPHY

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KEY MESSAGE

Salivary oestradiol testing correlates well to serum-based assessment. Saliva oestradiol and progesterone values, around the time of trigger, can vary in response to ovarian stimulation and vary from patient to patient. Saliva-based hormone tests may become the preferred method of hormone monitoring for fertility treatments in the future.

ABSTRACT

Research question: Ovarian stimulation during IVF cycles involves close monitoring of oestradiol, progesterone and ultrasound measurements of follicle growth. In contrast to blood draws, sampling saliva is less invasive. Here, a blind validation is presented of a novel saliva-based oestradiol and progesterone assay carried out in samples collected in independent IVF clinics.

Design: Concurrent serum and saliva samples were collected from 324 patients at six large independent IVF laboratories. Saliva samples were frozen and run blinded. A further 18 patients had samples collected more frequently around the time of HCG trigger. Saliva samples were analysed using an immunoassay developed with Salimetrics LLC.

Results: In total, 652 pairs of saliva and serum oestradiol were evaluated, with correlation coefficients ranging from 0.68 to 0.91. In the European clinics, a further 237 of saliva and serum progesterone samples were evaluated; however, the correlations were generally poorer, ranging from –0.02 to 0.22. In the patients collected more frequently, five out of 18 patients (27.8%) showed an immediate decrease in oestradiol after trigger. When progesterone samples were assessed after trigger, eight out of 18 (44.4%) showed a continued rise.

Conclusions: Salivary oestradiol hormone testing correlates well to serum-based assessment, whereas progesterone values, around the time of trigger, are not consistent from patient to patient.

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KEYWORDS

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INTRODUCTION

Standard monitoring of ovarian response during IVF treatment cycles involves close monitoring of circulating reproductive hormones, including oestradiol and progesterone levels, in association with measurements of ovarian follicle growth. Transvaginal ultrasound (TVUS) examination of follicular development is used in tandem with hormone monitoring. The standard in most IVF clinics (the need for both TVUS and serum oestradiol determinations in assisted reproduction) has been questioned, partly related to the inconvenience of phlebotomy. It has been suggested that the addition of serum oestradiol could be time consuming and expensive, and that simplification of IVF treatment by using TVUS alone should be considered (*Kwan et al., 2014*).

Many couples undergoing IVF report that the treatment regimen is demanding and creates anxiety owing to financial burdens, time commitments and emotional stress. Therefore, it is not surprising that treatment discontinuation is relatively common, with as many as 25–37% of couples stopping treatment after their first unsuccessful attempt (*Domar et al., 2018*). With such high drop-out rates, researchers worldwide have focused on the reasons behind these patient decisions. The most commonly cited reason for treatment discontinuation for non-insured patients is financial strain (*Domar et al., 2010*; *2018*; *Rooney and Domar, 2016*). For patients with insurance coverage, the most common reasons for treatment discontinuation are perception of poor prognosis and psychological burden (*Domar et al., 2010*; *Rooney and Domar, 2018*).

For patients undergoing IVF, salivary diagnostic testing may provide advantages over standard serum testing. Sampling saliva, in contrast to venipuncture, is non-invasive and can be carried out remotely by the patient herself at home, to be dropped off quickly or shipped, or can be carried out easily in the healthcare setting (*Lu et al., 1999*; *Hofman, 2001*; *Carroll et al., 2008*; *Groschl, 2008*; *Read, 2009*). Samples are stable at room temperature for a week and longer if refrigerated or frozen (*Gandara et al., 2007*). The ease of saliva sampling reduces blood draws and may provide a more 'patient friendly' approach to IVF, which, in turn, is associated with improved patient satisfaction, decreased stress and lower treatment drop-out (*Gerris, 2020*). This, however, should not compromise the chance of success (Flisser *et al*., 2007).

Sex steroids present in saliva, including oestradiol and progesterone, are unbound to carrier proteins and reflect free, bioactive hormone. Reproducible measurements of salivary oestradiol and progesterone have previously been established in premenopausal women during natural cycles, conception cycles and ovarian stimulation cycles using radioimmunoassay (RIA) techniques (*Belkien et al., 1985*; *O'Rourke and Ellison, 1990*; *Worthman et al., 1990*; *Lipson and Ellison, 1996*; *Lu et al., 1999*; *Ellison and Lipson, 1999*; *Gann et al., 2001*; *Groschl et al., 2001*; *Bao et al., 2003*; *Chatterton et al., 2005*; *Gandara et al., 2007*; *Celec et al., 2009*; *Dielen et al., 2017*). The average correlation coefficients reported for salivary and serum total oestradiol and progesterone range from 0.7 to 0.95 (*Belkien et al., 1985*; *Wong et al., 1990*; *Worthman et al., 1990*). Although the difficulty of use and unpredictability of the previously used RIAs limited their application, the newer generation enzyme-linked, competitive immunoassays are easy to administer and allow for accurate, quick and inexpensive measurements of salivary hormones (*Granger and Taylor, 2020*).

As an extension to preliminary pilot studies conducted by the present authors, the aim was to continue collection of concurrent saliva and serum samples to further analyse the relationship between saliva and serum reproductive hormone concentrations. The aim was to conduct a multi-centre blind validation of saliva samples collected during routine ovarian stimulation for IVF cycles. These saliva samples were then compared with samples of serum collected the same day. An additional objective was to analyse a second cohort of saliva samples collected more frequently around the time of human HCG trigger to ascertain whether more subtle changes in saliva oestradiol and progesterone values occurred during ovarian stimulation.

MATERIALS AND METHODS

Participants

The aim of this prospective study was to measure salivary and serum oestradiol and progesterone concentrations in patients undergoing monitored infertility treatment cycles with ovarian stimulation for IVF. Ovarian stimulation cycles involved the use of gonadotrophins, (i.e. FSH or human menopausal gonadotrophins) to stimulate the development of multiple ovarian follicles. Patients were aged between 21 and 43 years. Patients whose previous cycle was cancelled owing to hyperstimulation were excluded.

Enrolment

All patients planning to start ovarian stimulation for an IVF cycle at the participating IVF facilities (USA-SG [Shady Grove Fertility, Washington, DC], USA-RMA [Reproductive Medicine Associates of New York, New York, NY], Belgium, Italy, Spain and France) were asked if they wished to participate in this study. Separate approval was obtained in each country under the auspices of a multi-centre approval obtained in the USA through Western IRB (#1114402 [October 12, 2014] and #1173742 [28 February 2018]). The study was registered under clinicaltrials.gov [NCT02040545] (20 January 2014). A physician or nurse provided patients with an explanation of the study goals, methods and details of the subject involvement and obtained informed consent.

Number of participants

This prospective study was conducted in two phases. The first involved two centres in the USA whereby salivary oestradiol and serum measurements were assessed from 63 participants. In the second part of the study, 261 patients had saliva and serum collected. All 261 had serum oestradiol levels assessed, whereas 122 Spanish patients did not have serum progesterone evaluated. Concurrent samples were collected around three to five times between days 0 and 12 of ovarian stimulation.

In addition to the routine samples, a further series of samples was collected from 18 patients at Boston IVF when the patients' lead follicle had reached 16 mm. At this time, the patient collected a serum and saliva sample in the morning but was also asked to collect additional saliva samples at home at 16:00, 22:00, and on the following day at 08:00, 14:00 and 20:00. These patients were asked to follow the same collection routine until the day after HCG and gonadotrophin releasing hormone agonist trigger. These

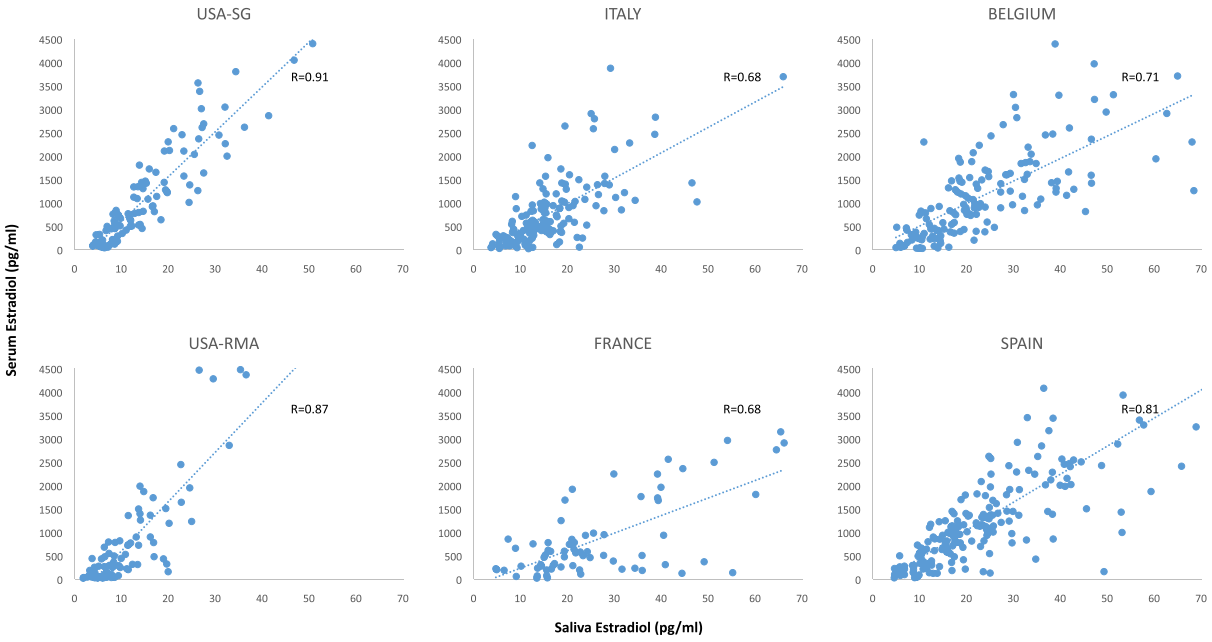

FIGURE 1 The correlation coefficients (R) and plots of saliva oestradiol to serum values from six independent IVF clinics based in the USA and Europe. USA-SG; participating US IVF facility Shady Grove Fertility, Washington, DC, USA; US-RMA, participating US IVF facility Reproductive Medicine Associates of New York, New York, NY, USA.

samples were frozen for later analysis as described below.

Sample collection

IVF cycle management and treatment proceeded according to standard techniques at the discretion of the treating physician. At every venipuncture during the treatment cycle, serum levels of oestradiol and progesterone were measured according to standard protocol, using an immuno-assay system. Patients collected a saliva sample via passive drool according to the assay specifications concomitantly. The Salimetrics assays have previously been optimized for consistent performance in saliva using passive drool and include diluents that properly control for inconsistency in the passive drool matrix. See online specifications for spike and recovery data (*Salimetrics Estradiol Assay Specifications, 2020*; *Salimetrics Progesterone Assay Specifications, 2020*). All patients were handed a collection instructions card at consent. Blood contamination was not measured; however, collection instructions were designed to minimize blood contamination. Collections were always made at least 30 min after a meal, and intake of a beverage or tooth brushing and mouths were rinsed with water 10 min before collection. No samples were excluded because of pre-analytic sampling requirements even though some were marked as discoloured. Twelve discoloured samples were subsequently excluded because they provided outlier saliva oestradiol levels of over 100 pg/ml. Samples were coded and stored at –20°Celsius until sent frozen for blinded analysis. Once the saliva samples were analysed, the corresponding serum results were then linked to the saliva analysis.

Salivary hormone assays

Salivary oestradiol and progesterone levels were measured on frozen–thawed saliva samples using a salivary 17B-oestradiol, and progesterone enzyme immunoassay kits (Salimetrics, Carlsbad, CA, USA), respectively. All samples were visually inspected after thawing to identify any abnormal colour. Immunoassay measurements of salivary hormones were conducted according to the assay specifications. Performance characteristics on the salivary oestradiol and progesterone assays have been previously assessed and published by the assay developer (Salimetrics, Carlsbad, CA, USA), including intra-assay precision, inter-assay precision, linearity of dilution, recovery, sensitivity, correlation with serum, cross reactivity and specificity of antiserum (*Salimetrics Estradiol Assay Specifications, 2020*; *Salimetrics Progesterone Assay Specifications, 2020*). Briefly, the functional sensitivity was determined by assaying 36 samples at a concentration level resulting in a coefficient of variation of 20%. The functional sensitivity of HC Salivary Estradiol EIA is 2.87 pg/ml and of progesterone 12.37 pg/ml. The lower and higher limits of the salivary oestradiol (1 pg/ml to 32 pg/ml) and progesterone (10 pg/ml to 2430 pg/ml) assays have been previously established. Assay performance was confirmed in the study setting. Performance characteristics of the serum assay are well established and clinically applied as the standard for circulating hormone measurement.

Statistical analysis

Saliva and serum Pearson Product Moment correlations were calculated using the matched mean saliva and serum assay results for all data points collected. Fisher's Z tests were used to test if the correlations were significantly different. SPSS software version 25.0 (Armonk, New York, USA) was used for all data analyses.

RESULTS

Saliva oestradiol values

Oestradiol evaluation at US sites

For the initial study, two US centres provided between one and seven salivary oestradiol samples per patient (n = 63 patients). In clinic USA-SG and USA-RMA, 30 and 33 patients, respectively, had concurrent saliva and serum samples collected. In Clinic USA-SG, 129 pairs

TABLE 1 SERUM AND SALIVA ESTRADIOL DESCRIPTIVE FOR THE EUROPEAN CLINICS

		Italy	France	Belgium	Spain
	Patients	**59**	**40**	**40**	**122**
Serum oestradiol (pg/ml)	Samples	152	77	149	211
	Minimum	20	22	26	28
	Maximum	3890	3921	4395	5570
	Mean	803.2	862.9	1174.9	1275.8
	SD	760.1	892.5	932.7	1026.3
Saliva oestradiol (pg/ml)	Samples	143	75	150	191
	Minimum	3.7	4.7	24.9	4.5
	Maximum	65.9	66.1	82.5	79.2
	Mean	16.7	26.4	24.0	23.9
	SD	9.4	15.5	14.3	14.4

TABLE 2 CORRELATIONS BY SITE AND PARTIAL CORRELATIONS CONTROLLING FOR CYCLE DAY

	Country	*n*[a]	R-value	Significance
Correlations by site	Italy	141	0.69	<0.001
	France	70	0.53	<0.001
	Belgium	144	0.75	<0.001
	Spain	186	0.79	<0.001
Partial correlations controlling for cycle day	Italy	140	0.64	<0.001
	France	68	0.51	<0.001
	Belgium	108	0.68	<0.001
	Spain	185	0.77	<0.001

Pearson product moment correlations of the serum and saliva were conducting using the natural log transformed data.
[a] Individuals who had matching serum-saliva data.

of saliva and serum oestradiol were evaluated with a correlation coefficient of 0.91. In clinic USA-RMA, 85 pairs were evaluated with a correlation coefficient of 0.87 (FIGURE 1). Over 87% of patients showed an individual within-cycle Pearson correlation of greater than 0.7 and 66% a correlation of greater than 0.9 (range 0.42–1.0).

Oestradiol evaluation at European sites

In the second part of the study, one to six salivary oestradiol samples were analysed for each patient from the four European clinical groups. The mean ± SD for serum and saliva for each clinic are shown in TABLE 1. The correlation coefficients and plots of saliva oestradiol to serum values are shown in FIGURE 1 and correlation coefficients in TABLE 2. Over

TABLE 3 SERUM AND SALIVA PROGESTERONE DESCRIPTIVES FOR THE EUROPEAN CLINICS

		Italy	France	Belgium	Spain*
	Patients	**59**	**40**	**40**	**122**
Serum progesterone (pg/ml)	Samples	152	78	146	
	Minimum	200	100	150	
	Maximum	4780	2840	17000	
	Mean	735	408	2294	
	SD	487	410	3662	
Saliva progesterone (pg/ml)	Samples	128	90	139	150
	Minimum	9	20	14	13
	Maximum	4482	1002	3244	4505
	Mean	168	183	183	188
	SD	539	168	362	410

[a]Serum values were not determined for progesterone in Spain.

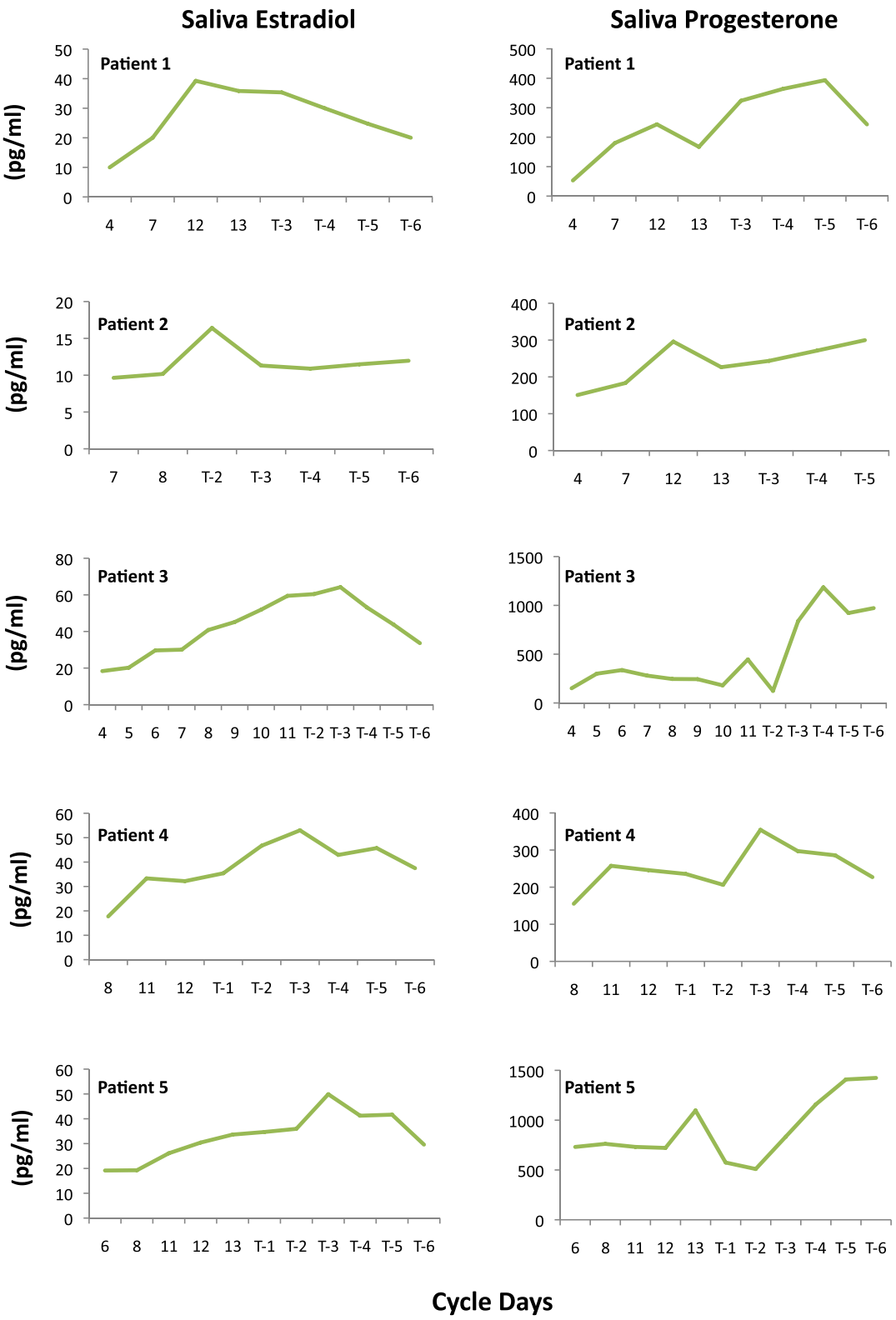

FIGURE 2 Representative plots of saliva oestradiol and progesterone values (pg/ml) of five different patients undergoing ovarian stimulation. Cycle days represent the day of cycle after commencement of FSH, whereas T-1 through to 6 represent the following: day of trigger afternoon (T1), evening (T2): day after trigger morning (T3), afternoon (T4), evening (T5): day of retrieval morning (T6).

75% of patients showed an individual within-cycle correlation of over 0.7 and 62%, a correlation of over 0.9.

When controlling for cycle day, the partial correlation coefficients remained similar for each site (TABLE 2). When a comparison was carried out between Italy and France and between Belgium and Spain, no significant difference was observed. When comparing Italy with Belgium, significance was found to be borderline ($P = 0.05$). Fisher's z test was used to determine if significant differences occurred in correlations between pairs of sites (two-tailed test). The comparisons are presented in Supplementary Table 1.

Saliva progesterone values

The descriptive values for progesterone samples collected from the four European sites are presented in TABLE 3. The mean serum values varied significantly between clinics, with the Italian and French groups having lower overall values. Pearson product moment correlations by each site for the serum and saliva were conducted using the natural log transformed data. The following values were obtained for each site: Italy: r (125) = 0.22, $P = 0.01$; France: r (64) = 0.12, $P = 0.34$; Belgium: r (99) = –0.02, $P = 0.87$.

Sequential saliva oestradiol and progesterone values to investigate variability after HCG trigger

The mean age (+SD) of the 18 patients providing sequential saliva samples was 37.4 + 2.9 years. Fifteen patients received an HCG trigger, two received leuprolide acetate (Lupron®) and one received a dual (Lupron® and HCG) trigger. Only three patients underwent a fresh embryo transfer as all others had all embryos frozen for a subsequent frozen embryo transfer as a result of either genetic testing or elevated serum oestradiol or progesterone. Saliva and serum oestradiol values were sampled concurrently before HCG trigger and showed a good correlation ($n = 55$; Pearson Correlation = 0.66). A smaller number of saliva and serum progesterone values were sampled concurrently before HCG and showed a weaker correlation ($n = 41$; Pearson Correlation = 0.30). The 18 patients collected on average 5.0 ± 0.7 samples after trigger. When oestradiol samples were assessed after trigger, five out of 18 patients (27.8%) showed an immediate decrease, two (11.1%) a delayed decrease and 10 (55.6%) remained relatively stable. Only one showed a rise in oestradiol levels. When progesterone samples were assessed after trigger, eight (44.4%) showed a continued rise, seven (38.9%) a delayed rise, whereas two remained stable and one decreased. Examples of the changing values are shown in FIGURE 2.

DISCUSSION

Ovarian stimulation for IVF and other treatments routinely involves TVUS with the addition of frequent venipunctures for hormonal assessments. Our multicentre data from US and European sites indicates that oestradiol monitoring of saliva correlates well with serum values. These data and previously published data correlating oestradiol with follicular measurement indicate that measuring oestradiol saliva may be a viable alternative to serum (*Rottiers et al., 2018*).

Testing of saliva has long been postulated as a source of hormone analytics in fertility testing (*LI et al., 1989*). Its use, however, has been surpassed by the availability of rapid immunoassays, even though they involve venipuncture. The multiple visits incurred during an IVF cycle, with coincident blood draws, make the ability to use a less invasive hormone monitoring system a potentially valuable option. Saliva testing is non-invasive, simple to perform, stress free and painless. Reduced stress by eliminating venipuncture has been proposed to be a major advantage of salivary testing (*Granger and Taylor, 2020*).

A further benefit of saliva values is that they reflect the biologically active (free) fraction of steroids in the blood (*Vining et al., 1983*; *Granger and Taylor, 2020*). The active fraction of steroid hormones is not bound by carrier proteins. One to 10% of the steroids in blood, leak into saliva from plasma. Albumin and sex hormone-binding globulin do not allow the bound fraction of the hormones to get into saliva owing to their high molecular weights (*Celec et al., 2009*; *Kells and Dollbaum, 2009*). Current serum immunoassays of oestradiol and progesterone only reflect the total amounts of these hormones. Numerous studies have shown that measurement of free hormone levels may be more sensitive indicators of the effect of the hormone. For example, in pre-pubertal girls, there is a progressive increase in the exposure of peripheral tissues to testosterone and oestradiol with advancing age. As sex hormones enhance tissue maturation, these incremental changes are thought to play a role in the somatic and psychological development of girls before the onset of the clinical signs of puberty (*Belgorosky and Rivarola, 1988*). Free testosterone has now gained more attention, and there are indications that it has a stronger clinical prediction use than total testosterone (*Goldman et al., 2017*). In relation to follicular development, little is known if free hormones, and, in particular free oestradiol, are better predictors of follicular growth than bound serum levels. It has previously been shown that salivary oestradiol concentrations are strongly correlated with the number of follicles that develop and their size (*Rottiers et al., 2018*).

In the present study, the correlation between saliva oestradiol and serum oestradiol was stronger than salivary and serum progesterone. In addition, the sequential assessment of oestradiol in relation to cycle day and time of maturation trigger were aligned to expected results. It was evident that different sites showed variability in saliva values (Supplementary Table 1). This was most likely due to differences in stimulation strategies as the serum values also differed (TABLE 1), with Italy and France showing lower mean serum oestradiol values than Belgium and Spain. Overall, previous studies examining saliva and serum oestradiol levels have shown good agreement (*Hull et al., 1986*; *Celec et al., 2009*; *Dielen et al., 2017*). In our previous study, statistical analysis showed a strong linear correlation between serum and salivary oestradiol (*Rottiers et al., 2018*). For every single unit increase in serum oestradio l (+ 1 ng/l), the estimated saliva oestradiol concentration increased with 0.011 pg/ml (95% CI 0.009 to 0.01). This strong linear correlation between serum and salivary oestradiol has been validated in this multi-centre trial spanning six different clinics.

Progesterone was, unfortunately, less conclusive, and brings into question a number of different issues in relation to testing progesterone in saliva. First, the assay may not be performing up to standard. All standards were always in range, and spiking of known progesterone

levels into saliva for validation of the assays showed excellent performance (data not shown). Second, progesterone may not be behaving similarly in saliva to oestradiol but, given overall similarities in their chemical structures, this would be hard to envisage. Finally, free progesterone itself may be behaving abnormally in certain infertility patients, and may undergo fluctuations during the day, or both. Good supporting data shows that elevated serum progesterone levels on the day of trigger during an IVF cycle have a negative effect on implantation rates in the fresh cycle (*Venetis et al., 2013*). This is presumed to be due to advancement of the endometrium, leading to embryo–endometrial asynchrony, with demonstrable changes in endometrial gene expression (*Labarta et al., 2011*). Specific serum progesterone cut-offs have long been debated and it has taken a long time to understand what these values may be, and a consensus has yet to be broadly reached (*Venetis et al., 2013*; *Huang et al., 2016*; *Siristatidis et al., 2018*; *Wu et al., 2019*). Progesterone values may also vary during the day (*Delfs et al., 1994*, *Veldhuis et al., 1988*). Recently it was shown that mean serum progesterone values varied between 08:00 and 20:00 by as much as a 44% (*Gonzalez-Foruria et al., 2019*). In their study, a mixed model analysis revealed that the progesterone reduction during the day was significantly associated with time and total recombinant FSH dose administered. This study suggested that a single progesterone determination on the final day of oocyte maturation is not reliable enough to make clinical decisions owing to the enormous variation in progesterone during the day. Our own data using saliva values taken before and after HCG trigger also showed a dramatic variability in progesterone values. Although this may represent some assay flux, it is plausible that free progesterone values of patients vary during the day (*Gonzalez-Foruria et al., 2019*). Being able to perform sequential oestradiol and progesterone assessments in patients could in the future aid in more accurate timing of retrievals, transfers in IVF cycles, or both.

The limitations of using saliva must be acknowledged. Although collection is easy, it is not always straightforward and, in both series of studies, a small number of patients with discoloured saliva samples showed poor correlations, indicating that they failed to collect according to protocol. Saliva-based hormone tests may, however, become the preferred method of ovarian stimulation monitoring in the future, in particular if rapid testing methods improve turnaround time for the assay. A further limitation is that saliva testing is unable to assay for other reproductive hormones, including LH and FSH. Finally, the possibility of allowing patients to collect at home or in private before attending clinic may also limit patient-staff contact in times of pandemics such as the current Covid-19 crisis. A further logistical limitation of the study is that, although serum assays are well established, the values used in this study were taken from different centres using different assay platforms.

In conclusion, salivary oestradiol provides a comparable alternative to serum-based assessment. In view of the limited knowledge of free progesterone, the results obtained by measuring saliva progesterone need to be further validated. Further studies are needed to create confidence in using salivary measurements alone and to establish whether key decisions during superovulation can be confidently made, in particular specific cut-off values for hyperstimulation. As we improve our understanding of free hormone levels, saliva-based testing may become a more viable method for hormone monitoring. The ease of saliva sampling may allow a reduction in treatment burden, improved patient satisfaction and decreased stress.

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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.2020.10.012.

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