**Hair cortisol concentration in patients with anxiety disorders: exploration of cross-sectional and longitudinal relationships with symptom severity and inflammatory markers**

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**Abstract**

*Background*. Hair cortisol concentration (HCC) can be used to periodically assess hypothalamo-pituitary-adrenal axis function, and appears correlated with prolonged exposure to stress.

*Methods*. Serial assessment (at Baseline, Week 6 and Week 12) of participants (n=35) with anxiety disorders by psychopathological rating scales, with assays of HCC and levels of peripheral anti- and pro- inflammatory cytokines. Patients underwent antidepressant treatment for an initial six weeks, followed by COX-2 inhibitor (celecoxib) augmentation or ‘treatment as usual’ for a further six weeks.

*Results*. At Baseline (n=35), HCC was elevated in patients with single-episode but not recurrent-episode anxiety disorders, mean IL-12p70 levels were low, and mean TNF-α levels were elevated. Following 6 weeks of antidepressant treatment (n=33), mean HCC was within the normal range but mean IL-2 level was low. Celecoxib augmentation (n=18) was associated with reduction in anxiety symptoms and normalisation of mean IL-2 levels.

*Limitations.* Small sample size. Not all participants were assessed at all time points.

*Conclusion*. Serial assessment of hair cortisol concentration is practicable in patients with anxiety disorders. These preliminary findings warrant further investigation in larger samples.

**Key words**: anxiety disorders, cortisol, HPA, hair, inflammation

**Background**

The hypothalamo-pituitary-adrenal (HPA) axis influences many body processes and haemostatic systems (including immune, metabolic, cardiovascular, reproductive and central nervous systems), and plays a major role in stress regulation and the sleep-wake cycle (Barrett et al., 2016). When compared to the extensive literature on HPA axis dysfunction in depressive illness, evidence relating to HPA function in patients with anxiety disorders is limited. There is no unifying disturbance of HPA axis function across anxiety disorders, and within each disorder the findings of investigations using similar methodology have produced inconsistent findings (Elnazer et al., 2014).

Assay of hair cortisol concentration (HCC) provides a measure of HPA function. Circulating cortisol is deposited from follicular capillaries, sweat glands and sebaceous glands into all layers of the growing hair shaft (Kapoor et al., 2018; Russell et al., 2012; Cook et al., 1964), which lengthens by approximately 1 cm. per month (with some ethnic variation), allowing determination of average HCC over a specific period (Loussouarn et al., 2001; Schütz et al., 1993). HCC ranges between 5-91 pg/mg (mean 18 pg/mg), and is not affected by hair colour or sex hormones (Raul et al., 2004), hairdressing treatments or light exposure (Kristensen et al., 2017). HCC is strongly associated with salivary cortisol area under the curve (AUC) over one month (r=0.61, p=0.01) and may be a more accurate reflection of overall cortisol output (Short et al., 2016).

HCC measurement facilitates management of Cushing syndrome, tracking monthly cortisol levels and capturing periodic hypercortisolemia (Manenschijn et al., 2012). Elevated HCC levels have been reported with ongoing stress (Vives et al., 2015), in athletes (Skoluda et al., 2012), victims of natural disasters (Gao et al., 2014), and in hospitalised infants (Yamada et al., 2007). In children, HCC is associated with post-stress salivary cortisol AUC (Vanaelst et al., 2012), AUC with respect to ground levels (AUCg) (Ouellette et al., 2015), and AUC with respect to increase (AUCi) (Kao et al., 2018). HCC levels may be elevated in depressed patients (Dettenborn et al., 2012) and in patients with post-traumatic stress disorder (Lou et al., 2012; Steudte et al. 2011). Findings from meta-analysis indicate a 22% higher median HCC in groups previously exposed to chronic stress, and 43% higher median HCC in groups experiencing ongoing stress, but no consistent correlations between HCC and self-reports of stress or depression (Stalder et al., 2017).

Inflammation in the periphery is ‘communicated’ to the brain via humoral and neural pathways. Resident microglia are activated and release pro-inflammatory cytokines which disrupt hippocampal neurogenesis. Peripheral cytokines activate the HPA axis, leading to increased levels of circulating glucocorticoids and suppression of neurogenesis, which may underlie some of the neurobehavioral changes associated with chronic inflammatory conditions (Chesnokova et al., 2016).

Some studies have found a positive effect for anti-inflammatory drugs on reducing depressive symptoms and levels of disrupted cytokines. A meta-analysis of clinical trials with non-steroidal anti-inflammatory drugs (NSAIDs) found beneficial effects in depressed patients (n=1497) (Iyengar et al., 2013); and a randomised double-blind controlled trial (n=40) of augmentation with the cyclo-oxygenase inhibitor-2 (COX-2) inhibitor celecoxib indicated it could enhance the response to antidepressants (Abbasi et al., 2012).

Less is known about circulating cytokines in anxiety disorders. Meta-analysis suggests differences in IL-10, TNF-α and IFN-α in patients with generalised anxiety disorder (GAD), when compared to controls (Costello et al., 2019). A case-control study of cytokine levels in patients with GAD found disturbances in the ratio of anti- and pro- inflammatory cytokines (with high ratios of TNF-α/IL-1, TNF-α /IL4, INF-γ/IL10 and INF-γ/IL4) (Hou et al.,2017). As little is known about potential interactions between HPA markers and circulating cytokines in anxiety disorders, we examined cross-sectional and longitudinal relationships between HCC and circulating cytokines in patients with a range of anxiety disorders: at Baseline, after six weeks of antidepressant treatment (Week 6), and after a further 6 weeks of either augmentation with the COX-2 inhibitor celecoxib, or a further 6 weeks of ‘treatment as usual’ (Week 12).

**Methods**

*Sample*. Study participants were recruited from primary care (general practice) and secondary care mental health services. Potentially eligible patients were sent a participant information sheet, reply slip and stamped addressed envelope. When a potential participant contacted the research team an appointment was arranged for screening with the Mini International Neuropsychiatric Interview (MINI) (Sheehan et al, 1998). Potential participants could ask questions about the study aims and procedures, and after providing consent underwent Baseline assessments. They were considered eligible for the study if aged between 18-70 years, with a primary diagnosis of an anxiety disorder or an anxiety-related disorder (post-traumatic disorder or obsessive-compulsive disorder), defined according to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5) criteria (American Psychiatric Association 2013), and competent to provide written consent. Patients were excluded from participation if they were outside the specified age range, had a primary diagnosis other than an anxiety disorder, if unable to provide written informed consent, had significant alcohol or substance use in the previous three months, had a physical illness that was unlikely to be stable over the course of the study, or were pregnant or breast feeding.

*Participant questionnaire assessments*. Patients were assessed using the Hospital Anxiety and Depression Scale (HADS) (Zigmond et al., 1983), Clinical Global Impression of Illness Severity (CGI-S) (Guy et al., 1976), Warwick-Edinburgh Mental Well-Being Scale (WEMWEBS) (Tennant et al., 2007), Arizona Sexual Experiences Scale (ASEX) (McGahuey et al, 2000) and Oxford Questionnaire of Emotional Side Effects of Antidepressants (OQuESA) (Price et al., 2012), at Baseline, Week 6 and Week 12. Treatment adherence was determined by patient report and tablet counts.

*Hair sampling.* Scalp hair samples (3 cm. long) were collected from study participants at Baseline, Week 6 and Week 12. Hair strands were cut with fine scissors as close as possible to the scalp from a posterior vertex position: the number of strands differed in accordance with participant permission. Each hair strand was repeatedly washed using isopropanol, then dried for 6 hours, and analysed performed on non-pulverized hair.

*Processing of hair samples*. Each specimen was incubated for 18 hours in 1800 μL methanol at room temperature. 1600 μL of the resulting suspension was purged with nitrogen at 50 °C and a pressure of 0.1 bar for at least 40 minutes. Samples were spun in a centrifuge at 10,000 r.p.m. for 2 minutes and 1 mL of the clear supernatant was transferred into a 2 mL tube. Methanol was evaporated at 650C under a constant stream of nitrogen until samples were completely dry (typically lasting around 20 minutes). Supernatants were re-suspended in 225 μL of distilled water and 50 microliters were submitted to liquid-chromatography coupled to tandem-mass spectrometry (LC-MS/MS). The lower limits of quantification of this assay method were below 0.1 pg cortisol per mg hair. The median coefficient of variation of all replicates was 4.9% (interquartile range: 1.3–13.9%) (Gao et al., 2013).

*Determination of hair cortisol concentration (HCC)*. Following milling of hair segments, two 50 mg aliquots of powdered hair from a single hair segment were processed in parallel. Twenty microliters were removed from the vial and used for cortisol determination with a commercially available immunoassay with chemiluminescence detection (CLIA, IBL-Hamburg, Germany). An unknown amount of antigen present in the sample and a fixed amount of enzyme-labelled antigen compete for the binding sites of the antibodies coated onto the wells. After incubation, the wells were washed to stop the competition reaction. After the substrate reaction, the intensity of the developed colour is inversely proportional to the amount of the antigen in the sample. Results of samples were determined directly using the standard curve. The reference normal range for HCC using this method in healthy volunteers (reported by the same lab) is 1-30 pg/mg.

*Blood sampling for pro- and anti-inflammatory cytokines.* Venous blood samples (8-10 ml) were collected into a vacutainer containing no preservative at Baseline and Week 6, sampling being performed in accordance with NHS procedures, observing sterile conditions, with infection control by either a nurse trained in phlebotomy or a qualified medical practitioner. Blood samples were centrifuged at 1600 g (4000 r.p.m., setting 4) on a Capricorn 2000 bench centrifuge for 10 minutes. The plasma fraction was transferred using a Pasteur pipette to vial tubes, then placed in a ziplock bag and airtight sealed container inside a lockable box for storage at -20oC prior to transfer to a -80oC freezer for longer-term storage.

*Cytokine quantification*. Luminex multi-analyte profiling (xMAP) technology employs proprietary bead sets distinguishable under flow cytometry. Each bead set is coated with a specific capture antibody, and fluorescence or streptavidin-labelled detection antibodies bind to the specific cytokine-capture antibody complex. Multiple cytokines in a fluid sample can be recognized and measured by the differences in both bead sets, with chromogenic or fluorogenic emissions detected using flow cytometric analysis: we used the Proinflammatory Panel 1 (human) Kit, (V-PLEX, MESO SCALE DISCOVERY, Rockville, MD, USA) to measure IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α.

*Data analysis.* Some participants preferred not to provide blood samples at some test points, but were prepared to participate with other tests. Normal reference ranges determined by the lab where the study samples were analysed were used (Table 1). Data were analysed using IBM SPSS® Statistics 26.0.

*Ethical aspects and research governance*. The project was approved by Hampshire Research Ethics Committee (REC reference 16/SC/0038); UK Health Research Authority (HRA) (IRAS project ID 170365) and the Medicines and Healthcare Products Regulatory Agency (EudraCT number:2016-000337-48), as part of a larger investigation of neuroinflammatory and endocrine factors.

**Results**

***Study sample*.** A total of 170 referrals were received from primary or secondary health services: 35 individuals (23 women, 12 men: mean age 32.46 years [SD 12.87 years]) provided consent to participate in the study and underwent Baseline assessment. All participants had English as their first language: 29 were White British, four Asian British, one Black British and one self-identified as Other White Caucasian. Twenty participants (57%) were professionally active at the time of enrolment. Reasons for non-participation included: feeling too unwell to take part in the study (22%); concern about time commitments (15%); lack of financial compensation (14%); discomfort in talking about sexual matters (10%); and no reason (39%).

***Treatment received*.** Two participants withdrew before the Week 6 review, and six participants before the Week 12 review. In the 33 participants who underwent ‘treatment as usual’ between Baseline and Week 6, 19 received a selective serotonin reuptake inhibitor, six received a serotonin-norepinephrine reuptake Inhibitor, three received a noradrenergic and specific serotonergic antidepressant, two underwent cognitive behavioural therapy, and a single participant received a beta-blocker: three patients had not undergone any new treatment by the Week 6 review. At Week 12, eighteen participants had undergone treatment celecoxib augmentation, and fifteen patients a further six weeks of continued antidepressant treatment.

***Clinical characteristics at Baseline*.** Interview with the MINI generated at least one DSM-5 diagnosis in each participant. Thirteen participants met diagnostic criteria for GAD, 8 for panic disorder with agoraphobia, 7 for social phobia, 4 for obsessive-compulsive disorder, and 3 for panic disorder. Nineteen participants (54.3%) were within the range of healthy body mass index (18.5-24.9), but 13 were overweight and three were underweight. The mean scores (and standard error of the means) on rating scales at were as follows: WEMWBS 27 (1.81); HADS-A 15.42 (0.88); HADS-D 13 (0.82); CGI-1 4.56 (0.22); OQUESA-GR 15.5 (1.17); OQUESA-RP 22.18 (0.75); OQUESA-ED 15.09 (3.17); OQUESA-NC 18 (1.27) and ASEX 17.40 (1.21) (Table 2).

***HCC at Baseline*.** Hair cortisol analyses were performed by chemiluminescence immunoassay on samples purified by LC-MS/MS from eleven participants: mean HCC was 26.66 pg/mg (median of 6.14 pg/mg). Mean HCC for male participants and female participants were 30.74 pg/mg [SEM14.79] and 24.19 pg/mg [SEM 19.963], respectively. Mean HCC was higher than the normal range (1-30 pg/mg) in patients with panic disorder with agoraphobia (72.37 pg/mg, [SEM 71.48]), but mean HCC in participants with other diagnoses were unremarkable: GAD, 25.32 pg/mg[SEM 17.30]; social phobia 14.07 pg/mg [SEM 9.51]; panic disorder 1.24 pg/mg (single patient). Mean HCC was elevated in ‘current’ cases (45.23 [SEM 22.21]) but within the normal range in recurrent cases (4.18 pg/mg [SEM 1.68]) (Table 3).

***Cytokines at Baseline***. Mean plasma cytokine concentrations were within normal ranges, other than a low mean concentration of IL-12p70 (0.11 pg/mL [SEM 0.04]) and high mean concentration of TNF-α (2.37 pg/mL [SEM 0.43]). Mean IL1 β concertation was low in male participants (0.005 pg/mL [SEM 0.005]). HCC levels were significantly correlated with plasma concentrations of IFN-γ (p=0.01), IL-10 (p=0.00), IL-2 (p=0.02) and IL-8 (p=0.02) (Figure 1). After adjusting for age, diagnosis and gender using logistic regression partial correlation, the correlation remained significant between HCC (pg/mg) and IL-10 mean concentration (pg/mL) (p=0.00), and there was a significant correlation between mean HCC (pg/mg) and IL-1βa mean concentration (pg/mL) (p=0.00). After adjustment for HADS-D score, there were no significant correlations between mean HCC and other parameters.

***Effects of treatment on rating scale scores***.Anxiety symptom severity (HADS-A) reduced from Baseline to Week 6 (mean 13.70 [SEM 0.73]), and quality of life (WEMWBS) improved from Baseline to Week 6 (mean 33.30 [SEM 1.75]).The severity of depressive symptoms (HADS-D) reduced from Baseline to Week 6 (mean 10.82 [SEM 0.68]).Mean OQUESA-ED declined from Baseline (20.25 [SEM 10.39]) to Week 6 (10.39 [SEM 0.947]), a non-significant change (p=0.373): but there was no further change in Week 12 mean OQUESA-ED score (10.39 [SEM 1.067]). Mean ASEX increased from Baseline to Week 6 (20.18 [SEM 0.96]), a significant change (p=0.01). Mean ASEX declined at Week 12 (17.26 [SEM 1.23]), a significant change (p=0.013).

Celecoxib augmentation was associated with significant improvement in quality of life (mean WEMWBS 7.50 increase [SEM 2.63, p=0.01]) and reductions in anxiety symptom severity (HADS-A 3.17 decrease [SEM 0.52, p=0.00]), and depressive symptom severity (HADS-D 2.11 [SEM 0.80, p=0.02]).A further six weeks of ‘treatment as usual’ was not associated with significant changes.

***Changes in HCC*.** Chemiluminescence immunoassay of hair samples (n=29) at Week 6 found a mean HCC of 5.94 pg/mg (SEM 1.21), within the normal range (1-30 pg/mg) and lower than the mean HCC at Baseline, although HCC values at Baseline and Week 6 were highly correlated (p=0.00). Paired sample t-test for HCC concentration found a non-significant change from Baseline (Week 0) to Week 6 (26.51 pg/mg, p=0.11). Celecoxib augmentation was associated with a non-significant reduction in HCC from Week 6 to Week 12 (-1.24 pg/mg, p=0.80), whereas treatment as usual group was associated with a non-significant increase (1.72 pg/mg, p=0.13). Post-treatment mean HCC (week 6 and Week 12) were found to be within normal range in both current and recurrent cases (Table 3).

We present HCC changes for individual participants at each test point (Table 4). The current/acute cases sub-group showed a reduction in mean HCC from Baseline to Week 12: by contrast, in the recurrent cases sub-group, mean HCC increased from Baseline to Week 12.

***Changes in inflammatory markers*.** Analysis of cytokine levels (of IFN-γ, IL-1β, IL-10, IL-12p70, IL-13, IL-2, IL-4, IL-6, IL-8 and TNF-α) in participants who provided blood samples at Week 6 (n=25) found a low mean concentration of IL-2 (0.22 pg/mL [median 0.17 pg/mL] and a high mean concentration of TNF-α (2.16 pg/mL [median 1.60 pg/mL]. Paired sample t-test for cytokine concentrations found that the changes from Baseline to Week 6 were not significant. Analysis of cytokine levels at Week 12 in the celecoxib augmentation group (n=18), found a low mean concentration of IL-1β (0.04 pg/mL [SEM 0.024], low mean concentration of IL-12p70 (0.12 pg/mL [SEM 0.015], low mean concentration of IL-13 (0.11 pg/mL [SEM 0.05] and a high mean concentration of TNF-α (2.33 pg/mL [SEM 0.21]. Concentrations at Week 12 in the celecoxib augmentation group were correlated with Week 6 concentrations for IL-10 (p=0.00); IL-13 (p=0.00); IL-2 (p=0.00); IL-6 (p=0.00); IL-8 (p=0.00) and TNF-α (p=0.00). Paired sample t-test found a significant change from Week 6 to Week 12 in the mean concentration of IL-2 0.06 pg/mL (SEM 0.02, p=0.00). Analysis of cytokine concentration in the ‘treatment as usual’ group at Week 12 (n=4) found low mean concentrations of IL-1β (0.43 pg/mL [SEM 0.018]); IL-12p70 (0.102 pg/mL [SEM 0.04]); IL-13 (0. 27 pg/mL [SEM 0.11]); and IL-2 (0.21 pg/mL [SEM 0.03]). A paired sample t-test found no significant change from Week 6 to Week 12.

Pro- and anti-inflammatory cytokine ratio analysis (Table 5) found elevated mean TNF-α/IL-10, TNF-α/IL-13, and INF-γ/IL-13, at all testing points. The analysis also found elevated INF-γ/IL-10 at Week 6, low IL-8/IL-10 at Baseline, and within range IL-8/IL-13 at all testing points. Current cases had low Baseline INF-γ/IL-10, within range INF-γ/IL-10 at Week 6 and low IL-8/IL-10 at Week 6 and Week 12. Recurrent cases had within range Baseline INF-γ/IL-10, elevated INF-γ/IL-10 at Week 12, and within range INF-γ/IL-13 at Baseline. Patients who underwent 6 weeks of augmentation by Week 12 had within range IL-8/IL-10. Patient who underwent 6 weeks of ‘treatment as usual’ by Week 12 had within range INF-γ/IL-10 and low IL-8/IL-10. Paired-t Test analysis found no statistically significant change in cytokines ratios from Baseline to Week 6 and to Week 12, except in the current cases who did not opt for augmentation at Week 6 (and seemingly had satisfactory response to treatment as usual) (n=4). This sub-group had significant Paired-T test change mean TNF-α /IL-10 from Week 6 to Week 12 (t 4.14, df 3, p=0.026).

**Discussion**

Recent evidence suggests HCC is a reliable measure of cumulative cortisol concentration over designated period (Short et al., 2018). We found some evidence of elevated HCC in patients with shorter-duration (current) illness but not in longer-duration (recurrent) illness. These findings might suggest a potential desensitisation of HPA response to stress in patients who suffer multiple episodes of anxiety disorders. We also found that changes in HCC were not correlated with changes in anxiety severity (HADS-A; CG-I) or mental wellbeing (WEMWBS).

The small number of participants represents a major limitation. Lack of financial compensation for offered time was mentioned by many referred patients as a reason for declining to participate. Further research and resources are needed to examine HCC in a larger clinical sample.

Pro-inflammatory and anti-inflammatory cytokines function in complementary and competing effects to produce a net-pattern response. The net-pattern response determines the onset, duration and severity of the inflammatory phase of healing. A net anti-inflammatory pattern is required for a less severe, shorter healing response (Figure 2). We found elevated TNF-α/IL-10 and TNF-α/IL-13 ratios at Baseline, Week 6 and Week 12. We found elevated INF-γ/IL-10 at Week 6 (and elevated INF-γ/IL-13 at Week 12). Patients with current anxiety disorders had low Baseline INF-γ/IL-10 and low IL-8/IL10 at Baseline, Week 6 and Week 12. Patients with recurrent anxiety disorders had elevated INF-γ/IL-10 at Week 6 and Week 12 and low IL-8/IL-10 at Baseline.

**Conclusion**

This study provides some evidence for elevated HCC in patients with a current anxiety disorder and a within-range HCC in patients with recurrent anxiety disorders, at Baseline. Elevated HCC at Baseline normalised after 6 weeks of treatment. This continued to be the case after a further 6 weeks of treatment (Week 12). These findings may suggest a potential desensitisation of HPA response to stress in patients who experience multiple episodes of anxiety disorders. Patients with current anxiety disorders had an anti-inflammatory cytokine response pattern compared to patients with recurrent anxiety disorders.

**Authors Contributions**

Authors meets the criteria of authorship described by The International Committee of Medical Journal Editors (ICMJE).

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**Statement of interest**

None.

**Ethical standards**

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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**Table 1. Normal ranges and ratios**

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| --- | --- |
| Biomarker | Range |
| HCC | 1-30 pg/mg |
| TNF-α | 0.10-1.75 pg/mL |
| IFN-γ | 0.64-14.4 pg/mL |
| IL-1β | 0.11-24.3 pg/mL |
| IL-2 | 0.22-2.68 pg/mL |
| IL-4 | NA |
| IL-6 | 0.16-27.2 pg/mL |
| IL-8 | 1.48-1720 pg/mL |
| IL-10 | 0.06-3.08 pg/mL |
| IL-12p70 | 0.26-0.38 pg/mL |
| IL-13 | 0.60-2.78 pg/mL |
| TNF-α/IL-10 | 0.57-1.67 |
| TNF-α/IL-13 | 0.167-0.522 |
| IFN-γ/IL-10 | 4.68-10.67  |
| IFN-γ/IL-13 | 1.067-5.18 |
| IL-8/IL10 | 24.667-558.442 |
| IL-8/IL-13 | 2.467-618.705 |

|  |
| --- |
| **Table 2. Rating scale and questionnaire scores at Baseline (Week 0)** |
|  | Mean | N | Std. Deviation | Std. Error of Mean |
| Age | 32.46 | 35 | 32.46 | 2.18 |
| WEMWBS-total W0 | 27.00 | 12 | 6.27 | 1.81 |
| HADS-D W0 | 13.00 | 12 | 2.83 | 0.82 |
| HADS-A W0 | 15.42 | 12 | 3.06 | 0.88 |
| EQRS-Total W0 | 18.14 | 29 | 5.63 | 1.04 |
| SAS-Total W0 | 7.88 | 33 | 3.33 | 0.58 |
| ASEX-Total W0 | 17.40 | 35 | 7.17 | 1.21 |
| CGI-S W0 | 4.56 | 34 | 1.31 | 0.22 |
| OQUESA-GR W0 | 15.55 | 11 | 3.88 | 1.17 |
| OQUESA-RP W0 | 22.18 | 11 | 2.48 | 0.750 |
| OQUESA-ED W0 | 15.09 | 11 | 10.51 | 3.17 |
| OQUESA-NC W0 | 18.00 | 11 | 4.22 | 1.27 |
| ASEX W0 | 17.40 | 35 | 7.17 | 1.21 |

|  |
| --- |
| **Table 3. HCC (pg/Ml)** |
|  Illness type | HCCW0 | HCCW6 | HCCW12 |
| Current  | Mean | 45.23 | 6.66 | 6.16 |
| N | 6 | 12 | 11 |
| Std. Error of Mean | 22.21 | 1.85 | 2.42 |
| Std. Deviation | 54.4 | 6.41 | 8.031 |
| Range | 141.61 | 22.25 | 28.44 |
| Recurrent | Mean | 4.18 | 5.42 | 8.83 |
| N | 5 | 17 | 14 |
| Std. Error of Mean | 1.68 | 1.62 | 5.68 |
| Std. Deviation | 3.76 | 6.7 | 21.27 |
| Range | 9.05 | 27.72 | 81.66 |
| Total | Mean | *26.57*♂ 14.72♀ 19.96 | 5.94 | 7.66 |
| N | *11*♂ 4♀ 7 | 29 | 25 |
| Std. Error of Mean | *13.3*♂ 14.72♀ 19.96 | 1.21 | 3.31 |
| Std. Deviation | ♂ 29.44♀ 52.82 | 6.49 | 16.54 |
| Range | ♂ 58.62♀ 142.96 | 27.72 | 81.66 |

**Table 4. HCC for all participants at Baseline, Week 6 and Week 12**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Participant gender, age | HCC W0 | HCC W6 | HCC W12 | ∆ HADS-A | ∆CGI-S |
| M,48 | - | 2.16 | 82.23 | -2 | -2 |
| M,48 | - | 28.28 | 6.75 | +1 | -1 |
| F,24 | 143.85 | 14.92 | 5.7 | 0 | -3 |
| F,23 | 2.29 | 6.29 | 7.69 | -4 | -1 |
| F,20 | - | 5.64 | 6.65 | -3 | 0 |
| F,21 | - | 4.97 | 1.73 | -2 | -1 |
| F,27 | - | 1.58 | 2.46 | +4 | 0 |
| F,23 | 3.07 | 2.61 | 0.71 | -10 | -1 |
| F,29 | - | 0.88 | 0.57 | -5 | -2 |
| F,24 | - | 0.56 | - | 0 | 0 |
| M,48 | - | - | - | -3 | -1 |
| F,21 | - | 3.06 | 0.57 | -4 | -1 |
| F,20 | 0.89 | 1.87 | 1.16 | -4 | 0 |
| M,27 | - | 4.8 | 3.17 | -2 | -3 |
| F,22 | - | - | - | -1 | 0 |
| F,22 | - | 2.75 | 1.02 | -4 | -1 |
| M,37 | 59.86 | 5.44 | 4.51 | -14 | -6 |
| F,22 | - | - | - | 0 | 0 |
| F,23 | 7.34 | 4.29 | 5.93 | -1 | -2 |
| M,24 | 9.95 | 3.08 | - | -3 | 0 |
| F,26 | - | 13.46 | 2.28 | -6 | 0 |
| M,24 | 51.94 | 5.65 | 7.68 | -2 | -2 |
| F,22 | - | 1.83 | 0.91 | -5 | -1 |
| F,23 | - | 8.37 | - | 0 | 0 |
| M,24 | - | - | - | 0 | 0 |
| M,56 | - | 2.91 | 7.65 | 0 | 0 |
| M,38 | 1.24 | - | - | 0 | 0 |
| F,38 | - | 3.09 | - | -1 | 0 |
| M,42 | - | 23.13 | 25.01 | -2 | -1 |
| F,49 | 6.15 | - | - | 0 | 0 |
| F,60 | - | 2.69 | 1.48 | -2 | -1 |
| F,24 | 5.76 | 2.11 | 1.69 | -1 | -2 |
| F,58 | - | 1.71 | 1.52 | -9 | -3 |
| M,48 | - | 4.17 | 5.66 | -2 | -1 |
| F,51 | - | 9.84 | 3.58 | -3 | 0 |
| HCC pg/mg approximated to two decimal pointsM: male; F: female; -: missing value |

**Table 5 Cytokine ratios**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | TNF-α/IL-10 | TNF-α/IL-13 | INF-γ/IL-10 | INF-γ/IL-13 | IL-8/IL10 | IL-8/IL13 |
| Total | Baseline | 10.11 SEM 2.84 | 7.3 SEM 1.5 | 5.77 SEM 0.72 | 8.34 SEM 5.22 | 12.08 SEM 4.03 | 12.83 SEM 6.86 |
| Week 6 | 12.77 SEM 1.95 | 12.25 SEM 2.7 | 13.55, SEM 2.9 | 15.83 SEM 4.15 | 32.21 SEM 8.44 | 24.76 SEM 6.31 |
| Week 12 | 11.05 SEM 1.55 | 10.7 SEM 2.26 | 9.36 SEM 2.22 | 11.12 SEM 4.46 | 30.98 SEM 8.04 | 24.15 SEM 5.87 |
| Current | Baseline | 5.65 SEM 4.28 | 10.59 | 4.01 SEM 0.91 | 23.91  | 9.07 SEM4.88 | 32.36 |
| Week 6 | 9.11 SEM 1.43 | 7.47 SEM 2.64 | 9.5 SEM 2.87 | 14.87 SEM 10.97 | 16.1 SEM 2.90 | 9.2 SEM 3.47 |
| Week 12 | 7.34 SEM 1.4 | 9.93 SEM 2.27 | 6.51 SEM 1.79 | 13.60 SEM 7.46 | 11.85 SEM 4.0 | 15.25 SEM 2.67 |
| Recurrent | Baseline | 12.34 SEM 3.5 | 6.2 SEM 1.44 | 6.66 SEM 0.64) | 3.15 SEM 0.8 | 13.58 SEM 5.9 | 6.32 SEM 3.04 |
| Week 6 | 14.83 SEM 2.84 | 14.37 SEM 3.53 | 15.821 SEM 4.28 | 16.26 SEM 4.14 | 41.28 SEM12.66 | 31.67 SEM 8.05 |
| Week 12 | 14.03 SEM 2.18 | 7.41 SEM 1.53 | 11.4 SEM 3.71 | 7.40 SEM 1.53 | 46.29 SEM 12.31 | 37.5 SEM 11.92 |
| W12 Augmentation | 12.33 SEM 1.82 | 11.04 SEM 3.23 | 10.01 SEM 2.73 | 7.65 SEM 1.17 | 37.35 SEM 9.73 | 30.97 SEM 8.62 |
| W12 treatment as usual | 6.57 SEM 1.62 | 10.18 SEM 3.49) | 7.08 SEM 3.25 | 16.3327 SEM 11.43 | 8.69 SEM 1.0 | 13.92 SEM 3.87 |
| Black: within reference rangeRed: higher than reference rangeBlue: below reference range |

**Figure 1. Correlations at Baseline (Week 0)**



**Figure 2. Cytokines response and inflammation**

|  |  |
| --- | --- |
| **Pro-inflammatory cytokines****IL-1, IL-8****TNF-α, INF-γ** | **Anti-inflammatory cytokines** **IL-4** **IL-10** **IL-11** **IL-13** |