Running title: Hair and salivary cortisol in pregnancy in IVF

Relationship between hair and salivary cortisol and pregnancy in women undergoing IVF

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Abstract

Evidence for an association between cortisol and clinical pregnancy in women undergoing In Vitro Fertilisation (IVF) is mixed with previous studies relying exclusively on short term measures of cortisol in blood, saliva, urine, and/or follicular fluid. Hair sampling allows analysis of systemic levels of cortisol over the preceding 3-6 months. The present study sought to explore the relationship between cortisol and clinical pregnancy outcome in women undergoing IVF utilising multiple indices of cortisol derived from both saliva and hair measured prior to commencing gonadotrophin treatment. A total of 135 women (mean age 34.5 SD +/-4.8) were recruited from an English fertility clinic (December 2012 to April 2014) 60% of whom became pregnant (n=81)). Salivary cortisol data were obtained over two days: upon awakening, 30 minutes post awakening, and at 22:00. A subsample (n=88) of the women providing salivary samples were approached consecutively to provide hair samples for the measurement of

cortisol. Independent Logistic regression analyses revealed that salivary cortisol measures including cortisol awakening response (CAR) (p=.485), area under the curve with respect to ground (AUCg) (p=.527), area under the curve with respect to increase (AUCi) (p=.731) and diurnal slope (p=.889) did not predict clinical pregnancy. In contrast, hair cortisol concentrations significantly predicted clinical pregnancy (p=.017). Associations between hair cortisol and clinical pregnancy remained when controlling for accumulations of salivary cortisol (p=.034) accounting for 26.7% of the variance in pregnancy outcome.

These findings provide preliminary evidence that longer term systemic cortisol may influence reproductive outcomes; and in turn suggests that interventions to reduce cortisol prior to commencing IVF could improve treatment outcomes.

Key words

Stress, Hair, Cortisol, Infertility, In Vitro Fertilisation, IVF

1. Introduction

The role that stress may exert on reproductive functioning has remained a topic of debate for decades (Boivin and Venetis., 2011; Mathiesen et al., 2011). This debate has been fuelled not least by the complexities of delineating plausible psychobiological pathways by which stress might influence reproductive outcomes (Whirledge and Cidlowski, 2010; Toufexis et al., 2014). Two main pathways have been considered: the hypothalamic pituitary adrenal (HPA) - hypothalamic pituitary gonadal (HPG) axis and the HPA – immune axis. The glucocorticoid cortisol, the main effector of the HPA axis, plays a central role in both pathways. With regard to the HPA-HPG pathway, elevated cortisol has been shown to inhibit sexual functions at all three levels of the HPG axis. At the level of the hypothalamus via inhibition of gonadotrophin releasing hormone (GnRh) secretion (Dubey and Plant, 1985; Kamel and Kubajak, 1987), at the level of the pituitary by interfering with GnRh induced luteinising hormone release (Briski and Sylvester, 1991), and at the level of the gonads by altering the stimulatory effect of gonadotrophins on sex steroid secretion (Bambino and Hsueh, 1981; Hsueh and Erikson, 1978).

With regard to the HPA-immune pathway it is well established that cortisol can regulate the activity of the immune system (Tsigos and Chrousos, 2002), including effects on the production of proinflammatory cytokines, tumour necrosis factor (TNF) and natural killer (NK) cell activity (Gatti et al. 1987; Mavoungou, 2006) all of which have been implicated in reproductive outcomes (Daher et al. 1999). However, the precise role of the immune system in pregnancy continues to be an area of debate. Taking the early phases of pregnancy as an example, it has been suggested that because the fetus is semi-allogenic to the

maternal host, that a degree of immune suppression may favour conception (Thornton, 2010; Nepomnaschy et al., 2007). In contrast, it has been argued that, rather than immune suppression, a strong inflammatory response is required for conception to occur (Mor, 2011). These competing views are likely to be due, in part, to the fact that as observed by Mor, (2011), pregnancy has often been conceptualised as a single immunological event. However, there is evidence that it consists of three distinct phases (characterised by a pro-inflammatory (first trimester), anti-inflammatory (second trimester) and then finally a return to a pro-inflammatory state (third trimester). Furthermore, as noted by Christian (2015), even though the immune system is highly regulated by the neuroendocrine system, to date, there has been little integration of these literatures in the context of fertility and pregnancy: further impeding attempts to delineate the significance of the HPA-immune pathway.

These contrasting views regarding the role of the immune system during conception are further complicated by the presence or absence of glucocorticoid resistance (GR). GR refers to the phenomenon whereby immune function fails to downregulate despite the presence of chronically elevated cortisol (Cohen et al., 2012; Barnes and Adcock, 2009). Thus, two scenarios are possible. In the first, elevated cortisol results in immunosuppression (in the absence of GR). In the second, elevated cortisol (in the presence of GR) fails to down regulate inflammatory responses (Nepomnaschy et al., 2007 Thornton, 2010). These opposing effects of cortisol on immunity have fuelled the debate about the role of the immune system in fertility and specifically whether and when immune

suppression, normal immune function or indeed heightened immune activity promotes or hinders reproductive outcomes.

Given what is understood about the direct effects of the HPA-HPG axis on reproduction, and the proposed role that immunosuppression may or may not play, divergent effects on pregnancy outcomes could be expected in women undergoing infertility treatment. Considering, for example, just the period prior to commencing treatment, increased cortisol, from the perspective of the HPA-HPG (and potentially also the HPA-immune axis in the presence of GR and excessive inflammation) could be expected to be associated with decreased likelihood of pregnancy. In contrast, from the perspective of an HPA-immune pathway (in the absence of GR), advocates of the view that immune suppression favours conception (e.g., Thornton, 2010; Nepomnaschy et al., 2007) may expect greater levels of cortisol to be associated with an increased likelihood of pregnancy. These contrasting outcomes were evident in a recent systematic review which reported that both high and low cortisol levels were associated with an increased likelihood of pregnancy in women undergoing IVF treatment (Massey et al., 2014). Twelve studies were identified that explored associations between cortisol (measured in blood, urine, saliva and follicular fluid) and establishment of clinical pregnancy. Three studies reported significant associations between elevated cortisol in follicular fluid and increased likelihood of clinical pregnancy (Andersen and Hornnes., 1994; Keay et al., 2002; Thurston et al., 2003). Four studies showed the opposite relationship albeit at different stages of treatment (Demytteneare et al., 1992; Micheal et al., 1999; An et al., 2011; An et al., 2013). The remaining five studies failed to find any significant associations between cortisol and pregnancy outcomes (Lovely et al., 2003; Lewicka et al., 2003;

Smeenk et al., 2005; Nouri et al., 2011; Csemiczky et al., 2000). These equivocal findings are in contrast to evidence from animal studies which have shown a more consistent relationship between elevated cortisol levels and impaired reproductive outcomes across a range of species (Dobson and Smith, 1995; Alejandro et al., 2014).

A number of methodological factors may have contributed to the findings in this review. First, the majority of studies failed to control for extraneous variables known to influence cortisol including time of day, caffeine consumption and body mass index (BMI) (Massey et al., 2014). Second, studies measured cortisol at different stages of treatment. Whilst IVF is a useful clinical model for exploring the effects of stress on reproductive outcomes, over half of the studies (7/12) measured cortisol following gonadotrophin administration (Andersen et al., 1994; Keay et al., 2002; Thurston et al., 2003; Micheal et al., 1999; Lovely et al., 2003; Lewicka et al., 2003; Nouri et al., 2011). Gonadotrophins have profound effects on the HPA axis and, therefore, are likely to have confounded any observed associations between HPA function and pregnancy. Third, studies relied exclusively on acute (saliva, blood) or short term (urine, follicular fluid) measures of cortisol. However, these measures are only able to provide information about cortisol levels over minutes (saliva/blood) to hours (urine/follicular fluid). At best, such measures may not accurately represent basal function or, at worst, may only capture transient levels of the hormone which are unlikely to influence health (Saxbe, 2008).

A recent methodological development that may prove promising for understanding HPA function is hair sampling which provides a retrospective index of cumulative cortisol exposure over extended periods of 3 to 6 months

(Gow et al., 2010). The validity and reliability of hair cortisol sampling has been supported in both animal (Davenport et al., 2006) and human studies (Sauve et al., 2007; Stalder and Kirschbaum, 2012). Furthermore, the utility of hair sampling as a marker of long term HPA function has been demonstrated in a range of health contexts (Yamada et al., 2007; Kalra et al., 2007; Dettenborn et al., 2011; Steudte et al., 2011; Pereg et al., 2011; Braig et al., 2015).

The aim of this study was, therefore, to build upon previous work by exploring the relationship between acute (saliva) and chronic (hair) cortisol measured in the 1-2 weeks prior to commencing IVF treatment and treatment outcomes, as measured by the absence/presence of a clinical pregnancy. In view of the continued uncertainty regarding the precise role of the HPA-immune pathway in reproductive outcomes, we hypothesised that, in support of the HPA-HPG pathway elevated levels of acute and chronic cortisol would be associated with a reduced likelihood of pregnancy.

2. Materials and Methods

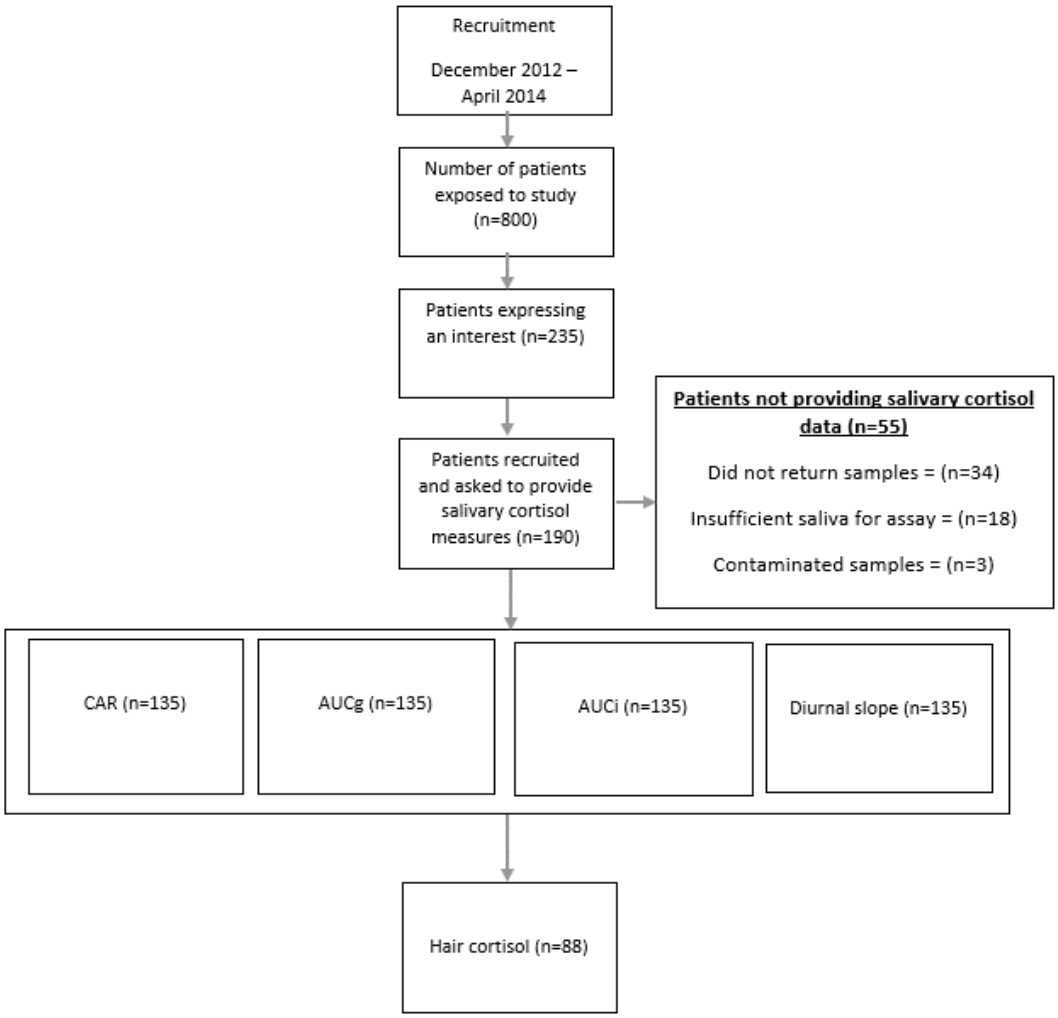
2.1 Recruitment

Recruitment took place at a single English fertility clinic between December 2012 to April 2014. Non-smoking women, with a body mass index (BMI) of 19 to 35, undergoing a long GnRH agonist protocol were eligible for participation. Study exclusion criteria included subjects with any recognised endocrinological health conditions (Kudielka et al., 2009). Patients taking concurrent corticosteroids were excluded from recruitment. Eligible patients received information regarding the study along with their initial appointment letters. During their initial consultation patients were asked whether or not they were interested in taking part in the study.

Those patients who were interested either met the study coordinator to discuss the study immediately after the consultation, or received a phone call at a later date. Participation in the study was optional and patients were assured that their decision to participate or not would not affect the quality of care provided by the clinic. Patients received no money or added benefits for taking part. Interested patients were asked to return to the clinic within seven days of their initial consultation to complete a study consent form and to collect a study participation pack. All patients provided written informed consent and the study was granted ethical approval by the NHS Health Research Authority, NRES Yorkshire and Humber Leeds East committee (Research Ethics Committee Reference 12/YH/0463).

Figure 1 summarises the participant recruitment in the study and number of patients providing cortisol data. Eight hundred patients were exposed to either a letter/study information leaflet, study poster and/or clinician/researcher invite. Two hundred and thirty-five patients expressed interest in the study. A total of one hundred and ninety patients were recruited. All patients were asked to collect saliva samples for the determination of salivary cortisol. After controlling for exclusions and missing data, complete data were available for n= 135 for the cortisol awakening response (CAR), area under the curve with respect to ground (AUCg) and increase (AUCi) and diurnal slope. Due to financial constraints hair samples were not collected from all participants. Instead, women providing saliva were approached consecutively to provide a hair sample (n=88).

Figure 1 summary of participant recruitment and number of patients providing cortisol data in the present study.



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2.2 Procedure

Patients who consented were given a study pack which included six salivettes (Salimetrics, UK) and a pre-paid envelope for the return of the salivettes to the clinic. Patients were instructed to provide samples using the salivettes on the two days prior to commencing their down regulation medication. Samples were collected at three times (upon awakening, 30 minutes post waking and 10pm)

over two consecutive days on weekdays and/or weekends resulting in six samples per patient. Each salivette device contains a cotton swab placed within a suspended insert in a capped plastic tube. A member of the research team demonstrated their use to each participant. In brief, this involved, removing the swab from the tube; placing it in the mouth and gently circulating it to stimulate saliva flow for a period of one to two minutes. Once saturated with saliva the swab was placed back in the tube and firmly capped. The samples were numbered and the time and date was recorded on the sample at the time of sample collection. Participants were instructed to refrain from caffeine, alcohol and the consumption of glucose based beverages on sampling days. Following the salivette demonstration, a hair sample was taken by the study coordinator within the clinic. Hair samples were cut with scissors as close as possible to the scalp from the vertex posterior of the head. This area has the least variation in cortisol (e.g. 16%) as compared to other areas (e.g. 31%) of the scalp (Sauve et al., 2007). Before cutting the hair participants were given the opportunity to feel or look at the amount of hair to be cut. Hair samples were wrapped in aluminium foil and stored in a dry and dark cupboard. It is generally accepted that hair grows on average 1cm per month therefore the most proximal 1cm of hair represents the previous month's cortisol exposure. Based on this, a 3cm segment of hair nearest to the scalp was examined. In line with previous research, this sample was assumed to represent the cumulative cortisol secretion over the previous three months (Wennig., 2000). Hair samples were taken 1 to 2 weeks prior to commencing treatment, while saliva samples were collected on the two days immediately prior to commencing downregulation treatment. At the end of the IVF cycle under observation medical and demographic variables were extracted

from the clinic's medical database including: *clinical pregnancy* - defined as a pregnancy diagnosed by ultrasonographic visualisation of fetal heart activity at 6 weeks post embryo transfer ; *Live birth* - defined as a birth in which a fetus was delivered with signs of life after complete extraction from the mother beyond 20 completed weeks of gestational age; *Number of oocytes* - defined as the amount of oocytes retrieved from the ovaries using an ultrasound-guided needle passed through the wall of the vagina to the ovaries; *Fertilisation rate* – defined as the percentage of oocytes fertilised in vitro via penetration of the ovum by the spermatozoon (Zegers-Hochschild et al., 2009).

2.3 Assay procedures

2.3.1 Saliva assay

An in-house radioimmunoassay (RIA) procedure utilizing commercial sources of 125l cortisol label (cat no 7121126; MP Biomedical UK) and cortisol rabbit polyclonal antibody (20-CR50; Stratech UK) was used to determine salivary cortisol concentrations. On the day of the radioimmunoassay, 100ul of standards were pipetted in triplicate into labelled plastic LP4 tubes, QC's and unknowns (100ul) were pipetted in duplicate. Using an Eppendorf multipette (100ul) antibody was added to all tubes except the Totals and NSB's. 100ul cortisol label was then added to all tubes. Samples were vortexed, covered with parafilm and left overnight to incubate at 4^{o}C.The following day 100ul (50ul of each) of pre diluted secondary antibody (Donkey Anti-Rabbit 1:80 and Normal Rabbit Serum 1:800) were added. Samples were vortexed, covered and left for a further 24 hours to incubate at 4^{o}C. The following day 1ml Assay Buffer was added to all tubes except

totals and centrifuged at 4000 rpm at 4^{o}C for 30mins. After aspirating the supernatant, the pellets were counted for 60 seconds on a Wizard 1471 Gamma Counter (Perkin Elmer UK) using the in-house cortisol protocol. Results were extrapolated from standard curves and concentrations from each of the sampling times expressed as nmol/l. The intra and inter-assay coefficients of variance for this assay were below 10%.

2.3.2 Hair assay

A high sensitivity in house radioimmunoassay was used to assess levels of cortisol in the hair. The hair cortisol extraction and assay used in this study was the procedure recommended by Meyer et al. (2014). Each hair sample was washed by placing each sample into a 15 ml screw-cap polypropylene centrifuge tube. Five ml of high performance liquid chromatography (HPLC)-grade isopropanol was then added to each tube followed by repeated inversion for 3 min using a rotator. Three centimetres of hair was cut from the root end of each sample. Samples were then put into 2ml Eppendorf SafeLock tubes with 5mm steel balls which were used to ground the hair for 15 minutes on the Tissue Lyser. One ml of Methanol was then added and the samples mixed overnight shaking gently. The next day the tubes were centrifuged for 3 mins at 10,000 rpm in a micro centrifuge and the supernatant transferred to clean Eppendorf tubes and centrifuged a second time. In the next step, 700ul of the methanol extract was pipetted into glass 75x12mm tubes which were then used to dry the solvent down under air using the sample concentrator. The dried extract was re-dissolved in 250ul of assay buffer and 2x 100ul taken for assay. The intra and inter-assay coefficients of variance for this assay were below10%. Hair cortisol

concentrations were expressed in pg/mg. Concentrations of hair cortisol (pg/mg) within the literature have been reported to range from 5pg/mg to 153/pg/mg for human hair (Raul et al., 2004; Suave et al., 2007; Kirschbaum et al., 2009).

2.4 Statistical analysis

2.4.1 Preparation of cortisol data prior to analysis

The cortisol awakening response (CAR) - In order to obtain a measure of the CAR, the second cortisol measure (sample taken 30 minutes after waking) was taken away from the first cortisol measure (sample taken on waking). This provided us with a measure of the absolute change in cortisol levels in the first 30 minutes following waking. This measure was calculated for each of the two days on which saliva was collected. Mean average levels of the CAR over the two-day period were then used in subsequent analysis.

Diurnal slope – The diurnal slope was calculated in this study by regressing the cortisol level onto the time of the sample, with the first sampling occasion treated as time 0, and later times being the number of hours after this time. The resulting slope measure was the expected amount that logged cortisol levels dropped per hour over the day for each individual. As with previous research (Vedhara et al., 2006), higher values or those closer to zero were indicative of more abnormal diurnal patterns. However, lower values or those further away from zero were indicative of more normal diurnal patterns. Mean average cortisol slope measures were computed across the two days and included in subsequent analysis.

Area under the curve (AUCg and AUCi) - The AUCg measure provides information on total hormonal output, thus, the basal activity of the HPA axis; while the AUC_i measure provides information on the reactivity of the system. Both areas under the curve (AUC) were calculated in this study using the formulae outlined by Pruessner et al., (2003). Mean AUC measures were calculated across the two days of cortisol sampling.

2.4.2 Approach to data analysis

All statistical analyses were conducted using SPSS version 21. Data screening was conducted to ensure data fitted the assumptions of parametric testing including homoscedasticity and homogeneity of variances and normal distribution. Outliers greater than +3 or less than -3 standard deviations were removed prior to analysis. Measures of salivary and hair cortisol were not normally distributed and therefore in accordance with recommendations log transformations were conducted prior to analysis (Miller and Plessow., 2013). Independent sample t tests were used to compare patient groups on clinical and demographic variables (age, number of oocytes, fertilisation rate, BMI and season of sampling) with effect size measures calculated using Cohen's d (Cohen, 1988). The variable 'season of sampling' was computed as follows: Spring (1 March to 31 May); Summer (1 June to 31 August); Autumn (1 September to 30 November); Winter (1 December to 1 28 February) (Staufenbiel et al., 2015). A priori power analysis revealed that for logistic regression with a binary dependent variable (clinical pregnancy) using several continuous independent variables, to achieve a power of .8 at a 0.05 significance a sample size of 130 was required (Hsieh et al., 1998).

Independent logistic regressions were conducted to examine the relationship of cortisol to pregnancy after controlling for clinical and demographic covariates. This analysis was approached in two ways. First, salivary cortisol indices and the hair cortisol measure were considered in separate models. This allowed us to examine the relationship of each individual measure separately. Second, given that chronic cortisol may be composed of accumulations of episodes of acute cortisol, a further model was constructed to identify the effects of chronic cortisol on pregnancy, after controlling for acute levels of the hormone. In step 1 relevant clinical and demographic covariates were entered (age, number of oocytes, fertilisation rate, BMI, season of hair sample) followed in step 2 by the addition of all salivary cortisol measures (CAR, AUCi, AUCg and diurnal slope) and in step 3 hair cortisol. The primary outcome for all analyses was clinical pregnancy.

3. Results

3.1 Comparing participants and non-participants on demographic and clinical variables

Independent sample t tests were conducted to compare patients providing salivary and or hair cortisol data (n=135) and the original sample who were recruited (n=190). For all measures, no statistically significant differences were evident between these groups on age, number of years infertile, number of previous attempts, fertilisation rate (%), number of oocytes or BMI (all p's> 0.05: data not shown).

3.2 Patient/treatment demographics and levels of cortisol

The mean age of the participants was 34 years (mean 34.5 SD 4.8). The study sample comprised of both first time patients and those who had treatment before with n=77 (57%) patients undergoing infertility treatment for the first time, n=26 (19%) patients were undergoing their second cycle and n=32 (24%) patients undergoing their third or more cycle of infertility treatment. The majority of couples had no children prior to treatment (n=117, 87%). The average duration of infertility was 2.4 years. Primary diagnoses varied across the sample with n=39 (29%) couples diagnosed with unknown infertility, n=35 (26%) diagnosed with male factor and n=55 (45%) diagnosed with female factor infertility. The sample comprised of both health authority (n=63) and self-funded (n=72) patients. The majority of women undergoing treatment were employed (n=119). Over half (n=75) of the sample obtained a clinical pregnancy during the IVF treatment cycle being observed in the study; with 89% (n=67)% of those becoming pregnant achieving a live birth. Patient/treatment demographics and cortisol

values are presented in Table 1 for patients who subsequently became pregnant and those who did not. Independent sample t tests compared these groups on the clinical and demographic data collected at baseline. There were no significant differences between the patient groups on mean age ($t_{181,157}$=.933, p=.322, d=.01), number of years infertile ($t_{188,184}$=.044, p=.965, d= .01) number of previous treatment attempts ($t_{188,154}$=.440, p=.660, d=.0) fertilisation rate (%) ($t_{161,140}$=.287, p=.775, d=.02) number of oocytes ($t_{141,120}$=1.317, p=.190, d=.02) nor BMI ($t_{188,170}$ =. 023, p=.982, d=.02).

Table 1: showing patient/treatment demographics and cortisol measures (means and standard deviations) between subsequently pregnant and not-pregnant groups.

Study Variable	Subsequently pregnant (n=75)	Subsequently not pregnant (n=60)
Patient and Treatment Demographics Means (SD)		
Age (years)	34.4 (4.9)	34.8 (4.6)
Number of years infertile	2.4 (.81)	2.4 (.91)
Number of previous treatment attempts	1.8 (1.2)	1.8 (1.5)
Fertilisation rate (% of oocytes fertilised)	44.3 (28.6)	44.5 (31.6)
Number of oocytes	10.8 (6.7)	10.8 (6.9)
Body Mass Index (BMI)	21.1 (1.2)	21.4 (1.2)
Cortisol levels Means (SD)		
Study Variable	Subsequently pregnant (n=80)	Subsequently not pregnant (n=55)
CAR (nmol/l)	-.133 (.169)	-.108 (.166)
AUCg (nmol/l)	43.5 (2.1)	43.8 (2.7)
AUCi (nmol/l)	4.3 (1.9)	4.0 (1.4)
Diurnal slope (nmol/l)	.691 (.367)	.689 (.282)
	Subsequently pregnant (n=48)	Subsequently not pregnant

		(n=40)
Hair cortisol pg/mg	19.4 (7.8)	24.9 (14.4)

Mean measures of salivary cortisol appeared comparable between patients subsequently becoming pregnant and those who did not (Table 1). Furthermore, as illustrated in Figure 2, there were no evident trends for any of the salivary cortisol indices to distinguish between the two groups. In contrast, levels of cortisol in hair appeared to be higher in women who subsequently did not become pregnant (see Table 1). Figure 3 shows the distribution of hair cortisol levels for the study sample.

Figure 2 illustrating mean and standard deviations for each salivary cortisol (nmol/l) measure (CAR, AUCi, AUCg and Diurnal Slope) for subsequently pregnant and not-pregnant groups.

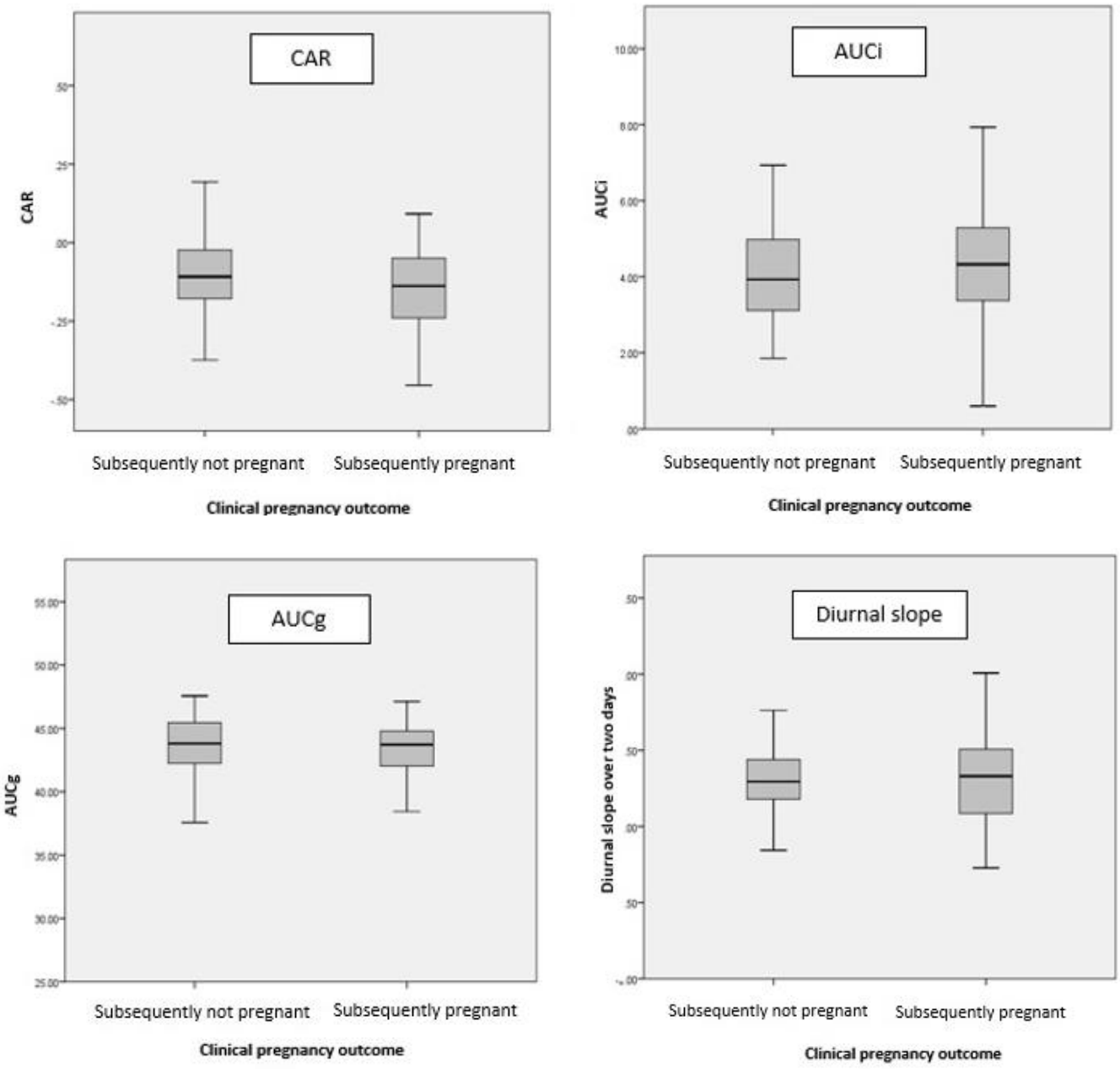

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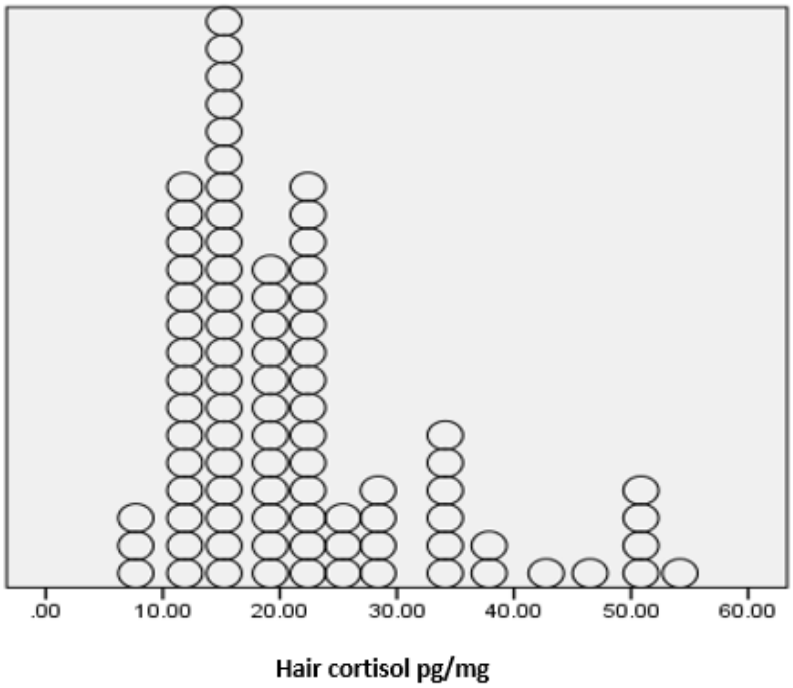
Figure 3 illustrating the distribution of hair cortisol concentrations (pg/mg)



3.3 Predicting clinical pregnancy

Independent logistic regression analyses (Table 2) revealed that, after controlling for the covariates age, number of oocytes, fertilisation rate, BMI and season of hair sampling, CAR (p=.485), AUCg (p=.527), AUCi (p=.731) and diurnal slope (p=.889) did not predict clinical pregnancy. Post-hoc analyses examined whether any of the salivary cortisol measures predicted clinical pregnancy in the sub-sample who provided both salivary and hair cortisol data (n=88). No significant associations were evident (all p's> 0.05: data not shown). In contrast, the analysis pertaining to hair cortisol demonstrated that hair cortisol concentrations were significantly negatively associated with clinical pregnancy (p=.017).

Table 2: Results of Hierarchical logistic regression run separately to explore the predictive effects of covariates, salivary cortisol and hair cortisol on clinical pregnancy.

Predictors	β	SE	df	Exp (β)	95% confidence intervals		Sig
CAR							
Step 1							
Age	-.025	.046	1	.975	.891	1.068	.587
Number of oocytes	-.037	.031	1	.963	.906	1.024	.233
Fertilisation rate	-.002	.009	1	.998	.981	1.015	.806
BMI	.181	.175	1	.835	.593	1.176	.302
Step 2							
CAR	-.981	1.404	1	.375	.278	.560	.485
AUCi							
Step 1							
Age	-.014	.047	1	.987	.899	1.082	.744
Number of oocytes	-.051	.031	1	.950	.895	1.009	.094
Fertilisation rate	.001	.009	1	1.000	.983	1.017	.966
BMI	-.205	.174	1	.814	.579	1.146	.239
Step 2							
AUCi	.043	.125	1	1.044	.817	1.335	.731
AUCg							
Step 1							
Age	-.011	.048	1	.989	.901	1.086	.824
Number of oocytes	-.053	.031	1	.949	.894	1.007	.085
Fertilisation rate	.001	.009	1	1.001	.983	1.018	.940
BMI	-.208	.174	1	.812	.577	1.143	.233
Step 2							
AUCg	-.058	.092	1	.943	.787	1.130	.527
Diurnal slope							
Step 1							
Age	-.018	.047	1	.982	.896	1.077	.705
Number of oocytes	-.041	.031	1	.960	.903	1.021	.196
Fertilisation rate	-.001	.009	1	.999	.982	1.016	.869
BMI	-.183	1.74	1	.833	.592	1.172	.294
Step 2							
Diurnal slope	.093	.663	1	1.097	.899	2.027	.889
Hair cortisol							
Step 1							
Age	.013	.066	1	1.013	.890	1.153	.846

Number of oocytes	-.026	.037	1	.974	.906	1.047	.477
Fertilisation rate	-.004	.012	1	.996	.974	1.019	.728
BMI	.031	.222	1	1.032	.668	1.594	.888
Season of sampling	.090	.221	1	1.094	.709	1.688	.685
Step 2							
Hair cortisol	-.068	.028	1	.934	.883	.988	**.017***

***Significant at the .05 level**

Given that chronic cortisol may be composed of accumulations of episodes of acute cortisol a further logistic regression was conducted to determine associations between hair cortisol and clinical pregnancy controlling for salivary cortisol. The results remained largely unchanged with elevated hair cortisol concentrations significantly predicting a reduced likelihood of clinical pregnancy (p=.034) accounting for between 26.7% (Cox and Snell) and 36.4% (Nagelkerke R^2) of the variance in pregnancy outcome (See table 3). The odds ratios indicated that for every unit reduction in hair cortisol the odds of clinical pregnancy increased by 1.09.

Table 3 Hierarchical logistic regression exploring the predictive effects of hair cortisol on clinical pregnancy whilst controlling for covariates and salivary cortisol indices.

Predictors	β	SE	df	Exp (β)	95% confidence intervals		Sig
Step 1							
Age	.054	.100	1	1.056	.868	1.285	.586
Number of oocytes	-.050	.060	1	.951	.846	1.070	.405
Fertilisation rate	-.013	.017	1	.987	.956	1.020	.445
BMI	.137	.362	1	1.147	.565	2.332	.704
Season of sampling	-.002	.263	1	.998	.597	1.670	.994
Step 2							
CAR	.008	.161	1	.003	.735	1.384	.959
AUCi	.461	.427	1	1.586	.687	2.665	.948
AUCg	.011	.163	1	1.011	.734	1.391	.948
Diurnal slope	.093	.663	1	1.097	.852	1.223	.889
Step 3							
Hair cortisol	-.091	.043	1	.913	.840	.993	**.033***

***Significant at the .05 level, Cox and Snell =.267, Nagelkerke = .364**

4. Discussion

To our knowledge, this is the first study to explore associations between both acute and chronic measures of cortisol exposure on the outcome of IVF. We examined whether levels of cortisol prior to IVF treatment were associated with the likelihood of clinical pregnancy. Measures of salivary cortisol (CAR, AUCg, AUCi and diurnal slope) did not predict clinical pregnancy. However, hair cortisol concentrations did, with greater levels associated with a reduced likelihood of conception. Our findings regarding hair cortisol add to a growing literature which suggests that chronic cortisol exposure may have important implications for health (Russell et al., 2012; Stalder and Kirschbaum, 2012) and highlights the use of hair sampling as a novel technique for understanding the role of cortisol on reproductive outcomes. Comparisons with other studies are,

however, difficult at this time, due to an absence of analogous research on the association between cortisol measured in hair and treatment outcomes following IVF.

Our findings provide partial support for our hypothesis that, in accordance with a HPA-HPG pathway, chronically elevated cortisol prior to commencing IVF treatment would be associated with a reduced likelihood of pregnancy. The absence of a relationship between salivary cortisol and clinical pregnancy should not necessarily be interpreted as evidence against the HPA-HPG pathway or cortisol influencing treatment outcomes in IVF. Rather, it may simply reflect the fact that hair derived assessments offer a more accurate picture of basal HPA activity as they capture cortisol exposure over a longer duration. In contrast, salivary derived assessments are, by definition, acute and therefore, a less precise indicator of basal activity. Indeed, the sampling protocol used in the present study (3 saliva samples over 2 consecutive days) could be considered the minimum standard for measuring cortisol and may have contributed to our null findings for salivary cortisol (Saxbe, 2008).The implications of our findings for the HPA-immune pathway are, however, more difficult to discern as we did not collect immunological measures and so cannot determine the relationship of cortisol to immunity or distinguish between the HPA-HPG and HPA-immune pathways.

It should be noted that the present study does not elucidate the factors that may cause chronic elevations in cortisol secretion (e.g., sleep, physical activity, stress) (Kudeilka et al., 2009). However, our data do suggest that interventions to reduce cortisol, pharmacological or behavioural, may be advantageous in the period prior to commencing fertility treatment. Stress in particular has established itself as a potent trigger of elevated cortisol secretion within the wider literature (Dickerson

and Kemeny, 2004) and therefore the focus of efforts to date has been on how psychological interventions may be used to mitigate the effects of long term cortisol exposure on health. Although evidence is not currently available from patients undergoing IVF, there are data from other populations suggesting that psychological interventions can significantly alter levels of cortisol measured in hair. For example, a recent study by Goldberg et al. (2014) explored the effects of mindfulness or a cognitive behavioural intervention for smoking cessation on hair cortisol concentrations and reported a significant decrease in hair cortisol following both interventions. There have, of course, also been studies which fail to find an effect of psychological interventions on hair cortisol (e.g., Young et al. 2015). Clearly, further research is required to examine the effectiveness of psychological and other interventions in reducing cortisol levels and their subsequent effects on IVF treatment outcomes.

A notable strength of this study was that both acute and chronic indices of cortisol production were examined in patients prior to IVF treatment whilst accounting for several confounds known to influence acute cortisol secretion (age, smoking, caffeine consumption, time of sampling). However, several methodological limitations are also worthy of note. First, as noted previously, the sampling protocol used in the present study is considered the minimum standard for assessing cortisol concentrations in naturalistic research (Saxbe, 2008; Clow et al., 2009). Second, several potential covariates were not accounted for including natural hair colour and frequency of hair washing which have been shown to influence hair cortisol concentrations (Staufenbiel et al., 2015). Similarly, data were not collected on shift work or whether samples were collected during weekdays or weekends. These factors have been shown to influence circadian

cortisol levels (Mirick et al., 2013; Thorn et al., 2006) and may have influenced our findings. It is also not clear how generalizable our results are to women trying to conceive naturally; also our findings extend only to clinical pregnancy. Therefore, it is unclear whether chronic cortisol is associated with live births following IVF although 89% of patients who became pregnant subsequently went on to have a live birth.

Despite these potential limitations, the present study extends the available evidence for the role of cortisol on clinical pregnancy in IVF patients (Massey et al., 2014) and provides the first evidence that elevated chronic cortisol levels are associated with a reduced likelihood of pregnancy. The pathways by which cortisol may influence reproduction, however, remain unclear, . Thus, further research is needed to not only corroborate these findings but also identify the pathways.

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