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Morning and evening salivary cortisol levels in patients with chronic widespread pain and those at high risk

Running Title

Salivary cortisol in chronic widespread pain

Authors

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Original Article

Funding

The study was funded by the University of Manchester.

Conflicts of interest

There are no conflicts to report.

Significance

This is the first study to examine neurobiological changes in chronic widespread pain and high risk individuals. One strength of the study is the absence of centrally-acting medication. We found high salivary cortisol common to Fibromyalgia and those at risk and identified contributing factors. Our results offer insight into the early mechanistic changes underlying Fibromyalgia development and open up possibilities for early diagnosis and prevention.

Abstract

Background: Hypothalamic-Pituitary-Adrenal (HPA) axis dysregulation has been implicated in chronic widespread pain (CWP); the hallmark of fibromyalgia (FM). This is the first study to compare HPA axis changes in individuals with CWP and those at high risk of symptom development.

Methods: We sought to determine differences in morning and evening salivary cortisol levels in FM (n = 19), those at-risk (n = 20), and pain-free controls (n = 17). Risk factors included non-CWP pain, somatic symptoms, illness behaviour and sleep disturbance. We conducted the study in the absence of centrally-acting medication, to address limitations of previous research.

Results: Repeated measures ANOVA revealed significant main effects of group ($p = 0.003$), and time of day ($p = 0.002$), with no significant interaction. Cortisol levels were higher in FM ($p = 0.027$) and at-risk ($p = 0.003$) groups, compared to controls, but there was no significant difference between FM and at-risk groups. The main effect of group remained significant with sleep problems ($p = 0.021$), and life events ($p = 0.007$), but was not significant with anxiety ($p = 0.076$) or depression ($p = 0.098$) scores as covariates. With sleep problems as a covariate, cortisol levels remained significantly higher only in the at-risk group ($p = 0.017$).

Conclusions: This study indicates elevated salivary cortisol in FM and those at high risk, and identifies anxiety, depression, and sleep problems as potential contributing factors. The results shed light on the dynamic relationship between stress, mood and sleep disorders and the brain's resilience to pain.

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Conclusions: This study indicates elevated salivary cortisol in FM and those at high risk, and identifies anxiety, depression, and sleep problems as potential contributing factors. The results shed light on the dynamic relationship between stress, mood and sleep disorders and the brain's resilience to pain.

Introduction

Chronic widespread pain (CWP), the distinguishing feature of Fibromyalgia (FM) is highly prevalent (Mansfield et al., 2016, Andrews et al., 2018), debilitating and life altering for those affected (Golden et al., 2015, Fitzcharles et al., 2016). FM is difficult to diagnose and manage, with pharmacological interventions demonstrating limited efficacy (Choy et al., 2010, Whibley et al., 2016, Häuser et al., 2018, Galvez-Sanchez and Del Paso 2020). The unknown aetiopathophysiology of FM hinders targeted treatment (Galvez-Sanchez and Del Paso 2020). Early diagnosis and management of regional and widespread pain show promising results towards prevention (White et al., 2002, Dickinson et al., 2003, van Koulil et al., 2010, Gatchel et al., 2014, Macfarlane et al., 2021). Regional or non-widespread pain, illness behaviour, somatic symptoms and sleep disturbance all constitute prominent risk factors for future CWP and FM onset (McBeth et al., 2001a, McBeth et al., 2001b, Gupta et al., 2007, Davies et al., 2008, Creed, 2020). Early mechanistic changes underlying the transition to CWP are not known, and a better understanding could guide more effective, early, targeted intervention (Creed, 2020).

One biomarker that shows alterations in FM is levels of the steroidal hormone cortisol, indicative of Hypothalamic-Pituitary-Adrenal (HPA) axis dysfunction. However, studies show contradictory results as to the direction of the effect (Catley et al., 2000, McLean et al., 2005, Wingenfeld et al., 2010, Riva et al., 2010, Riva et al., 2012, Úbeda-D'Ocasar et al., 2020), and may be affected by centrally-acting medication in most studies (Catly et al., 2000, McBeth et al., 2005, McBeth et al., 2007, Wingenfeld et al., 2010, Generaal et al., 2014).

Two studies investigated how changes in HPA axis function might underly the transition to CWP, in a cohort with somatic symptoms and illness behaviour as risk factors. The first study demonstrated lower salivary cortisol in a cohort with CWP and those at risk, compared to controls (McBeth et al., 2005). It is not clear how regional pain in the control group, and centrally-acting medication may have influenced their results. A follow-up prospective study within the at-risk group demonstrated that low morning and high evening salivary cortisol levels, and high serum cortisol (following overnight low-dose 0.25mg dexamethasone) were all highly predictive of new-onset CWP fifteen months later, controlling for psychological risk factors (McBeth et al., 2007). These studies highlight emergent evidence of the role of the HPA axis in CWP pathogenesis.

Here we sought to directly compare HPA axis function measured by salivary cortisol in patients with CWP, those at high risk of symptom development, and low-risk, pain-free controls. We designed the study to overcome previous research limitations by investigating

neurobiological mechanisms in the absence of centrally- acting medication, and by examining a wider range of risk factors, placing individuals at higher risk of symptom development (namely somatic symptoms, illness behaviour, non-CWP pain and sleep disturbance).

Methods

The study was approved by the regional Ethics Committee. The experimental protocol conformed to the Declaration of Helsinki, and all participants provided written informed consent.

Participants

All participants were recruited via advertising in the community, as well as in Rheumatology and Pain clinics in North West England. Nineteen patients diagnosed with FM (17 females; age, 41.5 ± 10.96 years; mean ± SD), 20 at-risk (16 females; age, 39.7 ± 9.5 years) and 17 pain-free, low-risk controls (14 females; age, 43.9 ± 12.1 years) took part in the study. Groups were matched for age ($\chi 2$ (2) = 1.097; p = 0.578) and sex (Pearson's $\chi 2$ (2) = 0.693; p = 0.707).

Inclusion and exclusion criteria

All participants were aged between 25 and 65. FM patients met the American College of Rheumatology criteria for FM (Wolfe et al., 2010). At-risk and healthy control groups were selected based on responses to a screening questionnaire, consisting of manikin drawings, where respondents indicated any sites of pain experienced for more than twenty-four hours, and if they had been aware of the pain for three months or longer. The screening questionnaire additionally comprised the following: the Somatic Symptom Checklist (Othmer and DeSouza 1985), the illness behaviour subscale of the Illness Attitude Scale (Kellner et al., 1987), and the Sleep Problems Scale (Jenkins et al., 1988).

The at-risk individuals had to fulfil the following criteria: (1) the presence of pain in the previous month, that lasted more than twenty-four hours, (2) the pain did not qualify as CWP- CWP was defined according to the Manchester definition, which requires pain for a minimum of three months in at least two sections, of two contralateral limbs, and in the axial skeleton (Hunt et al., 1999)- (3) they were required to report at least two of the following: two somatic symptoms and a minimum score of 4 on the illness behaviour subscale of the illness attitude scale, and the sleep problem scale in the previous month.

The healthy control group met the following criteria: (1) free from acute and chronic pain, and any other known disease and (2) they were excluded if they reported more than one of the following: two somatic symptoms, and a score of 4 or more on the illness behaviour and sleep problem scales.

The following exclusion criteria applied to all participants:

Neurological or morbid psychiatric illness, ischaemic heart disease, type 1 diabetes, epilepsy or anti-convulsant therapy, pregnancy, hormonal contraception or replacement therapy, oral or parenteral steroid therapy, previous adrenal or hypothalamic surgery, recent physical trauma, shift work, Adison's disease or Cushing's syndrome.

Medication

All subjects were either free from analgesics and antidepressants or were withdrawn from such medication. We estimated a minimum withdrawal duration of six half-lives for each drug, based on its pharmacokinetic properties (Electronic Medicines Compendium; last accessed 2020). See methods S1 for details of medication withdrawal in six FM patients.

Questionnaires

To assess the influence of factors that could impact HPA axis function, namely levels of anxiety and depression, sleep disturbance, and recent life events, all participants completed the following validated questionnaires: Hospital Anxiety and Depression Scale (HADS) (Zigmond and Snaith 1983), the Sleep Problem Scale (Jenkins et al., 1988), and the list of threatening life events (Brugha et al., 1985). Additionally, pain severity was assessed in all participants using the Brief Pain Inventory (Cleeland and Ryan 1991).

Diurnal salivary Cortisol

HPA axis function was assessed in all groups, based on measuring diurnal salivary cortisol levels. Upon acceptance into the study, participants were posted salivettes, along with standardised instructions for use. Saliva samples were acquired within four weeks after completing the study questionnaires. Two saliva samples were requested: the first at 10pm, and the second the following morning between 8 and 9am. As participants took samples at home, we could not control the time samples were taken. Almost all evening samples were reportedly acquired between 10 to 10.30pm, and the morning samples between 8 and 9am. Samples were immediately placed in the fridge, and returned to the study centre, where they were frozen and transported to the Pathology Laboratory for cortisol measurement.

Salivary cortisol Assay

Saliva samples were prepared using a modification of a previously published protein precipitation method (Jones et al., 2012).

A 50 µL of standard/QC/sample was added to 50 µL of 0.1 M zinc sulphate in a deep well microtitre plate and then 100 µL of working internal standard (50 µg/L D4-cortisol and 50 µg/L D7-cortisone). After mixing and centrifugation the supernatant was injected into the mass spectrometer.

Chromatography was carried out using a Waters Acquity ultra high pressure liquid chromatography system (Waters, Manchester, UK). 30uL of sample supernatant was injected onto a Waters Cortecs UPLC C18+ 1.6 µm, 2.1 x 50 mm column, using 60% of mobile phase A (deionised water containing 0.3 mmol/L (11 mg/L) ammonium fluoride in distilled Milli-Q water) and 40% of mobile phase B (methanol). The flow rate was 0.6 mL/min and the column temperature was 45°C. For chromatographic separation a linear gradient from 40 to 55% mobile phase B over 1.3 minutes was applied; followed by 0.5 minute at 100% B and 0.5 minute of re-equilibration at starting conditions. The eluate was directly injected into the mass spectrometer.

Quantification was performed on a XEVO TQ-S Micro mass spectrometer (Waters, Manchester, UK) operated in positive ion mode. The source offset was maintained at 30 V with a source temperature of 150 °C. The capillary voltage was 0.5 kV and the desolvation temperature and gas flow were 550°C and 1200 L/h. Multiple reaction monitoring mode was used with a dwell time of 0.016 s. Cortisol and cortisone were monitored using the following transitions for quantification and qualification respectively, 363.2>97.1 and 363.2>121.1; 361.3>145.1 and 361.3>163.1. Internal standard transitions were D_4-cortisol 367.2>121.1 and D_8-cortisone369.4>169.2. MassLynx NT 4.1 software was used for system control and the MassLynx TargetLynx programme for data processing. Peak height ratios of analyte and IS, 1/x weighting and linear least square regression were used to produce the standard curves.

Statistical Analysis

All statistical analyses were performed with SPSS version 22. Kruskal-Wallis H was performed to examine group differences in continuous variables (HADSA, HADSD, adverse life events, sleep problems scale and pain severity), given that data were not normally distributed. Mann-Whitney U Post-hoc tests were conducted to examine where differences lie, and results were Bonferroni corrected. Pearson's χ^2 was applied to assess group differences in categorical variables.

Morning and evening cortisol data were not normally distributed, and were therefore logarithmically transformed prior to analysis. The transformed data were closer to normal distribution. To detect differences between groups in cortisol levels, across morning and evening samples, a repeated measures ANOVA was performed, with two factors: Group with three levels (FM, at-risk and healthy controls) and Time of Day with two levels (am and pm). Age (mean centred) and sex were added as covariates to the model, and results were Bonferroni corrected for post-hoc comparisons.

We sought to examine the potential confounding effects of scores for anxiety, depression, adverse life events and sleep problems on cortisol. These factors were highly correlated, for example, HADSA and HADSD were significantly correlated with $r>0.7$; $p<0.001$ in both at-risk and healthy control groups. We therefore performed four 2-way ANOVAs, with the covariates introduced one by one (data were mean-centred), and Bonferroni correction for post-hoc tests was applied.

Results

Demographics and clinical scores

Table 1 shows mean, standard deviation and range in each group for age, HADSA, HADSD, sleep problems scale, adverse life events and pain severity, in addition to years since pain onset in FM (n = 17) and at-risk groups (n = 15), and years since FM diagnosis (n = 18).

Pain severity (BPI) significantly differed between groups ($\chi^2(2) = 36.890$; $p<0.001$). Post-hoc tests demonstrated significant differences between healthy controls and at-risk ($U = 25.500$; $p<0.001$), between healthy controls and FM ($U = 0.000$; $p<0.001$) and between at-risk and FM groups ($U = 87.000$; $p = 0.009$).

There were significant group differences in scores for anxiety ($\chi^2(2) = 27.530$; $p<0.001$), depression ($\chi^2(2) = 21.202$; $p<0.001$), and sleep problems ($\chi^2(2) = 27.559$; $p<0.001$). Groups did not significantly differ in the number of recent adverse life events, however ($\chi^2(2) = 2.941$; $p = 0.230$). Post-hoc tests revealed that healthy controls significantly differed from both FM patients in scores for anxiety ($U = 11$; $p<0.001$), depression ($U = 29$; $p<0.001$) and sleep problems ($U = 15$; $p<0.001$), as well as from the at-risk group in anxiety ($U = 36.5$; $p<0.001$), depression ($U = 62.5$; $p = 0.003$) and sleep problems ($U = 50.5$; $p<0.001$). FM patients only differed from the at-risk group in relation to sleep problems ($U = 94.5$; $p = 0.018$).

Salivary cortisol variation

Table 2 shows mean and standard deviation (SD), for morning and evening cortisol, in HC, AR and FM groups. Figure 1 represents box plots of log-transformed morning and evening cortisol by group. Mean cortisol levels were highest in the morning, and lowest in the evening across all groups, with highest morning levels in the at-risk group, and highest evening levels in the FM group, relative to the lowest levels in healthy controls consistent across both times of day.

The repeated measures ANOVA with morning and evening cortisol levels as the dependent variable, with age (mean-centred) and sex as covariates, revealed statistically significant effects of group $F(2, 51) = 6.550$; $p = 0.003$, and Time of Day $F(1, 51) = 10.285$; $p = 0.002$. There was no significant interaction between the two factors $F(2, 51) = 1.035$; $p = 0.363$, suggesting there were no differences between groups in diurnal variation of cortisol. Pairwise comparisons showed that cortisol levels among the healthy group were significantly different from both at-risk ($p = 0.003$) and FM groups ($p = 0.027$). There was no significant difference between at-risk and FM however ($p = 1.000$).

To control for the potentially confounding effects of group differences in scores for anxiety, depression, sleep problems and adverse life events, the ANOVA was repeated with each covariate separately. The within-subject factor, time of day, remained statistically significant across all models. There were no significant differences in group when anxiety or depression scores were entered as covariates. The main effect of group was statistically significant when sleep problems and adverse life events were accounted for. However, only the comparison between at-risk and healthy control groups remained significant when the sleep problems score was a covariate in the model, whereas both at-risk and FM groups significantly differed from healthy controls when the effect of adverse life events was accounted for. There were no significant differences between the at-risk and FM comparison in any of the models (see Table 3).

Discussion

To our knowledge, this is the first study to compare HPA axis biomarkers in CWP and those at high risk, compared to low-risk controls. Previous studies included patients on centrally-acting medication, and we avoided this limitation. We identified significantly higher levels of morning and evening Salivary cortisol, in both FM and at-risk individuals, reflecting a heightened physiological stress response. The diurnal cycle of cortisol was preserved in all

groups. We found that higher levels of anxiety and depression scores, accounted for elevated cortisol in FM and those at risk. Additionally, sleep disturbance contributed to increased cortisol in FM.

Our results are largely consistent with those of Catley et al., (2000) who identified higher overall salivary cortisol in patients with FM and rheumatoid arthritis (RA), compared to healthy controls, with no group difference in diurnal variation. Our study supports findings of increased salivary cortisol in FM (Wingenfeld et al., 2010) and chronic regional pain (Riva et al., 2012). Conversely, Riva et al (2010, 2012) detected lower cortisol levels in FM patients while McLean et al.,(2005) found no difference relative to controls. We did not confirm previous findings of lower morning and evening Salivary cortisol, or blunted diurnal rhythm in CWP and those with related risk factors, or in chronic multisite musculoskeletal pain compared to healthy individuals (McBeth et al., 2005, McBeth et al., 2007, Generaal et al., 2014, Paananen et al., 2015). A limited number of intervention studies have identified lower cortisol levels post-treatment in chronic musculoskeletal pain which supports our findings of higher baseline cortisol among our clinical groups (Vrbanović et al., 2019, Papandreou et al., 2020, Cheng et al., 2020).

A possible source of variation in findings among studies could stem from notable differences in patient characteristics, including medication use. In addition to somatic symptoms and illness behaviour (McBeth et al., 2005, McBeth et al., 2007), the present at-risk group reported pain that does not satisfy CWP criteria, and sleep disturbance. In contrast to previous research (Catley et al., 2000, McBeth et al., 2005, McBeth et al., 2007, Wingenfeld et al., 2010, Generaal et al., 2014), our study sample was not on centrally-acting medication, which exerts effects on the HPA axis (Gibb et al., 2016, Subramaniam et al., 2019).

Another explanation of the disparate findings could result from daily variance inherent in in-saliva-cortisol. Hair cortisol on the other hand, represents a more stable index of HPA axis function (Staufenbiel et al., 2013, Ross et al., 2014). Interestingly, Van Uum et al., (2008) reported increased hair cortisol in chronic pain patients relative to a healthy control group, in line with our findings. Furthermore, a meta-analysis reporting higher hair cortisol concentrations in chronic stress, identified a positive association between measures of salivary and hair cortisol (Stalder et al., 2017).

A further explanation of the divergent results relates to a hypothesis, previously posited for chronic stress (Miller et al., 2007) whereby high salivary cortisol could underlie an early stage of the condition, transitioning to an exhausted state of hypocortisolism in the long-term. For instance, Riva et al., (2010, 2012) reported elevated cortisol in chronic regional pain, and reduced cortisol in FM patients. Contrary to this hypothesis, in our study, both FM

and at-risk groups exhibited higher cortisol levels, though pain duration in FM was double that in the at-risk group. Future longitudinal studies with multiple follow-up visits are required to track salivary cortisol changes with symptoms of disease progression, along with more stable measures of cortisol in medication-free patients.

In the absence of a diagnosis for major depressive or anxiety disorders, our FM and at-risk patients exhibited significantly higher anxiety and depression scores compared to controls, which influenced the exaggerated cortisol levels. Our findings are largely consistent with the high salivary cortisol reported in patients with disorders of anxiety and depression comorbid with chronic multi-site and widespread musculoskeletal pain (Wingenfeld et al., 2010, Generaal et al., 2014) and in those without (Knorr et al., 2010, Stetler and Miller 2011, Faravelli et al., 2012, Elnazer and Baldwin 2014). Conversely, Catley et al., (2000) did not find significant covariation of positive and negative affect, with the increased cortisol in FM and RA, potentially due to similar levels of anxiety and depression in patients and controls. Our FM cohort however, exhibited higher levels of anxiety and depression, in line with the broader literature (Goldenberg et al., 2008, Løge-Hagen et al., 2019). There is evidence to suggest that depression represents a risk factor for CWP (Larsson et al., 2012, Mundal et al., 2014, Tan et al., 2019, Creed 2020). Future research should examine the role of mood disorders in the pathophysiological development of FM.

Insomnia constitutes a risk factor for both depression (Baglioni et al., 2011), and CWP (McBeth et al., 2015, Alföldi et al., 2017, Aili et al., 2018). We found that higher cortisol levels in FM were significantly affected by greater sleep disturbance, consistent with reports in chronic insomnia (Vgontzas et al., 1998, Rodenbeck et al., 2002, Shaver et al., 2002), and following sleep deprivation in healthy adults (Ekstedt et al., 2004, Kumari et al., 2009, Wright et al., 2015, Abell et al., 2016). A similar association was previously reported in FM (Riva et al., 2010); some studies did not find a significant link (McLean et al., 2005, McBeth et al., 2005, Riva et al., 2012), possibly due to differences in sleep assessment. In our study, the severity of sleep problems was significantly higher in the FM relative to the at-risk group. It is possible that the degree of sleep disturbance in the at-risk did not reach a threshold that induces a measurable effect on the HPA axis. Early management of sleep problems precluding HPA axis dysfunction may prevent progression of at-risk individuals to develop FM.

A notable strength of our study relates to the absence or withdrawal from centrally-acting analgesic and antidepressant medication, in addition to the contraceptive pill. The study is likely underpowered to detect a significant interaction between group and time of day. We did not account for the influence of some variables that could affect the cortisol results, such

as the phase of menstrual cycle, menopausal status, smoking and alcohol use, body mass index and level of physical activity (Hansen et al., 2008, Kudielka et al., 2009, Champaneri et al., 2013).

In conclusion, we found evidence of HPA axis hyperactivity in FM and those at high risk, and identified mood and sleep disturbance as contributing factors. Changes in resilience of the central nervous system and the dynamic effects of the HPA axis on this could further explain the transition from non-widespread pain to CWP. To this end we have conducted a brain imaging study in the same cohorts. Our findings may drive new strategies for early diagnosis and prevention of FM.

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Author Contributions

NB conceived the research idea, designed the study, carried out data collection and analysis and wrote the manuscript. AJ, JT and CB conceived the research idea, designed the study, reviewed and approved the analysis and the manuscript. TR and JR participated in data collection. BK and EP participated in data analysis. All authors discussed the results and commented on the manuscript.

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Legends for illustrations and tables

Fig. 1, Box-plots of log-transformed morning and evening salivary cortisol by group, where centre lines represent group median values, lines show range, and boxes contain interquartile range. Over all groups, cortisol levels were higher in the morning compared to the evening (ANOVA main effect of time of day: $p = 0.002$). Cortisol levels were also generally higher in the FM ($p = 0.027$) and AR ($p = 0.003$) groups relative to the HC group across both times of day. There were no differences between FM and AR groups. HC (healthy controls, blue), n = 17; AR (at-risk, red), n = 20; FM (fibromyalgia, purple), n = 19.

Table 1: Demographics and clinical scores in healthy controls (n = 17), at-risk (n = 20), and fibromyalgia (n = 19)

Table 2: Mean and SD for morning and evening cortisol (mmol/L) in healthy controls, at-risk and fibromyalgia

Table 3: Results of ANOVA with Covariates HADSA, HADSD, Sleep Problems, Adverse Life Events, Age, and Sex in HC, n = 17, AR, n = 20 and FM, n = 19

Table 1: Demographics and clinical scores in healthy controls (n = 17), at-risk (n = 20), and fibromyalgia (n = 19)

HC, Healthy controls; AR, At-risk; FM, fibromyalgia; HADSA, Hospital Anxiety and Depression Scale

		HC			**AR**			**FM**	
Variables	**Mean**	**(SD)**	**Range**	**Mean**	**(SD)**	**Range**	**Mean**	**(SD)**	**Range**
Age (years)	43.9	(12.1)	25-65	39.7	(9.5)	27-62	41.53	(10.96)	28-59
HADSA	1.35	(2.31)	0-7	7.35	(4.75)	0-18	9.05	(3.37)	2-15
HADSD	1.47	(2.15)	0-8	5.55	(4.94)	0-18	7.95	(3.89)	0-14
Sleep problems scale	2.65	(4.62)	0-17	9.05	(6.17)	0-20	14.84	(4.49)	4-20
Adverse life events	0.88	(2.42)	0-10	1.35	(1.87)	0-6	0.95	(1.08)	0-3
Pain severity (BPI)	0	(0)	0	3.45	(2.26)	0-8	5.58	(1.77)	3-8
Years since pain onset [a]	-	-	-	5.36	(7.13)	2-21	11.46	(7.12)	2-25
Years since FM Diagnosis [b]	-	-	-	-	-	-	3.43	(4.81)	0.07-20
Pain duration (Years)	-	-	-	-	(5.40)	-	-	(11.5)	-

for anxiety, HADSD, Hospital Anxiety and Depression Scale for depression; BPI, Brief Pain Inventory; SD, Standard Deviation.

[a] For AR (n = 15), for FM (n=17)

[b] For FM (n = 18)

Table 2: Mean and SD for morning and evening cortisol (mmol/L) in healthy controls, at-risk and fibromyalgia.

	HC (AM)			AR (AM)			FM (AM)			HC (PM)			AR (PM)			FM (PM)		
	All	F	M	All	F	M	All	F	M	All	F	M	All	F	M	All	F	M
N	17	14	3	20	17	3	20	17	2	17	14	3	19	17	3	19	17	2
Mean	6.08	6.69	3.23	15.03	15.94	9.83	0.88	7.74	7.55	0.42	0.44	0.33	7.72	0.95	0.43	1.93	2.08	0.65
SD	5.58	5.93	2.43	27.59	29.76	10.04	0.67	6.98	3.04	0.16	0.16	0.06	6.62	0.70	0.15	3.14	3.29	0.49

HC, Healthy controls; AR, At-risk; FM, fibromyalgia; N, number of participants; SD, Standard Deviation.

Table 3: Results of ANOVA with Covariates HADSA, HADSD, Sleep Problems, Adverse Life Events, Age, and Sex in HC, n = 17, AR, n = 20 and FM, n = 19

Covariate	Main Effect - Group	Main Effect – Time of Day	Post Hoc Results
HADSA	F(2, 52) = 2.704; p = .076	F(1, 52) = 148.589; p = .000	HC & AR p = .072 HC & FM p = .310 AR & FM p = 1.000
HADSD	F(2, 52) = 2.430; P = .098	F(1, 52) = 147.827; p = .000	HC & AR p = .104 HC & FM p = .890 AR & FM p = .871
Sleep Problems	F(2, 52) = 4.179; p = .021	F(1, 52) = 148.809; p = .000	HC & AR p = .017 HC & FM p = .151 AR & FM p = 1.000
Adverse Life Events	F(2, 52) = 5.493; p = .007	F(1, 52) = 147.962; p = .000	HC & AR p = .010 HC & FM p = .030 AR & FM p = 1.000
Sex	F(2, 52) = 6.054; p = .004	F(1, 52) = 10.223; p = .002	HC & AR p = .005 HC & FM p = .037 AR & FM p = 1.000
Mean-centered Age	F(2, 52) = 6.155; p = .004	F(1, 52) = 148.817; p = .000	HC & AR p = .005 HC & FM p = .023 AR & FM p = 1.000

Abbreviations: HC, Healthy Controls; AR, At Risk; FM, Fibromyalgia; HADSA, HADSD, Hospital Anxiety and Depression Scale.

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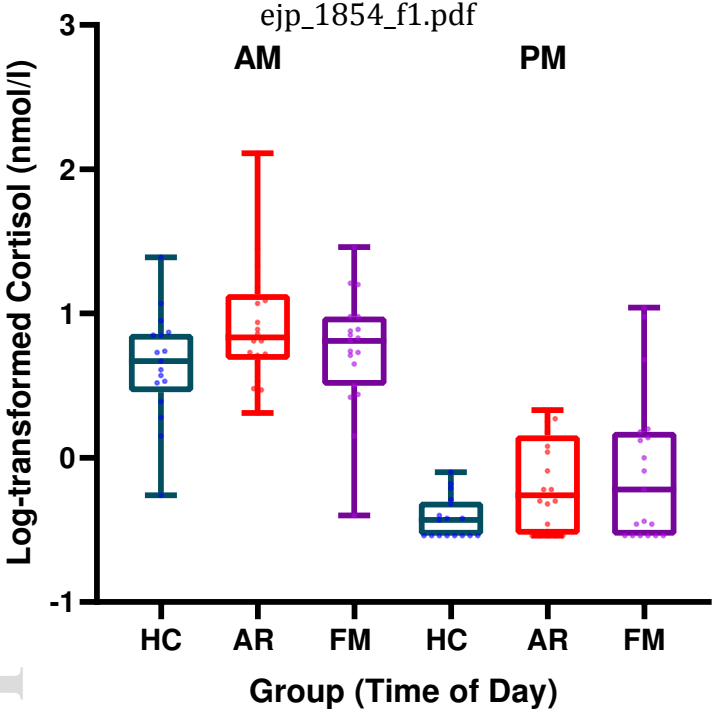

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HC (healthy controls, blue), n = 17; AR (at-risk, red), n = 20; FM (fibromyalgia, purple), n = 19.