Cortisol Levels in Chronic Fatigue Syndrome and Atypical Depression Measured Using Hair and Saliva Specimens

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Abstract:

Background: Several diagnostic criteria for major depressive disorder (MDE) overlap with those of Chronic Fatigue Syndrome (CFS). Furthermore, atypical MDE (A-MDE), a subtype of MDE characterised by profound fatigue and which has frequently been linked with CFS, exhibits similar low cortisol levels to CFS. However, this result has been only found in specimens designed for measuring acute cortisol levels. In this study, we measure cortisol levels in subjects with CFS and in subjects with atypical MDE (A-MDE), without psychiatric comorbidity, using both hair and saliva specimens, to gain a measure of both short and long-term cortisol levels in these two conditions.

Methods: Hair cortisol concentration, representing the cortisol concentration of the previous three months, and salivary cortisol, measured at six time-points across one day and including the cortisol awakening response (CAR), post-awakening delta cortisol and the total daily output, were assessed in an age and gender matched group of 34 controls, 15 subjects with A-MDE and 17 with CFS.

Results: CFS (92.2 nmol/l.h, s.d=33.2 nmol/l.h) and A-MDE (mean=89.1 nmol/l.h, s.d=22.6 nmol/l.h) subjects both showed lower cortisol total daily output in saliva (AUCg) in comparison to healthy controls (mean=125.5 nmol/l.h, s.d=40.6 nmol/l.h). However, hair cortisol concentration was not lower than that of controls in either patient group. CFS and A-MDE did not differ from one another on any cortisol measures. CFS subjects reported fewer daily hassles and less severe psychic anxiety symptoms in comparison to A-MDE subjects (all $p<0.05$). However, they did not differ in the severity of somatic anxiety symptoms. There was also no difference in the presence of overlapping symptoms such as fatigability and memory problems between A-MDE and CFS subjects.

Conclusion: Low levels of cortisol found using short-term measures of daily output may be transient, since cortisol levels were normal when a long-term measure (hair) was studied. This might be explained by a potential cortisol rhythm alteration. Although these disorders have their distinctive depressive and somatic features, they may from part of a wider group of Somatic Symptom Disorders (SSD), given the findings of the same pattern of cortisol secretion in both disorders and increased frequency of overlapping clinical features.

1. Introduction

Fatigue is a frequent symptom (20%) among patients seeking medical help. However, this tends to be transient, moderate and self-limiting (Afari & Buchwald 2014). In Chronic Fatigue Syndrome (CFS) fatigue is long-lasting and disabling, with no readily demonstrable alternative organic explanation (Papadopoulos & Cleare 2012).

The scientific debate in relation to the definition of CFS is continuous and at times controversial. CFS is not included within the most recent edition of the Diagnostic and Statistical Manual (*DSM-5;* 2013), although might fit within the new category of Somatic Symptoms Disorder (SSD), which explicitly includes patients with "medically unexplained symptoms" (Dimsdale & Creed 2010). For some authors, CFS has also been understood as part of a wider group of stress-related bodily disorders, which include fibromyalgia, lower back pain, irritable bowel syndrome, burn-out syndrome and atypical major depression (A-MDE) (Fries et al., 2005). This is supported by the observation of a number of shared characteristics between these conditions, including decreased cortisol (Heim, Ehlert, & D. Hellhammer 2000).

A previous study compared acute (saliva) and chronic (hair) cortisol levels between A-MDE and non-atypical depression (NA-MDE). Results suggested that A-MDE may have more in common with SSD as defined by DSM-5 than with NA-MDE (Herane-Vives et al. 2018). This conclusion was in part based on the results showing a higher association between A-MDE and common environmental disturbances ("daily hassles"). According to the authors of the Hassles Scale, subjects experiencing a higher frequency of these common environmental disturbances are under higher risk of developing stress-related bodily disorders or SSD (Kanner, 1981). Moreover, Herane-Vives et al (2018) also found that the prevalence of fatigability and concentration impairment symptoms – key features of SSDs and two of the diagnostic criteria for CFS (Fukuda & Straus 1994) – was higher in A-MDE than NA-MDE The final important finding of Herane-Vives et al (2018) was the absence of hypercortisolaemia – a very consistent biological biomarker in major depression (Theodor et al. 2016) – in both hair and saliva cortisol measures in A-MDE, but the presence of hypocortisolism in saliva measures, as has been found previously (Lamers et al., 2012).

Decreased cortisol has been a common finding SSDs, although not all studies have found decreased cortisol levels. One possible explanation for this inconsistency is because of differing methodologies for assessing cortisol levels, and in particular the absence of studies

using measures designed to measure accumulated cortisol levels. The use of hair sampling has the potential to overcome this issue.

There is some preliminary evidence to show that CFS shares several clinical and neurobiological features with A-MDE (Juruena & Cleare 2007). However, it is unclear the extent to which these common features are related to CFS itself or might be confounded by the high comorbidity with depression. Rates of diagnosable psychiatric disorder, and depression in particular, are high in CFS: Skapinakis et al (2003) and Field et al., (1997) for instance, found similar figures, showing that over 74% of the patients with CFS also had mainly depression or anxiety disorders, although other studies find lower rates (Matsuda and Matsui 2009). Whilst mild hypocortisolism has been a relatively consistent finding in CFS, meta-analysis suggests that this finding is in fact most apparent in those with rather than without comorbid depression (Tak et al 2011).

This study was designed to investigate cortisol levels using both short-term (saliva) and long term (hair) measures in a matched group of subjects with CFS, A-MDE, and healthy controls. The aim was to characterise these two disorders in terms of their acute and chronic cortisol secretion patterns. A detailed clinical comparison of these disorders was also planned. We hypothesised, based on recent results from Herane-Vives et al (2018), that A-MDE and CFS subjects would share the same pattern of cortisol secretion: normal cortisol level in hair and decreased cortisol in saliva. In addition, with regards to clinical characteristics, we hypothesised that CFS and MDE-A would show similarities in both symptom patterns, focussing on those symptoms common to the definitions of MDE and CFS, and in the presence of common environmental.

2. Methods:

2.1. Participants

Participants were recruited in UK and Chile. CFS Participants were recruited in UK from general practitioner referrals to the Chronic Fatigue Syndrome Research Unit at King's College Hospital in Camberwell, South London. Patients included both local catchment area and tertiary referrals from the South of England. In Chile, CFS patients were referred from a group of qualified psychiatrists and rheumatologists. CFS participants were assessed by a psychiatrist in UK and a trained researcher in Chile according to a standardised assessment protocol for CFS (Sharpe et al 1997). Depressed participants were recruited in the UK from public

advertisements (Wise et al. 2016) and local psychological therapy services. Depressed participants in Chile originated from referrals by a group of qualified psychiatrists at the Clínica Psiquíatrica Universitaria of University of Chile, Chile, and from public advertisements. All depressed participants were assessed by a psychiatrist in the UK and a trained researcher in Chile using the Mini International Neuropsychiatric Interview (Sheehan et al. 1998). Depressive and fatigue symptom ratings, evaluated on an independent set of patients, showed high inter-rater reliability [Intraclass Correlation Coefficient (ICC) = 0.96, $p<0.05$].

Depressed patients were required to meet axis I DSM-IV criteria for a major depressive episode as part either unipolar major depression or bipolar disorder, and to have ongoing depressive symptoms assessed using the HAM-17 (Hamilton 1960) with a score of ≥11. CFS patients were required to meet the most recent Centre for Disease Control (CDC) consensus criteria for CFS (Fukuda et al 1994); all also satisfied alternative consensus criteria for CFS (Sharpe et al 1991) and were assessed by a physician not to be experiencing fatigue secondary to another medical illness. All patients were medication free for ≥2 weeks (≥4 weeks for fluoxetine) and not receiving any psychological intervention at the time of the assessment. Patients were excluded if they reported any illicit substance use in the previous three months, had any unstable medical condition or were unable or unwilling to give hair, with a minimum hair length of 3 cm required. We also measured depression symptoms using the QIDS (Rush et al 2003) which unlike the HAM-D can assess reverse neurovegetative features (increased sleep and appetite/weight). Healthy controls were recruited in UK and Chile. Controls were required to have no current or past psychiatric diagnoses and no history of psychiatric illness in first-degree relatives.

The research was approved by the relevant local ethics committees in the UK and Chile and written, informed consent was obtained from each participant in both countries. All participants were compensated for their time in taking part in the research.

To investigate the relative frequency of those MDE symptoms (fatigability and impaired concentration) that are shared between SSDs such as chronic fatigue syndrome (CFS) (Fukuda & Straus 1994) and fibromyalgia (Leavitt & Katz 2002), we created a “Fatigability and memory factor” from the QIDS-C. We decided to use that scale, instead of the HAM-D, because it has two items that specifically inquire for these symptoms. It also gives more weight to these items allowing greater distinction between levels of the symptoms that are present

(Cusin et al. 2010). We also investigated anxiety symptoms through an "Anxiety Factor" that was constructed using the anxiety items of HAMD-17 scale (Levitt et al. 1993) and those specific psychic and somatic anxiety symptoms (Rassaby & Paykel, 1979) were studied using "Psychic Anxiety" and "Somatic Anxiety" factors, respectively, using their corresponding HAMD-17 items.

The frequency and severity of the most common day-to-day environmental disturbances during the month prior to study enrolment was measured using the Hassles Scale (Kanner & Coyne 1981) and the severity of more unusual environmental factors, such as major life events during the three months prior to the study, were assessed using the Recent Life Changes Questionnaire (RLCQ) (Miller & Rahe 1997). Early life trauma was assessed using the CTQ (Bernstein et al 1997). All psychometric scales had been previously validated in Spanish. The presence of an identifiable neurobiological response to environmental disturbances or life events was also measured. Due to the lack of an objective and agreed definition for stress, a recent proposed definition for this term was used, in which stress may be understood as the reaction to an event – biological or psychological – that causes a significant cortisol variation in hair (Herane Vives et al. 2015).

2.2 Biological specimens

Hair specimens

A trained practitioner collected hair samples of suitable participants. The presence and frequency of any biological confounders and procedures potentially affecting hair cortisol levels were measured, including cosmetic treatments (dyeing, bleaching, permanent straightening or waving) and frequency of hair washing. Collection procedure and analyses for each participant were standardised according to a strict protocol to collect approximately 3 months of hair growth equivalent to 3-month retrospective assessment of endogenous cortisol production. Cortisol levels were determined using a commercially available competitive ELISA (Salimetrics LLC, USA) and the results expressed in picograms of cortisol per milligram of hair (pg/mg). All hair samples were analysed at Salimetrics Laboratory, Cambridge, UK (www.salimetrics.com) (Albermann and Musshoff 2012) (See supplementary material for procedural details).

Saliva specimens

Saliva samples collection was taken at the time of the baseline assessment on a weekday Tuesday to Friday following hair sampling. Subjects were asked to provide six saliva samples using plain salivettes (Sarstedt, Leicester, UK) as per the protocol of Roberts et al (2004), with instructions given in writing at the time of the assessment. Samples were provided at awakening, 30 min and 60 min after awakening, and at 12:00, 16:00 and 20:00. Analyses of saliva cortisol concentrations were carried out in the Affective Disorders Laboratory at the Bethlem Royal Hospital, London UK. The area under the curve with respect to the ground (AUCg) was used for calculating the total daily cortisol output using all six samples. Two measures of cortisol reactivity in saliva were analysed in this study including the cortisol awakening response (CAR) and the delta post-awakening cortisol. The CAR was calculated as the area under the curve with respect to increase (AUCi) using the first three morning saliva samples collected over a one-hour period. The delta post-awakening cortisol was calculated as the difference between cortisol measured at awakening and the sample taken at 30-minutes. All measures were calculated in nanomoles per litre (nmol/l.h) (see Supplementary material for procedural details).

2.2 Subjects

15 A-MDE subjects (4 males, 11 female) and 17 CFS subjects (4 males, 13 female) were matched with a control group of 34 subjects (9 males, 25 female). Mean ages (± SD) were 41.1 ± 12.6 years in the CFS group, 35.5 ± 8.2 years in the A-MDE group and 35.2 ± 8.1 years in the control group (p=0.11) (see Table 1 for Demographic features). A-MDE subjects were included in a previous report comparing atypical and non-atypical subtypes of depression (Herane-Vives et al 2018). We previously compared cortisol levels, using hair and saliva specimens in a larger A-MDE sample (27) who were compared with 44 patients with non-atypical MDE and 40 controls (Herane-Vives, A et al. 2018). However, as part of this study, we had to reduce the number of A-MDE and controls in order to match participants as closely as possible for age, sex and body mass index. Since the group of A-MDE and control participants of that previous study were significantly thinner and younger than the CFS group, a number of them with those physical and demographic features were randomly selected and excluded from the analysis.

2.3 Statistical analysis:

Demographics, clinical features and questionnaire measurements were compared with one-way ANOVA or t-test for continuous variables and Chi-square or Fisher's exact test for categorical variables. Differences in cortisol levels in hair and saliva measures among CFS, A-MDE and healthy controls were tested using ANOVA with Bonferroni *post-hoc* test. The study groups were frequency matched in age, BMI and gender.

3 Results:

3.1 Clinical characterisation:

There were no differences across the three groups in terms of age, sex, BMI, waist circumference, frequency of hair washing, use of cosmetic treatment, phase of the menstrual cycle, proportion taking contraceptive pills, and alcohol and tobacco consumption (all p values >0.05) (Table 1)

3.2 Psychometric results

Whilst A-MDE subjects had a moderate depressive episode according to the HAMD17 (mean: 16.1), this episode was severe according to the QIDS-C (mean: 19.3). A-MDE and CFS subjects did not differ between them in the severity of anxiety symptoms (p=0.16), according to the HAMD-17 scale. However, they did differ when psychic and somatic anxiety symptoms were separately analysed. Indeed, while, they did not again show a significant difference in the severity of somatic anxiety symptoms (p=0.16), psychic anxiety symptoms were more severe in A-MDE in comparison to CFS (p<0.01). A-MDE and CFS patients did not differ in the frequency of concentration & fatigue symptoms (p>0.05) (Table 1).

A-MDE had experienced more early life trauma measurable through the CTQ than controls (p<0.01) but not than CFS subjects (p=0.16); CFS and control subjects did not differ between themselves (p=0.21) Control subjects had experienced fewer current life events than A-MDE (p=0.02) but not than CFS subjects (p=0.07). There were no differences between CFS and A-MDE subjects (p=1). There were also no differences between CFS and A-MDE subjects in terms of the number of current life events (p=1). Environmental disturbances in the form of daily hassles were less common and less severe in controls compared both to A-MDE (both p<0.01) and to CFS subjects (p=0.04 and p<0.01, respectively). These daily hassles were more common in A-MDE than in CFS subjects (p=0.03) but not more severe (p=0.37). See Table 2 for full psychometric results.

3.3 Hair cortisol results:

Hair cortisol measurements were obtained for 98.4% of the participants; one participant's hair sample was not able to be used. There were no significant differences in hair cortisol concentration across the three groups (p=0.91). Hair cortisol concentration (mean (s.d)) were: 8.7 (4.0) pg/mg hair in the control group, 8.1 (5.8) pg/mg hair in A-MDE and 8.4 (4.7) pg/mg hair in CFS.

3.4 Saliva cortisol results:

Saliva cortisol levels were available in 81.3% of the subjects because of a failure to return all samples and/or a significant violation of the saliva sampling protocol. A graph with the means of daily salivary cortisol levels over six time points by groups are seen in Figure 1. There was significantly lower daily cortisol output in both A-MDE and CFS groups in comparison to controls ($p<0.01$) (Figure 2). AUCg (mean (s.d)) were: 125.5 (40.6) nmol/l.h in the control group, 89.1 (22.6) nmol/l.h in A-MDE and 92.2 (33.2) nmol/l.h in CFS. This is illustrated in figure 1. There were no significant differences in other saliva measures (CAR or delta) across the three groups. CAR values (mean (s.d)) were: 1.6 (8.2) nmol/l.h in the control group, 0.3 (4.7) nmol/l.h in A-MDE and 0.9 (5.7) nmol/l.h in CFS (p=0.83). Delta cortisol values (mean (s.d)) were: 2.2 (7.2) nmol/l.h in the control group, 1.8 (6.1) nmol/l.h in A-MDE and 2.7 (9.2) nmol/l.h in CFS (p=0.94).

4. Discussion

These results showed a low total daily cortisol output but normal hair cortisol concentration in both A-MDE and CFS subjects in comparison to healthy participants, but no differences in either measure between CFS and A-MDE. CFS and A-MDE subjects did not differ in the frequency of fatigue and memory symptoms that are common to both MDE and CFS standard case definitions. However, CFS subjects had a significantly lower number of daily environmental disturbances and less severe psychic anxiety symptoms in comparison to A-MDE subjects.

Our findings suggest neurobiological overlap between A-MDE and CFS when cortisol levels are considered since they showed the same patterns of cortisol secretion in both hair and saliva measures. Of note is that in both conditions there is a reduction in a short-term measure of cortisol output in saliva, but no change in a long-term measure in hair. We could speculate that these two findings could be reconciled if subjects with these disorders experience episodic periods of hypercortisolaemia – perhaps at night time, on some days, or in response to stressful

triggers – which coupled with a more general decreased cortisol as we found in saliva would then average out to normal levels of cortisol accumulation in hair. This might suggest that both disorders have a cortisol rhythm alteration. However, the measure of acute cortisol reactivity that we did measure – the CAR and post-awakening delta cortisol – did not differentiate these two conditions from each other or controls. Thus, it may be that other more sustained measures of hyper-reactivity are involved, which would require confirmation in future studies.

Erratic patterns of cortisol secretion have previously been described in other conditions. For example, in a condition called as transient generalized glucocorticoid hypersensitivity (Nicolaides, 2015; Krysiak & Okopien, 2012) subjects can present with clinical manifestations of Cushing's syndrome, such as high blood pressure and diabetes, but show low cortisol levels when using acute measures such as saliva and blood (Nicolaides, 2015; Iida & Nakamura, 1990; Krysiak & Okopien, 2012). Such a pattern could be explained by increased tissue sensitivity to glucocorticoids and compensatory hypo-activation of the hypothalamic pituitary adrenal axis (Nicolaides, 2015).

Furthermore, heightened cortisol reactivity to stressors has been previously found in both A-MDE and CFS. For instance, O'Keane et al. (2005) found that after a corticotropin releasing hormone challenge, a situation that emulates stressful situations, subjects with atypical depression had higher levels of corticotropin (ACTH) than controls. Similarly, subjects with CFS showed a heightened salivary cortisol response to the insulin tolerance test, although not to the Trier Social Test or a standardized exercise test (Gaab et al. 2002). Against this, other studies using measures of reactivity such as the CAR or the corticotropin releasing hormone test have not shown be associated with increased in CFS (Papadopoulos and Cleare, 2010). Direct observation of periods of heightened cortisol release would be needed to confirm such a pattern is present.

The exploratory comparison between memory impairment and fatigability symptoms showed no differences between CFS and A-MDE subjects. This result is in line with our preliminary study comparing A-MDE and NA-MDE, which showed that these symptoms were more frequent in A-MDE. This result adds an overlapping pattern of clinical symptoms to the neurobiological findings, further strengthening the link between A-MDE, stress related disorders and CFS. There were however significant symptom differences, including not only the expected difference in depression severity, but also higher levels of psychic anxiety in A-

MDE. On the other hand, the somatic component of CFS was also reinforced, after observing that group of patients did not differ in the severity of somatic anxiety symptoms in comparison to A-MDE, conversely to those psychic anxiety symptoms that were more severe among A-MDE.

Contrary to the idea that stress plays a central role in A-MDE and CFS (Heim et al 2000), our data suggests that their shared clinical and neurobiological features may not be explained by environmental factors. First of all, although there was an association with several environmental factors, none of them qualified as stressors, according to our proposed stress definition (Herane Vives et al. 2015). Furthermore, CFS subjects had significantly fewer numbers of daily hassles than A-MDE subjects, which was the specific type of environmental factor that was significantly more associated with A-MDE than with other forms of depression. In addition to this, other environmental factors, such as early life trauma, were significantly less frequent (35.3%) in this sample of CFS than previous studies have shown (63%) (Heim & Wagner, 2006). In this context, Georgiades & Behan (2003) have provided evidence for a possible role of central nervous system in fatigue disorders.

Finally, it is conceivable to speculate that childhood trauma and daily environmental disturbances may be risk factors for developing comorbid depression in patients with CFS. Moreover, not only might environmental factors have a role in the association between CFS and comorbid depression, but also in the degree of decreased cortisol that these subjects may present. Tak et al. (2011) for instance, showed that subjects with CFS and comorbid depression had a deeper degree of decreased cortisol than those with CFS alone. Gracely and Schweinhardt (2015) described how childhood trauma is associated with both hypercortisolism and SSD, such as fibromyalgia, and comorbidities such as depression can also contribute to different HPA-axis dysfunctions. Moreover, some authors have found, for instance, that low cortisol in CFS is associated with a poorer response to Cognitive Behavioural Therapy (CBT) (Roberts et al. 2010).

Other than the lower rates of daily stressors in CFS, CFS and A-MDE present similarities in three key characteristics. First, they show no difference in the occurrence of fatigue and memory symptoms, both of which are defined features of somatic symptom disorders, CFS and A-MDE (Fukuda & Straus 1994; Leavitt & Katz 2002; DSM-5*;* 2013). Second, both disorders show lowered daily salivary cortisol output; this is especially relevant since it has

been suggested that decreased cortisol is a common neurobiological feature across the spectrum of SSDs (Griep & Boersma 1998; Roberts et al 2004; Pruessner 1999). Finally, hair cortisol and cortisol awakening responses were similar and did not differ from controls. These results may provide additional support for the view that A-MDE may be a subtype of SSD with a mood component rather than primarily an affective disorder.

4.1 Limitations and Future Directions

Limitations

Apart from the modest sample size, there are still some uncertainties in relation to the reliability of hair specimen for measuring accumulated cortisol levels. The role of the wash-out effect and sweat contamination is not entirely clear. Future well-designed hair studies may corroborate the role of these possible covariates. The methodology for assessing hair cortisol may be important. All hair cortisol studies, regardless of the hair cortisol extraction protocol used, have found significantly lower hair cortisol concentration in comparison to salivary cortisol levels. However, there are some difference. Thus, Balagova & Jezova (2018) used an increased volume of methanol for extracting cortisol and decreased the speed and duration of hair pulverization; they found a lower variability and higher cortisol concentrations compared to the method used by Xiang et al. (2016). We used a different protocol (Albermann & Musshoff, 2012), but our protocol's parameters were more similar to those of Xiang et al. (2016) than the Balagova & Jezova (2018) (see supplementary material). Therefore, future studies should pay more attention to these points with the aim for obtaining more accurate and comparable hair cortisol results.

Although the 1994 Fukuda et al CDC criteria that we used for the diagnosis our CFS patients remain the most widely used and validated in the literature, they are consensus case definitions for clinical and research purposes. There is no diagnostic test for CFS and other proposed but less widely used or validated case definitions exist (Brurberg et al. 2014).The discrepancy in depressive episode severity between the two depression scales (QIDS and Hamilton Depression Scale) may be explained by the fact that one of the main limitations of the Hamilton scale is that it fails to recognise all depressive domains, in particular reverse neurovegetative symptoms (Cusin et al. 2010), a key feature in A-MDE. The RCLQ scale was adapted to cover the 3 months corresponding to the period of hair cortisol accumulation; however, the Hassles Scale did not cover the same period. It was also not possible to differentiate the effect of severe

stressors on hair cortisol concentration from that of the disorder itself, another potential confounding factor.

Fatigue and memory symptoms were not measured with a specific scale designed for these symptoms. Instead, an exploratory analysis was used which incorporated a specific factor for measuring these symptoms. This is a potential limitation to our assessment of fatigue symptoms.

Finally, the sample size was modest, and although sufficient to detect changes in total cortisol output, may not have been as sensitive to smaller changes in the cortisol awakening response. We note that both CFS and A-MDE groups had numerically lower CAR values, and that a previous study which did find a lowered CAR in CFS had significantly higher number of patients (Roberts et al 2004).

Future directions

The use of antidepressants has not shown favourable outcomes in CFS (Afari & Buchwald 2014) and subjects with atypical depression show a worse response in the use of standard antidepressants in comparison to subjects with classic subtypes of depression (Thase 2009). If future studies confirm the presence of a cortisol rhythm alteration in these disorders and that cortisol has a pathophysiological role rather than only being epiphenomenological, the development of a drug with cortisol stabilization properties may become a valuable alternative to explore.

Certain kinds of specific physical and multidimensional treatments (e.g. cognitive and behavioural interventions), have shown positive results in CFS patients (Castell 2011; White et al. 2011; Chalder et al. 2012; Afari & Buchwald 2014, Whiting et al., 2001). and are recommended by the National Institute for Health and Care Exellence (2007). However, they have not been specifically studied in subjects with A-MDE. Finally, the combination of acute and chronic cortisol measures may provide additional information in the development of a future stratified medical practice specifically designed for providing individual solutions for each patient rather than a standardised treatment for all.

4.2 Conclusion

These results suggest that A-MDE and CFS subjects have very similar neurobiological features in terms of cortisol, with reduced daily salivary cortisol output but normal accumulated cortisol levels in hair. This pattern might be accounted for by a mid- to long-term cortisol rhythm alteration. Although, these two disorders have their own distinctive features, they also share important clinical features, such as fatigue and memory symptoms. Given also the differences between A-MDE and more classical subtypes of depression (Herane-Vives et al. 2018), A-MDE may be better characterised as a subtype of SSD with a mood component rather than primarily an affective disorder.

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Tables & Figures:

Table 1: Group characteristics of CFS, A-MDE and controls subjects

	Control	**A-MDE‡**	**CFS**		
Demographic & Health indicators	(N=34)	(N=15)	(N=17)	Overall p-value	*Post Hoc* test
Age (years); Mean (s.d)	35.2, (8.1)	35.5, (8.2)	41.1, (12.6)	0.11	∞
Female; n,(%)	23, (71.9)	11, (73.3)	13, (76.5)	0.94	∞
Single; n,(%)	13, (40,6)	5 (33.3)	7 (41.2)	0.87	∞
Unemployment (yes);n,(%)	1, (3.1)	2, (13.3)	1, (5.9)	0.34	∞
Tobacco (yes); n,(%)	5, (15.6)	4, (26.7)	1, (5.9)	0.27	∞
Alcohol (yes); n(%)	27, (84.4)	11, (73,3)	11, (68.8)	0.28	∞
BMI (Kg); mean, s.d	24.9, (3.7)	27.4, (5.2)	26.9, (5.9)	0.15	∞
Waist circumference (cm); mean, (s.d)	83.1, (10.8)	90.4, (14.8)	92.9, (21.4)	0.09	∞
Follicular phase; n,(%)	15, (60)	7, (70)	11, (91.7)	0.13	∞
Length of the episode (months); Mean,(s.d):	0,(0)	7.3 (5.9)	62.7 (58.8)	<0.0001*	B,C
Medical comorbidities; n (%)	2, (6.3)	4, (26.7)	7, (41.9)	<0.01*	B
Number of subjects taking:					
Medications; n(%)	13, (40.6)	11, (73.3)	7, (41.2)	0.08	∞
Contraception pills; n (%)	7, (21.9)	0, (0)	3, (17,7)	0.16	∞
Hair variables:					
Frequency hair washing per week; mean; n,(s.d)	4.6, (1.7)	3.8, (2.4)	3.4, (2.1)	0.15	∞
Cosmetic treatment (yes), n (%)∫	10, (31.3)	3, (23.1)	9, (52.9)	0.22	∞

‡: Subtypes based on ADDS scale.∫: dyeing, bleaching, permanent straightening or waving. A=Controls different from A-MDE. B=Controls different from CFS. C=A-MDE different from CFS ∞= no differences.*: P-value significant at p<0.05

Table 1 Psychometric results					
Depressive symptoms	**Control**	**A-MDE‡**	**CFS**	Overall p-value	*Post Hoc* test
HAMD-17; mean, (s.d)	0.3, (1.0)	16.1, (3.6)	5.9, (5.6)	<0.0001*	A, B, C
HAMD-21; mean, (s.d)	0.3, (1.1)	18.1, (4.9)	6.8, (7.8)	<0.0001*	A, B, C
QIDS; mean, (s.d)	0.5, (1.5)	19.2, (4.5)	6, (5.7)	<0.0001*	A, B, C
Concentration & fatigue factor; mean, (s.d)	0.09, (0.4)	4, (0.8)	3.7, (1.7)	<0.0001*	A, B
Anxiety factorΦ mean, (s.d)	0, (0)	4.4, (2.8)	2.9, (2.8)	<0.0001*	A, B
*Anxiety factor*Φ *Psychic* Mean, (s.d)	0.1, (0.3)	1.5, (0.9)	0.5, (0.7)	<0.0001*	A, B, C
*Anxiety factor*Φ *Somatic* Mean, (s.d)	0, (0)	2.2, (1.4)	1.5, (1.4)	<0.0001*	A, B
Environmental factors:					
Childhood trauma (yes); n, (%)	6 (18.8)	9 (60.0)	6 (35.3)	0.01*	A
Life events§(LUC); mean, (s.d)	93.4, (165.1)	333.9, (388.9)	266.8, (344.9)	0.01*	A
Severity of life events§; n,(%)	2, (6.3)	9, (60.0)	7, (41.2)	<0.0001**	A, B
Number of hassles¥; mean, (s.d)	18.0, (20.4)	123.7 (112.0)	66.4 (53.9)	<0.0001*	A, B, C
Severity of hassles¥; n, (%)	1, (3.1)	8 (53.3)	6 (35.3)	<0.0001**	A, B

‡: Subtypes based on ADDS scale, Φ: Anxiety factors were done using anxiety items of HAMD-17 scale, ¥: Hassles during the last month. *: P-value significant at 0.05 level. A=Controls different from A-MDE. B=Controls different from CFS. C=A-MDE different from CFS ∞= no differences.

Figure 1: Means of daily Salivary Cortisol Levels Over Six Time-Points by Groups.

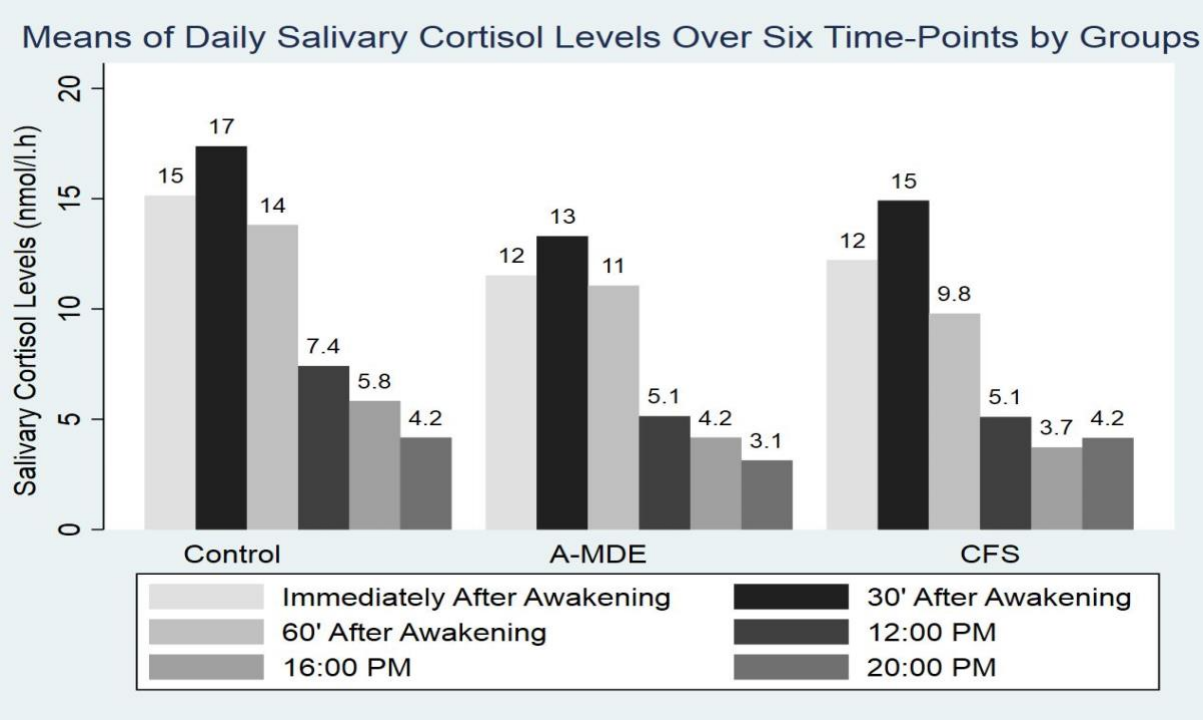
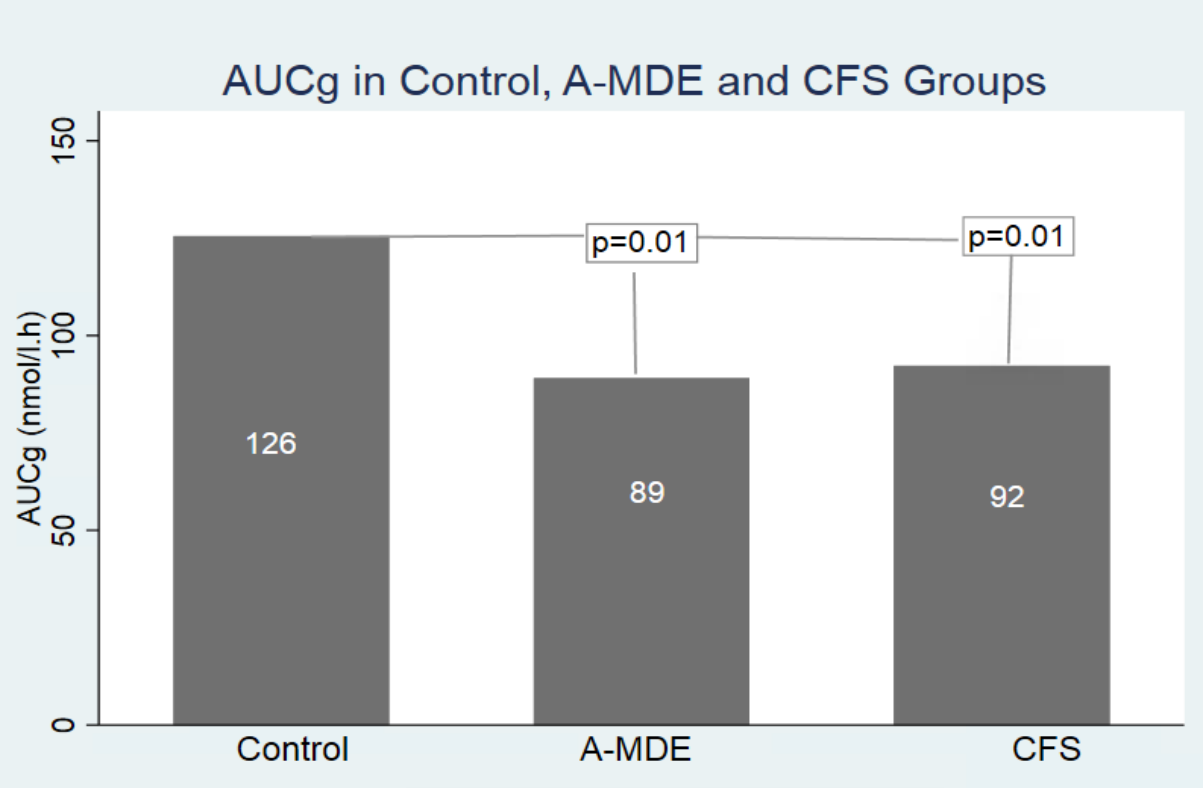


Figure 2: AUCg in Control, A-MDE and CFS Groups.



Supplementary material

Hair collection procedures

A) Collection

Hair samples were taken from the vertex at the back of the head and were cut with sterilised scissors as close to the scalp as possible. For this study, four locks of hair were required from different places from the vertex posterior, each to be the approximate thickness of a rubber band of 1 centimetre. At the laboratory, 3 cm of hair measured from the end to the scalp surface were cut from each lock, representing approximately 3 months of hair growth equivalent to 3-month retrospective assessment of cortisol production. The total weight of the four 3 cm segments from each lock is approximately equivalent to 60-80 mg of hair. Once collected, hair samples were stored at room temperature in the dark in a sealed container.

B) Analysis

Prior to analysis, the hair samples were washed in 1 ml of isopropanol to remove external contaminants, the isopropanol was removed from the vial and the hair allowed to dry in a clean air environment for 48 hours. Once fully dry five ceramic balls were added to each tube and the hair samples ground to a powder using am Fast Prep-24 (MP Biomedicals, LLC). To extract cortisol, 1.75 ml of methanol was added to each sample and the samples incubated for 20 hours whilst rotating the samples constantly.

The hair, methanol and ceramic balls were decanted into a polypropylene tube (Sarstedt AG & Co, Germany) that separated the ceramic balls from the rest of the mixture. The tube was centrifuged at 3000 RCF to separate the ground hair and methanol and 1.25ml of the clear methanol supernatant was decanted into a 2ml polypropylene cryovial. The methanol was then removed using a vacuum centrifuge (Scan Speed 40, Labgene) and the tubes frozen at -80°C until required for the cortisol ELISA. Cortisol levels were determined using a commercially available competitive ELISA (Salimetrics LLC, USA). Samples were thawed and reconstituted with 0.125ml of Salimetrics cortisol assay diluent and the samples were then assayed in accordance with the manufacturer's protocol. The results were expressed as picograms of cortisol per milligram of hair. All hair samples were analysed at Salimetrics Laboratory, Cambridge, UK (www.salimetrics.com).

Saliva specimen collection procedures

A) Collection

Subjects were instructed not to smoke, brush their teeth, or have anything to eat or drink for at least an hour before the collection of the samples. Samples were collected (1) immediately after awakening, (2) 30 minutes after awakening, (3) 60 minutes after awakening, (4) at noon, (5) at 4pm, and (6) at 8pm. Participants were instructed to avoid collections before 6 am and after 10 pm to minimise confounders. All participants filled out a questionnaire on socio-demographic details (gender, smoking habits, and health problems). Subjects were also instructed to specify whether they experienced any stressors and to provide any information which could be of relevance and/or interfere with the study. Moreover, they were asked to note the exact time for each saliva sample in a research log to assess self-reported compliance. Subjects were given instruction for storage and delivery the research team.

B) Analysis

Analyses of saliva cortisol concentrations were carried out in the Bethem Royal Hospital, London UK. On the arrival to the laboratory the salivettes were frozen at −20 ° Celsius. After thawing, they were centrifuged at 3500 rev/min for 10 min, which resulted in a clear supernatant of low viscosity. The saliva specimens were then frozen again in microtubes. Saliva cortisol concentrations were subsequently determined using the "Immulite" —DPC's Immunoassay analyser (www.diagnostics.siemens.com). To plot a calibration graph, set of 22 cortisol standards in saline were used in each assay. Results were highly reproducible with mean slope of 0.197 and standard error of the mean (SEM) ± 0.004 and the method correlated well with a previously published Time-Resolved fluorescence immunoassay (TR-FIA) (Mondelli et al. 2010). It had analytical sensitivity of 0.2 nmol/l and inter/intra assay precision-total imprecision in percentage (% CV) was less than 10% (cortisol concentration range 5 to 25 nmol/l)-. All samples from the same subject were analysed in the same run.

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