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Elevation of the ACTH/cortisol ratio in female opioid dependent patients: A biomarker of aging and correlate of metabolic and immune activation

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Abstract

BACKGROUND: Whilst the hypothalamic-pituitary-adrenal (HPA) Axis is a major stress axis, and is necessarily perturbed in opioid dependency, and stress is a major contributor to aging mechanisms, the HPA axis has not been studied in opioid dependency in an age-dependent manner.
OBJECTIVE: Hypothesis – Differences in age dependent levels of HPA components.
DESIGN: Cross-sectional comparison of general medical and opioid dependent patients (ODP, GMP). Setting – Primary Care. Patients – 51 GMC, 233 ODP. Ages 37.92+1.95 v. 37.12+0.62 years (P – N.S.) and 33.33% v. 71.67% male ($p<0.0001$). Intervention(s) – Measurement ACTH, cortisol and their ratio (ACR). Main Outcome Measure(s) – Pre-planned analysis ACR. Secondary outcomes: Impact of immune and metabolic markers.
RESULTS: ACTH/cortisol was a negative biomarker for age in female patients. Whilst the mean ACR were not different, the (log) ACTH/cortisol showed a positive relationship with age:sex:status ($p=0.0396$) and age:status ($p=0.0437$). The effect of addictive status was confined to hepatitis C (HCV) positive female ODP ($p=0.0355$), and the age:status interaction was also stronger in female HCV+ ($p=0.0075$) compared to HCV – ($p=0.0667$) patients. Multiple regression of ACR against age, status, ALT, CRP, and Globulins confirmed many significant interactions. ACTH/cortisol ratio interacted significantly from $p=0.0008$ in males and $p=0.0079$ in females, and in both sexes four terms included addictive status.
CONCLUSIONS: These data establish the ACTH/cortisol ratio as a negative biomarker of aging in females, and show that this decline is more pronounced in ODP an effect which is partly related to HCV seropositivity, immune and metabolic factors. Dementias are one of the most serious health and socioeconomic issues. Multi-infarct dementia (MID) and Alzheimer´s type dementia (AD) exhibit differences in cerebrovascular blood flow velocity profiles and in presence of microemboli, detected by transcranial Doppler sonography.

ORIGINAL ARTICLE

INTRODUCTION

Opioids have been noted to be the second most commonly prescribed class of drugs in the USA, behind only agents for controlling serum lipids (Younger *et al.* 2011). The stress system is attracting increasing research interest in many disciplines. Its major arms are the sympathoadrenomedullary and parasympathetic nervous systems, the hypothalamopituitary – adrenocortical (HPA) axis, and the immune system including immune active cells and cytokines.

In a Swedish study only 3 of 39 chronic pain patients treated with opioids did not show signs of endocrinopathy on stimulation testing and these three cases were all treated with low doses of medications (Brennan 2013). Several reports have documented various abnormalities of the HPA system in chronic clinical opioid dependency. Chronic systemic opioids have been shown to suppress the hypothalamus, pituitary and adrenal secretion (Abs *et al.* 2000; Palm *et al.* 1997; Geiss *et al.* 2005; Zgierska *et al.* 2007; Black *et al.* 2007) but there are conflicting reports of the effects of opioids on ACTH release (Pfeiffer & Herz 1984; Rhodin *et al.* 2010; Schluger *et al.* 2003; Veldhuis *et al.* 2013; Vuong *et al.* 2010). It is important to note that β-endorphin is a product of the CRH-ACTH pathway as it is also a breakdown product of proopiomelanocortin (POMC) the precursor molecule of both ACTH and β-endorphin (Kronenberg *et al.* 2007).

Interestingly the hypothalamus and pituitary are noted to have high concentrations of μ-, κ- and δ-opioid receptors (MOR, DOR and KOR respectively), and the arcuate nucleus of the hypothalamus, which is rich in MOR perikarya, has ramifications and connections throughout most of the CNS (Grossman 1983). Moreover opioid receptors are known to exist at high concentration on many immune cells, and several immune molecules including TNFα and the C3 complement component are known to directly determine brain structure (Deverman & Patterson 2009). Opioids therefore have important immunomodulatory functions and have been shown to control thymic lymphocyte maturation (Ninkovic & Roy 2013; McCarthy *et al.* 2001; Zhang *et al.* 2011).

Opioid withdrawal is known to be characterized by intense sympathoadrenal stimulation (Gold & Rea 1983; Kienbaum *et al.* 1998). Patients dependent on short term opioids such as heroin, morphine and oxycodone, can therefore be expected to be subject to the vagaries of the opioid withdrawal syndrome several times daily, including its intense sympathoadrenal activation. These acute stress responses in withdrawal have now been linked directly with a stimulation of organismal ageing (Reece & Hulse 2012). The administratoin of opioid antagonists to opioid naïve humans is associated with the release of inhibitory basal tone over the HPA axis, particularly marked in women (Conrad & Bimonte-Nelson 2010; Veldhuis *et al.* 2013).

Reports from several centres are consistent with acceleration of the ageing process in chronic opioid dependency (Degenhardt *et al.* 2009; Khademi *et al.* 2012; Reece & Hulse 2014; Lee *et al.* 2013). The plethora of endocrinopathies reported in opioid dependency (Brennan 2013; Grossman 1983; Vuong *et al.* 2010; Reece & Hulse 2014) and reports of neuroendocrine dysfunction (De Maddalena *et al.* 2012; Kronenberg H. M. *et al.* 2007) makes hypothalamic dysfunction likely, and this has in turn been convincingly linked with coordination and regulation of the systemic ageing process (Cai & Liu 2012; Li *et al.* 2012; Zhang *et al.* 2013; Zhang *et al.* 2008). Moreover opioids have been shown to rapidly and persistently alter human brain structure particularly in the amygdala, cingulate cortex and hypothalamus (Younger *et al.* 2011). Since age dependent changes in HPA activity have been described (Veldhuis *et al.* 2013), it follows therefore that HPA activity is an important marker of hypothalamic activity which is likely to be perturbed in opioid dependence and to have a role as an important and differential biomarker of aging in this population.

It is becoming increasingly clear that there are complex and interlocking interactions between metabolic states, immune dysfunction and the stress system (Foster & McVey Neufeld 2013; Clavel & Haller 2007; Rubino *et al.* 2012; Werts *et al.* 2011), which have wide ranging implications for increasing our understanding and treatment of a wide range of conditions including mood disorders, stress and anxiety (Werts *et al.* 2011), cancer (Reece 2007c; Reece 2007a), and obesity (Buhmann *et al.* 2014), to name but a few.

Despite these recent advances in closely related fields, a recent literature search did not identify any studies assessing HPA axis changes as age dependent biomarkers in patients dependent on non-medical sources of opioids, examining these changes by sex differentials, investigating the effects of hepatitis C seropositivity which is widespread amongst intravenous drug dependent populations, or examining the interlinking of HPA stimulation with those of metabolic or immune stimulation. As our centre sees both general medical control (GMC) and opiate dependent patients (ODP) we were ideally suited to assess and compare any important differences between the two groups.

METHODS

Patient selection

Patients were assigned to either the medical control group or the drug dependent group based upon whether or not they had had serology performed for Hepatitis C antibody. This test is only performed on drug dependent patients, so it is a useful marker for the drug dependent state. Patients were denoted as either general medical controls (GMC) or opioid dependent patients (ODP). There was no selection of patients based on age. Blood was taken in the period 1995–2014 as clinically indicated for patient care in the case of their

routine clinical care. No attempt was made to control patient diet or smoking prior to blood testing. Patients were not fasted.

Pathology analysis

All pathology was performed by Queensland Medical Laboratory (QML) according to National Association of Testing Authorities Australia (NATA) accredited methods to the Australian Laboratory standard AS-15189. QML is accredited both with NATA and to the international standard ISO 9001 the international laboratory clinical standardNote that different normal ranges are quoted for males and females for cortisol.

Statistics

The pathology results were downloaded as an Excel spreadsheet from QML. Data are presented as mean±S.E.M.. Categorical data were compared in Epi-Info 7.1.4.0 from Centres for Disease control in Atlanta Georgia, USA using the Mantel-Haenszel Chi squared test. Bivariate statistics were compared by categories in Statistica 7.1 from Statsoft, Oklahoma, USA. Continuous data was compared in "R" version 3.0.1 by linear regression. "R" was downloaded from the University of Melbourne Central "R" Archive Network (CRAN) mirror. Continuous variables such as age, ACTH, cortisol, the ACTH/cortisol ratio, ALT, Globulins, and CRP were log transformed as indicated by the Shapiro test. Multiple regression was performed and graphs were drawn in "R". The graphing package was ggplot2. Multiple regression was performed by the classical method with deletion of the least significant term until only significant terms remained. All t-tests were two tailed. A $p<0.05$ was considered significant.

Ethics

The study was given ethical approval by the Human Research Ethics Committee of the Southcity Medical Centre which has been accredited by the National Health and Medical Research Centre. All patient data was handled with strict confidentiality. The study was conducted in accordance with the Declaration of Helsinki.

RESULTS

51 GMC patients were compared with 233 ODP patients. The mean ages were 37.92±1.95 and 37.12±0.62 years respectively (t Sep. Var.=0.039, dF=60.65, $p=0.6952$, Table 1). There were 17 and 167 males in each group making them 33.33% and 71.67% male respectively (Mantel-Haenszel Chi Squ.=26.86, $p<0.0001$). Other parameters and their comparisons are presented in Table 1.

127/167 (75.60%) male opioid dependent patients were hepatitis C positive, as were 41/66 (62.142%) of the female ODP (Mantel-Haenszel Chi Squ.=4.54, $p=0.0331$).

Drug use data in these patients has been published on previous occasions (Reece & Hulse 2014; Reece 2007b; Reece 2007d; Reece 2007e). It has been shown to be typical of illicit opioid users in similar clinics for illicit drug users both nationally and internationally.

Figure 1 shows the components of the HPA axis (ACTH, cortisol, and ACTH/cortisol ratio) by chronologic age. Figure 2 presents similar data in a sex specific pattern. Figure 3 dichotomizes this data by addictive status.

None of the tests for HPA axis components dichotomized by addictive status were significant (Table 1). Neither were they significant when dichotomized by sex (data not shown).

As shown in Table 1 the mean serum albumin was not significantly different in GMC and ODP groups. The levels of albumin with age were not significantly different in any of the subgroup analyses (data not shown).

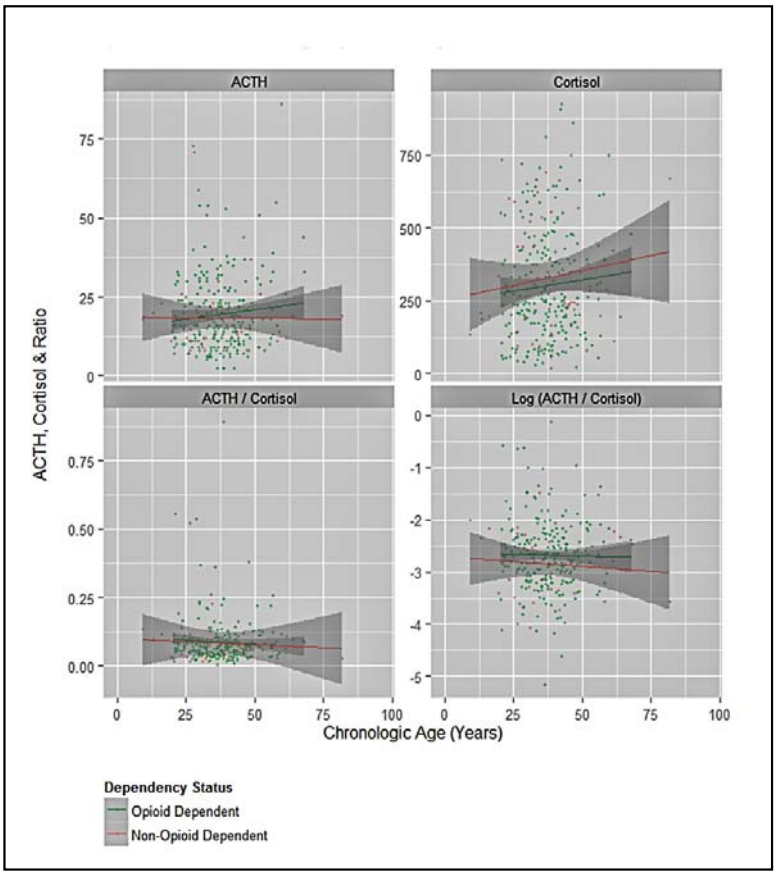

Fig. 1. ACTH, Cortisol ratio and logs by chronologic age by opioid dependency status.

Tab. 1. Bivariate comparisons.

Variable	N ODP's	N GMC's	Mean (SE) ODP's	Mean (SE) GMC's	*p*-value
Age (Years)	233	51	**37.12 (0.62)**	**37.92 (1.95)**	**0.6952**
Haemoglobin (g/dl)	230	43	146.04 (0.97)	138.81 (1.98)	0.0029
MCV (fL)	230	43	87.03 (0.3)	86.58 (0.7)	0.5527
Platelets $\times(10^9/L)$	228	43	254.54 (4.42)	260.77 (7.52)	0.5605
WCC $\times(10^9/L)$	230	43	8.26 (0.14)	7.09 (0.29)	0.0010
Neutrophil Absolute $(\times10^9/L)$	230	43	4.76 (0.12)	4.07 (0.24)	0.0185
Lymphocyte Absolute $(\times10^9/L)$	230	43	2.61 (0.06)	2.33 (0.12)	0.0635
Mono Absolute $(\times10^9/L)$	230	43	0.65 (0.02)	0.51 (0.03)	0.0000
ESR (mm/hr)	221	34	12.38 (0.81)	10.85 (1.68)	0.4839
High Sensitivity CRP (mg/dL)	233	31	6.27 (0.76)	5.51 (2.56)	0.7405
Sodium (mmol/L)	233	42	140.46 (0.16)	139.74 (0.35)	0.0774
Potassium (mmol/L)	233	42	4.26 (0.03)	4.39 (0.08)	0.0512
Glucose (mmol/L)	233	42	5.35 (0.16)	5.18 (0.19)	0.6603
Urea (mmol/L)	233	42	5.21 (0.1)	5 (0.26)	0.4012
Creatinine (mmol/L)	232	42	69.59 (0.93)	70.76 (3.07)	0.6454
Urate (mmol/L)	233	42	0.33 (0.01)	0.29 (0.01)	0.0025
Total bilirubin (mmol/L)	233	42	8.84 (0.41)	9.21 (0.8)	0.7108
Direct Bilirubin (mmol/L)	15	27	6.87 (1.25)	2.41 (0.28)	0.0031
Alkaline Phosphatase (IU/L)	230	42	80.4 (2.15)	85.48 (8.55)	0.5675
γ-Glutamyl Tranferase (IU/L)	233	42	54.84 (4.28)	27.90 (4.32)	0.0000
Alanine Aminotranferase (IU/L)	233	42	70.03 (8.25)	29.29 (3.16)	0.0000
Aspartate Aminotransferase (IU/L)	233	42	51.98 (4.41)	27.55 (2.05)	0.0000
Lactate Dehydrogenase (IU/L)	233	42	183.64 (3.1)	184.33 (7.22)	0.9306
Calcium (mmol/L)	232	42	2.38 (0.01)	2.35 (0.01)	0.0725
Corrected Calcium (mmol/L)	232	42	2.37 (0.01)	2.35 (0.01)	0.1414
Phosphate (mmol/L)	233	42	1.11 (0.01)	1.2 (0.03)	0.0137
Total Protein (g/L)	233	42	76.36 (0.34)	72.81 (0.75)	0.0000
Albumin (g/L)	233	42	42.79 (0.23)	42.43 (0.48)	0.5411
Globulin (g/L)	233	42	33.57 (0.29)	30.38 (0.57)	0.0000
Cholesterol (mmol/L)	233	43	4.56 (0.07)	5.14 (0.16)	0.0009
Triglyceride (mmol/L)	233	43	1.51 (0.07)	1.6 (0.17)	0.6501
LDL (mmol/L)	228	11	2.39 (0.05)	2.55 (0.31)	0.5261
HDL (mmol/L)	228	11	1.2 (0.02)	1.58 (0.09)	0.0005
ACTH (pg/ml)	228	46	19.54 (0.81)	18.41 (1.61)	0.5646
Cortisol (mcg/dL)	228	48	303.11 (12.87)	330.875 (26.95)	0.3660
ACTH/Cortisol	223	43	0.09 (0.01)	0.08 (0.02)	0.7567
Log ACTH	228	46	2.79 (0.04)	2.79 (0.07)	0.9870
Log Cortisol	228	48	5.47 (0.05)	5.61 (0.1)	0.2338
Log ACTH/Cortisol	223	43	−2.68 (0.04)	−2.85 (0.11)	0.1287

GMC – General Medical Controls; ODP – Opioid Dependent Patients

Tab. 2. Linear regressions of HPA Axis components in females against age.

Dependent variable	Variable	Variable parameters				Model parameters			
		Estimate	Std. Error	t value	*p*-value	R-Squared:	Model F	dF	*p*-value
~Age*Status									
Cortisol	Status(Adx)	–0.3194	0.1656	–1.929	0.0568	0.0679	4.4610	2, 93	0.0141
ACTH/Cortisol	Status(Adx)	0.3400	0.1478	2.300	0.0239	0.0943	5.5810	2, 86	0.0053
~Age*Status*Sex									
ACTH	Sex(Female)	–0.1351	0.0753	–1.795	0.0738	0.0081	3.2200	1, 272	0.0738
ACTH/Cort	Age: Sex(Female) :Status(Nadx)	–0.6319	0.3056	–2.0680	0.0396	0.0316	2.235	7, 258	0.0320
ACTH/Cort	Sex(Male):Status(Adx)	-2.7141	1.3390	-2.0270	0.0437				

Tab. 3. Linear regressions of HPA axis components against age & hepatitis C serostatus.

Group	Variable	Variable parameters				Model parameters			
Dependent variable	Parameter	Estimate	Std. Error	t value	*p*-value	R-Squared:	Model F	dF	*p*-value
All Patients									
ACTH/Cortisol	fHCV(Positive)	0.1770	0.1020	1.7360	0.0838	0.0125	2.677	2, 263	0.0707
Females									
ACTH/Cortisol	Age	–0.5567	0.2028	–2.7450	0.0074	0.0836	3.678	3, 85	0.0152
ACTH/Cortisol	fHCV(Negative)	0.3389	0.1888	1.7950	0.0762				
ACTH/Cortisol	fHCV(Positive)	0.3405	0.1594	2.1370	0.0355				
Opioid Dependent Females									
ACTH/Cortisol	fHCV(Positive)	4.2427	2.2695	1.8690	0.0667	0.0735	2.587	5, 57	0.0618
ACTH/Cortisol	Age: fHCV(Positive)	–1.1196	0.4040	–2.7710	0.0075				
Non-Dependent & HCV Negative Females									
ACTH/Cortisol	Age: fHCV(Negative)	0.08864	0.0501	1.7690	0.0836	0.0496	2.252	2, 46	0.1166

For males none of the comparisons by age were significant. Table 2 presents the significant comparisons for females from Figure 2 in quantitative form and shows that the addictive status shows an almost significant trend for cortisol with age (p=0.0568) and that the addictive status is a predictor of the ACTH/Cortisol ratio. The second part of this table shows that sex shows a non-significant trend as a determinant of ACTH (p=0.0738), but that both the age:sex:status and sex:status interactions are significant (p=0.0396 and 0.0437 respectively).

Figure 4 presents the sex dichotomized data for hepatitis C serostatus. The formal statistical analysis of this data is presented in Table 3. The ACTH/cortisol ratio is noted to show to be positively related to the hepatitis C positive serostatus (p=0.0355 compared to GMC), and to show an age related decline in females overall (p=0.0074). The age:status interaction was positive in hepatitis C positive females (p=0.0075). A non-significant trend was noted when HCV negative ODP were compared to GMC (p=0.0836).

Becasue of the above mentioned close interaction between metabolic states, immune dysfunction and the stress system, a linear regression was performed of the components of the HPA axis against age and status and various key metabolic (ALT) and immune indicators including c-reactive protein (CRP) and serum globulins to examine the interactions between metabolic states, immune dysfunction and the stress system. The resulting analysis is presented in quantitative form in Table 4 (for all patients and males) and Table 5 for females. Numerous significant interactions are noted between these various biomarkers and each component of the HPA axis in both sexes.

DISCUSSION

The main points shown in the present work are that the changes in the HPA axis are more pronounced in females than males. Although both the mean cortisol and ACTH levels were not dissimilar in each sex, the ACTH/cortisol ratio was elevated when regressed

against chronological age as a function of the addictive status only in females (Figure 2 and Table 2). Whilst the failure to demonstrate similar changes in men may be related to constraints of sample size in these subgroups, these changes are similar to those previously reported in non-opioid dependent clinical samples (Veldhuis *et al.* 2013). Similarly these changes were shown to be most pronounced in the HCV positive group of females (Figure 4 and Table 3), but again the statistical power to detect real differences was seriously limited in many subgroup analyses.

When the components of the HPA axis were regressed against major metabolic and immune parameters, which themselves have previously been shown to be very different in opioid dependent and control groups (Reece 2012b; Reece 2007c; Reece 2012), many statistically significant positive associations were demonstrated, and the addictive status was strongly and robustly significant in numerous interactive terms.

Whilst in some respects therefore these results can only be considered indicative and strongly suggest that more investigation requires to be performed in this important area, they are in themselves nevertheless of enormous interest. This body of work is very concordant with other larger analyses by both this group (Reece & Hulse 2013; 2014; Reece 2007c; Reece 2007d; Reece 2011; Reece & Davidson 2007) and others (Smyth *et al.* 2007; Degenhardt *et al.* 2009; Khademi *et al.* 2012) which show empirically that the pathological effects of opioid dependency in females is more pronounced than that seen in males. This is an intriguing finding whose explanation is not immediately apparent. Given the high levels of significance and the number of terms including immunological parameters in the present multiple regression studies (Tables 4 and 5) this may be linked to what has previously been observed to be a more aggressive immune system in females, which has been variously related to both hor-

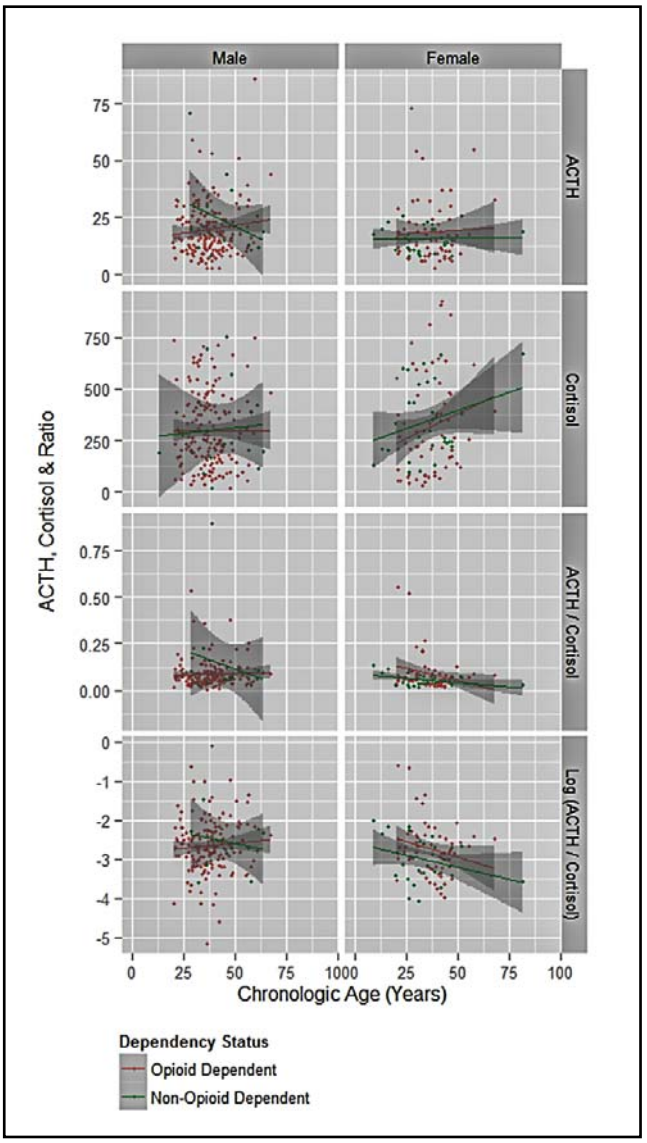

Fig. 2. ACTH, Cortisol ratio and logs by chronologic age, opioid dependency status and sex.

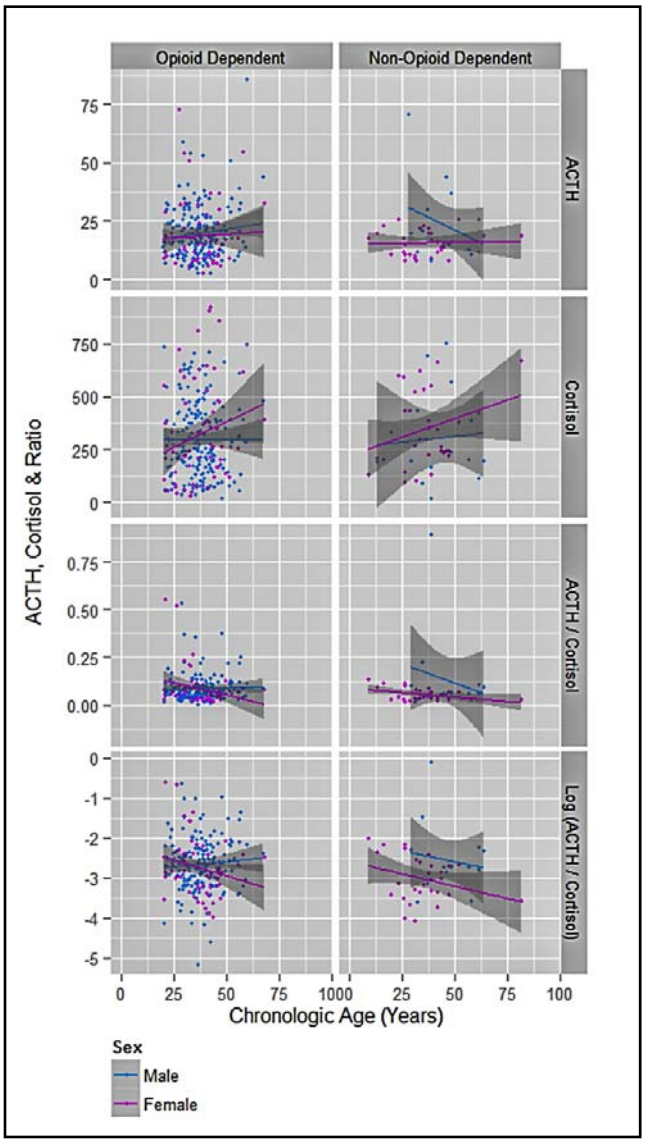

Fig. 3. ACTH, cortisol ratio and logs by chronologic age, sex and opioid dependency status.

monal (Klein 2012) and chromosomal (Migeon 2014) origins.

Indeed the high level of statistical significance between immune and stress hormonal parameters in both sexes is consistent with the close interrelationship of the stress and immune systems.

Hepatitis C hepatic infection is known to result in the production of a large number of proteins and immune active products from the liver (Yang *et al.* 2014). It is not unsurprising therefore that the present studies have documented that HCV seropositivity is associated with activation of the stress system in

Tab. 4. Linear regressions of HPA axis components against selected parameters – all patients & males.

Dependent Variable	Variable	Variable parameters				Model parameters			
		Estimate	Std. Error	t value	*p*-value	R-Squared:	Model F	dF	*p*-value
All Patients									
ACTH	Age:Globulin:CRP	0.0246	0.0101	2.441	0.0153	0.0123	1.7790	4, 247	0.1336
ACTH	Age:ALT:Status(Adx)	0.0454	0.0189	2.407	0.0168				
ACTH	Age:ALT:Status(NonAdx)	0.0492	0.0218	2.255	0.0250				
ACTH	Age:Globulin:CRP:ALT	–0.0063	0.0027	–2.342	0.0200				
Cortisol	CRP	–1.5331	0.5426	–2.826	0.0051	0.0238	4.0570	2, 249	0.0185
Cortisol	Globulin:CRP	0.4229	0.1513	2.795	0.0056				
ACTH/Cortisol	Globulin:CRP:ALT	–0.1537	0.0393	–3.909	0.0001	0.0681	3.2930	8, 243	0.0014
ACTH/Cortisol	CRP:ALT	0.4634	0.1328	3.489	0.0006				
ACTH/Cortisol	Age:Globulin:ALT:Status(Adx)	0.0163	0.0059	2.749	0.0064				
ACTH/Cortisol	Globulin:CRP	0.3038	0.1108	2.741	0.0066				
ACTH/Cortisol	Age:Globulin:CRP	–0.0556	0.0257	–2.160	0.0318				
ACTH/Cortisol	Age:CRP:Status(NonAdx)	–0.0661	0.0309	–2.138	0.0336				
Males									
ACTH	Globulin	3.6799	1.4984	2.456	0.0151	0.0239	2.3880	3, 167	0.0708
ACTH	Globulin:ALT	–0.7808	0.3725	–2.096	0.0376				
ACTH	ALT	2.7450	1.3260	2.070	0.0400				
Cortisol	CRP:ALT	–0.4171	0.1266	–3.294	0.0012	0.0582	2.3130	8, 162	0.0224
Cortisol	Age:Globulin:CRP:ALT	0.0314	0.0097	3.237	0.0015				
Cortisol	Age:ALT:Status(NonAdx)	0.5665	0.2238	2.531	0.0123				
Cortisol	Age:Globulin:CRP:ALT:Status (NonAdx)	–0.0822	0.0334	–2.464	0.0148				
Cortisol	CRP:Status(NonAdx)	3.8405	1.5937	2.410	0.0171				
Cortisol	Age:Status(NonAdx)	–1.9810	0.8380	–2.364	0.0193				
Cortisol	Age	–0.6719	0.2922	–2.299	0.0228				
ACTH/Cortisol	CRP:ALT	1.0441	0.3050	3.423	0.0008	0.1527	3.5530	12, 158	0.0001
ACTH/Cortisol	Age:CRP:ALT	–0.1686	0.0504	–3.343	0.0010				
ACTH/Cortisol	Age:Globulin	1.4258	0.4541	3.140	0.0020				
ACTH/Cortisol	Age:ALT	1.1196	0.4107	2.726	0.0071				
ACTH/Cortisol	Age	–4.3188	1.5896	–2.717	0.0073				
ACTH/Cortisol	Age:Globulin:ALT	–0.3014	0.1157	–2.606	0.0100				
ACTH/Cortisol	Status(NonAdx)	6.5141	2.5002	2.605	0.0101				
ACTH/Cortisol	ALT:Status(NonAdx)	–1.6613	0.6654	–2.497	0.0136				
ACTH/Cortisol	Age:CRP	0.1239	0.0572	2.166	0.0318				
ACTH/Cortisol	CRP:Status(NonAdx)	–20.5422	9.4961	–2.163	0.0320				
ACTH/Cortisol	Globulin:CRP:ALT	–0.1515	0.0702	–2.158	0.0325				
ACTH/Cortisol	Globulin:CRP:Status(NonAdx)	5.9955	2.8088	2.135	0.0343				

Tab. 5. Linear regressions of HPA axis components against selected parameters – females.

Dependent Variable	Variable	Variable parameters				Model parameters			
		Estimate	Std. Error	t value	*p*-value	R-Squared:	Model F	dF	*p*-value
ACTH	Age	83.2655	30.1131	2.765	0.0076	−0.0370	0.8713	23, 60	0.6325
ACTH	Globulin	103.9779	34.6816	2.998	0.0039				
ACTH	Status(Adx)	414.6038	125.4925	3.304	0.0016				
ACTH	Age:Globulin	−29.9164	9.9565	−3.005	0.0039				
ACTH	Globulin:CRP	−16.7822	6.3745	−2.633	0.0108				
ACTH	Globulin:ALT	−6.5799	3.2256	−2.040	0.0458				
ACTH	CRP:ALT	49.4797	16.6395	2.974	0.0042				
ACTH	Age:Status(Adx)	−118.1151	35.9685	−3.284	0.0017				
ACTH	Globulin:Status(Adx)	−141.4233	41.2181	−3.431	0.0011				
ACTH	CRP:Status(Adx)	−262.8384	73.3103	−3.585	0.0007				
ACTH	Age:Globulin:CRP	4.6400	1.7766	2.612	0.0114				
ACTH	Age:Globulin:ALT	1.8885	0.9262	2.039	0.0459				
ACTH	Age:CRP:ALT	−13.7161	4.7483	−2.889	0.0054				
ACTH	Globulin:CRP:ALT	−8.6519	3.6959	−2.341	0.0226				
ACTH	Age:Globulin:Status(Adx)	40.2900	11.7206	3.438	0.0011				
ACTH	Age:CRP:Status(Adx)	72.3319	20.5417	3.521	0.0008				
ACTH	Globulin:CRP:Status(Adx)	91.6054	25.3658	3.611	0.0006				
ACTH	Globulin:ALT:Status(Adx)	7.0144	3.3536	2.092	0.0407				
ACTH	Age:Globulin:CRP:ALT	2.4076	1.0847	2.220	0.0302				
ACTH	Age:Globulin:CRP:Status(Adx)	−25.2207	7.0675	−3.569	0.0007				
ACTH	Age:Globulin:ALT:Status(Adx)	−1.9951	0.9604	−2.077	0.0421				
ACTH	Globulin:CRP:ALT:Status(Adx)	−5.4269	2.0960	−2.589	0.0121				
ACTH	Age:Globulin:CRP:ALT:Status(Adx)	1.4918	0.5847	2.552	0.0133				
Cortisol	CRP	−170.0912	81.7379	−2.081	0.0411	0.1182	1.8560	13, 70	0.0510
Cortisol	Globulin:CRP	46.0763	23.0557	1.998	0.0495				
Cortisol	CRP:ALT	48.5289	22.6163	2.146	0.0354				
Cortisol	Globulin:CRP:ALT	−13.0014	6.3425	−2.050	0.0441				
Cortisol	Age:CRP:StatusNAdx	59.1008	24.6996	2.393	0.0194				
Cortisol	CRP:ALT:Status(Adx)	−1.0836	0.5261	−2.060	0.0432				
Cortisol	Age:Globulin:CRP:StatusNAdx	−16.3272	7.0120	−2.328	0.0228				
Cortisol	Age:CRP:ALT:StatusNAdx	−16.7226	6.8550	−2.439	0.0172				
Cortisol	Age:CRP:ALT:Status(Adx)	−12.7313	6.2947	−2.023	0.0469				
Cortisol	Age:Globulin:CRP:ALT:StatusNAdx	4.5841	1.9371	2.366	0.0207				
ACTH/Cortisol	Globulin	20.9761	7.6806	2.731	0.0079	0.1698	3.0460	8, 72	0.0052
ACTH/Cortisol	ALT	22.6100	8.1122	2.787	0.0068				
ACTH/Cortisol	Status(Adx)	71.7135	27.5489	2.603	0.0112				
ACTH/Cortisol	Age:Globulin	−0.1366	0.0645	−2.117	0.0377				
ACTH/Cortisol	Globulin:ALT	−6.6745	2.3881	−2.795	0.0067				
ACTH/Cortisol	Globulin:Status(Adx)	−21.3332	8.1087	−2.631	0.0104				
ACTH/Cortisol	ALT:Status(Adx)	−22.4311	8.5622	−2.620	0.0107				
ACTH/Cortisol	Globulin:ALT:Status(Adx)	6.6885	2.5101	2.665	0.0095				

the form of the ACTH/cortisol ratio. It is noteworthy that several non-significant trends of borderline significance (0.0762 and 0.0667 and 0.0836, Table 3) were seen when hepatitis C seronegative patients were considered in comparison with both HCV seropositive and GMC patients. It is our belief that when the sample size is expanded these probability values may well become more significant.

Another interesting point relates to the nature of the changes shown in Figure 2. Despite identity of the age dependent ACTH and cortisol levels the addictive status is very clearly a major determinant of the (log) ACTH/cortisol ratio in females. Table 2 shows that this graphical finding is associated with a *p*-value of 0.0239. This indicates that a higher level of ACTH is required centrally to maintain identical cortisol levels peripherally. This suggests a pattern of peripheral (adrenal) resistance to the effects of ACTH. Since opioids are known to trigger both central and peripheral innate immune pathways downstream of ligation of the Myeloid Differentiation Factor-2 (MD2) – Toll-Like Receptor 4 (TLR4, endotoxin receptor) heterodimer on both central and peripheral cells of the immune system (Hutchinson *et al.* 2010; Wang *et al.* 2012), this pattern suggests that at least for the HPA axis, it is the peripheral resistance which predominates over the central resistance arising from inflammation of the hypothalamic neuropil (Zhang *et al.* 2013).

An alternative explanation for this interesting finding may be that the hypothalamus is unusually sensitive to normal levels of peripheral cortisol due to opioid-induced hypothalamic inflammation. Exploration of these possibilities must await further investigations.

As a cholesterol derivate, cortisol is not soluble in the aqueous phase of blood. 75% of it is transported bound to transcortin (also known as cortisol binding globulin) and 25% is bound to albumin (Richard *et al.* 2010; Veldhuis *et al.* 2013). Transcortin assays were not available to the present study. The albumin levels were not different in any subgroups. It has been formally shown that disruptions of the level of transcortin is associated both with altered anxiety and stress responses and changes in the affective and depressive state in rodent models related to perturbations of the level of free and active cortisol, which is normally around 5% of the total (Richard *et al.* 2010; Veldhuis *et al.* 2013) . The possibility exists therefore that major disruptions of the major plasma binding protein for cortisol may importantly perturb the scenario described herein. Serum transcortin levels have also been shown to fluctate in a diurnal cycle (Barrientos *et al.* 2015), and the gene for the glucocorticoid receptor has also been shown to undergo circadian cycling (Kim & Forger 2012; Korencic *et al.* 2014).

Work from the St Louis Washington School of Medicine on transcortin in rodents is of particular relevance to these results. These workers showed in male rats that opioid exposure resulted in a rapid increase in beginning three days after morphine exposure reaching

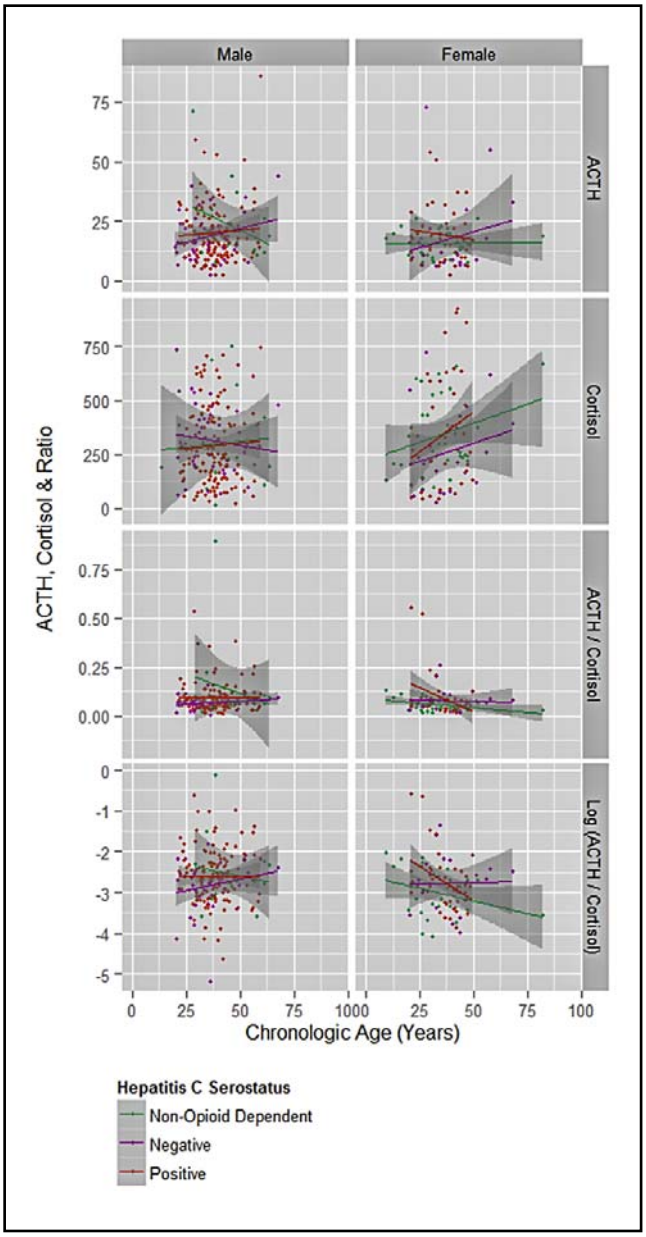

Fig. 4. ACTH, cortisol ratio and logs by chronologic age, hepatitis C serostatus and sex.

a peak of 160% of baseline at seven days, and declining again as morphine levels fell, and associated with a reduced level of available free cortisol (Nock *et al.* 1997). They subsequently showed however that whilst morphine had no effect on transcortin levels in females, the amount of physiologically active corticosterone was dramatically reduced in both sexes (Cicero *et al.* 1997). They noted also that the basal level of transcortin in females was twice that in males (Nock *et al.* 1998), and that the sex differential effect on transcortin was dependent on the adolescent testosterone surge (Nock *et al.* 2000). It may be that some similar mechanisms operate also amongst humans.

The importance of immune and metabolic activation, and their complex and interdependent interrela-

tionships are increasingly being appreciated in many disease states (Foster & McVey Neufeld 2013; Buhmann *et al.* 2014; Caracciolo *et al.* 2014; Vadnie *et al.* 2014; Werts *et al.* 2011). Similarly both immune changes in opioid dependency (McCarthy *et al.* 2001; Reece 2012b; Reece 2012a; Reece 2007c; Reece 2012) and dysmetabolic changes are well recognized (Cooper *et al.* 2003; Ceriello *et al.* 1988). Several reports exist of immune stimulatory and reciprocal immune suppressive effects of opioid dependency and their age dependent alterations, which indicate that immune dysregulatory changes are an important biomarker of ageing (Franceschi *et al.* 2000). Opioids themselves together with some of their major metabolites, have recently been shown to be directly immunostimulatory (Hutchinson *et al.* 2010; Wang *et al.* 2012). This effect is likely to occur both in peripheral tissues and centrally at many sites throughout the neuraxis. The relative contributions of these various changes and their various contributions to the multifarious long term pathophysiology of opioid dependency (Reece & Hulse 2014) are only beginning to be considered.

Important interactions between the opioid, $GABA_A$ and glutamate systems have also been demonstrated (Younger *et al.* 2011). Both ACTH and cortisol have been implicated in the proper maturation of the adrenal medulla and the expression of mature levels of the enzymes responsible for adrenaline (epinephrine) synthesis (Huang *et al.* 2012). Conversely in the brain stem the adrenergic neurons of the nucleus of the solitary tract are controlled by opioidergic signalling (Brunton & Russell 2008). Endogenous opioids are known to play an important role in the development of neural stem cells, and their fate specification and particularly in the decision to differentiate either along a neuronal or astroglial pathway (Giuliani *et al.* 2011). This is believed to occur both in the neuraxis and along peripheral nerves (Ter Laak *et al.* 2003; Plantinga *et al.* 1995). GABA, cannabinoid and cytokine (especially TNFα) signalling is also involved. Since opioids themselves interact with all of these systems (Scavone *et al.* 2013), the above findings relating to altered brain structure are of particular interest (Younger *et al.* 2011). Cortisol is also a potent and well known inhibitor of neuronogenesis (Eisch *et al.* 2000; Eisch 2002). It is possible then that studies showing increase in size of limbic and paralimbic structures (Younger *et al.* 2011) and the pituitary gland (Lorenzetti *et al.* 2010) in opioid dependency may reflect increased gliogenesis. ACTH also controls adrenocortical production of DHEA, an important precursor to androgen synthesis (Brennan 2013).

Immune stimulation has been linked with changes in brain structure (Frodl & Amico 2014). Moreover HPA activation levels has been shown to be linked with telomere length, a major marker of cellular aging and replicative potential (Tomiyama *et al.* 2012). It is likely that bidirectional relationships exist between HPA and immune dysregulation on the one hand and brain aging on the other (Garrido *et al.* 2012; Veldhuis *et al.* 2013). These changes may in turn then become self-reinforcing(Conrad & Bimonte-Nelson 2010). This process has important implications both for treatment decision in opioid dependency syndromes, and for the optimal duration of that treatment (Reece & Hulse 2013; 2014).

Interestingly, glucocorticoid pre-treatment prior to an immune challenge has been shown to *enhance* rather than *suppress* microglial immune activation in some circumstances, and the rise is cortisol seen in aging has been shown to parallel that seen in stress (Barrientos *et al.*). These observations place the present clinical data in a fascinating context, and are consistent with the elevated level of hippocampal glucocorticoid receptor and its activation observed by these workers, at least in the female group.

There were several limitations of the present work. As mentioned above the limited sample size was a significant constraint and indicates larger groups in future iterations of this work. Drug use data was not available to this analysis. Its inclusion would allow cumulative dose effect relationships to be examined.

In conclusion these data show that there is a relative elevation of the ACTH/cortisol ratio in opioid dependent females which is not seen in this sample of males; that this ratio declines in an age dependent manner in both opioid dependent and non-dependent females in a manner not seen in males, and is therefore a useful biomarker of aging in females. Whilst this change was significant in hepatitis C seropositive females the statistical analysis suggested it may also be present in Hepatitis C seronegative females. Numerous highly significant associations were shown on multiple regression between the components of the HPA axis and markers of systemic metabolic and immune activation.

AUTHORS CONTRIBUTIONS

ASR designed the study, treated the patients, analyzed the data and wrote the initial draft of the paper. GKH gave advice on study design, and data analysis, wrote the paper and assisted with literature review.

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